COMPLEMENTARY AND CONVENTIONAL ANTIMICROBIALS FOR INTEGRATIVE TARGETED CONTROL OF BACTERIAL VAGINOSIS ASSOCIATED PATHOGENS

By

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ABSTRACT OF THE DISSERTATION

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Bacterial vaginosis, the polymicrobial vaginal infection, occurs in women of an adolescent and childbearing age and associated with numerous gynecological and obstetric complications.This infection is characterized by the presence of thick-adherent vaginal biofilms, composed mainly of *Gardnerella vaginalis, Atopobium vaginae, Mobiluncus curtisii, Prevotella bivia* and *Peptostreptococcus anaerobius*. *Gardnerella vaginalis* is thought to be one of the primary etiological agent of the infection paving the way for various opportunists to colonize the ecological niche. The failure of conventional treatment with synthetic antibiotics is largely due to antibiotic tolerance of biofilm-associated cells and infection recurrence with antibiotic-resistant mutants. The dissertation-related articles include four major components of the study. First one is a review on importance of natural derived antimicrobials (alone and particularly in combination with antibiotic) as an effective strategies for combating the tolerance of biofilm-associated pathogens to antibiotic treatment. Second component is our study aimed at evaluation of antimicrobial activity of natural derived substances, subtilosin, ε-poly-L-lysine and lauramide arginine ethyl ester against established biofilms of *G. vaginalis*, using three commonly utilized methods (plate counts, ATP viability and resazurin assays) to assess cell viability in the antimicrobial-treated *G. vaginalis* biofilms.

Subtilosin and lauramide arginine ethyl ester showed the strongest biofilm bactericidal effect in comparison to the other tested antimicrobials. The plate count was reported as the best method for estimating the bactericidal effect of the studied antimicrobials. The study's third component is a research article reporting on elucidation of antimicrobial activity of subtilosin and lauramide arginine ethyl ester in combination with commonly prescribed antibiotic clindamycin and metronidazole against bacterial vaginosis-associated pathogens. All tested antimicrobial combinations were inhibitory for BV-associated *Mobiluncus curtisii* and *Peptostreptococcus anaerobius*. LAE and subtilosin synergized ith clindamycin and metronidazole against biofilms of *G. vaginalis* but not biofilm-associated vaginal lactobacilli. Last but not least, the fourth component of our study is a report on safety and putative probiotic properties of *Bacillus amyloliquefaciens* B-1895 and subtilosin-producing *Bacillus subtilis* KATMIRA1933. The cell-free supernatants of both *Bacillus* strains were non-mutagenic and having antimicrobial properties against human pathogens *Listeria monocytogenes, Streptococcus intermedius* and *Porphyromonas gingivalis*. The two strains were strongly co-aggregating with pathogenic *Escherichia coli* and *Pseudomonas aeruginosa*. In addition, the endospores of *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 were tolerant to 0.3% (w/v) bile salts and survived when incubated for 4 h in MRS broth at pH 2.0 to 3.0. The chapters are linked through the idea of using natural derived antimicrobial (particularly, subtliosin and LAE) alone (II) and in combination with the commonly prescribed antibiotics metronidazole and clindamycin (III) to counteract biofilm formation by BV-associated pathogens. Since LAE is already certified as GRAS for certain applications,

we evaluated safety of a putative probiotic *Bacillus subtilis* KATMIRA1933 (IV), the producer of subtilosin which was used as anti-biofilm agent.

Dedications

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PREFACE

Chapter 1 has been submitted for publication as "**Control of biofilm formation: antibiotics and beyond**.", **Algburi, A., Comito, N., Kashtanov, D., Dicks, L.M.T., Chikindas, M.L.** *Applied and Environmental Microbiology,* 2016. AEM01664-16. I was the first author who wrote the first draft and took care of reviewers' comments.

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Chapter 3 has been published as "**Natural antimicrobials subtilosin and lauramide arginine ethyl ester synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli.**", **Algburi, A., Volski, A. and Chikindas, M.L.** 2015. *FEMS Pathogens and disease*, 73 (5), p.ftv018. I was the first author who did 100% of bench work and wrote the first draft with taking care of the reviewers' comments.

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Chapter 1: Control of biofilm formation: antibiotics and beyond.[1]

This review chapter aims at the analysis of a novel approach for control of biofilm-forming infectious microorganisms. This effort is in line with the strategy, which recently received strong encouragement from the National Center for Complementary and Integrative Health of the National Institute of Health (U.S.); i.e. combining conventional treatments with complementary methods to uncover “potential usefulness and safety issues of natural products”.

[1] This chapter was submitted for publication in *Applied and Environmental Microbiology*, AEM01664-16, 2016. A new version has been created after taking care of reviewers' suggestions and comments. All references and formatting within follow the specifications of the journal.

Control of Biofilm Formation: Antibiotics and Beyond

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ABSTRACT

Biofilm-associated bacteria are less sensitive to antibiotics than free-living (planktonic) cells. Furthermore, with variation in concentration of antibiotics throughout a biofilm, microbial cells are often exposed to levels below inhibitory concentrations and may develop resistance. This, and the irresponsible use of antibiotics, lead to the selection of pathogens that are difficult to eradicate. The challenge is to develop antimicrobial compounds with a mode of activity different to those of most antibiotics. This review addresses the reasons for persistent biofilm-associated infections when treated with antibiotics and evaluates the effect natural antimicrobials have on pathogens in biofilms when used instead of, or in combination with, commonly prescribed antibiotics.

INTRODUCTION

According to some authors, eighty percent of pathogens that survive in biofilms are associated with persistent infections (1, 2). Typical examples are *Pseudomonas aeruginosa* associated with cystic fibrosis (3), *Staphylococcus aureus* responsible for most wound infections (4) and *Gardnerella vaginalis*, one of the major causative agents of bacterial vaginosis (5). About ninety percent of a biofilm is composed of extracellular polysaccharides (EPS), proteins and DNA (6). The EPS provides stability, mediates surface adhesion, and forms a scaffold that immobilizes cells, enzymes and antibiotics (7 -11).

Cells in biofilms experience stringent growth conditions. Survival depends on the ability of cells to mutate and exchange genetic information, including horizontal gene transfer (7, 12). Resistance to antibiotics may thus be seen as a phenotypic shift in behavior when cells adapt to a sessile life style (13). This hypothesis is supported by cells developing tolerance to antimicrobial peptides and phagocytosis (14). Some staphylococci produce poly-γ-DL-glutamic acid (PGA) that binds to antimicrobial peptides and protects bacterial cells form neutrophil phagocytosis (15). Other physiological changes occur due to oxygen deprivation or nutrient deprivation, especially in deeper layers of the biofilm. Oxygen deprivation and low metabolic activity in biofilms rendered *P. aeruginosa* more tolerant to antibiotics (16). Rapid changes in pH between layers in a biofilm may lead to the accumulation of organic acids and deactivate penetrating compounds (16). Complex (polymicrobial) biofilms composed of multiple species are generally more resistant to antibiotics than biofilms composed of a single

species (17, 18). The diversity and metabolic state of cells in a biofilm plays a key role in antibiotic resistance. Persister cells are generally more resistant to antibiotics, survive treatment and re-establish the biofilm community (11, 19, 20). Antibiotics that target growing bacteria may not be effective in dormant sections of a biofilm. Some antibiotics that are typically less effective against metabolically less active cells include β-lactams, ciprofloxacin, tetracycline and tobramycin (3, 21).

Studies of *P. aeruginosa* showed an increase in antibiotic tolerance when the cells were immobilized in a biofilm. Zhang and Mah (22) showed that the efflux pump PA1874-1877 in *P. aeruginosa* was more actively expressed by biofilm-associated cells when compared to planktonic cells. The efflux pumps in *Pseudomonas* spp. are also used in the secretion of biocides such as glutaraldehyde (23). Cells with inactive efflux pumps may have diminished ability to form biofilms (24). Therefore, antimicrobial agents that inactivate efflux pumps such as thioridazine and Phe-Arg -naphthylamide (PAN) (24), might be helpful in the prevention of biofilm formation.

Biofilm formation is a complex activity that involves a number of simultaneous occurring mechanisms. Failure to develop new antibiotics, combined with the spread of resistance and improper use of antibiotics, will lead to an increase in morbidity and mortality across the globe. Challenges to the discovery of alternative treatments have been mentioned in other reviews (10, 25, 26). Estrela et al. (27) discussed the potential of combining antimicrobial compounds with antibiotics to inhibit quorum sensing in a biofilm. In this review, the combination of different classes of antimicrobial compounds with antibiotics to control biofilm formation is discussed (Figure 1). This is in line with

the recent approach taken by the National Center for Complementary and Integrative Health (NCIH) of NIH, i.e. combining conventional treatments with complementary methods to uncover “potential usefulness and safety issues of natural products” (28). We will address the theoretical (assumption-based) and experimentally detected reasons behind the failure of treatments and the tolerance of persistent infections to traditional treatments.

ANTIBIOTICS COMBINED WITH ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are naturally produced by eukaryotes and prokaryotes as part of the innate immune/defense system (29). The unique feature of most AMPS is their small size (15-30 amino acids), charge (amphitatic or cationic), and that they target cell membranes (29, 30). The positively charged peptides are attracted to the negatively charged cell membranes of bacteria and biofilm surfaces. Active and slow-growing bacteria in biofilms are killed by AMPs (31) and manipulation of the AMPs’ amino acid composition may result in increased antimicrobial activity (32-34). One example of genetic manipulation is the construction of the broad-spectrum bactericidal peptide R-FV-I16 by removing the functional defective sequence RR7 and by inserting the anti-biofilm sequence FV7 embedded in peptide RI16 (33). The specificity of AMPs can also be changed, as demonstrated by Li et al. (35) and He et al. (36). These authors designed specifically targeted AMPs (STAMPs) highly selective in the killing of a cariogenic *Streptococcus mutans* strain, but with no effect on non-cariogenic oral streptococci.

AMPs either form pores in the cell membrane or act as membrane perturbers. Several variations of each mechanism are discussed in the review by Wimley and Hristova (37).

At low concentrations, AMPs may act bacteriostatically (10). De la Fuente-Nunez and co-workers (38) have shown that AMP 1037 stimulates the swarming of *P. aeruginosa* PA2204 cells, but inactivates twitching motility and biofilm formation. The Peptide antimicrobial NA-CATH:ATRA1-ATRA1, a synthetic cathelicidin, inhibited *S. aureus* biofilm formation, and the peptide LL-37 *P. aeruginosa* biofilm formation when used at levels below MIC (39, 40). The authors concluded that the two AMPs prevented the expression of genes encoding proteins involved in biofilm formation. In *P. aeruginosa* the down-regulated genes are coding for Type IV pili, rhamnolipid synthesis, quorum sensing, and the assembling of flagella (39). Some AMPs have specific antimicrobial features, for example lactoferrin chelates iron and inhibits biofilm formation by *P. aeruginosa* (41). In another study (42), binding of AMPs to eDNA enhanced the detachment of biofilms.

To survive in the presence of AMPs, bacteria utilize various approaches, e.g. mutation that changes the structure and charge of the cytoplasmic membrane, modification of lipopolysaccharides in the cell wall, secretion of AMPs by specific efflux pumps, etc. (43). Herbert and co-workers (44) discovered that the regulatory system in *S. aureus* involved in biofilm formation (GraRS) plays an important role in resistance to AMPs. Resistance was reversed when AMPs were added in combination with other antimicrobial compounds. The combination of AMPs with antibiotics prevented biofilm formation by *P. aeruginosa* (45-49). In these studies, AMPs from various sources were combined with commonly prescribed antibiotics against *P. aeruginosa*. Eckert et al. (45) found that the STAMP, G10KHc, was as active as tobramycin against planktonic- and biofilm-associated cells of *P. aeruginosa*. Increased antimicrobial activity was observed when

G10KHc was combined with tobramycin. The peptide destabilized the cell membrane, which enhanced the penetration of tobramycin into cells. In a similar study, Hirakura et al. (50) reported that enhanced antimicrobial activity of the broad-spectrum AMP tachyplesin III against biofilm-associated *P. aeruginosa* when combined with the antibiotic piperacillin tazobactam (TZP). The same results were obtained with an in vivo urethral stent infection model (46). Inhalation of the amphipathic polypeptide colistin, combined with ciprofloxacin, killed biofilm-associated *P. aeruginosa* and improved the lung functions of CF patients (47) over a 4-week treatment. Colistin inhibited the growth of slow-growing persister cells. While 32 μg/ml of AMP GL13K did not eradicate biofilms, the same concentration of GL13K, in combination with tobramycin (1 μg/ml), eradicated 67.5% of *P. aeruginosa* biofilms (48). Dosler and Karaaslan (49) reported an 8-fold increase in *P. aeruginosa* biofilm destruction when a combination of peptides CAMA and LL-37 were used, compared to most of the antibiotics they have tested, including ciprofloxacin.

Research on biofilms of *S. aureus* also indicated that AMPs combined with conventional antibiotics may be a better alternative than antibiotics alone. In vivo studies conducted by Mataraci and Dosler (51) and Dosler and Mataraci (52) showed a synergistic effect on biofilms of methicillin-resistant S. aureus (MRSA) when nisin was combined with daptomycin/ciprofloxacin, indolicidin with teicoplanin, and CAMA with ciprofloxacin. Pre-treatment of central venous catheters (CVC) with cathelicidin peptide BMAP-28, in combination with traditional antibiotics quinupristin/dalfopristin (Q/D), linezolid (LZD) and vancomycin, reduced *S. aureus* on CVC and prevented bacteremia (53). To effectively eradicate biofilms on CVC, the "antibiotic-lock” technique (also

called "intraluminal therapy") was suggested, which involves the filling of CVCs with a predetermined concentration of AMPs. A combination of the cationic peptide IB-367 and LZD, in the antibiotic-lock technique eradicated *S. aureus* biofilms on CVC (54). Cirioni et al. (55) reported a reduction in biofilm-associated *S. aureus* on vascular grafts when sub-MIC levels of vancomycin were combined with the lipopeptides Pal–Lys–Lys–NH2 and Pal–Lys–Lys. Some AMPs with broad anti-biofilm activity, e.g. peptide 1018, blocks or degrades (p)ppGpp, which is essential for biofilm formation, within 30 min. At low concentrations, peptide 1018 inhibits biofilm formation, but eradicates pre-formed biofilms when applied at higher concentrations (56). In a separate study, the same authors reported on the *in vivo* and *in vitro* anti-biofilm activity of newly synthesized broad-spectrum D-enantiomeric AMPs (57). These peptides acted in synergy with antibiotics in the inhibition and eradication of pathogenic biofilms of *P. aeruginosa*. Synergy of AMPs and antibiotics against biofilm-associated pathogens should attract the attention of scientists to explore the mechanistic actions of these combinations.

Anti-biofilm activity of AMPs alone, or in combination with antibiotics, against *G. vaginalis* was recently reported. While being non-bactericidal, retrocyclins (RC-101) prevented biofilm formation by *G. vaginalis* (58). Turovisky et al. (59) evaluated the antibacterial activity of the natural AMPs subtilosin, ε-Poly-L-Lysine (PL) and Lauramide Arginine Ethyl Ester (LAE) against biofilm-associated *G. vaginalis*. Of all the tested combinations, subtilosin and LAE were more most effective against *G. vaginalis* biofilms. In fact, subtilosin synergizes with clindamycin (protein synthesis inhibitor) and metronidazole (inhibits nucleic acid synthesis) against biofilm associated pathogens, but not biofilm-growing vaginal lactobacilli (60). Therefore, synergistic activity of AMPs

with commonly used antibiotics justifies the importance of considering them as alternatives in the control of biofilms.

ANTIBIOTICS COMBINED WITH BIOFILM-DEGRADING ENZYMES

The enzymatic degradation of biofilms is associated with the chemical composition of EPS produced by microbial cells. Adhesion to surfaces stimulates bacterial cells to produce EPS (61), which is mainly composed of polysaccharides, proteins, and nucleic acids (62). These components play a key role in cell-cell or cell-surface attachment, supporting the integrity of biofilm architecture and protecting biofilm cells from the shearing stress factors (63, 64). Enzymes could inhibit and disrupt the EPS matrix formation and then facilitate the detachment of biofilm. However, a second antimicrobial substance is required to target the detached cells (65, 66).

The biofilm-degrading enzymes DNase I, α-amylase and dispersin B (DspB) showed high activity in reducing EPS mass and biofilm cell numbers (66- 69). However, the older the *P. aeruginosa* biofilm, the more difficult it dissolves with DNase I treatment The production of high quantities of EPS and proteolytic exo-enzymes by the mature biofilms inactivates DNase I (68). In the same regard, purified recombinant DNase1-like 2 (DNase1L2), extracted from human stratum corneum, effectively prevented biofilm-associated *P. aeruginosa* and *S. aureus* (67). *Bacillus subtilis* S8-18α-amylase was evaluated against biofilms of a clinical strain of methicillin resistant *S. aureus* (MRSA) and *P. aeruginosa* ATCC10145 (69). An efficient biofilm inhibition and degradation of mature biofilms were reported due to disruption of EPS. Craigen et al. (70) found that α-amylase, derived from *Bacillus subtilis,* was more effective in degrading the EPS in

biofilms of *S. aureus* and *P. aeruginosa* compared to amylase from human saliva and sweet potato. In addition, Singh et al. (71) noticed a strong degradation activity of α-amylase (82 % biofilm reduction) against biofilm-associated *P. aeruginosa*. Biofilm-degrading enzymes such as lysostaphin (72) and alginate lyase (73) showed anti-biofilm activity against various pathogenic bacteria. Although these enzymes function to destroy and detach biofilms, the possiblility of preventing biofilm re-establishment is not guarenteed.

Treatment of *S. aureus* biofilm with combinations of recombinant human DNase I (rhDNase I) and topical antiseptics (chlorhexidine gluconate and povidone iodine) reduced cell by 4 to 5 logs more when compared to using antibiotics alone (74). Tetz et al. (75) noticed a modification in the biomass, texture, morphology, and number of cells in a biofilm when DNase was applied. The alteration of biofilm structure enhanced the activity of antibiotics against biofilms of *P. aeruginosa* and *S. aureus*. Studies conducted with a murine vaginal model showed that DNase increased the activity of metronidazole against *G. vaginalis* in biofilms ten-fold (76). Donelli and co-workers (77) found that DspB, produced by *Actinobacillus actinomycetemcomitans*, alone or synergized with cefamandole nafate, hydrolised the EPS of a staphylococcal biofilm, promoted antibiotic penetration, and augmented the killing of cells. Moreover, reported a synergistic anti-biofilm activity has been reported when triclosan and DspB were used in combination against *S. aureus* in biofilms formed on vascular catheters (65). Using a continuous flow culture, alginate lyase showed a remarkable sensitization and elimination of mucoid biofilm-associated *P. aeruginosa* when administrated with gentamycin, compared to inactivity with only gentamycin (73). The enzyme lysostaphin, extracted from

Staphylococcus simulans, eliminated S. *aureus* cells from biofilms in vitro (78). Furthermore, the in vivo work has demonstrated the biofilm killing activity of lysostaphin (15 mg/kg) combined with (50 mg/kg) nafcillin against methicillin-resistant *S. aureus biofilms growing on a medical device* (72). Lysostaphin has shown synergy with five of nine antibiotics, with highest eradication of MRSA in biofilms when used in combination with clarithromycin (79). The latter suppressed hexose polymerisation. In addition, proteinase K showed an inhibition of early adhesion and dispersion of *S. aureus* in a mature biofilm (80). Despite the high cost of production, biofilm eradicating enzymes could possibly be used an alternative to antibiotics against persistent infections, especially if the enzymes synergize with commonly used antibiotics.

ANTIBIOTICS AND QUORUM SENSING INHIBITORS

Quorum sensing (QS) regulates virulence behaviors, including biofilm formation (81). QS compounds include N-acyl homoserine lactones (AHLs) produced by gram negative and auto-inducing peptides by gram positive bacteria. In both Gram-negative and Gram-positive bacteria, auto-inducer-2 (AI-2) molecules are used for inter- and intra-species communications in biofilms and are involved in regulation of biofilm formation. Inhibition of this process by QS quenchers or inhibitors (QSI or QSQ) may play a key role in preventing the production of virulence factors and inhibit biofilm formation by many pathogens. In addition, enzymatic degradation of QS signals such as lactonase, acylase, oxidoreductase and paraoxonase has also been reported (82). These quenchers attenuate QS by blocking or shutting down the expression of QS-genes in pathogens,

which leads to biofilm inhibition without killing planktonic cells or influence normal growth.

Recently, inhibition of QS and biofilm formation has been reported in several studies (83-86). The injection of RNAIII-inhibiting peptide (RIP) as QSI in rats with MRSA graft infection repressed staphylococcal RNAIII-activating protein and *agr* systems, which are required for staphylococcal biofilm formation (84). The autoinducer RNAIII-activating protein RAP played a role in the QS mechanism of *S. aureus* by stimulating the phosphorylation of the target of RAP (TRAP) protein. TRAP regulates the expression of several virulence factors and their regulator, *agr*, could be inhibited by RIP (85). In addition, Usnic acid, a secondary metabolite of lichen loaded with polymers, also interfered with QS which prevented *S. aureus* biofilm formation and changed the morphology of *P. aeruginosa* biofilm (83). A pungent oil of fresh ginger (6-gingerol) has been shown to bind to QS receptors in *P. aeruginosa* and prevented the production of several virulence factors and paused biofilm maturation. Transcriptomic analysis confirmed that 6-gingerol inhibits QS-induced gene expression and the production of virulence factors (87). Several compounds were reported to have QS inhibitory effects, including penicillic acid, solenopsin A, catechin, ellagic acid derivatives and curcumin (88). Using QS inhibitors alone, or in combination with antibacterials, creates an opportunity for their use in biofilm controlling formulations.

Rasmussen et al. (89) referred to the activity of patulin and penicillic acid, which were isolated from *Penicillium* species as active QS inhibitor compounds that control QS-gene expression in *P. aeruginosa*. Also, Balaban et al. (85) reported anti-biofilm activity

RIP against biofilms of MRSA as well as its synergistic activity when combined with antibiotics and cationic peptides. Interestingly, a clearance of *P. aeruginosa* biofilm and reduction in pyocyanine production was reported by Roy et al. (90) when phenyl-DPD, the AI-2 analog, was combined with gentamicin indicating the possible role of QS system in biofilm maturation and/or dispersion. Furthermore, Kiran et al. (91) reported that lactonase from *Bacillus* spp. did not effect the growth of *P. aeruginosa*, but reduced biofilm formation. The perturbation of biofilm formation by lactonase increased the susceptibility of biofilms to antibiotics and significantly reduced the production of virulence factors when lactonase was used in combination with ciprofloxacin and gentamicin. It is not known if QS inhibitors from various sources possess the same or different more anti-biofilm activity.

Plant-derived QS inhibitors often exhibit remarkable biofilm reduction ability, especially when combined with antibiotics. Brackman and co-workers (92) conducted an in vivo study to assess the activity of tobramycin against *P. aeruginosa* biofilm, in addition to clindamycin and vancomycin against *S. aureus* biofilm, alone and in combination with QS inhibitors (baicalin hydrate, cinnamaldehyde and hamamelitannin). The combined treatments strengthened the antibiotic potential against biofilms (92). Zeng et al. (93) reported anti-biofilm activity of the traditional Chinese medicine baicalein. Due to the proteolysis of signal receptor TraR protein by baicalein, it was suggested as a QS quencher. Another compound, 14-alpha-lipoyl andrographolide (AL-1), inhibited the *Las* and *Rhl* QS systems by repressing the transcriptional level of QS-regulated genes (94). Both baicalein and AL-1 synergized with the tested antibiotics against *P. aeruginosa* biofilm. Furthermore, fruit extract of *Lagerstroemia speciosa* (LSFE) is

reported as a down-regulator of (QS) genes (*las* and *rhl)* and N-acylhomoserine lactones of *P. aeruginosa* PAO1 (95). Also, LSFE increased the antibiotic potential of tobramycin on PAO1 biofilms. Garlic extract fractionation, ajoene, was determined as a QS inhibitor controlling QS-associated virulence factors of *P. aeruginosa*, such as rhamnolipids (96). In the same study, ajoene, which has been found to possess proteolytic activity (97), synergized with tobramycin to efficiently kill *P. aeruginosa* biofilm and prevent the lytic necrosis of PMN cells. Recently, a compression in vivo study was performed by Christensen et al. (98) between one single treatment (ajoene or horseradish juice extract) and combination treatment (QS inhibitors with tobramycin) of BALB/c mice in which wild-type *P. aeruginosa* were injected into the peritoneal cavity. The antimicrobial combinations treated-mice group showed a significant decrease in the number of biofilm-associated *P. aeruginosa* compared to the groups treated with a single treatment. Overall, QS inhibitors combined with antibiotics could have a great impact on future applications for preventing biofilm formation of pathogens, especially *P. aeruginosa*.

To our knowledge, there are no published reports on whether *G. vaginalis* recruits the quorum sensing system to regulate its biofilm formation and/or virulence factors, such as vaginolysin production. Because it is a fastidious microorganism (99), growing *G. vaginalis* in auto-inducer assay medium made of several salts is challenging. Modified methodology is required to investigate whether QS mechanism are associated with biofilm formation in *G. vaginalis*, especially since the *luxS* gene that is involved in the synthesis of autoinducer-2 is reported (http://www.ebi.ac.uk/QuickGO/GProtein?ac=I4LKI6, accessed on March 01, 2016). QS inhibitors represent one of the effective alternative approaches to inhibit biofilm

formation. These quenchers have been found to possess “high species specificity”, effectively act against certain pathogens, and do not influence the normal growth of other microorganisms (100). Unless they have bactericidal activity, QS inhibitors may not be effective enough for killing pre-formed biofilm-associated pathogens on its own. Therefore, a second antimicrobial agent is required to ensure the bacterial killing process is successful.

ANTIBIOTICS AND ESSENTIAL OILS

Essential oils (EOs) are natural antimicrobial formulations with broad-spectrum activity against bacteria, fungi, and viruses (101). EOs act on cytoplasmic enzymes and membrane proteins by attacking cellular ATP and ATPase. Moreover, EOs disrupt membrane permeability, modify protein motive forces and membrane fatty acids, leading to the leakage of metabolites and ions. Some EOs are QSI by interfering with and regulating QS genes, leading to reduction of biofilm formation and virulence factor production (102). The easiness of EOs extraction, non-toxicity to the tissue culture, quick degradation in water and positive health impacts (103-105) may increase the value of EOs as alternative antimicrobial agents.

