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Research paper

Technological and biological evaluation of tablets containing different strains of lactobacilli for vaginal administration

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Abstract

Ten strains of lactobacilli were evaluated for the administration of viable microorganisms to restore the normal indigenous flora in the treatment of urogenital tract infections (UTI) in women. As the strains considered are facultative anaerobes, optimization of the production process was particularly critical to preserve bacterial viability. The microorganisms were formulated in single- and double-layer vaginal tablets. The two layers were characterized by different release properties: one is an effervescent composition that ensures a rapid and complete distribution of the active ingredient over the whole vaginal surface; while the second is a sustained release composition capable of releasing the lactobacilli over a longer period of time. Three different retarding polymers were tested, and all the formulations and tablets were evaluated in terms of technological processability, bacterial viability and stability, and cell adhesion properties of the microorganisms. From the results obtained, three out of ten strains appear particularly suitable for their application in the treatment of UTI. A larger batch of tablets made with a mixture of the three strains was then evaluated, confirming the feasibility of their industrial production and a good bacterial viability in the final dosage form.

Keywords: Vaginal tablets; Bioadhesive polymers; Lactobacilli; Urogenital tract infections

1. Introduction

Recently, a new approach for therapy and prevention of urogenital tract infections (UTI) in the female population is directed towards the restoration of the normal vaginal flora which is competitive to an extensive colonization of pathogens [1,2].

The vaginal flora of healthy women is dominated by different species of lactobacilli which produce large amounts of lactic acid, lowering the vaginal pH (3.5–4.5) [3]. In women suffering from vaginal and urinary tract infections, several pathogens are observed [3,4]; in bacterial vaginosis, an increase in the pH values (>5.0) of the vaginal environment underlines the alteration of the microbial balance [5].

Lactobacilli dominate a healthy flora, but they coexist with a multitude of other species, including potential pathogens [6]. A disruption of this balance could be a factor that leads to infections, while the presence of lactobacilli in the vagina seems to be essential to prevent overgrowth of other bacteria [4,7–9].

Several microbial products, including hydrogen peroxide (H_2O_2), bacteriocins and lactic acid, among others, have been considered to have a protective effect against intravaginal invasion by potential pathogenic bacteria. H_2O_2-generating lactobacilli are bactericidal to *Gardnerella vaginalis* in vitro [10], and their presence in the vagina has been shown to be related to decreased frequencies of bacterial vaginosis and trichomoniasis [11,12].

The possibility of utilizing lactobacilli in the maintenance of a healthy state in the human female urogenital tract is based on the capacity of these probiotic microorganisms to produce a barrier population. Lactobacilli could inhibit the adhesion of pathogenic microorganisms to vaginal epithelial cells by a competitive exclusion process. This mechanism may involve both steric hindrance and competition for receptors [8].

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Particular attention should be given to the design and production of pharmaceutical dosage forms intended for the administration of living cells, since bacterial suspensions show poor stability and are quickly cleared after vaginal instillation [4,13]. In continuation to previous studies, we designed a new pharmaceutical formulation for the administration of viable microorganisms in the treatment and prevention of UTI in women. This dosage form was designed to enhance the adhesion of the microorganisms to the mucosa and a proper colonization of the vaginal epithelium. It consists of a double-layer vaginal tablet which combines an effervescent layer for the immediate release of a fraction of the dose and a slow-release layer to increase the residence time of the microorganism preparation in the vaginal environment [13,14]. To verify the efficiency of the two formulations separately, single-layer tablets were also produced and tested.

To obtain the microbial preparation in a powdered form that can be used in tablet production, the bacterial suspensions were freeze-dried. The lyophilized powders were characterized both from technological and biological viewpoints to select the most suitable product for activity, processability and stability requirements.

2. Materials and methods

2.1. Strain and culture conditions

Ten strains of lactobacilli obtained from international and our own collections were used. They were referred to the species *Lactobacillus brevis* (CD2), *Lactobacillus salivarius* (FV2, FV3), *Lactobacillus crispatus* (FV4), *Lactobacillus gasseri* (FV5, FV6, FV7, FV8, FV9, FV10). The organisms were inoculated from frozen (−80°C) vials onto de Man–Rogosa–Sharpe (MRS) broth (Merck). Following 48 h of culture at 37°C in anaerobic conditions (Gas-Pak System, BBL) the organisms were checked for purity and subcultured in MRS broth.

