ANTIMICROBIAL AND RHEOLOGICAL PROPERTIES OF BENZOYL PEROXIDE FORMULATED POLYCARBOPHIL/CARBOMER 934P HYDROGEL

By

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ABSTRACT OF THE THESIS

Antimicrobial and Rheological Properties of Benzoyl Peroxide Formulated

Polycarbophil/Carbomer 934P Hydrogel

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BACKGROUND: The human vagina is colonized by a variety of indigenous microflora; in healthy individuals *Lactobacillus* is a predominant bacterial genus while those with bacterial vaginosis (BV) carry a variety of anaerobic bacteria. The disruption of healthy microflora and shift to primarily facultative anaerobic bacteria leads to microbial disease. Benzoyl peroxide (BPO) is chosen as a new antimicrobial agent since it is listed as safe for use in bleaching in food product and as an active ingredient of acne topical treatments. Polycarbophil/Carbomer 934P are chosen to be the network polymers of the hydrogel to encapsulate BPO, due to their good muco-adhesion.

EXPERIMENTS: In this study, the antimicrobial activity of BPO against *Gardnerella vaginalis*, one of the primary causative agent of BV, and their safety for vaginal lactobacilli such as *L. acidophilus, L. gasseri, L. plantarum,* and *L. vaginalis* is evaluated using a well diffusion assay, direct contact assay and diffusible agent assay. The rheological properties of the formulations are measured using an oscillation frequency sweep, an oscillation shear stress sweep and a flow shear rate experimental design.

RESULTS: It is shown that in the well diffusion assay *G. vaginalis* is inhibited at 0.01% (w/w) hydrogel-encapuslated BPO and that the tested *Lactobacillus* spp. are inhibited at 2.5%.
In direct contact assays (cells grown in a liquid culture containing hydrogel, hydrogel containing 1.0% BPO or BPO particles), hydrogels loaded with 1.0% BPO caused 6-log reduction of *G. vaginalis*. Conversely, three of the lactobacilli spp. are not inhibited while *L. acidophilus* growth is delayed. Encapsulation of BPO into polycarbophil/Carbomer 934P hydrogel improves its antimicrobial activity against *G. vaginalis*.
The rheological properties of the hydrogel formulation are not greatly compromised by encapsulation of 1.0% BPO. The new formulation still possesses a solid-like behavior, with a low yield stress and a high viscosity after diluted.
CONCLUSION: Hydrogel containing BPO can be a new drug in BV treatment based on its antimicrobial activity on BV-associated pathogen/not on healthy vaginal lactobacilli when it is encapsulated into polycarbophil/Carbomer 934P hydrogel**,** and its good rheological properties.

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1 INTRODUCTION

1.1 Bacterial vaginosis

Bacterial vaginosis (BV) was one of the most common vaginal diseases. Among child-bearing age women, it affects 29% women in North American [1], 12% in Australia [2] and 20% in sub-Saharan Africa [3]. Amsel criteria and Nugent Score were the most widely accepted clinical criteria in BV diagnosis [4], [5].

BV could cause some serious risks to human health. It increases women's susceptibility to other sexual transmitted diseases (STDs) such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), Chlamydia and gonorrhea [3], [6], [7], [8]. It also caused pregnancy complications including preterm birth of low-birth-weight infant [9].

1.1.1 *Gardnerella vaginalis*

It was extensively shown that when the natural vaginal microflora shifts from healthy lactobacilli to mainly anaerobes, *Gardnerella vaginalis*, *Bacterides* spp., *Prevotella* spp., and *Mobiluncus* spp., with an elevated vaginal pH, microbial disease such as BV can occur [10]. Though *G. vaginalis* could also be found in women with or without BV, there was an increase of prevalence and concentration of *G. vaginalis* among women diagnosed with BV. From 117 patients, *G. vaginalis* was found from 87.5% of women with BV, while 26.4% of healthy women [11]. A diagnostic indicator of BV is based on the concentration of *G. vaginalis* exceeding 2×10^7 CFU/ml combined with vaginal pH>4.5 [12]. Therefore it was regarded as a major pathogenic microorganism in Nugent Score criteria for BV diagnosis [5]. *G. vaginalis* was adherent to vaginal epithelial cells. Examined by transmission electron microscopy, the adherence was mediated by an outer fibrillar coat [13]. By adherence, *G.*

vaginalis colonized while minimizing their contact with defense factors from healthy vaginal bacteria and avoiding being washed by vaginal fluid [14].

1.1.2 Healthy lactobacilli

A healthy human vagina was dominated by healthy lactobacilli. From a healthy vagina, *Lactobacillus acidophilus* and *Lactobacillus gasseri* are most frequently isolated, as well as other species such as: *Lactobacillus plantarum*, *Lactobacilli vaginalis*, *Lactobacillus jensenii* and *Lactobacillus crispatus*, etc. [15], [16], [17]. These lactobacilli competed anaerobic pathogens by producing natural antimicrobials such as hydrogen peroxide (H_2O_2) and bacteriocins, reducing pH by organic acids and adherence to epithelium cells [18], [19], [20]. H_2O_2-producing lactobacilli was considered as a defense mechanism of healthy human vagina ecosystem [21]. They could protect women from acquisition of BV [20], [22], as well as HIV-1, gonorrhea and trichomoniasis [23]. H_2O_2 alone or with addition of myeloperoxidase and chloride was reported toxic to *G. vaginalis* [24].

Lactobacilli protected vaginal ecosystem by producing bacterioncins. Bacteriocin, antimicrobial protein or peptide produced by bacteria was another defense factor in the human vagina. While antibiotics were secondary metabolite products, bacteriocins were ribosomally synthesized. Lactobacilli were found to be the primary producer of bacteriocins, which possess a wide range of inhibitory activity [25]. Bacteriocin produced by *L. acidophilus* 160 was purified and proven to be inhibitory against 9 isolates of *G. vaginalis* [26]. Bacteriocin gassericin A was purified from *L. gasseri* LA39 culture and found inhibitory against enteric pathogens [27], [28].

Lactobacilli protected vaginal ecosystem by producing organic acids. A healthy vaginal pH was maintained at around 4.5 [29]. Lactic acid and C_3-C_5 aliphatic acids produced by lactobacilli and vaginal epithelial cells secretions contributed to vaginal acidity, thus controlling the composition of microflora, limiting the growth environment suitable to lactobacilli [30], [31].

Lactobacilli also protected vaginal ecosystem by adherence to vaginal epithelial cells. *L. acidophilus, L. gasseri* and *L. jensenii* were found able to self-aggregate, displacing *G. vaginalis* in the competition for the vaginal cells receptors glycolipids and adhered to epithelial vaginal cells. Proteins and lipoproteins were responsible for self-aggregation of lactobacilli. Glycoproteins and carbohydrate were responsible for the adherence of lactobacilli to the vaginal epithelial cells [32].

1.1.3 Current treatment against bacterial vaginosis

1.1.3.1 Antibiotics

Centers for Disease Control and Prevention (CDC) recommended treatments for BV were a 7-day 500.0 mg twice a day oral intake of metronidazole, a 7-day 2.0% intravaginal clindamycin cream application or a 5-day 0.75% intravaginal metronidazole gel application [33]. The effectiveness of these treatments were proven in many clinical and laboratory studies. In a double-blind, randomized *in-vivo* trial, intravaginal treatment of clindamycin eradicated *G. vaginalis* and other major BV-associated anaerobes [34]. In a clinical trial, cure rate of intravaginal metronidazole treatment against BV was 92.5%, compared to 89.9% of oral metronidazole administration, with significantly less adverse effects [35].

1.1.3.2 Drawback of antibiotic treatments

High recurrence rate after metronidazole and clindamycin treatment was a serious drawback of the current antibiotic treatment. Recurrence rate of BV after antibiotics in either oral or intravaginal therapy was 30% within one month [36]. Though the oral metronidazole therapy was able to significantly improve BV symptoms, in 12 months, it failed to recover healthy vaginal microflora in 84% patients or prevent BV from recurrence among 58% patients [37]. A long-term study showed a 52% BV recurrence rate and most BV occurred within one year after antibiotic treatment [38].

High Recurrence rate of metronidazole and clindamycin treatment could be resulted from several factors, including antibiotic resistance, residue of *G. vaginalis* biofilm and impact on healthy lactobacilli recovery. In a randomized clinical trial, clindamycin resistance of 1059 anaerobic bacteria isolates increase from 17% to 53% after therapy [39]. Among 50 strains of *G. vaginalis* collected from 321 specimens, 68% were resistant to metronidazole and 24% were not sensitive to clindamycin [40]. After oral metronidazole treatment, a dense and active *G. vaginalis* biofilm was found adherent on the vaginal mucosa [41]. In a recent cohort study, one month post-treatment colonization of H_2O_2-prodcucing *L. crispatus* and *L. jensenii* was recovered only in few women [42].

The consistency of sexual partners and contraceptive practices could also be a factor to BV recurrence. A variety of BV-associated microflora could be colonized from the penis and the urethra and some of them were resulted from partnered sexual activity [43]. Patients with same pre-/post-treatment sexual partner and inconsistent condom use were under higher risk of BV recurrence [44].

1.1.4 Alternative treatments

In recent studies, some effort has been done on potential alternative treatments for BV, mainly including lactobacilli therapies in oral or intravaginal applications, alone or with extended antibiotic, and intravaginal gels containing acids.

1.1.4.1 Oral lactobacilli treatment

The oral treatment containing viable cells of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 were shown to be effective against BV in several clinical studies. Probiotic dosage regimens of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 converted 7 out of 11 patients with BV to normal or intermediate Nugent Score and the required dose was 10^8 viable microorganisms per day [45]. In a short-term study, 20 subjects with BV who received oral treatment of these two strains showed a higher cure rate (80%, 85% and 90%) compared to the other 20 subjects who received metronidazole gel treatment (45%, 45% and 55%) at 6, 15 and 30 days [46]. In another double-blind study among 544 BV diagnosed subjects, oral capsules of the two same strains established balanced vaginal microbiota in 51.1% subjects in a 13-week follow-up period, compared to 20.8% in placebo group [47]. The success of the two strains was developed into commercial available products as probiotic supplement aiming vaginal health, such as Fem-Dophilus® and RepHresh Pro-B®.

Lactobacilli tablets containing *L. brevis* CD2, *L. salivarius* FV2 and *L. plantarum* FV9 were proven to be effective in treating BV in a study with 39 subjects. Lactobacilli tablets cured 100% (18 out of 18) subjects with BV and converted 83% to normal vaginal flora after treatment, compared to 6% and 0% of placebo. In two weeks, the cure rate dropped to 50%

and subjects with normal vaginal flora counted 67%, compared to 12% and 6% cure rate from placebo group [48].

1.1.4.2 Intravaginal lactobacilli treatment

A 3-day lactobacilli liquid culture (pH 4.1~4.5) intravaginal application was used as BV treatment among 20 women. Significant decreases in vaginal pH, discharge and redness, and improvements in bacterial ecosystem eradication were found after treatment, but no follow-up monitor was conducted [49]. Vaginal tablets containing at least 1×10^7 CFU/tablet viable *L. acidophilus* and 0.03 mg estriol were tested as 6-day treatment or 32 women with BV. The cure rates in 2 and 4 weeks after treatment (88% and 77%) were significantly higher than the cure rate among placebo group (25% and 22%) [50].

1.1.4.3 Lactobacilli combined with antibiotic treatment

Aggressive treatments of the clindamycin gel, the clindamycin oral intake, the lactobacilli vaginal gelatin capsules, the metronidazole gel and another lactobacilli instillation, the extending across three menstruation periods, were applied to 63 women with BV, whose partner also received oral clindamycin treatment. Lactobacilli capsules contain strains of *L. gasseri, L. crispatus, L. jenseneii, L. rhamnosus* and *L. reuteri*. The cure rate dropped from 74.6% after 6 months, to 65.1% after 12 months and 55.6% after 24 months, while only 17 out of 43 established received lactobacilli strains [51].