Kavanaugh and Ribbeck (106) referred to the high biofilms eradication effect of three EOs: cassia, Peru balsam, and red thyme when compared to ofloxacin and gentamicin against biofilms of *Pseudomonas* and *S. aureus*. Biofilm formation was also inhibited when oregano essential oils, carvacrol and thymol were used against *S. aureus* (107). Nostro et al. (107) found that two to four fold greater than the MIC was sufficient for killing biofilm-associated *S. aureus*. Five of nine biofilms formed by coagulase negative

staphylococci (CoNS) strains were completely eradicated when 5% tea-tree oil (TTO) was used while the same concentration of TTO achieved complete eradication of MSSA and MRSA biofilm growth as microcolonies in glycocalyx during 1h treatment (108). The antimicrobial function of TTO was attributed to the disruption of the hydrophobic phospholipid bilayers of bacterial cell membrane.

Thymol has been found to interfere with adhesion of *G. vaginalis* to human vaginal cells (109). The combination of thymol and eugenol showed synergistic activity, interfering with newly established and matured *G. vaginalis* biofilm and reducing the microbial adhesion to the human vaginal epithelial cells (109). In the in vivo study, a synergistic activity between thymol and eugenol vaginal douche was reported reducing the recurrence rate of BV infection (110). To our knowledge, the nature of EOs efficacy has not been explained yet, but it is possibly associated with the biofilm structure, physiology, and/or the chemical composition of EOs.

Few studies have focused on the antimicrobial combinations of EOs and antibiotics have been performed. EOs modify the tolerance of bacterial cells to antibiotics (111). In this regard, synergistic activity was reported when *P. graveolens* essential oil was used in combination with norfloxacin against two strains of *S. aureus* (112). In the same study, EOs increased the norfloxacin's uptake by bacterial cells. For future applications, this may reduce the antibiotic's side effect(s) in order to obtain a safer treatment. Moreover, the anti-biofilm potential of several EOs including eugenol, cinnamaldehyde, citral and geraniol, has been screened by Jafri et al. (113). The data from Coelho and Pereira (114) study showed synergistic activity of three essential EOs, cinnamon (*Cinnamomum*

zeylanicum), TTO (*Melaleuca alternifolia*) and Palmarosa (*Cymbopogon martini*) when combined with ciprofloxacin against pre-formed biofilm of *P. aeruginosa*. EOs, especially TTO, synergized with ciprofloxacin and exerted a remarkable reduction in biofilm mass and viable biofilm cell numbers. The anti-biofilm action was noticed even when very low concentrations of TTO with ciprofloxacin were used, compared with using them individually. The two antimicrobials in combination targeted more than one component of the bacterial cell (115) including DNA synthesis (116) and the cytoplasmic membrane (117), counteracting the tolerance of *P. aeruginosa.* In agreement with this study, EOs of *Origanum vulgare* L., carvacrol and thymol were identified as putative efflux pump inhibitors facilitating the uptake of antibiotics, norfloxacin, erythromycin and tetracycline (118). More in vitro and in vivo studies are required to verify the safety and efficacy of EOs as "modulating drug resistance" alone or in combination with conventional antibiotics.

ANTIBIOTICS AND NANOPARTICLES

Nanoparticles (NPs) showed an inhibitory effect against planktonic and biofilm cells. This activity is related to ATP-associated metabolism, permeability of the outer membrane and generating hydroxyl radicals, which are induced by bactericidal compounds (119). Silver nanoparticles (Ag NPs) at concentration 100 mg/ml showed anti-biofilm activity by producing 4-log reduction of *P. aeruginosa* (120). Moreover, there was 95% inhibition in biofilm formation by *P. aeruginosa* when Ag NPs was used as an anti-biofilm agent during 24 h of treatment (121). A synergism was noticed when NPs were combined with antibiotics against *S. aureus*, leading to disruption of the

biofilm architecture and modulation of the antibiotic resistance of pathogens. In this regard, data of Chaudhari et al. (122) referred to the inhibition of quorum sensing by Ag NPs and prevention of biofilm formation by *S. aureus*. In the same study, a synergistic anti-biofilm effect was noticed when Ag NPs was combined with chloramphenicol and gentamicin. Antimicrobial activity of Ag NPs was influenced by their net charge and their diffusion through the biofilm (123).

In the same regard, Cihalova et al. (124) showed more MRSA biofilm disruption and inhibition (94%) when antibiotic complexes (ampicillin, oxacillin and penicillin) were combined with selenate NPs (Se NPs) compared to the control groups, which were exposed to tested antibiotics only. A higher expression of *mecA*, the cassette *mec* responsible for staphylococcal resistance in β-lactam antibiotics (125), was identified in MRSA biofilm treated with 50 μM of ampicillin, oxacillin or penicillin. Using mass spectra, the protein profile was changed in MRSA treated with Se NPs alone, comparable to when Se NPs were used together with penicillin. Like Ag NPs, the changing of protein profile by Se NPs may interfere with MRSA pathogenesis through interaction of NPs with bacterial DNA and modifying bacterial pathogenesis (126). Recently, Gurunathan et al. (127) generated new, cost effective Ag NPs prepared by combining silver ions with leaf extract of *Allophylus cobbe*. This NPs showed a higher antibacterial and anti-biofilm activity against both *P. aeruginosa* and *S. aureus* when combined with ampicillin and vancomycin, rather than using NPs or antibiotics alone. The interaction of Ag^{+} with the bacterial cell membrane disrupted membrane permeability, inhibiting their respiratory enzymes and thus production of reactive oxygen species (ROS) (128). It has been suggested that the higher production of ROS, the higher damage of cellular membrane

and more ampicillin and vancomycin uptake in the presence of NPs. In an attempt to improve bactericidal activity of NPs, Habash and his coworkers (129) evaluated different sizes of citrate-capped Ag NPs alone and in combination with aztreonam against *P. aeruginosa*. Habash et al. found that 10 nm capped Ag NPs synergized with aztreonam, efficiently disrupting the biofilm structure of *P. aeroginosa*. Overall, the antimicrobial potential of NP compounds may depend on their sizes, charges and stability in order to enhance antibiotics and control biofilm. However, the safe consumption of NPs must be established before using them in pharmaceutical formulation as antibacterial agents.

CONCLUSION

Finding an effective strategy to control biofilm formation remains a challenge (Table 1). Antibiotic resistance and the recurrence of infections reflect the failure of conventionally used antibiotics for the treatment of biofilm-associated persistent infections. Alternative methods for biofilm prevention and/or eradication are urgently required to modify the traditional treatments. The efficacy of several novel, natural antimicrobial compounds has been identified to efficiently control biofilm formation on biotic and abiotic surfaces. In addition, the anti-biofilm activity of these antimicrobials is more potent when they are combined with conventional antibiotics. Compared to the activity of each one individually, a stronger anti-biofilm activity (synergistic or enhancement) is obtained when traditional antibiotics are used in combination with antimicrobials reviewed here, or when used in the presence of other recently reported compounds such as chitosan (130-135), nitric oxide (141) and Cis-2 (136). The potency of antimicrobial combinations is ultimately determined by the synergy of interacting antimicrobials where each one of

them is acting on different targets (Table 2 and Figure 1). The beneficial properties of this method on the consumer's health make it a promising strategy could be used in industrial and pharmaceutical applications to control pathogenic biofilms.

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Tables and figures

Fig. 1. Complementary approaches for controlling bacterial biofilms.

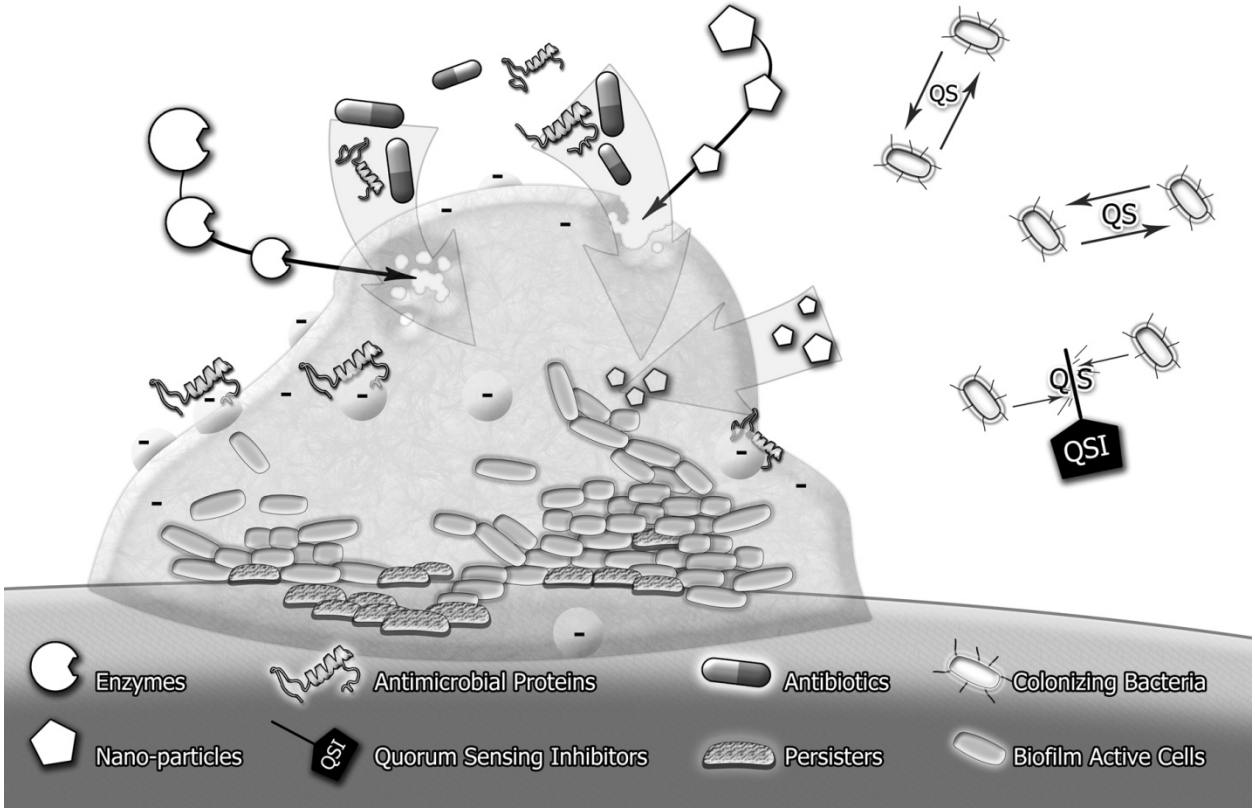

TABLE 1 Mode of action, advantages and limitations of the reviewed anti-biofilm agents.

Antimicrobial compounds	Proposed Mode(s) of Action on Biofilm	Advantage(s)	Limitation(s)
Antimicrobial Peptides	▪ Cationic AMPs interact with anionic biofilm surface (38). ▪ AMPs inactivate the twitching motility of *P. aeruginosa* and inhibit biofilm formation (38). ▪ Disrupt expression of biofilm formation essential genes, down-regulate expression of type IV pili, rhamnolipid, quorum sensing, ands flagella assembly genes (39). ▪ Bind to eDNA and accelerate detachment of the biofilm (42).	▪ Relative selectivity, broad-spectrum activity, cationic and amphipathic properties, disruption of bacterial cell membrane with low frequency and slow emerge of bacterial tolerance (29, 30). ▪ Bactericidal activity against slow growing bacteria within biofilm (31). ▪ Antimicrobial activity improved and cytotoxicity reduced by modifying and hybridization the sequences of primary amino acids (32, 33).	▪ Development of resistance to AMPs via modification of the bacterial lipopolysaccharides or using efflux pumps (44). • Susceptibility of some AMPs to proteolytic enzymes as well as their high cost of purification and sequences (142). ▪ Insufficient selectivity of STAMPs (142). ▪ Possible hemolytic and potential cytotoxic effect (142).
Biofilm Degrading Enzymes	▪ D*egradation* of extracellular matrix components (polysaccharides, protein and eDNA) (67).	▪ Efficient inhibition of biofilm formation, disruption of the EPS production , dispersing the pre-formed biofilm (68, 70). ▪ Reduces the number of biofilm viable cells [70].	▪ High quantities of EPS and proteolytic exoenzymes by mature biofilm counteract the enzymatic activity (68).
Quorum Sensing Inhibitors	▪ Inhibition of cell-to-cell or cell-to-surface attachment. ▪ Inhibition of binding of QS signals to receptor proteins, antagonizing quorum signal biosynthesis, or degrading QS signals (143).	▪ Inhibition of phosphorylation of the target of autoinducer RNAIII-activating protein (TRAP) protein by QSI. TRAP regulates the expression of virulence factors (biofilm formation, essential proteases, toxins), and their regulator, agr (85). ▪ QSIs possess “high species specificity” and effectively act against certain pathogens (100).	▪ Lack of bactericidal activity (91). ▪ Reported toxicity of and resistance to QSI (143).
Essential oils	▪ Attack cellular ATP and ATPase, acting on cytoplasmic enzyme and membrane proteins /fatty acids leading to the leakage of metabolites and ions (102). ▪ Anti-quorum sensing activity by down regulation of QS genes leading to reduce virulence factors production and biofilm formation (102). ▪ Putative efflux pump inhibitors facilitating the uptake of antibiotics (118).	▪ Have a broad-spectrum activity against a wide range of pathogenic microbes (101). ▪ Have been used as ethnomedicine against bacterial infection and cancer for a long time (103, 104). ▪ Simple extraction, non-toxic to the tissue culture, quick degradation in water and positive health impacts (105). ▪ Biofilm inhibition is noticed at the sub-MICs of many-tested EOs, such as *T. vulgaris* EO (113).	• Some EOs produce oxidative stress and possess toxic properties inducing killing activity against eukaryotes (144). • Increased albumin level and skin irritation (see review of Patel [144]).
Nanoparticles	▪ Interference with ATP-associated metabolism, change of the outer membrane’s permeability and generation of hydroxyl radicals (119). ▪ Inhibition of quorum sensing and prevention of biofilm formation (122). ▪ Changing of bacterial protein profile and modifying their pathogenesis by interaction with bacterial DNA (126).	▪ New technique, simple method, cost effective compounds, and delivers strong antimicrobial activity (127). ▪ As antimicrobial carriers (145): • High stability in the biological environment and high carrier capacity. • The possibility to incorporate both hydrophilic and hydrophobic molecules. • Its viability using different courses (oral, parenteral and inhaled) of administration. ▪ Design nanoparticles (NPs) to ensure release of an efficient drug concentration from the matrix (145).	▪ The antimicrobial potential of NPs depends on size, charge, stability and biocompatibility (129). ▪ Cytotoxicity (145).

TABLE 2 In vivo and in vitro studies of natural anti-biofilm agents in combination with antibiotics for combating biofilm-associated pathogens

	Natural anti-biofilm compounds	Antibiotics in combination	Interaction activity	Study type(s)	Biofilm-associated pathogen(s)	Reference(s)
Antimicrobial Peptides	▪ Cathelicidin BMAP-28	Quinupristin/dalfopristin (Q/D), linezolid, vancomycin	AMPs enhance antibiotics activity.	In vivo and in vitro	*S. aureus*	53
	▪ Indolicidin, CAMA [cecropin (1-7)-melittin A (2-9) amide], nisin	Daptomycin, linezolid, teichoplanine, azithromycin, ciprofloxacin	Synergistic effect	In vitro	MRSA *S. aureus*	51, 52
	▪ Citropin 1.1	Rifampin, monocycline	AMPs enhance hydrophobic activity of antibiotics	In vivo and in vitro	*S. aureus*	138
	▪ Colistin	Tobramycin, aminoglycoside	AMPs enhance antibiotics activity	In vivo and in vitro	*P. aeruginosa*	47
	▪ G10KHc (STAMP)	Tobramycin	Synergistic effect	In vitro	*P. aeruginosa*	45
	▪ IB-367	Linezolid	AMPs enhance antibiotics activity	In vivo and in vitro	*S. aureus*	54
	▪ Tachyplesin III	Piperacillin-tazobactam (TZP)	AMPs enhance antibiotics activity	In vivo and in vitro	*P. aeruginosa*	46
	▪ Plus Pal–Lys–Lys–NH2 or Pal– ▪ Lys–Lys-soacked graft	Vancomycin	AMPs enhance antibiotics activity	In vivo and in vitro	*S. aureus*	55
	▪ BMAP-27, BMAP-28	Tobramycin	AMPs enhance antibiotics activity	In vitro	*S. aureus*, *P. aeruginosa*	139
	▪ LL-37	Tobramycin	AMPs enhance antibiotics activity	In vivo	*P. aeruginosa*	140
	▪ LAE, Subtilosin	Clindamycin, metronidazole	Synergistic effect	In vitro	*G. vaginalis*	60
	▪ GL13K	Tobramycin	AMPs enhance antibiotics activity	In vitro	*P. aeruginosa*	48
	▪ LL-37, CAMA: cecropin(1-7)-melittin A(2-9) amide, melittin, defensin, magainin-II)	Ceftazidime, tobramycin, ciprofloxacin, doripenem, piperacillin, colistin	Synergistic effect	In vitro	*P. aeruginosa*	49
Biofilm Degrading Enzymes	▪ Dispersin B	Cefamandole Nafate	Synergistic effect	In vitro	*S. aureus*	77
	▪ DispersinB	Triclosan	Synergistic effect	In vivo and in vitro	*S. aureus*	65
	▪ DNase I	Metronidazole	DNase I enhances antibiotics activity	In vitro	*G. vaginalis*	76
	▪ DNase I, RNase A, proteinase K	Ampicillin, cefotaxime, rifampin, levofloxacin, azithromycin	DNase I enhances antibiotics activity	In vitro	*P. aeruginosa*, *S. aureus*	75
	▪ Proteinase K	Streptomycin, gentamycin, ampicillin	Proteinase K enhances antibiotics activity	In vitro	*S. aureus*	80
	▪ Lysostaphin	Nafcilin	Lysostaphin enhances antibiotics activity	In vivo and in vitro	MRSA *S. aureus*	72
	▪ Lysostaphin	Clarithromycin, levofloxacin, linezolid	Synergistic effect	In vitro	MRSA and MSSA *S. aureus*	79
	▪ Recombinant human DNase I (rhDNase I)	Povidone iodine, chlorhexidine gluconate, benzalkonium chloride	RhDNase I enhances antibiotics activity	In vitro	*S. aureus*	74
	▪ Alginate lyase	Gentamycin, Ceftazidime	Alginate lyase enhances antibiotic activity	In vitro	*P. aeruginosa*	73
Quorum sensing inhibitors	▪ *Lagerstroemia speciosa* extract (LSFE)	Tobramycin	LSFE enhances antibiotic activity	In vitro	*P. aeruginosa*	95
	▪ Lactonase	Ciprofloxacin, gentamicin	Lactonase enhances antibiotic activity	In vitro	*P. aeruginosa*	91
	▪ Baicalein	Ampicillin	Synergistic effect	In vitro	*P. aeruginosa*	93
	▪ 14-alpha-lipoyl andrographolie	Azithromycin, ciprofloxacin, streptomycin, fosfomycin, erythromycin, gentamicin	Synergistic effect	In vitro	*P. aeruginosa*	94
	▪ Phenyl DPD	Gentamicin	QSI enhances antibiotic activity	In vitro	*P. aeruginosa*	90
	▪ Patulin, penicillic acid	Tobramycin	QSIs enhance antibiotic activity	In vitro	*P. aeruginosa*	89
	▪ Ribonucleic-acid-III-inhibiting peptide (RIP)	Cefazolin imipenem teicoplanin, levofloxacin	Synergistic effect	In vitro	MRSA *S. aureus*	85
	▪ Ajoene	Tobramycin	Synergistic effect	In vivo and in vitro	*P. aeruginosa*	96
	▪ Baicalin hydrate, cinnamaldehyde, hamamelitannin	Tobramycin Clindamycin, vancomycin	QSIs enhance antibiotic activity	In vivo and in vitro	*P. auruginosa*, *S. aureus*	92
Essential Oils	▪ Thyme oil, eugenol	Penicillin, ampicillin, cloxacillin, cephalathion, methicillin, novobiocin, vancomycin	EOs enhance antibiotic activity	In vitro	*S. aureus*	113
	▪ TTO (Terpinen-4-ol (T4ol))	Ciprofloxacin	Synergistic effect	In vitro	*P. aeruginosa*	114
	▪ *P. graveolens* essential oil	Norfloxacin	Synergistic effect	In vitro	*S. aureus*	112
Nanoparticles	▪ Silver Nanoparticles (AgNPs)	Chloramphenicol, gentamicin	Synergistic effect	In vitro	*S. aureus*	122
	▪ Selenate NPs	Ampicillin, oxacillin, penicillin	NPs enhance antibiotics activity	In vitro	MRSA *S. aureus*	124
	▪ Green AgNPs	Ampicillin, vancomycin	NPs enhance antibiotics activity	In vitro	*P. aeruginosa* and *S. aureus*	127
	▪ Citrate-capped AgNPs	Aztreonam	Synergistic effect	In vitro	*P. aeruginosa*	129
Other antimicrobial agents	▪ Chitosans	Streptomycin	Chitosan enhances antibiotic activity	In vitro	*S. aureus*	133
	▪ Buffered chitosan sponge	Vancomycin, amikacin	Chitosan enhances antibiotics activity	In vivo	*S. aureus*, *P. aeruginosa*	134
	▪ Antibiotic-loaded chitosan microspheres	Tetracycline	Chitosan enhances antibiotics activity	In vitro	*P. aeruginosa*	135
	▪ Ultrasonic exposure	Gentamicin	Ultrasonication increased transport of gentamicin	In vitro	*P. auruginosa*	137
	▪ Cis-2-decenoic acid	Daptomycin, vancomycin	C2D enhances antibiotic activity	In vitro	MRSA S. aureus	136
	▪ Nitric oxide	Tobramycin	Nitric oxide enhances antibiotic activity	In vitro	*P. auruginosa*	141

Chapter 2: Susceptibility of *Gardnerella vaginalis* biofilms to natural antimicrobials subtilosin, ε-poly-L-lysine and lauramide arginine ethyl ester [2].

In this chapter, three methods were used to evaluate the antibiofilm potential of naturally derived compounds and the antibiotic, clindamycin against planktonic and biofilm cells of BV-associated bacteria. Subtilosin, the bacteriocin and lauramide arginine ethyl ester, the GRAS substance, were more active as compared to ε-poly-L-lysine (another GRAS substance) and clindamycin against biofilms of *G. vaginalis*. Interstingly, subtilosin showed a selective mode of action by inhibiting *G. vaginslis* but not vaginal lactobacilli. These data strengthen the importance of utilizing selective-action antimicrobials in medical applications for treatment of BV infection. In addition, this chapter addressed comparison of plate counts against ATP measurement and resazurine assays to identify most sensitive, accurate and reproducible method for estimation of the number of live cells in biofilms treated with antimicrobials. According to our results, ATP measurement and resazurine assays underestimate the bactericidal effect of stressors on biofilm-associated cells.

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Susceptibility of *Gardnerella vaginalis* biofilms to natural antimicrobials subtilosin, ε-poly-L-lysine and lauramide arginine ethyl ester.

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ABSTRACT

Bacterial vaginosis is a common vaginal infection associated with numerous gynecological and obstetric complications. This condition is characterized by the presence of thick adherent vaginal biofilms, composed mainly of *Gardnerella vaginalis.* This organism is thought to be the primary aetiological cause of the infection paving the way for various opportunists to colonize the niche. Previously, we reported that the natural antimicrobials subtilosin, ε-poly-L-lysine and lauramide arginine ethyl ester selectively inhibit the growth of this pathogen. In this study we used plate counts to evaluate the efficacy of these antimicrobials against established biofilms of *G. vaginalis*. Additionally, we validated and compared two rapid methods (ATP viability and resazurin assays) for the assessment of cell viability in the antimicrobial-treated *G. vaginalis* biofilms. Out of the tested antimicrobials, lauramide arginine ethyl ester had the strongest bactericidal effect, followed by subtilosin, with clindamycin and polylysine showing the weakest effect. In comparison to plate counts, ATP viability and resazurin assays considerably underestimated the bactericidal effect of some antimicrobials. Our results indicate that these assays should be validated for every new application.

INTRODUCTION

Bacterial vaginosis (BV) is the most common vaginal infection in women of childbearing age [1, 2]. This condition is characterized by the replacement of vaginal lactobacilli with a variety of predominantly-anaerobic pathogens, such as *Gardnerella vaginalis*, *Prevotella*, *Peptostreptococcus* and *Bacteroides* spp., with total bacterial numbers often rising 100- to 1000-fold compared to the normal levels in the vagina [3-8]. These changes within the vaginal microbiota are frequently (but not always) accompanied by an elevation in vaginal pH and by an abundance of vaginal secretions that have a typical amine odor [9]. Aside from being a major nuisance due to its symptoms, BV (even in its asymptomatic form) has been associated with serious gynecological and obstetric complications [10-13]. In particular, BV may lead to preterm birth in pregnant women, a major risk factor for perinatal mortality and morbidity [14-16]. BV is a risk factor for the development of post-abortion endometritis and pelvic infection following gynecologic surgery [17, 18]. There is also evidence that BV increases the chance of transmission and acquisition of sexually-transmitted infections, such as a HIV [19, 20] and HSV-2 [21, 22].

Due to the complex polymicrobial nature of this disorder, conventional treatments for BV, with the antibiotics clindamycin and metronidazole, are notorious for their low (60%) efficacy and high (30-40%) rates of recurrent infection [23-27]. The exact aetiology of BV remains unclear despite decades of intense research, making it a challenge to design effective treatment [28]. Since most BV-related species are frequently

isolated from the vaginas of healthy women, many researchers view BV as a microbial imbalance rather than an infection [29-31]. Conversely, there is also evidence that at least some BV-related pathogens can be transmitted sexually [32-33]. Ultimately, most researchers agree that the aetiology of BV is complex and that the outcome of the infection depends not only on the pathogens but also on the indigenous vaginal microflora and the host's immunity [28].

