Viability of lactobacillus acidophilus in various vaginal tablet formulations

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ABSTRACT

The lactobacilli which are present in vaginal fluids play an important role in prevention of vaginosis and there are considerable interests in formulation of these friendly bacteria into suitable pharmaceutical dosage forms. Formulating these microorganisms for vaginal application is a critical issue as the products should retain viability of lactobacilli during formulation and also storage. The aim of this study was to examine the viability and release of *Lactobacillus acidophilus* from slow-release vaginal tablets prepared by using six different retarding polymers and from two effervescent tablets prepared by using citric or adipic acid. The Carbomer–based formulations showed high initial viablility compared to those based on HPMC-LV, HPMC-HV, Polycarbophil and SCMC polymers which showed one log decrease in viable cells. All retarding polymers in slow release formulations presented a strong bacterial release at about 2 h except Carbomer polymers which showed to be poor bacterial releasers. Although effervescent formulations produced a quick bacterial release in comparison with polymer based slow-release tablets, they were less stable in cold storage. Due to the strong chelating characteristic of citric acid, the viability was quickly lost for aqueous medium of citric acid in comparison with adipic acid based effervescent tablets.

Keywords: *Lactobacillus acidophilus*, Probiotic, Vaginal tablet.

INTRODUCTION

Bacterial vaginosis is characterized by replacement of *lactobacillus* predominant vaginal flora by pathogenic bacteria (1). Restoration of normal flora of vagina is a key factor in prevention and therapy of vaginosis (2). Lactobacilli are the most important components of vaginal normal flora which are present at 10^7-10^8 CFU/ml of vaginal fluids in healthy pre-menopausal women (3-6). These bacteria are believed to protect vagina from invading pathogens such as *Gardenella vaginalis* and *Trichomonas* (7, 8). They protect the vaginal epithelium through a series of barriers such as self aggregation, adherence (9, 10) and also interfere with potential pathogens (9, 11). Production of antimicrobial compounds such as hydrogen peroxide (12-14), lactic acid, bacteriocin-like substances and biosurfactants (15-17) also contribute to growth inhibition of pathogenic microorganisms (18, 19).

Because of the great importance of lactobacilli's role in prevention and treatment of vaginal infections (20, 21) there has been considerable interest in formulating these friendly bacteria into suitable pharmaceutical dosage forms (21-24). The aim of this study was to investigate the viability of lyophilized *Lactobacillus acidophilus* in various vaginal tablet formulations. To increase the residence time of bacteria, various bio-adhesive polymers were evaluated for formulation of retarding vaginal tablets. To produce a high initial load of bacteria in vagina before application of slow-release tablets, two effervescent tablets of *L. acidophilus* were also prepared and evaluated for viability.

MATERIALS AND METHODS

Strain and culture conditions

Lactobacillus acidophilus PTCC 1643 (DSM 20079, ATCC 4356) was purchased from Persian Type Culture Collection (PTCC), Tehran, Iran.

The microorganism was regenerated into Man-Rogosa-Sharp (MRS) broth from the lyophilized vial and several stock cultures were prepared in PBS containing 20% glycerol and stored at-70^0C.

Preparation of freeze-dried cells

One hundred and fifty ml aliquots of MRS broth medium were inoculated with 7.5 ml of

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Table 1. Formulation used for preparation of *L. acidophilus* slow-release tablets

Formulation	Amount (mg)
Lyophilized powder	250
Mannitol	260
Talc	10
Magnesium stearate	9
Colloidal silicon dioxide	1
Retarding polymer[a]	45

[a] Six different retarding polymers were tested which included: HPMCs of low (HPMC-LV) and high (HPMC-HV) viscosity, SCMC, Carbomer 394P, Carbomer 974P and Polycarbophil

Table 2. Formulation used for preparation of *L. acidophilus* effervescent tablets