In another study aiming at preventing BV recurrence, 16 women with BV who were once failed to be cured by 7-day oral metronidazole were separated into two groups. 8 of them were treated by intravaginal 0.75% metronidazole gel, while the other 8 women received a 3-month oral probiotics tablet in addition to the metronidazole treatment as above. The cure

rate of BV from metronidazole and probiotics combination treatment was 100% at 4 weeks, 75.0% at 8 weeks and 62.5% at 12 weeks, compared to 75.0% at 4 weeks, 50.0% at 8 weeks and 37.5% at 12 weeks among metronidazole alone group. The combination of metronidazole gel and oral probiotics tablet was more effective against BV in this 12-week study [52]. There was negative report of this combination treatment as well. After treatment of clindamycin ovules, 255 patients used either freeze-dried lactobacilli loaded (*L. fermentum, L. caseivar, L. rhamnosus* and *L. gasseri*) or placebo tampons during their following menstruation period, while no significant difference was found among 4-week cure rates between lactobacilli and placebo tampons [53].

1.1.4.4 Intravaginal lactic acid gel

In a study involving 42 subjects who were seriously affected with BV, the clinical and bacterial effects of an initial 7-day lactic acid intravaginal gel, followed by a 3-day monthly lactic acid or placebo inserts treatments were assessed. The lactic acid gel eradicated signs of BV from 88% subjects and established vaginal lactobacilli microflora among 83% among subjects, compared to 10% and 16% in placebo group [54]. In another study involving 90 subjects with BV, the effect on lactobacilli re-colonization, change of pH, BV symptoms, tolerability and safety of lactic acid gel treatment was assessed. Lactic acid gel was reported as safe, well-tolerated and as efficacious in curing BV. It also performed better in promoting lactobacilli colonization when combined with metronidazole, and reducing BV recurrence rate, compared to oral metronidazole treatment [55].

1.2 Benzoyl peroxide

1.2.1 Physical and chemical properties

Benzoyl peroxide (BPO) was consisted of two benzoyl groups bridged by a peroxide link. It was colorless to white crystals or granular powders. Its solubility was inversely related to solvent's polarity. It was sparingly soluble in water (0.000155 mg/g). It was slightly soluble in ethanol (17.90 mg/g) and propylene glycol (2.95 mg/g). It was soluble in chloroform, acetone and ether [56].

BPO was unstable when heated or dissolved in solvents due to the instability of O-O bond [56]. When BPO was heated dried and over its melting point (104~106 ℃), explosion might occur [57]. Therefore, commercially available BPO particles were combined with 30% moisture as hydrous benzoyl peroxide for safety, and most BPO existed in suspension formats such as gel or lotion in market.

The degradation of BPO in solution followed a radical mechanism. It degraded into benzoate radicals, attacking other free radicals from decomposition of benzoyl peroxide and the solvent. The degradation could be highly induced and accelerated by free radicals, and strongly influenced by the type of solvents (degradation rate at 79.8 ℃: highly halogenated solvents < most aromatics < most aliphatic < ethers, alcohols, monohydric phenols < amines) [58], [59]. In pharmaceutical gel formulation, the degradation rate of BPO could be markedly reduced by substitution of acetone for ethanol, the elimination of chelation agents and the addition of sodium hydroxide [60].

Peroxide chemicals were very effective antimicrobials and bleaching agents due to their strong oxidative properties. BPO was an important member of the peroxide family and it was

widely applied in many commercially available products, i.e., acne treatment and bleaching processes in food (flour and milk) due to its relevantly low irritation, toxicity and cost.

1.2.2 As acne treatment

Benzoyl peroxide was listed in WHO Model List of Essential Medicine as a form of 5% in cream or lotion to improve skin differentiation and proliferation [61]. BPO was made into a lot of commercially available products for acne treatment in a form of gel, lotion or wash foam from 5% to 10%, such as DDF® 5% BPO gel, Walgreens® 5% BPO Acne Medication Gel, Zapzyt® Acne Treatment 10% Gel, Jan Marini® BPO Acne Treatment 10% Lotion, Rugby® Acne Medication BPO 10% lotion, PanOxyl® 10% BPO Acne Foaming Wash, and etc.

1.2.2.1 Antimicrobial effect

BPO was proven to be effective in reducing acnes and inhibiting acne pathogen *Propionibacterium acne.* In clinical studies, BPO, alone or in combination with clindamycin [62], erythromycin [63], adapalene [64] or salicylic acid [65], was demonstrated to be effective in reducing inflammatory *P. acne*/noninflammatory acne lesions with high patients' satisfaction [66]. It also effective inhibited antibiotic-resistant *P. acne* with no BPO-resistance reported [67], [68], which might result from its multiple cell protein targets oxidized by free radicals [69], [70].

1.2.2.2 Skin penetration and in vivo metabolism

Studies were conducted on the penetration of BPO through skin and its metabolism *in vivo* and the summarized in Figure 1. When applied to skin, BPO penetrated into skin through the stratum corneum or the follicular opening very quickly, wherein converting to benzoic acid

without demonstrated depot. Benzoic acid is rapidly excreted by urine through renal clearance, precluding hepatic conjugation and systemic toxicity due to drug accumulation [71]. While the trans-epidermal penetration rate of BPO was concentration-dependent, its metabolic disposition was not affected by concentration [72], [73].

Benzoic acid was also widely applied in food as preservatives. The pK_a of benzoic acid is 4.19. When $pH<pK_a$, it was mostly in an undissociated form and easily penetrate bacteria cell membrane. Since the pH of the bacteria cell was neutral and much higher than pK_a of benzoic acid, benzoic acid dissociated into proton and benzoate, thus increasing the concentration of proton and decreasing the internal pH of the cell. In order to extrude the protons from benzoic acid to maintain a neutral pH, energy in the form of adenosine triphosphate (ATP) would be using. A constant influx of proton would eventually exhaust cellular energy [74].

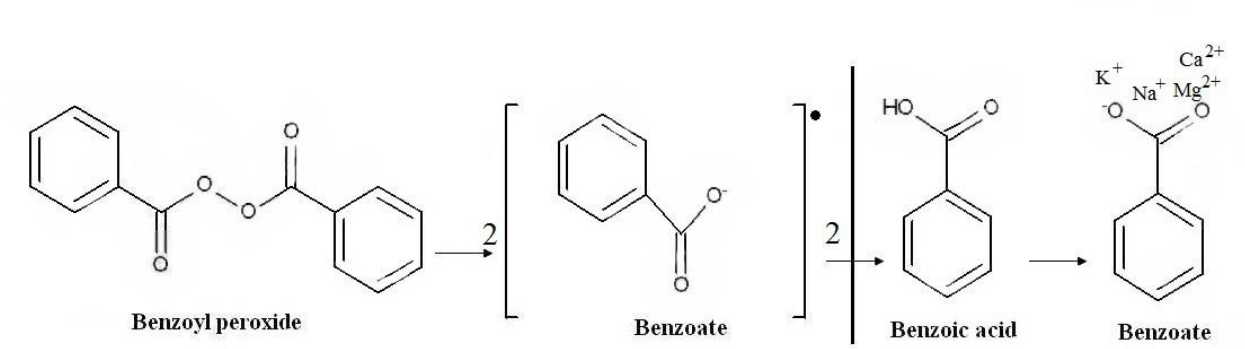

FIGURE 1 The breakdown and metabolism of benzoyl peroxide[a]

[a]This figure was a collaboration effort from Veronica Cavera, who is a M.S. student in Biochemistry and Microbiology department in Rutgers.

1.2.2.3 Adverse effects

1.2.2.3.1 Irritation

Most complains from patients about BPO topical treatment were irritation, erythema, burning, peeling and dryness [66], [75], which were also found related to BPO concentration in the formulation. In a study involving 153 patients with mild to moderate acnes received BPO topical gel treatment, 2.5% BPO gel was reported to cause less frequent peeling, erythema and burning symptoms as compared to 10.0% BPO, but no significant difference from 5.0% BPO [76]. Treatments of 5.0% BPO gel formulation was reported to be well tolerant and most irritations were minor [77].

New formulations were made to eliminate irritations in a laboratory studies. Carbopol® gel containing 5.0% BPO was emulsified with different vegetable oils and applied to male albino rats' skin. No redness or dryness was observed from sesame oil/BPO gellified emulsion [78]. In another study involving 31 subjects, BPO was incorporated into microspheres, made into 5.5% wash. In a combination with clindamycin and tretinoin, it was used to treat acne and its tolerance was assessed. Compared with a gentle cleanser, BPO microsphere offered a same tolerance profile at day 0, 14 and 21 [79].

A new lotion containing BPO which was entrapped in a controlled–release styrene-divinylbenzene polymer system was evaluated *in vitro* and *in vivo*, compared to a lotion containing freely dispersed BPO. *In vitro*, less BPO was absorbed through rhesus monkey skin from a polymeric system. *In vivo*, the new formulation was proven to induce reduced skin irritation, while significantly reduced P. acne and aerobic bacteria counts. The

lotion containing microsphere entrapped BPO reduced skin irritation without comprising its antimicrobial efficacy [80].

1.2.2.3.2 Sensitization

It was reported in 1982 that a 13-year-old girl developed allergic contact to 5.0% BPO topical product after a four-month treatment, which was later confirmed by closed patch test [81]. In a 10-year period study involving 272 children, 101 had positive reaction to 1 or more allergens, while BPO was listed as one of the main allergens [82]. In a patch test involving 59 dental technicians and 732 patients, BPO ranked 2nd in positive allergic reaction. Among dental technicians who were exposed to BPO in their working environment, the allergic reaction was more frequent [83]. In a clinical study involving 59 patients with acne, after a mean 10.7-month treatment with commercial 5% or 10% BPO gel, 3 of them developed sensitization. Compared to patch test, the rate of sensitization in clinical test was dependent on many factors including penetration, sweat, instability of the material, inflammation grade and application duration [84].

1.2.3 In bleaching process

BPO was used for flour, milk and whey bleaching agents for many decades. BPO was generally recognized as safe (GRAS) and allowed to be used in food with no limitation other than good manufacturing practice (GMP) by food and drug administration (FDA) [85]. Its acceptable concentration as food additives is 40 mg/kg in flour and 100 mg/kg in whey, by the joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) committee on food additives [86].

BPO oxidized and accelerated the natural oxidation process of carotenoid and annatto in flour and cheese-making as free radical initiator, therefore bleaching and sterilizing food. Most BPO was converted to benzoic acid during oxidation, while the remaining part is converted during further process, such as baking and drying. In a study using high-performance liquid chromatography (HPLC) for detection, 150 ppm of BPO added in freshly milled wheat flour dropped to 11 ppm after 9 days contact, and afterward to nondetectable levels. Benzoic acid was found 16 ppm 12 hours after bleaching and dropped to 6 ppm in 3 months [87].

BPO was also used to bleach milk products. Heating cream at 125 and 145 ℉ with 0.0009% BPO for 90 to 120 minutes efficiently oxidized 50% carotenoid with sufficiently bleaching and no formation of objectionable oxidized and tallow flavors [88]. In another two studies using 34% whey protein concentrate and 80% serum protein concentrate, compared to protein bleached by H_2O_2, less lipid oxidation and subsequent off-flavor was produced by BPO bleaching [89], [90].

1.2.4 Toxicity

Overall information about BPO toxicity could be found from a report published by screening information data set program (SIDS)/ economic cooperation and development (OECD) [91]. From this report, the toxicity of BPO on human health could be concluded as the following 8 points:

1) BPO applied to skin was absorbed and converted to benzoic acid in dermis, without systemic circulation. Benzoic acid enters circulation and is rapidly cleared through the kidnies.

2) The acute oral toxicity of BPO, shown as 50% lethal dosage was over 2000 mg/kg body weight in mice and 5000 mg/kg in rats. Following inhalation of 24.3 mg/l BPO caused no death in male rats.

3) BPO could cause irritation upon 24-hour contact to skins and eyes. But skin irritation was slight, while 5-minute contact didn't cause eye irritation.

4) BPO was proven to cause sensitization by studies in animals and humans.

5) Repeated dose below 500 mg/kg/day BPO was suggested to be at no-observed-adverse-effect level (NOAEL).

6) No positive result supports that BPO was genotoxic.

7) Though BPO couldn't be demonstrated to be a carcinogen, some studies suggest that it was a skin tumor promoter.

