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THE BACTERIOCIN SUBTILOSIN A AND SYNERGISTICALLY-ACTING CONVENTIONAL ANTIMICROBIALS FOR CONTROL OF BACTERIAL VAGINOSIS ASSOCIATED PATHOGENS

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ABSTRACT OF THE THESIS

The bacteriocin subtilosin A and synergistically acting conventional antimicrobials for control of bacterial vaginosis associated pathogens

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Bacterial vaginosis is one of the most prevalent vaginal infections in women of child-bearing age and is caused by the shift of healthy lactobacilli to anaerobic pathogenic bacteria including *Gardnerella vaginalis.* While current antibiotic treatments were initially effective there is increasing evidence of resistance as well as damage to normal flora. The papers herein address the usage of bacteriocins for the purpose of indicating methods of more efficiently inhibiting *G. vaginalis* while having little effect on normal flora. (i) is a review on the current landscape of antibiotics and posits that bacteriocins present a viable option as either synergists to current antibiotics or as potential next generation option. (ii) is a research paper on the interaction of four antimicrobials (lauramide arginine, ε-poly-L-lysine, clindamycin phosphate and metronidazole) and subtilosin against *G. vaginalis* and four clinical isolates of lactobacilli. The combinations of CS and MS were synergistic in terms of FICI while all tested combinations displayed Bliss synergy against *G. vaginalis*. (iii) is a research paper in which a polyethylene glycol (PEG) gel with passively entrapped subtilosin was shown to cause a 8 $\log_{10}$ CFU/ml reduction of *G. vaginalis* with quantities $\leq$ 15 μg/mL of subtilosin in a bimodal release kinetic in OD_{595} endpoint experiments. No inhibition of lactobacilli spp. was observed. Rheological properties were also recorded, indicating the

gel is appropriate for vaginal application. (iv) is a polycarbophil Carbopol® 934P hydrogel which encapsulates benzoyl peroxide capable of inhibiting *G. vaginalis* but not normal vaginal lactobacilli. Gels with 0.01% v/v of BPO inhibited *G. vaginalis* while gels with 2.5% v/v of BPO inhibited tested lactobacilli in direct contact time kill assays and well diffusion assays. Rheological tests indicated that the gel was ideal for human usage and that the addition of BPO did not alter its physiochemical properties. (v) is a review that discusses bacteriocins and their usage in delivery systems. Each paper is linked through the ideas that of bacteriocins (i, ii, iii, v), bacteriocins and BV (ii, iii), BV (ii, iii, iv) or drug delivery systems (ii, iii, iv, v).

DEDICATION

This thesis is dedicated to **Veronica Loscalzo** and **Salvatore Cavera** who, without their love, compassion, and humor, this thesis would not have been possible.

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nil sine labore

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FIGURES

[Ψ] For brevity, figures are organized in order in which they appear and are listed by the appearance in manuscript number.

Bacteriocins and their position in the next wave of conventional antibiotics

This review focuses on current discussion of the antibiotic crisis and posits that bacteriocins serve as an attractive alternative. It does so by first discussing the broader reasons behind the antibiotic crisis, e.g. the economic, regulatory and scientific causes as well as their interrelatedness. Following this, the review expands upon the scientific advantages of bacteriocins; first by discussing how bacteriocins target four of the five classic drug targets (bacterial peptidoglycan/cell wall synthesis, DNA replication and transcription, and disruption of the bacterial membrane). As of writing this review bacteriocins have not been identified as having any interaction with folate biosynthesis. It then discusses how bacteriocins have a novel target, septum formation. Following this is a section on bacteriocin engineering which discusses multiple mechanisms and instances of increased effectiveness are discussed. Bacteriocins as immunomodulators, two-component regulatory system manipulations and the effects in Lotka-Volterra iterative dynamics are discussed. The review concludes with a discussion limitations and suggestions for future research.

The review was written by the candidate with advice and direction from the thesis advisor. The table was designed by Timothy Arthur.

Bacteriocins and their position in the next wave of conventional antibiotics

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Abstract

Microorganisms are capable of producing incredible diverse selection of defense mechanisms including antibiotics, bacteriocins, lytic agents, protein exotoxins, etc. Such mechanisms have been identified in nearly 99% of studied bacteria. The multiplicity and diversity of bacteriocins and the resultant effect of these interactions on microbial ecology has been thoroughly studied. However, the use of incorporating bacteriocins into drug delivery systems to be used in conjunction with or in order to replace conventional antibiotics the field is rapidly emerging since the last decade. The extensive array of bacteriocins positions them as one of the most promising options in the next wave of antibiotics. The goal of this review is to explore the three dimensions of the current antibiotic crisis and to determine the candidacy of bacteriocins by their ability to fulfill multiple classic drug targets as well as novel targets, improved efficacy when engineered, their natural usages in competition arrays, use in synergy, and research in drug delivery systems. The final portion of this review will discuss the current research landscape as well as limitations.

Introduction

The rate at which conventional antibiotics are becoming increasingly less effective in treating common infections is greater than ever. The result of resistance stems from multiple causes which can be subdivided by the country's income. In high-income countries, persistent, frequent administration of antibiotics in hospitals, the community, and agriculture has lead to the usage of more broad-spectrum antimicrobials. Failure to follow regimens properly and use of suboptimal doses has lead

to a selective pressure for resistant strains (1, 2). In low-income countries, antibiotic usage is increasing in conjunction with the relative income of the nations' citizens, high rate of hospitalizations, and increased prevalence of nosocomial infections (3). In many cases, there is poor access to essential surgical services which results in excessive mortality from commonplace injuries, complications of pregnancy and congenital abnormalities (3, 4). Further, current existing drug programs often fail due to inadequacies in therapy, poor patient compliance, interrupted drug supply and inappropriate treatment regimens or misunderstandings of the regimen.

The two mechanisms of resistance are interconnected by interspecies gene transmission, lack of sanitation, and the ever-increasing global dynamics, whether it is through travel, trade or disease transmission. Disease resistance is not new; it is a phenomenon that has been documented since the discovery of penicillin (5, 6). What makes this current wave of resistance problematic is both the rate at which resistance is occurring as well as the widespread nature of resistance.

The antibiotic crisis results from scientific, economic and regulatory causes.

The reason for this crisis can be further divided into three separate topics. The first is scientific/biological; all seemingly obvious drug targets have been identified (7). Often referred to as "low hanging fruit being plucked" there is an increased rate of resistance and instances of total drug resistance. The second is economic; the development of antibiotics is a poor return on investment as compared to other classes of drugs. The third is regulatory; the system of antibiotic approval has become increasingly complex and infeasible, particularly in the last decade (for review on these

issues see: 8, 9). The particulars of these two branches will not be discussed in as much detail as seen in these reviews.

It is imperative to recognize that these causes interact and influence one another and cannot be considered independent of one another. For example, scientific and regulatory challenges increase both cost and development time which exacerbates economic disadvantages.

Bacteriocins are one of the most appealing options to solving the antibiotic crisis.

While bacteriocins may not initially appear as capable of solving the three-fold crisis of classic antibiotics, they do offer a step in the right the direction. Bacteriocins are defined as ribosomally-synthesized substances which inhibit the growth of closely related species through numerous mechanisms which will be discussed in detail in this review (10, 11). What will not be discussed in detail are the arguments over classification schema. The scheme used here divides bacteriocin by modifications; in bacteriocins of Gram-positive microorganisms, such as those produced by lactic acid bacteria (LAB) class I undergo post-translational modifications while class II undergo either none or minimal modifications. Bacteriocins from Gram-negative bacteria are divided (1, 12) into small peptides, such as microcins and large peptides, such as colicins (13–15). Further subdivisions exist within these broader categories including instances of homology in motifs but will not be discussed here. Truly, the amount of research on categorization indicates the amount of interest in bacteriocins. Individuals interested in bacteriocin organization are suggested to the program BAGEL3 which uses FASTA DNA sequences to characterize and identify homology between bactercinogenic bacteria and protein production (16).

Currently, there is extensive research being performed on bacteriocins, and the FDA regulates their usage as a food preservative. As of 2012, 62 genera encompassing 195 bacterial species are considered as microbial food cultures with a history of safe use for fermentation purposes (17). Of these, many listed are bacteriocingenic, although only one (*Lactobacillus hordei)* is specifically identified as such. The overwhelming use of bacteria in food preservation and methods used to regulate them, indicate that it may be possible to extrapolate the success of these in food as possible success in human infections.

Comparatively, antibiotics will never have the same high return of investment as compared to medications that individuals have to take every day, or for their entire lives, however this should not diminish their overall necessitation. Antibiotics capable of effectively combating bacterial infections are a critical aspect of our lives; one which have been taken for granted since the discovery of penicillin in 1942. It is important that it be remembered that having effective antibiotics is something that we have not always had and that microorganisms can easily and often quite efficiently eliminate humans.

Bacteriocins utilize some of the classic drug targets.

Given this landscape it is clear that novel drugs need to be developed. Current methods of identifying novel antibiotics generally fall into one of two categories: synthetic chemical efforts or isolation of new natural product scaffolds. Examples of more recent synthetic chemical efforts include high throughput screening of chemical libraries and targeted structure-guided experiments (18).

Classic antibiotics fall into five major categories. These targets include: i) bacterial peptidoglycan/cell wall, ii) Protein biosynthesis, iii) folate biosynthesis,

iv)DNA replication and transcription, and v) disruption of the bacterial membrane (1, 19–21). These are considered the five major clinically validated antibacterial targets. Bacteriocins can inhibit closely related bacterial species, spore-formers, and have even shown instances of fungicidal activity (22, 23). Inhibition of spore-formers, fungicidal activity, and more broad-spectrum activities have also been observed. Antibiotics comparatively tend to inhibit in a broad-spectrum manner with numerous side effects. The side effects and increased incidence of bacteriocin resistance are two topics that need to be researched further. There are reports of bacteriocins affecting four of the aforementioned clinically relevant antibiotic targets. This is critical, indicating that bacteriocins are capable of multiple mechanisms of action.

i. Cell wall biosynthesis is inhibited through the binding of lipid II by class IA bacteriocins

The cell wall is critical to the overall bacterial survival in that it regulates cellular integrity and morphology particularly in cases of internal osmotic pressure fluctuations. Therefore, prevention of cell wall biosynthesis is a critical target. Current studies in the development of the rod-shaped bacteria *Escherichia coli* and *Bacillus subtilus* have indicated MreB, a bacterial actin homolog, as critical for maintenance of shape and penicillin-binding proteins (PBPs) as enzymatic regulators. These MreB structures rotate for maximum uniform distribution of peptidoglycan insertion sites (24). Motion is dependent on availability of these subunits. PBPs, particularly PBP2 is responsible for covalent cross-linking of glycan strands during growth (25). Current antibiotics target cell wall synthesis at four different stages of peptidoglycan development: i) inhibition of the synthesis of lipid II, ii) inhibition of the undecaprenol carrier lipid, iii) binding of

lipid II, and iv) binding and blocking of the active sites of PBPs (6). The coupling of MreB motion and PBP2 regulation appears highly conserved among bacterial species (24–26).

Nisin A, produced by *Lactococus lactis,* one of the most frequently referenced bacteriocins, possesses multiple modes of action. This lantibiotic docks to lipid II, a membrane-bound precursor of the cell wall and thus inhibits cell well synthesis. In addition, following lipid II docking, pore formation by nisin molecules arranged as a pore-forming "unit" can be induced, which rapidly kills cells. At high quantities, this process can be divided into two stages with the first being bacteriostatic and the second bactericidal (6, 27, 28). Nisin has also been found to act as a lytic agent (29). Nisin has been implicated in the inhibition of numerous Gram-positive bacteria leading to its usage in the food industry (28, 30, 31). Similarly, nukacin ISK-1, produced by *Staphylococcus warneri,* inhibits cell wall synthesis by binding lipid II but has not been shown to induce pore formation (32–34). This bacteriocin has been shown to inhibit MRSA biofilms (32). It has been found that ring A is responsible for binding lipid II (34). *Microbispora* spp. ATCC-PTA-5024 produces NAI-107 which also binds to lipid II leading to inhibition of vancomycin-resistant enterococci and methicillin-resistant *S. aureus* (35, 36).

The three aforementioned bacteriocins show great promise in preventing cell wall biosynthesis through the blocking of lipid II. Future research may be directed towards reconstruction studies in which there is better understanding into the molecular mechanism of development and the linkage of MreB, PBPs and lipid II. Understanding how these interact could potentially indicate novel antimicrobial targets.

ii. Inhibitory and destructive effects on DNA replication and DNA structure

Replication is one of the criteria of life and within the process lies the relaxation of positive supercoils which allows for superhelical tension preceding polymerase and the continuation of the process. Due to differences in the structure of DNA gyrase between eukaryotic and prokaryotic organisms, this is an excellent target, often by one of two methods. The first is through competitive inhibition of the ATPase active site on the GyrB subunit and the second is by binding and preventing decatenation replicating DNA.

Microcin B17 has been found to have a decatenation mechanism of action (1). The structural features of this bacteriocin position it as potentially useful in DNA gyrase inhibition but it is currently not suitable for human usage. Microcin B17 represents an important mechanism of action which, using the motif data could help in the design of engineered bacteriocins (the importance of which is discussed later in this review).

DNase and RNase mechanisms of action have been identified in the colicin family (E2, E7, E8 and E9, and, D, E3, E4, E5, E6, and cloacin DF13, respectively) (14, 37–41). Members of the E-group endonuclease colicins bind the BtuB/Tol translocation machinery in order to cross the outer membrane and have the H-N-H motif (37, 38).

iii. Protein synthesis is inhibited by the E-group ribonuclease colicins

Protein production is another critical cellular process frequently targeted by antibiotics. Often, protein synthesis is inhibited at the formation of the 30S initiation complex, the 70S ribosome or during the elongation process (42, 43). Inhibition at each of these specific stages leads to a shortened or malformed protein and eventual cellular death. Specifically, colicins E3, E4, E6, and cloacin DF13 show 16S rRNase activity

(40, 41). These bacteriocins cleaves the 16S rRNA at the 3'-end of the coding sequence which inhibits translation (13, 38, 40). It does so by accumulation of sequential impaired decoding events which results in low occupancy at the A site and inability to elongate the peptide past the first few codons. In short, it cleaves stop codons into the A site. It decreases the stability of codon-recognition complex, slowing aminoacyl-tRNA accommodation at the A site (13, 40).

Similar to the RNase colicins are the tRNases which act by accelerating the exhaustion of tRNA in the cytoplasmic pool and limiting protein synthesis. tRNases (D and E5) (15). Ogawa et al. 2006 demonstrated that $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$ molecules are digested by colicin D and E5 in the susceptibility order of $tRNA^{Tyr} > tRNA^{Asp} > tRNA^{His} > tRNA^{Asn}$ (15). Colicin D has also been shown to have a similar ribonuclease activity to that of E3 (44, 45).

Outside of the colicin family, the bacteriocin carocin S2, produced by *Pectobacterium carotovorum* has been shown to have a ribunclease mode of action against *P. carotovorum* SP33 (46).

iv. Disruption of bacterial membrane integrity

A number of bacteriocinogenic -producers use lipid II as a docking site, one of the classic examples is nisin. Other bacteriocins which use this binding site include: Bac-GM17, Pln E/F, Pln J/K, and geobacillin I. Bac-GM17 is produced by *B. clausii* StrainGM17 and is found to be both heat and pH stable (between pH 3 and 9). It was noted as having a bactericidal mode of action against numerous Gram-positive and Gram-negative bacteria as well as a fungistatic mode of action against *C. tropicalis* R2 CIP203 (42). Geobacillin I, produced by *Geobacillus thermodenitrificans* NG80-2,

contains several structural motifs similar to nisin; leading to its similarity in how it binds to lipid II and in its subsequent pore formation (19). Four plantaricins (Pln E, F, J and K) have been identified as having anti-Candida activity (47). Among these, PlnJ was identified as the most inhibitory and PlnK was least inhibitory. When used in combination it was found that PlnJ/K and Pln E/F were most effective at inhibiting *C. albicans C. glabrata, C. parapsilosis, Aspergillus fumigatus* and *A. terreus* (47).

Not all bacteriocins which cause membrane damage bind to lipid II. Dysgalacticin, produced by *S. dysgalactiae* supsp. *equisimilis* strain W2580, binds to membrane-bound glucose and/or mannose phosphotransferase system (man-PTS). Once dysgalacticin has docked to the man-PTS it disrupts the cytoplasmic membrane by causing an efflux of potassium ions (K+), which dissipates the membrane potential as seen in *S. pyogenes* (48). Lactococcin A, B and some *Listeria*-active pediocin-like bacteriocins similarly bind man-PTS, permeabilizing the membrane in a proton-motive-force (PMF) independent manner which causes cellular leakage (49). Currently one of the exact targets of man-PTS has been identified as Ell_t^{Man} for *L. monocytogenes* inhibition by mesentericin Y105 (50). Further research is necessary to identify the exact targets of the man-PTS.

Bacteriocins which act on the targeted cells by forming pores in their membranes, do not always dock to either lipid II or man-PTS. Lacticin Q, produced by *L. lactis* QU 5, forms toroidal pores due to lipid flip-flop which causes protein leakage and cell death without a specific receptor (51–54). It has variable degrees of activity among Gram-positive bacteria which is also dependent on the accumulation of hydroxyl radicals (51). Further, lacticin Q shows selectivity in inhibition for Gram-positive

bacteria but not for Gram-negative bacteria, due to phyisiochemical differences of the outer membrane (53).

v. Folate biosynthesis pathway represent mechanisms of actions not currently targeted by bacteriocins.

Similarly, there are multiple targets within the folate synthesis pathway including the dihydrofolate reductase (DHFR), a precursor shared between eukaryotic and prokaryotic organisms, as well as dihydropteroate synthase (DHPS), an enzyme unique to prokaryotic organisms. Current inhibitors of these two targets result in interactions with enzymatic neighbors in the folate pathway. As of the writing of this review, there are no currently characterized bacteriocins which inhibit bacteria using either of these modes of actions. This is not to say that bacteriocins are not inherently incapable of utilizing these mechanisms, it does in fact, offer a possibly untested avenue of research which could prove fruitful.

Septum formation is a novel drug target utilized by garvicin A and lactococcin 972

While it is of significance that bacteriocins hit four of the classic drug targets, it is important to recognize that it is becoming increasingly clear that novel targets using novel systems may be a more effective method of treating infections. Bacteriocins represent one such novel target in the inhibition of septum formation.

Septa consist of an in-growth of the cytoplasmic membrane and the mucopeptide layer during the final stages of mitosis. Addition of antibiotics during cytokinesis results in the development of a bulge and inhibition of the cell cycle (6). Two bacteriocins have thus far been shown to have this mechanism of action; garvicin A and lactococcin 972 (55, 56). Garvicin A is specifically active against other *L. garvieae* strains while

lactococcin 972 inhibited only closely related lactococci spp. The exact mechanism of action in lactococcin 972 is through the blocing of septum invaginations which results in cellular elongation and widening (56).

This target is similar to the inhibition of cell wall synthesis but its mechanism is wholly different. It uses wholly different target machinery and mechanisms of action positioning it as a crucial field of study. It is important that other targets, such as the FtsZ, FtsA and ZipA rings be considered as new and potential targets for these bacteriocins (57).

Engineering bacteriocins represent a method of improving stability and inhibition capabilities.

One of the ways to address novel targets and to improve the current efficacy and stability of current bacteriocins is through the manipulation of bacteriocins. Some bacteriocins which have been engineered include: microcin B17, geobacillin I, nisin, and lacticin 3147.

Microcin B17 acts a DNA gyrase inhibitor. It was found that the polyglycine in the N-terminus is used as an anchor for microcin synthase and the terminal Ser-His-Ile contribute to critical stabilisation interactions in the bacteriocin (1). The derivative Mcc[Gly46-Ile69] showed similar cleavage activity to the wildtype.

Geobacillin I, the analogs with NVA and P as the linker between the C and D rings displayed an eightfold and twofold increase in MIC values, respectively. It is further postulated that stability could be improved through the mutation of Dha5 based on the nisin analog I4K/Dha5F/L6I which had increased stability and antimicrobial activity (19, 58). This sort of thinking is vital; by using observed homology it may be

possible to consider analogs which improve structural problems in multiple related bacteriocinogenic producers.

The “popularity” of nisin has lead to numerous mutations being generated in the FNDL box, the ring positions, and the hinge regions. It has been found that mutations of D-19A, F-18H, F-18M, L-16D, L-16K, and L-16A enhanced production of nisin (59). The N-terminal thioester ring positions have been randomized and removed in combinations (58). It has been found that mutating ring A results in an increased activity, removal of ring D and E results in the inability to make cell pores and that the opening of ring B eliminates antimicrobial activity while retaining autoinducer activity (58). In hinge experiments, N20/M21P and M21P/K22 mutants were still capable of binding lipid II but were unable to form cellular pores but had potent antimicrobial activity against vegetative *B. anthracis* cells but not spores (28). Mutants with hinge regions of AAK, NAI and SLS have also been produced and have increased antimicrobial activity against *L. lactis, S. agalactiae, Mycobacterium smegmatis* MC2155 and *S. aureus* RF122 (60).

Lacticin 3147 is a two-peptide lantibiotic in which fourteen subclones, containing different combinations of the genes (61). It was found additional copies of genes which encoded for the biosynthetic/production machinery and the regulator LtnR results in high-level overproduction while additional copies of structural genes, such as *ltnA1A2* results in reduced produced.

These different strategies represent potential methods of manipulating bacteriocins to improve their overall efficacy as well as their stability. Such strategies

are being more readily applied and represent means of addressing current failing in bacteriocins.

Bacteriocins are potent synergists with other bacteriocins and with conventional antibiotics

Bacteriocins possess numerous potential for therapeutic applications, not only as alternatives, but as synergists to antibiotics. There are several scenarios that antibiotic-bacteriocin synergy could act to enhance current, insufficient infection therapies. In cases in which the bacteriocin is not as efficacious on its own it has been indicated that it is possible to improve the overall inhibitory effect by combining the bacteriocin with other bacteriocins or conventional antibiotics.

i. Instances of synergy between bacteriocins

The four plantaricins (Pln E, F, J and K) were identified to have anti-Candida activity on their own(47). PlnJ was identified as most inhibitory while PlnK was least inhibitory. When used in combination it was found that the combinations of PlnJ/K and Pln E/F were most effective at inhibiting *C. albicans* (47). Similarly, it has been found that lacticin Q when combined with nisin could overcome certain hurdles, such as the inactivation of nisin at alkaline pH values while improving overall efficacy since these two bacteriocins are in the same category (54).

ii. Instances of synergy between bacteriocins and conventional antibiotics

The bacteriocin subtilosin A has been shown to be a potent synergist with clindamycin, metronidazole, LAE and polylysine against the bacterial vaginosis (BV)-associated pathogen *Gardnerella vaginalis* (71–73, unpublished data). Further, it has

been shown that the two peptide lacticin 3147 synergistically interacts with polymixin in order to inhibit *Cronobacter* spp. and *E. coli* (21).