2.2. Cell production and freeze-dried preparations

For the batch production of CD2, FV2 and FV9, the strains were grown in a 12 l fermentor jar equipped with a combination pH electrode connected to an automatic pH controller (Applikon dependable Instruments bv–Schiendam–The Netherlands). Prior to use, the entire fermentor assembly was autoclaved for 15 min at 121°C. Ten litres of medium (whey permeate, 40 g/l; casein peptone, 20 g/l; yeast extract, 5 g/l; glucose, 20 g/l; Tween 80, 1 g/l; sodium acetate, 5 g/l; $(NH_4)_2SO_4$, 2 g/l; sodium citrate, 2 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; $MnSO_4 \cdot 4H_2O$, 0.05 g/l) was added to the sterilized fermentor jar and sterilized for 20 min at 115°C.

The temperature of the medium was set at 37°C, the pH was adjusted to 6.5 with 30% NaOH, and the automatic pH controller was adjusted to maintain the growth medium at a pH of 6.5 with a 30% NaOH neutralizer.

The medium was then inoculated with 500 ml of MRS culture, previously incubated for 16 h at 30°C for CD2 and 16 h at 37°C for FV2 and FV9. After 8 h of fermentation, the fermentor was refrigerated and cells were harvested by centrifuging for 20 min at $6700 \times g$ at 4°C.

The pellet was resuspended at 50% in cryoprotecting medium (skim milk powder, 10%; malt extract, 10%; water, 80%), previously sterilized for 20 min at 115°C, and the pH was adjusted to 6.4 with 30% NaOH. The concentrated cultures were then freeze-dried at 25°C subsequent to previous freezing at −40°C.

2.3. Pharmaceutical formulation and tablets production

The lyophilized powder was calibrated through a 300 μm screen (ATSM, 50 mesh). The technological behaviour of the different freeze-dried powders obtained from the ten strains considered was characterized in terms of bulk density, tapped density (at constant volume reduction, generally after 500 stroke) and repose angle. All these characteristics are critical in industrial tablet production.

Table 1
Formulations used for single- and double-layer tablets preparation[a]

Formulation	Fast-release layer (mg)	Slow-release layer (mg)
Lyophilized powder (FV)	500	500
Lactose (C. Erba, Milan, I)	429	
Maize starch (C. Erba, Milan, I)	86	
Adipic acid (C. Erba, Milan, I)	67	
Sodium bicarbonate (C. Erba, Milan, I)	53	
Ascorbic acid (C. Erba, Milan, I)	50	50
Stearic acid (C. Erba, Milan, I)	3	
Mannitol (C. Erba, Milan, I)		520
Retarding polymer		90
Talc (C. Erba, Milan, I)		20
Magnesium stearate (C. Erba, Milan, I)	10	18
Colloidal silicon dioxide (Grace, Worms, D)	2	2
Weight of the tablet layer	1200	1200

[a] Three different retarding polymers were tested: HPMCs of low (HPMC-LV) and high (HPMC-HV) viscosities; and CP.

The compositions of the fast- and slow-release formulations used for the production of single- and double-layer tablets are reported in Table 1.

Since the *Lactobacillus* strains used are facultative anaerobes, great attention is devoted to formulation design and technological processes to guarantee and maintain the bacterial viability and stability in the final dosage form.

The formulation designed for a prompt release of the microorganisms contains a granulated base to improve powder flow-ability and the effervescent mixture, while the lyophilized powder is added only at the end of the process to avoid exposure of the microorganisms to the wetting step and excessive technical manipulations. For preparation of the granulated base lactose, adipic acid and 85% of the total amount of maize starch was wetted with a 10% w/v starch paste (made with the remaining quantity of starch). The wetted mass was forced through a 710 μm screen (25 mesh). The granules were dried in a circulating air oven (30°C), calibrated through the same screen and dried up to a constant weight. Sodium bicarbonate, magnesium stearate, ascorbic acid, stearic acid and colloidal silicon dioxide were added to the granules and mixed for 15 min. The weighed amount of lyophilized powder was then mixed with the proper amount of effervescent granulate in a Turbula apparatus for 15 min.