Formulation	Amount (mg)
Lyophilized powder[a]	250
Lactose	175
Maize starch	100
Acid source	
Adipic Acid	33.5
Citric acid	29.5
Sodium bicarbonate	33.5
Magnesium stearate	5.5
Stearic acid	1.5
Colloidal silicon dioxide	1

[a] Lyophi;ized powder of *Lactobacillus acidophilus* was used

Lactobacillus acidophilus acidophilus stock vials and incubated at 37^0C under 5% CO_2 for 24 h. The lactobacilli vegetative cells were harvested by spinning the culture at 5000 g for 15min at 4°C. Cells were washed with phosphate buffer, pH 7 and were spinned at 5000 g. Washed cells were freeze dried after re-suspension (50% w/v) in sterile cryo-protecting medium containing 10% w/v skim milk powder and 10% w/v malt extract.

Formulation of slow-release powder

The lyophilized powder containing *L. acidophilus* cells were calibrated by forcing the spongy mass onto a 300 µm screen (ATSM, 50 mesh). All six slow-release formulations had the ingredient depicted in Table 1 which were different in retarding polymer. The polymers used included; two types of hydroxypropylmethylcellulose (HPMC) low and high viscosity grades (HPMC-LV from Shin-Etsu, Japan and HPMC–HV from Merck, Germany), two acrylic acid polymers (Carbomer 934P and Carbomer 974P, BF Goodrich, USA), sodium carboxymethylcellulose (SCMC from Aqualon, USA) and Polycarbophil (Noveon AA-1 from BF Goodrich, USA) as a cross-linked polymer of acrylic acids. Although these polymers are known for their bio-adhesive properties but due to their high molecular weights and viscosities they are also capable to hydrate and make gel slowly. This facilitates tablet dissolution over a longer period and provides a prolonged release of active ingredient embedded in a polymeric matrix. The efficiencies of the polymers were tested in terms of their performances in prolonging the disintegration time and the rate of lactobacilli release from slow release tablets. The formulated powder was prepared by mixing ingredients with the lyophilized bacteria.

Formulation and preparation of fast-release granules

The composition of fast-release formulations were identical (Table 2) with exception that either citric or adipic acid was used in each formulation. To improve powder flow-ability, the granulated base was made first and added to the effervescent mixtures. The granulated base was prepared by wetting lactose, acid source and 85% of the total amount of maize starch with a 10% w/v starch paste (made with the remaining of starch). The wetted mass was forced through a 710 µm screen (25 mesh) and the granules were dried in an oven (30^0C). Sodium bicarbonate, stearic acid and colloidal silicon dioxide were added to the granules, mixed thoroughly and then were treated with certain amount of the lyophilized powder containing about 3.8×10^9 CFU of lactobacilli. The lyophilized powder was included at the end, in order to avoid exposure of microorganisms to the wetting step.

Tablet preparation and analysis

A single punch tableting machine (Korsch AR 400, Germany) was used for preparation of vaginal tablets, with circular shape of die and punch. The possible adverse effects of pressing on lactobacilli's viability were investigated by comparing the viability of bacteria in tablets with those in formulated powders and granules. The disintegration time of tablets were tested according to the guidelines of British Pharmacopoeia (BP) 1998 (25) using a special apparatus for disintegration test of vaginal tablets and suppositories.

Dissolution and rate of microbial release by the slow-release formulations were measured according to Maggi et al. (26) by the paddle method, 30 rev/min, in 400 ml of deionized sterile water at 37^oC in aseptic condition under a laminar air flow cabinet. Samples were obtaine aseptically at different time intervals and plated on the

Table 3. Number of viable *L. acidophilus* in various slow-release formulated powders and tablets

	Polymer type					
	HPMC[a] $_{LV}$	HPMC[b] $_{HV}$	Carbomer 934P	Carbomer 974P	Polycarbophil	SCMC[c]
CFU[d]/575 mg formulated powder	2.70×10^{8}[e]	1.67×10^{8}	1.72×10^{9}	3.28×10^{9}	2.24×10^{8}	2.64×10^{8}
CFU/tablet	2.9×10^{8}	1.6×10^{8}	2.7×10^{9}	4.4×10^{9}	1.5×10^{8}	1.6×10^{8}