Limitations

Despite the remarkable potential for the incorporation of bacteriocins in synergistic applications, there are some pitfalls that have not been tested. By introducing bacteriocins into novel infection therapies, there is a risk that pathogens can form a resistance to applied bacteriocins, similarly to what is happening to antibiotics. Many would argue that the application of another antimicrobial may postpone issue of resistance, rather than resolving it. Furthermore, synergistic therapies could create a dual-resistant pathogen epidemic.

A natural concern about using bacteriocins for the use preservation of food is the selection of resistant strains. Studies in LAB have shown that resistances carries a significant fitness cost with resistant strains having a slower growth rate than their sensitive ancestor. Treatment with a combination of bacterions, for instance nisin and a class IIa bacteriocin, would theoretically reduce the incidence of resistance. There is currently conflicting evidence as to whether resistance to one class of LAB bacteriocin can result in cross-resistance to another class.

Conclusion

The era of the most successful infection prevention and treatment therapies is coming to an end. For approximately a century, antibiotics have been a reliable and effective method for treating almost all documented infections. However, after continuous use and careless regulation, antibiotics have driven the evolution of resistant organisms. Nosocomial environments serve as a repository for these organisms.

Resistant strain infection rates in immunocompromised and susceptible individuals in nosocomial settings are commonly exposed to and infected with these strains. Once contracted, the antibiotic resistant infections are significantly more difficult to treat with traditional antibiotics. Since the emergence of this dilemma, a variety of novel therapeutic treatments have been explored.

Bacteria have a number of methods to allow for improved and successful allelopathy. Bacteriocins have been of particular interest as they been heavily used in the food industry and have shown promise in the medical industry. Bacteriocins position themselves as fullfilling three of the five classic drug targets, novel targets, acting as potent synergists

Recommendation for Future Research

As it stands, bacteriocins represent one of the most-studied microbial defense systems. It is clear from both the abundance, and multiplicity of, that bacteriocins are the weapons of choice in the microbial world. Understanding the evolutionary relationships and ecological functions of such successful toxins could answer enormous and interesting questions. Although it is largely agreed upon that bacteriocins play a role in mediating microbial dynamics and maintaining diversity the mechanism of how is poorly defined. Again, the ramifications of further research into these and other mechanisms would greatly improve our understanding of microbial interactions. Research into mechanisms of action is not entirely academic; bacteriocins are already enormously useful in food preservation and maintain the potential to be equally important as alternatives, synergists or part of a multiple hurdle approach with antibiotics.

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Table 1: Bacteriocins and their targets				
Bacteriocin	**Target**	**Mechanism of Action**	**Primary Producer(s)**	**Reference(s)**
Nisin A	Lipid II	Inhibition of cell wall formation	*Lactococcus lactis*	6,27,28
Nukacin ISK-1	Lipid II	Inhibition of cell wall formation	*Staphylococcus warneri*	32-34
NAI-107	Lipid II	Inhibition of cell wall formation	*Microbispora spp.* ATCC-PTA-5024	35, 36
Microcin B17	DNA gyrase	Prevent DNA decantenation	*E. coli*	1
Colicin family	DNA and RNA	DNase and RNase	*E. coli*	14, 37-41
Carocin S2	RNA	RNase	*Pectobacterium carotovorum*	46
BAC-GM17	Lipid II	Membrane disruption	*Bacillus clausii* GM17	42
Geobacillin I	Lipid II	Membrane disruption	*Geobacillus thermodenitrificans* NG80-2	19
Pln E, F, J and K	Lipid II	Membrane disruption	*Lactobacillus plantarum*	47
Dysgalacticin	Mannose phosphotransferase system	Membrane disruption	*Streptococcus dysgalactiae* subsp. *equismilis* strain W2580	48
Lactococcin A, B	Mannose phosphotransferase system	Membrane disruption	*L. lactis* subsp.	49
Pediocin-like bacteriocins	Mannose phosphotransferase system	Membrane disruption	Unnamed producer(s)	49
Mesentericin Y105	Mannose phosphotransferase system	Membrane disruption	*Leuconostoc mesenteroides* Y105	50
Lacticin Q	Cellular membrane	Torodial pore formation, protein leakage	*L. lactis* QU 5	51-54
Garvicin A	Not determined	Inhibition of septum formation	*Lactococcus garvieae*	55
Lactococcin 972	Not determined	Inhibition of septum formation	*L. lactis*	56

The natural antimicrobial subtilosin A synergizes with lauramide arginine ethyl ester (LAE), ε-poly-L-lysine (polylysine), clindamycin phosphate and metronidazole, against the vaginal pathogen *G. vaginalis* 140180 and selected isolates of lactobacilli

The following research paper uses the microdilution method concurrently with the checkerboard assay in order to determine the interaction of two antimicrobials against four species of *Lactobacillus* and one strain of *G. vaginalis*. This work uses Bliss independence and fractional inhibitory concentration index (FICI) to interpret the results as synergistic, antagonistic or as having no interaction in an *in vitro* array. The combinations of metronidazole and subtilosin, and clindamycin and subtilosin and clindamycin were found to be synergistic against *G. vaginalis* in terms of FICI while all tested combinations were found to have Bliss synergy. The results of these experiments will be applied to future biofilm experiments as well as *in vivo* arrays.

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The candidate designed and performed all experiments described herein and wrote the manuscript. Experiments were performed under the direction and guidance of the thesis advisor who also assisted in editing the manuscript.

The natural antimicrobial subtilosin A synergizes with lauramide arginine ethyl ester (LAE), ε-poly-L-lysine (polylysine), clindamycin phosphate and metronidazole, against the vaginal pathogen *G. vaginalis* 140180 and selected isolates of lactobacilli

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ABSTRACT

Bacterial vaginosis (BV) is a common, recurrent vaginal infection, linked to increased chances of preterm delivery, incidence of sexually transmitted infections and fertility problems. BV is caused by a shift of the vaginal ecosystem from predominately *Lactobacillus* to a multispecies biofilm that consists of *Actinomyces* spp. The most common species identified include *Gardnerella vaginalis* and *Prevotella* spp. Current treatments have been associated with increased resistance as well as negative effects on healthy microbiota. The objective of this study was to evaluate the synergistic potential of ten two-antimicrobial combinations against *G. vaginilis* and four representative lactobacilli. The four tested antimicrobials were lauramide arginine ethyl ester, ε-poly-L-lysine, clindamycin phosphate, metronidazole and the bacteriocin subtilosin A. The use of bacteriocins as either synergist or alternative treatment positions bacteriocins as an excellent alternative to current antibiotics. The microdilution method was used to determine the minimum inhibitory concentration (MIC) of each of the antimicrobials individually and the checkerboard assay was used to evaluate these MICs in combination. Clindamycin and subtilosin (CS), and metronidazole and subtilosin (MS), were synergistic against *G. vaginalis* in terms of fractional inhibitory concentration index (FICI). All tested combinations were found to have Bliss synergy. The combination of clindamycin and polylysine (CP) was identified as antagonistic against *L. acidophilus* in terms of both FICI and Bliss synergy. The combination of clindamycin and metronidazole (CM) was antagonistic against *L. vaginalis* for both FICI and Bliss synergy. The combinations of clindamycin and polylysine (CP), clindamycin and LAE

(CL), clindamycin and subtilosin (CS), and LAE and polylysine (LP) were identified as Bliss antagonistic against *L. vaginalis* but did not indicate FICI antagonism.

INTRODUCTION

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

electron-transport proteins with a sufficiently negative redox potential, therefore positioning it as active against organisms with anaerobic metabolisms (6–8).

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

promising synergist in assisting in the improvement and overall health-maintenance of the vaginal ecosystem.

In addition, we reported on the inhibitory properties of glycerol monolaurate (GML), LAE and polylysine alone against *G. vaginalis* (13). Lauramide arginine ethyl ester (hereafter referred to as LAE) (Table 1) was given GRAS (generally recognized as safe) status in the United States in 2005 and is currently allowed as a food preservative at quantities up to 225 mg/kg bw/day for individuals over the age of two (15, 16). LAE rapidly hydrolyzes to form N-ε-lauroyl-L-arginine (LAS) which hydrolyzes to arginine, which is converted into urea and ornithine. These are catabolised via the urea and citric acid cycles to form carbon dioxide and urea to be excreted through respiration or urination, respectively. The mechanisms of action of LAE is through the disruption of the plasma membrane bilayer without causing cellular lysis which leads to reduced cellular growth (15–17). Similarly, ε -poly-L-lysine (hereafter referred to as polylysine) (Table 1) received GRAS status in 2010 and has been identified as safe up to 250mg/kg bw/day (18). Polylysine is a homopolymer of the amino acid L-lysine produced through fermentation of the bacterium *Streptomyces albulus* subsp. *lysinopolymerus* (18). The mechanism of action is through one of two physical ionic interactions within microbial cell membranes; one is through the induction of pore formations while the second is through the disintegration of the cellular membrane. Both mechanisms increase permeability of other antimicrobials, positioning polylysine as a potent synergist (4, 18).

The study presented here builds off of combination work previously performed by our group as well as that performed by Draper et al. (2013) in which it was indicated that the two peptide lantibiotic lacticin 3147 and polymyxins acted synergistically

against Gram-negative microorganisms (19). This group has looked further into the action of lacticin 3147, having recently identified it as capable of preventing *Streptococcus mutans* biofilm formation (20).

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

MATERIALS AND METHODS

Preparation of antimicrobial solutions. MIRENAT-CF (LAE) was a gift from Vedeqsa Corp (Vedeqsa, Barcelona, Spain) and contained 1 mg/mL stock of LAE. A 25% (250 μg/mL) stock solution of polylysine was a gift from the Chisso America, Inc. Corporation (lot 2090501; Rye, NY). A 98% stock of clindamycin phosphate was obtained from Tokyo Chemical Industry (TCI, Tokyo, Japan). A 99% (M.W. 171.16) stock of metronidazole was purchased from Acros Organics (Acros Organics, New Jersey, USA). All antimicrobial solutions were filter sterilized using a 0.45-μm filter (Nalgene, Rochester, NY) prior to use.

Subtilosin was obtained in house through fermentation of *Bacillus amyloliquefaciens* KATMIRA1933, as previously described (4). The stock solution was stored at 4 °C until needed for experiments.

Bacterial growth conditions. *G. vaginalis* ATCC 14018 was maintained as previously described (4). Briefly, bacterial cultures were stored at -80 °C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) and 15% (v/v) glycerol. Frozen stocks were cultured on human blood bilayer-Tween (hereafter referred to as HBT) agar (Remel, Lenexa, KS) and grown in anaerobic conditions in a Type A Coy Laboratory Vinyl Anaerobic Chamber (Coy Laboratory Products, Grass Lake, MI) at 37 °C. The anaerobic environment considers the following air composition: 2.5% hydrogen gas, 5% CO_2. Isolated colonies were inoculated in Brain Heart Infusion supplemented with 3% horse serum (hereafter referred to as BHI+3%HS) media for 48 hours. Initial cultures were subcultured twice before use. Cultures used for checkerboard assays were grown to 10^8 CFU/mL and then diluted 100-fold in growth medium for a working concentration of 10^6 CFU/mL. All media and agar were preincubated in the aforementioned anaerobic conditions overnight to remove oxygen-related stress.

The four reference species of lactobacilli were selected as they represent clinical isolates that have been found in healthy women (*Lactobacillus gasseri* ATCC 33323 and *L. plantarum* ATCC 39268) and those with recurrent vaginal infections (*L. acidophilus* ATCC 4356 and *L. vaginalis* ATCC 49540) (21, 22). All bacteria were stored at -80 °C in DeMan, Rogosa and Sharpe (MRS) broth (Difco, Sparks, MD) containing 15% glycerol (v/v) until use. The cells were cultured on MRS agar and grown anaerobically

in the same anaerobic chamber used for *G. vaginalis* experiments. Single colonies were inoculated in MRS broth and grown anaerobically for 24 hours without agitation. Cells were subcultured twice before use.

Determination of minimal inhibitory concentration (MICs). Stock solutions of all antimicrobials were prepared in 100mM phosphate buffer saline (PBS) solution. Antimicrobials were filter-sterilized using 0.45 μm syringe filters (Fisher, Pittsburgh, PA), and diluted to 2X final working concentration. This was done in order to prevent further dilution upon addition of media. MICs were determined as follows. Briefly, 24 hr cultures of *G. vaginalis* in BHI+3%HS were transferred to fresh media to obtain an optical density at 595 nm (OD_{595}) of 0.2. From the stock solutions, 10-fold serial dilutions of each antimicrobial were made, following the microdilution method as described by Amrouche et al. (23) and adapted further by Noll et al. (13). A sterile, 96-well microplate (Corning, Inc., Corning, NY) was prepared by adding the serial dilutions of antimicrobials horizontally from the highest to lowest concentration tested. Antimicrobials were tested in 20 μL increments in duplicate. The volume of the well was increased to a total volume of 100 μL with the addition of PBS buffer, with the contents mixed by an Eppendorf Xplorer automatic pipette (Eppendorf Hauppauge, NY). Equal volumes (100 μL) of bacteria and serial dilutions of each antimicrobial were mixed into the wells. Control wells were those with neither bacteria nor any of the dilutions of antimicrobials. Heavy mineral oil was placed on top of each well to prevent evaporation during optical density readings. The use of mineral oil also assists in the prevention of evaporation-induced spikes in data. This procedure was identical for the four tested lactobacilli species except for the use of MRS broth instead of BHI+3%HS

broth. Experiments were performed four times in duplicates. Plates were prepared in the anaerobic chamber to prevent oxygen related stress from interfering with the experiment.

Checkerboard assays. The interaction between all the antimicrobials was tested via the checkerboard assay as described by Badaoui Najjar et al. (24) with some modifications. For each combinatorial experiment, antimicrobial A was placed in horizontal rows while antimicrobial B was placed into the vertical columns. Using the stock solution that was 10-fold-higher than the respective MIC, each compound was aliquoted to test concentrations above, equal to or below the individual MIC of each tested antimicrobial (Table 2). The checkerboard assay was carried out in a manner identical to the MIC experiments (Table 3). Experiments were performed four times in duplicates.

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

Synergy interpretation using FICI. There are numerous models and approaches which assess *in vitro* drug interactions. Currently, the two prevailing theories are the Loewe additivity (LA) and Bliss independence (BI) models because they fulfill the no-interaction theory in which it is stated that only synergy or antagonism, respectively, can be claimed. In LA, it is posited that an agent should not have synergistic interactions with itself or similar agents. It is further stated that if two similar drugs are given in equal concentrations the effect of the drug will be doubled (25, 26).

The MICs of each single antimicrobial agent and all combinations were determined in a checkerboard assay. For each antimicrobial combination, the fractional inhibitory concentration index (FICI) was determined by computing the ratio of the MIC of the combination divided by the MIC of the antimicrobial alone, and then adding these two ratios together. Equation 1 defines the LA model: $FICI = FIC_A + FIC_B$ in which: $FIC_A = (MIC_{\text{ANTIMICROBIAL A IN COMBINATION}}/MIC_{\text{ ANTIMICROBIAL A ALONE}}) + FIC_B = (MIC_{\text{ ANTIMICROBIAL B IN COMBINATION}}/MIC_{\text{ ANTIMICROBIAL B ALONE}})$ (Equation 1). FICI data were interpreted using the following criteria: a FICI ≤ 0.5 is defined as synergistic while a value that is ≥ 0.5 and ≤ 4.0 is identified as having no effect (no interaction). A FICI of > 4.0 is identified as antagonistic (27).

Synergy using Bliss independence-based drug interaction analysis. Bliss independence (BI) considers two drugs that have reached their maximal effect (growth inhibition) and, once doing so, not contribute to the overall effect of the other drug. In BI, the two agents are considered as independent, i.e. as having different targets. BI is described by the following equations: $E_{IND} = E_A + E_B - E_A \times E_B$ (Equation 2). E_A is defined as the percentage of bacterial growth inhibition from of *x* mg/liter of drug A. E_B is defined as the percentage of bacterial growth inhibition at *y* mg/liter alone. E_{IND} is the expected percentage of bacterial growth which results from a noninteractive, thus independent, theoretical combination of drugs A and B. The difference between the observed (E_{OBS}) and expected (E_{IND}) percentage of growth inhibition from these drug combinations is described by equation 3: $\Delta E = E_{OBS} - E_{IND}$ (Equation 3). If ΔE and its 95% confidence level (CI) were >0 (i.e. $E_{OBS} > E_{IND}$, more growth inhibition was observed than if the two drugs were acting independently), Bliss synergy was concluded

for the drug combination. If ΔE and its 95% CI were <0 (i.e., $E_{OBS} < E_{IND}$, less growth inhibition was observed than if the two drugs were acting independently), Bliss antagonism was concluded for the particular drug combination. In cases in which the 95% CI of ΔE=0, the conclusion was deemed Bliss independence (28).

Graphical representation, statistical analysis, and presentation of data. All statistical analyses were performed in Sigma Plot 11.0 except for LA and BI data analysis which was performed using MS Excel 2007. All statements regarding statistical significance are at the 95% level (29). Chart representation of data was created in MS Word 2007. All figures were drawn using Instant JChem 6.2.1 (MathWorks).

RESULTS

Antimicrobial susceptibility of tested lactobacilli. The MICs of clindamycin, LAE, polylysine, metronidazole, and subtilosin against the four tested lactobacilli were determined by the microdilution method. As seen in Table 2, much of the data are confirmatory from our previously published results (4). It expands upon these data by indicating that *L. gasseri* is inhibited by 77.5 μg/mL of clindamycin, and identifying that the MICs of subtilosin appeared to be greater than originally suspected. The MICs of metronidazole did not vary much between the four tested lactobacilli species, ranging between 50 μg/mL-100μg/mL.

The MIC of clindamycin against *L. acidophilus* was 9 μg/mL and differed greatly from the other observed clindamcyin MIC values. The MIC of polylysine (133.33 μg/mL) was similar to that of *L. gasseri* (111.6 μg/mL) but varied from that of *L. plantarum* (1768 μg/mL) and *L. vaginalis* (55.8 μg/mL).

Antimicrobial susceptibility of *G. vaginalis*. The MICs of clindamycin, LAE, polylysine, metronidazole and subtilosin against the BV-associated pathogen *G. vaginalis* was determined via the microdilution method. As seen in Table 2, all tested antimicrobials inhibited *G. vaginalis* at concentrations generally lower as compared to the four tested lactobacilli. Clindamycin inhibited *G. vaginalis* at 16.67 μg/mL, which is lower than *L. gasseri* and *L. plantarum* (77.5 μg/mL and 25 μg/mL, respectively) while *L. acidophilus* and *L. vaginalis* were inhibited at significantly lower concentrations (9 μg/mL and 0.78 μg/mL, respectively). All other tested antimicrobials were found to inhibit *G. vaginalis* at concentrations lower as compared to tested lactobacilli (Table 2).

Determination of synergy between two antimicrobial substances. Having identified all individual MICs against the five tested bacteria, a checkerboard assay was performed with all possible combinations of antimicrobials. Assays were designed to test a wide range of concentrations; starting with above each individual antimicrobial's MIC and decreasing in a serial dilution to zero μg/mL (negative control). Wells that resulted in complete inhibition of the bacterium had the concentrations of each antimicrobial recorded (Table 3). These values were then analyzed using FICI and BI so as to determine their interaction.

Identification of interaction against tested lactobacilli. The combinations of metronidazole and LAE (ML), and metronidazole and subtilosin (MS) were synergistic against all tested lactobacilli in terms of both FICI and BI. The combination of LAE and subtilosin (LS) was synergistic against all tested lactobacilli except *L. plantarum*, for which there was no interaction. Similarly, the combination of polylysine and subtilosin

(PS) indicated synergy against all tested lactobacilli expect for *L. acidophilus* which also indicated no interaction (Table 4).

The combination of clindamycin and metronidazole (CM) was found to be synergistic in terms of FICI and indicated Bliss synergy against *L. gasseri* while having no interaction against *L. acidophilus* or *L. plantarum* and being antagonistic against *L. vaginalis*. Bliss antagonism was also noted for the combination of CM against *L. vaginalis*. The combination of clindamycin and polylysine (CP) was found to have no interaction against all tested lactobacilli except *L. acidophilus* for which it was antagonistic. All other tested combinations indicated combinations of synergism and no interaction in terms of FICI. Bliss antagonism was also found for the combinations of clindamycin and LAE (CL), clindamycin and subtilosin (CS) and LAE and polylysine (LP) against *L. vaginalis* (Table 5).

Identification of interaction against *G. vaginalis*. None of the tested combinations antagonized in their action against *G. vaginalis* in terms of FICI values or BI (Tables 4 and 5). Four of the tested combinations were synergistic, clindamcyin and subtilosin (CS), metronidazole and polylysine (MP), metronidazole and subtilosin (MS) and polylysine and subtilosin (PS) in terms of FICI. All combinations tested indicated Bliss synergy.

DISCUSSION

G. vaginalis is one of the primary causes of BV, the most common vaginal infection identified in women of childbearing age. Its prevalence and the associated ramifications of this infection indicate the necessity to effectively inhibit the growth of the causative bacteria and assist in the recovery of the healthy microbiota. Currently

used antimicrobials lead to AMR and inhibition of normal microbiota. The inhibition of healthy microorganisms lengthens the overall recovery time of the natural vaginal ecosystem (30). The aim of this study was two-fold. First, it determined whether combinations of commercial antibiotics acted synergistically against the BV-associated pathogen *G. vaginalis* while also evaluating the interaction of these same combinations against clinical isolates of lactobacilli. Second, the interaction of the bacteriocin subtilosin in combination with four antimicrobials was observed against all five bacteria. It was found that subtilosin is an excellent synergist and could serve as an alternative option to conventional antibiotics, particularly the combination of CP, which was found to be antagonistic against *L. acidophilus* with both FICI and BI. The combinations of CM, CP, CL, CS and LP were found to have Bliss antagonism against *L. vaginalis* but these combinations were not found to be antagonistic in terms of FICI except for CM. The combination of ML was found to be synergistic against all tested lactobacilli while having no interaction again *G. vaginalis*.