Historically, *G. vaginalis* was thought to be the sole causative agent of this condition [34, 35], however its role in the aetiology of BV was downgraded over the years as the plethora of other bacterial species was gradually linked to the condition [28, 34, 36]. Recent evidence has once again placed *G. vaginalis* in the spotlight. In particular, studies of vaginal biopsy samples revealed that dense adherent biofilms of *G. vaginalis*, in contrast to the sparse cells, were detected only in the vaginas of BV patients and not in healthy women [37]. *In vitro* studies assessing adherence, biofilm formation capabilities, and cytotoxicity among BV-related anaerobes indicated that *G. vaginalis* has the highest virulence potential [38]. Finally, vaginal biofilms composed mainly of *G. vaginalis* were shown to persist following standard antibiotic therapy [39]. Presumably, bacteria within these biofilms serve as a reservoir for the recovery of BV microbiota after the cessation of antibiotic therapy, leading to recurrence of BV [39]. These findings suggest that *G. vaginalis* may have a leading role in the BV infection process, paving the way for various opportunists to colonize the vagina [38].

The less than satisfactory performance of antibiotics is thought to be due to their inability to fully eradicate BV-associated pathogens (partly because of emerged resistance), and to their negative impact on healthy vaginal microbiota [37, 39, 40]. For

this reason, novel antimicrobials, with the ability to selectively target vaginal pathogens, particularly biofilms, are critically needed.

The bacteriocin subtilosin is a promising alternative treatment for BV, especially when used as part of a multiple-hurdle approach, a tactic well-known to drastically hinder microbial resistance mechanisms [41, 42]. Subtilosin (subtilosin A) is a cyclic 34-amino acid peptide produced by a dairy-derived strain, *Bacillus amyloliquefaciens* KATMIRA1933. This peptide was shown to inhibit the growth of BV-associated *G. vaginalis*, *Mobiluncus curtisii* and *Peptostreptococcus anaerobius* [41]. Sutyak et al. [41] reported that natural antimicrobials ε-poly-L-lysine (polylysie) and lauramide arginine ethyl ester (LAE) synergized with subtilosin in inhibiting *G. vaginalis*. Importantly, the subtilosin-based antimicrobial formulations involving polylysine and LAE did not inhibit the growth of vaginal lactobacilli strains [41]. Polylysine is cationic polypeptide consisting of 25-35 L-lysine residues. Numerous *in vivo* studies indicated that this antimicrobial is safe for human consumption and it is currently on the commercial market in Japan as a food preservative [43-45]. LAE is a derivative of lauric acid, L-arginine, and ethanol [46] with the generally recognized as safe (GRAS) status for use in meat, poultry, and other food products (GRAS Notice No. GRN 000164). To this point, only the inhibitory activity of subtilosin, polylysine and LAE have been evaluated against BV-related pathogens. Prevention of pathogenic growth is a model reflective of prophylaxis but not necessarily of treatment of BV, since this condition is characterized by the presence of already-established pathogenic vaginal biofilms [37]. Due to protection of exopolysacharide matrix and other factors, biofilm cells are generally more resistant to stresses than their planktonic counterparts [47, 48]. Therefore concentrations of

antimicrobials that are effective against biofilms are expected to be higher than the concentrations effective against planktonic cells.

This study assessed bactericidal properties of subtilosin, polylysine, and LAE against established *G. vaginalis* biofilms in comparison to clindamycin. The activity of each antimicrobial was evaluated by three different methods (plate counting, ATP viability, and resazurin assays) to determine the advantages and limitations of each method when used to study *G. vaginalis* biofilms.

MATERIALS AND METHODS

Media, strains, and growth conditions

G. vaginalis ATCC 14018 was stored at -80°C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) and with 15% glycerol added to the total volume. The cells were propagated anaerobically at 37 °C in BHI with 3% horse serum. For experimental procedures, *G. vaginalis* was sub-cultured at least once in BHI broth supplemented with 1 % glucose (BHIG). Media used for all procedures were pre-incubated overnight at 37 °C in an anaerobic environment to minimize any stress to the cells (i.e. oxygen, low temperature).

Frozen stocks of *Lactobacillus vaginalis* ATCC 49540, *Lactobacillus gasseri* ATCC 33323, and *Lactobacillus plantarum* ATCC 39268 were stored at −80 °C in MRS broth containing 15 % glycerol (v/v). The cells were propagated in DeMan, Rogosa and Sharpe (MRS) broth at 37 °C under aerobic conditions and were subcultured at least twice prior to being used in the experiments.

Preparations of antimicrobials

The antimicrobials used were subtilosin, ε-poly-L-lysine (polylysine), lauramide arginine ethyl ester (LAE) and clindamycin. Subtilosin was produced through fermentation of *B. amyloliquefaciens* KATMIRA1933 and purified as described previously [42]. The aqueous stock solution of subtilosin contained 2.65 mg/mL protein as determined by Micro BCA^{TM} Protein Assay Kit (Thermo Scientific, Rockford, IL) and produced a single band on a silver stained SDS-PAGE gel indicating its purity. Polylysine (250 mg/mL) and LAE (100 mg/mL, MIRENAT-CF) were gifts from Chisso America, Inc. (Rye, NY) and Vedeqsa Corp. (Barcelona, Spain), respectively; clindamycin phosphate was purchased from TCI America (Portland, OR). The aqueous solutions of all the antimicrobials were filter-sterilized through 0.2 μm syringe filters (NALGENE, Rochester, NY) prior to use. The antimicrobials were then serially diluted with BHIG broth to attain the desirable concentrations.

Minimial Inhibitory Concentrations

Minimal Inhibitory Concentrations (MICs) of the tested antimicrobials were determined using the assay described by Sutyak Noll et al. [41] with minor modifications. Briefly, serial 0.67-fold dilutions of each antimicrobial were prepared in BHIG broth using a 96-well microplate (BD, Franklin Lakes, NJ). The overnight culture of *G. vaginalis* was added to each well of the plate at 1% of the total volume (200 µl). The plate was incubated anaerobically at 37 °C for 24 hours. Lactobacilli plates were incubated at 37 °C for 24 hours under aerobic conditions. Bacterial growth was evaluated following the incubation period by taking an endpoint reading at OD_{595} with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA).

Growth of biofilms

Unless stated otherwise, all biofilm-related procedures were conducted at 37 °C within the anaerobic glove box (Coy Laboratory Products Inc., Grass Lake, MI) which was supplied with a gas mixture containing 10% hydrogen, 5 % carbon dioxide and 85% nitrogen. To grow biofilms, BHIG broth was inoculated with an overnight culture (1%) and dispensed into a 96-well microplate (200 μL in each well). Transparent MICROTEST tissue culture plates with flat bottoms (BD) were used to grow biofilms for all the experiments involving antimicrobials. Biofilms were grown in opaque tissue culture plates (BD) to compare the ATP content of intact and disrupted biofilms. The plates were incubated for either 25 or 50 hours (depending on the experimental objective) with the growth medium being replaced every 25 hours.

The activity of the antimicrobials was evaluated using 25-hour biofilms. The supernatant covering the biofilms was removed using a micropipette, and each well of the plate was gently washed with 200 μL of BHIG broth. Then 200 μL of BHIG broth containing the selected antimicrobial was dispensed over each biofilm. After 25 hours of incubation the antimicrobial-containing medium was removed with a micropipette. Each well was gently washed with 200 μL of BHIG broth, and 200 μL of BHIG broth was dispensed over each biofilm. The cell viability of each biofilm was then quantified using the following three methods.

Plate counting

Biofilms were first disrupted by vigorous pipetting. The cell suspension was then serially diluted using BHIG broth and 10 μL of each dilution was plated in four replicates (40 μL

in total) on BHI agar plates using the drop plate method described by Hoben and Somasegaran [49] and Herigstad et al. [50]. Colonies on the plates were counted after 72 hours of incubation under anaerobic conditions at 37 °C.

ATP viability assay

The ATP viability assay was conducted using the method described by Patterson et al. [48] with minor modifications. Briefly, biofilms were turned into cell suspensions by vigorous pipetting. Each suspension was diluted ten-fold with BHIG broth and 270 μL of the dilution was transferred into a well of a white opaque tissue culture plate (BD). The plate was centrifuged for five minutes (1238 *g*, 22 °C) and the liquid in each well was carefully removed with a micropipette. The wells were then gently washed with 200 μL of PBS buffer. After this procedure, 50 μL of BacTiter-Glo (Promega, Madison, WI) reagent was dispensed over the cells in each well. Following five minutes of incubation at ambient temperature, luminescence readings (integration time 500 ms) were taken using Luminoscan Ascent (Thermo Scientific, Barrington, IL). Aside from experimental samples, each plate contained standards which were used to construct standard curves relating the measurements of luminescence to viable cell count (VCC). These standards were prepared by vigorously pipetting untreated biofilms and by making serial dilutions of the cell suspension.

Resazurin assay

The assay was conducted using methods described by Extremina et al. [51] and Pettit et al. [52] with some modifications. Biofilms were disrupted by pipetting, and 24 μL of 2 μM resazurin solution was added to 170 μL of the cell suspension. The change in

absorbance at OD_{595} was monitored every 120 seconds using a Bio-Rad microplate reader, Model 500 (Bio-Rad, Hercules, CA). Kinetic curves were generated with the Microplate Manager 5.2.1 software (Bio-Rad). This software was also used to determine the inverse slope (change in OD_{595} over time, AU/sec) of each curve during a 600 second incubation interval. The inverse slope is predicted to be proportional to the number of viable cells because it corresponds to the rate of resazurin reduction.

Microscopy

For microscopic imaging, cells were grown in Lab-Tek II Chambered Coverglass System (NUNC™, Rochester, NY) for 25 hours. Biofilms were handled as described above, and were then stained with LIVE/DEAD® BacLight Bacterial Viability Kit for microscopy (Molecular Probes, Eugene, OR) by following the manufacturer's instructions. The imaging was performed with LSM 710 Confocal Microscope (Carl Zeiss, New York, NY) under 1000x magnification using 488 nm laser and two detection channels with spectra ranging between 493-526 nm and 598-633 nm, respectively.

Data analysis and statistics

All experiments were conducted at least three times in duplicate. The standard deviation is represented in the figures by error bars. The efficacy of the antimicrobials (Figure 2) was evaluated using cumulative data from three independent experiments. The cell viability of each biofilm was assessed simultaneously by the three methods. The methods were compared within a single experimental set (Figure 4). Unless stated otherwise, calculations were carried out in Microsoft Excel, and the results were graphed using

SigmaPlot 11.0 (Systat Software Inc., Chicago, IL). Statistical analysis was performed with SigmaPlot 11.0 (Systat Software Inc.) using the Student's *t*-test ($P \leq 0.01$).

RESULTS AND DISCUSSION:

General description of *G. vaginalis* biofilm

G. vaginalis formed confluent multi-layered biofilms on both polystyrene and glass surfaces. Microscopic examination of the single-layered region near the edge of the slide revealed cells densely packed within an exopolysaccharide matrix (Fig 1). As expected, exposure to oxygen and ambient temperatures had a detrimental effect on *G. vaginalis* [53]. Mature (25-hour) *G. vaginalis* biofilms that were washed and plated on the bench as described by Patterson et al. [48] (under aerobic conditions at ambient temperatures) had 100-fold fewer viable cells compared to the biofilms handled in the anaerobic chamber at 37 °C; in both cases agar plates were incubated anaerobically. Therefore, to minimize stress to the cells, all procedures, except for the luminescence measurements, were conducted in the anaerobic chamber at 37 °C.

Bactericidal effect of four antimicrobials on biofilms of *G. vaginalis*

The minimal inhibitory concentrations of the antimicrobials in our system were similar to those reported for *G. vaginalis* in the literature (Table 1). The discrepancies between the previously reported MIC values and the ones we measured can be attributed to differences in bacterial growth media and other conditions of the assay. For each antimicrobial, three concentrations covering the 100-fold range were tested against *G. vaginalis* biofilms. The comparison between the antimicrobials was made at 10x the MIC concentration reported in the literature.

After 25 hours of incubation the VCC in *G. vaginalis* biofilms reached 10^8 CFU/cm^2. The VCC did not change following the additional 25 hours of incubation in BHIG broth (the duration of antimicrobial exposure) (Fig 2). Therefore, any decrease in VCC following exposure of the biofilm to the antimicrobials signifies cell death.

Clindamycin and polylysine produced only up to a 2-log reduction in the VCC of *G. vaginalis* (Fig 3b and 3c, respectively). The effect of clindamycin remained constant within the tested range (16-1600 µg/ml) suggesting that it reached its threshold of activity. Only the highest concentration of polylysine (2500 µg/ml) reduced the VCC by more than 1 log. The antimicrobial activity of polylysine is related to its electrostatic adsorption to a cell's surface causing cell clumping and ultimately the cessation of protein synthesis [54]. Similarly, clindamycin is a protein synthesis inhibitor [55]. The cellular functions affected by these two antimicrobials may not be essential for survival of established biofilms in the absence of other stressors.

In contrast to clindamycin and polylysine, LAE reduced the VCC in biofilms of *G. vaginalis* by up to 5 logs with a clear dose response within the tested range (10-1000 µg/ml) (Fig 3d). It is likely that the effectiveness of LAE against biofilms of *G. vaginalis* is at least partly related to the detergent properties of this compound [46]. Dose response within the tested range (1-100 µg/ml) was also observed for subtilosin with about 3-log reduction in VCC at concentration of 10x MIC (Fig 3a). Although both subtilosin and LAE target bacterial cytoplasmic membranes, these two antimicrobials have different molecular mechanisms of action [46, 56]. When compared at 10x MIC, subtilosin was less effective in reducing the number of viable biofilm cells than LAE but more effective than clindamycin and polylysine.

Most investigators agree that effective treatment for BV should selectively target BV-related pathogens, while allowing healthy vaginal microbiota to proliferate and recover. Swidsinski et al. [37] reported that in vivo vaginal lactobacilli do not form confluent biofilms; instead, they are sparsely distributed on vaginal epithelium. Therefore, in vitro studies involving lactobacilli biofilms may not be reflective of the situation in vivo. For these reasons, in our preliminary investigation we evaluated safety of the selected antimicrobials against commonly isolated vaginal *Lactobacillius* spp. (*L. vaginalis*, *L. gasseri*, and *L. plantarum*) by determining the MIC values.

The MICs of clindamycin greatly varied between the lactobacilli species, ranging from 0.78 to >50 μg/mL (Table 2). Earlier reports also suggested that clindamycin (much like metronidazole) can be harmful to healthy vaginal microflora [40]. In contrast, subtilosin was not inhibitory to any of the selected *Lactobacillus* spp. even at the highest tested concentration (100 μg/mL).

MICs of polylysine and LAE varied greatly among the tested species. Generally, concentrations of these two antimicrobials that were modestly effective against biofilms of *G. vaginalis* when used alone were also inhibitory to vaginal lactobacilli. Therefore, high concentrations of LAE and polylysine may influence vaginal microbial balance restoration in women affected by BV. However, LAE and polylysine may be used in lower concentrations in combination with synergistically acting agents such as subtilosin.

Previously, subtilosin was shown to have synergistic interactions with polylysine and LAE in inhibiting the growth of *G. vaginalis* [41]. Due to the major differences in the mode of action of these substances [46, 54, 56], subtilosin, LAE and polylysine are also expected to work synergistically against the biofilms of *G. vaginalis* when used in

combination with each other and, perhaps, with conventional antibiotics. Our future work will focus on the combinatorial effect of these substances on biofilms. However, it is technically challenging to test large number of samples using plate counting. Therefore, we evaluated and compared two simpler methods for the enumeration of viable *G. vaginalis* cells in antimicrobial-treated biofilms.

Comparison of ATP viability and resazurin assays to plate counting

Patterson et al. [48] reported the use of an ATP viability assay to study biofilms of *G. vaginalis*. However, to the best of our knowledge the assay has not been validated, for this specific microorganism, against other methods. The assay is rapid and convenient, and it relies on the assumption that the ATP content of a bacterial population is proportional to the number of viable cells [57]. This assumption is generally true for an exponentially-growing bacterial population. However, it is well-known that antimicrobials may have very diverse effects on the ATP content of their target cells [28, 56, and 58]. Additionally, due to a unique cell wall structure, *G. vaginalis* is notorious for being difficult to lyse. Therefore it is possible that the lysing component of the assay kit cannot be effectively used to extract ATP from the cells. Cell-lysis may be further hindered by the biofilm matrix.

Initially, we used the BacTiter-Glo assay kit to compare the ATP content of intact biofilms with a cell suspension derived from the same biofilms by vigorous pipetting. The estimates of viable cells in intact biofilms and in the derived cell suspension were comparable (data not shown), suggesting that the biofilm matrix does not interfere with the assay. Furthermore, serial dilutions of the biofilm cell suspension had ATP contents proportional to their viable cell counts with a linear range between 10^3-10^8 CFU/mL (Fig

4a). In contrast, the linear range for resazurin reduction (Fig 4b) was rather narrow (between 10^7-10^8 CFU/mL). To the best of our knowledge, the use of resazurin assay with *G. vaginalis* has not been reported.

The ATP viability and resazurin assays generally revealed the same trend as plate counting for the activities of the antimicrobials. However, the actual log reduction estimated by these two methods differed from the numbers obtained by plate counting (Fig 5). One major discrepancy between the methods is the 100-fold reduction in the VCC caused by clindamycin which was not revealed by the ATP viability assay (Fig 5b). Clindamycin inhibits protein synthesis [55]. A plausible explanation for the discrepancy is that clindamycin's activity does not necessarily affect the cellular ATP content.

The effect of subtilosin was also severely underestimated by the ATP viability assay (Fig 5a). This underestimate is probably related to the fact that subtilosin (at its MIC) induces only a mild efflux of ATP (<25%) from cells of *G. vaginalis* and does not induce intracellular hydrolysis of ATP [56]. Something very similar might be true for other antimicrobials; i.e. the antimicrobials may kill their target cells without depleting their ATP, thus giving false negative results in the ATP viability assay and possibly also in the resazurin assay.

It is also important to remember that although plate counting is a well-accepted method for enumerating viable cells, it has certain limitations, especially when used on antimicrobial-treated biofilms. This method is based on the assumption that each viable cell gives rise to a single colony, which may not be true due to cell clumping. Cells derived from biofilms treated with antimicrobials may clump differently than those in

untreated biofilms. Additionally, cells injured by antimicrobials might be viable but not culturable (VBNC), which would result in an underestimate using plate counts [59].

Ultimately, the information collected by all three methods complement each other. Both ATP viability and resazurin assays are simple and rapid methods. However, the estimates of viable cells provided by these methods can be significantly different from plate counts. We recommend validating these methods for every new application. Nonetheless, both methods may still be useful for a quick, conservative (compared to plate counting) assessment of antimicrobial activity, especially when numerous samples have to be evaluated at once.

CONCLUSION:

Plate counts revealed that at 10x MIC, LAE had the strongest bactericidal effect on biofilms of *G. vaginalis*. Subtilosin was slightly less effective, while polylysine and clindamycin induced only a mild reduction in the VCC. Compared to plate counts, ATP viability and resazurine assays can considerably underestimate bactericidal effect of certain antimicrobials against *G. vaginalis*. Therefore, these assays must be validated for every new application.

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FIGURE LEGENDS

Table 1. Minimal inhibitory concentrations of the antimicrobials tested against *G. vaginalis*

Table 2. Minimal inhibitory concentrations of the antimicrobials tested against commonly isolated vaginal *Lactobacillus* spp.

Figure 1. The 24-hour biofilm of *G. vaginalis* on a glass surface.

Figure 2. The viable cell counts in 25 and 50-hour biofilms of *G. vaginalis*.

Figure 3. Bactericidal effects of the antimicrobials subtilosin (a), clindamycin (b), polylysine (c) and LAE (d) against *G. vaginalis* biofilms as assessed by plate counting. Bars in each figure represent cumulative data from three independent experiments conducted in duplicate. Data sets that are statistically different from controls ($P \leq 0.01$) are designated with asterisks (*).

Figure 4. Standard curves relating measurements obtained by ATP viability (a) and resazurine (b) assays to the number of viable biofilm cells. The linear range is between 10^3-10^8 CFU/ml for the ATP viability assay (a) and between 10^7-10^8 CFU/ml for the resazurine assay (b).

Figure 5. Viability of *G. vaginalis* biofilm cells assessed by ATP viability and resazurin assays in comparison to plate counts. ATP viability (open circle) and resazurin assays (closed circle) reveal the same trend as plate counting (closed reverse triangle) for the activities of subtilosin (a), polylysine (c) and LAE (d) but not clindamycin (b). The actual

log reduction estimated by these two assays was considerably different from that obtained by plate counting.

TABLES AND FIGURE

Table 1

Antimicrobial	MIC (*μ*g/ml)
Subtilosin	7.2[1] /9.2[2] [41]
ε-poly-L-lysine	33[1] /25[2] Sutyak et al [41]
lauramide arginine ethyl ester	13.3[1] /10[2] Sutyak et al [41]
Clindamycin	1.9[1]/16 [3, 4]

[1] MIC in our study, 2 MIC in Sutyak et al. [41] study, 3 MIC in Catlin [34] and Martens [60] studies

Table 2

Lactobacilli spp.	Antimicrobial agent (μg/mL)			
	Subtilosin	Clindamycin	Polylysine	LAE
L. vaginalis	>100	0.78	55.8	15.63
L. gasseri	>100	>50	111.6	31.25
L. plantarum	>100	25	1786	62.5

Figure 1

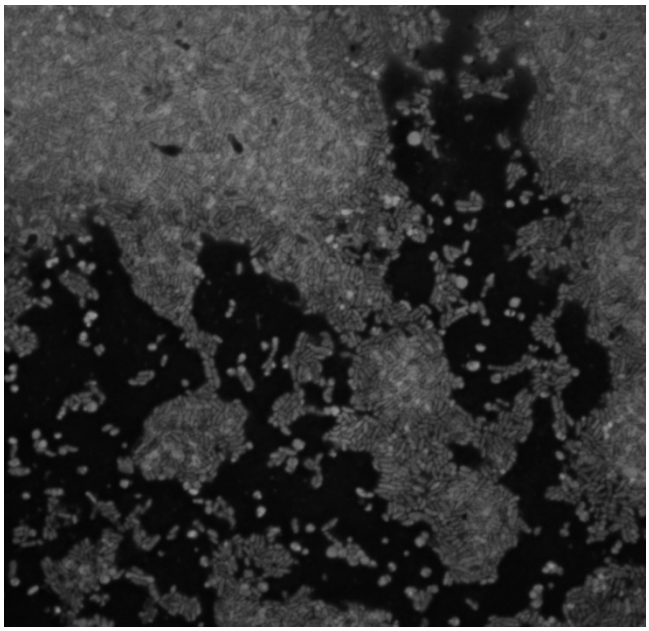


Figure 2

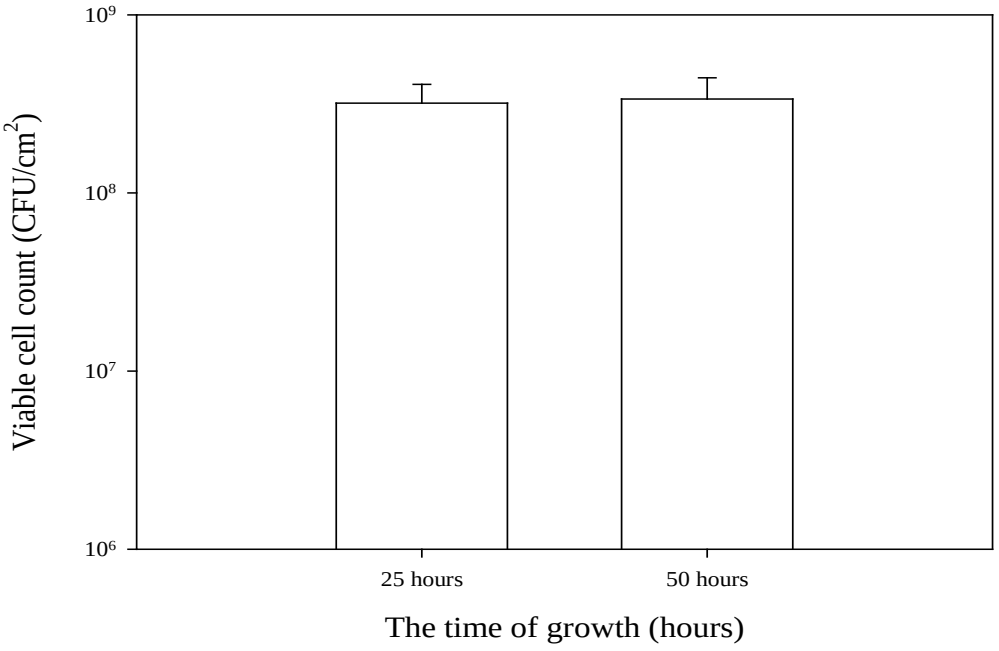
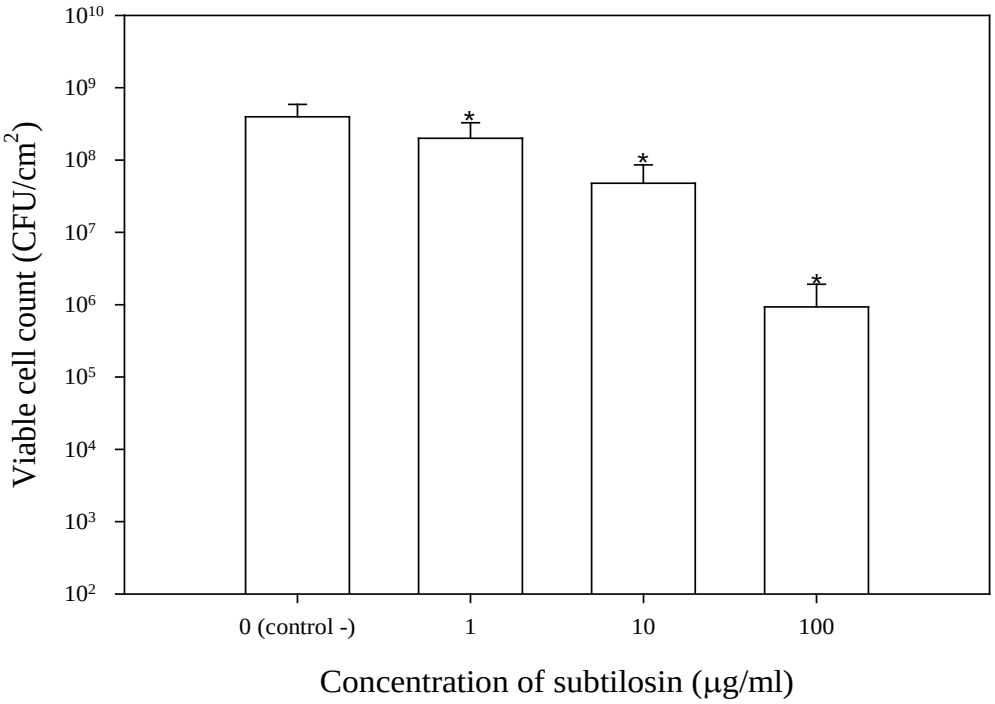
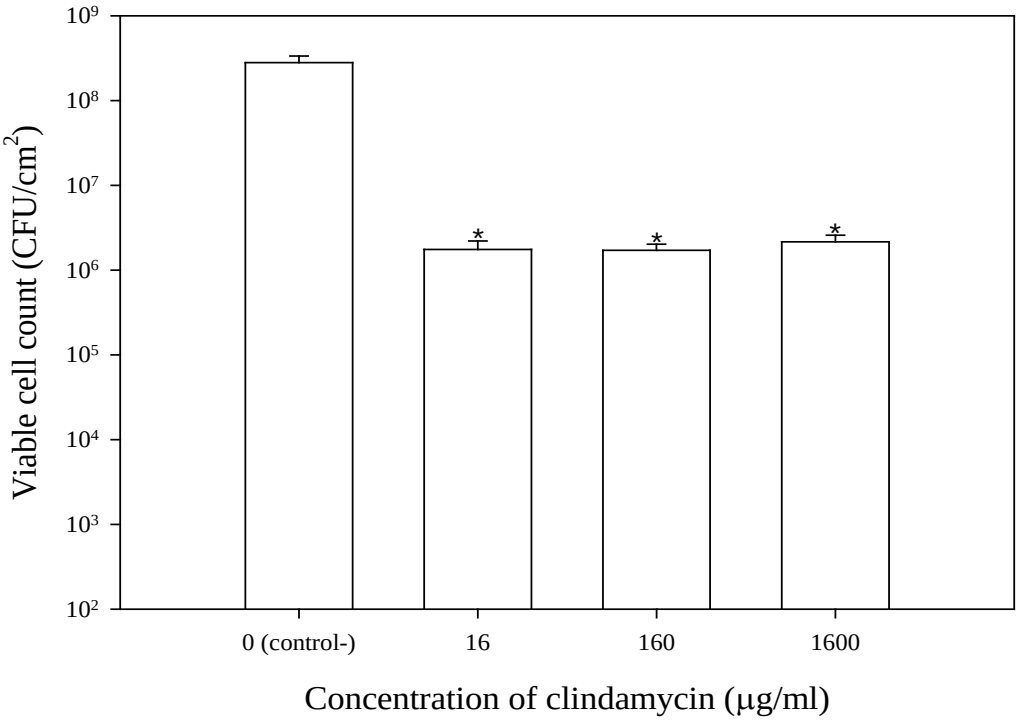


