



Original Article

Evaluation of Lactobacilli Containing Suppository Formulation for Probiotic Use

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Abstract Human vagina represents a dynamic ecosystem dominated by certain species of *Lactobacillus*. This microorganism restricts the growth of pathogens by using properties of steric exclusion and inhibitory substance production. Serious complications including bacterial vaginosis and vaginal cancer are often determined in women with reduced numbers of lactobacilli. Local application of *Lactobacillus* is consequently promising to keep the vagina colonized by this strain, which consequently reduces the infections. In the present work, two vaginal formulations, *e.g.* effervescent tablet and PEG-based suppository, containing *Lactobacillus* were prepared. The hollow type PEG-based suppository was satisfied according to physical and biological characterizations. The lactobacilli inoculated had released from the formulation within 30 minutes. More than 90% of the bacteria released were viable and remained demonstrating good adherence to HeLa cells. Moreover, the abilities on bacteriocin and H₂O₂ productions were not deteriorated by the suppository manipulation. ©All right reserved.

Keywords: bacterial vaginosis, probiotics, vaginal *Lactobacillus*, vaginal suppository

INTRODUCTION

Bacterial vaginosis (BV) is characterized by an alteration of normal vaginal microflora in which a mixed anaerobic bacterial flora becomes prevalent over the population of lactobacilli. Apart from a malodorous vaginal discharge that causes irritating symptoms, BV has been associated with pelvic inflammatory disease,¹ infections following gynaecological surgery,² and pre-term birth.³ Although effective, oral and intra-vaginal administrations of metronidazole and clindamycin have been followed by frequent recurrences of BV.⁴ Because of its high morbidity and frequent recurrence following treatment, alternative therapeutic agents are developed for BV treatment.

Probiotics are defined as “live microorganisms which, when administered in adequate amount, confer a health benefit on

the host”.⁵ The possibility on utilizing lactobacilli as vaginal probiotics is based on the capacity of these microorganisms to produce a barrier population, which subsequently inhibits the adhesion of pathogenic bacteria to vaginal cells. The mechanism of which has been found to be mediated by a competitive exclusion process involving both steric hindrance and competition for receptors. Other interfering mechanisms revealed by *Lactobacillus* are the production of lactic acid, H₂O₂, and bacteriocins.⁵ Lactic acid is produced from metabolisms of carbohydrate. It helps to maintain the vaginal pH at < 4.5, thereby creating an inhospitable environment for the growth of most endogenous pathogenic bacteria. H₂O₂ is appeared to be bactericidal on *Gardnerella vaginalis*, and mostly absent in genital tracts of women with BV. Bacteriocins are proteinaceous molecules produced by bacteria and exert a wide inhibitory range on

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bacteria and fungi.⁶ Due to the bacterium's benefits, it is promising to locate the vagina of women with BV by *Lactobacillus*, aiming to maintain vaginal healthy state which consequently prevents the disease recurrence.

To confer healthy function, *Lactobacillus* to be used as probiotics should be viable upon administration. In addition, they are with antagonistic activity against pathogens, and ability to adhere to vaginal epithelium of the host.⁷ To date, numerous probiotic products are available in liquid form such as yogurt, and solid form such as powder, granule, and tablet. Higher bacterium's viability has appeared for liquid products, but they have been poorly stable and quickly cleared from vaginas of animal models.⁸⁻¹⁰ In this work, alternative formulations were developed aiming to maintain as high as possible the live cells with proper probiotic properties. Freeze-dried powders of lactobacilli were prepared and characterized based on their antagonistic activity, process-ability and stability. The suitable powders were selected for dosage preparation. Two formulations including effervescent tablet and PEG-based suppository were prepared and investigated. Disintegration was performed in a 10-ml synthetic vaginal fluid. The *Lactobacillus* released was examined for viability, antagonistic activity, and adhering capacity to vaginal epithelium cell model.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Freeze-Dried Preparation

Lactobacillus jensenii 5L08 and *Lactobacillus crispatus* 21L07 were obtained from the previous study.⁶ They were separately sub-cultured twice in LAPTg medium for 12 hours.¹¹ The second culture was used to prepare a 1%-inoculum in LAPTg, and grown to early stationary phase. The cells were harvested by centrifugation (6,000 x g, 10 minutes), washed with saline solution (0.9% NaCl), and resuspended in 10% skim milk to obtain approximately 1×10^9 cfu ml⁻¹. The suspended cells were frozen at -80°C for 24 hours followed by freeze drying at -50°C for 20 hours under 0.01 mbar vacuums (Model

Lyph Lock 4.5, Labconco Corporation, KS, USA). The lyophilized powders were calibrated through a 300 µm screen (ATSM, 50 mesh) and technologically characterized in terms of bulk density and repose angle. By the plate counting method,¹² the bacterial powder was examined to contain approximately 1×10^7 cfu g⁻¹.