This study utilized FICI and BI, two of the most common parametric models concurrently in order to determine possible synergy in action of ten antimicrobial combinations against five bacteria in an *in vitro* system. In the described array design, these approaches are considered to be valid and utilized by many investigators (27). The use of both of these tools allows for an analysis of the concentration effect of two antimicrobials in combination and an evaluation of the effectiveness against these bacteria. Previously, our group used isobolograms to determine such pharmocodynamic interactions but since the tested combinations included a maximum of two antimicrobials it was not appropriate. The data represented here indicates both the

reproducibility of using subtilosin in combination while also expanding upon its synergy using two different analysis methods. This includes the synergy of subtilosin with polylysine (13) which is confirmed here as a control. However, in the same study the synergism of LAE and subtilosin (LS) was also indicated which was not observed in this study. The two pharmocodynamic interaction methods used indicated that overall most of the tested combinations were synergistic while the combination of CP was antagonistic against *L. acidophilus* and CM was antagonistic against *L. vaginalis.* The synergy of subtilosin with three of the four tested antimicrobials through FICI and its Bliss synergy with all of the antimicrobials indicate its potential as a potent synergist.

BV is a polymicrobial infection; however, the cytotoxicity and biofilm-forming potential of *G. vaginalis* largely position it as the causative agent of the disorder, which is why it was the only BV-associated pathogenic bacterium tested in this study. Future synergy studies will incorporate other essential microorganisms associated with BV as well as biofilm studies. While the study is a continuation of our group's investigation of synergistically-acting antimicrobials, it is also influenced by the growing interest in improving conventional antibiotic by combining them with nature-derived and synergistically-acting antimicrobials. Doing so may act as a novel and more effective treatment method (19). Further, some synergistic combinations against *G. vaginalis* were found to be highly effective against the tested vaginal lactobacilli thus indicating that these combinations would, in practice, be rather detrimental to the individual and impede recovery of the vaginal ecosystem (31, 32).

The outcome of this study is two-fold. It provides a foundation into the effects of the studied antimicrobials in particular which combinations are synergistic against the

tested pathogen and which should not be considered because of their activity against the healthy microbiota.

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TABLE 1 Antimicrobial agents used in this study

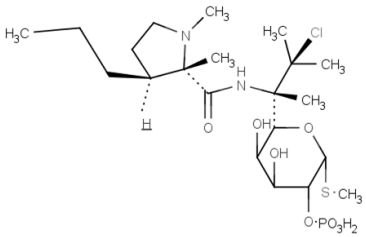
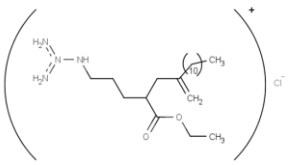
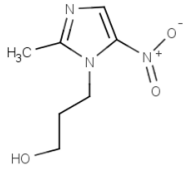
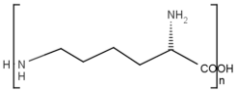
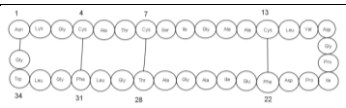
Name	Mechanism of Action	Chemical Structure	References
Clindamycin phosphate	Lincosamide; prevents replication by inhibiting protein synthesis by binding the 23s portion of the bacterial ribosome resulting in premature dissociation of the peptidyl-tRNA from the ribosome.		1,4,5
Lauramide arginine ethyl ester	Disrupts the plasma membrane leading to inhibited cellular growth.		14,15
Metronidazole	Nitroimidazole derivative; disrupts an organism's redox system.		6-8
ε-poly-L-lysine	Causes physical ionic interactions with the cell membrane. Induces pore formations which disintegrate the cellular membrane.		14-16
Subtilosin A	Creates pores in the cytoplasmic membrane which leads to an efflux of intracellular ions and ATP.		4, 10-14

TABLE 2 The MIC$^{\alpha}$ of clindamycin, polylysine, LAE, metronidazole and subtilosin against *G. vaginalis* and four clinical lactobacilli spp.

	Antimicrobial compound				
Species	Clindamycin	Polylysine	LAE	Metronidazole	Subtilosin
G. vaginalis	16.67	25	10	50	12
L. acidophilus	9	133.33	50	100	1000
L. gasseri	77.5	111.6	31.25	75	825
L. plantarum	25	1786	62.5	100	785
L. vaginalis	0.78	55.8	15.63	50	725

Table 2. MIC of the four tested antimicrobials and one bacteriocin against the BV-associated pathogen *G. vaginalis* and four clinical isolates of lactobacilli. Some of this data is confirmatory while other data expands upon original data (4, 13). All MIC$^{\alpha}$ provided in μg/mL. All data is the average of four separate experiments in duplicates. All assays conducted resulted in identical results for all substances (no standard deviation).

TABLE 3 Combinatorial antimicrobial susceptibility of *L. acidophilus*, *L. gasseri*, *L. plantarum*, *L. vaginalis* and *G. vaginalis*

	Tested bacterium				
Combination$^{\beta}$	*L. acidophilus*	*L. gasseri*	*L. plantarum*	*L. vaginalis*	*G. vaginalis*
CM	30/2.56	64/4.8	1.92/2.56	3/4.8	7/8
CP	30/160	12/160	30/160	1.2/60	10/2.5
CL	4.8/50	4.8/20	4.8/50	1.56/3.908	7/6.14
CS	12/400	12/400	30/400	1.56/400	2/1.28
MP	2.56/25.6	2.56/25.6	2.56/25.6	2.56/25.6	8/5
ML	2.56/1.28	2.56/1.28	6.4/3.2	6.4/3.2	20/9.6
MS	6.4/160	16/64	16/160	6.4/160	3.2/3.2
LP	1.28/160	8/160	20/160	8/160	7.68/2.5
LS	5/500	3.125/275	6.25/785	1.563/72.5	25/4.6
PS	66.66/500	55.8/82.5	357.2/78.5	27.9/145	2.5/4.6

Table 3. The MIC$^{\alpha}$ of clindamycin, polylysin, LAE, metronidazole and subtilosin in combination against four clinical isolates of lactobacilli and the BV-associated pathogen *G. vaginalis*. MIC$^{\alpha}$ provided in μg/mL.

$^{\beta}$To save on space antimicrobial combinations are listed by their first letters. Combinations are as follows: CM = clindamycin/metronidazole, CP =

clindamycin/polylysine, CL = clindamycin/LAE, CS = clindamycin/subtilosin, MP = metronidazole/polylysine, ML= metronidazole/LAE, MS = metronidazole/subtilosin, LP= LAE/polylysine, LS = LAE/subtilosin, PS = polylysine/subtilosin.

TABLE 4 FICI$^{\nu}$ Values for combinatorial data from Table 3

	Tested bacterium				
Combination$^{\beta}$	*L. acidophilus*	*L. gasseri*	*L. plantarum*	*L. vaginalis*	*G. vaginalis*
CM	3.36$^{\sigma}$	0.89$^{\kappa}$	0.10$^{\sigma}$	4.01$^{\rho}$	0.62$^{\sigma}$
CP	4.53$^{\rho}$	1.59$^{\sigma}$	1.29$^{\sigma}$	1.48$^{\sigma}$	0.70$^{\sigma}$
CL	1.53$^{\sigma}$	0.70$^{\sigma}$	0.70$^{\sigma}$	2.25$^{\sigma}$	0.73$^{\sigma}$
CS	1.73$^{\sigma}$	0.64$^{\sigma}$	1.71$^{\sigma}$	2.55$^{\sigma}$	0.23$^{\kappa}$
MP	0.51$^{\sigma}$	0.26$^{\kappa}$	0.04$^{\kappa}$	0.51$^{\sigma}$	0.36$^{\kappa}$
ML	0.05$^{\kappa}$	0.08$^{\kappa}$	0.11$^{\kappa}$	0.33$^{\kappa}$	1.36$^{\sigma}$
MS	0.22$^{\kappa}$	0.29$^{\kappa}$	0.36$^{\kappa}$	0.35$^{\kappa}$	0.33$^{\kappa}$
LP	1.23$^{\sigma}$	1.69$^{\sigma}$	0.41$^{\kappa}$	3.38$^{\sigma}$	0.87$^{\sigma}$
LS	0.60$^{\sigma}$	0.43$^{\kappa}$	1.10$^{\sigma}$	0.20$^{\kappa}$	2.88$^{\sigma}$
PS	1.00$^{\sigma}$	0.60$^{\sigma}$	0.50$^{\kappa}$	0.70$^{\sigma}$	0.48$^{\kappa}$

Table 4. The FICI numbers generated from the data included in Table 3. $^{\nu}$FICI numbers are rounded to two decimal places. Combination efficacy is denoted as such: (κ) denotes synergy (σ) no effect, (ρ) denotes antagonism. $^{\beta}$To save on space antimicrobial combinations are listed by their first letters. Combinations are as follows: CM = clindamycin/metronidazole, CP = clindamycin/polylysine, CL = clindamycin/LAE, CS = clindamycin/subtilosin, MP = metronidazole/polylysine, ML= metronidazole/LAE, MS = metronidazole/subtilosin, LP= LAE/polylysine, LS = LAE/subtilosin, PS = polylysine/subtilosin

TABLE 5 BI Data from combinatorial data from Table 3

	Tested bacterium				
Combination [β]	*L. acidophilus*	*L. gasseri*	*L. plantarum*	*L. vaginalis*	*G. vaginalis*
CM	10.13 [ϑ]	54.22 [ϑ]	23.75 [ϑ]	-0.18 [ρ]	7.54 [ϑ]
CP	-38.19 [ρ]	67.12 [ϑ]	382.29 [ϑ]	-0.24 [ρ]	3.45 [ϑ]
CL	2.06 [ϑ]	22.42 [ϑ]	12.90 [ϑ]	-0.05 [ρ]	1.41 [ϑ]
CS	36.03 [ϑ]	586.47 [ϑ]	72.45 [ϑ]	-3.83 [ρ]	1.44 [ϑ]
MP	130.62 [ϑ]	81.46 [ϑ]	1766.77 [ϑ]	26.47 [ϑ]	10.13 [ϑ]
ML	64.84 [ϑ]	22.38 [ϑ]	60.77 [ϑ]	7.05 [ϑ]	4.36 [ϑ]
MS	1313.39 [ϑ]	600.31 [ϑ]	752.31 [ϑ]	346.17 [ϑ]	5.29 [ϑ]
LP	48.06 [ϑ]	3.25 [ϑ]	1067.57 [ϑ]	-3.11 [ρ]	2.11 [ϑ]
LS	469.55 [ϑ]	248.70 [ϑ]	441.00 [ϑ]	105.52 [ϑ]	0.13 [ϑ]
PS	661.37 [ϑ]	77.66 [ϑ]	13718.35 [ϑ]	358.02 [ϑ]	2.59 [ϑ]

Table 5. BI data generated from Table 3. Combination responses are denoted as either Bliss synergy (ϑ) or Bliss antagonism (ρ). BI responses are rounded to two decimal places.

Poly(ethylene glycol) (PEG)-based hydrogels for the controlled release of the antimicrobial subtilosin, for prophylaxis of bacterial vaginosis (BV)

The following is on the development of a 8-armed poly(ethylene glycol) (PEG) hydrogel which displayed a two phase kinetic (burst release rate of 4.0 µg/hr (0-12 h) and slow sustained release rate of 0.26 µg/hr (12-120 h)) release of subtilosin for the inhibition of *G. vaginalis.* It was found that a 8 $\log_{10}$ CFU/ml reduction was seen in time kill and OD_{595} experiments in PEG gels containing $\geq$15 µg. No inhibition was observed in any of the tested lactobacilli species.

This paper serves as an example of an instance of a bacteriocin being successfully encapsulated into a drug delivery system for the purpose of inhibiting pathogenic bacteria while not inhibiting normal flora in an *in vitro* system. It is a direct application discussed in the review "On Bacteriocin Delivery Systems and Potential Applications" which is co-authored by the candidate which is why it is included as an effort in this thesis.

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Poly(ethylene glycol) (PEG)-based hydrogels for the controlled release of the antimicrobial subtilosin, for prophylaxis of bacterial vaginosis (BV)

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ABSTRACT

Current treatment options for bacterial vaginosis (BV) have been shown as inadequate at preventing recurrence and do not provide protection against associated infections such as HIV. This study examines the feasibility of incorporating the antimicrobial peptide subtilosin within covalently cross-linked PEG-based hydrogels for vaginal administration. The PEG-based hydrogels (4% and 6%, w/v) provided a two-phase release of subtilosin with an initial rapid release rate of 4.0 µg/hr (0-12 h) followed by a slow sustained release rate of 0.26 µg/hr (12-120 h). The subtilosin-containing hydrogels inhibited the growth of the major BV-associated pathogen *Gardnerella vaginalis* with a reduction of 8 $\log_{10}$ CFU/ml with hydrogels containing $\geq$15 µg entrapped subtilosin. In addition, the growth of four common species of vaginal lactobacilli was not significantly inhibited in the presence of the subtilosin-containing hydrogels. The above findings demonstrate the potential application of vaginal subtilosin-containing hydrogels for prophylaxis of BV.

INTRODUCTION

Bacterial vaginosis (BV) is a common and often recurrent vaginal pathology in women of childbearing age (1-3). BV is characterized by overgrowth of anaerobic pathogens such as *Gardnerella vaginalis*, *Prevotella*, *Peptostreptococcus*, *Mobiluncus* and *Bacteroides spp.* (1, 2). Women with BV display reduced vaginal *Lactobacillus spp.* and high vaginal pH (up to 7.0) (4). The disrupted vaginal microbiome in women with BV significantly increases risk to sexually transmitted diseases such as HIV, in addition to pre-term birth and infertility (5-9).

The current recommended treatment regimen for BV is oral or vaginal administration of metronidazole or clindamycin (10, 11). However, the recurrence rate for BV is high with cure rates of only 60-70% after a month of treatment (12-15). Another concern with the current antibiotic treatments for BV is the development of antimicrobial resistance (AMR) which is defined as the evolution of resistant strains to a given set of antimicrobials (16). AMR may result from horizontal gene transfer of plasmids carrying multiple drug resistance (MDR) markers (for review please see: **Hawkey PM, Jones AM**. 2009) (17). While AMR is less readily observed with metronidazole, recent reports have suggested the development of resistance with clindamycin treatment (18-21). Prolonged treatment with antibiotics may also result in associated problems such as systemic toxicity and inhibition of healthy vaginal lactobacilli (22). Therefore alternative options are being explored for the prophylaxis and treatment of BV, such as oral/vaginal administration of probiotics and vaginal acidification (23-27). In addition, vaginal microbicide formulations designed for the prevention of HIV are currently being examined for their inhibitory effect on BV-associated pathogens given the significant correlation between BV and HIV incidence (28-30). The Phase I safety trial of a microbicide candidate BufferGel showed a reduction in the incidence of BV from 30% at enrollment to 6% after one week (28). Another study examining the effect of polystyrene sulfonate (PSS) and cellulose sulfate (CS) on BV showed that the above compounds inhibited several BV-associated anaerobic bacteria at concentrations below 10 mg/ml (30). Currently, a Phase 3 trial for assessing the efficacy of 1% SPL7013 Gel (VivaGel) for the treatment of BV is underway (ClinicalTrials.gov Identifier NCT01577537).

Recently, bacteriocins have been suggested as potential alternatives to antibiotics for the treatment of BV (31-36). Unlike broad-spectrum antibiotics, some bacteriocins selectively target pathogenic microorganisms without disturbing the healthy vaginal flora. One such bacteriocin, subtilosin A (referred to hereafter as subtilosin) has demonstrated antimicrobial activity against several BV-associated pathogens including *G. vaginalis* (34, 37, 38). Subtilosin is a cyclic anionic peptide produced by *Bacillus subtilis* and *Bacillus amyloliquifaciens* (33, 39, 40). Subtilosin inhibits *G. vaginalis* by forming transient pores in the cell membrane resulting in an efflux of ATP and subsequently cell death (41). In addition to its antimicrobial properties, subtilosin has antiviral activity against herpes simplex virus type 1 (HSV-1) inhibiting HSV-1 replication in a dose-dependent manner (42). The antimicrobial and antiviral properties of subtilosin make it a good candidate for development as a vaginal microbicide for the prophylaxis of BV. Therefore, the feasibility of incorporating subtilosin in poly(ethylene glycol) (PEG)-based hydrogels for vaginal administration is investigated in this study.

In the current study, PEG-based hydrogels were developed for the sustained release of subtilosin. The hydrogels were formed *in situ* by the covalent cross-linking of 8-arm PEG-SH and 4-arm PEG-NHS polymers. Subtilosin was incorporated into the hydrogels by passive entrapment within the polymer matrix and its release profile examined. The antimicrobial activity of the subtilosin-containing hydrogels against the predominant BV pathogen *G. vaginalis* was evaluated using an endpoint assay and by observing death kinetics in the presence of a stressor. These data was collected through plate counting. The effect of the subtilosin-containing hydrogels on the normal vaginal

flora was assessed by examining the growth of four reference species of healthy human vaginal lactobacilli in the presence of the PEG-based hydrogels.

MATERIALS AND METHODS

Materials

The 8-arm PEG-SH (20 kDa) and 4-arm PEG-NHS (20 kDa) polymers were obtained from NOF Corporation (White Plains, NY). The micro-BCA protein assay kit was obtained from Thermo Fisher Scientific Inc. (Rockford, IL) and the Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Clindamycin (CLI) was obtained from Tokyo Chemical Industry (Tokyo, Japan). The p24 protein assay kit was obtained from Advanced BioScience Laboratories, Inc. (Kensington, MD).

Production of subtilosin

Subtilosin was isolated and purified through fermentation of *Bacillus amyloliquefaciens* KATMIRA1933, as previously described (33, 37). Briefly, the cell-free supernatant was collected, filter-sterilized and then purified using ammonium sulfate (30%) precipitation and affinity chromatography with a C18 column. The concentration of subtilosin was determined using the micro-BCA protein assay kit (as per the manufacturer's instructions) and found to be 3.14 mg/ml. The subtilosin solutions were stored in sterile distilled water at 4 °C until further use.

Stability of subtilosin

The stability of subtilosin was determined at pH 7.4 and 9.0 using the following procedure. Aqueous subtilosin solutions were lyophilized using a centrifugal evaporator

and re-suspended in sodium phosphate buffer (PB, 20 mM, pH 7.4 and 9.0). The solutions were then diluted to a protein concentration of 200 µg/ml (in the PB buffer) for the appropriate pH condition and incubated at 37 °C. Aliquots were withdrawn at pre-determined time intervals over a period of two weeks, and subtilosin concentration was analyzed by HPLC using a Waters XSELECT™ HSS T3 2.5 µm (3.0 x 50 mm) column. The mobile phase consisted of water with 0.05% TFA (solvent A) and acetonitrile with 0.05% TFA (solvent B). A gradient from 5% to 100% B was applied over 6.5 min, at a flow rate of 0.5 ml/min. The subtilosin concentration at each time point was expressed as a percentage of the initial concentration (t=0 min) and plotted over time. The experiment was done in triplicate and data expressed as mean±SEM.

Preparation of PEG-based hydrogels with subtilosin

The PEG-based hydrogels were prepared by passively entrapping subtilosin within the polymer matrix. Hydrogels were prepared by mixing varying amounts of 8-arm PEG-SH (4%, 6% and 8%; w/v) with 2 equiv. of 4-arm PEG-NHS and subtilosin in PB (pH 7.4) at RT. The amount of subtilosin loaded into the hydrogels was verified by Bio-Rad protein assay. The time of formation of the hydrogels was determined using the "inverted tube method" and was noted as the time when the solution ceased to flow, upon inversion of the tube (43-45).

Release of subtilosin from hydrogels

The release of subtilosin from the hydrogels was determined in phosphate buffered saline (PBS, 10 mM, pH 7.4). The hydrogels with passively entrapped subtilosin were placed in vials and immersed in PBS. The vials were incubated at 37 °C,

on an orbital shaker. At pre-determined time intervals, the PBS was removed and replaced with an equal volume of PBS, in order to maintain sink conditions throughout the study. The amount of subtilosin in the release medium was determined using the Bio-Rad protein assay, as per the manufacturer's instructions (O.D. 595 nm). The cumulative release of subtilosin was expressed as a percentage and plotted over time (n=3, mean±SEM).

Bacterial strains and growth conditions

Gardnerella vaginalis ATCC 14018 was the reference BV-associated strain used in these studies. The cells were stored at -80 °C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) and 15% glycerol. For *in vitro* studies, cells from frozen stocks were cultured on human blood bilayer-Tween (HBT) agar (Remel, Lenexa, KS) and grown at 37 °C in 5% CO_2 and 2.5% H_2 for 48 h using EZ Anaerobe Container System GasPaks (Becton, Dickinson and Co., Sparks, MD). Colonies were inoculated in BHI medium supplemented with 3% horse serum for 24-48 h and then serially diluted and plated until counts were observed at 10^8 CFU/ml. These cells were then subcultured at least twice before use. Briefly, the overnight culture was transferred to fresh BHI medium supplemented with 3% horse serum and incubated anaerobically at 37°C. All media and agar were pre-incubated for at least 24 h in the above-mentioned anaerobic conditions to remove oxygen.

The four reference species of human lactobacilli used in this study were *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus gasseri* ATCC 33323,

Lactobacillus plantarum ATCC 39268 and *Lactobacillus vaginalis* ATCC 49540. These four were chosen as they represent a wide selection of species seen in both healthy and women with recurrent BV infections. Non-pregnant women with largely normal pathologies have predominantly one or two species of lactobacilli (generally *L. crispatus*, *L. gasseri* or *L. iners*) (46, 47). While as individuals with recurrent infections have been found with a wider array of lactobacilli including all of those tested in this study. However, the quantity of lactobacilli is comparatively lower in those with recurrent infections. Therefore, by testing species identified in both pathologies, our study indicates the effect of our delivery system on a wider variety of microbiomes. The lactobacilli *spp*. were stored at -80°C in DeMan, Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) containing 15% glycerol. The cells were cultured on MRS agar and grown aerobically at 37 °C overnight. Single colonies were inoculated in MRS broth and grown aerobically for 24 h with agitation. These cells were subcultured twice before use.

End-point evaluation of *G. vaginalis* growth on subtilosin-containing hydrogels

The growth of *G. vaginalis* on hydrogels with varying amounts of passively entrapped subtilosin was evaluated by endpoint analysis. This procedure was chosen with the particular aim of evaluating the final effect of the stressor on the targeted microorganism rather than to study the kinetics of the bacterial growth in the presence of the antimicrobial. Hydrogels (4%, w/v) with varying amounts of subtilosin (8, 12, 15 and 20 µg per 50 µl of gel) were prepared in a 96-well plate. In addition, hydrogels with no entrapped subtilosin were prepared in order to evaluate the possible effect of the hydrogel alone on *G. vaginalis* growth. The vaginal pathogen (10^8 CFU/ml) was added

to the wells (200 µl/well) and incubated anaerobically at 37 °C for 48 h. Medium alone was used as the positive control and CLI (100 µg/ml) was used as the negative control for growth. Following the incubation period, growth was evaluated by performing viable cell counts using the drop plate method (34, 48). Cells were mechanically separated from the hydrogels by making a slit in the hydrogels using a pipette tip. The cell counts were performed in duplicate from each well and the data expressed as the Mean ±SD of three experiments (n=9). *G. vaginalis* growth in the presence of subtilosin alone (without hydrogel) was evaluated by incubation in the same medium supplemented with pre-determined concentrations (8-20 µg/ml) of subtilosin using the procedure described above (n=3).