8) Based on the dosage for significant weight decrease, the NOAEL level for reproduction toxicity and development toxicity was 500 mg/kg.

1.2.5 Potential application in intravaginal application against bacterial vaginosis

In a *in vitro* antimicrobial study, *L. casei* and *L. acidophilus* were found most resistant to peroxide-based bleaching agents among oral strains [92]. Due to the fact that BPO share a same functional group as H_2O_2, it was hypothesized, in this study, that BPO had a selective antimicrobial effect on vaginal microflora, by inhibiting BV-associated pathogen *G. vaginalis* without influencing the growth of healthy lactobacilli.

1.3 Intravaginal delivery gel

The vagina was treated as a route for drug delivery for many years. Most of the intravaginal delivery treatments aimed at local conditions, including anti-bacterial/anti-fungal infection, spermicide, contraception and cervical ripening; It was also discovered to be a route for systematic treatment delivery, such as urogenital tract infections, hormone therapy, induction of labor and diabetes mellitus [93].

The advantages of intravaginal delivery compared to oral therapy [94]:

1) Gastrointestinal absorption (potential vomiting, drug-drug interference or decreased hepatic metabolism) and the first-pass hepatic metabolism effect could be avoided.

2) Side effects resulted from drug fluctuations of oral daily intake could be avoided.

3) Selective regional therapeutic administration could be achieved with little or no change throughout the rest of the body.

1.3.1 Introduction of vaginal delivery systems

Vaginal delivery systems could be divided to semi-solids, solids and liquids by their physical status before administration. Characteristics, advantages, disadvantages and functions of common commercially available intravaginal delivery products were summarized in Table 1-3 [93].

TABLE 1 Summary of semi-solid vaginal delivery systems

Delivery system	Gel	Cream/Ointment
Characteristics	Cross-linked network dispersed in large amount of liquid, solid-like behavior; Dispersion phase is continuous	50% water and 50% oil dispersion as emulsion; Dispersion phase is not continuous
Function (active component/ product name)	Anti-bacterial (metronidazole/ Vandazole®, Metrogel-vaginal®), (clindamycin/ Clindesse®), (lactic acid/balance™ activ); Contraception (nonoxynol-9/Advantage-S®, Conceptrol®, Gynol II®); Pregnancy support (progesterone/Crinone®) Maintenance of pH (-/RepHresh®); Moisturizer (-/Replens®);	Anti-fungal (clotrizazole/Trivagizole®), (miconazole/Monistat®); Anti-bacterial (clindamycin/Clindesse®) Anti-fungal, vaginal candida infection (tioconazole/Vagistat-1®)
Advantage	acceptable, feasible, easy to use, non-toxic, non-irritating to vaginal mucosa	
Shortage	Messy to apply, embarrassing leakage	

TABLE 2 Summary of solid vaginal delivery systems

Category	Solid		
Delivery system	Suppository	Tablet	Vaginal ring
Characteristics	Inserted as solid, melts or dissolves under body temperature to deliver drug	May contain excipients in oral tablets preparation and muco-adhesive materials, compacted from powder into solids	Polymeric (silicon) flexible ring, with 5.5 cm diameter and 4-9 mm circular cross section diameter
Function (active ingredient/ brand name)	Labor inducer (dinoprostone/Cervidil®); Anti-bacterial (clindamycin/Cleocin®); Anti-fungal (miconazole nitrate/Walgreen®) (tea tree oil/Tea Tree Therapy™);	Contraception (desogestrel&ethinyl estradiol/Desogen®);	Contraception (Etonogestrel&ethinyl estradiol/NuvaRing®); Hormone replacement therapy (estrogen/Ferming®, Estring®)
Advantage	Long-term drug release	Easy to manufacture	Continuous controlled drug release, not interfere with coitus
Shortage	Discomfort to insert	Cannot be used to deliver hydrophobic drug	Need to be removed in weeks or months

TABLE 3 Summary of liquid vaginal delivery system

Category	Liquid
Delivery system	Douche
Characteristics	Liquid used to flush into and wash away discharge or other fluids from vagina, very widely applied in USA
Function (active component/ product name)	Cleaning after menstrual periods or residue of other vaginal drugs (water/Summer's eve®)
Advantage	Low cost, easy to apply
Shortage	Increase the risk of vaginal infection

1.3.2 Crosslinked poly (acrylic acid) hydrogel

Gels were the most widely applied vaginal delivery system due to their acceptability, feasibility and low cost, while their main disadvantages were messiness and leakage. In order to eliminate their shortfalls, vaginal gels formulations with muco-adhesive polymers, such as polycarbophil and carbomer had been developed.

1.3.2.1 Polycarbophil and Carbomer 934P

Both polycarbophil and Carbomer 934P were high molecular weight, crosslinked, acrylic acid-based polymers (PAA). Their standards were listed in US Pharmacopeia (USP) monographs and approved by FDA as inactive ingredients in drug products for oral, buccal, topical, ophthalmic and vaginal application in a form of gel, capsule, tablet, suspension, cream, lotion, tablet, etc. In FDA drug database, as gel to be delivered in vaginal route, the maximum potency is 2.25% for polycarbophil and 2% for Carbomer 934P.

Polycarbophil was the product of poly (acrylic acid) crosslinked with divinyl glycol. Commercial name of polycarbophil was Noveon® AA-1 Polycarbophil, while its calcium salts were Noveon® CA-1 and Noveon® CA-2 Polycarbophils [95].

Carbomer homo-polymer, commercially available as Carbopol®, was a group of polymers of acrylic acid crosslinked with allyl sucrose or allylpentaerythritol. Carbomer 934P was the product of poly acrylic acid crosslinked with allyl ethers of sucrose. Dried carbomer contained 56% to 68% of carboxylic acid (-COOH) groups, therefore the pH of 1% carbomer dispersion in water was 2.5~3.0 [96], [97].

1.3.2.2 Crosslinked poly (acrylic acid) hydrogel network

When exposed to an environment with pH from 4.0 to 6.0, these crosslinked polymers could swell in water up to 1000 times their original volume to form a hydrogel. When neutralized by sodium hydroxide (NaOH) and the pH is increased above their pKa (6.0±0.5), -COOH groups on crosslinked polymers' backbone became anions and repulsing each other between the negative charges and increasing its swelling capacity [97].

The roles of processing conditions such as the ionic strength and pH in controlling the network structure of crosslinked PAA were studied by Elliott *et al.* [98]. It was concluded that 1) dilution during polymerization decreased the degree of crosslink and increased the extent of primary cyclization, 2) during pH was increased from 2.2 to 6.0 by NaOH, the degree of negatively ionized of -COOH chains were increased and the extent of cyclization was decreased, 3) when pH exceeded pK_a 6.0, -COOH groups were fully ionized. Further addition of NaOH increases the ionic strength and leads to ionic shielding, diminishing the repulsion forces between crosslinked PAA molecular and allows more cyclization.

1.3.2.3 Effect of Crosslinked poly (acrylic acid) hydrogel against bacterial vaginosis

Several currently market available vaginal hydrogels (Advantage-S®, Crinone®, Replens™, RepHresh®, Miphil®, etc.,) were composed of polycarbophil and carbomers. Some of them contained active ingredients for contraception or pregnancy support, while some of these formulations were also proven to be effective in improving abnormal vaginal conditions.

In a clinical study, 30 women with vaginal pH>4.5, plus vaginal discharge or a positive whiff test or presence of clue cells were treated with Miphil® (main ingredient is polycarbophil) twice weekly for 6 weeks, compared to a vaginal douche of lactic acid. Miphil® reduced

vaginal pH from 5.4±0.4 to 4.7±0.6 at week 3 and 4.6±0.6 at week 6, compared to 5.3 in lactic acid group. It also acted better in eliminating positive whiff test results (7 out of 7 subjects) and clue cells (6 out of 7 subjects) compared to lactic acid (1 out of 7 subjects and 0 out of 7 subjects) [99].

In another clinical study involving 17 patients diagnosed with BV, Replens^{TM} (composed of polycarbophil, Carbomer 974P, glycerin, mineral oil and sorbic acid) was self-administrated every 3 days for 4 weeks. Four weeks after treatment, improvements were found. 11 patients converted to amines free. 8 converted to whiff negative, with a decline of clue cells number and Nugent scores. Six weeks after treatment, 6 reported complete resolution of vaginal odor and 5 reported complete resolution of vaginal discharge. But there was no decrease in vaginal pH, nor was placebo or control treatment conducted [100].

1.3.3 Rheological properties of hydrogel

It was important to measure the rheological properties of a new vaginal hydrogel before clinical studies were conducted. Features essential to clinical outcome of gel formulations, such as spreadability and retention, were partially governed by their rheological properties, though the specific values of these properties remained unclear. A relationship between viscosity and bio-adhesion was found in Carbomer gel formulation [101]. An excellent gel formulation should be easy to spread and cover the vaginal surface, while obsesses adequate retention to maintain *in situ* [102]. Adjustment and optimization on formulations could be guided by the results of rheological measurement *in vitro*. Measurements should be conducted on both the new formulation and the placebo, differing in the presence or absence of the active compound [103].

By oscillatory measurements, a complete characterization of both elastic and viscous properties of the network could be learnt. An oscillatory frequency needed to be conducted to determine the range of frequency in which the gel formulation exhibits gel-like properties. Under a fixed frequency within this range, an oscillatory shear stress could be conducted and information such as the microstructure and yield stress could be acquired.

Since gels were non-Newtonian fluids, their viscosity varied with shear rate. It was not sufficient to evaluate a single viscosity at a defined shear rate. Therefore, a flow measurement of viscosity conducted in a range of shear rate was required [104].

The effects of temperature and interaction of formulation with vaginal fluid or semen on viscosity should also be considered. In laboratory studies, the simulant of vaginal fluid (pH=4.2) and semen (pH=7.7) was developed and used to dilute formulations for rheological measurements [105], [106], [107]. In this study, the physiological temperature 37 ℃ was determined and formulations were diluted with vaginal fluid.

Rheological properties of vaginal gels composed of hydrophilic polymers were measured in some previous work. Compared to sodium carboxymethyl cellulose gels (Conceptrol®, GynoII®), crosslinked PAA gels (Replens™, RepHresh®) were found easier to spread along vagina mocosa, retain in the vagina and possess a more rigid, more solid-like structure [102]. Owens *et al.* also did a series of works on the rheological properties of the commercially available contraceptive gels [103], [105], [108]. They confirmed that the rheological properties of intravaginal hydrogel formulations could be influenced by dilution with fluid, temperature and the environmental pH.

1.4 Objectives

Due to the high recurrence rate after antibiotic treatment of BV, a new treatment with selective antimicrobial activity is needed. When applied to the patients, it should inhibit BV-associated pathogen *G. vaginalis* without influencing the recovery of healthy vaginal lactobacilli, in order to re-establish a healthy vaginal microflora to prevent recurrence.

In this study, BPO is selected as the antimicrobial agent. It possesses a same functional group as H_2O_2. Since H_2O_2 is a natural defense factor of healthy vaginal lactobacilli, they are expected to be more tolerant to BPO than *G. vaginalis*. Moreover, BPO has been used in commercial products for acne treatment and in food production for bleaching, its toxicity and in vivo metabolism has been well studied.

Because the effective application of BPO cannot be conducted orally, an intravaginal delivery system is required. Based on the overview, gels are the most accepted and widely used intravaginal delivery systems. To overcome the shortage of intravaginal gel such as leakage and discomfort, a muco-adhesive and safe hydrogel formulation composed of cross-linked poly (acrylic acid) is selected.

After hydrogels with different concentrations are formulated, their antimicrobial activities against BV-associated pathogen *G. vaginalis* and four healthy vaginal lactobacilli strains are tested to find out the difference between the strains' tolerance to BPO hydrogels. Rheological properties of the hydrogel formulation, in the presence or absence with BPO are measured *in vitro*, in order to evaluate the rheological behaviors of the formulation when applied.