Figure 3

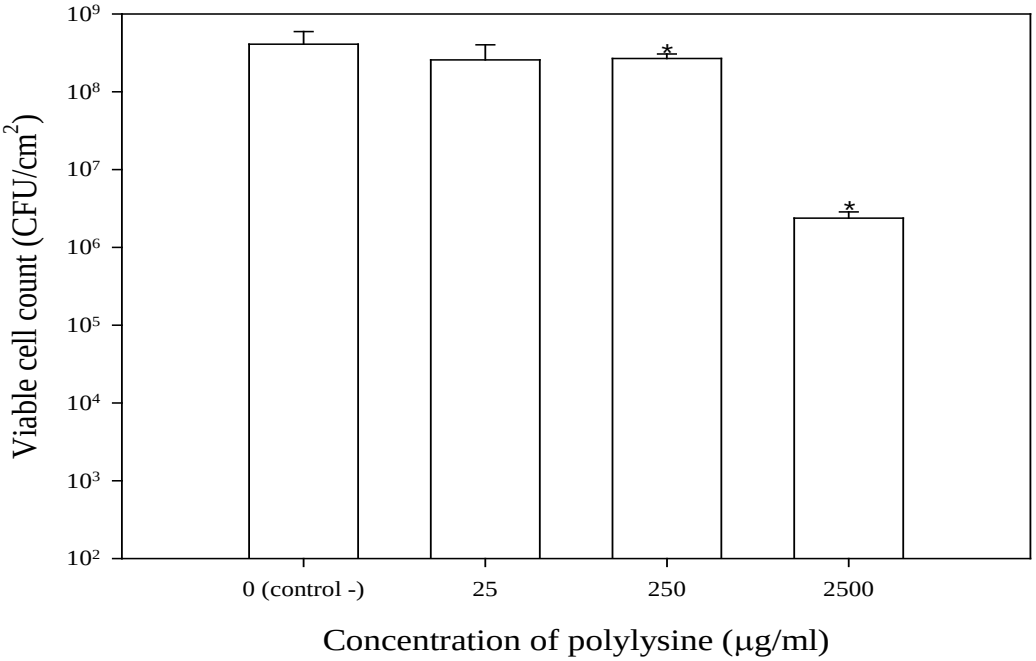
a.



b.


Viable cell count (CFU/cm2)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
*
*
*
0 (control-)
16
160
1600
Concentration of clindamycin (µg/ml)

c.


Viable cell count (CFU/cm2)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
*
*
0 (control -)
25
250
2500
Concentration of polylysine (µg/ml)

d.

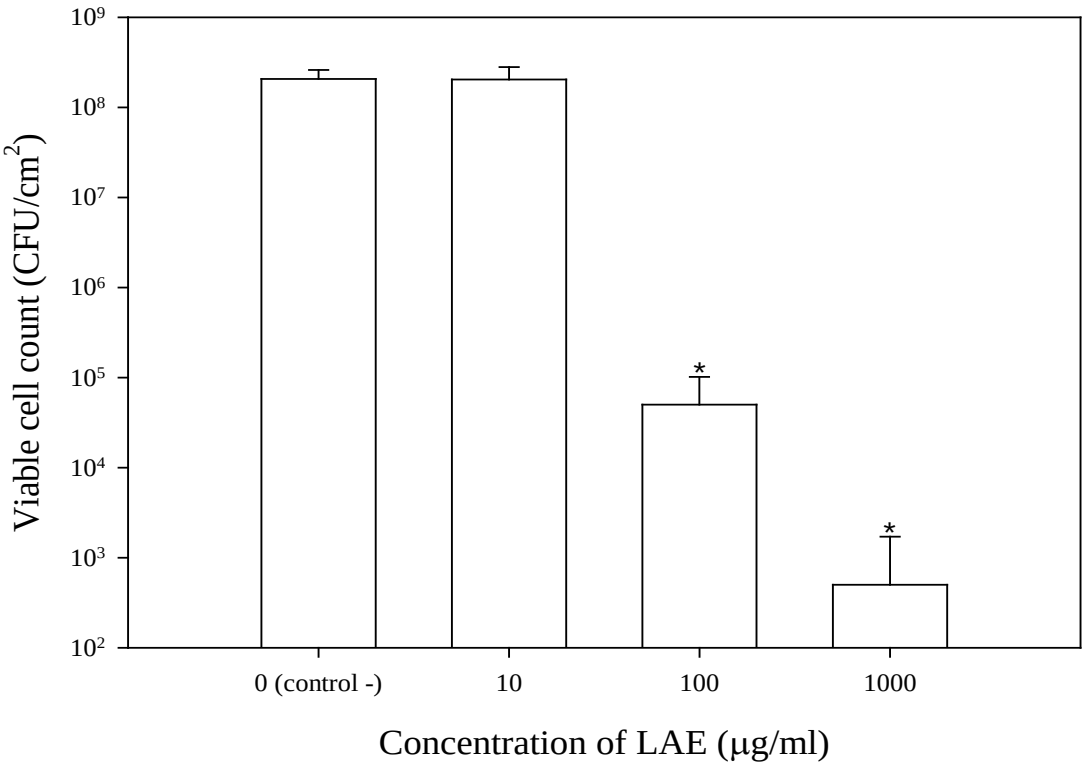
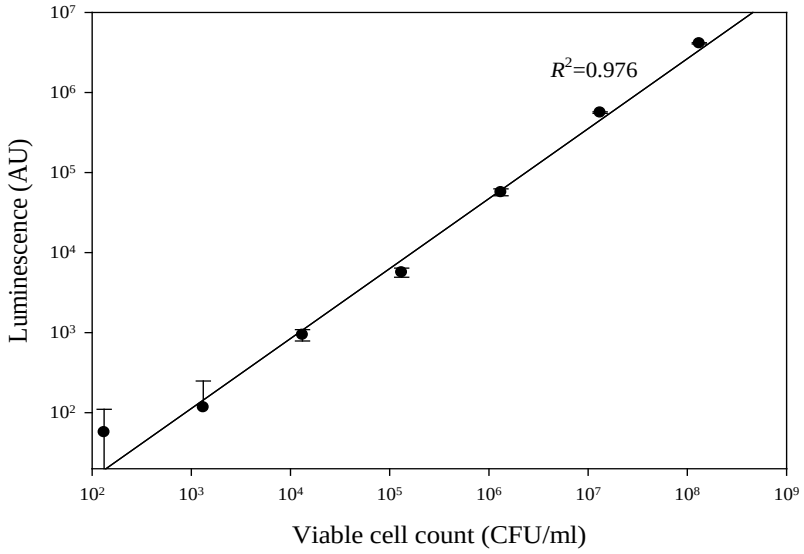

Viable cell count (CFU/cm2)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
*
*
0 (control -)
10
100
1000
Concentration of LAE (μg/ml)

Figure 4

a.


Luminescence (AU)
10⁷
10⁶
10⁵
10⁴
10³
10²
R²=0.976
10²
10³
10⁴
10⁵
10⁶
10⁷
10⁸
10⁹
Viable cell count (CFU/ml)

b.

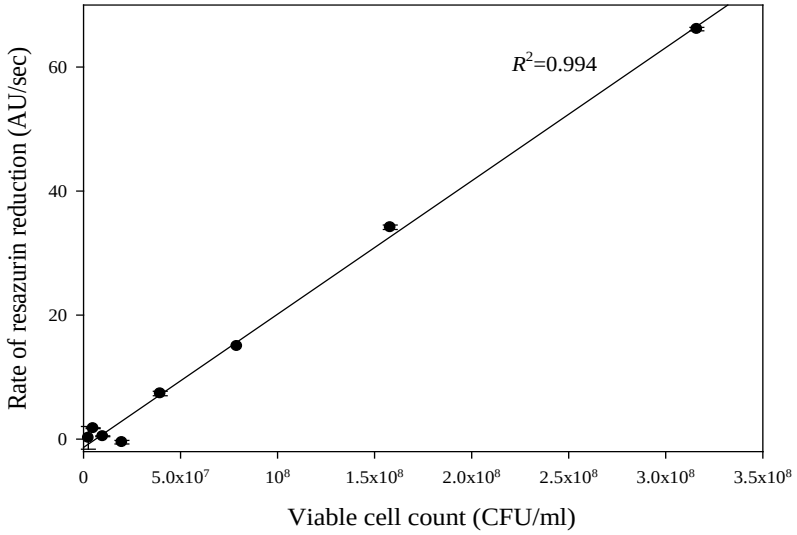
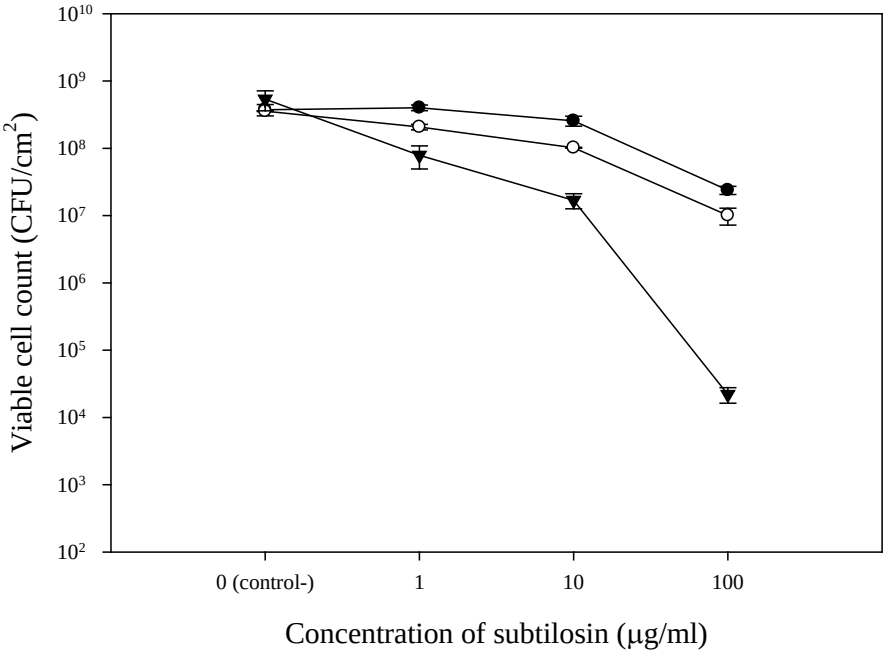
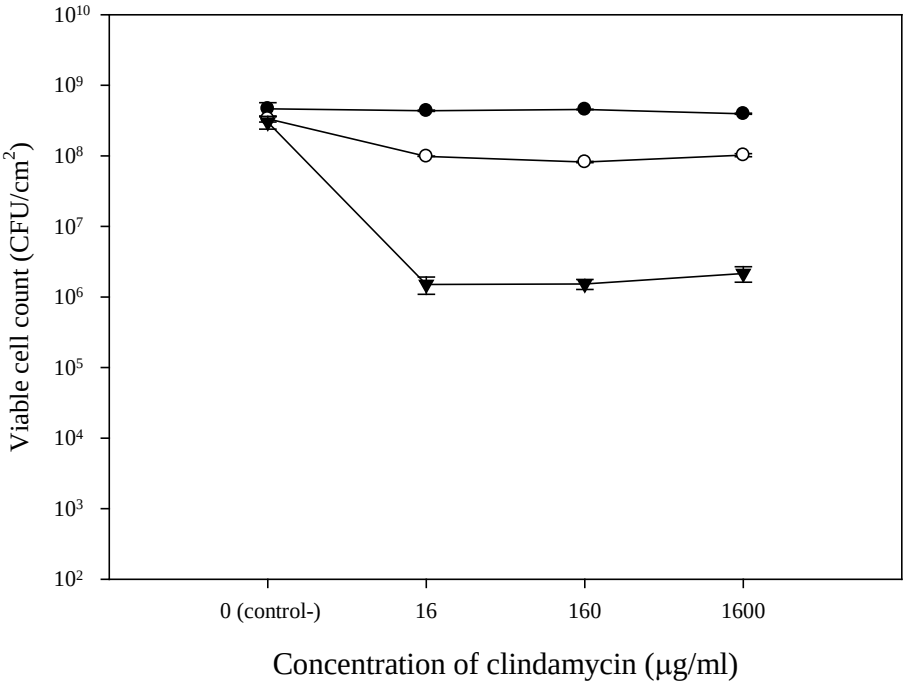

R^2=0.994
Rate of resazurin reduction (AU/sec)
60
40
20
0
0
5.0x10^7
10^8
1.5x10^8
2.0x10^8
2.5x10^8
3.0x10^8
3.5x10^8
Viable cell count (CFU/ml)

Figure 5

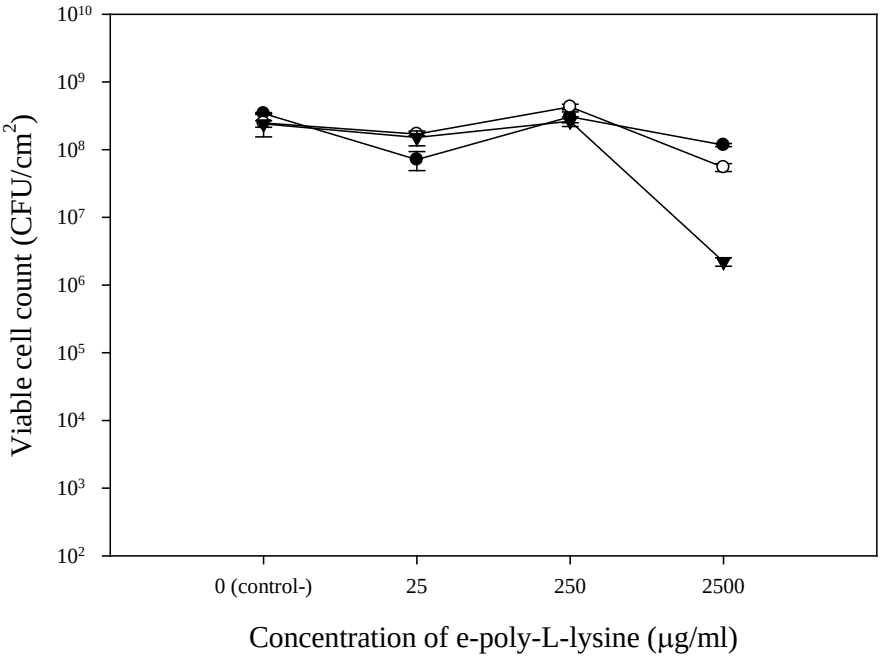
a.


Viable cell count (CFU/cm^2)
10^{10}
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0 (control-)
1
10
100
Concentration of subtilosin (μg/ml)

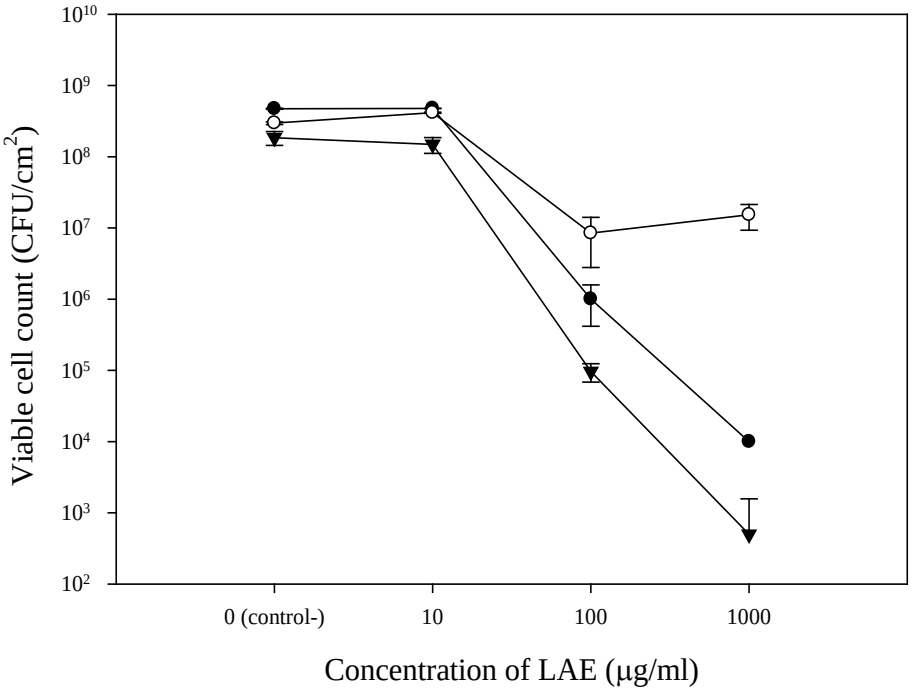
b.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
Viable cell count (CFU/cm^2)
0 (control-)
16
160
1600
Concentration of clindamycin (μg/ml)

c.


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
Viable cell count (CFU/cm^2)
0 (control-)
25
250
2500
Concentration of e-poly-L-lysine (μg/ml)

d.


Viable cell count (CFU/cm2)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0 (control-)
10
100
1000
Concentration of LAE (μg/ml)

Chapter 3: Natural antimicrobials subtilosin and lauramide arginine ethyl ester (LAE) synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli [3]

As described in Chapter 2, subtilosin and LAE were more effective antimicrobials against biofilm-associated *G. vaginalis* but not against healthy vaginal lactobacilli. This finding may justify the use of selectively-acting compounds in medical and personal care products. In this chapter, we found that subtilosin and LAE synergized with conventional antibiotics, metronidazole and clindamycin against biofilms of *G. vaginalis* but not against the biofilms of vaginal lactobacilli. In addition, all tested antimicrobial combinations were inhibitory for *Mobiluncus curtisii* and *Peptostreptococcus anaerobius*, the BV- associated anaerobic pathogens. This study's selected results were presented at the 5th International Symposium "Autotroph Microorganisms" (12.21-24.2015, Moscow State University, Moscow, Russia) dedicated to the 90th anniversary of academician E.N. Kondratieva (Russian Academy of Sciences), Maks Press, Moscow, 192 pages, ISBN 978-5-317-05141-9 and were published in the book of the symposium's abstracts **Algburi A., Volski A. and Chikindas M.L.** 2015. "Natural antimicrobial subtilosin A from probiotic strain *Bacillus subtilis* KATMIRA1933 synergizes with antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis*, one of the causative agents of bacterial vaginosis." Page 96. Published in: Proceedings of the 5th International Symposium.

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

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Running title: Synergistic antimicrobials against vaginal biofilms

Abstract

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

Key words: Natural antimicrobials, *Gardnerella vaginalis*, biofilm, antimicrobial synergy

Introduction

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

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

The loss of vaginal lactobacilli protection, elevation of vaginal pH above 4.5, and increasing number of anaerobic pathogens are the most common characteristic features of BV (Syed & Braverman, 2001). Nugent *et al*. (1991) proposed a scoring system for the diagnosis of BV by analyzing the bacterioscopy of a vaginal smear. On a scale from 0 to 10, the score of bacterial vaginosis is equal to seven points or higher. While the symptoms can be mild, BV is in fact health and life threatening when it persists, causing gynecological and obstetrical complications (Scott & Smyth, 1987; Graham *et al*., 2009).

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

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

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

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

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

Materials and methods

Bacterial strains, culture media and growth conditions

From the frozen stock (-80^{o}C), *G. vaginalis* ATCC 14018 was inoculated in three culture media and propagated at 37^{o}C for 48 h. Brain-Heart Infusion broth (BHI) (Difco BD, Franklin, NJ, USA) supplemented with horse serum 3% (JRH Biosciences, KS) was used to maintain microbial growth. Human Blood Tissue (HBT) agar (Remel, Lenexa, KS, USA) was used to confirm the purity of the frozen stock. The inoculated broth and HBT agar were incubated anaerobically (10% hydrogen, 5% carbon dioxide, and 85%

nitrogen) using an anaerobic gloves box (Coy Laboratory Products, Inc., Grass Lake, MI, USA). After the incubation period, the bacterial cells were transferred to BHI broth supplemented with 1% glucose (Fisher Scientific, Waltham, MA, USA) (BHIG), every 24 h/ twice prior to the initiation of an experiment. In order to provide suitable conditions for *G. vaginalis* anaerobic growth and to avoid oxidative stress, culture media were pre-incubated in the anaerobic chamber at least overnight before bacterial inoculation.

Five species of representative vaginal *Lactobacillus* were used in this project to evaluate the possible effect of antimicrobial combinations. *L. vaginalis* ATCC 49540, *L. plantarum* ATCC 39268, *L. acidophilus* ATCC 4356, *L. rhamnosus* 160 (gift of Dr. Aroutcheva, Rush University Medical Center) and *L. gasseri* ATCC 33323 were taken from the frozen stock and suspended into DeMan, Rogosa and Sharpe broth (MRS Difco BD, Franklin Lakes, NJ, USA). After overnight incubation under anaerobic conditions at 37^{o}C, lactobacilli were re-inoculated twice, every 24 h, into a fresh medium before starting the experiment. For lactobacilli biofilm formation, MRS broth supplemented with 1% glucose and 2% sucrose (Fisher Scientific, Waltham, MA, USA) was used.

The antimicrobial interactions were also evaluated against *Peptostreptococcus anaerobius* ATCC 27337, *Mobiluncus curtisii* ATCC 35241 and *Prevotella bivia* ATCC 29303, the predominant anaerobes that have been identified in vaginal samples taken from BV-infected women. The anaerobes were maintained in the anaerobic chamber and transferred daily using BHI supplemented with 3% horse serum (BHIH).

Stock solutions of antibacterial agents

Four antimicrobials were evaluated for their activity against biofilm-associated *G. vaginalis*. Lauramide arginine ethyl ester (LAE) (Vedeqsa, Inc, L- Lamirsa, LAE-CF) was a gift from Vedeqsa, Inc (Barcelona, Spain). Clindamycin phosphate and metronidazole were purchased from TCI America (Portland, OR, USA). Subtilosin was produced as previously described by Sutyak Noll *et al*.(2008) and stored as a stock solution containing 5.56 ± 0.23 mg mL^{-1} of the protein. The stock solution of LAE was 10 mg mL^{-1}. Clindamycin phosphate and metronidazole were prepared as a stock solution of 10 mg mL^{-1}. The stock solutions of antimicrobials were dissolved in double deionized water (ddH_2O), sterilized using syringe filter 0.45 µm and kept in the refrigerator for a maximum of three weeks. On the day of the experiment, the stock solutions were diluted in the anaerobic chamber (to avoid oxidative stress) with pre-incubated BHIG, BHIH or MRS broth (according to the bacterial species) to avoid changing the concentrations of nutrients of growth media.

Determination of minimum biofilm inhibitory concentrations (MICs-B)

MIC determination was performed according to Sutyak Noll *et al*. (2012) with minor modifications. Briefly, the antimicrobials were diluted (a series of two-fold dilutions) with an appropriate volume of fresh BHIG in 96-well tissue culture plate (Falcon, Corning Incorporated, Corning, NY, USA). The final volume of antimicrobial agents diluted into the broth was 100µL in each well. The overnight cell culture at $3\pm2x10^8$ CFU mL^{-1} was diluted in BHIG to the final $5x10^6$ CFU mL^{-1}. From the diluted bacterial cells, 100 µL was transferred in the wells containing pre-determined concentrations of antimicrobials. Plates were incubated under anaerobic conditions at 37°C for 24-28 h. Mineral oil (Sigma-Aldrich chemical, St. Louis, MO) was added (75 µL) to each well to

avoid evaporation. The MIC was determined by taking the endpoint reading using a plate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). The MIC was defined according to CLSI (Clinical and Laboratory Standards Institute) guidance (2010) as the lowest concentration of antimicrobial in wells with absorbance A_{595} equal to or less than 20% of the control's mean absorbance (bacterial growth without antimicrobial addition).