End-point evaluation of *Lactobacillus spp.* in the presence of subtilosin-containing hydrogels

The growth of four strains of vaginal lactobacilli (*L. acidophilus, L. gasseri, L. plantarum* and *L. vaginalis*) in the presence of subtilosin-containing hydrogels was evaluated as follows: hydrogels (4% w/v) with subtilosin (8, 12, and 20 µg per 50 µl of hydrogel) and subtilosin solutions (50 µl/well in PB, final concentrations of 8- 20 µg/ml per well) were prepared in a 96-well plate. In addition, hydrogels with no entrapped subtilosin were prepared in order to evaluate the effect of the hydrogel alone on lactobacilli growth. The selected *Lactobacillus* spp. were added to the wells (200 µl/well of overnight culture) and incubated in anaerobic conditions at 37 °C for 48 h. Medium with no antimicrobial added was used as the positive control and CLI (100 µg/ml) was used as the negative control for growth. Enumeration was performed in conditions identical to *G. vaginalis* experiments. Briefly, growth was evaluated through the use of

viable cell counts using the drop plate method. Following 48 hours of incubation cells were mechanically removed from the hydrogels using a sterile pipette tip. The cell counts were performed twice in triplicates. Data is expressed as the Mean ±SD of two experiments (n=6).

RESULTS

Stability of subtilosin

Subtilosin is a relatively hydrophobic, cyclic peptide with a molecular weight of 3.4 kDa (33, 39, 40). The stability of subtilosin was investigated under varying pH conditions (pH 4.0-9.0) before incorporation into PEG-based hydrogels. Having a pI of 3.88, subtilosin is poorly soluble at pH 4.0-5.0. Therefore, its stability was not investigated at this pH range. The stability of subtilosin was evaluated in PB at pH 7.4 and 9.0 over a period of 2 weeks using HPLC. No change in the concentrations of subtilosin was observed indicating that subtilosin is stable under these conditions (Figure 1).

The stability of peptide drugs is especially important as peptides often undergo conformational changes, hydrolysis and oxidation when incorporated in a formulation (49, 50). This can affect the activity of the peptide and thus the efficacy of the formulation. Sutyak et al. (2008) indicated that subtilosin (at pH ~6.5) is heat-stable, with no change in antimicrobial activity following incubation at 100 °C for one hour (33). The current study examines the long-term stability of subtilosin in aqueous buffer at 37 °C under varying pH conditions. Since subtilosin did not undergo degradation

under the above-mentioned conditions, it is likely to retain its activity when incorporated in the hydrogel formulation.

Preparation of PEG-based hydrogels and release profile of subtilosin

The PEG-based hydrogels were prepared by cross-linking the 8-arm PEG-SH and 4-arm PEG-NHS polymers via thioester bonds (Figure 2). Subtilosin was loaded into the hydrogels by passive entrapment within the polymer matrix. The concentration of subtilosin prior to incorporation in the hydrogels was determined using both micro-BCA assay and Bio-Rad protein assay. The micro-BCA assay was found to interact better with subtilosin given the anionic nature of the peptide. However, unlike the Bio-Rad protein assay, the micro-BCA assay was found to interfere with the PEG-SH polymer. Hence, the Bio-Rad protein assay was used to determine subtilosin concentration before incorporation into hydrogels and in the release medium. The hydrogels were prepared by mixing 8-arm PEG-SH (4%, 6% and 8%; w/v) with 2 equiv. of 4-arm PEG-NHS and subtilosin in PB (pH 7.4) at RT. The hydrogels formed within 30 min and increasing the polymer concentration did not significantly alter the gelation time (Table 1).

The release of subtilosin from the hydrogels (4% and 6%, w/v) was evaluated in PBS (10 mM, pH 7.4) at 37 °C. The amount of subtilosin released at each time point was determined using the Bio-Rad protein assay and percentage cumulative amount released plotted over time. The release of subtilosin from the hydrogels was two-phase with an initial rapid release phase (47% and 42% release in 24 h from the 4% and 6% w/v hydrogels, respectively), followed by a slow sustained release phase (Figure 3). The

average release rate during the rapid phase (0-12 h) was 3.96 and 4.04 µg/hr for the 4% and 6% w/v hydrogels, respectively. The release rate declined to 0.28 and 0.24 µg/hr from 12-120 h for the 4% and 6% w/v hydrogels, respectively.

Growth of *G. vaginalis* on subtilosin-containing hydrogels

Subtilosin has been previously reported to inhibit *G. vaginalis* with a minimum inhibitory concentration (MIC) of 7.2 or 9.2 µg/ml depending on whether the pathogen was grown planktonically (former case) or in biofilms (latter case) (34, 37). The MIC of subtilosin for the growth conditions used in our study was therefore determined and found to be 12 µg/ml, which is within the range of previously reported MICs. The inhibitory effect of the hydrogels with varying amounts (8-20 µg per 50 µl of hydrogel) of entrapped subtilosin on *G. vaginalis* growth was determined using endpoint analysis. Growth was evaluated by performing viable cell counts after the incubation period of 48 h. A 3 $\log_{10}$ reduction in viable cell count was observed on hydrogels with 12 µg of subtilosin and 8 $\log_{10}$ reduction was observed on hydrogels with 15 µg and 20 µg of subtilosin (Figure 4). No reduction in cell count was observed on the control hydrogels (0 µg subtilosin) indicating that the inhibition of bacterial growth was due to the antimicrobial activity of subtilosin alone (Figure 4).

Growth of *Lactobacillus spp.* in the presence of subtilosin-containing hydrogels

The growth of four vaginal *Lactobacillus spp.* (*L. acidophilus, L. gasseri, L. plantarum* and *L. vaginalis*) in the presence of subtilosin (8-20 µg/ml), and subtilosin-containing hydrogels (8-20 µg per 50 µl of hydrogel) was evaluated using plate counting following 48 h of incubation. In these experiments, less than one log viable cells reduction was

observed which is considered as microbiologically insignificant change in the cell numbers (Figure 5A-D) This is consistent with the earlier observed values of MIC for subtilosin (>100 µg/ml) against *L. vaginalis*, *L. gasseri* and *L. plantarum* (37).

DISCUSSION

The current study examined the feasibility of incorporating subtilosin in PEG-based hydrogels for sustained vaginal drug delivery. Controlled drug release is particularly important for vaginal microbicides since it is essential that therapeutic drug concentrations be maintained in the vagina for a prolonged period of time (51). Toward this end, intravaginal rings (IVRs) are being developed for the sustained release of antiretroviral drugs in the vagina for a period of 30-40 days (51, 52). However, IVRs pose the problems of high initial burst release of drug and drug instability due to high manufacturing temperatures of 80-90 °C (52-54). Therefore, there is a need for developing alternative vaginal drug delivery systems for application as microbicides. Temperature and pH sensitive hydrogels and nanoparticles are currently being explored as potential microbicide delivery systems (55-58).

In our laboratory, PEG-based hydrogels were previously developed for ocular drug delivery and dermal wound healing (43-45). The controlled release of pilocarpine and doxycycline from the hydrogels was achieved by passive entrapment of the drugs within the polymer network (43-45). The current study examines the feasibility of achieving controlled release of subtilosin from PEG-based hydrogels formed via thioester cross-links. The hydrogels are degradable under physiological conditions since the thioester cross-links are hydrolytically labile (43).

The release of subtilosin from the PEG-based hydrogels into the bulk medium was biphasic, with an initial rapid phase followed by a slow sustained release phase. The relative influence of diffusion and polymer relaxation on subtilosin release from the PEG-based hydrogels was determined by fitting the first 60% of the total amount of subtilosin released to the

The diffusion exponent n was calculated from the slope of the plot of log $(\frac{M_t}{M_\infty})$ vs. log (t) and was found to be 0.59 and 0.46 for the 4% and 6% w/v hydrogels respectively. Since $0.45< n<0.89$, the release mechanism was non-Fickian or anomalous indicating that subtilosin release was controlled by both the rate of diffusion through the polymer matrix and polymer relaxation (59).

Controlled release of antimicrobials has been shown to be effective in inhibiting microbial growth for a prolonged period of time (60-62). Dang et al. showed that polycaprolactone matrices loaded with ciprofloxacin and miconazole nitrate provided sustained release of the antimicrobials with activity against the vaginal pathogens *Neisseria gonorrhea* and *Candida albicans* for 13-30 days (61). Moreover, controlled release of antimicrobials has been shown to be more efficacious than a single continuous dose release. The controlled release of the bacteriocin nisin was found to effectively inhibit the growth of a reference microorganism, *Micrococcus luteus* for a period of 48 h when compared to the instant addition of nisin, which inhibited *M. luteus* for only 12 h (60). Similarly, Abdelghany et al. showed that the controlled release of gentamicin from PLGA particles inhibited biofilms of *Pseudomonas aeruginosa* with improved efficacy over a single dose of free gentamicin (62). The results of the current study demonstrate

that subtilosin retains its antimicrobial activity against *G. vaginalis* when formulated as PEG-based hydrogels. A subsequent evaluation of the growth kinetics of *G. vaginalis* in the presence of free subtilosin and subtilosin-containing hydrogels will give further insight into the time course of inhibition with controlled release in comparison with a single dose of subtilosin.

Vaginal microbicides must only minimally affect healthy vaginal lactobacilli since a compromised vaginal environment increases susceptibility to HIV infection and other STDs (63). The failure of nonoxynol-9, an early microbicide candidate in clinical trials was partially due to its inhibitory effect on lactobacilli *spp.* (64). Therefore, current microbicide candidates are being screened for their effect on multiple vaginal lactobacilli *spp*. prior to clinical testing (64-65). Anderson et al. evaluated the inhibitory effect of the microbicides sulfuric acid-modified mandelic acid (SAMMA), polystyrene sulfonate (PSS) and cellulose sulfate (CS) on commercially available lactobacilli *spp.* and vaginal isolates (66). Their findings indicated selective inhibition of *Lactobacillus crispatus* and *Lactobacillus acidophilus* with SAMMA and CS, suggesting differential sensitivity of *Lactobacillus spp*. to the microbicide candidates (66). More recently, a clinical study by Ravel et al. indicated a shift in vaginal microbiota from predominantly *Lactobacillus spp*. to anaerobic *spp*. with twice-daily application of CS and nonoxynol-9 gel (65). Another study by Fichorova et al. showed that CS selectively inhibited epithelium-associated and planktonic *L. crispatus*, while hydroxyethylcellulose (HEC) had no effect (67-68). The current study indicated no significant inhibition of healthy vaginal lactobacilli in the presence of subtilosin-containing hydrogels, suggesting that the PEG-based hydrogels do affect the normal vaginal flora.

CONCLUSION

In this study the feasibility of incorporating the antimicrobial peptide subtilosin in PEG-based hydrogels for vaginal drug delivery was demonstrated. The hydrogels with passively entrapped subtilosin showed a two-phase release of subtilosin with an initial rapid phase (4.0 µg/hr), followed by a sustained release phase (0.26 µg/hr) over several days. Subtilosin released from the hydrogels retained activity against the primary BV pathogen *G. vaginalis*. A reduction of >3 $\log_{10}$ CFU/ml was observed on the subtilosin-containing hydrogels in comparison with the medium control. The hydrogels did not inhibit the growth of four strains of healthy human vaginal lactobacilli *spp*. Collectively, the above results suggest the potential application of subtilosin-containing hydrogels as vaginal microbicides for BV prophylaxis.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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TABLES AND FIGURES

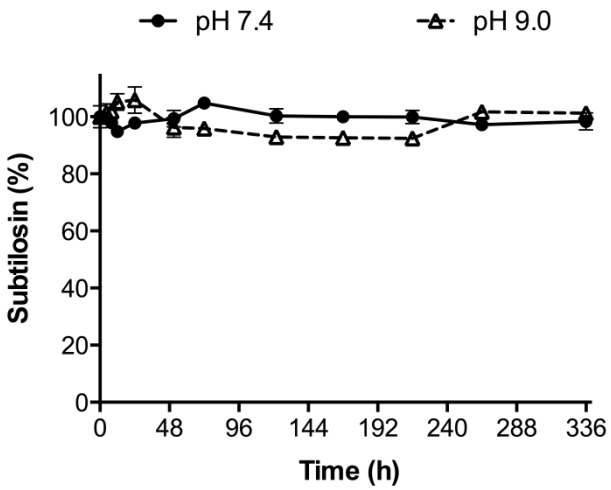

Figure 1. Stability of subtilosin at 37 °C in PBS (pH 7.4 and 9.0); mean±SEM, n=3. Subtilosin concentration at each time point was determined using HPLC. Subtilosin was found to be stable under both pH conditions over a period of two weeks.

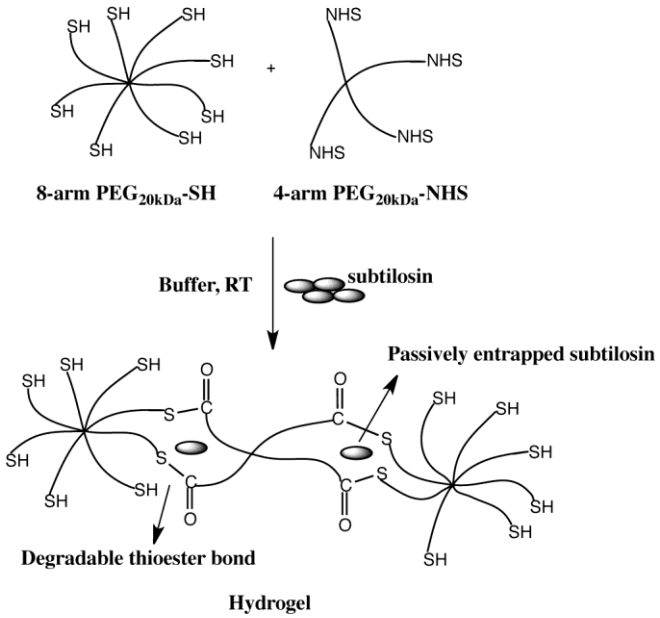

Figure 2. Schematic representation of hydrogel formation using 8-arm PEG-SH and 4-arm PEG-NHS polymer

8-arm PEG-SH (mg/0.05 ml)	**4-arm PEG-NHS (mg/0.05 ml)**	**Time of hydrogel (0.1 ml) formation (min)**
4	8	26.7 ± 1.6
6	12	25.6 ± 0.3
8	16	25.6 ± 1.1

Table 1. Time of formation of hydrogels with passively entrapped subtilosin; mean±SD, n=3

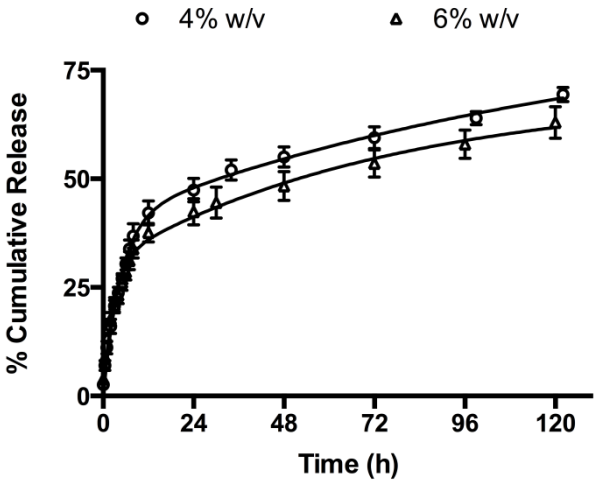

Figure 3. Release of subtilosin from 4% w/v and 6% w/v PEG-based hydrogels in PBS at 37 ºC; mean±SEM, n=3. The release of subtilosin from the hydrogels was two-phase,

with an initial rapid release phase (47% and 42% release in 24 h from the 4% and 6% w/v hydrogels, respectively), followed by a slow sustained release phase. The average release rate for the first 12 h was 3.96 and 4.04 µg/hr for the 4% and 6% w/v hydrogels, respectively. The average release rate from 12-120 h was 0.28 and 0.24 µg/hr for the 4% and 6% w/v hydrogels, respectively.

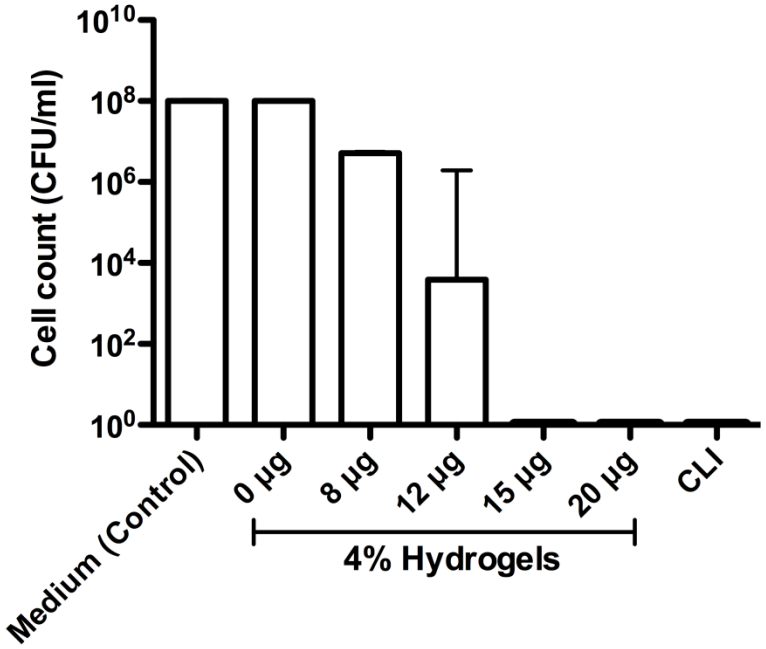

Figure 4. Growth of *G. vaginalis* on hydrogels with varying concentration of subtilosin, determined by endpoint analysis; mean±SD n=9. *G. vaginalis* was plated on wells containing 4% w/v hydrogels with 8-20 µg entrapped subtilosin (per 50 µl of gel). The plate was incubated at 37 °C for a period of 48 h. Medium was used as the positive control for growth and CLI (100 µg/ml) was used as the negative control. Cell counts indicated a reduction of 3 $\log_{10}$ CFU/ml on hydrogels with 12 µg subtilosin and 8 $\log_{10}$ CFU/ml on hydrogels with 15 µg and 20 µg subtilosin compared to the medium control.

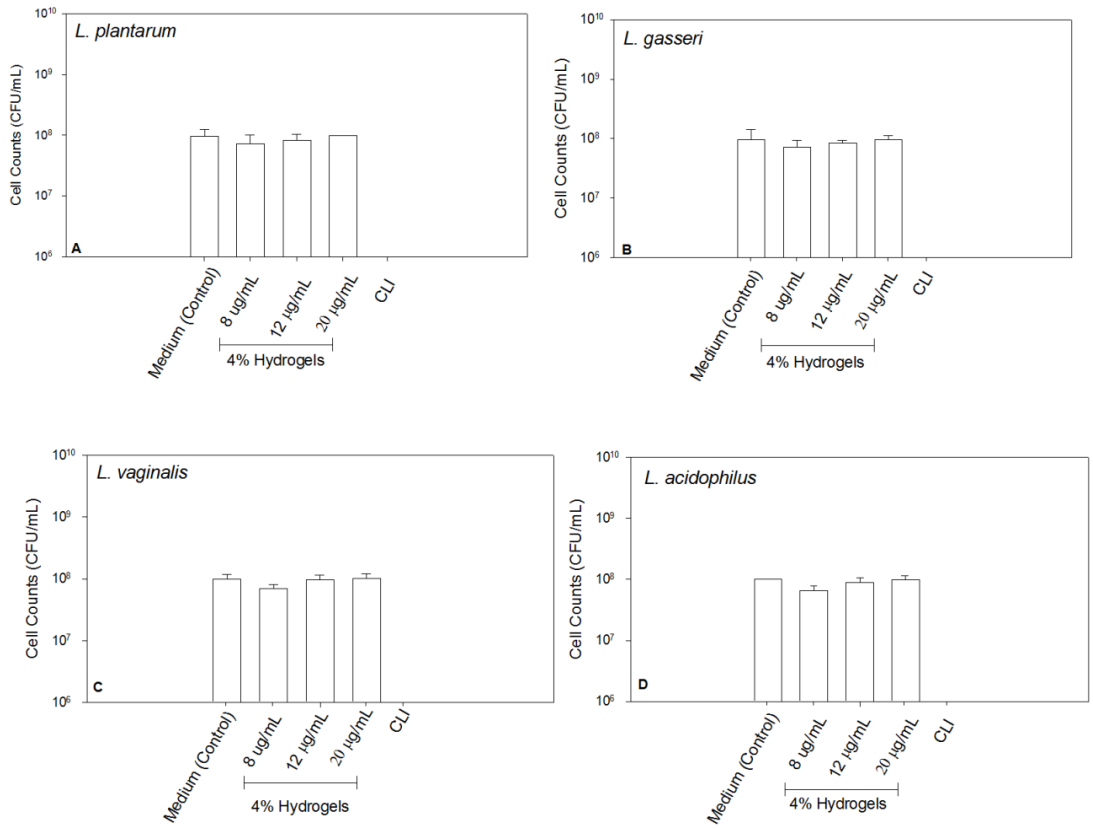

Figure 5 A-D. Growth of lactobacilli *spp.* (A. *L. plantarum*, B. *L. gasseri*, C. *L. vaginalis* and D. *L acidophilus) in* medium with various concentrations (8- 20 µg/ml) of subtilosin, and on 4% w/v hydrogels with 8-20 µg passively entrapped subtilosin (per 50 µl of gel); mean±SEM n=6. CLI (100 µg/ml) was used as a negative control and bacteria in medium was used as a positive control. Following 48 h, all lactobacilli were enumerated using the drop plate method. Cell counts indicated no significant reduction in $\log_{10}$ CFU/ml for any tested concentrations of subtilosin hydrogels as compared to the medium control.