1.5 Research approach

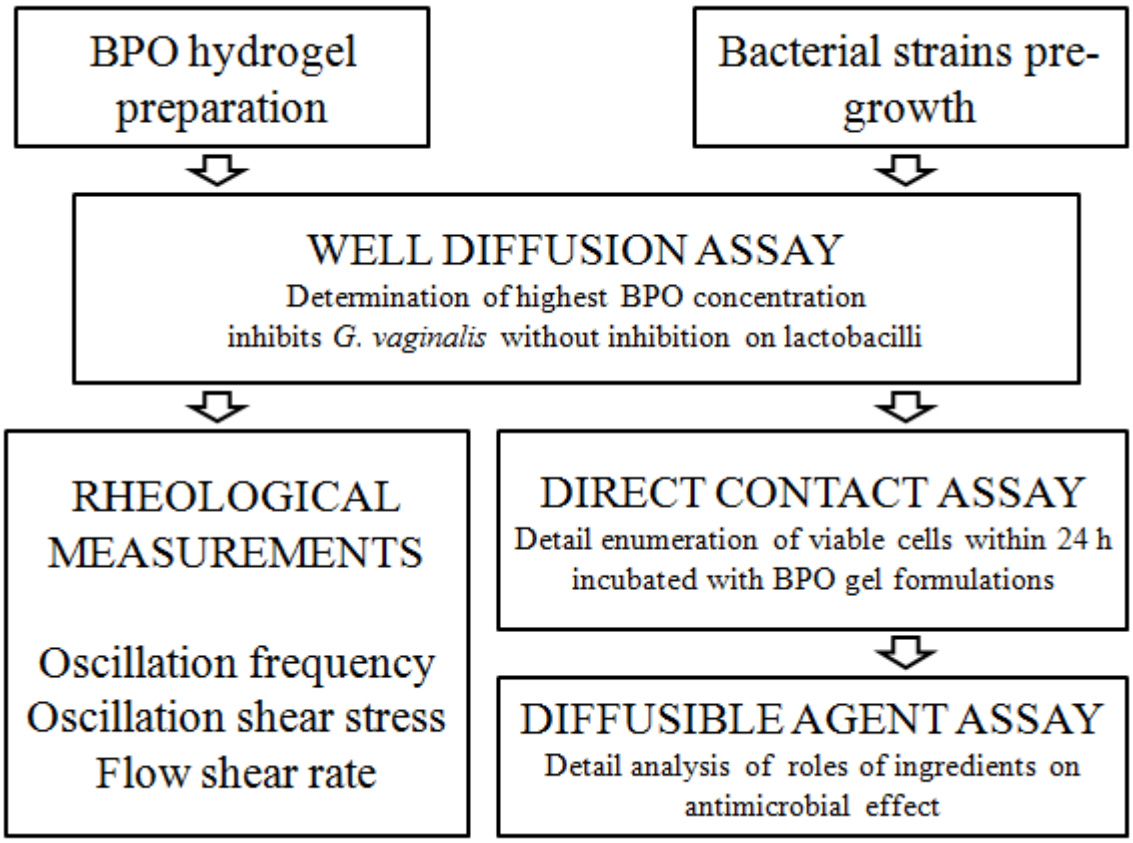

FIGURE 2 Research approaches of this study

This work is a part of the study “Benzoyl Peroxide Formulated Polycarbophil/Carbopol® 934P Hydrogel with Selective Antimicrobial Activity, Potentially Beneficial for Treatment and Prevention of Bacterial Vaginosis.” focused at elucidative of possible use of BPO in feminine health care formulations, which was submitted and accepted for publication in “Infectious Diseases in Obstetrics and Gynecology” in October, 2013.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical compounds

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

Vaginal fluid simulant (VFS), used in rheological evaluation, was comprised of (g/L ddH_2O): 3.51 sodium chloride, 0.22 calcium hydroxide, 3.40 hydroxide potassium, 0.018 bovine serum albumin, 2.00 lactic acid, 1.00 acetic acid, 0.16 glycerol, 0.40 urea and 5.00 glucose. 6N hydrochloric acid added to adjust pH to 4.20 [106]. Sodium chloride, bovine serum albumin, lactic acid and glucose were purchased from Sigma-Aldrich® Co. (St. Louis, MO). Calcium hydroxide, glycerol and urea were purchased from Fisher Scientific Inc. (Waltham, MA). Hydroxide potassium and hydrochloric acid were purchased from VWR® International LLC. (South Plainfield, NJ).

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).

2.1.2 Bacterial growth conditions

G. vaginalis ATCC 14018 was used as the BV-associated pathogen. It was stored at -80 ℃ in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (HS) (JRH Biosciences, KS) and 15% w/v glycerol. Frozen stocks were cultured on human

blood bilayer-Tween (HBT) agar (Remel, Lenexa, KS) and grown at 37 ℃ in 5.0% CO_2 and 2.5% H_2 for 48 hours using EZ Anaerobe Container System GasPaks (Becton, Dickinson and Co, Sparks, MD). When necessary, 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 healthy vaginal microflora. They were stored at -80 ℃ in DeMan, Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) containing 15% glycerol by volume. The cells were plated on MRS agar (1.5% w/v) and grown aerobically at 37 ℃. For experimental procedures, single colonies were inoculated in 20 ml of MRS and grown aerobically for 24 hours with shaking. Cells were subcultured twice before use. Briefly, 200 µl of the overnight culture were vortexed and transferred to 20 ml of fresh broth and inoculated for use in experiments.

2.2 Methods

2.2.1 Hydrogel preparation

Base gel was defined as hydrogel without BPO encapsulated within it. The base gel is prepared as follows (w/w): polycarbophil/Carbomer 934P was separately hydrated in double distilled water. Sodium acetate, acetic acid, Carbomer gel and glycerol were slowly added to polycarbophil gel while stirring. The final concentrations of these components were: 2.0% polycarbophil, 1.0% Carbmer 934P, 15.0% glycerol, 0.049% sodium acetate and 0.038% acetic acid. To elevate pH of base gel to 4.50, 5 M sodium hydroxide solution was used.

Benzoyl peroxide particles were evenly dispersed in base gel at pre-determined concentrations from 0.01% to 10.0% in Nasco WHIRL-PAK bags. 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).

2.2.2 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 [109]. Briefly, colonies isolated from frozen stock were re-streaked on pre-incubated (i.e. kept in the anaerobic environment overnight) HBT plates. For the *Lactobacillus* spp. MRS soft (0.7% w/v) agar was seeded with overnight cultures (~10^7 CFU/ml). Soft agar overlay plates were dried for approximately 30 minutes in a Purified Class II Safety Cabinent (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 ℃. After incubation, zones of inhibition were measured with Vernier calipers (Nova-Tech, Houston, TX). Experiment was performed four times in duplicate with *G. vaginalis* and twice in triplicates with lactobacilli strains.

2.2.3 Inhibition by direct contact assay

Inhibition studies was determined using the time kill studies method as described by Liang et al. [110] with modifications. All bacteria were grown in contact with 5 ml 1.0% BPO gel, 5 ml base gel or 0.067 g hydrous BPO (containing 0.05 g BPO) particles. BPO particles were defined as the hydrous BPO compound without encapsulation in the base gel. BPO was not dissolved in ethanol or dimethyl sulfoxide (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 sterile centrifuge tube, followed by 40 ml of either BHI+3% HS or MRS broth. Overnight cultures of *G. vaginalis* were diluted to 10^6 CFU/ml while the four lactobacilli spp. were diluted to 10^3~10^4 CFU/ml. Cells were grown in direct contact with base gel, BPO gel or BPO particles and incubated anaerobically at 37 ℃. 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. [111]. Colonies were counted and calculated. The experiment was carried out twice in duplicates with *G. vaginalis* and at least three times in duplicates with lactobacilli strains. Q test was conducted on results and $Q_{90\%}$ was set as rejection level.

2.2.4 Inhibition by a diffusible agent assay

The inhibition of *G. vaginalis* by the BPO gel through a 0.45 µm diffusible membrane was tested using the insert plates. Briefly, an overnight culture of *G. vaginalis* was diluted to 10^6 CFU/ml then 600 µl was transferred into 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 1.0% 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 [111]. This experiment was carried out twice in duplicates. Q test was conducted on results and $Q_{90\%}$ was set as rejection level.

2.2.5 Rheological measurements

Rheological evaluations of base gel and BPO gel formulations were conducted 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 temperature at 37 ℃.

Oscillation frequency sweeps and oscillation shear stress sweeps were evaluated on the following formulations: base gel and 1.0% BPO gel. In oscillation frequency sweeps, the shear stress was fixed at 10 Pa, within the linear viscoelastic region and G' and G" were measure between 0.5 to 100 Hz. In oscillation shear stress measurements, the frequency was fixed at 1 Hz, and shear stress was from 1 to 1000 Pa.

Flow shear rate measurements at a variable shear rate from 0.05 to 1000 1/s were conducted on the formulation. After gelation, base gel and 1.0% BPO gel was diluted with VFS at 25%, 50%, 75% and 100% (gel/gel+VFS; v/v) and kept in 37 ℃ incubator overnight prior to test. At 25% VFS dilutions, since the formulations did not fully absorbed the liquid, shear rate sweep was conducted only on the semi-solid portion. Viscosity using the flow shear measurements of diluted gel formulation was measured at a fixed frequency 1 Hz.

All experiments were conducted at least in triplicates. Q test was conducted on data and $Q_{90\%}$ was set as rejection level.

2.2.6 Statistics and figure design

All the statistical analysis was performed and figures in result section were graphed in Sigma Plot 11.0. The benzoyl peroxide structure and breakdown were made in MarvinSketch 5.12.1.

3 RESULTS AND DISCUSSION

3.1 Inhibition effect by well diffusion assay

Well diffusion assays are performed to identify the lowest concentration of BPO (w/w) at which the BV-associated pathogen *G. vaginalis* can be inhibited. Mean values and standard deviations of the inhibition zones from six experiments are shown in table 2.

Zones of inhibition are observed in *G. vaginalis* containing plates following exposure to 0.01% (w/w) and to all higher concentrations. Inhibition distance increases from 0.61 to 3.06 mm when the BPO gel concentration in the well increases from 0.01% to 10.0%. Zones are observed at BPO concentrations of 2.5% or higher for all lactobacilli strains. The sizes of inhibition distance are all below 1.00 mm. No zones of inhibition are observed with the base gel, indicating no associated antimicrobial properties.

Based on this result, four tested lactobacilli strains are more tolerant to BPO. The highest concentration of BPO that inhibits *G. vaginalis* but not lactobacilli is 1.0%. In further tests, 1.0% BPO hydrogel is studied.

TABLE 4 Zones of inhibition (mm) from well diffusion assay (gel formulations and clindamycin) [a,b]

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	0.61±0.22	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.1	0.94±0.63	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.25	1.33±0.56	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
0.5	1.44±0.63	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1.0	1.89±0.42	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
2.5	2.11±0.60	0.50±0.00	0.50±0.00	0.50±0.00	0.50±0.00
5.0	2.44±0.58	0.50±0.00	0.50±0.00	0.50±0.00	0.67±0.26
7.5	2.72±0.65	0.50±0.00	0.50±0.00	0.50±0.00	0.83±0.52
10.0	3.06±0.81	0.50±0.00	0.75±0.27	0.50±0.00	0.50±0.00
Clindamycin (100 μg/ml)	7.11±2.03	3.58±0.58	4.83±1.25	5.67±0.60	6.67±2.06

[a] The distance from the edge of gel loading well to the edge of inhibition zone is calculated from the diameter the inhibition zone in millimeters using Vernier calipers. Mean values and their standard deviations are provided.

[b] This part of the study was with collaboration effort from Veronica Cavera, who is a M.S. student in Biochemistry and Microbiology program in Rutgers.

3.2 Inhibition effect by direct contact assay

The 1.0% BPO gel was chosen for these tests as it represents the highest concentration that inhibited *G. vaginalis* but had no effect on the lactobacilli spp. by the well diffusion assay.