Bacterial biofilm formation assay

The biofilm formation assay was performed using the method described by Turovskiy *et al*. (2012) with minor modifications. Briefly, from an overnight culture of *G. vaginalis* in BHIHS, 200 µL were transferred into a 50 mL test tube (Thermo Scientific, Rochester, NY, USA) containing 20 mL of BHIH broth. The tube was incubated overnight at 37^{o}C under anaerobic conditions using the anaerobic chamber. Following incubation, 750-1000 µL of the cell suspension was transferred into a new tube with 20 mL of fresh BHIG broth to achieve $5x10^{6}$ CFU mL^{-1}. Then, 200 µL of the cell suspension was pipetted into a sterile 96 well polystyrene flat bottoms tissue culture plate (Falcon, Corning Incorporated, Corning, NY, USA). An amplification tape (Nalge Nunc International, Rochester, NY, USA) was used to cover the 96-well plate to avoid medium evaporation. The culture plate was incubated anaerobically for 24-27 h at 37^{o}C.

For vaginal lactobacilli biofilm, we followed the Jones & Versalovic (2009) method with minor modifications. Briefly, frozen stock lactobacilli was taken with a disposable loop (Fisher Scientific, Pittsburgh, PA, USA) and directly inoculated into MRS broth. After overnight incubation at 37^{o}C, 200 µL of bacterial suspension was transferred into 50 mL tube containing 20 mL MRS broth supplemented with 1% of glucose and 2%

sucrose (MRS-GS) and incubated aerobically at 37^{o}C for 24 h without agitation. To assure consistency in the number of cells used in the study, optical density ($O.D_{600}$) of a second overnight bacterial culture was measured (SmartSpec 3000 Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA) and adjusted, if necessary, to OD_{600} = 3.03 ± 0.043 which was ~10^9 CFU mL^{-1}. Then, 200 µL of cell suspension was inoculated into 20 mL MRS-GS, mixed by Vortex and incubated for 15 min at 37^{o}C. Then 200 µL of the last suspension (≈ 10^7 CFU mL^{-1}) was added to the wells of a 96 well tissue culture plate. Amplification tape was used to cover the 96-well micro-plate and avoid medium evaporation. The micro-plates were incubated at 37^{o}C under aerobic conditions for 24 h. After the incubation period, each well was gently washed twice with 200 µL of fresh medium (BHIG was used for *G. vaginalis* and MRS-GS for vaginal lactobacilli) to remove non-adhered bacteria. To disrupt biofilm, vigorous pipetting was performed with 200 µL of fresh broth. For each well, six ten-fold dilutions (10^1-10^6 CFU mL^{-1}) were made with a fresh culture media. Then 20 µL from each dilution was plated in duplicates on agar plate (BHI was used for plating *G. vaginalis* and MRS was used for vaginal lactobacilli). The plates were incubated for 72 h at 37^{o}C. The grown colonies were enumerated using the colony counter (Corporate Headquarters Reichert, Inc., Buffalo, New York, USA).

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

First, supernatant was discarded and the biofilm was gently washed twice with fresh BHIG broth to remove non-attached cells. The concentrations of antimicrobials in the wells were as following: LAE 1000 µg mL^{-1}, subtilosin 138.9 µg mL^{-1}, clindamycin and metronidazole were added at 2000 µg mL^{-1}. These concentrations were chosen according

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

Plate counting method

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

then incubated for 72 h at 37^{o}C under anaerobic conditions. The number of colonies between 2 to 20 CFU per spot was regarded as a quantifiable number.

Checkerboard assay

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

Checkerboard assay, data analysis

In our study, isobologram was used to analyze the interactions of natural antimicrobial agents with commonly used antibiotics. This method is based on the comparison of the MBC-B value of each individual antimicrobial with its MBC-B value when used in combination. Axis (X) represents MBC-B of antimicrobial (A) with the coordinates (0, x) and axis (Y) represents antimicrobial (B) with the coordinates (y, 0). The two points (A) and (B) are connected by a line (Turovskiy & Chikindas, 2011). Each MBCs-B value of

two combining antimicrobials is represented as a point on the graph. In this study, three of these points were selected and plotted. Results are expressed according to locations of MBCs-B points on the line that connects (A) and (B) as following: when MBCs-B points are located under or above the line, the two combining antimicrobials are synergized or antagonized respectively, against the tested micro-organism.

Statistics

Each antimicrobial combination was conducted at least three times in duplicate. The results illustrate the average of three experiments unless it is mentioned otherwise.

Results

Evaluation of antimicrobial activity against biofilm-associated *G. vaginalis* and planktonic lactobacilli

MIC-B was determined using the broth micro-dilution method to evaluate the activity of antimicrobials against biofilm-associated *G. vaginalis*. Low concentrations of natural antimicrobials were effective against biofilm- associated *G. vaginalis* but not against vaginal lactobacilli which were tolerant to the much higher concentrations.

Clindamycin at 1.56 μg mL^{-1} inhibited the growth of *G. vaginalis.* At this concentration it was bactericidal for *L. vaginalis* and *L. plantarum* but not for other vaginal lactobacilli. While the MIC of metronidazole for lactobacilli was relatively high (> 200 μg mL^{-1}), the MIC-B of metronidazole for *G. vaginalis* biofilm was 6.25 μg mL^{-1}.

The MIC-B of LAE and subtilosin for *G. vaginalis* were 6.25 and 3.7 μg mL^{-1}, respectively. Vaginal lactobacilli planktonic growth was inhibited only when high concentrations of these antimicrobials were used (Table 1).

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

To determine the time required for the antimicrobial agents to efficiently inhibit biofilms-associated *G. vaginalis* at pre-determined concentrations, survivability of antimicrobials-treated biofilms was evaluated at 0, 1, 3, 8, and 18 h of incubation, in duplicates (Fig. 2). The concentrations of antimicrobials in this experiment were as following: LAE, 1000 μg mL^{-1}, subtilosin, 138.9 μg mL^{-1}; and clindamycin and metronidazole, 2000 μg mL^{-1}. Clindamycin alone at concentration 2000 μg mL^{-1} produced a 2.65±0.17 log reduction after 8 h with no further growth inhibition of biofilm-associated cells after this time point. In order to determine the MBC-B of clindamycin, several concentrations of this antibiotic were tested (4, 6, 8, 16, and 20 mg mL^{-1}). Only the 20 mg mL^{-1}, the MBC-B, caused a 7 log reduction (killing effect ≥ 3 log reduction) in the number of viable cells (Table 2).

Metronidazole 2000 μg mL^{-1} killed biofilm-associated cells to the point of no detection (by plating) after 8 h incubation. Similarly, 500 μg mL^{-1} of metronidazole had a bactericidal effect against biofilm-related *G. vaginalis* after 8 h (Table 2). LAE 1000 μg mL^{-1} and subtilosin 138.9 μg mL^{-1} killed 100% of biofilm cells during the first hour of treatment. We found that the MBCs-B of LAE and subtilosin were 50 μg mL^{-1} and 69.5 μg mL^{-1}, respectively (Table 2).

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

Based on the previously observed synergy in action of subtilosin and clindamycin against planktonic cells of *G. vaginalis* (Turovskiy *et al.* 2012), we proposed that a combination

of subtilosin with clindamycin could also decrease the high MBC-B value of clindamycin. According to our data (Fig. 3), subtilosin synergizes with clindamycin against biofilms of *G. vaginalis*. The MBC-B of subtilosin in combination with clindamycin decreased eight-fold from when it was used alone (8.6 µg mL^{-1} in combination instead of 69.5 µg mL^{-1} when it was used alone). The MBC-B of clindamycin in combination decreased more than six-fold from when it was used alone (2.9 mg mL^{-1} in combination instead of 20 mg mL^{-1} alone).

We noticed that metronidazole inhibited the growth of planktonic cells and biofilm formation of *G. vaginalis* with MIC-B 6.25 µg mL^{-1}, which was much lower than the concentration that inhibited the growth of vaginal lactobacilli, > 200 µg mL^{-1}. The combination of subtilosin with metronidazole was acting synergistically against biofilm-associated *G. vaginalis* (Fig. 5). The MBC-B of subtilosin in combination was sixteen folds lower than when it was used alone (4.3µg mL^{-1} in combination instead of 69.5 µg mL^{-1} alone). The MBC-B of metronidazole in combination was eight folds lower than what it was used alone (62.5 µg mL^{-1} in combination instead of 500 µg mL^{-1} alone).

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

LAE was combined with the two synthetic antibiotics to evaluate their interactions. Synergistic activity was found when LAE was combined with clindamycin and with metronidazole (Fig 4 and 6). The MBC-B of LAE in combination with clindamycin was eight times lower (6.25 µg mL^{-1}) than the MBC-B of the antimicrobial alone (50 µg mL^{-1}). In the same combination, MBC-B of clindamycin was almost seven folds lower than

when it was used alone (2.9 mg mL^{-1} in combination instead of 20 mg mL^{-1} alone). Similar MBC-B value of LAE was found when it was combined with metronidazole (6.25 µg mL^{-1} in combination instead of 50 µg mL^{-1} alone). The MBC-B of metronidazole in this combination was eight folds lower than when it was used alone (62.5 mg mL^{-1} in combination instead of 500 µg mL^{-1} alone).

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

We noticed that when clindamycin was combined with subtilosin or with LAE, the growth of planktonic lactobacilli was inhibited. However, almost all combinations of metronidazole with subtilosin or with LAE did not inhibit normal growth of vaginal lactobacilli (Table 4).

While combinations of clindamycin with subtilosin or with LAE inhibited the growth of planktonic lactobacilli, they were ineffective against biofilm-associated lactobacilli at concentrations inhibitory for *G. vaginalis* biofilms (data not shown).

Discussion

Recently, a new trend emerged focused on evaluation of conventional antibiotics as formulations synergistically acting with bacteriocins (Naghmouchi *et al*. 2011; Draper *et al*. 2013). Consequently, this study focused on combinations of natural antimicrobial LAE and subtilosin with clindamycin and metronidazole to assess synergistic activity against biofilms of *G. vaginalis.* The importance of exploring antimicrobial proteins, both alone and in combination with conventional drugs, is recognized by many investigators and as such was recently reviewed by Di Luca *et al.* (2014). Clindamycin and

metronidazole are the commonly prescribed antibiotics for treatment of BV (Workowski & Berman, 2010). Development of antimicrobial resistance (Beigi *et al.,* 2004) and infection recurrence (Barbieri, 2013) are the most dangerous side effects of abusing or overusing these antibiotics. The ability to use alternative medications such as bacteriocins, alone or in combination with synthetic antibiotics, is highly desirable for avoiding the disadvantages that come from using antibiotics alone. In our study, we evaluated the anti-biofilm activity of natural antimicrobials alone and in combination with the most commonly used antibiotics against biofilm -associated *G. vaginalis*. To evaluate the activity of antimicrobials against these biofilms, the labor intensive direct plate count was used instead of more 'advanced methods', which appear to be less reliable according to our thorough investigation (Turovskiy *et al.*, 2012).

The bactericidal activity of the selected antimicrobial agents against *G. vaginalis'* biofilm was evaluated for 18 h and confirms 8 h as a preferential time of exposure for the most efficient inhibition of the targeted micro-organism by the studied antimicrobials and their formulations. When clindamycin 2000 µg mL^{-1} was used, about 2.65±0.17 log reductions were achieved after 8 h and the viable cell numbers remained unchanged after the next 10 h of incubation. When LAE 50 µg mL^{-1}, subtilosin 69.5 µg mL^{-1} and metronidazole 500 µg mL^{-1} were used, again the bactericidal activity reached its peak after 8-9 h. This finding is in accord with what we see as a convenient application time of antimicrobial agents in the vaginal environment during the night resting period (about 8-9 h). In our previous work, we reported that MBC-B of both subtilosin and LAE against biofilm-associated *G. vaginalis* at concentration 100 µg mL^{-1} produced 3 and 5 log reductions, respectively, while clindamycin at concentration1600 µg mL^{-1} caused only a 2

log reduction (Turovskiy *et al.*, 2012). Discrepancies had been detected when MBC-Bs were compared between our data and what Turovskiy *et al.* (2012) found. They were due to the differences in brand names of the used antimicrobials, the exposure time to antimicrobials and the experimental method that was followed to determine the MBC-Bs.

To avoid the undesirable side effects of using the conventional antibiotics alone for BV treatment (Bradshaw *et al.*, 2006; Oduyebo *et al.*, 2009), innovative solutions are urgently required. Synergistically-acting combinations of natural antimicrobials and synthetic antibiotics are promising solutions for future pharmaceutical formulations (Baker *et al*., 2007; Yang *et al.*, 2014). In our study, four combinations were evaluated. The natural antimicrobials subtilosin and LAE were combined with antibiotics, clindamycin or metronidazole, using a checkerboard assay.

After identifying the MBC-B value of each compound, a checkerboard assay was performed to evaluate the nature of combination between two antimicrobial agents. The linear logistical isobologram was used to analyze the nature of antimicrobial combinations and determine if they are synergistic or antagonistic. Isobolograms are commonly used to strengthen graphical and statistical design and support the data mathematically and biologically (Chen &Pounds, 1998).

In this study, synergistic activity was found when antimicrobials subtilosin and LAE were combined with clindamycin and metronidazole against biofilm-associated *G. vaginalis*. Subtilosin forms temporary pores leading to cell death (Sutyak Noll *et al*., 2012). A solid-state NMR experiment using model phospholipid bilayers showed that when subtilosin is used at high concentrations, it binds to the lipid head group region and

then partially embeds in the lipid bilayer membrane causing lipid perturbation and a permeabilization defect (Thennarasua *et al*., 2005).

Many studies reported dangerous side effects of metronidazole, such as carcinogenicity in animals, genotoxicity in humans, and *in vitro* mutagenicity (Stranz & Bradley, 1981; Bendesky *et al*., 2002; Koss *et al*., 2012). Importantly, the synergy between natural antimicrobials and metronidazole may help to avoid the undesirable side effects which may be caused by high dosage of the antibiotic alone, and decrease the opportunity of bacterial mutation that leads to antibiotic resistance.

LAE, the cationic surfactant, altered the permeability of cytoplasmic membrane to ions, such as potassium ions, in both Gram positive and negative bacteria (Rodriguez *et al*., 2004). The antimicrobial activity of LAE against bacterial biofilm is not fully understood yet. Bonnaud & co-workers (2010) noticed a strong interaction between LAE and anionic polymers such as alginate. Cotton *et al.* (2009) found that the anionic polysaccharide alginate is required for biofilm's tolerance to antimicrobials. Therefore, it is possible that LAE interacts with alginate at the surface of biofilm, modifying its biopolymer structure and enhancing the biofilm's susceptibility to clindamycin and metronidazole.

Our findings are in accord with Cavera *et al*. (2015), who analyzed the combination of natural antimicrobials with each other and with antibiotics using a fractional inhibition concentration index (FICI). Cavera *et al*. (2015) noticed that subtilosin and LAE synergized with clindamycin as well as metronidazole to inhibit the growth of planktonic cells of *G. vaginalis*. Synergistic activity was detected when subtilosin was combined

with the natural antimicrobials LAE, glycerol monolaurate, and PL against BV-associated pathogen *G. vaginalis* (Sutyak Noll *et al.*, 2012). These data and ours confirm synergistic activity when natural antimicrobials combined with each other and/or with the synthetic antibiotics against *G. vaginalis* and its biofilm.

It is imperative for any study focused on inhibition or killing of BV-associated pathogens to identify if these treatments influence non-pathogenic vaginal lactobacilli due to the importance of this microbiota in maintaining vaginal heath. While there were many studies focused on the control of BV-associated pathogens, all of them neglected elucidation of the activity of antimicrobial compounds on vaginal lactobacilli (Hubrechts *et al*., 1984; Braga *et al*., 2010; Schwebke *et al*., 2011; Henriques *et al*., 2012; Brocklehurst *et al*., 2013; Hymes *et al*., 2013; Kandimalla *et al*., 2013; Pathak *et al*., 2014). On the contrary, we tested the selected combinations of antimicrobials (Table 3) against five species of vaginal lactobacilli using the broth micro-dilution method. Our study showed that only planktonic cells of vaginal lactobacilli were inhibited by combination of clindamycin with either LAE or subtilosin. However, these combinations were harmless for biofilm-associated lactobacilli. At the same time, combinations of metronidazole with subtilosin or with LAE did not reduce the normal growth ability of either planktonic or biofilm-associated lactobacilli. Lack of metronidazole's activity at concentration >200 $\mu g\ mL^{-1}$ against lactobacilli as observed in this study, is in agreement with previously published reports (Simoes *et al*., 2001: Austin *et al*., 2006). Moreover, Anukam & Reid (2008) found that metronidazole at 1 $mg\ mL^{-1}$ does not inhibit normal growth of *L. rhamnosus* GR-1 and *L. plantarum* KCA which was in agreement with the study by Ocana *et al*. (2006). Finally, according to Martín *et al.* (2008), healthy vaginal

isolates of *L. gasseri* and *L. plantarum* were more resistant to metronidazole than to clindamycin, gentamicin, ciprofloxacin, trimethoprim, and sulfametoxazole.

The mechanism(s) of resistance to metronidazole is not fully understood. Church *et al.* (1996) explained that lactobacilli as facultative aerobic bacteria lack ferredoxin-linked hydrogenase, which is an essential enzyme for metronidazole-intracellular activity. According to previous studies and our data, using lower concentrations of metronidazole combined with the natural antimicrobials such as LAE and subtilosin may lead to enhance the antibiotic susceptibility of BV-associated pathogens, while keeping the probiotic vaginal lactobacilli alive.

Clindamycin is a protein synthesis blocker (Chambers, 2003). Our data illustrated that vaginal lactobacilli were sensitive to clindamycin 0.78 μg mL^{-1}; however, higher concentrations of subtilosin (> 500) μg mL^{-1} and LAE (16-62.5) μg mL^{-1} were required for bacterial growth inhibition. Our findings are in agreement with the studies showing sensitivity of planktonic but not biofilm-associated lactobacilli to clindamycin (Coppola *et al*., 2005; Klare *et al*., 2007).

In regard to vaginal lactobacilli biofilm, we found that the antimicrobial combinations (which were bactericidal to *G. vaginalis*–associated biofilm) had no effect on the normal growth of lactobacilli-formed biofilm. Biofilm-associated cells are often reported as being 100 to 1000 times more tolerant to various stresses than planktonic cells due to different mechanisms used to withstand these factors. Kubota *et al.* (2009) found that antimicrobial tolerance of biofilm-associated lactobacilli with different growth phases was higher than resistance in planktonic cells.

Biofilm formation by lactobacilli (Ocãna & Nader-Macias, 2004) confers positive health effects for the vaginal environment. These advantages include a replacement of biofilm formed by pathogenic bacteria (Woojin *et al*., 2011), improvement of the production of anti-pathogenic agents and increasing tolerance of biofilm-associated cells to antimicrobial factors. In our study, *L. acidophilus* and *L. vaginals* formed rather thin and patchy biofilms when grown in a 96-well tissue culture plate. However, *L. rhamnosus, L. plantarum* and *L. gasseri* were capable of biofilm formation with a bacterial count of 5×10^{8} - 10^{9} CFU mL^{-1}. The ability of some lactobacilli species to attach on surfaces and establish their biofilms may depend on the cell hydrophobicity and the charge of the vaginal epithelial surface (Millsap *et al*., 1997). It is also believed that the establishment of biofilm by lactobacilli is genetically-encoded. Sturme *et al*. (2005) found that production of cyclic thiolactone autoinducing peptide, which is encoded by *lam*, the *L. plantarum* regulator, is associated with bacterial adherence. A *luxS* knockout in *L. rhamnosus* GG showed a defect in biofilm formation and metabolic activity (Lebeer *et al.*, 2007). The genome sequences of *L. plantarum* WCFS1 (GenBank accession no. NP_784522) and *L. gasseri* (GenBank accession no. ZP_00046310) confirmed the presence of *luxS* homologues.

Also, it has been found that pili and cell surface proteins play an important role in lactobacilli adhesion and biofilm formation. Pili-coding genes were identified in genome sequences of some lactobacilli species (Forde *et al*., 2011), enhancing bacterial adhesion and biofilm formation (Danne & Dramsi, 2012; Lebeer *et al.*, 2012). The S-layer cell surface proteins of lactobacilli and their role in biofilm formation have been studied. Lortal *et al*. (1992) and Golowczyc *et al*. (2007) referred to the role of the S-layer in

enhancing bacterial cell adhesion during lactobacilli aggregation and biofilm development.

Biofilm formation may be influenced by the culture media that are used to grow lactobacilli. The MRS-GS broth was used as growth medium in our experiment in order to obtain a developed biofilm of lactobacilli in the 96-well tissue culture plate. The number of bacterial cells in biofilms was very low when lactobacilli were grown using MRS alone or supplemented with either sucrose or glucose. In addition to sucrose, glucose is considered as a main carbon source for lactobacilli (Kandler & Weiss, 1986). Tenuta *et al*. (2006) found that when growth medium was supplemented with glucose, fructose and sucrose, the number of lactobacilli in biofilm became higher than in the medium without these sugars. *L. rhamnosus* was unable to form biofilm when MRS broth or MRS broth without glucose was used as the growth medium (Lebeer *et al*., 2007). Ismail *et al*. (2006) reported that using medium supplemented with glucose and sucrose could promote lactobacilli biofilm formation and development. While glucose is considered as an essential part of exopolysaccharides (EPS) (de Vuyst *et al*., 2001), addition of sucrose to TY medium increased the adhesion and biofilm formation abilities of the cells (Shemesh *et al*., 2007).

BV is associated with multi-species biofilms (Swidsinski *et al.*, 2005). However, isolation of *G. vaginalis* from 99% of women who have the BV-infection (Hillier *et al*., 1990) does not neglect the role of other anaerobic pathogens, such as *M. curtisii* and *P. anaerobius* in establishment of vaginal biofilm and its antimicrobial tolerance. Both *M. curtisii* and *P. anaerobius* were incapable of forming a developed biofilm on their own when grown in BHIG broth. Our observation was in agreement with Swidsinski *et al*.

(2005) who found that a thick and adherent biofilm was formed only by *G. vaginalis*. Machado *et al*. (2013) reported that *G. vaginalis* biofilm "encouraged" other anaerobes to "participate" in a formation of a multispecies biofilm.

The combinations of antimicrobials synergistic against biofilm-associated *G. vaginalis* were evaluated against planktonic cells of *M. curtisii*, and *P. anaerobius*, the predominant BV-associated anaerobes (Hillier *et al*., 1990). In this study, all combinations (Table 3) caused partial inhibition of *M. curtisii* growth and were bactericidal for *P. anaerobius*.

Recurrence and antibiotic tolerance of BV-associated micro-organisms may be connected with the prevalence of other BV-causing anaerobes such as *Mobiluncus* species. Schwebke & Lawing (2001) found *Mobiluncus* in 84.5% of samples, in which 77.3% were *M. curtisii.* Unlike bacterial sensitivity to clindamycin, *M. curtisii* were resistant to metronidazole and its hydroxyl metabolite (Spiegel, 1987). According to Joesoef *et al*. (1999), after antibiotics treatment the percentage of BV-associated infection recurrence was generally about 50%. Michelle *et al.* (2008) suggested that the reason behind 67.9% of BV recurrence (with high Nugent scores) is in the presence of *M. curtisii.* A relationship has been found between high Nugent scores and *M. curtisii* resistance to initial treatment.

Sensitivity of *P. anaerobius* to clindamycin and metronidazole has been reported by (Könönen *et al*., 2007). It is known that metronidazole works by selectively targeting anaerobic microbes, including *Peptostreptococcus* species. The presence of nitroimidazole-resistance encoded *nim* genes was considered the cause of

Peptostreptococcus resistance to metronidazole (Theron *et al*., 2004). Könönen *et al*. (2007) reported the antimicrobial resistance of *P. anaerobius* to some of the used β-lactam antibiotics, although bacterial cells were unable to produce β-lactamase. The slow growth of *Peptostreptococcus* may explain their resistance to antimicrobials that target the growth factors (Higaki *et al*., 2000).

Synergistic activity was reported when natural antimicrobials were combined with conventional used antibiotics against planktonic and biofilm-associated pathogens (Choi & Lee, 2012a; Choi & Lee, 2012b; Kaur & Sharma, 2013; Minahk *et al.*, 2004). Minahk *et al*. (2004) found that the cationic peptide enterocin CRL35, which is produced by *Enterococcus mundtii,* was synergized with tetracycline, erythromycin and chloramphenicol against *Listeria innocua*. Using the checkerboard assay, Choi & Lee (2012) reported synergistic activity when pleurocidin was combined with ampicillin, chloramphenicol and erythromycin against six of the tested bacterial species. Pleurocidin is a positively charged and amphipathic antimicrobial peptide extracted from mucus secretions of *Pleuronectes americanus,* a winter flounder (Choi & Lee, 2012a). In a separate publication, Choi & Lee (2012b) found that arenicin-1, the positively charged antimicrobial peptide isolated from *Arenicola marina,* strongly synergized with antibiotics against studied pathogenic bacteria. Choi & Lee (2012b) noticed that arenicin-1 enhanced the penetration of erythromycin and chloramphenicol by perturbing the permeability of the cytoplasmic membrane. Pleurocidin and arenicin-1 induced the formation of hydroxyl radicals when they combined with antibiotics and exerted anti-biofilm activity (Choi & Lee, 2012a; Choi & Lee, 2012b). Kaur & Sharma (2013) evaluated the antimicrobial combinations of cell free supernatants (CFS) of vaginal

lactobacilli with ciprofloxacin, streptomycin, moxifloxacin and rifampicin against *Salmonella typhimurium* and *Pseudomonas aeruginosa*. They found that CFS synergized with antibiotics and increased sensitivity of *P. aeruginosa* to antibiotic treatment (Kaur & Sharma, 2013). Since most natural antimicrobials target the cytoplasmic membrane or bacterial cell envelope, they are paving the way for antibiotics to finalize their mechanism of action and avoid the problematic issue of antibiotics resistance. In the future, an *in vivo* study needs to be conducted to ensure the safe use of these combinations without any harmful side effects. Our future studies will follow the suggested strategy in experimental design and in the format of the data report (Lourenço *et al.*, 2014).