Benzoyl Peroxide Formulated Polycarbophil/Carbopol® 934P Hydrogel with Selective Antimicrobial Activity, Potentially Beneficial for Treatment and Prevention of Bacterial Vaginosis

This following discusses the production of and rheological properties of a polycarbophil/carbopol 934P hydrogel which passively entrapped benzoyl peroxide for the purpose of inhibiting *G. vaginalis.* It was found that 0.01% BPO-encapsulated gel was inhibited *G. vaginalis* in both well diffusion assays and direct contact assays. In time-kill assays, 1% BPO-encapsulated gel caused a 6 log reduction of *G. vaginalis. L. acidophilus, L. gasseri, L. plantarum* and *L. vaginalis* were inhibited at 2.5% in well diffusion assays. The rheological properties indicated that the gel is suitable for vaginal application and that the addition of BPO did not significantly alter rheological properties.

The candidate performed all of the microbiological work on *G. vaginalis* assisted in the lactobacilli work, production of the hydrogel, assisted in the transwell plate experiments as well as writing the final manuscript.

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Benzoyl Peroxide Formulated Polycarbophil/Carbopol® 934P Hydrogel with Selective Antimicrobial Activity, Potentially Beneficial for Treatment and Prevention of Bacterial Vaginosis

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Abstract

The human vagina is colonized by a variety of indigenous microflora; in healthy individuals the predominant bacterial genus is *Lactobacillus* while those with bacterial vaginosis (BV) carry a variety of anaerobic representatives of the phylum *Actinobacteria*. In this study, we evaluated the antimicrobial activity of benzoyl peroxide (BPO) encapsulated in a hydrogel against *Gardnerella vaginalis*, one of the causative agents of BV, as well as indicating its safety for healthy human lactobacilli. Herein it is shown that in well diffusion assays *G. vaginalis* is inhibited at 0.01% hydrogel-encapuslated BPO and that the tested *Lactobacillus* spp. can tolerate concentrations of BPO up to 2.5%. In direct contact assays (cells grown in a liquid culture containing hydrogel with 1% BPO or BPO particles), we demonstrated that hydrogels loaded with 1% BPO caused 6-log reduction of *G. vaginalis*. Conversely, three of the tested *Lactobacillus* spp. were not inhibited while *L. acidophilus* growth was slightly delayed. The rheological properties of the hydrogel formulation were probed using oscillation frequency sweep, oscillation shear stress sweep and shear rate sweep. This indicating the gel as suitable for vaginal application and that the encapsulation of BPO did not alter rheological properties.

1. Introduction

The healthy human vagina is colonized by a variety of bacterial species with lactobacilli being a predominant group of microorganisms. The cause and etiopathogenesis of bacterial vaginosis (BV) is still poorly understood; however, it has been extensively shown that when the natural ecology shifts to mainly Gram-negative *Actinobacteria,* microbial disease such as BV can occur [1]. BV affects one in three women in the

United States. Initially, infection leads to discomfort and a foul discharge while long term infection has been correlated with increased risk of pelvic inflammatory disease, sexually transmitted infections, and pregnancy complications including preterm birth [2, 3]. Advanced methodological approaches utilizing qPCR and deep sequencing confirm BV as a multi-species infection [4, 5]. However, *G. vaginalis* is observed in approximately 70% of tested women regardless of whether the individual is considered positive for BV based on the Nugent criteria [5-7].

Current Food and Drug Administration (FDA)-approved treatments include nitroimidazoles (i.e. metronidazole) or the lincosamide clindamycin [8]. These antibiotics alter the indigenous microflora by inhibiting both the problematic and healthy bacterial species. *G. vaginalis* and other anaerobic bacteria grow in complex biofilms; therefore, a high dose of antibiotics is required to inhibit their growth. This high-dose requirement causes wide-spectrum microbial elimination and arrests the competitive exclusion capability of *Lactobacillus* leading to increased tolerance of BV-associated microorganisms [9]. Recurrence of resistant BV-associated pathogens is seen in more than 50% of women up to a year following treatment from metronidazole or clindamycin [10, 11]. Although there is no established causation between antibiotic usage and recurrence incidence it is plausible that the lack of selectivity in available antibiotic treatment options may severely disrupt restoration of normal vaginal microbiota, which could prevent BV recurrence [8]. Lactobacilli are naturally occurring representatives of the vaginal microbiota, beneficiary to overall vaginal health. These bacteria effectively protect the vaginal environment against pathogens by producing natural antimicrobials such as hydrogen peroxide, bacteriocins, and weak organic acids

[9, 12]. Usually, healthy vaginal environments are colonized by predominantly one or two species of lactobacilli (generally *L. crispatus* or *L. iners)*. Women with recurrent BV infections have been documented to have a wider variety of lactobacilli including but not limited to *L. gasseri, L. plantarum, L. jensenii,* and *L. vaginalis* [13, 14]. Due to this wide variety of non-pathogenic bacteria it is imperative to treat the pathogenic microorganisms with a more selective antimicrobial which is harmless to lactobacilli and does not promote resistance in pathogens..

BPO is an organic peroxide included on the World Health Organization (WHO) Model Lists of Essential medicines [15, 16]. It is identified as safe as for human use and is found in a variety of products ranging from flour bleaching agents to cleaning products. Further, BPO is often used in topical formulations because it is reasonably stable in gel solutions. Free radical generation from BPO has not been associated with acquired resistance in acne-associated cases [16, 17]. Resistance from BPO usage has not been reported for *G. vaginalis*; moreover, naturally occurring *Lactobacillus* spp. produce hydrogen peroxide which also utilizes a homolytic fission to competitively inhibit other bacteria. When used in topical medicinal or cosmetic formulations, such as in a hydrogel treating acne, BPO undergoes homolytic fission resulting in two benzoate radicals due to the perester nature of the compound. As benzoate passes through the epithelium, a proton is acquired, altering the compound into benzoic acid which diffuses freely into the bloodstream where it is deprotonated due to its low pKa [18]. Free cations in the blood will be attracted to benzoate and the resultant structure is excreted without further alteration (Fig. 1).

Antibiotics targeted at vaginal pathogens are administered either orally or intravaginally. The latter requires a delivery system which provides physical contact of the antimicrobial agent with the mucosa. Gels represent one of the most frequently used intravaginal delivery systems due to their acceptability, feasibility, and low cost [19]. This delivery system reduces the bacterial load through the formation of a physical barrier with the vaginal topography which enhances natural defenses of the vaginal environment [12, 20]. The most effective gels also need to be mucoadhesive; this allows for an extended period of contact between the encapsulated drug and the vaginal epithelium while maintaining its properties [21, 22].

Several currently available vaginal hydrogels (Crinone®, Replens®, RepHresh ®, Advantage-S®, Miphil® etc.,) are utilizing bioadhesive properties of polycarbophil which belongs to the poly (acrylic) acid group.

The underlying hypothesis for this study was that the BPO encapsulated in bioadhesive hydrogel would have a selective antibacterial profile required for a sustained treatment effect in BV, making it a good candidate for further development. In this study, we developed a polycarbophil/Carbopol® 934P hydrogel with encapsulated BPO . The resulted gel formulation was capable of controlling the BV-associated pathogen *G. vaginalis* while not inhibiting four healthy vaginal microorganisms (*L. acidophilus, L. gasseri, L. plantarum* and *L. vaginalis*). This gel is conceptually close to already marketed products with proven safety features, mimics the function of the peroxide-producing lactobacilli and therefore could be a promising candidate for treatment of BV. The rheological properties of the formulation evaluated *in vitro* indicate that the gel will maintain its structure within the vaginal environment.

2. Materials and Methods

2.1. Chemical compounds. Noveon® AA-1 polycarbophil was obtained from Lubrizol Advanced Materials, INC (Cleveland, OH 44141). Hydrous BPO (74% BPO, 26% water), Carbopol® 934P, acetic acid and sodium acetate were from Spectrum Chemical Manufacturing Corp. (New Brunswick, NJ).

Vaginal fluid simulant (VFS) used in rheological evaluation was composed of (g/L ddH_2O): 3.51 sodium chloride, 0.222 calcium hydroxide, 3.41 potassium hydroxide, 0.018 bovine serum albumin, 2.0 lactic acid, 1.0 acetic acid, 0.16 glycerol, 0.4 urea and 5.0 glucose. To adjust pH to 4.2, 6N hydrochloric acid was added [23]. Sodium chloride, bovine serum albumin, lactic acid and glucose were purchased from Sigma-Aldrich® Co (St Louis, MO 63103). Calcium hydroxide, glycerol and urea were purchased from Fisher Scientific Inc (Pittsburgh, PA15275). Potassium hydroxide and hydrochloric acid were purchased from VWR® International LLC (Randor, PA 19087). Clindamycin (Tokyo Chemical Industry, Tokyo, Japan) was used as a positive control for well diffusion experiments. Aqueous stock solutions of clindamycin were filter-sterilized through 0.2 μm syringe filters (NALGENE, Rochester, NY).

2.2. Bacterial strains, growth, and conditions. G. vaginalis ATCC 14018 was used as the BV-associated pathogen and was stored at -80 °C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) and 15% (by volume) glycerol. Frozen stocks were cultured on human blood bilayer-Tween (HBT) agar (Remel, Lenexa, KS) and grown at 37 °C in 5% CO_2 and 2.5% H_2 for 48 hours using EZ Anaerobe Container System GasPaks (Becton, Dickinson and Co, Sparks, MD). Experiments were performed in Type A Coy Laboratory Vinyl

Anaerobic Chamber (Coy Laboratory Products, Grass Lake, MI). The anaerobic conditions are identical to those provided by the EZ Anaerobe Single colonies were streaked onto HBT plates for the modified agar-streak well diffusion assay. All media and agar for *G. vaginalis* were preincubated in the aforementioned anaerobic conditions for 24 hours to remove oxygen-related stress.

L. vaginalis ATCC 49540, *L. gasseri* ATCC 33323, *L. plantarum* ATCC 39268 and *L. acidophilus* ATCC 4356 were representative of the normal flora of a healthy vagina [13, 14]. *L. gasseri* and *L. vaginalis* are representative of vaginal flora of healthy women while *L. acidophilus* and *L plantarum* have been isolated in women with recurrent BV infections. These were selected as they represent a wider net of non-pathogenic bacteria that should not be inhibited to ensure continued health and maintenance of the vaginal environment.They were stored at -80 °C in DeMan, Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) containing 15% glycerol by volume. The cells were plated on 1.5% w/v MRS agar and grown aerobically at 37 °C. For experimental procedures, single colonies were inoculated in 20ml of MRS and grown aerobically for 24 hours with agitation (100 RPM). Cells were subcultured twice before use. For all experiments, 200 μL of the overnight culture was transferred into 20 mL of fresh broth.

2.3. Hydrogel preparation. Base gel was defined as hydrogel without BPO encapsulated within it. The base gel is prepared as follows (w/w): polycarbophil/ Carbopol® 934P were hydrated in double distilled water. Sodium acetate, acetic acid, Carbopol® gel and glycerol were slowly added to polycarbophil gel while stirring. The final concentrations of these components were: 2% polycarbophil, 1% Carbopol® 934P, 15% glycerol,

0.049% sodium acetate and 0.038% acetic acid. To elevate pH of base gel from 3.20 to 4.50, 5 M sodium hydroxide solution was used.

BPO particles were evenly dispersed in base gel at pre-determined concentrations from 0.01% to 10% in Nasco WHIRL-PAK bags (Fisher Scientific Inc. Pittsburgh, PA15275). The gel was then placed into 50 ml tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 720 relative centrifugal force (RCF in *g* forces) at ambient temperature for 10 minutes in an Allegra 21R Centrifuge (54180 fixed angle rotor) (Analytical Instrument Brokers, LLC, MN).

2.4. Agar-streak and soft agar overlay well diffusion assays. A modified agar streak method described by Waksman and Reilly was used for *G. vaginalis* as the microorganism does not grow well in soft agar [24]. Briefly, colonies isolated from frozen stock were re-streaked on pre-incubated (i.e. kept in the anaerobic environment overnight) HBT plates. To obtain a homogenous lawn of *Lactobacillus* spp.,5 ml ofMRS soft (0.7% w/v) agar was ionculated with overnight cultures (10^7 CFU/ml). and evenly distributed over 1.2% MRS agar plate. Soft agar overlay plates were dried for approximately one hour in a Purified Class II Safety Cabinet (LabConco Co., Kansas City, MI).

Wells were then aseptically punched through the soft agar overlay and the hard agar with the back of a 1000 µl tip. Each well was filled with 100 µl of gel formulation. Fifty µl of 100 µg/ml clindamycin was used as a positive control. Plates were incubated in an anaerobic jar (Sigma Aldrich, St. Louis, MO) for 24 hours at 37 °C. After incubation, zones of inhibition were measured with Vernier calipers (Nova-Tech, Houston, TX). This experiment was performed six times in duplicates.

2.5. Direct contact inhibition studies. Inhibition studies were conducted using the time kill method as described by Liang et al. [25] with modifications. All bacteria were grown in contact with 5 ml 1% BPO gel, 5 ml base gel or 0.05 g BPO particles (0.067 g hydrous BPO). BPO particles are defined as the hydrous BPO compound without encapsulation in the base gel. BPO was not dissolved in ethanol or DMSO as these do not simulate what would be used in an actual product. Each assay included a growth control without a test sample as a negative control. The test sample was placed at the bottom of a 50 ml tube, followed by 40 ml of either BHI+3% horse serum (HS) or MRS broth. Overnight cultures of *G. vaginalis* were diluted to 10^6 CFU/ml while the four *Lactobacillus* spp. were diluted to 10^3 CFU/ml. Cells were grown in direct contact with base gel, BPO gel or BPO particles and incubated anaerobically at 37 °C. At 0, 1, 3, 6, 9, 12 and 24 hour time intervals, 300 µl of the culture was taken out for viable cell enumeration by the drop plate counting method as described by Herigstad et al. [26]. The experiment was carried out four times in duplicates. Q test was performed and $Q_{90\%}$ was set as rejection level.

2.6. Inhibition by BPO released from the gel. To avoid direct contact between the targeted cells and the released antimicrobial, the inhibition of *G. vaginalis* by the BPO gel through a 0.45µm diffusible membrane was tested via a control insert assay as described by Rajan et al. (manuscript submitted for publication). Briefly, an overnight culture of *G. vaginalis* was diluted to 10^6 CFU/ml, then 600 µl was transferred onto the bottom of a 24 well control insert plate (Becton, Dickinson and Co., Bedford, MA). The control inserts were then placed into the wells and 50 µl of 1% BPO gel, base gel, or 0.5 mg BPO particles was placed on the top of each membrane. At the 0, 1, 3, 6, 9, 12 and

24 hour time intervals 200 µl of culture was removed for enumeration by the drop plating method on HBT agar plates [26]. This experiment was carried out twice in duplicates for a total of 4 replicates. Q test was performed and $Q_{90\%}$ was set as rejection level.

2.7. Microbial growth in pH adjusted media. All cultures were grown in aforementioned, standard conditions for 24 hours at which time 200 µl of overnight culture was transferred to 20 ml of pH adjusted media (MRS for *Lactobacillus* spp. and BHI+3% HS for *G. vaginalis*). Media pH was adjusted to 4.5 (the average pH of all gels) through simple titration using either 0.1 M hydrochloric acid or 30% lactic acid solution. Prior to adjustment, the pH of BHI+3% HS is 7.05 while the pH of MRS is 6.16. Medium was filter-sterilized using 0.45 µm filters (NALGENE, Rochester, NY). Two hundred µl of culture was transferred into a sterile, 96-well microplate (Corning, Inc., Corning, NY). Wells containing bacteria in non-pH adjusted media and pH adjusted media without culture were used as controls. To prevent evaporation, 50 µl of sterile mineral oil was pipetted gently on top of each well to allow for anaerobic growth and Microplates were prepared anaerobically and turbidity was measured at 595 nm (Bio-Rad model 550 microplate reader, Bio-Rad Life Sciences, CA) at 0, 1, 3, 6, 9, 12, 18 and 24 hours at 37 °C in anaerobic conditions. This was performed twice in quadruplicates.

2.8. Rheological measurements. Rheological evaluations of base gel and BPO gel formulations were measured using Hybrid Discovery HR-2 Rheometer (TA Instruments, New Castle, Delaware, USA) equipped with a 25 mm cross-hatched parallel steel plate

and a temperature controlled parallel plate. The gap was maintained at 1.0 mm and the temperature control was set at 37 °C.

Oscillation frequency sweeps and oscillation shear stress sweeps were evaluated on the following formulations: base gel and 1% BPO gel. In oscillation frequency sweeps, the shear stress was fixed at 10 pascal (Pa), within the linear viscoelastic region, and G' and G" were measure between 0.5 to 20 hertz (Hz). In oscillation shear stress measurements, the frequency was fixed at 1 Hz, and shear stress increased from 10 to 1000 Pa.

Flow shear rate measurements at a variable shear rate from 0.1 to 1000 /second were conducted on the formulation. After gelation, the base gel and 1% BPO gel was diluted with VFS at 25%, 50%, 75% and 100% (gel/gel+VFS; v/v) and kept at 37 °C in an incubator overnight prior to test. Viscosity using the flow shear measurements of diluted gel formulation was measured at a fixing frequency of 1 Hz. All experiments were conducted in triplicates.

2.9. Statistics and figure design. All statistical analysis was performed and figures in result section were graphed in Sigma Plot 11.0. The BPO structure and breakdown were made in MarvinSketch 5.12.1.

3. Results and Discussion

3.1.Influence of BPO gel formulations on microbial growth in well diffusion assay.

Well diffusion assays were performed to identify the lowest concentration of BPO (w/w) at which the BV-associated pathogen *G. vaginalis* could be inhibited. Mean values and associated standard deviation of the inhibition zones are shown in Table 1. Zones of inhibition were observed in *G. vaginalis* containing plates following exposure to BPO as low as 0.01% (w/w). Zones were also confirmed in all tested higher concentrations.

Zones of inhibition were observed at BPO concentrations of 2.5% or higher for all lactobacilli strains. No zones of inhibition were observed with the base gel, indicating no associated antimicrobial properties.

3.2.BPO gel formulation selectively inhibits G. vaginalis in direct contact assay.

The 1% BPO gel was chosen for these tests as it represents the highest concentration that inhibited *G. vaginalis* but had no effect on the tested *Lactobacillus* spp. in the well diffusion assay (Figure 2A-E).

To determine survivability of *G. vaginalis,* following 24 hours of direct exposure to 1% BPO (w/w), the drop plating technique was used. Following 24 hours of exposure, a six log reduction of the viable *G. vaginalis* cells was observed when the microorganism was grown in contact with the 1% BPO gel., *G. vaginalis* cells were not inhibited by the base gel and grew up to 7.9 $x10^7$ CFU/ml in the indirect inhibition assay or up to 7.3 $x10^7$ CFU/ml in direct contact with the base gel. Therefore, we conclude that 1% BPO gel is effective at inhibiting *G. vaginalis*. By comparison, free BPO particles were less effective than the base gel, only reducing viable cell counts by approximately three logs (Figure 2A).

To determine the possible effect of direct contact with BPO on the viability of vaginal *Lactobacillus* spp. the direct contact assay was repeated under the same anaerobic conditions outlined for *G. vaginalis*. Following 24 hours of incubation, no significant inhibition was noted in direct contact assays in which cultures were grown in direct contact with BPO particles, the base gel, or the 1% BPO gel (Figure 2B-D). A two log reduction was noted in *L. acidophilus* cultures grown in contact with the 1% BPO gel or the base gel (Figure 2E).

The 1% BPO hydrogel formulation is capable of inhibiting the growth of the BV-associated pathogen *G. vaginalis* while having little to no impact on the growth of selected vaginal *Lactobacillus* spp. Continued maintenance of the vaginal ecology may improve the rate of recovery from BV. As indicated by Mitchell et al., following antibiotic treatment, vaginal lactobacilli recovery is directly proportional to cure success rate [8]. Given that most antibiotics are non-selective in nature, it is imperative to consider the continued impact on the health of the individual by establishing methods that continually maintain a stable vaginal microbiome. The hydrogel described is capable of serving such a function. It is more selective, eliminating the tested pathogenic vaginal bacterium while supporting the growth of a healthy vaginal microbiome and reducing the recurrence rate of BV without supplementary lactobacilli treatment. The suggested and described approach is a technique that is a valuable preliminary assay that addresses the complex problem of BV. Further experiments based on our study will further elucidate the interaction within polymicrobial infections and help identify methodologies to further prevent inhibition of healthy microorganisms.

3.3.Effect of BPO on G. vaginalis viability in an 'indirect contact' assay.

Once the effect of the BPO formulations on the selected microorganisms was studied by the direct contact method, the effect in indirect exposure was evaluated using a control insert plate (Figure 3). The trials were conducted only with *G. vaginalis* at concentrations proven to be inhibitory to this vaginal pathogen because of the expense of the assay. Under conditions identical to those used in the indirect contact experiments, when exposed to gel-diffused BPO, *G. vaginalis* was inhibited similarly to what was observed in direct contact with 1% BPO gel and BPO particles (a six log

reduction in viable cell count). In this, cells were placed at the bottom of the well while the effect of placing cells on top of the inserts was not assessed.

3.4.Growth of vaginal microorganisms in pH adjusted media.

It was observed that in the 'indirect contact' experiments the base gel did not influence *G. vaginalis* viability while BPO particles caused a 3 log reduction in the number of viable cells. The base gel did however cause a 2 log reduction in *L. acidophilus* cultures. Therefore, one of the properties of the base gel (low pH of 4.5) was tested for possible effect on the growth of all tested microorganisms. *L. plantarum, L. gasseri* and *L. vaginalis* grew normally while there was a slight inhibition of *L. acidophilus* and *G. vaginalis*, confirming that pH may play some role in reducing the bacterial viability (Fig 4A-E).

3.5.Rheological properties of gel formulations.

The results of oscillation frequency are shown in Figure 5. In both tested formulations, from 0.5 to 20 Hz, the storage modulus (G') was always greater than loss modulus (G"), indicating that this material exhibits 'gel-like' properties. When frequency varied from 0.5 to 20 Hz, G' of base gel was significantly reduced by 1% BPO ($P<0.01$). When frequency varied from 1.2 to 20 Hz, G" of base gel was significantly reduced by 1% BPO ($P<0.05$). But within measured range, G' of 1% BPO gel was above 600 Pa, indicating that the formulation still possesses a solid-like behavior.

The results of oscillation shear stress are shown in Figure 6 and Table 2. Yield stress was defined as the shear stress at which its corresponding storage modulus was less than 95% of the average value from the first three detected storage modulus within the viscoelastic region, which represents initial gel elasticity. The yield stress of both

formulations was approximately 30 Pa, indicating that the gel deforms upon addition of stress causing it to shear thin, which allows it to be injected intravaginally.