[a] low viscosity hydroxypropylmethylcellulose; [b] high viscosity hydroxypropylmethylcellulose; [c] sodium carboxymethylcellulose; [d] CFU, colony forming unit; [e] the numbers quoted are means of duplicate experiments

surface of selective medium for Lactobacilli strains (MRS agar). The number of survived bacteria was determined after incubation at 37^0C under 5% CO_2 for 48 h and the purity of the cultures was examined microscopically.

The viability of the *L. acidophilus* of effervescent tablets in aqueous medium was tested by plating the samples onto the MRS agar medium in consecutive times.

Viability and stability tests of lactobacilli

Freeze–dried powder and pharmaceutical preparations were stored in plastic containers in dry place at 4-6 ^{o}C and were tested for lactobacilli viability after 1 and 6 months of storage. Viability of the cells were examined using a serial dilution method and subsequent plating in duplicate onto MRS agar medium following incubation at 37^{o}C for 24 h under 5% CO_2.

RESULTS AND DISCUSSION

The number of viable bacteria remaining after lyophilization process was about 1.5×10^{10} CFU/g. Both slow-release and fast-release formulations contained equal amounts of bacteria (0.25g or 3.75×10^{9} CFU/tablet). The number of bacteria in various slow-release formulated powders and tablets are shown in Table 3. The Carbomer–based formulations showed high viability, almost close to those in pre-formulated lyophilized powder but about one log decrease in viable cells was observed in HPMC-LV, HPMC-HV, Polycarbophil and SCMC based formulations. Tabletting had no adverse effect on the viability of bacteria. Bacterial viability in the citric acid as well as adipic acid–based formulations is depicted in Table 4. Both effervescent formulations retained bacterial viability almost at the same level as in lyophilized powder.

Disintegration times of slow and fast release formulations using the apparatus described in British Pharmacopoeia are shown in Table 5. The HPMC–LV and HPMC-HV based formulations showed disintegration times of 3.25 and 5.75 h respectively, while those of Carbomers were around 8 h. A disintegration time of 30 min for HPMC based tablet using the apparatus described in the Farmacopea Ufficiale Italiana has been reported (27). The difference in HPMC-based formulations disintegration time may be due to the method of testing or commercial grade of retarding polymer.

Dissolution rates of different polymer–based tablets by determining Lactobacilli's release are shown in Figure 1. Almost all polymers showed a bacterial release of about 10^7 CFU/tablet after 10 h, those of Carbomers 934P and 974P were about 10^6 and 10^5 CFU/tablet respectively. The highest bacterial release for SCMC, Polycarbophil and HPMC-LV based formulations reached within 2 h, while HPMC-HV showed an approximately 0.5 log increase in the subsequent 4 h. Maggi et al. (26) obtained disintegration/dissolution times for slow-release vaginal tablets which were prepared using HPMC-LV, HPMC-HV and Carbopol 934 as retarding polymers. Similarly they found unsteady dissolution times for Carbopol, but found HPMC-LV as the most suitable retarding polymer by disintegration/dissolution time of about 6-7 h. In our experiments HPMC-LV, SCMC and Ploycarbophil released a high initial bacterial load during the first 15 min. However HPMC-HV was found to be a suitable retarding polymer for vaginal formulations, showing a more continuous bacterial release and suitable disintegration time of 5.75 h.

The viability of the *L. acidophilus* in effervescent tablets in aqueous medium is reported in Figure 2. Although the initial release of bacteria was almost the same in both citric and adipic acid-based tablets and 10^9 CFU of bacteria were released after 15 min, the viability was quickly lost in citric acid based tablets and reached to about 10^7 CFU/tablet after 10 h. These results were expected since citric acid is a strong chelating agent. The concentration of citric acid used in the effervescent formulation was around 5% which is well above the reported bacterial inhibitory concentrations (28).