The slow-release formulation was prepared by mixing mannitol, the retarding polymer, talc, magnesium stearate and the freeze-dried microorganisms in a Turbula apparatus for 20 min.

Three types of polymers were evaluated: two hydroxypropylmethylcelluloses (HPMC), one of low and one of high viscosity grade, HPMC-HV and HPMC-LV, respectively, (Methocel® K, Colorcon, Orpington, UK); and a carboxyvinyl polymer (CP; Carbopol® 934 PH, Goodrich, Cleveland, OH). These polymers are known to show bioahdesive properties, but they are also able to hydrate and gel very slowly, due to their high molecular weights and viscosities; for this reason, the tablet dissolves over a longer time and provides a prolonged release of the active ingredient embedded in the polymeric matrix. The efficiencies of the polymers were tested in terms of their performance in prolonging the disintegration/dissolution time of the slow-release layer and their ability to enhance in vitro bacterial adhesion to cultured cells.

A single punch tabletting machine (Korsch EK0, Berlin, Germany) was used for preparation of the vaginal tablets. It is equipped with an almond-shaped set of die and punches. For each strain and formulation considered, single-layer tablets were produced to verify bacterial viability in each layer separately. Then the final dosage form, consisting of double-layer tablets, was produced by layering in sequence the two different formulations (fast-release and slow-release) in the die of the tablet press before compression.

The disintegration time of the fast-release layer was tested according to the United States Pharmocopoeia (USP) 23 test method, in 900 ml of deionized water at 37°C. The time needed for complete disintegration/dissolution of the slow-release layer was measured using the USP dissolution test apparatus n.2, paddle, 30 rev./min, in 400 ml of deionized water at 37°C.

2.4. Viability test and stability of lactobacilli

Freeze-dried powder and pharmaceutical preparations were stored in plastic containers at 4–6°C and examined for a period of 18 months in order to establish their stability. The serial dilution method, by plating in duplicate onto MRS agar medium (Oxoid), was used for counting total lactobacilli.

2.5. Adhesion test

2.5.1. Cells

HeLa cells were grown in 75 cm^2 flasks (Falcon) to confluent monolayers in 5% CO_2 at 37°C in Eagle's minimal essential medium (MEM; Gibco laboratories), containing 6% foetal bovine serum (FBS), 2 mM glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin.

2.5.2. Preparation of microorganisms for adherence assay

Lactobacilli were stored at 4°C as lyophilized powder in anaerobic conditions until use. Before the adherence assay, all bacterial strains were cultured in MRS broth (Oxoid). After 18 h incubation at 37 or 30°C (*L. brevis*) in anaerobic conditions (15% CO_2), bacteria were refreshed in newly made medium and then incubated overnight in the same experimental conditions. The anaerobic environment was obtained by incubating the bacteria in a jar containing specific 'Anaerogen' bags (Oxoid). Cultures of lactobacilli were washed twice at 2200 g for 10 min in phosphate-buffered saline (PBS; pH 7.2). The working dilution of the microorganism suspensions was determined by performing sequential measurements of optical densities of cultures at 600 nm and quantitation of viable microorganisms by colony counts. For viable counts, appropriate dilutions of lactobacilli suspension were spread in triplicate onto MRS agar (Oxoid). Colonies were enumerated after incubation of plates at 37°C for 48 h in appropriate atmospheric conditions. For each strain, the correlation between the optical density of microorganism dilutions and colony forming units (CFU) was established.