3.2.1 Inhibition effect by direct contact assay on *G. vaginalis*

To determine the microbial survivability of the BV-associated pathogen *G. vaginalis* following 24 hours of direct exposure to the highest concentration of BPO that inhibited the pathogenic bacteria but did not affect the lactobacilli spp. (1.0% BPO w/w), drop plating is used to enumerate surviving colonies. Mean values and standard deviations of two experiments are shown in figure 3. Given that initial counts were similar (7.4×10^5 CFU/ml), following 24 hours of exposure, a six log reduction is observed when grown in contact with the 1.0% BPO gel, as compared to incubation in the absence of the formulation (7.9×10^7 CFU/ml) or in direct contact with the base gel (7.3×10^7 CFU/ml). The concentration of viable cells when incubated with 1.0% BPO gel for 24 h is below detectable limitation (33 CFU/ml), indicating that 1.0% BPO gel was effective at inhibiting *G. vaginalis*, as well as no inhibition effect of base gel on *G. vaginalis*. The free BPO particles are less effective, reducing viable counts by less than three log (8.2×10^4 CFU/ml).

3.2.2 Inhibition effect by direct contact assay on healthy vaginal lactobacilli

Following the observation that *G. vaginalis* growth is greatly reduced, this experiment is repeated with representative vaginal lactobacilli spp. Mean values and standard deviations of three experiments are shown in figure 4-7.

Following 24 hours of incubation, no significant inhibition is noted in direct contact assays in which cultures are incubated with BPO particles, the base gel or the 1.0% BPO gel on *L.*

vaginalis, *L. gasseri* or *L. plantarum* (figure 4-6).

Started from 7.6 $x10^2$ CFU/ml, the growth of *L. acidophilus* is slowed when incubated with the base gel or 1.0% BPO gel from 6 hours. A less than two log reduction is noted in *L. acidophilus* cultures grown in contact with the 1.0% BPO gel (2.0 $x10^7$ CFU/ml) or the base gel (5.5 $x10^7$ CFU/ml), compared to incubation in absence with the two formulations (8.2 $x10^8$ CFU/ml) or with free BPO particles (1.7 $x10^9$ CFU/ml). But neither the formulation stops *L. acidophilus* from growing (figure 7).

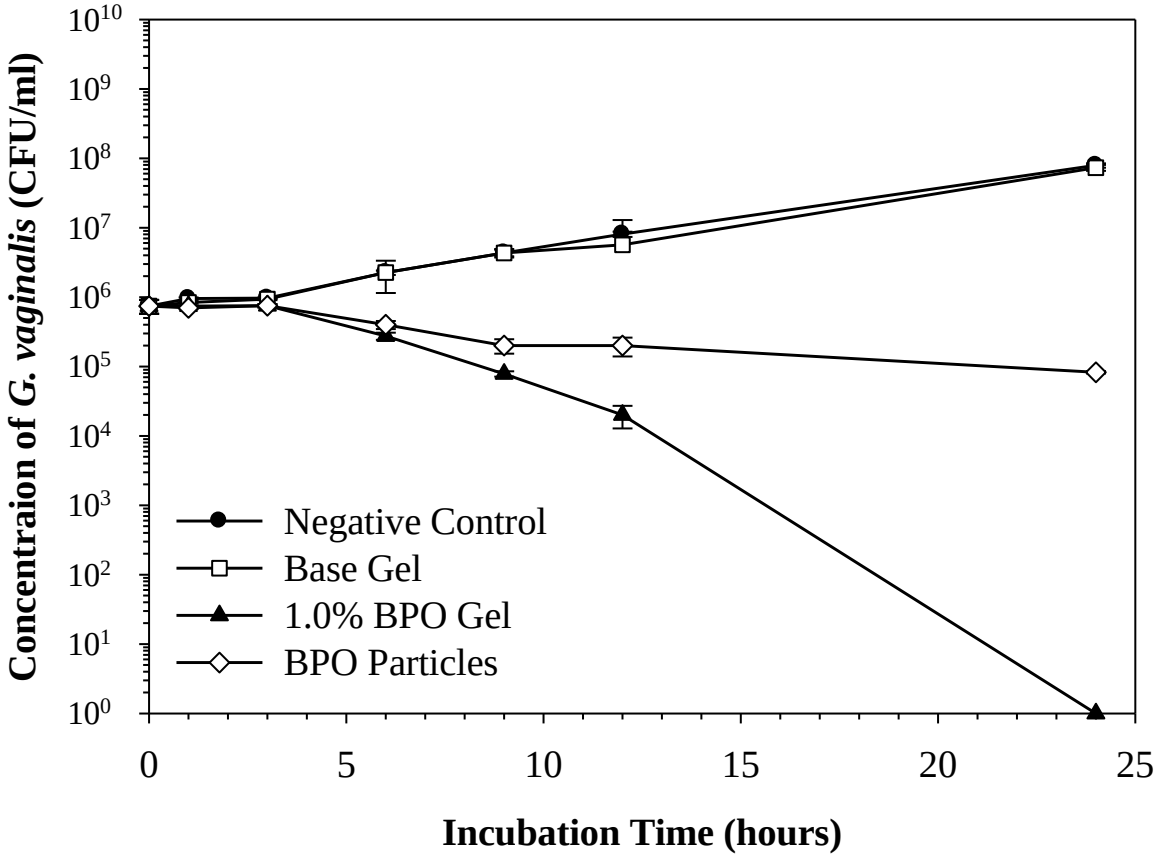

FIGURE 3 BPO inhibits *G. vaginalis* by direct contact assay [a,b]

[a] Experiments were conducted twice in duplicates. Mean values and their standard deviations are provided.

[b] This part of the study was with collaboration effort from Veronica Cavera, who is a M.S. student in Biochemistry and Microbiology program in Rutgers university.

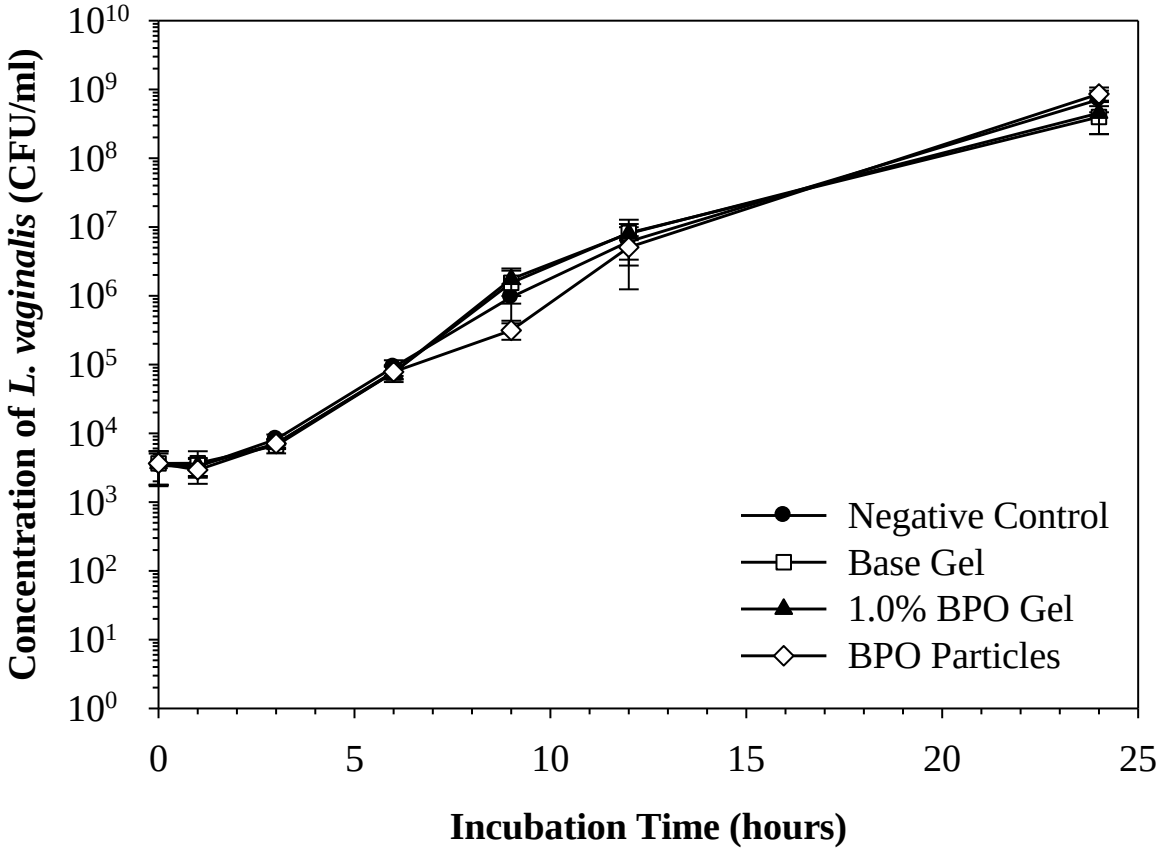

FIGURE 4 BPO formulations effect on *L. vaginalis* by direct contact assay [a]

[a] Experiments were conducted at least three times in duplicates. Mean values and their standard deviations are provided.

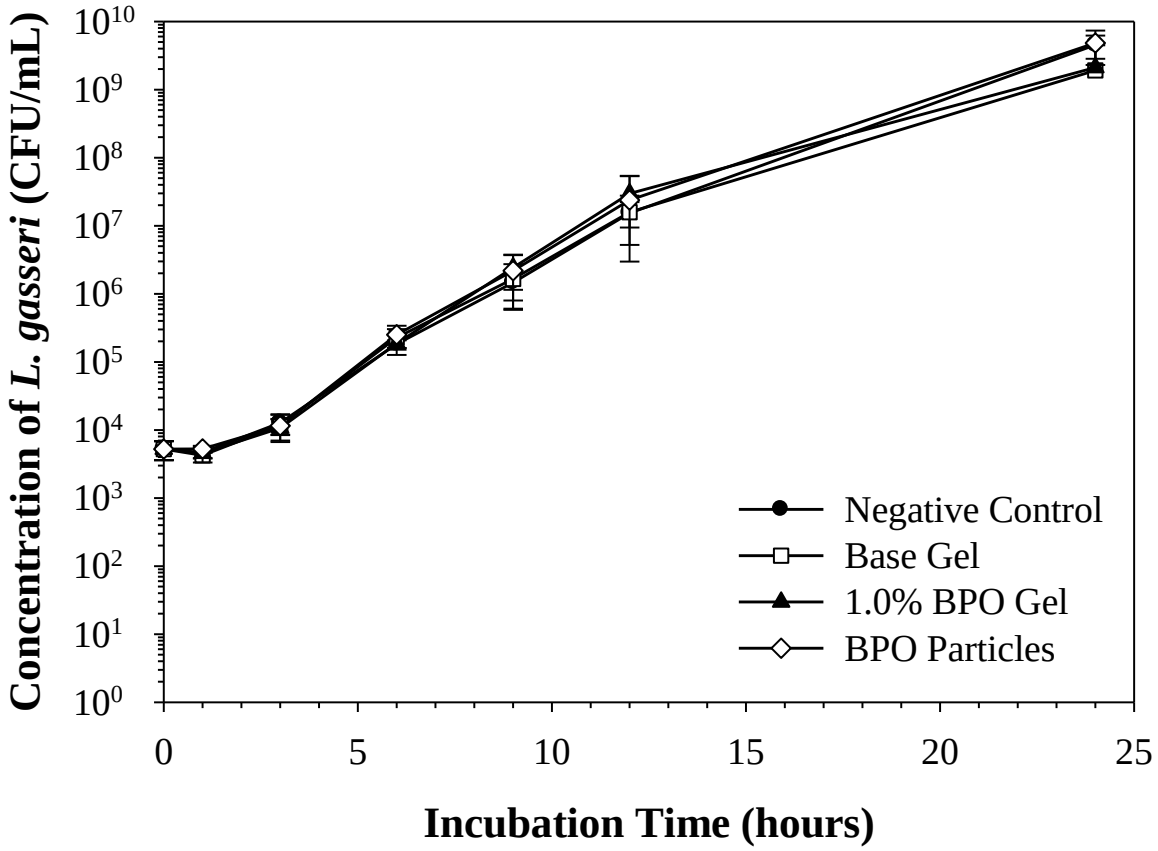

FIGURE 5 BPO formulations effect on *L. gasseri* by direct contact assay [a]

[a] Experiments were conducted at least three times in duplicates. Mean values and their standard deviations are provided.