Table 4. Number of viable *L. acidophilus* in citric and adipic acid-based formulated powders and tablets.

	Acid type	
	Citric acid	Adipic acid
CFU[a]/596 or 600 mg formulated powder of citric or adipic acid respectively	2.62×10^{9} [b]	2.46×10^{9}
CFU/tablet	4.6×10^{9}	2.5×10^{9}

[a] CFU, colony forming unit; [b] the numbers quoted are means of duplicate experiments

Table 5. Disintegration time of various polymer or acid-based tablets

	Polymer type					
	HPMC[a] LV	HPMC[b] HV	Carbomer 934 P	Carbomer 974 P	Polycarbophil	SCMC[c]
Disintegration time (h)	3.25	5.75	8.5	8	5	6
	Acid type					
	Citric acid			Adipic acid		
Disintegration time (h)	1			1		

[a] low viscosity hydroxypropylmethylcellulose; [b] high viscosity hydroxypropylmethylcellulose; [c] sodium carboxymethylcellulose

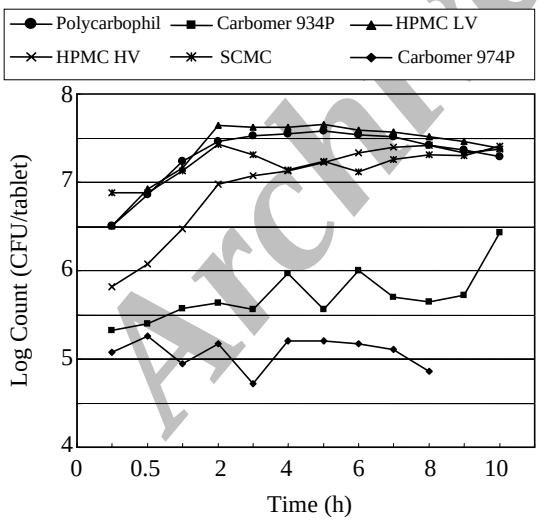

Figure 1. Release of *L. acidophilus* from different slow-release vaginal tablets. HPMC-LV, low viscosity hydroxypropylmethylcellulose; HPMC-HV, high viscosity hydroxypropylmethylcellulose; SCMC, sodium carboxymethylcellulose

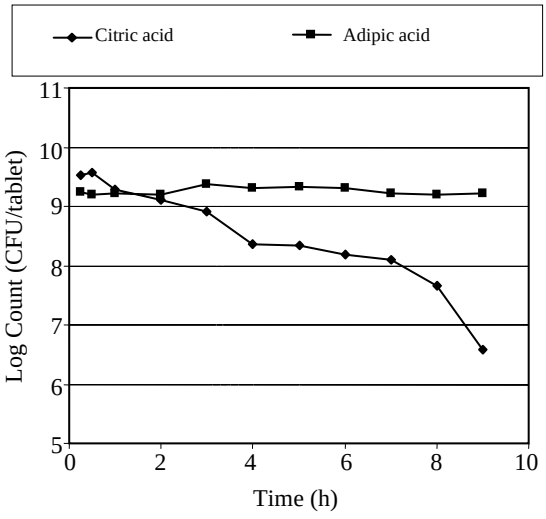

Figure 2. *L. acidophilus* viability of citric and adipic acid-based effervescent vaginal tablets in aqueous medium.