The cross-over of G' and G" represents breakdown of the gel microstructure allowing the material to flow as a viscous liquid. The cross-over of G' and G" of both formulations were all above 300 Pa, indicating that they possess a rigid microstructure [27].

The results of flow shear rate are shown in Figure 7. The viscosity of all tested formulations decreased with increasing shear rate, indicating a shear thinning behavior and their injectability will be enhanced at higher shear rates. A high viscosity, at low shear rates, makes the formulations easier to stay along vagina mucosa [28]. The dispersion of BPO did not influence the viscosity of 50%, 75% and 100% dilution of the base gel. However, 1% BPO greatly increased the viscosity of base gel when it was diluted with VFS at 25%, which could be a result from the decrease in water component from 1% BPO encapsulation. Both the base gel and the 1 % BPO gel, the viscosity, as a function of shear rate ranging from 0.1 to 1000 /s, decreased when the formulation was diluted with VFS, indicating that the formulation will flow more easily when diluted with vaginal fluids *in vivo*. Viscosity significantly decreased upon dilution from 50 % to 25%, which may allow leakage to occur. Therefore, the presented intravaginal drug delivery system presents multiple advantages over oral drug usage in its potential ability to control BV infection. On a much larger scale, the BPO-encapsulated hydrogel conceptually mimics the pathogen-inhibiting function of healthy peroxide-producing lactobacilli. This unique antibacterial profile supports its candidacy as a viable option for treatment and, perhaps equally importantly, for prevention of recurrence of BV. Prevention of the pathogen’s recurrence is crucial for effective treatment and

prophylaxis of bacterial vaginosis; this can be achieved with the gel's ability to sustain healthy microbiota thus preventing the suppressed pathogens from dominating the environment. BPO has been used in flour bleaching and acne treatment for decades. It is permitted by the FDA for use as a topical drug up to 10% as active ingredient in products as acne treatment, based on the studies proving the safety of BPO [29]. There is no evidence which suggests that the topical application of BPO gel or lotion is directly carcinogenic [30-32]. While realizing the danger of oxidative agents when used inappropriately [33], the commonly acknowledged role played by the lactobacilli produced hydrogen peroxide in controlling vaginal pathogens should not be neglected [9]. Since most current studies focus on its safety when applied topically and exposed to ultraviolet light radiation, the safety of BPO when applied intravaginal should be assessed in other models before it is approved as a BV treatment.

Future studies will include gel delivery analysis in a mouse model and identification of possible irritation of vaginal epithelium at 1% BPO, which we propose as a possible dosage level.

4. Conclusion

In this study, we defined and studied a BPO-encapsulated hydrogel formulation capable of inhibiting the growth of the BV-associated pathogen *G. vaginalis* while having a limited effect on healthy lactobacilli in the vaginal ecosystem. The rheological properties of the gel indicate it to be suitable for the suggested application.

Disclaimer

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TABLE 1 Zones of inhibition (mm) from well diffusion assay[a]

BP % (w/w)	*G. vaginalis*	*L. vaginalis*	*L.gasseri*	*L. planturm*	*L. acidophilus*
0 (Base Gel)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.01	0.50±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.10	0.67±0.26	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.25	1.08±0.20	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.50	1.17±0.26	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1.00	1.67±0.26	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
2.50	1.92±0.38	0.50±0.00	0.50±0.00	0.50±0.00	0.50±0.00
5.00	2.25±0.42	0.50±0.00	0.50±0.00	0.50±0.00	0.50±0.26
7.50	2.42±0.38	0.50±0.00	0.50±0.00	0.50±0.00	0.58±0.20
10.00	2.58±0.38	0.50±0.00	0.75±0.27	0.50±0.00	0.67±0.26
Clindamycin (100 μg/ml)	22.20±0.80	17.30±0.50	19.00±0.60	21.80±0.80	26.00±0.90

[a]The distance is measured from the edge of the loading well to the edge of the inhibition zone in millimeters using Vernier calipers. Experiments were conducted six times in duplicates. Mean values and their standard deviations are provided.

TABLE 2 Yield stress and cross-over of G' and G" of tested formulations[a]

Formulation	Yield Stress (Pa)	Cross-over of G' and G" (Pa)
Base gel	32.42±1.73	345.96±6.56
1% BPO gel	29.85±2.72	332.58±12.01

[a] Experiments were conducted in triplicates. Mean values and their standard deviations are shown.

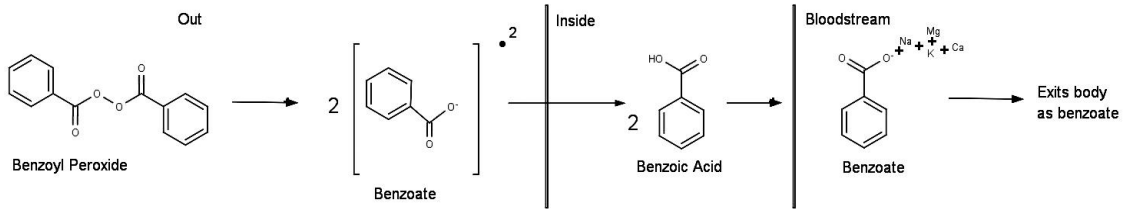
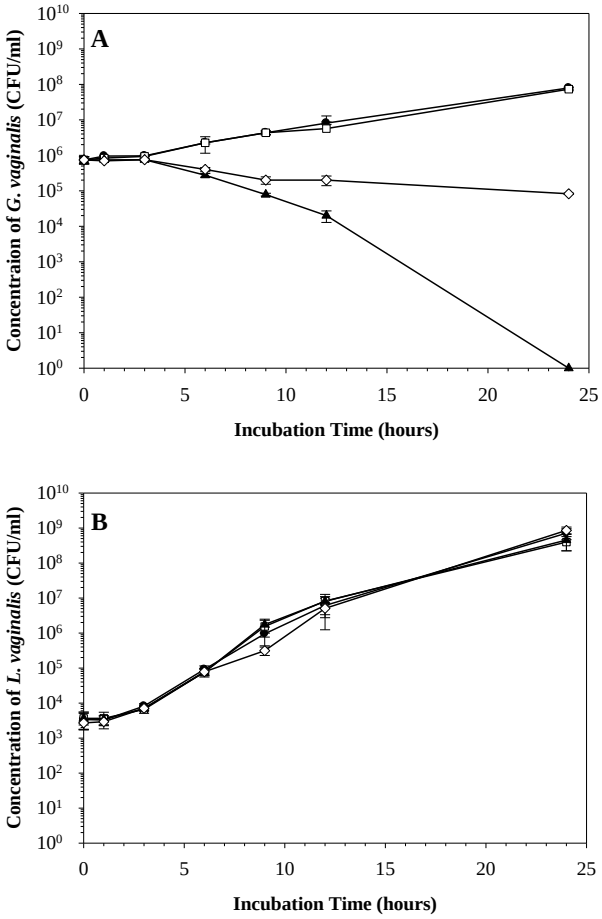


FIGURE 1. The breakdown of BPO upon contact with epithelium. BPO undergoes a homolytic fission resulting in two benzoate radicals which pass into the bloodstream where it is protonated into benzoic acid then deprotonated. Free cations in the blood will be attracted but no further modifications will occur while in the body.



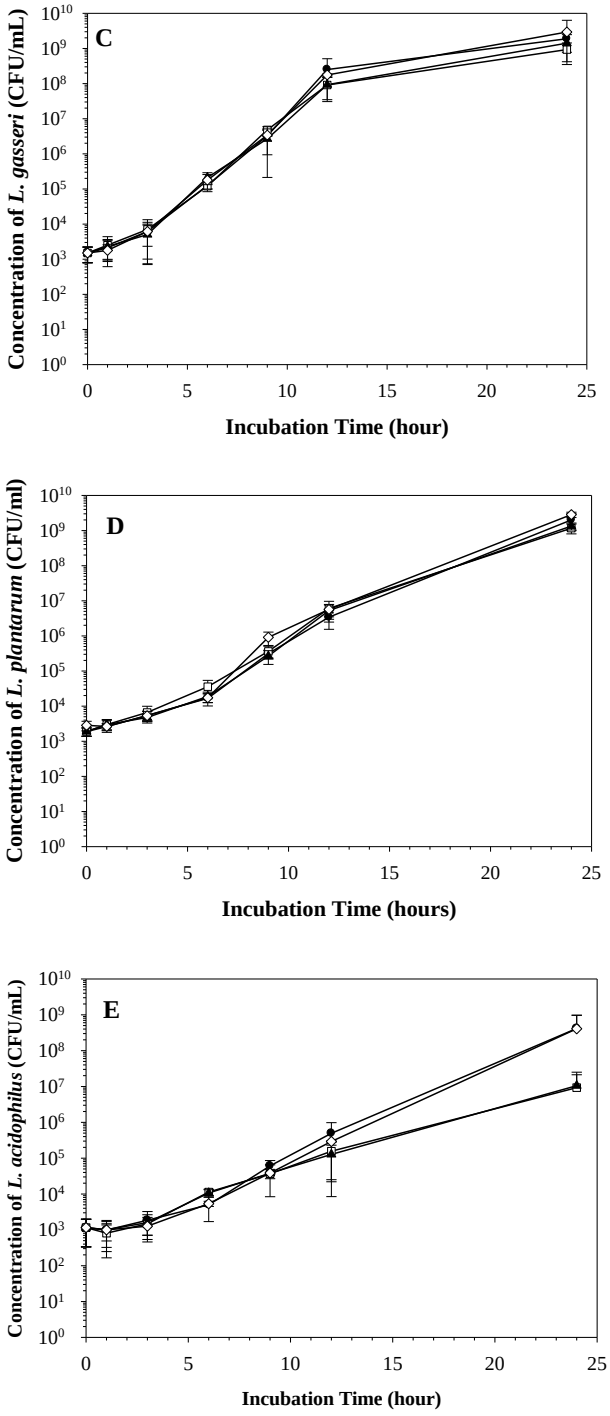


FIGURE 2. BPO inhibits *G. vaginalis* (A) but not healthy vaginal lactobacilli *L. vaginalis* (B), *L. gasseri* (C), *L. plantarum* (D) and *L. acidophilus* (E) in direct exposure experiments. (●) represents the negative control, (□) represents the base gel, (▲) represents 1% BPO hydrogel, and (◊) represents BPO particles. Experiments were conducted twice in duplicates. Mean values and standard deviations are shown.

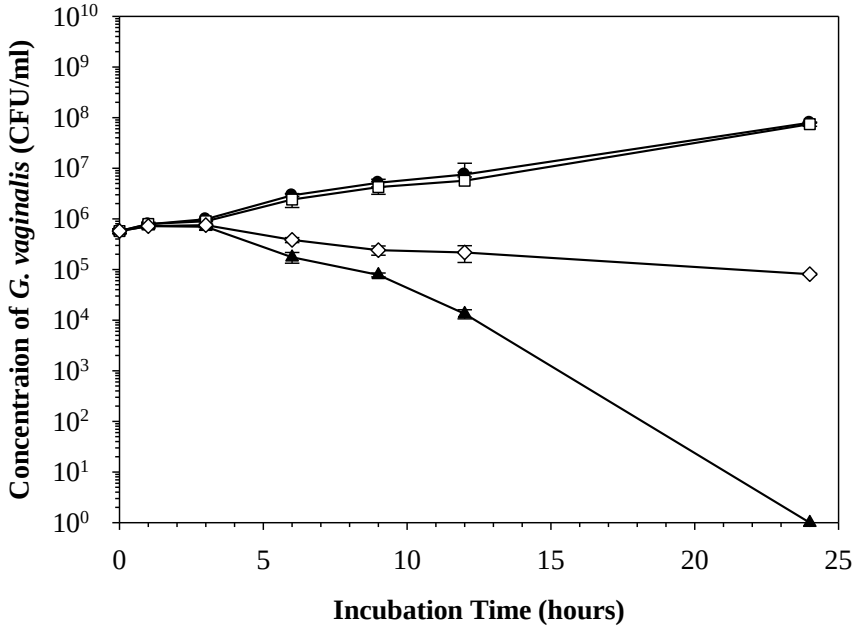
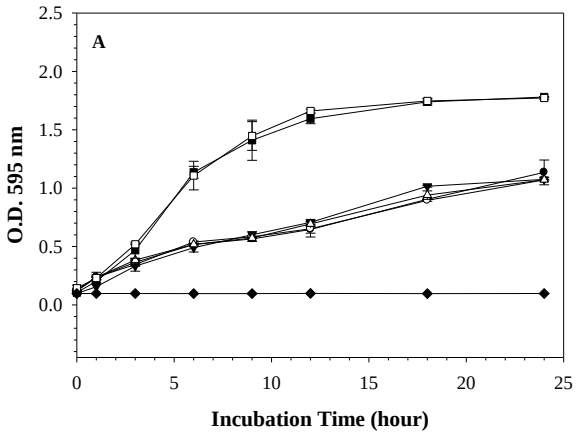


FIGURE 3 The effect of base gel, 1% BPO gel and BPO particles on *G. vaginalis* in an indirect contact assay. All experiments were conducted twice in duplicates; (●) represents the negative control, (□) represents the base gel, (▲) represents 1% BPO hydrogel, and (◊) represents BPO particles. Mean values and standard deviations are shown.


A
O.D. 595 nm
Incubation Time (hour)
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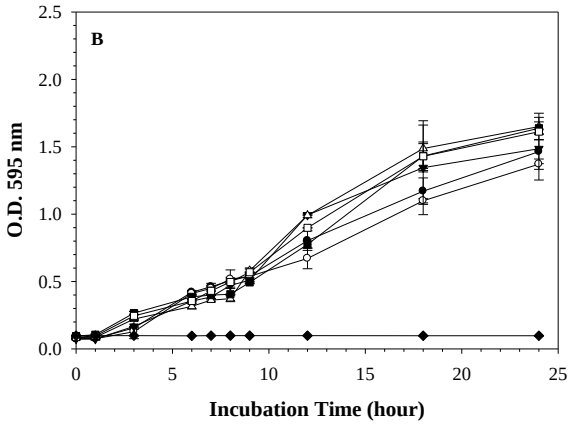
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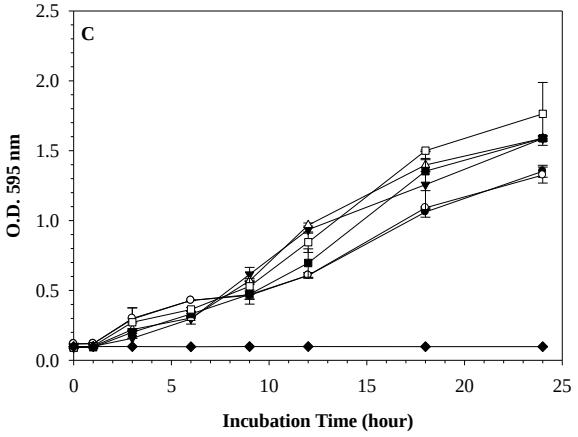
C
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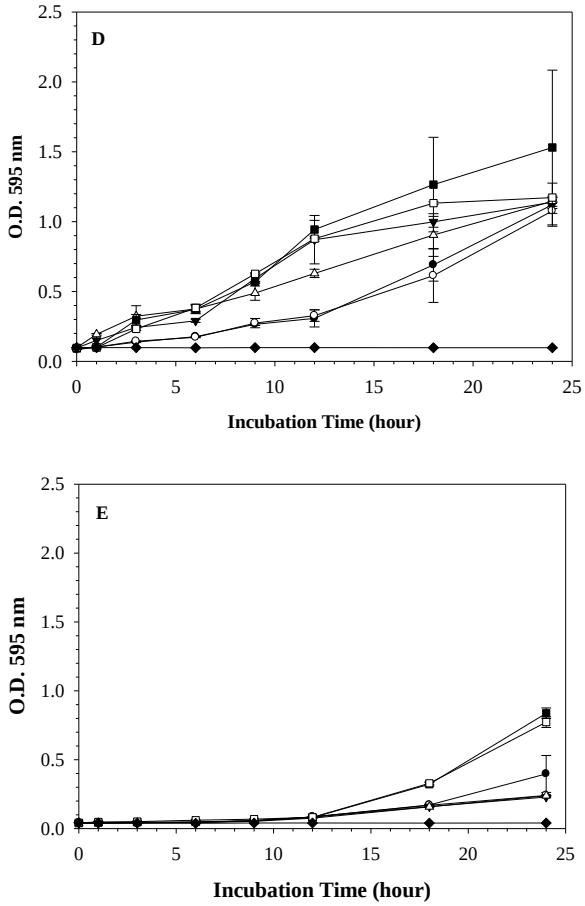

FIGURE 4. Influence of pH on growth of *G. vaginalis* (A), *L. vaginalis* (B), *L. gasseri* (C), *L. plantarum* (D) and *L. acidophilus* (E). Microbial growth was evaluated in media with pH altered to the average pH of the gels (4.5) by HCl (●,○) or lactic acid (▲, Δ), and normal growth medium (■,□) (MRS for lactobacilli spp. and BHI+3%HS for *G. vaginalis*). Sterile broth (♦) was also shown as negative control. Data were collected hourly (shown only 0, 1, 3, 6, 9, 12, 18, and 24h measurements). Experiments were conducted twice in quadruplicates.

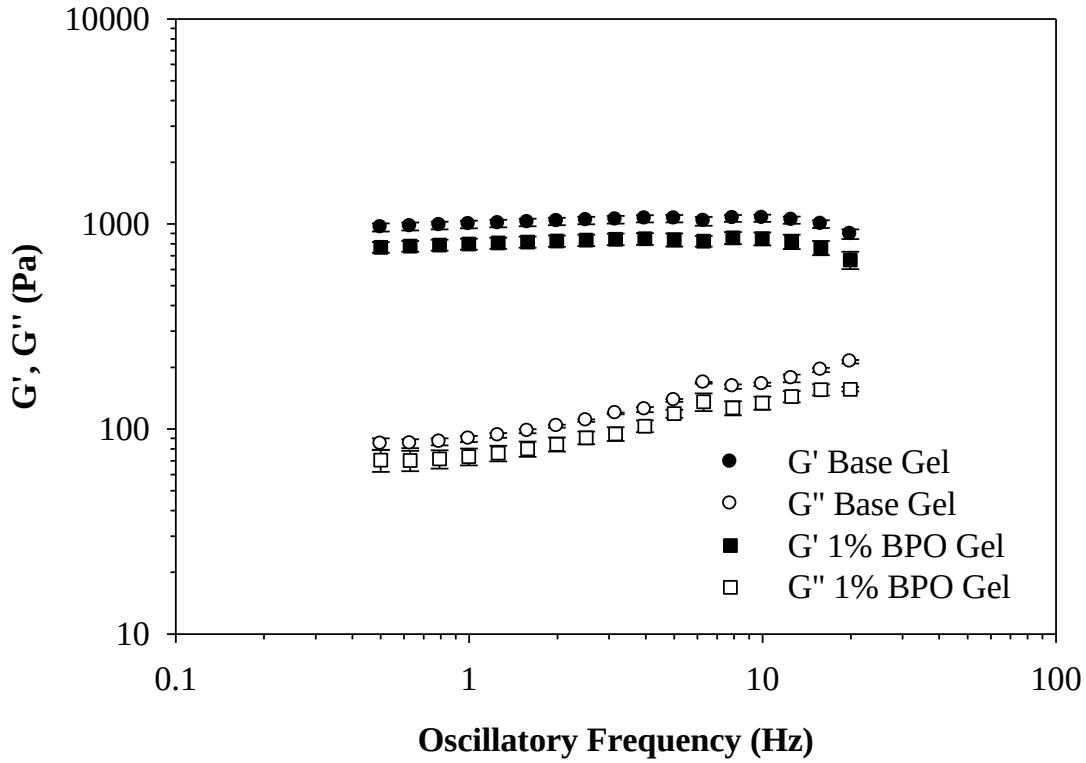

FIGURE 5. Storage modulus (G') and loss modulus (G") as a function of oscillatory frequency (Hz) on the base gel and 1% BPO gel. Experiment was conducted in triplicates. Mean values and standard deviation of three experiments are shown.

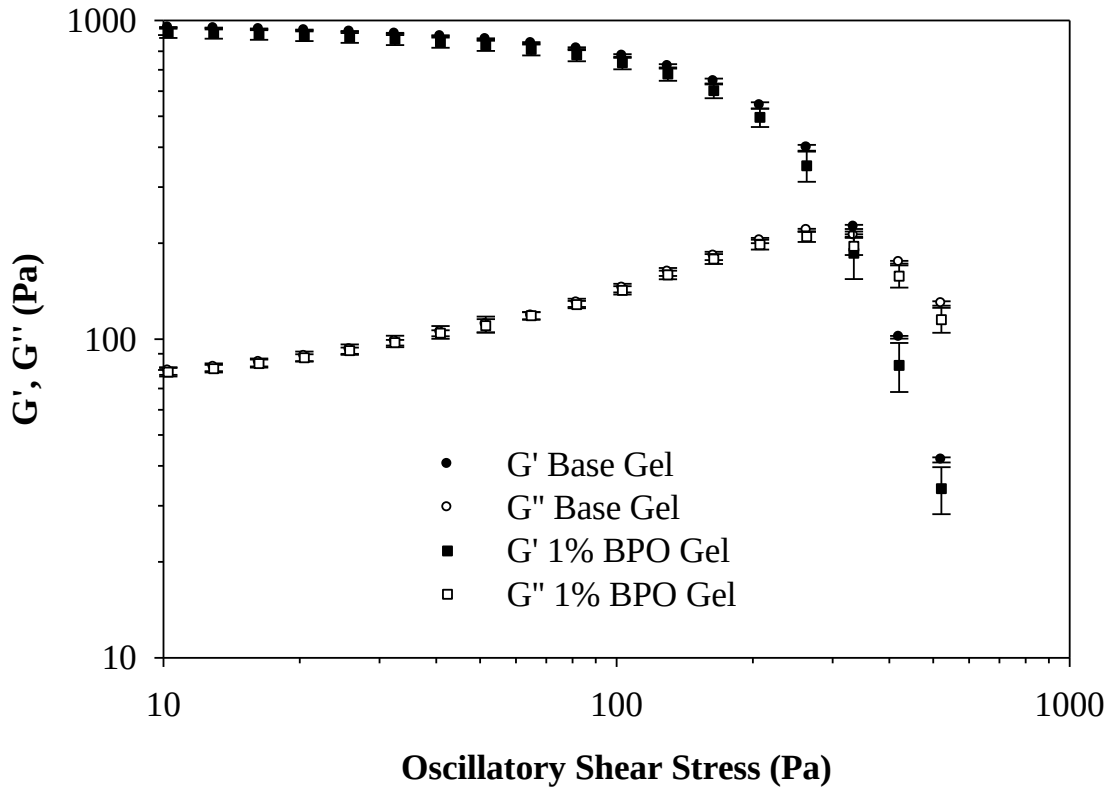

FIGURE 6 Storage modulus (G') and loss modulus (G'') as a function of oscillatory shear stress (Pa) on the base gel and 1% BPO gel. Experiment was conducted in triplicates. Mean values and standard deviation of three experiments are shown.