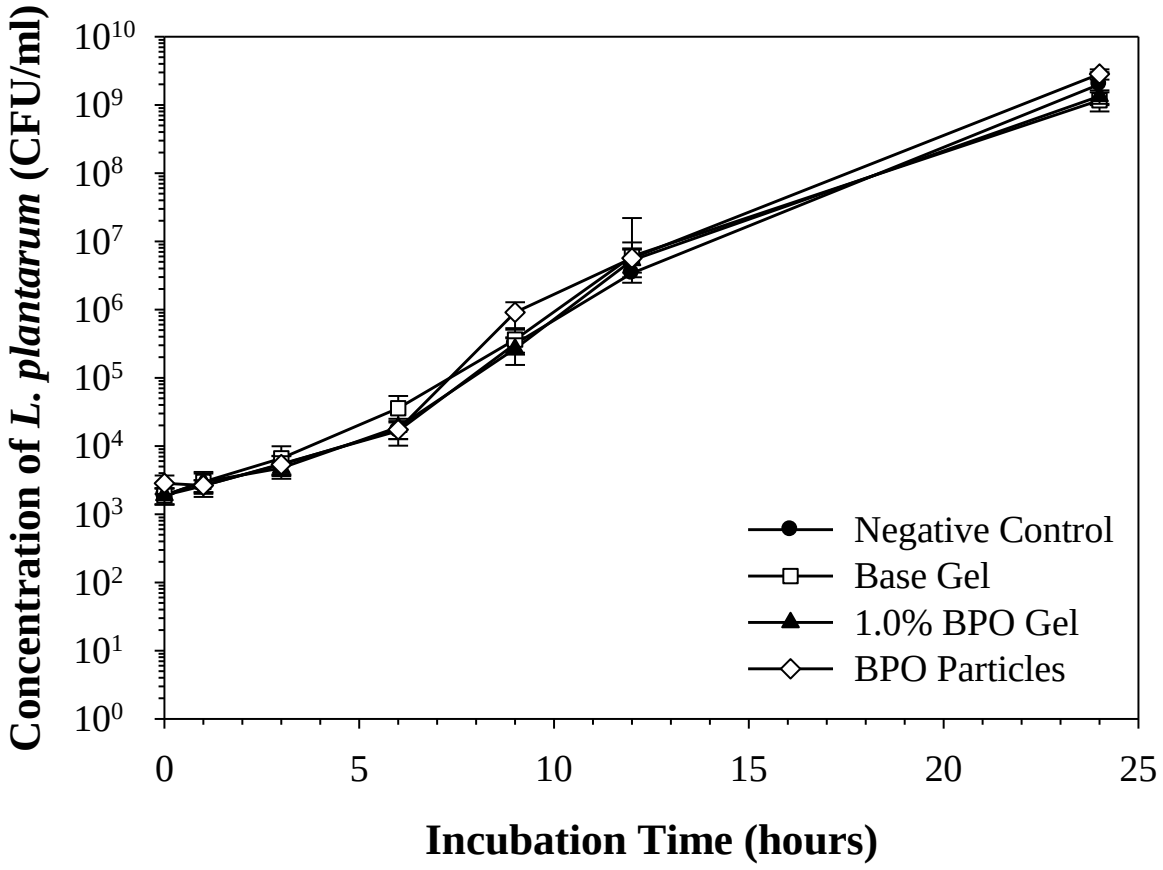

FIGURE 6 BPO formulations effect on *L. plantarum* by direct contact assay [a]

[a] Experiments were conducted at least three times in duplicates. Mean values and their standard deviations are provided.

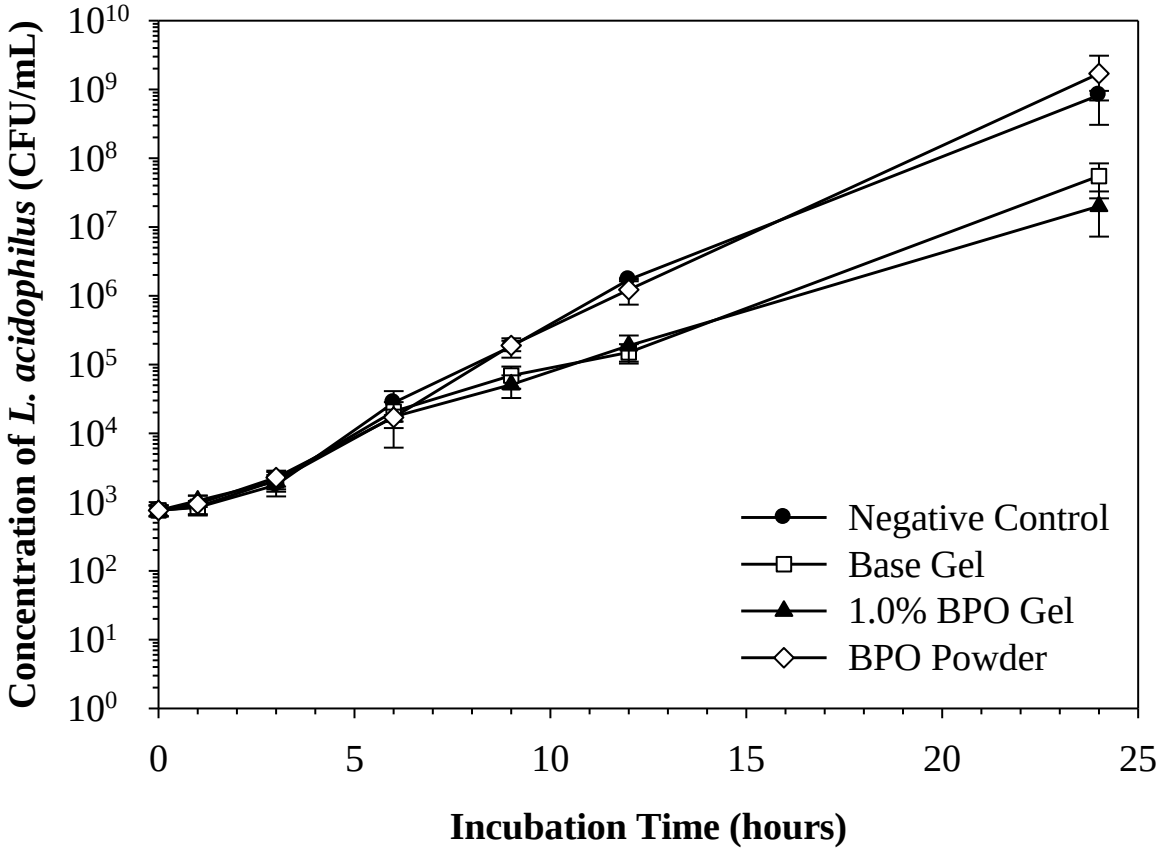

FIGURE 7 BPO formulations effect on *L. acidophilus* by direct contact assay [a]

[a] Experiments were conducted at least three times in duplicates. Mean values and their standard deviations are provided.

3.2 Inhibition effect by diffusion agent assay on *G. vaginalis*

Once the effect of the formulations on the strains is implicated by the direct contact method, the effect in indirect contact is evaluated using a control insert assay (figure 8). The mean values and the standard deviations from two experiments are shown in figure 8. In identical conditions, when exposed to gel-diffused BPO, *G. vaginalis* is inhibited similar to the result observed where it is in direct contact with 1.0% BPO gel and BPO particles (a six log reduction). The concentration of viable cells when incubated with 1.0% BPO gel for 24 h is also below detectable limitation (33 CFU/ml).

Based on the result, it can be assumed that in 1.0% BPO hydrogel, it is the BPO that diffused out from the hydrogel formulations that inhibits the growth of *G. vaginalis*. Other non-diffusible ingredients such as polycarbophil or Carbomer 934P are not antimicrobial. The fact that BPO performs a stronger inhibition effect on *G. vaginalis* after encapsulated into hydrogel formulation can be resulted from the alternation of its diffusion rate.

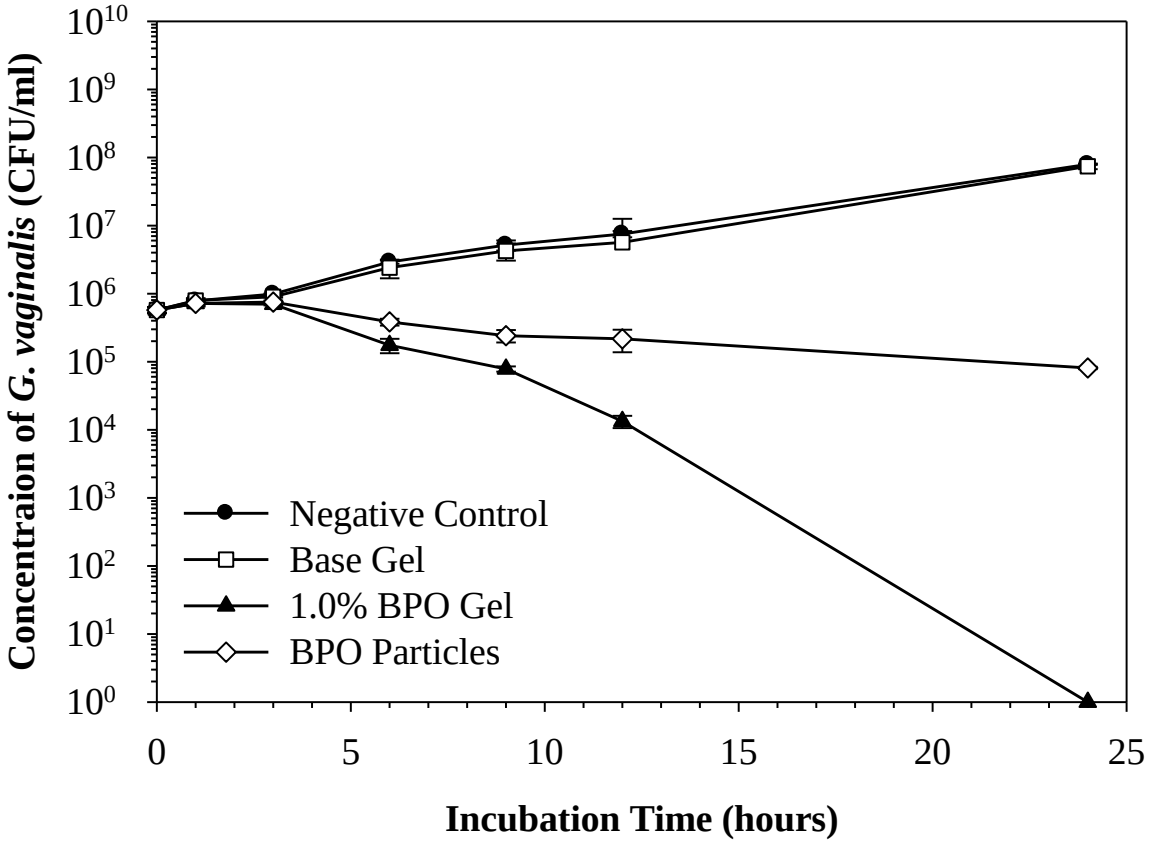

FIGURE 8 The effect of base gel, 1.0% BPO gel and BPO particles on *G. vaginalis* in diffusible agent assay [a,b]

[a] Experiments were conducted twice in duplicates. Mean values and their standard deviations are provided.

[b] This part of the study was with collaboration effort from Veronica Cavera, who is a M.S. student in Biochemistry and Microbiology department in Rutgers.

3.3 Rheological properties

3.3.1 Oscillation frequency measurements

The results of oscillation frequency measurements are shown in figure 9. Under frequency higher than 20 Hz, since the gel formulations yield and bring in too much noise, data is not shown in the figure. Of both tested formulations, from 0.5 to 20 Hz, the storage modulus (G') was always greater than the loss modulus (G"), indicating that this material exhibits gel-like properties. Within the measured range, G' of 1.0% BPO gel was above 600 Pa, indicating that the formulation still possesses a solid-like behavior.