Table 6 shows the stability of *L. acidophilus* in lyophilized powder and pharmaceutical preparations during 6 months of storage at 4°C. The lyophilized form of *L. acidophilus* retained its viability of almost 10^{10} CFU/g after 6 month storage at 4°C. All the slow-release tablets were stable within the tested period but in both citric and adipic acid-based effervescent vaginal tablets,

Table 6. Stability of pre-formulated lyophilized powder, slow-release and effervescent tablets of *L. acidophilus* after six months storage at 4°C

	Time of storage at 4°C (month)	Total count (CFU[a]/g)
Lyophilized bacteria	0	1.5×10^{10} [b]
	1	1×10^{10}
	6	9×10^{9}
Slow- release tablet	Time of storage at 4 °C (month)	Total count (CFU/tablet)
HPMC-LV	0	2.9×10^{8}
	1	1.9×10^{8}
	6	1.7×10^{8}
HPMC-HV	0	1.6×10^{8}
	1	1.4×10^{8}
	6	1.0×10^{8}
SCMC	0	1.6×10^{8}
	1	1.6×10^{8}
	6	8.0×10^{7}
Polycarbophil	0	1.5×10^{8}
	1	1.4×10^{8}
	6	1.1×10^{8}
Carbomer 974P	0	4.4×10^{9}
	1	2.3×10^{9}
	6	1.5×10^{9}
Carbomer 934P	0	2.7×10^{9}
	1	1.85×10^{9}
	6	2.5×10^{8}
Fast-release tablet	Time of storage at 4 °C (month)	Total count (CFU/tablet)
Adipic acid	0	2.5×10^{9}
	1	8.5×10^{8}
	6	2×10^{8}
Citric acid	0	4.6×10^{9}
	1	3.5×10^{8}
	6	1.4×10^{8}

[a] CFU, colony forming unit; [b] the numbers quoted are means of duplicate experiments

one log decrease in viabilities were observed. These results are in accordance with those of Maggi et al. (26) who have shown that stability of vaginal tablets of lactobacilli depend to both bacterial strain and also type of polymer used.

CONCLUSION

Although fast-release formulations produced a quick bacterial release in comparison to polymer based slow-release tablets, but they were less stable upon storage. All the slow-release formulations showed a strong bacterial release except Carbomers with poor bacterial shedding probably due to their high viscosity characteristics. HPMC-HV with a more continuous bacterial release and suitable disintegration time could be a suitable retarding polymer for vaginal formulations.

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REFERENCES

1. Elmer GM, Surawicz CM, McFarland LV. Biotherapeutic agents. A neglected modality for the treatment and prevention of selected intestinal and vaginal infections. J Am Med Assoc 1996; 275: 870-876.
2. Charteris WP, Kelly PM, Morelli L, Collins JK. The role and therapeutic potential of *Lactobacillus* species in female urogenital tract infection. Microecol Ther 1997; 26: 59-96.
3. Nezdarilova J. Interaction between vaginal Lactobacilli and other microorganisms in the vaginal flora. Scripta Medica 1992; 65: 135-141.
4. Sobel JD. Vaginitis and vaginal flora. Curr Opin Infect Dis 1996; 9: 42-47.