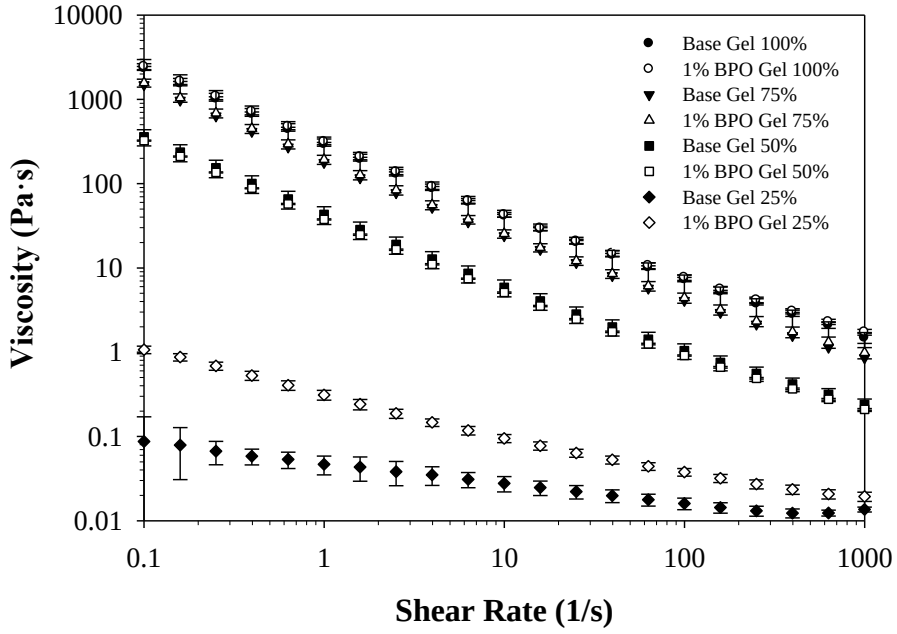

FIGURE 7 Viscosity (Pa·s) as a function of shear rate (1/s) of 100%, 75%, 50% and 25% dilutions in VFS of base gel and 1% BPO gel. Experiment was conducted in triplicates. Mean values and standard deviation of three experiments are shown.

On Bacteriocin Delivery Systems and Potential Applications

This review discusses one of the possible applications of bacteriocins; the encapsulation of bacteriocins into devices for inhibition for pathogenic bacteria. In this review, current research in the field as well as the limitations are discussed. There are sections on the use of bacteriocins in conjunction with both inorganic metal and organic nanoparticles in order to induce cellular leakage as well as how implant impregnation appears to reduce surgical site infection by reducing subsequent biofilm formation. It discusses loaded PEG gels and PET fibers for the inhibition of *S. aureus.* There are discussions into the dental, veterinary, livestock, aquaculture and food industry and implications of placing bacteriocins into animal as well as human foodstuff. It provides future directions and suggestions for how this technology could be applied. It is a fitting conclusion to this thesis.

The candidate wrote the gels, food industry, dental and future perspectives section, designed the chart on encapsulation methods, assisted with sources as well as proofread the paper.

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On Bacteriocin Delivery Systems and Potential Applications

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Abstract

Bacteriocins are antimicrobial peptides produced by a variety of bacteria. These peptides can act as antibiotic synergists or alternatives to enhance the therapeutic effects of current infection treatments and decrease the prevalence of resistant strains. Two bacteriocins, namely nisin and pediocin PA-1, are currently being used by the food industry; however, the introduction of these and other into the biomedical industry and further development of food applications have been challenged by the slow development of reliable delivery systems. For bacteriocins, these systems rely on novel and preexisting technologies. Many essential variables need to be accounted for to formulate successful delivery methods. In this review, documented and potential bacteriocin delivery systems are examined with special attention paid to how those systems are being implemented in the food and medical industries.

Keywords: bacteriocin, antimicrobial peptides, drug delivery systems, gels, nanotechnology, oral systems, probiotics

Introduction

Antimicrobial peptides are produced by organisms across all three domains of life. In the past two decades, ribosomally-synthesized antimicrobial peptides of bacterial origin commonly referred to as bacteriocins, have become the focus of many biomedical and food-based research groups. Bacteriocins are produced by virtually all bacterial species. While Gram-positive and Gram-negative microorganisms produce numerous bacteriocins, the only commercially used bacteriocins are produced by Gram-positive lactic acid bacteria (LAB). Therefore, the majority of this review is dedicated to this group of molecules. Arguably the most credible, early classification system proposed four distinct classes of lactic acid bacteria derived bacteriocins; lantibiotics, non-lanthionine, heat labile and lipid or carbohydrate complexes [1]. Within Class II, the non-lanthionine class, there are three subclasses segregated by functional sequences [1]. Since then, there have been several modifications that have molded the system to better encompass and separate bacteriocins into proper classifications. One of the current classification methods added two classes to the three class system and extensively expanded the subclasses [2]. Despite numerous proposed modifications to bacteriocin classification systems, there is still much uncertainty on how to accurately distinguish and characterize bacteriocins [3]. Such discussions into taxonomy will not be addressed in this review. Also, bacteriocins from Gram-negative bacteria are usually grouped into two distinctive categories: low molecular weight microcins (1-10 kDa) and high molecular weight colicins (30-80 kDa) [4].

A bacteriocin's action against targeted microorganisms can be bactericidal [5-7], bacteriostatic [8, 9] and lytic [10] thus qualifying them as polymodal [11, 12] at different

concentrations. A bacteriocins' activity is dependent on a target organism and vary between species [11]. Some Gram-positive bacteriocins are effective against viruses [13, 14], Gram-positive [15, 16], Gram-negative [17], and Gram-variable [18] organisms. Bacteriocin-producing lactic acid bacteria (LAB) are widely studied for application as probiotics, food preservatives and as potential infection treatments [18-20]. Bacteriocinogenic strains are immune to the bacteriocins that they produce, however closely related strains are highly susceptible [1]. In a complex environment colonized by a consortium of microorganisms, such as the gastrointestinal tract, bacteriocin production is an extremely advantageous property that increases the survivability of the producer. Despite the marked competitive advantage of bacteriocin production, it is not essential for the survival of LAB within their niches [21].

Due to their selective antimicrobial activity, bacteriocins are being studied as possible alternatives [22] or synergists [23] to modern antibiotics. They also present a means of improving food preservation due to their impressive *in vitro* and *in situ* efficacy against a large panel of human [24, 25] and foodborne [26] pathogens. Antibiotic synergism with bacteriocins is a particularly effective measure of reducing the minimum inhibitory and bactericidal concentrations [27]. By synergizing two or more antimicrobial compounds, multiple metabolic pathways can be targeted and inhibited which enhances efficacy of the treatment [23]. Additionally, synergy reduces the risk of cytotoxic effects of bacteriocins and antibiotics [28] on mammalian cells by reducing the effective dose. Whether it is as the primary treatment or as a synergist, the objective of bacteriocin application is to decrease the dependence on modern antibiotics,

combat the emerging issue of multidrug resistant pathogens and to develop more effective infection treatment options.

Despite the evidence of noticeable *in vitro* antimicrobial efficiency, bacteriocins have not been fully integrated at the clinical level. Some promising drug delivery methods have recently been investigated [15, 29-31], but there are more options which have yet to be explored. Bacteriocin delivery focuses mainly on ensuring that the antimicrobial is administered at the site of the infection or susceptible areas at a controlled rate, evading an immune response and maintaining the stability of the peptides. Due to their physical characteristics, such as: heat stability, size and charge, some bacteriocins are more suitable to treat certain infections than others. Additionally, bacteriocins have to be introduced in a way that will not trigger an immune response. Due to the vast diversity of bacteriocins and infections, formulating optimal combinations of drug and delivery systems has been complicated. Other issues have been noted which have slowed the incorporation of bacteriocins into the biomedical field. Herein we diagram the encapsulation methods discussed in this review paper (Fig. 1).

Limitations

Bacteriocins demonstrate an enormous potential to significantly impact the food and biomedical industries, however there are non delivery-based limitations to their application. One such limitation is the phenomenon of bacteriocin resistance [32, 33]. Like antibiotics, bacteria can develop resistances to bacteriocins via spontaneous mutations [34]. Studies have indicated that resistance may stem from production of

proteolytic enzymes that degrade the antimicrobial peptides [35] and from the presence of two-component systems that regulate ABC transporters [35, 36]. The controlled release of bacteriocins may circumvent the acquisition of resistance; this field is underdeveloped and has yet to prove this theory.

Additionally, bacteriocin cytotoxicity has been questioned and thoroughly assessed, particularly in nisin and pediocin. There are isolated reports on cytotoxicity of certain bacteriocins, including commercially used nisin and pediocin [37]. However, these reports should always be passed through the prism of a particular application of the tested molecule(s). Indeed, some eukaryotic cells are more sensitive to bacteriocins than other; however this is true for pretty much any biologically active substance: even regularly consumed with numerous foods substances such as saponins are lethal if administered intravenously. As for bacteriocins, both nisin and pediocin are harmless for humans and animals when used as food preservatives [38]. Also, nisin, being reported as promising spermicidal antimicrobial, had no negative effect on vaginal mucosa when studied in a rabbit model [39]. In addition, when studied for oral applications, nisin was found to be safe in animal trials – in fact, dietary level of 5.0% (about 3g/kg/day delivered to the trial rats) was reported as causing no negative effect [40]. When studied for vaginal application as a potent antimicrobial, a bacteriocin subtilosin A was found safe for human vaginal tissues as compared to marketed drugs [41].

Nanotechnology

There are several nanotechnologies currently being utilized as antimicrobial peptide and bacteriocin drug delivery systems. According to the National

Nanotechnology Initiative (NNI), nanotechnology is a scientific paradigm focused on the science of 1 to 100 nanometer substances that are manipulated to perform a specific task. Drugs are loaded on to nanomaterials to improve pharmacokinetics by altering physical characteristics, such as; solubility, half life, and bioavailability [42]. In many instances, bacteriocin nanoencapsulation protects the peptide from proteolytic degradation and undesirable *in vivo* and *ex situ* interactions [42]. Additionally, nanomaterials can be formulated to deliver the payload to specific tissue or infected sites, therefore reducing the amount of antimicrobial required for effective treatment. The development of nanotechnology has proven to be a promising redesign of traditional drug delivery systems which are becoming increasingly ineffective due to bacterial resistance.

Metal nanoparticles systems

Nanoparticles encompass a large range of nanometer compounds that bind to bioactive molecules to enhance or prolong their efficacy. Metal nanoparticles, primarily gold [43], palladium [44] and silver [45] are among the most commonly used in established drug delivery systems. Due to size and positive charge, the metal ion acts as a free radical diffusing into and disrupting the cellular membrane [46]. Additionally, metal nanoparticles can be molded into different shapes and sizes to enhance the overall therapeutic effect. Although the physical characteristics are advantageous in some respects, metal nanoparticles can diffuse into healthy human cell membranes and have displayed broad-range activity against both pathogens and commensal flora [47]. Peptide attachment or capping can reduce overall healthy cell toxicity in addition to providing selectivity to nanoparticles by incorporating desired properties that direct the

complex to the target [48]. A recent study has indicated that capping silver nanoparticles with bacteriocin enterocin minimized the effective concentration for inhibiting food-borne pathogens while reducing red blood cell lysis [48]. Additionally, the synthesis of bacteriocin-capped metal nanoparticles is cost-effective and eco-friendly [48]. Beyond the report by Sharma et al. [48], there are few supporting publications that investigate the effects of bacteriocin-capping metal nanoparticles. Considering the results of this study, the efficacy of this technology should be scrupulously examined with other bacteriocin candidates as well as with other metal nanoparticle combinations.

Organic nanoparticle systems

Most nanoparticle technologies are not viable options for oral delivery because they utilize typically inedible inorganic or synthetic molecules [49], such as; gold [43], polystyrene and polytetrafluoroethylene [50]. The lack of safe to eat nanoparticle formulations has greatly impacted the bacteriocin application in the food industry. Nanoparticles can stabilize and prolong the efficacy of bacteriocins through controlled release motifs [49]. Recently, a novel carbohydrate-based nanoparticle delivery system has been developed by Bi and colleagues [49]. This technology is based on a colloidal emulsion where phytoglycogen octenyl succinate carbohydrate complexes assemble on an oil droplet and stabilizes free nisin, prolonging its efficacy against foodborne pathogens [49]. While in the carbohydrate emulsion, nisin's antimicrobial activity against *L. monocytogenes* was significant after 40 days of storage [49]. Once again, research in the field of carbohydrate-based nanotechnology is limited. The results of the initial studies demonstrate that there is much potential for this technology in the food industry.

Bacteriocin scaffolding with nanofiber technology

Nanofiber technology is prominent in wound care and biodegradable dressing formulations. In healthcare environments, exposed chronic wounds are extremely susceptible to nosocomial infections [51]. Wound dressings are formulated to reduce the risk of infection by inhibiting the colonization of pathogenic strains. Most wound dressings are produced by nanofiber technology and loaded with encapsulated antimicrobials and hemostatic agents to promote wound healing. Once applied to a wound, antimicrobials elute from the nanofiber for several days at a controlled rate [52, 53], offering continuous exposure which reduce the risk of for the development of bacterial resistance. Bacteriocins and antimicrobials are loaded onto nanofibers in a process called electrospinning [53-55]. In electrospinning, a combination of polymers, typically poly(ethylene oxide) and poly-(L-lactide) [55], and the bacteriocin are dissolved in a solvent and are loaded into a syringe. A high voltage is applied to electrodes placed in polymer-bacteriocin solution and a rotating metal screen collector. When the applied current exceeds the charge of the solution, a Taylor cone forms and the bacteriocin-loaded polymer propels from the syringe tip. As the polymer jets from the syringe, the solvent dissolves and polymer nanofibers are collected by a metal screen collector. The resulting nanofibers have nano- to micrometer diameters and are near uniformly loaded with bacteriocins [54]. In a recent study, encapsulated plantaricin 423 demonstrated efficacy against a common nosocomial infection, *Enterococcus faecium* HKLHS for up to 6 days following wound dressing [55].

Furthermore, bacteriocin scaffolding technologies have been employed for antiviral therapies [13]. In a recent study, a subtilosin loaded onto a poly(vinyl alcohol) nanofiber

showed virucidal activity against Herpes simplex virus type 1 [13]. Modern antiviral treatments require concentrations of drugs that are cytotoxic to nephritic tissue [56]. Subtilosin-loaded nanofibers demonstrated significant i*n vitro* efficacy without exhibiting cytotoxicity [13]. These characteristics suggest that antiviral nanofiber therapies are better alternatives as compared to current drug therapies [13]. *Nano- and microspheres*

Liposome technology takes advantage of the remarkable biophysical properties of amphipathic phospholipids. When dissolved in polar solvents, these molecules spontaneously undergo a process called micellization and assemble into unilamellar (ULVs) and mutlilamellar vesicles (MLVs), also known as liposomes, which mimic the cellular phospholipid bilayer. In the presence of appropriate ratios of phospholipids, bacteriocins are encapsulated in liposomes. Loaded liposomes integrate in the bacterial cellular membrane and release the bacteriocin payload into the cytoplasm. Specificity of liposome systems can be enhanced by adjusting lipid ratios, adding cholesterol to increase membrane leakage by incorporating membrane proteins to promote bacterial membrane fusion. Liposome delivery systems are advantageous because bacteriocins are stabilized and protected from undesired interactions [57].

Liposome-based bacteriocin delivery systems are more prominent in the food industry than in the biomedical industry. Several bacteriocin-like inhibitory substances (BLISs) have also successfully been encapsulated in liposomes [58]. BLISs are bacterial-produced molecules that are not classified by the bacteriocin criteria, but otherwise display bacteriocin-like antimicrobial activity [59]. Recently, nisin and (BLIS) P34 were encapsulated in phosphatidylcholine liposomes and incorporated into

Minas freschal cheese [58]. Liposome encapusulation prolonged the nisin and P34 antilisteria activity because both substances were released gradually as compared to free nisin [58]. This application is particularly useful because Minas freschal cheese contains no natural preservatives and is permissible for *L. monocytogenes* growth. Other bacteriocins such as peodocin AcH (also known as pediocin PA-1) [60] have also displayed significant antilisterial activity.

Although the aforementioned studies demonstrate great potential for liposome-packaged bacteriocins, the subject remains largely unstudied in the biomedical field. Recently, a study describes a panel of five bacteriocins isolated from *Lactobacillus salivarius, Streptococcus cricetus* and *Enterococcus faecalis* that are effective against *Mycobacterium tuberculosis*, the causative agent of tuberculosis [62]. The bacteriocins were encapsulated in liposomes composed of 3 parts phosphatidylcholine to 1 part cardiolipin and tested against *M. tuberculosis* in *in vivo* rat models [62]. Four of the five bacteriocins were more effective than rifampicin, the antibiotic traditionally used to treat *M. tuberculosis* infections [62].

Surgical Devices

Implant Impregnation

Bacterial colonization of implants is an increasingly persistent concern with the emergence of resistant nosocomial strains. Colonization of metal and bone implants often leads to biofilm formation due to the net positive charge on the surfaces. Biofilm treatment is complicated by the resultant architecture and requires significantly higher doses of antibiotics and antimicrobials. The bioengineering industry has countered this

problem with loading bone and metal implants with exceedingly high concentrations of antibiotics. Unfortunately, resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), continued to cause infections post surgery. Bacteriocin-loaded, specifically nisin and ST4STA [63], bone implants have displayed remarkable anti-biofilm and MRSA inhibition rates [64]. *In vitro* studies demonstrated that bacteriocin release is dose-dependent, but, in general, most concentrations were released slowly and inhibited MRSA growth up to 48 hours. Many nisin variants have shown low minimum inhibitory concentrations against VRE in various *in vitro* studies [63]. In addition to prolonged antimicrobial efficacy, bacteriocin loading was not detrimental to the implant structure or function. Given these advantages, bacteriocin- impregnated implants are a better option than antibiotic-loaded implants.

Coated Medical Devices

The medical device coating industry is one of the fastest growing global industries. The emergence of resistant surgical site infections has been pressing for the development of more efficacious antimicrobial coatings on catheters, sutures and other medical devices. Due to the high prevalence of infectious strains in hospitals, patients are extremely susceptible to acquiring nosocomial infections during or after surgery [65]. According to the World Health Organization, catheter-associated infections rates are among the highest in healthcare setting. They are responsible for a marked increase in urinary tract and bloodstream infections which lead to prolonged hospitalization and elevated mortality rates [66]. Currently, inorganic antimicrobials, such as silver alloys, are the most commonly used catheter coating [67]. However, catheters coated with modern antibiotics, such as nitrofurazone, have outperformed the silver alloy coating

[68]. The catheter coating paradigm is shifting towards antibiotic coatings, this is however only a temporary solution due to the considerable rate of resistant strains emergence.

Continuous release of bacteriocins offers a better biofilm formation prevention strategy. *Pseudomonas aeruginosa* and *Escherichia coli* are among the most common catheter-associated infections [69]. *P. aeruginosa* forms biofilms and possesses multidrug efflux pumps that allow it to withstand significantly higher doses of frequently used antimicrobials [70, 71]. In combination, these two characteristics have made the treatment of *P. aeruginosa* infections nearly impossible. Recently, a bacteriocin produced by *Lactobacillus acidophilus* has demonstrated significant anti-*Pseudomonas* activity *in vitro* [72]. This currently unnamed bacteriocin may serve as the best catheter coating to date. Additionally, other studies have shown that catheters colonized by colicin-producing *Escherichia coli* K12 inhibit the growth of infectious clinical *E. coli* isolates [73]. Although selective colonization may not be a conventional approach, it could offer continuous production of effective bacteriocins and, in theory, would reduce pathogen colonization and infection rates.

Gels

Gels are defined as a substantially dilute cross-linked system incapable of flow in a steady-state environment [74, 75]. Crosslinking within the fluid causes the gel to behave in a thixotrophic manner. Gels are a dispersion of liquid molecules within a solid. Particulates can be dispersed readily through the gel matrix allowing for even distribution. Gels are defined primarily as either physical or chemical based on the

method of gelation (further segregation of gels will not be discussed in this review.) Bacteriocins have been encapsulated in hydrogels for use as topical treatments and in food preservation. Gels provide direct stabilized contact with the surface which may allow for an improved controlled release profile.

Nisin has been encapsulated in PET (polyethylene terephthalate) fibers. The textile was then encapsulated into a hydrogel and found to have antibacterial properties against strains of *S. aureus* [76]. Subtilosin, a bacteriocin produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens*, has been successfully incorporated into a PEG-based gel and shown to inhibit the bacterial vaginosis associated pathogen *Gardnerella vaginalis* with little associated impact on selected, healthy strains of lactobacilli (unpublished data).

Hydrogels can be formed through physical, ionic or covalent interactions in crosslinked polymer chains. The variation in their bulk properties provides a variety of uses. Although gels have not been extensively examined in conjunction with bacteriocins, such usage could provide more targeted strategies to dealing with problematic bacteria such as those that form biofilms. Bacteriocins have been used in other capacities to help regulate the oral microbiome.

Dental Applications

The human oral cavity houses an astounding 700 bacterial species [77]. Diversity and the emergence of predominant species differ between healthy individuals, appearing to be both site and subject specific. Over 141 different bacterial taxa representing six

different bacterial phyla have been found. Common genera include *Gemella, Granulicatella, Streptococcus* and *Veillonella* [78].

With the diversity and multiplicity of the oral microbiome, novel methods of inhibiting problematic bacteria, such as those that cause periodontal disease, pharyngitis or tonsillitis, requires the use of specific drug delivery systems. *Streptococcus salivarius* M18 produces multiple bacteriocins including the lantibiotics salivaricin A and salivaricin B, streptin and SA-FF22 [79-81]. Further, *S. salivarius* harbors bacteriocin-encoding transmissible DNA particles known as megaplasmids. Transmission of such megaplasmids has been indicated *in vivo* and provides a highly-flexible propensity for the acquisition, expression and deactivation of many bacteriocin loci which may account for both *S. salivarius* predominance as well as providing insight into how this bacterium maintains a balanced oral ecosystem. *S. salivarius* provides beneficial modulatory activities through the inhibition of pathogenic bacteria such as *S. pyogenes* and serves as a repository for bacteriocin determinants acquired from other oral bacterial species through the transport of IS elements. It is important to note, that like bacteriocins, bacteriocinogenic strains require GRAS status in order to be sold on the U.S. market.