2.5.3. Adherence assay

The adhesion reaction was performed in a 24-well tissue culture plate containing a sterile coverslip in each well. One millilitre of HeLa cell suspension at a concentration of 4.5×10^4 cells/ml was seeded onto each well and incubated in a 5% CO_2 atmosphere at 37°C. After 48 h, the cells, grown to approximately 60% confluence, were washed twice with PBS and added with a 0.1 ml suspension of lactobacilli (in MEM) at a concentration of 5×10^9 bacteria/ml. The plates were then incubated for 1 h at 37°C under microaerophilic conditions to allow attachment.

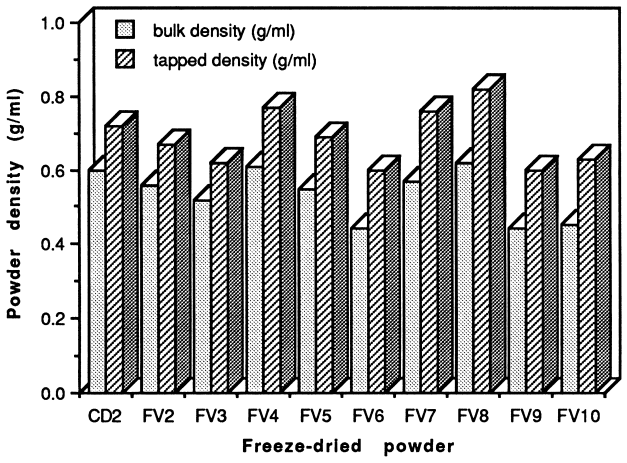

Fig. 1. Bulk and tapped density of the different freeze-dried powders.

Table 2
Disintegration/dissolution time (h) of the slow-releasing tablets containing the different freeze-dried products as a function of the retarding polymers[a]

Lyophilized strains	HPMC-LV	HPMC-HV	CP
CD2	6.7	15.0	6.2
FV2	7.2	15.5	6.8
FV3	7.6	16.3	7.4
FV4	6.2	15.4	5.1
FV5	6.6	15.3	6.0
FV6	6.3	14.9	5.5
FV7	6.8	15.0	5.7
FV8	7.1	16.0	6.0
FV9	7.5	16.5	6.9
FV10	7.6	17.0	8.1

[a] Polymers: HPMCs of low (HPMC-LV) and high (HPMC-HV) viscosities; and CP.

The medium containing the planktonic bacteria was then discarded and the monolayers were washed several times in PBS, fixed with 0.4 ml/well May–Grünwald for 4 min, washed with water, Giemsa stained for 15 min, water-washed again and air dried. By microscopy, each HeLa cell was scored for the presence and the number of bacteria attached. Each adherence assay was conducted in duplicate and 500 randomly chosen cells were evaluated for micro-organism adhesion.

3. Results and discussion

3.1. Technological characterization

The bulk and tapped density of the ten freeze-dried products are generally rather low (FV6, FV9, FV10; Fig. 1) and most of them show scarce packing properties (tapped to bulk density ratio), in particular CD2, FV2 and FV3. This parameter suggests that these materials also have poor flowability as confirmed by the repose angle that is very high for all the products considered, and is comprised from a minimum of 52.1°, for CD2, to a maximum of 61.3°, for FV3. These poor technological characteristics have been corrected using some suitable pharmaceutical excipients, such as glidants and lubricants, and by granulating part of the components, because the lyophilized powder could not be granulated. The repose angle of the final formulations was reduced below 50°, and a bulk and tapped density in the range of 0.4–0.5 g/ml was obtained in all cases.

As expected, the disintegration time of the fast-releasing layer is very short, at about 3 ± 1 min. This parameter is not influenced by the presence of the different freeze-dried products thanks to the presence of the effervescent mixture that acts as driving force for the disintegration process.

Also, the time needed for complete dissolution of the slow-release layer is only slightly affected by the presence of the different lyophilized powders; in this case, the dissolution process is strongly controlled by the type and viscosity grade of retarding polymer used (Table 2). Both HPMCs are quite efficient in reducing the disintegration/dissolution rate of the dosage form, but the tablets containing HPMC-LV show the most suitable dissolution time of about 6–7 h. The results of the CP containing systems are rather unsteady, from 5 to 8 h.