Pharmaceutical Formulations and Productions

Two dosage forms including effervescent tablet and PEG-based suppository were prepared. To prepare granulated base for the tablets, 316 g of lactose, 79 g of microcrystalline cellulose, 125 g of citric monohydrate, 142 g of sodium bicarbonate, and 50 g of Explotab[®] were mixed thoroughly in porcelain. The mixture was transferred to a hot casserole and agitated on a hot plate until granules were apparent. After cooling, the granules were forced through a 300 µm screen (50 mesh) and dried at 37°C in a circulating air oven until a constant weight was approached. To make one tablet, the mixture of 0.3-g bacterial powder and 1.2-g granulated powder was placed on a single punch machine equipped with a capsule-shaped set of die and punches (Korsch EKO, Berlin, Germany). Pressing method was performed according to manufacturing recommendations.

The suppository was prepared by melting 20 g of PEG 4000 at 50°-60°C in a water bath. Then, 20 g of PEG 400 was added and agitated until thoroughly melted. After congealed, the mixture was poured into suppository molds (Alex Peck Antique Scientifica, USA). A hollow centered to the mold was made by pressing sterile stirring rod into the mixed base prior setting. A 0.3-g bacterial powder was filled into the hollow followed by capping with the mixed PEG. All products were refrigerated at -20°C until use.

Characterization of Finished Products

Physical properties including uniformity of weight, surface texture, and disintegration were determined by applying the USP 27.¹³ The uniformity of weight was performed by weighing twenty tablets or suppositories separately. The average weight and percentage of deviation were calculated. Surface texture

of the finished products was observed visually and recorded as smooth, rough, or uniform surface. Disintegration time was determined in a 10-ml lactate buffer pH 4.5. The time period needed for complete disintegration was recorded.

Viability and Stability Tests

Both *Lactobacillus* powders and the pharmaceutical products were stored in sterile plastic wears at 4°C for the periods of 15 days, 2 and 6 months. Physical stability was then visually investigated. It was different in sample preparation for viability test. One gram of the bacterial powder was uniformly re-suspended in 10 ml of LAPTg, whereas each unit of the preparations was disintegrated and homogenized in 10 ml of the medium. At specific time period, viable cell count was determined by the plate dilution method and reported in the term of percentage viability. All experiments were done in triplicate.

Testing for the Remaining Probiotic Properties

HeLa (ATCC) is often used as modal vaginal epithelium, and was kindly obtained from the National Cancer Institute of Thailand. Following pharmaceutical formulations, the persistence of beneficial properties including HeLa-adhesion and producing capabilities of bacteriocin and H₂O₂ of *L. jensenii* 5L08 or *L. crispatus* 21L07 was investigated.

Culturing of HeLa Cells

Eagle's minimal essential medium (MEM, Sigma) containing 6% foetal bovine serum (FBS), 100 IU ml⁻¹ penicillin G, and 100 µg ml⁻¹ streptomycin was used for culturing HeLa cells. The cells were grown in 75 cm² flasks (Falcon) at 37 °C with 5% CO₂.

Preparation of Lactobacillus Cells

Bacterial lyophilized powders and the formulation products were either resuspended or disintegrated in small volumes of LAPTg. The cell suspensions were separately plated on LAPTg agar plates and incubated at 37 °C for 48 hours under anaerobic atmosphere.⁶ Colonies were selected and cultivated twice

for 18 hours in LAPTg. Bacterial cells were harvested, and washed and re-suspended with saline solution. The optical density at 540 nm (OD₅₄₀) of the bacterial suspensions was determined using UV-Vis spectrometer (Hewlett Packard 8452A Diode Array). Viable cells were determined by colony counts as described previously. The correlation between OD₅₄₀ and colony forming units (cfu) was established.

Adherence Assay

The adhesion reaction was performed in a 6-wells tissue culture plate. To each well, one milliliter of HeLa cell suspension (1 x 10³ cells ml⁻¹) was seeded and grown to 80% confluence. After washing the cell monolayer with phosphate buffer saline (PBS), a 2-ml bacterial suspension (1 x 10⁸ cells ml⁻¹ in MEM) was added. The wells were incubated for 1 hour at 37°C under microaerophilic conditions to allow bacterial attachment. The non-attached cells were discarded by washing several times with PBS. The adhered cells were fixed with 95% methanol for 4 minutes, washed with deionized water, and then air dried. The assay was conducted in triplicate. The average numbers of *Lactobacillus* attached to each HeLa cell was calculated from randomly chosen ten HeLa cells.