5. Larsen B. Vaginal flora in health and disease. Clin Obstet Gyneocol 1993; 36: 107-121.
6. Mehta A, Talwalker J, Shetty CV, Motashaw ND. Microbial flora of the vagina. Microecol Ther 1995; 23: 1-7.
7. Puapermpoonsiri S, Kato N, Watanabe K, Ueno K, Chongsomchai C, Lumbiganon P. Vaginal microflora associated with bacterial vaginosis in Japanese and Thai pregnant women. Clin Infect Dis 1996; 23: 748-752.
8. Blackwell AL, Fox AR, Phillips J, Barlow D. Anaerobic vaginosis (non-specific vaginitis): clinical, microbiological, and therapeutic findings. Lancet 1983; 2: 1379-1382.
9. Boris S, Suarez JE, Vazquez F, Barbes C. Adherence of human vaginal Lactobacilli to epithelial vaginal cells and interaction with uropathogens. Infect Immun 1998; 66: 1985-1989.
10. Mardh PA, Westrom L. Adherence of bacteria to vaginal epithelial cells. Infect Immun 1979; 13: 661-666.
11. Chan RCY, Reid G, Irvin RT, Bruce AW, Costerton JW. Competitive exclusion of uropathogens from human uroepithelial cells by *Lactobacillus* whole cells and cell wall fragment. Infect Immun 1985; 47: 84-89.
12. Eschenbach DA, Davick PR, Williams BL, Klebanoff SJ, Young-Smith K, Critchlow CM, Holmer KK. Prevalence of hydrogen-producing *Lactobacillus* species in normal women and women with bacterial vaginosis. J Clin. Microbiol 1989; 27: 251-256.
13. Hawes SE, Hiller SL, Benedetti J, Stevens CE, Koutsky LA, Wolner-Hanssen P, Holmes KK. Hydrogen peroxide-producing and acquisition of vaginal infections. J Infect Dis 1996; 174: 1058-1063.
14. Klebanoff SJ, Hiller SL, Eschenbach DA, Waltersdorph AM. Control of the microbial flora of the vagina by H_2O_2-generating lactobacilli. J Infect Dis 1991; 164: 94-100.
15. Reid G, McGroarty JA, Angotti R, Cook RL. *Lactobacillus* inhibitor production against *Escherichia coli* and coagregation ability with uropathogens. Can J Microbiol 1985; 34: 344-351.
16. Boris S, Barbes C. Role played by Lactobacilli in controlling the population of vaginal pathogens. Microb Infect 2000; 2: 543-546.
17. Sieber R, Dietz U. *Lactobacillus acidophilus* and yogurt in the prevention and therapy of bacterial vaginosis. Int Dairy J 1998; 8: 599-607.
18. Mombeli B, Gismonda M. The use of probiotics in medical practice. Int J antimicrob agents 2000; 16: 531-536.
19. Piard JC, Desmazeaud M. Inhibition factors produced by lactic acid bacteria. Bacteriocins and other antibacterial substances. Lait 1992; 72: 113-142.
20. Hallen A, Jarstrand C, Pahlson C. Treatment of bacterial vaginosis with lactobacilli. Sex Trans Dis 1992; 19: 146-148.
21. Parent D, Bossens M, Bayot D, Kirkpatric C, Graf F, Wilkinson FE, Keiser RP. Therapy of bacterial vaginosis using exogenously-applied *Lactobacilli acidophili* and allow dose of esteriol: a placebo-controlled multi centric clinical trial. Arzneim Forsch 1996; 46: 68-73.
22. Maggi L, Brigid P, Matteuzzi D, Conte U. Pharmaceutical formulations for the vaginal administration of viable microorganisms. Eur J Pharm Biopharm 1994; 40: 176-178.
23. Mastromarino P, Brigidi P, Macchia S, Maggi L, Pirovano F, Trinchieri V, Conte U, Matteuzzi D. Characterization and selection of *Lactobacillus* strains for the preparation of vaginal tablets. J Appl Microb 2002; 93: 884-893.
24. Shalev E, Battino S, Weiner E, Colodner R, Keness Y. Ingestion of yogurt containing *Lactobacillus acidophilus* compared with pasteurized yogurt as prophylaxis for recurrent candidal vaginitis and bacterial vaginosis. Arch Fam Med 1996; 5: 593-596.
25. British Pharmacopoeia. London: The Stationary Office; 1998. p. A188-189.
26. Maggi L, Mastromaria P, Macchia S, Brigid P, Pirovano F, Matteuzzi D, Conte U. Technological and biological evaluation of tablets containing different of lactobacilli for vaginal administration. Eur J Pharm Biopharm 2000; 50: 389-395.
27. Cavalier Ved. Vesely, Renata Maria Anna, De Simone, Claudio, inventors. Pharmaceutical compositions containing Lactobacilli for treatment of vaginal infections and related method. US Patent 6,277,370. 2001 Aug 21.
28. Phillips CA. The effect of citric acid, lactic acid, sodium citrate and sodium lactate, alone and in combination with nicin, on the growth of bacteria. Lett Appl Microb 1999; 29: 424-428.