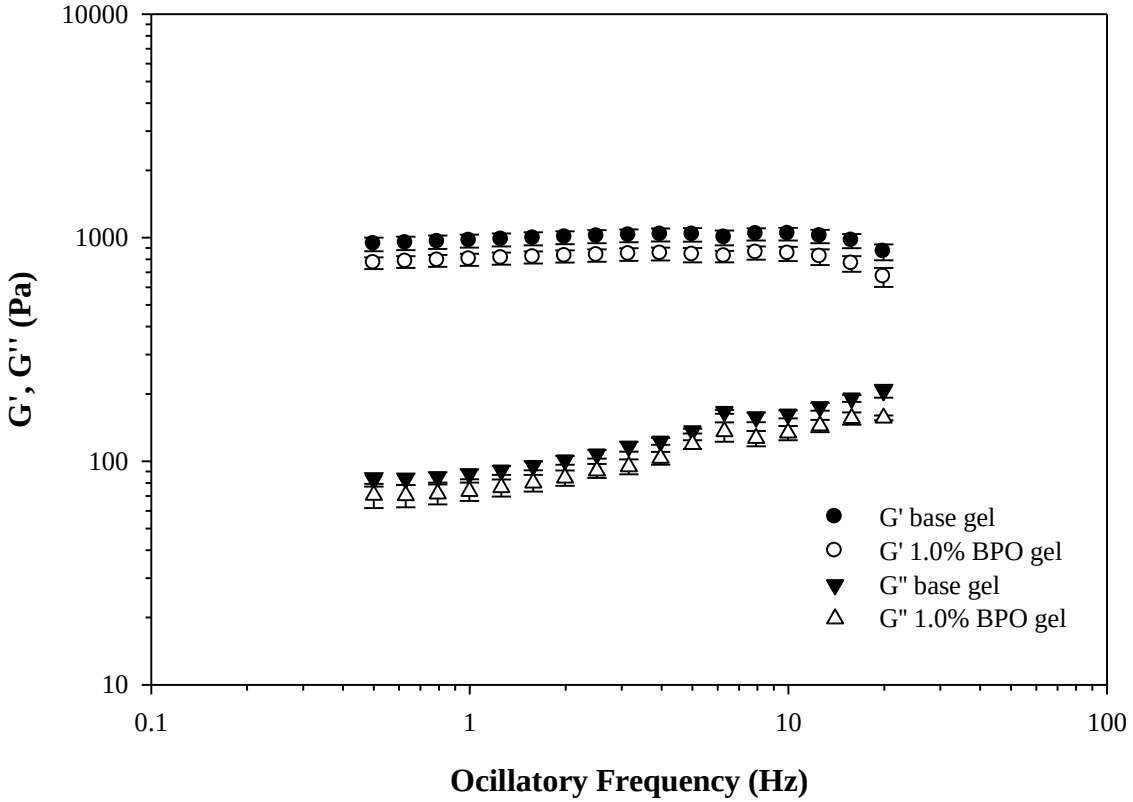

FIGURE 9 Storage modulus (G') and loss modulus (G'') as a function of oscillatory frequency (Hz) on the base gel and 1.0% BPO gel [a]

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

3.3.2 Oscillation shear stress measurement

The results of oscillation shear stress are shown in figure 10 and table 5. Under shear stress higher than 600 Pa, since the gel formulations yield and bring in too much noise, data is not shown in the figure 10.

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 deformation. Yield stress of both formulations was around 30 Pa, indicating that the gel deforms upon addition of stress allowing it to be injected into vagina via shear thinning.

The cross-over of G' and G" represents the breakdown of the gel microstructure, under shear stress larger than the cross-over, the formulation behaves liquid-like. It also represents the stress required to inject the gel formulation. Cross-over of G' and G" of both formulations was both above 300 Pa, indicating that they possess a relatively rigid microstructure [112].

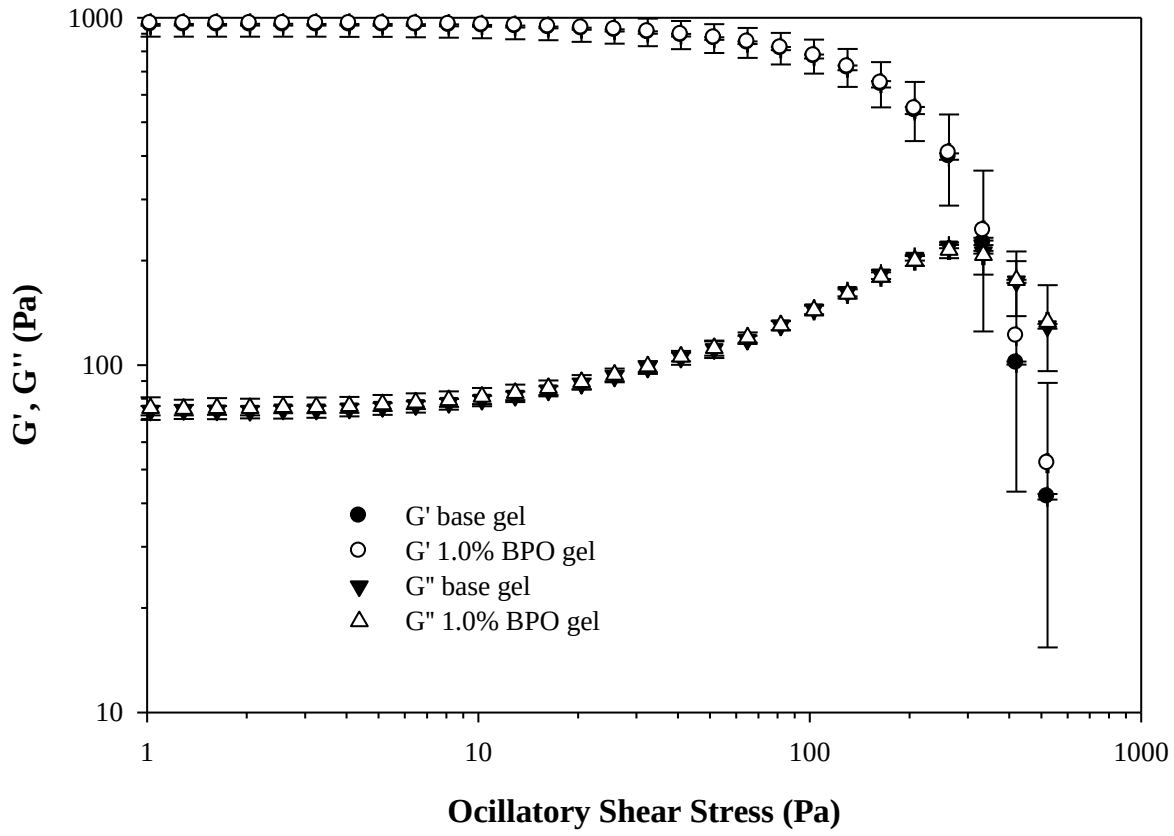

FIGURE 10 Storage modulus (G') and loss modulus (G'') as a function of oscillatory shear stress (Pa) on the base gel, and 1.0% BPO gel [a]

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

TABLE 5 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	347.81 ±3.37
1.0% BPO gel	31.39 ±3.80	357.14 ±49.55

[a] Values are calculated from the results from at least three experiments. Mean values and standard deviations are shown.

3.3.3 Flow shear rate measurements

The results of flow shear rate are shown in figure 11. The elastic component of 25% dilution is too weak, so data at lower shear rate is not shown. The viscosities of all tested formulations decreased with increasing shear rate, indicating their shear thinning behavior and their injectability will be increased by sustained higher shear rates [108].

Both the base gel and the 1.0% BPO gel, the viscosity, as a function of shear rate ranging from 0.05 to 1000 1/s, decrease when the formulation is diluted with VFS, indicating that the formulation will be easier to flow when diluted with vaginal fluids *in vivo*. The high viscosities under low shear rate of the formulations make them easier to stay along vagina mucosa.

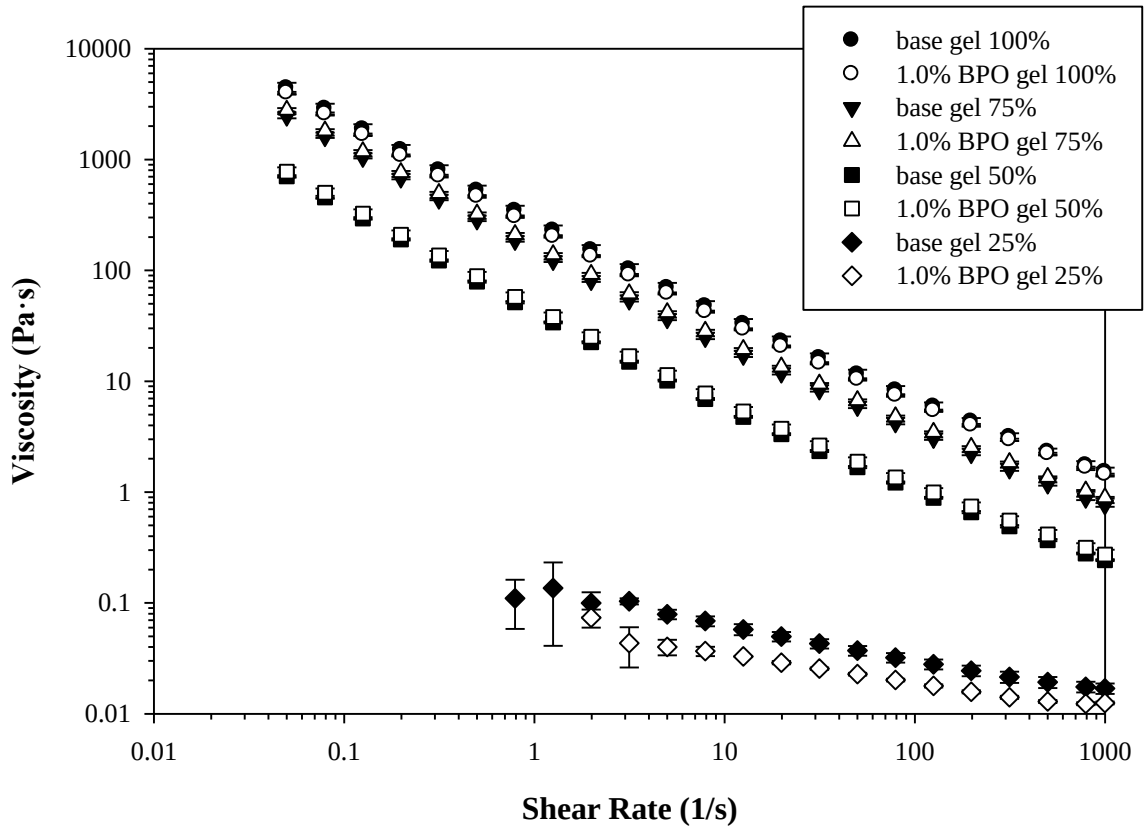

FIGURE 11 Viscosity (Pa s) as a function of shear rate (1/s) of 100%, 75%, 50% and 25% dilutions in VFS of base gel and 1.0% BPO gel [a]

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

4 CONCLUSIONS

The new formulation of BPO hydrogel is proven to inhibit *G. vaginalis* without inhibiting four healthy lactobacilli. By well diffusion assay, when the concentration of BPO encapsulated in polycarbophil/Carbomer 934P hydrogel is within 1.0%, BV-associated pathogen *G. vaginalis* is inhibited, while four healthy lactobacilli are not inhibited. By direct contact assay, 1.0% BPO hydrogel causes a 6-log reduction on *G. vaginalis* growth within 24 hrs, without inhibiting four healthy lactobacilli, and also shows a stronger antimicrobial activity than free BPO particles. By diffusible agent assay, the antimicrobial activity is contributed to diffusible ingredients from the hydrogel formulation.

1.0% BPO hydrogel formulation has good rheological properties for intravaginal application. It possesses a solid-like behavior, a low yield stress and maintains high viscosity after diluted. It should be easy to be injected and retain in the vagina.

5 FUTURE WORK

All tests and measurements of this study are conducted *in vitro*. In the further process of drug product development, a serial of *in vitro, in vivo* and clinical tests are required. *In vitro* tests should be conducted on the cytotoxicity and muco-adhesion of the formulations. *In vivo* tests should be conducted in animal models to test the distribution and retention properties of the formulations. Clinical tests can be focused on prevention, cure rate and recurrence rate of BPO hydrogel treatment against BV, including its effect on colonization of patients' microflora after treatment.