Interference Assay

Two formats of the interference assay were conducted; the exclusion and the displacement tests. *Gardnerella vaginalis* and *Candida albicans* were used as test pathogens.⁶ In the exclusion test, HeLa cell monolayer was incubated with *Lactobacillus* (1 x 10⁸ cells ml⁻¹) for 1 hour, followed by washing the non-adhered cells with PBS. The test pathogen (1 x 10⁵ cells ml⁻¹) was subsequently added and incubated further for 1 hour. Extensive washing and fixing were performed as previously described. For the displacement test, in contrast, the pathogen was first incubated with HeLa cells for 1 hour which was followed by the addition of lactobacilli. Each test was done in triplicate. The lactobacilli attached per HeLa cell were calculated and compared with those acquired from the adherence assay.

Production of Lactic Acid, H₂O₂ and Bacteriocin by the Released Lactobacilli

Separation of *Lactobacillus* cells from lyophilized samples and the formulation products was done as described above. The colonies were selected and evaluated for the productions of lactic acid, H₂O₂, and bacteriocin by using the methods of Kaewsrichan et al.⁶

Statistical Evaluation

The means from three replicates were analyzed using single factor ANOVA ($\alpha = 0.05$) Sigma Plot 8 software. Significant differences between individual means were determined using Student *t*-test.

RESULTS

Physical Characteristics of the Formulation Products

Surface texture, uniformity of weight, and disintegration were physical properties to be investigated. Surfaces of the effervescent tablets were rough with several fractures, whereas those of PEG-based suppositories were smooth but appeared waxy. Accounting from 20 units each, the average weights were of 1.34 g and 1.86 g for the tablet and the suppository, respectively. Weight variation of both formulations appeared to be within $\pm 5\%$. Product's liquefaction time was measured

by disintegration test. In 10 ml of synthetic vaginal fluid, the average disintegration times of the tablet and the suppository were of 26 ± 2 and 33 ± 2 minutes, respectively.

Viability of the Released Lactobacilli

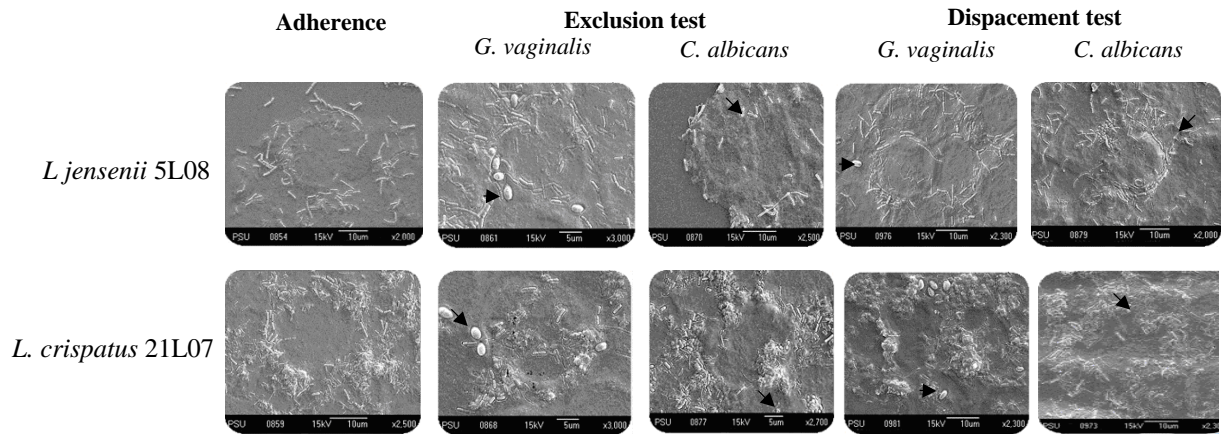
Lactobacillus freeze-dried powders were stored at 4°C under two different conditions, air and nitrogen gas. When exposed to air, most of *L. crispatus* 21L07 (98%) and 70% of *L. jensenii* 5L08 died within 2 months. In contrast, more than 90% of the lactobacilli were viable during 6 months of storage under nitrogen gas. Then, the suitable freeze-dried powders were used for dosage preparations after which viable cells were enumerated. Recently after making the tablets, cells of *L. jensenii* 5L08 and *L. crispatus* 21L07 that viable were not detected. But, approximately 90% of the *Lactobacillus* in the suppository's hollows was survival although stored for a period of 6 months (Table 1).