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables and Figures

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

Antimicrobial agents	MIC-B of antimicrobials (µg mL^{-1}) /*G. vaginalis*	MIC of antimicrobials (µg mL^{-1})/ Vaginal lactobacilli
LAE	6.25[1]/ 10[2]	15.36-62.5[1], 15.36-62.5[3]
Subtilosin	3.7[1]/ 9.2[2] and 12[3]	> 500[1], 725-825[3]
Clindamycin	1.56[1]/16[3]	0.78->50[1], 0.78-77.5[3]
Metronidazole	6.25[1]/ 50[3]	>200[1], 50-100[3]

[1]Data from this study, [2]data from Sutyak Noll *et al.* (2012), [3]data from Cavera *et al.* (2015)

Table 2 Minimum biofilm bactericidal concentrations (MBCs-B) of antimicrobials against biofilm-associated *G. vaginalis* after 8 h incubation

Antimicrobial agents	MBC-B (µg mL^{-1})
Subtilosin	69.5
LAE	50
Metronidazole	500
Clindamycin	20000

Table 3 The MBCs-B of antimicrobial combinations which synergized against biofilm-associated *G. vaginalis*

Antimicrobials Combinations	MBC-B (µg mL^{-1})
Subtilosin + Clindamycin	34.7+2900, 17.3+4400, 8.6+6600
Subtilosin + Metronidazole	4.3+250, 17.3+ 62.5
LAE + Clindamycin	25+2900, 6.26+10000
LAE + Metronidazole	6.25+250, 12.5+125, 25+62.5

Table 4 Antibacterial effect of synergistically acting combinations (against biofilm-associated *G. vaginalis*) on the growth ability of vaginal lactobacilli

		Growth ability of vaginal lactobacilli				
Antimicrobial combinations	The MBCs-B (μg mL^{-1})	L.v[a]	L.p[b]	L.g[c]	L.a[d]	L.r[e]
Subtilosin + Clindamycin	34.7+2900	-	-	-	-	-
	17.3+4400	-	-	-	-	-
	8.6+6600	-	-	-	-	-
Subtilosin + Metronidazole	4.3+250	+	+	+	+	+
	17.3+ 62.5	+	+	+	+	+
LAE + Clindamycin	6.25+10000	-	-	-	-	-
	25+2900	-	-	-	-	-
LAE + Metronidazole	6.25+250	+	+	+	+	+
	12.5+125	+	+	+	±	+
	25+62.5	-	+	+	-	±

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

Figure 1 Checkerboard assay, an example for two antimicrobial combinations

Metronidazole Concentrations (µg mL^{-1})	Lauramide Arginine Ethyl ester concentrations (µg mL^{-1})										
	0	**100**	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.56**	**0.78**	**Controls Antimicrobials And broth**	**Controls Biofilm + broth**
	1000	**1000/100**	**1000/50**	**1000/25**	**1000/12.5**	**1000/6.25**	**1000/3.125**	**1000/1.56**	**1000/0.78**		
	500	**500/100**	**500/50**	**500/25**	**500/12.5**	**500/6.25**	**500/3.125**	**500/1.56**	**500/0.78**		
	250	**250/100**	**250/50**	**250/25**	**250/12.5**	**250/6.25**	**250/3.125**	**250/1.56**	**250/0.78**		
	125	**125/100**	**125/50**	**125/25**	**125/12.5**	**125/6.25**	**125/3.125**	**125/1.56**	**125/0.78**		
	62.5	**62.5/100**	**62.5/50**	**62.5/25**	**62.5/12.5**	**62.5/6.25**	**62.5/3.125**	**62.5/1.56**	**62.5/0.78**		**Controls Both only**
	31.25	**31.25/100**	**31.25/50**	**31.25/25**	**31.25/12.5**	**31.25/6.25**	**31.25/3.125**	**31.25/1.56**	**31.25/0.78**		
	15.6	**15.6/100**	**15.6/50**	**15.6/25**	**15.6/12.5**	**15.6/6.25**	**15.6/3.125**	**15.6/1.56**	**15.6/0.78**		

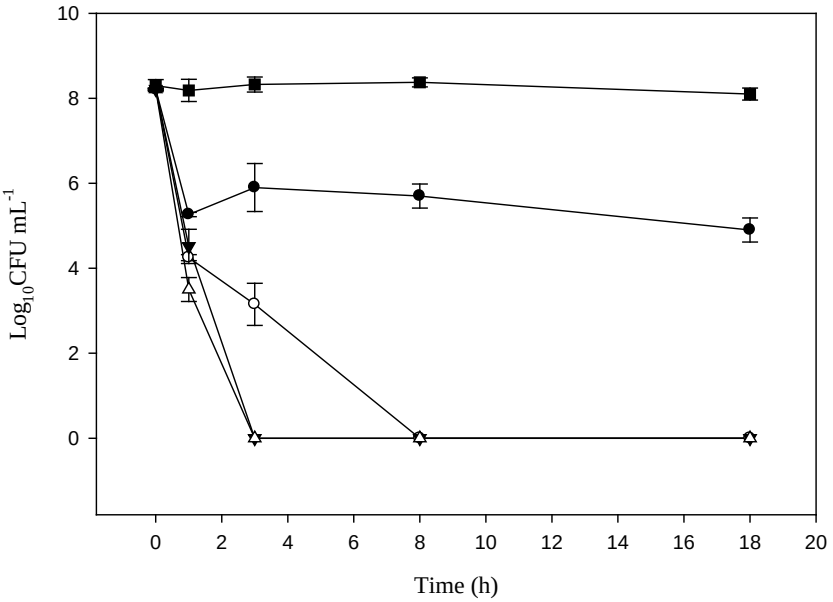

Figure 2. Bactericidal activity of antimicrobial agents against biofilm-associated *G. vaginalis* during 18 h incubation. LAE, 1000 µg mL^{-1} (reversed triangle) and subtilosin, 139 µg mL^{-1} (open triangle) killed the biofilm cells during first hour of treatment, clindamycin, 2000 µg mL^{-1} (closed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

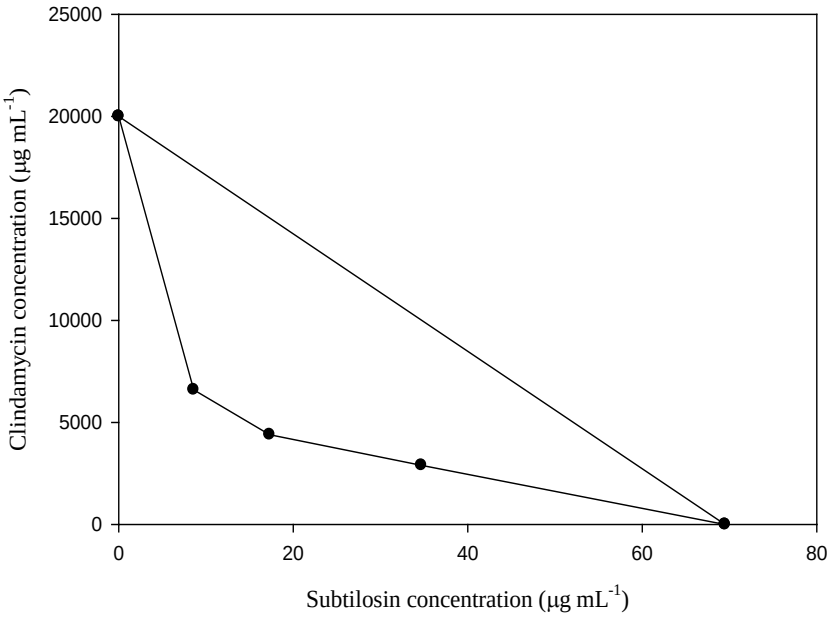

Figure 3. Isobologram of interaction between subtilosin and clindamycin. Subtilosin synergized with clindamycin against biofilm-associated *G. vaginalis*. The MBC-B of subtilosin in combination with clindamycin was eight folds lower than when it was used alone (8.6 µg mL^{-1} in combination instead of 69.5 µg mL^{-1} alone). The MBC-B of clindamycin in combination was more than six folds lower than when it was used alone (2.9 mg mL^{-1} in combination instead of 20 mg mL^{-1} alone).

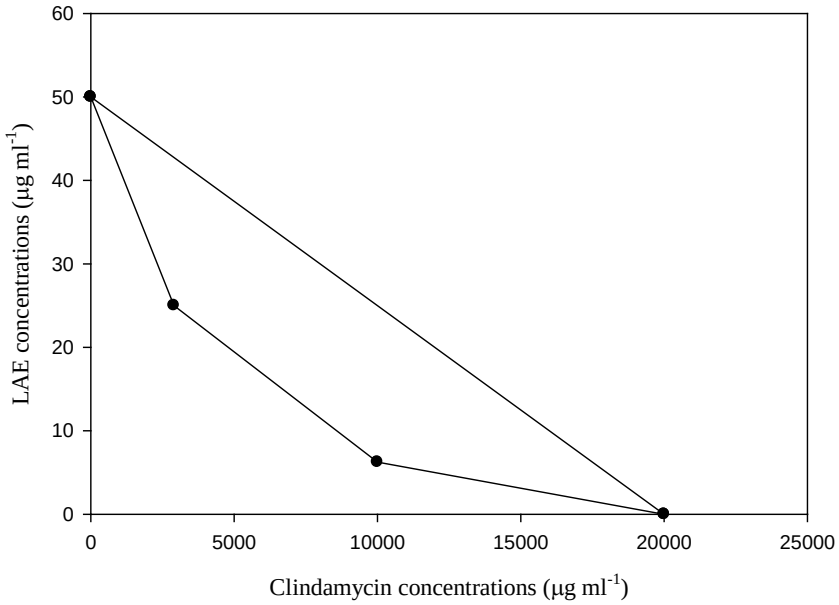

Figure 4. Isobologram of interaction between LAE and clindamycin. LAE synergized with clindamycin against biofilm-associated *G. vaginalis*. The MBC-B of LAE in combination was eight folds lower than when it was used alone (6.25µg mL^{-1} in combination instead of 50 µg mL^{-1} alone). The MBC-B of clindamycin in combination was more than six folds lower than when it was used alone (2.9 mg mL^{-1} in combination instead of 20 mg mL^{-1} alone).

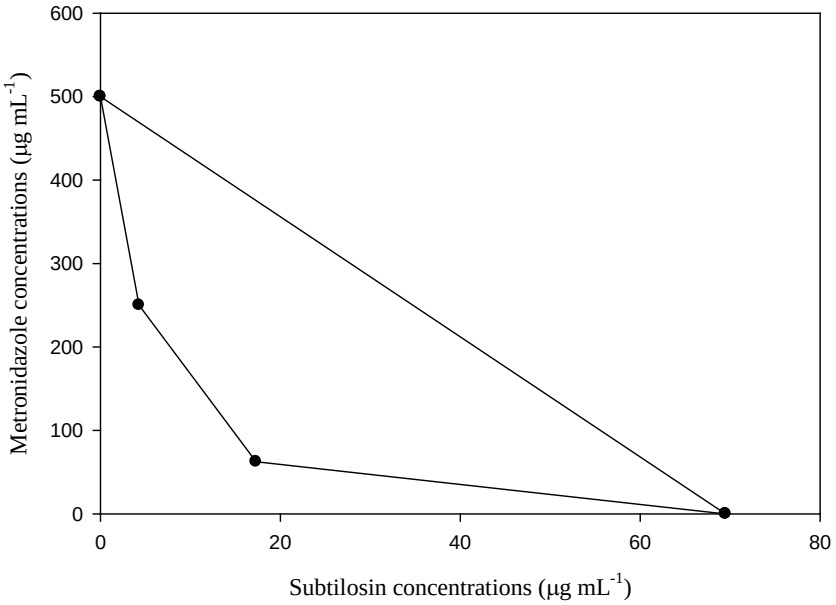

Figure 5. Isobologram of interaction between subtilosin and metronidazole. Subtilosin synergized with metronidazole against biofilm-associated *G. vaginalis*. The MBC-B of subtilosin in combination was sixteen folds lower than when it was used alone (4.3µg mL^{-1} in combination instead of 69.5 µg mL^{-1} alone). The MBC-B of metronidazole in combination was eight folds lower than what it was used alone (62.5 µg mL^{-1} in combination instead of 500 µg mL^{-1} alone).

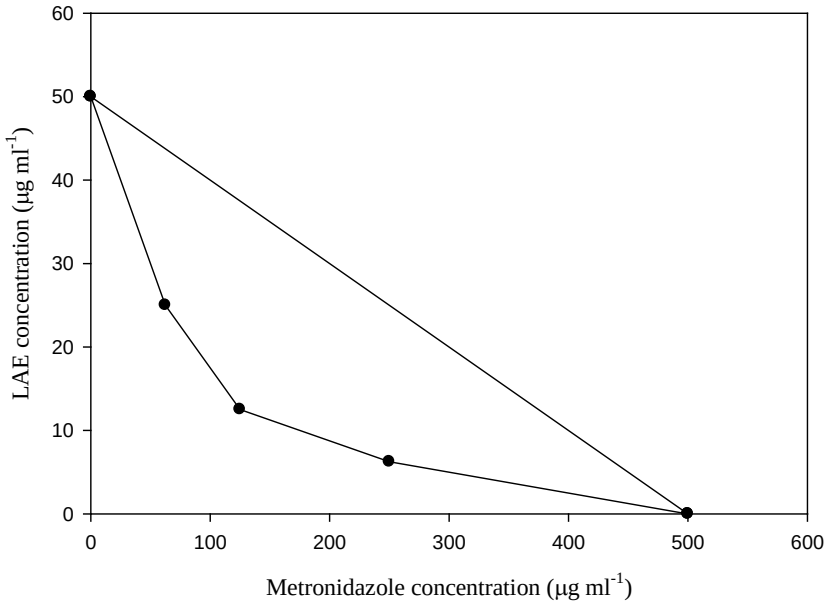

Figure 6. Isobologram of interaction between LAE and metronidazole. This figure shows the synergistic activity between LAE and clindamycin against biofilm-associated *G. vaginalis*. The MBC-B of LAE in combination was eight folds dilutions lower than when it was used alone (6.25 µg mL^{-1} in combination instead of 50 µg mL^{-1} alone). The MBC-B of metronidazole in combination was eight folds lower than when it was used alone (62.5 µg mL^{-1} in combination instead of 500 µg mL^{-1} alone).

Chapter 4: Safety properties and probiotic potential of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895 [4].

Probiotic capacity of several bacilli strains have been studied. Some of them are utilized in health-promoting formulations for humans and in agriculture. However, there is still a need for new strains with various health-promoting activities. This chapter examines safety and probiotic properties of *Bacillus subtilis* KATMIRA1933and *Bacillus amyloliquefaciens* B-1895. The safety features of these strains such as, absence of hemolytic activity, antibiotic resistance, carcinogenic and mutagenic frequency were determined. In addition, in the tested microorganisms' antimicrobial potential, protease activity, autoaggregation/coaggregation capability and tolerance to bile salts and acidity were evaluated. Furthermore, the anti-oxidant, immunomodulatory and DNA-protection activities of these strains were previously reported by our multi-disciplinary international team of collaborators. All these benifecial properties together potentiate the probiotic role of the studied bacilli for various applications in humans and agriculture

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Safety Properties and Probiotic Potential of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895

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Running title: safety of two *Bacillus* spp. with probiotic properties

Abstract

This study reports on the safety and putative probiotic properties of *Bacillus amyloliquefaciens* B-1895 and *Bacillus subtilis* KATMIRA1933. According to the bacterial reverse mutation (Ames) test, cell-free supernatants of *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 were not mutagenic. The two strains co-aggregated with *Escherichia coli* and *Pseudomonas aeruginosa*, and cell-free supernatants inhibited the growth of *Streptococcus intermedius* and *Porphyromonas gingivalis*. Endospores of *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 were tolerant to 0.3% (w/v) bile salts and survived incubation for 4 h in MRS broth at pH 2.0 to 3.0. The ability of the two strains to produce antimicrobial compounds potentiates their application in health care formulations, personal care products, food and animal feed.

Keywords: *Bacillus*, Safety, Probiotics, Antimicrobials, Bacteriocins, Spores

1. Introduction

According to the World Health Organization, probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [1]. Probiotic strains with the ability to produce antimicrobial compounds are often used to control the growth of pathogenic microorganisms in fermented food and animal feed. The strains may also be incorporated into personal care products [2][3]. Daily supplementation with probiotics proofed effective in the alleviation of cold and flu symptoms [4], and as food supplement to patients on cancer treatment [5]. Many probiotic bacteria produce a broad range of effective antimicrobials, including lactic acid, hydrogen peroxide and bacteriocins.

Bacteriocins are commonly defined as genetically encoded substances of a proteinaceous nature produced by virtually all bacteria and are active against various, most closely-related, microorganisms [6]. This positions them as a very appealing alternative to antibiotics and chemical stressors, especially in an age when alternative bacterial infection therapies are being widely investigated [7]. Several strains of *Bacillus* spp. have been recognized as safe for food or industrial applications and, importantly, have been documented as probiotics [8].

This study investigates several characteristics of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895, both of which are suspected to have probiotic properties. *B. subtilis* KATMIRA1933, isolated from a dairy product called YoguFarm, has been unwittingly consumed by humans for years without harmful effects [9]. The strain produces antimicrobial proteins, including subtilosin A [9]. Genomic analysis has shown that *B. amyloliquefaciens* B-1895, isolated from soil, has the potential to produce a

number of proteolytic enzymes and subtilin, an antimicrobial peptide active against foodborne pathogen *Listeria monocytogenes* [10]. The strain is used as a probiotic in royal fish [11]. Inclusion of *B. amyloliquefaciens* B-1895 into bird feed enhanced immunity to the Newcastle virus [12] and improved the body weight of the birds [13]. In addition, fermentates of both strains were reported as having antioxidant and DNA protective activities [14].

Any microorganism considered for use as a probiotic must be tested for specific advantageous characteristics, balanced by a thorough evaluation of its safety. The antibiotic susceptibility of bacterial cells to routinely prescribed antibiotics is essential for the putative probiotic's safety evaluation. Microbial resistance to antibiotics in part is a gene-encoded mechanism that occurs either by genetic mutation or by gaining resistance via horizontal or vertical gene transfer [15]. It is important to find out if antibiotic-resistance gene(s) are transferable from probiotics to the commensal microorganisms or to pathogenic bacteria. New forms of resistant pathogens may emerge if such genes are transferred from probiotics to pathogens [16]. Many studies have been conducted to identify the antibiotic-resistance genes in *Bacillus* species [17]-[20].

Some products produced by bacteria have the potential to damage host cells. It is thus important to screen for such products when assessing the safety of a strain. In particular, hemolysin production by bacteria has been identified as a virulence-associated feature [21]. Various bacterial species, including *Bacillus cereus* and group A streptococci, that produce hemolysin BL and streptolysin-o, respectively, are considered as pathogens due to their potent hemolytic activity [22]. The hemolytic mechanism in *Bacillus* is not fully understood. However, recent studies have been performed to identify the gene (s)

responsible for hemolytic activity [23] [24]. Even though the hemolytic activity of some *Bacillus* spp., such as *Bacillus subtilis,* is less than in pathogens [25], these isolates may be considered unsafe for food or personal health care applications until the effect of this virulence factor is either eliminated, modified, or confirmed as causing no harm to the eukaryotic host.

Bacillus species have a long history of use in biotechnology and as dietary supplements for humans and animals of agricultural importance. Also, bacilli have been engineered to produce biologically active substances such as antibiotics and enzymes [26]. A distinctive feature of the *Bacillus* spp. is high proteolytic activity [27]. The advantages and importance of proteolytic enzymes have been widely reported. Briefly, they include the activation of regeneration processes, the enhancement of normal digestion, and degradation of allergic compounds [27]-[29].

Mutagenicity and carcinogenicity assessments of the antimicrobial substances are the harbinger to efficiently evaluating bacterial products possessing antibacterial activity prior to their use in pharmaceutical applications. The Ames test is used to evaluate the mutagenic potential of substances by determining if the chemical causes DNA damage that leads to genetic mutations in *Salmonella* spp. [30]. Association between mutagens and cancer induction [31] raises the importance of recruiting a screening test for mutagenicity to ensure the safe consumption of such chemicals.

As many probiotics are to be consumed, their selection depends on bacterial tolerance to acids and bile salts. Candidacy of a probiotic depends on the ability of bacterial cells or their spores to survive and grow at the high acidity (pH 3 or below) of the stomach [32]

and with the detergent-like activity of intestinal bile salts that disrupt the cellular membrane [33]. *In vitro*, the tolerance of *Bacillus* species to acids and bile salts reflects their survival rates and viability through the gastrointestinal tract [32] [34].

Coaggregation is the adherence of genetically distinct bacteria and is considered a desirable characteristic in a probiotic microorganism [35]. It is believed to facilitate the integration of exogenous bacteria, which is important for the development of multispecies biofilms [35]. Coaggregation allows a beneficial organism to adhere to a pathogenic organism. Aggregation may also help the bacterium adhere to different surfaces, which is very relevant to human health. Adhesion may also allow probiotic organisms to create a barrier, which could effectively prevent colonization by pathogens [36]. Auto-aggregation of probiotic strains may be required for adhesion to intestinal epithelial cells, which would keep them from being flushed out by the body, and would in such a way give them an advantage over other organisms [36]. Though not all mechanisms of action have been determined, further research can reveal the more complex aspects and implications of this ability.

The objective of this study was to investigate whether *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 possess additional health benefits that would qualify them to be probiotic candidates. A battery of tests is commonly employed and has been addressed in the study on *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895. Antibiotic susceptibility, hemolytic/ fibrinolytic activity, proteolytic activity, bacterial reverse mutation, tolerance to acids and bile salts, and bacterial auto-aggregate/co-aggregate abilities were evaluated and analyzed for both bacilli. The antimicrobial activity of cell-free supernatants (CFSs) of tested bacilli against pathogens was also

evaluated. Regarding the ability to auto-aggregate and co-aggregate, the most appropriate method for the mathematical interpretation of collected data was identified. A visual analysis utilizing microscopy was used as a mode of comparison for evaluating two methods of mathematical analysis. It was determined that both the extracts possessed unique antibacterial capabilities along with the desirable traits that would allow them to progress as candidates for probiotics therapies.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Frozen stocks of *B.* subtilis KATMIRA1933 and *B. amyloliquefaciens* B-1895 were inoculated in De Man, Rogosa and Sharpe (MRS) medium (Becton Dickinson and Company, Sparks, Maryland, USA) and incubated aerobically with shaking at 250 rpm for 24 h at 37 °C. *M. luteus* ATCC10240 was used as a reference microorganism. The pathogenic bacteria included in our study are listed in Table 1. These bacterial strains were inoculated into tryptic soy medium (Becton Dickinson and Company, Sparks, Maryland, USA) and incubated aerobically for 24 h at 37 °C. The oral pathogens, upon revival from −80°C DMSO stocks, were maintained on trypticase soy broth (TSB) and agar plates containing haemin 1 $\mu g \cdot mL^{-1}$, menadione 1 $\mu g \cdot mL^{-1}$, 20% defibrinated sheep's blood (BAPHK) at 37 °C under anaerobic conditions (5% H_2, 10% CO_2, 85% N_2). *S. intermedius* strain F0413 was maintained on Brain Heart Infusion broth at 37 °C under anaerobic conditions, while *S. mutans* strain 25175 was incubated aerobically. Broth cultures of *Porphyromonas gingivalis* strains, *Prevotella intermedia* strains, and *Fusobacterium nucleatum* were grown in Todd–Hewitt broth (THB) containing haemin 1

$\mu g \cdot mL^{-1}$ and menadione 1 $\mu g \cdot mL^{-1}$ (designated THBHK) at 37 °C under anaerobic conditions.

2.2. Antimicrobial Activity of CFS of Studied *Bacillus* Strains

Cell free supernatant of two tested bacilli strains were prepared as previously described by Sutyak et al. [9]. Broth cultures (1.5 mL) of oral pathogens were grown for 24 h in the appropriate atmosphere and media. Culture density for each strain was determined using OD_{600} values. All strains were adjusted to an OD_{600} of 0.1 to correspond to 1×10^6 $CFU \cdot mL^{-1}$. Cultures were swabbed to the appropriate solid support agar media, which had been dried for 20 min in a tissue culture hood. Plates that were to be used under anaerobic conditions were placed in the chamber and all further steps were carried out within. Using the wide end of a 200 µL yellow pipette tip, holes were bored into the media, creating a well to accommodate supernatants. Cell-free supernatant (120 µL) from *B. subtilis* KATMIRA1933 and B-1895 were added to each well, in triplicate. Plates were incubated for 5 days at 37 °C under anaerobic conditions and the diameter of the zones of inhibition were measured in millimeters (mm) with a digital caliper.

2.3. Antibiotic Susceptibility of Studied Bacilli

B. subtilis KATMIRA1933, *B. amyloliquefaciens* B-1895, and *M. luteus* ATCC 10240 were included in this assay. Bacterial strains grown overnight were diluted 1:100 with corresponding fresh media to yield approximately 10^6 $CFU \cdot mL^{-1}$. This was verified by the plate counting method. The disc diffusion test was conducted according to the (CLSI) Performance Standards for Antimicrobial Disk Susceptibility Tests [37]. The tested antimicrobial discs included ampicillin (10 µg), erythromycin (15 µg), and tetracycline

(30 µg) from Becton Dickinson and Company (Sparks, Maryland, USA), while bacitracin (10 µg), chloramphenicol (30 µg), kanamycin (30 µg), penicillin (10 IU), streptomycin (10 µg) and oxytetracycline (30 µg) were from Benex Limited (Shannon, Co. Clare). Plates were incubated for 24 h at 37 °C. Radii of zones of inhibition were measured in millimeters (mm) with a digital caliper (Fischer Scientific, Pittsburg, PA, USA) from the edge of the disk to the edge of the inhibition zone.

2.4. Bacterial Reverse Mutation (Ames) Assay

The mutagenicity assessment of subtilosin, CFSs of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 was carried out according to Maron and Ames [38], Cappuccino and Sherman and [39] with minor modifications. Briefly, *Salmonella typhimurium* K-6 TA1535 overnight growth was prepared according to the manual a day before performing experiment. Top agars were melted using a hot water bath (45 °C). Then, 300 µL of histidine/biotin and 100 µL of the overnight culture of *S. typhimurium* K-6 TA1535 were added to the top agars. Top agar contents were gently mixed and immediately poured over the minimal agar plates. A sterile forceps was used to pick up a filter paper disc and dip it into the two microcentrifuge tubes containing different concentrations of tested samples. Positive and negative controls were included. After filter paper discs were saturated with tested chemicals, they were placed into the center of a minimal agar plate that was laid out with top agar containing biotin and bacterial growth suspension. All the plates were incubated aerobically at 37 °C for 48-72 h in an inverted position. The results were evaluated by counting the number of colonies that grew on the agar plates. The mutant frequency was calculated for tested samples. Mutant

frequency was calculated as the number of revertant colonies in treated plates divided by their numbers in the negative control.

$$MF = \frac{NT}{NC}$$

Where MF= Mutant Frequency, NT= Number of revertant colonies on treated plates, NC= Numbers of colonies on negative control plate.