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7 APPENDIX

Encapsulation and Release of Subtilosin A in Poly(vinyl alcohol) Electrospun Nanofibers

This work is a part of the study "Safety, Formulation and *In Vitro* Antiviral Activity of the Antimicrobial Peptide Subtilosin against Herpes Simplex Virus Type 1." which was submitted and accepted for publication in "Probiotics and Antimicrobial Protein" vol. 5, issue 1, pp. 26-35, in 2013.

7.1 Abstract

Poly(vinyl alcohol)-based subtilosin nanofibers with a width of 278 nm were produced by the electrospinning process. The retained antimicrobial activity of the subtilosin-based fibers was determined via an agar well diffusion assay. The loading capacity of the fibers was 2.4 mg subtilosin/g fiber, and loading efficiency was 31.6 %.

7.2 Introduction

Subtilosin A (subtilosin) was a cyclical class II peptide [1], [2], produced by *Bacillus subtilis* [3] and *Bacillus amyloliquefaciens* [4]. It was proved to be effectively inhibiting human pathogens, including bacterial vaginosis associated *Gardnerella vaginalis* [5] with spermicidal activity [6], food-borne pathogen *Listeria monocytogenes* [7] and Herpes simplex virus type 1 [8].

Nanofibers were widely studied due to its potential as delivery systems in many applications including medical tissue engineering [9], [10]. Among the fiber spinning techniques, electrospinning was a novel and efficient procedure to fabricate non-woven nanofiber matrix [11].

Antimicrobial peptides were successfully encapsulated into electrospun nanofibers. *Lactobacillius plantarum* 423 and its bacteriocin were fabricated into polyethylene oxide nanofibers without severe compromise of its antimicrobial activity. *L. plantarum* cells after encapsulation were still producing

bacteriocin [12]. Nisin was also fabricated into gelatin electrospun fiber and able to inhibit the growth of *Staphylococcus aureus* and *L. monocytogenes* after 5-month storage at 25 ℃ [13].

7.3 Materials and methods

7.3.1 Compounds

Subtilosin was isolated and purified from cultures of *B. myloliquefaciens* KATMIRA1933, following the protocols previously described by Sutyak et al. [6]. Poly(vinyl alcohol) (PVOH, Mw = 61 kDa, Sigma-Aldrich) was chosen as a carrier polymer due to its high biocompatibility [16]. Trypticase™ soy broth (TSB), trypticase™ soy agar (TSA), agar and yeast extract were purchased from Becton, Dickinson and Company (Sparks, MD, USA). Yeast extract was added into TSB and TSA in the amount of 0.6% as a nutritional supplement to improve microbial growth. The indicator organism *Micrococcus luteus* ATCC 10240 was chosen due to its high sensitivity to bacteriocins and its widespread used as a bacteriocin sensitive indicator strain by academia and industry [14].

7.3.2 Preparation of antimicrobial fibers

PVOH (1.5 g) was dissolved in 10 ml Millipore water and heated at 80 ℃ for 8 h. After cooling, the PVOH solution was blended with a purified 4.6 mg/ml subtilosin solution for a final concentration of 0.12 g/ml PVOH and 0.9 mg/ml subtilosin. The PVOH/subtilosin solution was then transferred into a 10 ml plastic syringe with a blunt-end metal needle (O.D. 1.27 mm). The syringe filled with stock solution was mounted onto a syringe pump (New Era Pump Systems Inc., Farmingdale, NY, USA). A high-voltage external electric field was applied to the polymer solution under which a Taylor cone [15] formed at the needle end. As electrostatic repulsion on the liquid surface surpassed the surface tension, a polymer liquid jet

was then erupted from the Taylor cone. After experiencing jet whipping [16] and solidification, polymeric fibers were deposited onto the grounded aluminum foil collector. The whole process was maintained at the condition of 15 kV for voltage supply, 0.2 ml/h for feeding rate and 10 cm for needle-to-collector distance. As a negative control, 0.12 g/ml PVOH solution without subtilosin was electrospun by the same procedure as above. After 5 h of processing, the fibrous mat was directly peeled off from the grounded collector covered with aluminum foil. Fibers were then dried under vacuum for 4 h prior to further use.

7.3.3 Morphology characterization of antimicrobial nanofibers

Surface images of the antimicrobial PVOH fibers with and without subtilosin were collected with a commercial Nanoscope IIIa Multi-Mode AFM (Veeco Instruments, Plainview, NY, USA) equipped with a J scanner, which was operated in tapping mode using a silicon cantilever. The scanned images were obtained at the scan size of 5 μm × 5 μm and 50 μm × 50 μm. The scan frequency was set at 0.1 Hz. The section analysis embedded in the software Nanoscope 3.0 was utilized to calculate the fiber diameter distribution.

7.3.4 Assessment of subtilosin loading capacity and efficiency via well diffusion assay

The evaluation of subtilosin loading capacity and efficiency was conducted by agar plate well diffusion inhibition assays [17]. All nanofiber mats were sterilized by direct exposure to UV light (257 nm) in a biosafety cabinet (Forma Class II, A2 Biological Safety Cabinet, Thermo Fisher Scientific, Pittsburgh, PA, USA) for 10 min per side prior to use. Twenty milligrams of PVOH/ Subtilosin fibers was cut with sterile tweezers, immersed in 200 μl sterile ddH_2O and kept at 4 ℃ for 24 h. Twenty milligrams of PVOH fibers was also dissolved as a negative control to eliminate the possibility of PVOH inhibition. An overnight culture of M.

luteus in TSB (c. 10^9 CFU/ml) was diluted 100-fold by blending with ~55 ℃ soft agar (TSB supplemented with 7 g/l agar). Then, 4 ml of the soft agar containing *M. luteus* was transferred onto the surface of a TSA plate. After 30 min of solidification, a sterile glass pipette (approx. 5 mm diameter) was used to create wells in the agar plate. Fifty microliters of purified subtilosin, PVOH/subtilosin fibers (24 h water solution), negative control and their double dilutions were injected into the wells in duplicate. After 24 h incubation at 37 ℃, minimum inhibitory concentration (MIC) was defined as the lowest concentration to form a visible inhibition circle on the *M. luteus* growth layer. Tests were repeated three times.

7.4 Results and discussion

7.4.1 Nanofiber morphology

Figure 15 shows the tapping mode-atomic force microscopy (TP-AFM) images of antimicrobial PVOH fibers. Relatively straight fibers were formed from the pure PVOH solution (Fig. 12A), and an individual PVOH fiber's diameter was 567 nm (Fig. 12B) as calculated by the section analysis embedded in the Nanoscope software. Addition of subtilosin affected the fiber's morphology, resulting in thinner fibers from the electrospinning process (Fig. 12C). An individual PVOH/subtilosin fiber diameter was determined to be 278 nm (Fig. 12D). This phenomenon is likely due to subtilosin's interaction with PVOH, resulting in weakened entanglement between the PVOH polymer chains, shifting the critical entanglement concentration of PVOH solution toward a higher value and subsequently reducing fiber diameter.

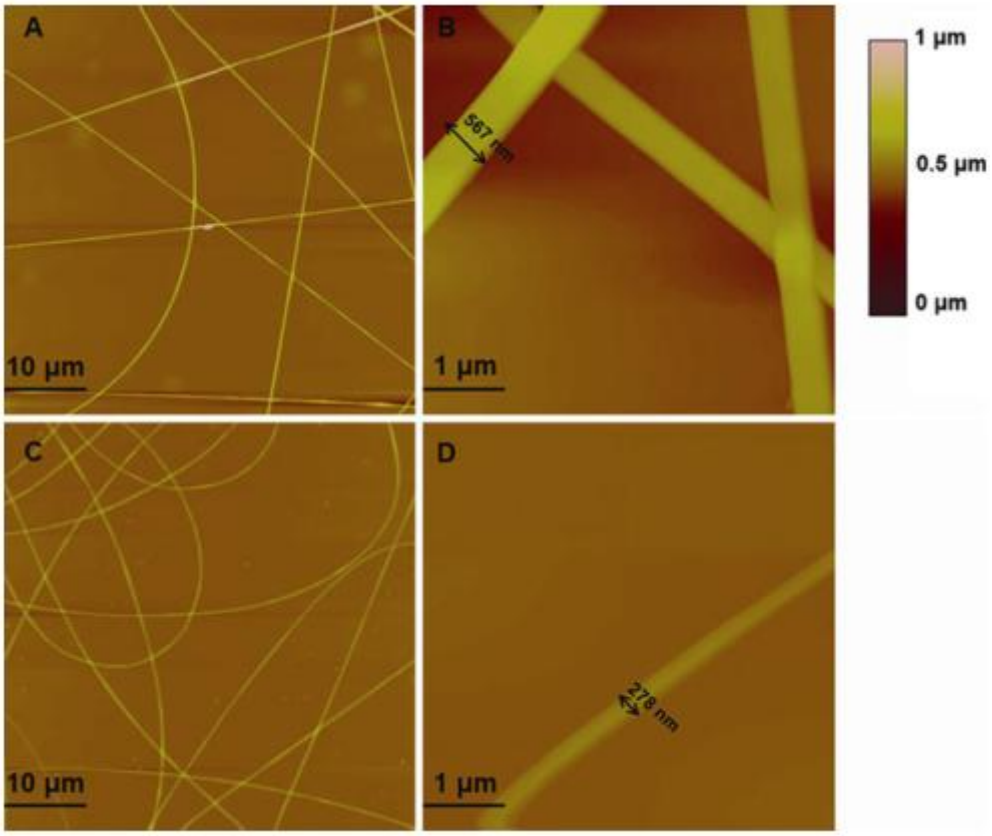

FIGURE 12 Surface morphology of PVOH-based nanofibers with and without incorporated subtilosin [a]

[a] This part of the study was with collaboration effort from Dr. Ji Li, who was a Ph.D. student in Food Science department in Rutgers.

Tapping mode-atomic force microscopy (AFM) images of PVOH electrospun fibers

including:

A: 50 μm × 50μm height image of PVOH electrospun fibers;

B: 5 μm × 5μm height image of PVOH electrospun fibers;

C: 50 μm × 50 μm height image of PVOH/ subtilosin electrospun fibers;

D: 5 μm × 5 μm height image of PVOH/ subtilosin electrospun fiber.

7.4.2 Loading capacity and loading efficiency

Loading capacity was defined as the amount of subtilosin released from a gram of

subtilosin/PVOH nanofiber. Loading efficiency was defined as the ratio of loading capacity

to the amount of subtilosin used in the blended solution to produce 1 g of nanofiber.

First, the MIC of subtilosin against *M. luteus* was determined by well diffusion assay as 60 mg/l. The fourfold dilution of a 20 mg nanofiber per 200 µl ddH_2O solution, equivalent to 25 mg fiber/ml ddH_2O, was the lowest dilution that retained antimicrobial activity, establishing it as the MIC. The concentration of subtilosin released from 25 mg of fiber in 1 ml of sterile ddH_2O was equal to 60 µg/ml. Thus, the loading capacity was calculated to be 2.4 mg subtilosin per gram of fiber.

Since the water acting as a solvent in the subtilosin/PVOH solution was evaporated and dried during the electrospinning and vacuum processes, 1 g of nanofiber was assumed to be primarily composed of PVOH. Since 8.3 ml of 0.12 g/ml PVOH solution was required to form 1 g of fiber, the amount of subtilosin used in the blended solution was 7.6 mg. Thus, loading efficiency was calculated to be 31.6 %. The loss of subtilosin may be the result of the high voltage applied [12] or the difficulty of subtilosin release from encapsulation.

7.5 Conclusion

PVOH electrospun nanofiber is an effective system to deliver subtilosin. The blend solution of PVOH/subtilosin was effectively electrospun into nanofiber with a diameter of 278 nm, evaluated by AFM. By agar well diffusion assay and result of bioactivity against *M. luteus*, 31.6% of subtilosin was encapsulated and released from the nanofibers.

7.6 Reference

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