Adhesion of the Released Lactobacilli to HeLa Cells

Following released from the formulations, the *Lactobacillus* was tested for the adherence capability to HeLa cells. As indicated in Figure 1 and Table 2, a large number of *L. crispatus* 21L07 adhered to the cell culture. In addition, this strain was very well in competition with *G. vaginalis* and *C. albicans* for vaginal receptors. However, decreased adherence and interference properties were observed for *L. jensenii* 5L08.

Table 1. Percentage of viable *Lactobacillus* for the freeze-dried powders and the pharmaceutical products, the effervescent tablets and the PEG-based suppositories, stored in air or under nitrogen gas for the specified period

Strain of <i>Lactobacillus</i>	Duration of storage (months) at 4 °C	% Viability		% Viability	
		O ₂ presented	N ₂ atmosphere	Tablets	Suppositories
<i>L. jensenii</i> 5L08	0.5	84.8	97.3	Not detected	92.3
	2	30.0	95.0	Not detected	90.3
	6	10.0	93.7	Not detected	89.0
<i>L. crispatus</i> 21L07	0.5	10.0	92.5	Not detected	92.6
	2	1.82	91.0	Not detected	91.0
	6	~ 0	89.0	Not detected	90.0



Arrows indicated the pathogen used in each experiment.

Figure 1. Scanning electron micrograph (SEM) showing the adhesion to HeLa cells of the *Lactobacillus* following released. The lactobacilli that obtained from fresh cultures were used as the controls. Adhesion, exclusion and displacement assays were performed according to “Materials and Methods”.

Table 2. Percentage of *Lactobacillus* able to adhere to HeLa cells following challenged with *G. vaginalis* and *C. albicans* compared with that of the non-treated controls. Adhesion, exclusion and displacement assays were performed as indicated in “Materials and Methods”

Strain	% Adhesion of <i>Lactobacillus</i>					
	Control ^a	Adhesion	Exclusion test		Displacement test	
			<i>G. vaginalis</i>	<i>C. albicans</i>	<i>G. vaginalis</i>	<i>C. albicans</i>
<i>L. jensenii</i> 5L08	33.0 ± 2.8	24.1 ± 0.5	22.9 ± 1.9	22.3 ± 0.3	21.9 ± 0.2	10.0 ± 0.4
<i>L. crispatus</i> 21L07	35.0 ± 0.5	34.2 ± 1.2	32.6 ± 3.5	36.0 ± 2.2	33.7 ± 1.6	34.8 ± 2.5

^a The cells obtained from fresh cultured medium.

Lactic Acid, H₂O₂ and Bacteriocin Productions of the Released Lactobacillus

Lactobacillus releasing from lyophilized samples and pharmaceutical products was examined for the productions of lactic acid, H₂O₂ and bacteriocin using the previously described method.⁶ By growing *L. jensenii* 5L08 and *L. crispatus* 21L07 overnight in LAPTg (pH 6.8), the cultured media were changed to the pH of 4.1 ± 0.1. Lactic acid was a dominant acid determined, while acetic acid was produced a little bit (3 g l⁻¹). Approximately 14 and 16 g l⁻¹ of lactic acid were produced by *L. jensenii* 5L08 and *L. crispatus* 21L07, respectively.¹⁴ On the determination of H₂O₂ and bacteriocin, it was revealed that these inhibitory substances were remained detecting in the *Lactobacillus* cultures (data not shown).

DISCUSSION

Since *L. jensenii* 5L08 and *L. crispatus* 21L07 are facultative anaerobes, great attention has been devoted to formulation design and technological processes in order that viable lactobacilli in the final products are maintained and guaranteed. A dosage of effervescent tablet was chosen because of promptly releasing of the microorganisms. Besides, the effervescent granules could improve powder flow-ability resulting in the uniform weight. For PEG-based suppository, the bacterial lyophilized powders were added to suppository's hollows only after the mixed base was set. This could avoid the exposure of microorganisms to wetting steps and excessive technical manipulations.

Formulation's physical properties including surface texture, uniformity of weight, and disintegration were investigated. Surfaces of the effervescent tablets were rough and fractured. But, smooth and waxy surfaces were revealed for the PEG suppositories. There was not different in % weight variation between the tablets and the suppositories, including that the variation was within the acceptable limit of the USP 27 ($\pm 5\%$). By comparing with the suppository, the tablet was simply disintegrated in the synthetic vaginal fluid, indicated within 33 ± 2 and 26 ± 2 minutes, respectively. This suggested that tablet's disintegration might be accelerated by the presence of effervescent granules. In contrast, the mixed PEG base of the suppositories had to be dissolved prior bacterial releasing. This took the time longer. However, disintegration times of both formulations were not varied although using different lots of the freeze-dried powders.