The results were expressed as following: the substance considered has mutagenic activity when its MF value is ≥2, is a possible mutagen when the value ranges of 1.7 to 1.9, and has no mutagenic activity when the frequency ranges ≤ 1-1.6 [40].

2.5. Determination of Protease Activity by the 'Stabbing' Method

Detection of the proteolytic activity of *Bacillus* strains, *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895, was performed as described by Ponmurugan [41] with minor modifications. Briefly, from the frozen stocks, bacilli were maintained in MRS broth aerobically with agitation (150 rpm), for 24 h at 37 °C. From the last overnight culture, 10 µL was spread on MRS agar by streaking with a loop and plates were incubated aerobically for 24 h at 37 °C. After the incubation period, one colony was picked up using an inoculating loop and stabbed into a milk agar plate. A milk agar composed of peptone (0.1%), NaCl (0.5%) and skim milk (10%) was prepared according to Uyar et al. [42] with some minor modifications. The components of the milk agar medium were mixed thoroughly with double deionized water and autoclaved for 15 min. The inoculated plates were incubated for 24 h at 37 °C under aerobic conditions. The results were reported as following: a clear zone of proteolytic activity around inoculated colonies

represented protease positive and if the clear zone did not appear, it was protease negative.

2.6. Hemolytic Activity on Whole and Defibrinated Blood Agar

A hemolytic assay was performed as described by Luo et al. [43] with minor modifications. Instead of inoculating blood agar with 10 µL of bacterial suspension using a disposable loop, a polyester tipped applicator (Fischer scientific, Pittsburg, PA) was used to spot-inoculate onto the whole and defibrinated blood agar. This was achieved by touching the tip of the applicator to one bacterial colony before using the tip to lightly touch the fresh blood agar while rotating the applicator. By using the polyester applicators, circular inoculation sites of about 5 mm in diameter were formed. Each bacterial strain was inoculated onto the blood agar using this method, and sufficient space was given between each spot. Inoculated blood agar plates were then incubated aerobically for 24 h at 37 °C. Plates were then checked for hemolytic activity.

2.7. Fibrinolytic Activity Test

One milliliter of LB broth was inoculated with *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 and incubated for 18 h at 37 °C with agitation. Each culture was adjusted to one unit of the MacFarland Turbidity standard. Then, 1 mL of the adjusted cell suspensions and 24 mL of fresh LB were mixed in 50 mL culture flasks, and incubated 5 days at 37 °C. The CFSs were collected by centrifugation (4480 *g*, 15 min, and 4 °C). Plates containing fibrin were prepared accordingly, the 1.5% agar-based formulation (10 mL) consisting of 0.4% fibrinogen in 50 mM sodium phosphate buffer at pH 7.4 was mixed with 0.1 mL of thrombin (10 NIH units) poured into the Petri dishes

(15 mL), and left to polymerize and to dry at room temperature. Then, 7 mm diameter holes were punched in the solidified agar. These holes were filled with 30 µL of the CFSs and incubated overnight. The clear zones of fibrinolytic activity were measured with a digital caliper.

2.8. Coaggregation Test

The coaggregation assay was performed to evaluate the coaggregation ability of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 strains with select pathogens following the method described by Cisar et al. [44] with some modifications. Bacterial cultures were harvested from the planktonically grown cells incubated at 37 ^{o}C by centrifugation (4480 *g*, 15 min, 23 ^{o}C); they were washed with sterile phosphate buffered saline (PBS) twice. After the second wash, the harvested cells were re-suspended in PBS and the optical density (OD_{600}) was adjusted to 0.25. In a 96-well microtiter plate, 100 µL of each test strain was mixed with 100 µL of *Bacillus* strain, while 200 µL of each bacterial suspension in monoculture was used as controls. The plate was placed in a micro-titer plate spectrophotometric reader (SmartSpecTM 3000) and kept at 30 °C. Measurements of OD_{600} were taken once per hour for 24 h and calculated for coaggregation. Each experiment was performed in quadruplicate. Samples of 100 µL were taken after 2 h reading for Gram staining and observed microscopically for coaggregation (Fig. 1).

2.9. Mathematical Analyses

Two methods were employed to calculate the percent of coaggregation after 2 h incubation in each of the mixtures, for the purpose of comparison. In the first method, the

percent auto-aggregation of each bacterium and the percent coaggregation in each mixture was calculated as described by Ledder et al. [35] with the following equation:

Method 1:

$$\text{Coaggregation \%} = \left(\frac{x-y}{x}\right) \times 100$$

Where x is the pre-incubation value and y is the post-incubation value at a certain time point.

The second method employed the equation described by Handley et al. [45]:

Method 2:

$$\text{Coaggregation \%} = \left[1 - \frac{2(x+y)}{(Ax+Ay)}\right] \times 100$$

Where Ax and Ay are the organisms as controls and (x+y) is a mixture of the two.

Both methods used optical density data obtained after 2 h, at OD_{600}. The data from the two methods were compared and the more appropriate method was chosen from the analysis of 2 h and used for the 8 h analysis.

2.10. Microscopy

Bacterial interactions at the 2 h time points were visualized on slides using histological techniques. To visualize, bacteria were stained with Gram stain (BD, Becton and Company, Maryland, US). Images were obtained with a Nikon DS-Fi1 camera mounted on a Nikon Eclipse 80i compound microscope using the 100x/ 1.25 oil objective. Images were analyzed using Nikon, NIS- Elements D3.0 software. The amount of coaggregation

was visually analyzed and scored with a scoring system, with 0 being the absence of coaggregation and 4 being an abundance of coaggregation (Fig. 1).

2.11. Production of *Bacillus* Spores

Sporulation-inducing esculin agar was composed of esculin hydrate (E8250-5G) (Sigma-Aldrich), 1 g, ferric citrate (Fisher scientific), 0.5 g, and BHI, 40 g. These components were mixed and completely dissolved in deionized water up to 1 L. The pH of the mixture was adjusted to 7.0, agar (1.5%) was added and the medium then was autoclaved. *Bacillus* sporulation was achieved following Franklin and Clark [46] with minor modifications. Briefly, the esculin agar medium was poured into 150×15 mm Petri dishes to achieve about a 10 mm thick layer. *Bacillus* grown on MRS plate was scraped and seeded onto an esculin plate. The inoculated agar was incubated for 15-25 days aerobically at 37 °C. After the first five days of incubation, spore production by the *Bacillus* strains was monitored daily using light microscope. Once sufficient numbers of spores were produced in the grown colonies, they were harvested using sterile inoculating loops. The spores were washed with sterile distilled water and pelleted by centrifugation (5444.5 *g*, 20 min at 4 °C). The pellets were re-suspended with 20 mL sterile distilled water, glass beads were added, and incubated with agitation at 75-80 °C for 25 min to ensure the killing of the vegetative cells. Following that, tubes were placed on ice for 10 min and then the glass beads were discarded. The suspension was collected by centrifugation (5444.5 *g*, for 20 min, at 4 °C) and washed three times with ice-cold sterile water. The spores were re-suspended in a minimum volume of sterile ice-cold water and counted by plating. The spore suspension was aliquoted and stored at -80 °C for future use.

2.12. Acid and Bile Tolerance of *Bacillus* Spores

The acid and bile tolerance method was performed according to Hyronimus et al. [47] with minor modifications. Briefly, a frozen stock of *Bacillus* spores was diluted with PBS to achieve 10^8 spores·mL^{-1}. Tubes containing spores were incubated at 80 °C for 20 min with agitation to get rid of remaining vegetative cells. After heat treatment, the tubes were placed on ice for 10 min. Ten milliliters of MRS broth was transferred into sterile tubes and the broth pH was adjusted to different pH values: 2.0, 2.5, 3.0 using 0.1 N HCl (Sigma Aldrich, St. Louis, MO). Ten milliliters of MRS broth containing 0.3% bile salts (Oxoid Ltd, Basingstoke, UK) was prepared and transferred into 50 mL test tubes. Control tubes containing broth medium only without adding acid or bile salts were included in this experiment. A 100 µL of diluted spore suspension contain 5×10^7 Spore Forming Unit per milliliter (SFU·mL^{-1}) was dispensed into each tube (acid, bile, and control tubes). At each time interval, 0, 0.5, 1, 2, and 4 h, the spread–plate method was used to enumerate the numbers of surviving spores after acid and bile treatment on MRS plates. The inoculated plates were incubated aerobically at 37 °C for 24-30 h. For each treatment, the survival rates were measured. Survival rate was defined as the percentages of the logarithmic number of SFU at each time point (s) divided by SFU numbers at 0 time point (control). For example, the survival rates after 4 h is (($\log_{10}$ SFU·mL^{-1} at 4 h)/($\log_{10}$ SFU·mL^{-1} at 0 h)) × 100.

2.13. Statistical Analysis

For the antibiotic susceptibility assay and antimicrobial activity of CFS of studied *Bacillus* strains, experiments were performed at least three times in triplicate. Co-

aggregation and auto-aggregation experiments were conducted three times; the collected values were then analyzed mathematically and visually. Acid and bile tolerance studies were repeated three times in duplicate and the results shown were expressed as mean (%) ± SD. Student's *t*-test with two-tailed distribution (Excel, Microsoft Corporation, US) was used to compare the survival rates (%) of the spores/ vegetative cells of bacilli strains in the 3 sets of pH during the 4 h incubation.

3. Results

3.1. Antimicrobial Activity of CFS of the Studied Bacilli

Selected pathogens were tested for their sensitivity to the CFS of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 strains (Table 2). All *P. gingivalis* isolates were sensitive to *B. subtilis* KATMIRA1933 CFS, as indicated by a zone of clearing of the bacteria (Table 2). *S. intermedius* was susceptible to CFS of *B. amyloliquefaciens* B-1895 (Table 2). *P. intermedia*, *F. nucleatum* and *S. mutans* were not inhibited by the CFS at the concentrations tested (data not shown).

3.2. Antibiotic Susceptibility of *Bacillus* Strains

The sensitivity of the *Bacillus* strains to nine antibiotics was assessed according to CLSI [48]. The antibiotics were ampicillin 10 µg, erythromycin 15 µg, tetracycline 30 µg, bacitracin 10 µg, chloramphenicol 30 µg, kanamycin 30 µg, streptomycin 10 µg, oxytetracycline 30 µg, and penicillin 10 IU. Antibiotic susceptibility test results revealed that *B. subtilis* KATMIRA1933 was tolerant to bacitracin and streptomycin, and more susceptible to penicillin, ampicillin and chloramphenicol than other tested antibiotics. The tolerance of *B. amyloliquefaciens* B-1895 strain to bacitracin, streptomycin, tetracycline and oxytetracycline was more than other antibiotics, and its susceptibility to

ampicillin and chloramphenicol was the highest. Antibiotic susceptibility of *Bacillus* strains was compared to that of *M. luteus*, which is a frequently used Gram-positive reference bacterium (Table 3).

3.3. Hemolytic and Fibrinolytic Activity Test

Hemolytic activity of the *Bacillus* strains was determined using MRS medium, supplemented by whole blood and MRS, supplemented with defibrinated blood. While *B. amyloliquefaciens* B-1895 produced weak hemolysis on whole blood agar plates, a zone of clearance was observed with *B. subtilis* KATMIRA1933 (Table 4). On defibrinated blood agar, both *Bacillus* strains displayed no hemolytic activity. *S. aureus* and *M. luteus* were used as positive and negative controls, respectively, for this set of experiments. Because hemolytic activity of *B. subtilis* KATMIRA1933 was observed on whole blood agar, we hypothesized that the predominant is the fibrinolytic activity. Using a fibrinolytic activity assay, it was demonstrated that *B. subtilis* KATMIRA1933 CFS produced a fibrinolytic zone of 14 mm diameter, while *B. amyloliquefaciens* B-1895 CFS formed a zone of 11 mm diameter.

3.4. Proteolytic Activity Test

To determine whether the *Bacillus* strains possess proteolytic activity, the presence or absence of a zone of clearance around bacterial growth and/or bacterial CFS on milk agar was determined; clearance is indicative of protein hydrolysis. *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 cells or CFS showed proteolytic activity with clear zone areas of 13±0.5/5±0.3 mm and 15±0.6/3±0.3 mm, respectively, after 24 h incubation. *E. coli* O157:H7 and its CFS were used as negative controls (data not shown).

3.5. Bacterial Reversal Mutation (Ames) Assay

To evaluate the mutagenic potential of the *Bacillus* extracts and purified compounds, with the hope of ensuring these natural products were free of mutagenic factors, the Ames test was conducted using *S. typhimurium* strain TA1535. In addition to the isolated subtilosin, CFS of the *Bacillus* strains were tested. The number of revertant colonies was counted on glucose minimal agar and the mutant frequency (MF) was calculated. The number of revertant colonies when MRS broth (negative control) was tested were 15-16 CFU per plate (Table 5); similar results were obtained when phosphate buffer saline (PBS; negative control) was tested (18-20 CFU per plate) (Table 5). According to Kirkland [40], a frequency that ranges from ≤ 1-1.6 for the tested substances indicates no mutagenic activity. The mutant frequency of 50 $\mu g \cdot mL^{-1}$ subtilosin was 1.4 higher when compared with concentration of 100 $\mu g \cdot mL^{-1}$ and 550 $\mu g \cdot mL^{-1}$ (Table 5). The MF of 100%, 50%, 25% and 12.5% CFS of *B. subtilis* KATMIRA1933 were 0.87, 0.9, 0.7 and 1, respectively. A low mutant frequency was determined for CFS of *B. amyloliquefaciens* B-1895, 0.56, 0.5, 0.7 and 0.13 when 100%, 50%, 25% and 12.5% respectively, were assessed.

3.6. Auto-aggregation and Coaggregation of Bacterial Strains

Kinetic measurements of auto-aggregation and coaggregation of the bacilli with pathogenic bacteria was determined during an 8 h time period using an automated micro-titer plate reader to quantitatively evaluate aggregation efficiency. Bacterial strains varied with respect to the time required to observe a high aggregation value. Two methods were used to evaluate the auto-aggregation and coaggregation percentages (Table 6). The highest percentages were obtained using the calculation of method 1, which was

previously described by Ledder et al. (2010). Method 1 was chosen in this study as the more convincible method, compared with method 2, after mathematical interpretation of optical density data; the method was similar in its application and more closely matched conclusions made from the microscopic analysis (Fig. 1 and Table 6). The percentage of auto-aggregation of *B. amyloliquefaciens* B-1895 was the highest (89.5%), while the lowest was of *S. aureus* and *S. enterica* (14.3% and 15.4%), respectively (Table 7). *B. amyloliquefaciens* B-1895 strain was highly co-aggregative with *E. coli* (47.1%), *P. aeruginosa* (46.9%), *S. enterica* (43.9%) and *L. monocytogenes* (41.9%), but it poorly co-aggregated with *S. aureus* (29.9%). In the case of *B. subtilis* KATMIRA1933, the high coaggregation percentage was observed with *E. coli* (50.3%) followed by *P. aeruginosa* (49.7%), *L. monocytogenes* (48.2%) and *S. enterica* (47.4%), while low coaggregation was observed with *S. aureus* (34%) and *S. mutans* (31.8%) (Table 8).

3.7. Tolerance of *Bacillus* Spores and Vegetative Cells to Acids and Bile Salts

To evaluate the acid and bile salt tolerance of the spores and vegetative cells, the bacilli were exposed to a range of acid (pH 2.0, 2.5 and 3.0) and 0.3% bile salts during 4 h. The survival rates (SR) (%) of *B. subtilis* KATMIRA1933 spores were constant (unchanged) during the incubation period at all pHs tested. At pH 2.0, the final SR was 98.4±2.3%, and slightly higher in pH 2.5 and 3.0, which were 98.75± 1.76% and 99.15±1.2%, respectively. The 4 h SRs for *B. amyloliquefaciens* B-1895 spores similarly were nearly constant across all pH conditions; in the pH 2.0 and 3.0 environment, the SR was 96.45±5.02%, while at pH 2.5 it was 97.3±3.81%. A significant difference was found between the survival rates of spores and vegetitative cells of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 into the three sets of pH values for 4 h. Bile tolerance

of the *Bacillus* spores was 88±1.27% for *B. subtilis* KATMIRA1933 and 84.85±2.05% for *B. amyloliquefaciens* B-1895.

As expected, the tolerance of vegetative cells of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 strains to acidity was much less than those of the spores. In the case of the *B. subtilis* KATMIRA1933 strain, vegetative cells were incapable of surviving at pH 2.0 and pH 2.5 after 1 h incubation, however at pH 3.0 the SR was 23.9% at 4 h. *B. amyloliquefaciens* B-1895 vegetative cells were more tolerant to acidic conditions than those of *B. subtilis* KATMIRA1933. About 30% of vegetative cells of *B. amyloliquefaciens* B-1895 were able to survive after 4 h at MRS with the different pH values. There was no difference in the tolerance of vegetative cells of studied Bacillus strains to bile salt when compared with the tolerance of their spores. Statistically significant differences in acid tolerance between the *Bacillus* strains were observed after 30 min of incubation (Data not shown). While there was not a statistically significant difference between the vegetative cells of both *Bacillus* strains ($P > 0.05$), the survival rates (%) in 0.3% bile salts exhibited a statistically significant difference between the spores of the two bacilli strains during the 4 h incubation ($P < 0.05$) (Data not shown).

4. Discussion

The *B. subtilis* KATMIRA1933 strain was used as a source of subtilosin A and studied for various applications such as for control of food-borne and other human pathogens [9]. *B. amyloliquefaciens* B-1895 Strain was reported as a putative probiotic in poultry and fish [13]. Based on the beneficial properties of both microorganisms, we hypothesize on their possible use as probiotics for human and/or agricultural applications. Therefore, the

effort was made to look into these strains' safety and some of their probiotic-related capacities.

Positive health effects were noticed such as increment food consumption and increasing of broilers' body weight [13]. An association between *Bacillus*-food supplements and immune system stimulation was identified in chicken. When birds were vaccinated against the Newcastle virus, the antibody titers in chicken with a *B. subtilis*-direct feed were greater than the control group without bacilli supplement-food [12].

The soil isolate, *B. amyloliquefaciens* B-1895 showed probiotic potential in Azov-Chernomoskaya royal fish by eliminating pathogens and increasing survival rate of fish [11]. Using the RAST server [49], the protein-encoding genes that are responsible for bacitracin-like antibiotics biosynthesis were identified [50].

B. subtilis KATMIRA1933 was isolated from a dairy product [9] and it has been extensively consumed by people without any negative reported side effects. *B. subtilis* KATMIRA1933 was detected as an antimicrobial-producer strain. The bacteriocin subtilosin, which is a secondary metabolite of *B. subtilis* KATMIRA1933, showed selective antimicrobial activity against pathogens such as *Gardnerella vaginalis* but not against protective (beneficial) lactobacilli [51][52].

The antimicrobial activity of CFSs of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 against oral pathogens was reported in this study. These results attracted our attention moving toward evaluating the probiotic potentials of these bacilli. Safety tests were performed to ensure the innocuous side effects of the utilization of such microorganisms in food and medical applications.

An active area of oral health research has been aimed at the identification of natural products to either kill or attenuate pathogenicity of the key bacterial strains that contribute to oral infections such as caries and periodontal disease. Much of the effort has focused on finding plant-based compounds that are able to act as antimicrobials or bacteria-modulating compounds. An alternative active area of research is the identification of bacterial factors that can act as therapeutics, and as presented here, products of soil isolates that display antimicrobial properties against two classes of sub-gingival plaque organisms that have been associated with periodontitis and second-site infections such as brain or liver abscess, as in the case of *S. intermedius*.

As demonstrated here, *B. subtilis* KATMIRA1933 extract has anti-microbial effects against all *P. gingivalis* strains tests; these represent a combination of fimbrinated, afimbrinated, encapsulated, and non-encapsulated strains. Previous studies addressing *P. gingivalis* susceptibility to *Bacillus* extracts identified that only those without a capsule were susceptible, however, the researchers used different growth conditions than were presented here [53]. In our experience, *P. gingivalis* growth in liquid culture is suboptimal and there is mixed success in diluting into liquid media with insufficient $CFU \cdot mL^{-1}$ concentration. The measure is growth over starting inoculum, but we are not told if there was a control well with no subtilosin addition, which would have been the appropriate comparison [53]. Presented here regardless of capsule formation, we observed inhibition of *P. gingivalis* growth; however we did observe that those strains without or with little capsule, 381 and 33277 respectively, were slightly more susceptible to killing by the extract, which is in agreement with the previous finding [53].

Interestingly, Lys-gingipains of *P. gingivalis* do not inhibit activity of subtilisin as reported by Shelburne et al. [53]. However, the studied *P. gingivalis* strain 33277 was already remarkably sensitive to subtilosin. It will be interesting to elucidate susceptibility of *P. gingivalis* W83 to subtilosin, since this strain is lacking some proteases and is likely to be more sensitive to the antimicrobial protein. The presence of the protease on the surface is likely to degrade subtilosin. However, it was shown that the mutant lacking the proteolytic enzyme coding gene displayed similar MIC to the parent strain. Arg-gingipain absence failed to induce further sensitivity to subtilosin. The authors state that this could be due to the lack of arginine residues in proteins. Subtilosin contains lysine residues, however it was not noted if the correct gingipain cleavage site is present in subtilosin, therefore the lys-gingpain also has the potential to be ineffective at cleavage. It should be noted that *P. gingivalis* has a host of proteases, and given the robust inhibition of growth, it would appear that the agent responsible for activity is not susceptible to proteolysis by *P. gingivalis* proteases. To confirm, this hypothesis would need to be formally tested.

Previous studies by Tsubura et al. [54] have demonstrated that Extraction 300E (E-300, AHC Co. Gunma, Japan), a preparation from the culture medium supernatant of Japanese soil *B. subtilis* isolate and the commercially available VITALREX (AHC Co., Gunma, Japan), a stable oral tablet of lyophilized *B. subtilis* DB9011, were both effective in reducing periodontitis levels in patients in comparison to controls [54][55]. The BANA tests that were used to assay for periodontitis showed reduced levels in patients treated with the compounds; this test indicates that there are organisms that are able to hydrolyze the synthetic peptide benzoyl- DL-arginine-naphthylamide, which are attributed to the Gram negative anaerobes present in the subgingival plaque, of which *P. gingivalis* is a

member. A reduction in BANA levels also coincided with a reduction in a number of target bacteria, including *P. gingivalis* and *P. intermedia* [55].

It is being debated which tests may serve as a “minimum requirement” to characterize a putative probiotic’s safety and value [1][56][57]. Most agree that the antibiotic susceptibility of bacterial cells is the test of priority to identify if they are tolerant or sensitive to commonly prescribed antibiotics. Resistance of probiotics to antibiotics has both positive and negative impacts on human health. When bacterial resistance is intrinsic, it helps and supports the restoration of intestinal microbiota after a course of antibiotics that are administrated to the host for infection treatment [57]. At the same time, it is problematic when antibiotic resistance-genes in probiotics are transferable to other microbiota or pathogens leading to the appearance of new resistant strains [58]. In our study, we evaluated the antibiotic susceptibility, using a disc diffusion test, of the two *Bacillus* strains to nine antibiotics. The tested bacilli were more tolerant to bacitracin and streptomycin but susceptible to penicillin, ampicillin, erythromycin and chloramphenicol more than other used antibiotics. Also, tolerance of *B. amyloliquefaciens* B-1895 to tetracycline and oxytetracycline was higher than of *B. subtilis* KATMIRA1933.

Bacitracin production by the *Bacillus* themselves reflects the natural resistance of *Bacillus* strains to these antibiotics. *Bacillus* resistance to bacitracin occurs either through the specific transporter protein, BcrABC, which takes bacitracin out of the cell [59] or by an undecaprenol kinase, which provides C_{55}-isoprenyl phosphate, the BacA [60]. A putative bacitracin transport permease has been identified in *Bacillus subtilis*. This protein encoded by the *B. subtilis bcrC* (*ywoA*) gene is associated with bacitracin resistance [61]. Our data were in agreement with Senesi et al. [62] and Adimpong et al.

[63] who found that all tested *Bacillus* strains were resistant to streptomycin and tetracycline at certain concentrations. Resistance of *B. subtilis* KATMIRA1933 to several antibiotics was not a surprising finding and it was confirmed by genome annotation data that was performed by Karlyshev et al. [64] for the studied bacilli using RAST analysis [49]. In addition to multidrug resistance efflux pumps encoding genes in *B. subtilis* KATMIRA1933, genes coding for resistance to vancomycin, fluoroquinolones, fosfomycin, and β-lactam antibiotics were also identified. Importantly, these studies referred to the fact that the probiotic bacilli that are used in animal and human food industries have shown multi-drug resistance behavior, especially toward streptomycin and tetracycline [65]-[67]. In the case of *B. amyloliquefaciens* B-1895, the β-lactamase gene, the streptothricin acetyltransferase-biosynthesis gene, and genes for resistance to fluoroquinolone and tetracycline antibiotics were also detected [50].

Antibiotic resistance of bacterial species is either intrinsic or acquired by the transfer of a gene from plasmids, transposons, or the mutation of the bacterial gene [68][69]. Mazza et al. [70] tested the resistance stability of antibiotic resistance markers existing in *B. subtilis* O/C, T, N/R, and SIN strains. Four therapeutic antibiotics were considered (chloramphenicol, tetracycline, rifampicin and streptomycin). They noticed that the resistance stability to tetracycline, rifampicin, and streptomycin existed for at least 200 generations without selective pressure. *In vivo* and *in vitro* studies explained the “absence of homologous transfer of resistance markers among the resistant strains” [70]. *Bacillus* species such as *B. subtilis* have been included on the Qualified Presumption of Safety (QPS) microbes list. The QPS list was generated including microbial taxonomic units in which the acquired antibiotic resistance-genes are absent [3][71].