3.2. Viability of the ten strains

Table 3 shows the viability of the strains of lactic acid bacteria tested in different pharmaceutical preparations during 18 months of storage at 4°C. Concerning the viability and the stability, several strains examined, including CD2, FV2, FV3 and FV9, maintained a high viability for at least 18 months.

3.3. Adhesion of lactobacilli to HeLa cells

Adhesion of microorganisms to epithelial cells represents an essential step for colonization and persistence in a specific site. Since adhesive properties vary considerably between lactobacilli strains [8,15–17], we first studied the adhesion capacity of the strains of lactobacilli to HeLa cells, a cell line which originated from a human carcinoma of the cervix. The adhesion of lactobacilli, reported in Table 4, is expressed as the percentage of infected cells and the average number of adherent bacteria/infected cell and per cell. The last value was calculated on the basis of the data obtained by microscopic examination reported in the first two columns and represents a global evaluation of the adhesion. The results obtained indicated that all strains of lactobacilli were able to adhere to cells, exhibiting different degrees in their attachment to cell membranes. The adherence of the bacteria varied from an overall mean of 5 to 29 bacteria/infected cell and a percentage of 19–95% of cells with adherent bacteria. Four strains (FV2, FV3, FV4 and FV7) were very poorly adhesive (1–3 adherent bacteria/cell), two strains (FV6 and FV10) showed an intermediate adhesiveness (6–9 adherent bacteria/cell), whereas *L. brevis*

Table 3
Viability of the examined lactobacilli strains in freeze-dried powder and different pharmaceutical formulations

Strains	Time of storage at 4°C (months)	Freeze-dried powder (cells/g)	Tablets (cells/g)		
			Fast-release	Slow-release	Double-layer
CD2	1	8.0×10^{9}	5.0×10^{8}	7.0×10^{7}	6.0×10^{8}
	6	2.3×10^{8}	3.6×10^{8}	3.0×10^{7}	1.7×10^{8}
	12	1.1×10^{8}	2.0×10^{8}	8.0×10^{6}	1.0×10^{7}
	18	1.0×10^{8}	6.0×10^{7}	5.0×10^{6}	8.0×10^{6}
FV2	1	17.0×10^{9}	7.0×10^{9}	1.2×10^{9}	5.0×10^{9}
	6	6.8×10^{9}	1.0×10^{9}	2.0×10^{8}	1.0×10^{9}
	12	5.6×10^{9}	1.0×10^{8}	1.0×10^{7}	1.9×10^{8}
	18	1.1×10^{9}	8.0×10^{7}	8.0×10^{6}	1.0×10^{8}
FV3	1	3.0×10^{9}	16.0×10^{9}	9.2×10^{9}	12.0×10^{9}
	6	1.5×10^{9}	1.0×10^{9}	4.0×10^{9}	4.5×10^{9}
	12	3.0×10^{9}	2.0×10^{8}	2.0×10^{7}	1.0×10^{8}
	18	1.5×10^{8}	4.0×10^{7}	4.0×10^{6}	1.0×10^{7}
FV4	1	7.0×10^{7}	2.0×10^{6}	3.0×10^{6}	6.0×10^{6}
	6	3.0×10^{4}	4.0×10^{4}	4.0×10^{4}	5.0×10^{4}
	12	1.0×10^{4}	6.0×10^{3}	4.5×10^{3}	4.0×10^{3}
	18	5.0×10^{3}	4.2×10^{3}	4.0×10^{3}	2.1×10^{3}
FV5	1	3.5×10^{9}	3.0×10^{8}	4.0×10^{7}	9.0×10^{7}
	6	3.0×10^{8}	1.0×10^{8}	3.0×10^{5}	1.5×10^{7}
	12	8.3×10^{7}	6.5×10^{7}	2.3×10^{4}	7.8×10^{6}
	18	3.0×10^{7}	1.2×10^{7}	2.0×10^{4}	4.0×10^{5}
FV6	1	50.0×10^{9}	35.0×10^{9}	4.8×10^{9}	4.0×10^{9}
	6	41.0×10^{9}	29.0×10^{9}	3.0×10^{8}	8.2×10^{8}
	12	12.0×10^{9}	8.7×10^{8}	7.7×10^{8}	2.4×10^{8}
	18	11.0×10^{9}	8.3×10^{8}	1.4×10^{6}	7.0×10^{7}
FV7	1	14.0×10^{9}	3.0×10^{9}	6.7×10^{9}	4.2×10^{9}
	6	6.0×10^{8}	1.0×10^{8}	4.0×10^{8}	3.0×10^{8}
	12	5.3×10^{7}	2.4×10^{7}	4.6×10^{7}	4.7×10^{7}
	18	2.0×10^{7}	1.0×10^{7}	8.0×10^{6}	2.0×10^{7}
FV8	1	5.0×10^{9}	3.0×10^{9}	6.3×10^{8}	6.0×10^{8}
	6	1.6×10^{9}	9.0×10^{7}	1.0×10^{8}	2.4×10^{8}
	12	8.6×10^{8}	2.0×10^{7}	3.8×10^{7}	5.1×10^{7}
	18	3.1×10^{8}	1.1×10^{7}	2.2×10^{7}	8.3×10^{6}
FV9	1	4.0×10^{9}	2.8×10^{9}	9.6×10^{8}	3.2×10^{9}
	6	1.5×10^{9}	1.3×10^{9}	9.0×10^{8}	2.0×10^{9}
	12	4.0×10^{8}	5.0×10^{8}	5.0×10^{8}	1.0×10^{9}
	18	7.0×10^{7}	4.0×10^{7}	1.2×10^{8}	8.5×10^{8}
FV10	1	40.0×10^{9}	2.4×10^{9}	3.7×10^{9}	8.9×10^{9}
	6	2.2×10^{9}	2.0×10^{8}	6.0×10^{7}	8.0×10^{8}
	12	8.3×10^{8}	7.4×10^{7}	1.0×10^{7}	2.3×10^{8}
	18	8.1×10^{8}	5.3×10^{7}	2.0×10^{6}	7.0×10^{7}