Further, the bacteriocins produced by *S. salivarius* have been encapsulated into Bactoblis®, a lozenge that releases the bacterium into the mouth over the course of ninety days. Following the treatment in adults, an 80% reduction was noted in episodes of streptococcal pharyngitis in the first few months and a 60% drop six months after the product [81-83]. Similar results were seen in children; following 90 days of treatment, a 90% drop was seen in episodes of streptococcal pharyngeal infections, 65% drop six

months after the product [79]. *S. salivarius* has also been shown to help reduce plaque content following three months of treatment [81].

The efficacy of LAB produced bacteriocins has recently been evaluated for the use in regulating oral biofilms. It has thus far been indicated that, although only a small percentage of food-related LAB producers are active against oral bacteria (~1.3%), they cause major shifts in cellular chemistry [84].

Recently, the U.S. Army has been researching and formulating a method to incorporate KSL-W, a synthetic antimicrobial peptide into chewing gum to inhibit the formation of biofilms and dental plaque for prolonged periods [85]. KSL-W reduces the adhesion and growth of *Candida albicans* [86] and other biofilm-forming bacteria [87]. There are many bacteriocins that display anticaries activity that would serve as viable candidates for loaded chewing gum technologies. Recently, anti-caries antimicrobial peptides (including bacteriocins) were reviewed by Pepperney and Chikindas [87].

Veterinary and Livestock Applications

Bacteriocins have also been incorporated into many veterinary applications. *E. coli* serovar O157:H7 is the one of the most prominent livestock pathogens and the focus of bacteriocin treatment in this environment [88]. Like the use of probiotics in human consumption, livestock feed is often supplemented with bacteriocinogenic probiotics to promote healthy flora that inhibit pathogens, such as *E. coli* O157:H7 and *Streptococcus bovis* [88]. Colicin-producing *E. coli* strains are of particular interest in livestock applications and several feed patents that incorporate these strains been developed. Effective bacteriocins and bacteriocin-producing strains are still being discovered, so the

potential for feed-based patents is increasing. Garvicin, a bacteriocin recently isolated from *Lactococcus garvieae* 21881, demonstrated significant activity against pathogenic serovars of the same species [89]. The discovery of novel bacteriocins that are applicable in veterinary and livestock systems provide efficient candidates for strain replacement technologies which promote healthy flora.

Bacteriocins have surfaced as an effective treatment of bovine mastitis [90]. Bovine mastitis is a potentially fatal disease caused by *E. coli*, *Streptococcus uberis* and multi-drug resistant *Staphylococcus aureus* which infects the mammary gland and udder tissue in cows [90, 91]. The infection is often difficult to treat due to the diversity of causative agents and inadequate activity of modern antibiotics [91]. Additionally, the causative strains often produce bacteriocins which give them a competitive advantage to successfully colonize the environment [92]. However, bacteriocinogenic strain supplementation into feed has shown promising results in strain replacement efforts. The release of probiotics may be improved by developing controlled release delivery systems, rather than the sustained release observed in current feed supplementation. These discoveries harbor much potential, but are limited by the GRAS status. Substantial support for the safety of bacteriocin administration must be present to obtain GRAS status.*Aquacultural biotechnologies*

According to the Food and Agriculture Organization (FAO), fish farming or aquaculturing is one of the fastest growing sectors within the food industry. Estimates put the global distribution of farmed aquatic life at 110 million metric tons (FAO). Finfish, mollusks and crustaceans are among the most commonly farmed aquatic organisms. Infections and food spoilage are particularly costly issues in the aquacultural

industry. Opportunistic aquatic pathogens thrive in fish cultures and increase high mortality rates at the early developmental or larval stage [93]. Diseases, such as; fish tuberculosis, frunculosis and hemorrhagic septicemia are persistent and devastating for farmed fish [94-96]. Additionally, once harvested, the farmed fish are exposed to a wide variety of microorganisms that significantly accelerate spoilage [97]. Several aquatic species have been identified as bacteriocinogenic or BLIS producers [98, 99]. Multiple approaches have been implemented to promote healthy fish-microbe interactions with bacteriocins.

In most countries, aquacultures are frequently treated with prophylactic antibiotics to combat bacterial infections [100]. However, prolonged exposure to subinhibitory concentrations of antibiotics has only applied a selective pressure for more resistant pathogens which continue to infect the farmed aquatic animals [101]. Antibiotic treatment of aquaculture is not only detrimental to the farmed organism, but to the consumer and the microbial ecology of the aquatic farm and surrounding waterways [102]. The most conventional aquaculture application takes advantage of the probiotic effects of bacteriocinogenic strains [103]. Although it is not currently practiced, studies show that dry-spraying fish with bacteriocinogenic strains has inhibited the growth of *Listeria* [103].

Food Applications

In addition to all of the apparent and aforementioned applications of bacteriocins in the biomedical industry, bacteriocins have a great potential in the food industry. In 1988, the Food and Drug Agency granted GRAS status to nisin and it has been used as

an antimicrobial food additive ever since its classification [104]. Among other uses, nisin was one of the primary antimicrobials used to combat the series of *Listeria monocytogenes* outbreaks in the late 1990s and 2000s. In addition to nisin [105] and pediocin PA-1 [106], there are numerous bacteriocins that are gradually moving towards GRAS status. Many of the current bacteriocin food biotechnologies deliver the antimicrobials in a continuous or sustained release [107]. Studies have shown that controlled release may be more effective than the sustained delivery [108]. Controlled delivery improves the efficacy of bacteriocins by ensuring the peptides successfully overcome physiological barriers and preserve structure and functionality [109]. Although this paradigm is still in its infancy, novel controlled delivery systems are slowly being developed to enhance the antimicrobial capacity of bacteriocin food additives. There are three main methods of including bacteriocins in food, first through the addition of purified bacteriocin to food products, the inoculation of a food with LAB which produce the bacteriocin itself or the incorporation of an ingredient that has been previously fermented with the bacteriocin-producer bacterium [110].

Bacteriocin-producers: live "delivery systems"

Fermented milk products have been consumed for thousands of years. Mixed cultures of yeast, LAB and bifidobacteria ferment the milk and remain in the product. The live bacteria are considered to be probiotics when they confer a health benefit for the host [105]. Probiotics promote the growth of healthy intestinal flora while inhibiting harmful proteolytic bacteria by reducing the pH and producing bacteriocins. By ingesting live cultures, bacteriocins are produced *in vivo* and do not need to obtain GRAS status. Live cultures have been used in conjunction with non-discriminatory

antibiotic regimes to help replace the host microbiome following antibiotic usage. Further, the use of LAB in fermented dairy products has been shown to markedly improve bowel regularity and indigestion. The presence of *Saccharomyces cerevisae* in the oral cavity, gut or vaginal environment has been linked to an increased incidence of yeast infections. LAB selectively inhibit these microorganisms providing an improved microbiome.

Increasingly more food products have been encapsulating probiotics as a means of conferring further health benefits but have not been simply passively entrapping live cultures. In some cases the cell-free supernatant (CFS) of the bacteriocin producer is placed into the water or food [111]. In some cases the CFS has caused a direct health benefit such as a reduction in total cholesterol counts in rats. In other cases the bacteria is freeze-dried and trapped in protein-carbohydrate matrices allowing for continued bacterial survivability [112]. Both methods provide enhanced techniques for encapsulating the bacteriocins which allow for further controlled release and improved viability during storage.

Bacteriocins in the control of food-associated biofilms

There is limited data reported on targeted delivery of bacteriocins for control of biofilms in food systems. Most of these studies are limited to modified packaging materials and their use as active packaging systems for surface-inactivation of foodborne pathogens and spoilage bacteria, as discussed in this review; these studies are still in their infancy and often use model systems with planktonic cells [113]. However, some investigators suggest combinational approach where antimicrobials active against

biofilms are used in combination with bacteriocins. For instance, when combined with lysozymes, nisin has been shown to be remarkably effective in controlling biofilm formation by food-associated *S. aureus* [114]. Still, study of bacteriocins' controlled delivery as an effective approach for preventing food-associated biofilms is still in its infancy.

Furthermore, live bacteriocinogenic strains encapsulated in PET films have demonstrated the ability to inhibit the growth of *L. monocytogenes* [115]. Challenge studies of *L. monocytogenes* against both biofilm- and non-biofilm forming strains of *Lactobacillus plantarum* and *Enterococcus* encapsulated in active PET films [115]. At the optimal biofilm forming *L. plantarum* colonization, 5,4 and 3.9 log reductions were observed in planktonic and biofilm-forming *L. monocytogenes* populations, respectively [115]. This innovative food safety biotechnology may provide a cost effective solution for food spoilage and proliferation of food pathogens.

Bacteriocin-loaded plastic packaging and films

Antimicrobial packaging films are indirect food additives that act as another line of defense against food contamination [116]. These films are designed to release antimicrobial agents, commonly nisin, into the food to inhibit the growth of *L. monocytogenes* and other foodborne pathogens for extended periods [117]. Antimicrobial packaging films are primarily used to package cheeses and hot dogs [118]. A low-density polyethylene packaging film is coated with a solution of cellulose and an antimicrobial agent [119]. The focus of this field is to regulate the controlled release of nisin from the coated packages into the food and maintain bioavailability. The

contact angle of the film to the food product is also an important consideration when formulating new films. Although nisin is the sole GRAS status bacteriocin used in the food industry, groups are using other bacteriocin candidates to validate their potential to obtain GRAS status. Enterocin 416K1, a bacteriocin produced by *Enterococcus casseliflavus* IM 416K1, is a promising candidate for nisin alternative food packaging films [119]. A marked decrease in *L. monocytogenes* growth was observed after 24 hours of exposure to enterocin 416K1 coated packaging [119]. Additionally, this decrease was observed, though to a lesser extent, up to 28 days [119].

Multilayered antimicrobial packaging has recently been formulated to contain several bacteriocins [120]. Partially purified active bacteriocins isolated from *Lactobacillus curvatus*, lactocin 705 and lactocin AL705, were loaded on multiple layers of packaging films and retained activity against *Listeria innocua* and *Lactobacillus plantarum* for up to 45 days [120]. The extended efficacy of the multilayered films may serve as a viable technology coupled with food protection measures.

Conclusion

Bacteriocins have the potential to revolutionize or significantly impact the biomedical industry by diminishing antibiotic dependence, but there is disconnect between *in vitro* and *in vivo* efficacy. *In vitro* studies demonstrate that some bacteriocins are acutely active against human pathogens; however *in vivo* applications require exceedingly higher concentrations. Theoretically, the development of novel or improved drug delivery systems would enhance the *in vivo* efficacy of bacteriocins. There are

several unexamined drug delivery systems that can be applied to bacteriocins. All options should be investigated to harness the full potential of bacteriocins.

Here, we have documented a variety of drug delivery systems that have only begun to explore the possibilities associated with bacteriocin usage. Each system has its own associated challenges, however as microorganisms continue to become tolerant to synthetic antibiotics it is increasingly apparent that the only logical method for dealing with microorganisms is to use their own style of weaponry against them. In this thought, bacteriocins seem not only logical but pragmatic, and represent, in our minds, the next step in drug development ideology.

Future Perspective

Antibiotic drug resistance is a persistent issue in the food and biomedical industries. The development of effective, alternative drug therapies will eliminate our dependence on resistance-inducing antibiotics. To date, bacteriocins and antimicrobial peptides have indicated a promising prospective as antibiotic alternatives or synergists. The bacteriocin paradigm is in the process of moving from *in vitro* to *in vivo* studies. If *in vivo* studies prove to be as successful as the *in vitro* results, bacteriocins and antimicrobial peptides will likely be incorporated into infection and antiviral therapies. Although bacteriocins will combat the antibiotic dependence, other technologies must be explored as a long term answer because they too can induce resistance evolution. However, the longevity of effectiveness can be prolonged by monitoring prescriptions which will prevent overuse.

Executive Summary

Introduction

- Bacteriocins are antimicrobial peptides of a bacterial origin that can be bactericidal, bacteriostatic, lytic and/or virucidal.
- Bacteriocins are commonly used in the food industry and show great potential for the biomedical and healthcare industries.
- Incorporation of bacteriocins into treatment plans could possibly eliminate healthcare's dependence on antibiotics.
- Although *in vitro* results are impressive, reliable drug delivery systems need to be developed to improve *in vivo* efficacy.

Nanotechnology

- Drug delivery nanotechnologies improve the pharmacokinetics of bacteriocins by increasing bioavailability, solubility and half life.
- Both inorganic metal and organic nanoparticles diffuse through or into the cell membrane to cause leakage. Bacteriocin-capped nanoparticles provide specificity and reduce cytotoxicity as compared to nanoparticles alone.
- Electrospun nanofibers loaded with bacteriocins are well suited for novel wound dressing formulations. Prolonged controlled release and a favorable concentration to surface area ratio make them an ideal candidate for effective infection prevention measures and treatments.
- Nanoencapsulation of bacteriocins in liposomes provide controlled release for bacteriocins in food based and biomedical technologies.

Surgical Devices

- Impregnating implants with bacteriocins will reduce the incidence of surgical site infections by preventing colonization and subsequent biofilm formation. Implant integrity is not compromised by bacteriocin impregnation.
- Bacteriocin coating of medical devices can prevent bacterial colonization and reduce the prevalence of catheter infections. Additionally, selective catheter colonization of bacteriocinogenic strains has tested well *in vitro.*

Gels

- Gels are thixotrophic substances capable of evenly distributing particles within its matrix.
- PET fibers have successfully encapsulated nisin within a hydrogel system and has been shown to have antibacterial properties against *S. aureus*.

Dental Applications

- The human oral microbiome is multitudinous.
- *S. salivarius* M18 is a predominant bacterium in healthy oral environments, providing regulation through the production of lantibiotics and megaplasmids.
- These bacteriocins have been encapsulated into Bactoblis®, a lozenge that has indicated the aforementioned modulation capacity.
- KSL-W, a synthetic antimicrobial peptide, has been integrated into chewing gum as a means to prevent dental plaque and the growth of *C. albicans.*

Veterinary and Livestock Applications

- Advancement of feed supplementation technologies may increase animal productivity, as well as reduce transmission of common livestock associated pathogens.
- Bacteriocins may also serve as viable alternative treatments of multi-drug resistant infections that are common and often fatal in livestock.
- The aquacultural industry could benefit from the use of bacteriocins. Antibiotic resistant strains are rampant in farmed fish and probiotic or bacteriocin supplementation would reduce resistance evolution.

Food Applications

- Nisin was the first bacteriocin granted GRAS status in 1988, and remains one of the most widely used bacteriocins in the food industry.
- Live cultures of LAB assist in the maintenance of a healthy gut and vaginal environment by selectively inhibiting pathogenic bacteria and assisting in the reestablishment of host microorganisms.
- Probiotics are provided in foods in one of three ways: through the CFS of the bacteriocin producer, through freeze-drying the bacterium, or by passively entrapping the live culture into a food product.
- Bacteriocin-loaded packaging serves as another layer of protection against foodborne pathogens. Technologies are focused on improving the controlled release of bacteriocins and extending the efficacy.

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Figure 1: A summation of the main encapsulation approaches discussed in the review paper

Encapsulation Method	Description	Benefits	Bacteriocin	Reference
Silver nanoparticles	Silver particles capped with bacteriocins	reduce overall healthy cell toxicity, reduce red blood cell lysis	Enterocin	47, 48, 50
Carbohydrate-based nanoparticles	Bacteriocin stabilizing carbohydrate emulsion	Prolongs and increases efficacy, organic	Nisin	49
Nanofiber scaffolds	Electrospun fibers loaded with bacteriocin	Direct contact, sustained release profile	Plantaricin 423	53-55
Nanospheres	Liposome encapsulation	Controlled release, specificity can be engineered	Nisin, BLIS P34	58, 59
Implant impregnation	Implant prepared with bacteriocins integrated into formulation	Inhibits biofilm formation	Nisin and ST4STA	63
Catheter coating	Bacteriocin coating of catheters	Inhibits biofilm formation, promotes selective colonization	Unnamed bacteriocin prod. by *L. acidophilus*	67-69, 72
Hydrogel	Gel matrices loaded with an even distribution of bacteriocins	Even distribution, stabilized contact, controlled release profile	Nisin and subtilosin	14, 76
Oral tablet	Lozenge	Inhibits oral biofilms, periodontal diseases	Unspecified *S. salivarius* bacteriocin	82-84, 86
Gum technology	Chewing gum	Inhibits the adhesion of *C. albicans* and other oral biofilms	KSL-W	86, 87
Livestock Feed Supplementation	Probiotics for animal feed	Increase animal productivity and decrease transmission of pathogens	Colicins, Garvicin	88-92
Aquaculture Dry Spray	Prophylactic spraying with bacteriocinogenic dry spray	Inhibits growth of *L. monocytogenes*	Unspecified	99-102
Addition to food product with bacteriocin or bacteriocin-producer	Probiotic drinks and food products	Allows for sustained release, competitively inhibits the growth of *S. cerevisae* in the gut or vaginal environment, improves viability during storage	LAB, Nisin	105, 108, 109
Incorporation into food packaging	Melt-processing incorporation, multi-layer incorporation	Surface-inactivation of foodborne pathogens and spoilage bacteria, specifically *L. monocytogenes* and	Nisin, *Lactobacillus curvatus*, lactocin 705, lactocin AL705, Enterocin 416K1	113- 115, 117, 119, 120

	L. innocua, improved food protection		

APPENDICES

Appendix A: Equations

Paper ii.

Equation 1: Fractional inhibitory concentration index (FICI) is determined:

$FICI = FIC_A + FIC_B$ which is further broken down to

$$(MIC_{\text{ANTIMICROBIAL A IN COMBINATION}}/MIC_{\text{ ANTIMICROBIAL A ALONE}}) + (MIC_{\text{ ANTIMICROBIAL B IN COMBINATION}}/MIC_{\text{ ANTIMICROBIAL B ALONE}})$$

A FICI ≤ 0.5 is defined as synergistic while a value that is ≥ 0.5 and ≤ 4.0 is identified as having no effect. A FICI of > 4.0 is identified as antagonistic.

Equation 2: Bliss independence (BI) is determined:

$E_{IND} = E_A + E_B - E_A \times E_B$

E_A is defined as the percentage of bacterial growth inhibition from of *x* mg/liter of drug A. E_B is defined as the percentage of bacterial growth inhibition at *y* mg/liter alone. E_{IND} is the expected percentage of bacterial growth which results from a noninteractive, thus independent, theoretical combination of drugs A and B. The difference between the observed (E_{OBS}) and expected (E_{IND}) percentage of growth inhibition from these drug combinations is described by equation 3: $\Delta E = E_{OBS} - E_{IND}$ **(EQUATION 3).** If ΔE and its 95% confidence level (CI) were >0 (i.e. $E_{OBS} > E_{IND}$, more growth inhibition was observed than if the two drugs were acting independently), Bliss synergy was concluded for the drug combination. If ΔE and its 95% CI were <0 (i.e., $E_{OBS} < E_{IND}$, less growth inhibition was observed than if the two drugs were acting independently), Bliss

antagonism was concluded for the particular drug combination. In cases in which the 95% CI of ΔE=0, the conclusion was deemed Bliss independence.

Paper iv.

Ritger-Peppas equation:

$$\frac{M_t}{M_\infty} = kt^n$$

where $\frac{M_t}{M_\infty}$ is the fraction of drug released at time t, k is the proportionality constant and n is the diffusion exponent indicative of the mechanism of release.

Appendix B: Acronyms

AMP antimicrobial peptide

AMR antimicrobial resistance

BHI brain heart infusion

BI Bliss independence

BV bacterial vaginosis

FICI fractional inhibitory concentration index

GML glycerol monolaurate

GRAS generally recognized as safe

HBT human blood bilayer-Tween

HS horse serum

LA Loewe additivity

LAE lauramide arginine ethyl ester

LAS N-ε-lauroyl-L-arginine

MIC minimal inhibitory concentration

MRS deMan Rogosa Sharpe

PEG poly ethylene gylcol

OD optical density

STI sexually transmitted infection

Appendix C: Color Figures

Figure 1: Clindamycin Phosphate

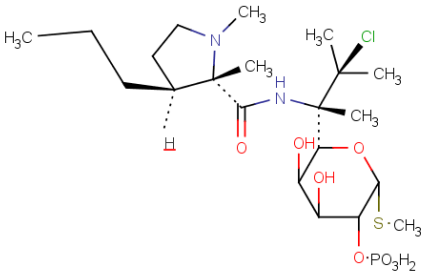


Figure 2: Lauramide arginine ethyl ester

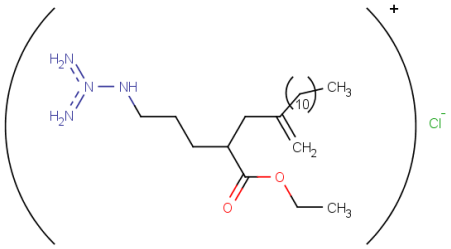


Figure 3: Metronidazole

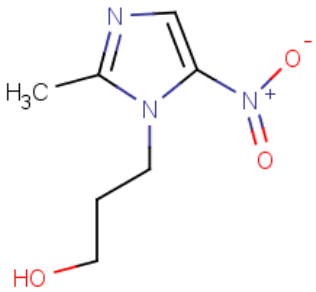


Figure 4: Poly-L-lysine

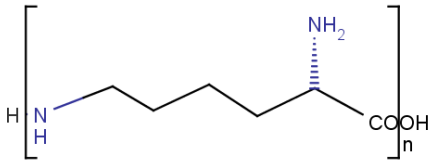
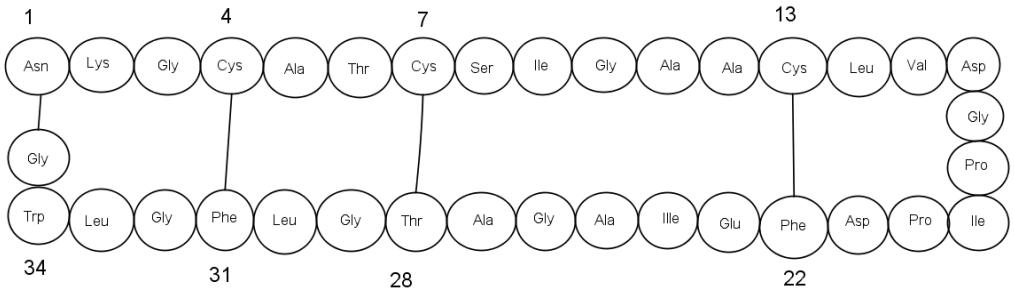


Figure 5: Subtilosin A



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