In addition to antibiotic resistance, toxigenic potential such as the hemolytic activity [72] of *Bacillus* was evaluated in this study. It is known that hemolysin enzyme production is one of the virulence factors of pathogenic microorganisms. In whole blood agar, a weak hemolytic zone around *B. subtilis* KATMIRA1933, but not *B. amyloliquefaciens* B-1895, was observed and compared with the clearer zone of hemolysis produced by *S. aureus*, a positive control. No hemolytic activity of both *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 was detected on defibrinated blood agar. Therefore, we assumed that the activity of *B. subtilis* KATMIRA1933 on the whole blood agar was fibrinolytic, not hemolytic action. To confirm our assumption, a fibrinolytic assay was performed for both tested bacilli. The clear zone of fibrinolytic activity produced by *B. subtilis* KATMIRA1933 was wider than the zone generated by the *Bacillus* strain *B. amyloliquefaciens* B-1895. The question that remains is why *B. amyloliquefaciens* B-1895, which has fibrinolytic activity like *B. subtilis* KATMIRA1933, did not produce the zone of hemolysis on the whole blood agar. However, hemolytic activity was determined in commercial human *Bacillus* species commonly used as probiotics [72][73]. Hemolytic activity is a highly recommended test by EFSA to ensure that the bacterial strain was free of the toxigenic potential [74]. Although studies on fish and pigs reported that the hemolytic activity of microbial enzymes *in vitro* does not necessarily produce any negative effect *in vivo* [75][76], hemolytically active bacteria are not recommended as feed additives according to EFSA guidelines [71]. Based on our results, we propose the evaluation of bacterial hemolytic activity accompanied by the assessment of fibrinolytic activity.

A bacterial reverse mutation (Ames) assay was performed to evaluate the mutagenic potential and ensure safe utilization of *Bacillus*' metabolites. The mutant frequency value confirmed that CFSs of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 were free from mutagenic substances. In this work, we used *S. typhimurium* K-6 TA1535 as *his*-mutant strain without using a metabolic activator (S9 mix). Mortelmans and Zeiger [30], Vijayan et al. [77], and Lupi et al. [78] found no significant differences in the number of revertant colonies of *S. typhimurium* TA1535 in the presence or absence of S9. The results of the Ames test were considered as primary data but did not guarantee that bacilli cell products do not have any mutagenic or carcinogenic active substances [79]. We agree that some supportive tests are needed to strengthen our finding, such as the Micronucleus assay, *in vitro* chromosomal aberration assay, and oral toxicity studies in rats to identify genotoxicity and clastogenicity [79].

According to the WHO definition that probiotics produce health benefits in the host, proteolytic activity of the tested bacilli was evaluated and identified. Our data showed that *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 behaved like many previously studied *Bacillus* species, showing a clear zone of proteolytic activity when they were inoculated on milk agar medium. Sevinc and Demirkan [80] found that utilizing skim milk agar for the qualitative test of protease is better than casein agar. The advantages of proteolytic enzymes have been reported in many studies [27]-[29]. In these studies, the importance of proteolytic enzymes includes the activation of regeneration processes, the enhancement of fibrinolytic activity in the plasma, the enhancement of normal digestion processes, and degradation of allergic and chemical compounds.

The ability of probiotics to co-aggregate and auto-aggregate is considered an advantageous characteristic feature. Their adhesion to a pathogenic organism can facilitate the elimination of the organism from the body and its ability to self-aggregate gives it an advantage in a competitive environment. The microorganisms employed in this study were chosen as common pathogenic organisms found in products of consumption. Two mathematical analyses of the coaggregation data were evaluated, Method 1 was described by Ledder et al. [35] and Method 2 was described by Handley et al. [45]. The more comprehensive method for mathematical interpretation of optical density data was chosen on the basis of two requirements. Foremost, the calculated percentage of coaggregation had to make sense in its application. A mathematical form of analysis may often fall short when the parameters of a biological system must be taken into account, not quite "fitting" in a representative attempt. Second of all, the percentages that better reflected the visual analysis were identified. The extent of coaggregation and auto-aggregation was determined from the data that was found using the more appropriate method.

Method 1 appeared to be most appropriate for the data analysis in this study, for it agreed with the microscopic (visual) observations (Fig. 1). Method 2 failed to adequately reflect the adhesion in a number of mixtures of microorganisms that were easily observed microscopically. For instance, some Method 2 derived values were <0, although coaggregation was clearly visible under the microscope (Fig. 1). This method error could be due to any number of factors that affected the microorganisms, if the equation reflects a specific state. Method 1, however, gave percentages of coaggregation that more closely

resembled the scores given during visual analysis, signifying that it was more appropriate for the data at hand.

After 2 h, *B. amyloliquefaciens* B-1895 adhered most to *P. aeruginosa* and *E. coli* more than other bacterial cells. Though these data did ideally match the coaggregation percentages of *B. amyloliquefaciens* B-1895 with *P. aeruginosa and E. coli,* which were found to have 31.5% and 31.4%, respectively, at this optical density when method 1 was applied (Table 6).

B. subtilis KATMIRA1933 adhered mostly to *E. coli* and *P. aeruginosa*, but less to *S. enterica*, *S. aureus,* and *L. monocytogenes* (Table 8*)*. Similarly, *B. subtilis* KATMIRA1933 mostly coaggregated with the above-mentioned bacterial cells. Both *Bacillus* strains were found to have auto-aggregating abilities after 2 h, especially *B. amyloliquefaciens* B-1895 (86%), which was also reflected in the visual analysis (data not shown).

According to the percentages of auto-aggregation (Table 7), *B. amyloliquefaciens* B-1895 showed greater instances of auto-aggregation than *B. subtilis* KATMIRA1933, at a more significant level, during 8 h. For *B. amyloliquefaciens* B-1895, the highest percentage of coaggregation was noticed with *E. coli* and *P. aeruginosa* and the lowest was with *S. aureus* (Table 8). *B. subtilis* KATMIRA1933 was highly coaggregated with *E. coli*, *P. aeruginosa*, and *L. monocytogenes* but less with *S. mutans* (Table 8).

Spores of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 showed high tolerance to different pH values of 2.0, 2.5, and 3.0 that simulate gastric conditions. The survival rates (%) of SFU in various acidic conditions during 4 h of incubation were

either similar or less than 1-log reduction of the viable spore count when compared with a control (at time zero), and more than 80% of *Bacillus* spores survived in 0.3% bile salts (Data not shown). Duc et al. [81] stated that not all the spores of *Bacillus* probiotic strains were tolerant to gastric acidity and bile compounds in the upper gastrointestinal tract. Diversity in acid and bile tolerance of *Bacillus* species spores has been detected. When *B. coagulans* Unique IS-2 spores were evaluated by Sudha et al. [82], a 2-log reduction of $SFU \cdot mL^{-1}$ was identified after exposure to pH 1.5 and a 1-log reduction at both pH 2.0 and 3.0 at 3 h. Also, three strains of *B. coagulans* (BCI4 LMAB, CIP5264 and CIP6625) were tested by Hyronimus et al. [47], who reported the high susceptibility of these strains to acid (pH 2.5) and 0.3% bile after 3 h incubation. Our findings were in agreement with Guo et al. [83] who found that the number of *B. subtilis* MA139 that exerted probiotic potential in the gastrointestinal tract of pigs, was steady (unaffected) at pH 2.0 and in nutrient broth supplemented with 0.3% bile for 3 h. The high tolerance of *Bacillus* spores to gastric acidity and bile salts of the proximal intestine, compared with vegetative cells, are assorted properties required for the selection of probiotics [84] and are promising in acidified food packaging and oral pharmaceutical applications.

Germination of *Bacillus* spores in the intestinal environment has been mentioned in many studies [73][85][86]. Studies such as Barbosa et al. [87], Duc et al. [81] and Fakhry et al. [88] were conducted to evaluate the tolerance of vegetative cells of *Bacillus* to acids and bile salts. Our data were in agreement with these studies in which high susceptibility of cells, in contrast to spores, to both conditions was reported. Studies of spore structures are required to determine the correlation with the susceptibility of some *Bacillus* spores to acids or bile salts. Evaluation of the survivability of spores in the presence of

gastrointestinal tract enzymes, lysozymes, acids and bile salts [84][86] need to be conducted *in vivo* to reflect the real assessment of *Bacillus* stability (sporulation or germination) under such conditions.

Before the establishment of probiotic capacity, other properties of *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 should be further evaluated such as cell adhesion, hydrophobicity, and genotoxicity.

To conclude, safety of the studied bacilli to human health should be established and completely confirmed due to the exciting potential of *Bacillus* for personal care, food and medical applications.

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Conflict of interest

The authors declare no conflict of interest.

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Tables and Figures

Table 1. Bacterial strains and culture conditions used in this study.

Bacterial Species and Origin	Source / culture medium	Importance	References
Bacillus subtilis KATMIRA1933	Dairy products / MRS	Bacteriocin-producer bacteria	[9]
Bacillus amyloliquefaciens B-1895	Soil isolate/ MRS	Proteolytic enzyme-producer, Probiotics in royal fish and food supplement	[11][12][50]
Micrococcus luteus ATCC10240	Skin of humans and other animals and in soil, marine and fresh water, plants, fomites, dust, and air/ TSB	Opportunistic pathogens for the immunocompromised	[89][90]
Escherichia coli O157:H7	Food and human pathogen/ TSB	Intestinal infection andfood-related outbreaks	[91]
Staphylococcus aureus ATCC 13565	Food and human pathogen/ TSB	Staphylococcal food poisoning, nosocomial infections and infections on indwelling medical devices	[92][93]
Streptococcus mutans ATCC 33402 and *Streptococcus mutans* strain 25175	Human-Oral pathogen/ BHI	Dental decay	[94]
Streptococcus intermedius strain F0413	Human CNS[a] and pulmonary pathogen/BHI	CNS abscesses, pulmonary infection	[95][96]
Salmonella enterica Stanley 7308	Intestinal pathogen/ TSB	Contaminated seeds sprout-bacteria	[97][98]
Listeria monocytogenes Scott A	Foodborne and human pathogen/ TSB	Food-borne and human disease (human Listeriosis)	[99][100]
Campylobacter jejuni ATCC33560	Human and nimal pathogen / TSB	Gastroenteritis (zoonotic infection)	[101][102]
Pseudomonas aeruginosa ATCC15442	Human pathogen/ TSB	Contamination of ophthalmic phanmaceuticals and nosocomial pathogen (cystic fibrosis)	[103]-[105]
Porphyromonas gingivalis strains 381, W83, 33277, A7A1-28 (ATCC 53977)	Human-oral pathogen/ TSB-THB	Periodontal breakdown and disease and acute necrotizing ulcerative gingivitis	[106][108]
Prevotella intermedia strains 25611, 17	Human-oral pathogen/ TSB-THB	Development and progression of periodontal disease	[109][110]
Fusobacterium nucleatum strain ATCC 25586	Human commensal and pathogen/ TSB-THB	Oral infections, adverse pregnancy outcomes, [b]GI disorders, colorectal cancer	[111]-[114]

[a]CNS: Central Nervous system, [b]GI: Gastrointestinal

Table 2. *Bacillus* extract-induced zones of inhibition of oral pathogens.

***Bacillus amyloliquefaciens* B-1895 extract**	**Diameter (mm)**
Streptococcus intermedius F0413	14.8±2.0
***Bacillus subtilis* KATMIRA1933 extract**	**Diameter (mm)**
Porphyromonas gingivalis 381	15.8±0.6
Porphyromonas gingivalis 33277	15.6±0.6
Porphyromonas gingivalis W83	13.7±1.0
Porphyromonas gingivalis A7A1-28	13.6±0.9

Table 3. Antibiotic susceptibility test of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895.

Antimicrobial Disc	Dose (µg)	*Bacillus subtilis* KATMIRA1933	*Bacillus amyloliquefaciens* B-1895	*Micrococcusluteus* ATTC1420
Ampicillin	10	16±0.4 mm	18±0.3 mm	14±0.4 mm
Erythromycin	15	15±0.2 mm	15±0.4 mm	15±0.2 mm
Tetracycline	30	13±0.6 mm	8±0.2 mm	14± 0.9 mm
Bacitracin	10	2±0.3 mm	6±0.2 mm	18 ±0.3 mm
Chloramphenicol	30	16±0.3 mm	17±0.4 mm	13 ±0.4 mm
Kanamycin	30	13±0.3 mm	11±0.7 mm	7 ± 0.4mm
Penicillin[a]	10	17±0.4 mm	15±0.4 mm	14±0.7 mm
Streptomycin	10	2±0.7 mm	7±0.3 mm	7 ±0.4 mm
Oxytetracycline	30	14±0.6 mm	9±0.3 mm	15±0.3 mm

[a]Penicillin was 10 IU

Table 4. Hemolytic Activity of *Bacillus* strain and their Cell free Supernatants (CFS).

Bacterial species	Blood hemolysis (Whole blood)	Blood hemolysis (Defibrinated blood)
Micrococcus luteus	-	-
Staphylococcus aureus	++	++
Bacillus subtilis KATMIRA1933	++	-
Bacillus amyloliquefaciens B-1895	+	-

++=Complete β-hemolysis, +=Weak hemolysis, -=no hemolysis.

Table 5. Mutagenic and carcinogenic assay (Ames test).

Chemical substances	No. of CFU	Mutant frequency
4-NOPD Crystals (Positive Control)	30-38	2-2.4
Subtilosin	26	1.4
50 µg/mL	20	1.1
100 µg/mL	14	0.87
550 µg/mL		
CFS of *Bacillus subtilis* KATMIRA1933		
100%	14	0.87
50%	14	0.9
25%	11	0.7
12.5	15	1
CFS of *Bacillus amyloliquefaciens* B-1895		
100%	9	0.56
50%	8	0.5
25%	10	0.7
12.5%	20	1.3
PBS (Negative Control)	18-20	1
MRS (Negative Control)	15-16	1
Disc only	0	0

Table 6. Comparison between method 1 (as described by Ledder et al. [35]) and method 2 (as described by Handley et al. [45]) to calculate the Co-aggregation values of *Bacillus* strains with tested pathogens after 2 h incubation.

Bacillus strains	Bacterial species	Method1 (%)	Method2 (%)	Microscopic analysis
Bacillus subtilis KATMIRA1933	*Escherichia coli*	16.5	8.1	3
	Pseudomonas aeruginosa	15.6	6.5	3
	Staphylococcus aureus	14.4	5.8	1-2
	Listeria monocytogenes	14.6	1.8	2-3
	Salmonella enterica	17.1	3.6	3-4
	Streptococcus mutans	12	1.8	2
	Campylobacter jejunii	14.5	1.9	2
Bacillus amyloliquefaciens B-1895	*Escherichia coli*	31.4	5.9	3
	Pseudomonas aeruginosa	31.5	<0	3-4
	Staphylococcus aureus	29.9	<0	1
	Listeria monocytogenes	30.3	<0	3
	Salmonella enterica	31.6	<0	2
	Streptococcus mutans	28.9	<0	3
	Campylobacter jejunii	25.5	<0	2

Table 7. Highest auto-aggregation of the tested microorganisms as observed during 8 h of incubation.

Bacterial strains	Auto-aggregation (%)	Time (h)
Listeria monocytogenes	28.08	6
Streptococcus mutans	29.4	8
Escherichia coli	32.8	8
Staphylococcus aureus	14.3	4
Pseudomonas aeruginosa	20.5	8
Salmonella enterica	15.4	8
Campylobacter jejunii	64.9	8
Bacillus subtilis KATMIRA1933	59.5	8
Bacillus amyloliquefaciens B-1895	89.5	8

Table 8. The highest co-aggregation % of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895 with the selected pathogens during 8 h of incubation.

Pathogens	*Bacillus subtilis* KATMIRA1933 Cells %	Time (h)	*Bacillus amyloliquefaciens* B-1895 Cells %	Time (h)
Escherichia coli	50.3	8	47.1	6
Pseudomonas aeruginosa	49.7	8	46.9	8
Salmonella enterica	47.4	8	43.9	7
Campylobacter jejunii	38.7	8	33.3	4
Staphylococcus aureus	34	8	29.9	2
Streptococcus mutans	31.8	8	35.5	4
Listeria monocytogenes	48.2	8	41.9	5

Table 9. Survival rate (%) of spores and vegetative cells (VC) of *Bacillus* strains under acidic conditions during 4 h

Time (h)	*B. subtilis* KATMIRA1933 Spores			*B. subtilis* KATMIRA1933 Vegetative cells			*P* value
	pH2	pH2.5	pH3	pH2	pH2.5	pH3	
0.5	99.2±1.1	97.15±4.03	99.5±0.7	25.1±1.69	47.3±0.84	45.45±6.85	<0.001
1	98.95±1.5	98.75±1.77	99.4±0.84	23.9	27.5	26.3	<0.001
2	100	97.8±3.1	99.35±0.91	0	0	26.3	<0.001
4	98.4±2.3	98.7± 1.76	99.15±1.2	0	0	23.9	<0.001
Time (h)	*B. amyloliquefaciens* B-1895 Spores			*B. amyloliquefaciens* B-1895 Vegetative cells			*P value*
	pH2	pH2.5	pH3	pH2	pH2.5	pH3	
0.5	98.2±2.54	97.3±3.81	96.6±4.8	43.15±7.1	47±1.69	71.4±4.38	0.004
1	97.3±3.81	96.7±4.66	96.45±5.02	43.68±0.98	33.1±4.2	38.8±1.41	<0.001
2	96.6±4.8	97.25±3.88	96.6±4.8	33.7±3.39	34.9	36.15±3.46	<0.001
4	96.45±5.02	97.3±3.81	96.45±5.02	28.9	35.5±0.84	31.9±5.93	<0.001

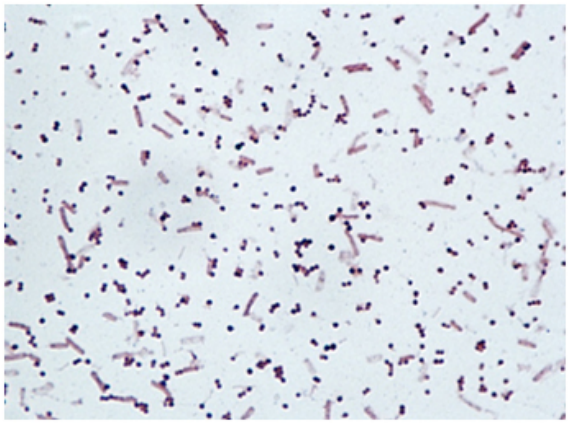
Table 10. Survival rate (%) of spores and vegetative cells (VC) of *Bacillus* strains in the presence of 0.3% bile salts during 4 h

Bacillus strains	Time				*P* value
	0.5	1	2	4	
B. subtilis KATMIRA1933 spore	96.1±5.51	91.95±2.75	90.3±2.68	88±1.27	0.17
B. subtilis KATMIRA1933 / Vegetative cells	96.1±1.83	97.4±2.75	94.35±0.63	89±1.69	
B. amyloliquefaciens B-1895 spore	88.05±0.07	87.4±0.56	87.45±1.76	84.85±2.05	0.11
B. amyloliquefaciens B-1895 Vegetative cells	95.45±1.62	94.8±0.7	90±4.66	83.35±4.59	

Figure 1. Microscopic analysis of auto-aggregation and coaggregation. A 0-4 scoring system was utilized, with 0 representing no adhesion between similar or different microorganisms, and 4 representing maximum aggregation after 2 h incubation.

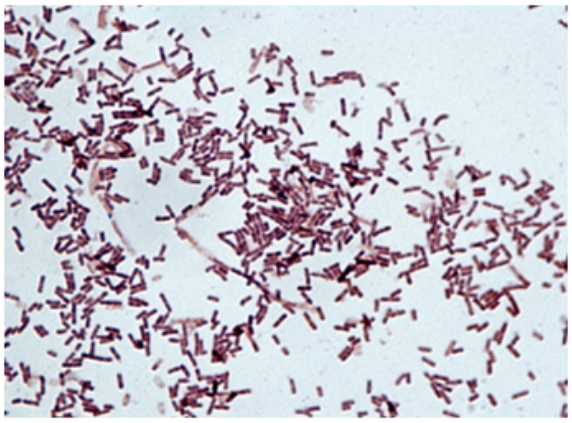
Bacillus amyloliquefaciens B-1895 and *Staphylococcus aureus* ATCC 13565

Minimal co-aggregation: 1



Bacillus subtilis KATMIRA1933

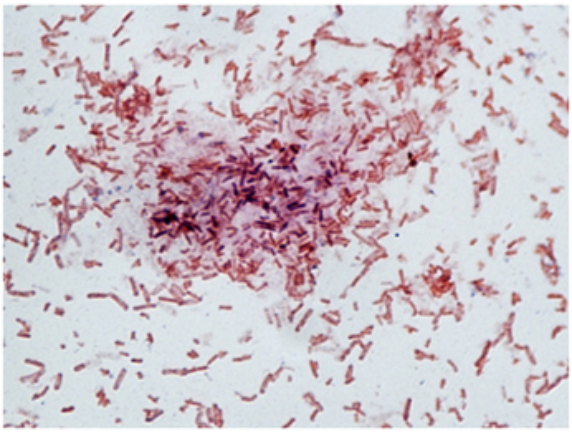
Some auto-aggregation: 2



Bacillus amyloliquefaciens B-1895

and *Pseudomona aeruginosa* ATCC15442

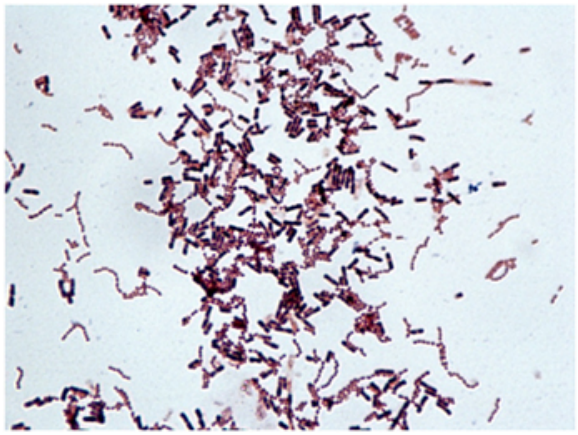
Significant co-aggregation: 3



Bacillus subtilis KATMIRA1933

and *Streptococcus mutans* ATCC 33402

Abundance of co-aggregation: 2



Chapter 5: SUGGESTIONS FOR FUTURE STUDIES

The reported studies (Chapters II and III) were focused on evaluation of in vitro efficiency of natural antimicrobials, alone and as multi-synergistic formulations against BV-associated pathogens. We reported on subtilosin's ability to selectively kill vaginal pathogens but not healthy vaginal lactobacilli, both alone and in combination with antibiotics. This finding should attract the attention of those who are interested in possible use of this kind of antimicrobials include such antimicrobial in pharmaceutical application for treatment BV infection. In this regards, several future studies are suggested.

1. To elucidate mechanism of synergistic action of subtilosin and selected antimicrobials against pathogenic biofilms. In this regards, the biofilm will be treated with the assayed antimicrobial combinations. Then, Laser Scanning Confocal Microscopy will be used for 3D imaging of biofilm structure, and Electron Microscopy/ Transmission Electron Microscopy for observing biofilm-associated cell features.

2. In vivo study needs to be performed to estimate anti-biofilm activity of subtilosin and tested natural antimicrobials alone and in combination with conventionally used antibiotics metronidazole or clindamycin. This study will be preceded by evaluation of each combination's safety in vivo using animal models and in vitro using cytotoxicity assay on EpiVaginal (VEC-100) ectocervical tissue.

3. Substances such as peptides, enzymes, essential oils and nanoparticles should be elucidated in combinations with commonly prescribed antibiotics for combating persistent infections in synergistic action. The importance of combination studies is in

reducing the cost, the doses, and the harmful side effects when each antimicrobial would be used individually.

4. Biofilm prevention is as important as biofilm removal. Inhibition of biofilm initiation increased the possiblity of attacking and killing the bacterial cells easily (compared to biofilm cells). Prevention of biofilm formation can be achieved through disarming of physical (bacterial cell attachment mechanism, organells, etc.) or chemical (quorum signalling compounds) factors. Benzoyl peroxide (BP) and salicylic acid (SA), for example, have been previously reported as inhibiting biofilm formation in *S. typhimurium*. We suggest to evaluate the inhibitory effect of BP and SA against planktonic and biofilm-associated *G. vaginalis* (work in progress). In addition, the mechanism of quorum sensing inhibition and its role in control of biofilms will be studied using colorimetric methods for quantification of Acyl Homoserine Lactones using *Chromobacterium voilaceum* (for Gram negative bacteria) and evaluation of Fe(III) ion reduction for quantification of autoinducer-2 (for Gram positive and Gram variable bacteria). The safety assessment of BP and SA substances should be established and ensured for the possible commercial applications. In addition to these compounds, the QS inhibition activity of subtilosin will be evaluated as well.

5. In chapter (IV), initial safety evaluation of *B. subtilis* KATMIRA1933 CFS have been performed. Further quantitative assessments of *Bacillus* metabolites are still required to ensure safety of these substances in the future applications. These tests include evaluation of hemolytic activity, cytotoxicity on human fibroblast, in addition to mutagenicity and carcinogenicity screens

6. Various cost effective commercially available substrates should be evaluated as a source of nutrients for biological production of natural derived antimicrobials.

Curriculum Vitae

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Education

October 1999 - October 2004	College of Veterinary Medicine, University of Baghdad, Iraq.
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Publications:

1. **Turovskiy, Y., Cheryian, T., Algburi, A., Wirawan, R.E., Takhistov, P., Sinko, P.J. and Chikindas, M.L.** 2012. Susceptibility of *Gardnerella vaginalis* biofilms to natural antimicrobials subtilosin, ε-poly-L-lysine, and lauramide arginine ethyl ester. *Infectious diseases in obstetrics and gynecology*, 2012, 284762. http://doi.org/10.1155/2012/284762.

2. **Algburi, A., Volski, A. and Chikindas, M.L.** 2015. Natural antimicrobials subtilosin and lauramide arginine ethyl ester synergize with conventional antibiotics clindamycin and metronidazole against biofilms of *Gardnerella vaginalis* but not against biofilms of healthy vaginal lactobacilli. *FEMS Pathogens and disease*, 73(5), p.ftv018. http://dx.doi.org/10.1093/femspd/ftv018.

3. **Algburi, A., Volski, A., Cugini, C., Walsh, E.M., Chistyakov, V.A., Mazanko, M.S., Bren, A.B., Dicks, L.M.T. and Chikindas, M.L.** 2016**.** Safety properties and probiotic potential of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895. *Advances in Microbiology*, 6, 432-452. http://dx.doi.org/10.4236/aim.2016.66043.

4. **Algburi, A., Comito, N., Kashtanov, D., Dicks, L.M.T. and Chikindas, M.L.** 2016. Control of biofilm formation: antibiotics and beyond. Submitted to *Applied and Environmental Microbiology*. AEM01664-16.