Since *L. jensenii* 5L08 and *L. crispatus* 21L07 have been sensitive to oxygen,⁶ lines of the freeze-drier were plugged with nitrogen gas in order to protect the cells from oxygen exposure. It was found that $> 98\%$ of viable cells was obtained upon freeze-drying. Their viability of up to 90% was maintained for 6 months under nitrogen atmosphere (Table 1). It was noted that the lactobacilli that viable were not determined for tablet preparation although viable bacterial powders were used at start. On the contrary, approximately 95% viability was apparent for the *Lactobacillus* in the suppositories. The components of freeze dried powders and of effervescent granules, as well as the granulation procedure were not causing of cell death, because viable lactobacilli were remained demonstrating at the steps indicated. While, high pressure and excessive heat generated during tablet making were proven to be the causes (data not shown). Increased viable cells were thus revealed for the suppository because of its gentle preparing procedures. But, the method will be applied only if small scale of the products is required. The tablet formulation was indicated to be more optimal for commercial. However, ingredients of the tablets should be modified and developed by replacing with others that generate low heat.

Adhesion of *Lactobacillus* to vaginal epithelial cells has been considered as an important step for colonization and persistence in human vagina.⁵ Although adhesive property varies considerably among the probiotic strains,¹⁵ 36% of *L. jensenii* 5L08 and *L. crispatus* 21L07 have been showed to adhere to the modal vaginal epithelium (HeLa).⁶ The degree of adhesion was similar to that presented by *Lactobacillus rhamnosus* GG, the strain commonly included in commercial products.¹⁶ To test whether ingredients of lyophilized powder and those within the formulations influenced their adhesion, this property was determined for the *Lactobacillus* following released. It appeared that a number of *L. crispatus* 21L07 adhered to HeLa cells and strongly competed with *G. vaginalis* and *C. albicans* for the cell's receptors (Figure 1 and Table 2). In comparison, reduced adhesion and interference was revealed for *L. jensenii* 5L08. This suggested that either formulation components or technical manipulations or both affected adhesion property of *L. jensenii* 5L08 to some extent. Since *L. jensenii* 5L08 showed good exclusion property, it was optimal for prophylaxis use rather than for treatment of vaginal infections (Table 2). On the other hands, the adhesion and interfering mechanisms used by these microorganisms might be different.

Vaginal pH of > 5 is commonly detected in women with BV. Besides, lactic acid produced by lactobacilli has been thought as fundamental factor that protects the vagina form infections.¹⁷ *L. jensenii* 5L08 and *L. crispatus* 21L07 have been capable to acidify growing environments to the pH of 3.8. Bacteriocin has been strongly produced by *L. jensenii* 5L08, while moderate production has revealed for *L. crispatus* 21L07. However, small amount of H_2O_2 has been synthesized by both strains.⁶ It is necessary to ensure that the viable lactobacilli remain producing these antagonistic compounds upon manipulations, storage, and administration. By growing the released lactobacilli in LAPtg, the cultures were acidified to the pH of 4.1 and determined to mainly composing of lactic acid. The growth of *G. vaginalis* was inhibited by lactic acid at levels they

produced, whereas *C. albicans* was tolerant. However, *C. albicans* was effectively killed by the synergistic actions of lactic acid, H₂O₂, as well as bacteriocin.⁶

CONCLUSION

The PEG-based suppository containing lyophilized *L. jensenii* 5L08 or *L. crispatus* 21L07 has revealed as a good candidate for probiotic formulation. Following released, a number of lactobacilli were viable. They were able to adhere to vaginal epithelial cells and compete with *G. vaginalis* and *C. albicans* for vaginal receptors. In addition, the abilities on H₂O₂ and bacteriocin productions made the strains beneficial for use in prophylaxis and treatment of vaginal infections. The suppository completely dissolved within 30 minutes in synthetic vaginal fluid. The suppository's ingredients and its production method described herein were not affected the *Lactobacillus* viability. In contrast, viable *Lactobacillus* was not detected for the tablets. This was caused by high pressure and heat generated on the technical process. Other ingredients that produce low heat should be used to develop the new tablet's formulas for keeping as high as possible the viable lactobacilli.

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