and three different strains of *L. gasseri* were highly adhesive (19–27 bacteria/cell).

3.4. Effect of the presence of different polymers on the adherence of lactobacilli to HeLa cells

Reid and co-workers [8] analyzed different characteristics that could be involved in the inhibition of uropathogens by lactobacilli. Bacterial size, adherence capability, competitive exclusion and inhibition of pathogenic growth were examined in several strains of lactobacilli to assess their relative importance. It was concluded that adherence is an essential factor for the antimicrobial activity of lactobacilli, since it is responsible for competitive exclusion by masking specific receptor sites on cell surfaces for pathogenic microorganisms by steric hindrance.

In order to achieve the best degree of lactobacilli adhesion to the cell surface, the effect of the different retarding polymers on the adhesion of lactobacilli was examined. The compounds were used at half of the maximal concentration at which these could be solubilized, and were added to the medium during the adhesion step. In Table 5, the effect of HPMC with different viscosity grades on lactobacilli adhesion is reported. The results obtained showed that the presence of HPMC (HPMC-LV and HPMC-HV), at a concentration of 1 mg/ml, produced an enhancement in the adhesion of five strains (CD2, FV2, FV6, FV8 and FV9), including either highly intermediate or poorly adhesive microorganisms. On the other hand, the same

Table 4
Adhesion of different strains of lactobacilli to HeLa cells[a]

Strains[b]	% Cells with adherent bacteria	Mean number of adherent bacteria/ infected cell	Mean number of adherent bacteria/ cell
CD2	94.8	28.5	27.0
FV2	28.5	5.3	1.5
FV3	19.3	7.3	1.4
FV4	32.2	7.1	2.3
FV5	93.9	25.2	23.7
FV6	72.0	11.8	8.5
FV7	37.4	6.4	2.4
FV8	90.5	23.2	20.9
FV9	83.7	22.2	18.6
FV10	59.1	9.6	5.7

[a] Quantitative bacterial adhesion was analyzed on 500 randomly chosen cells, in three independent experiments, conducted in duplicate.
[b] Lactobacilli were used at a concentration of 5×10^9 bacteria/ml.

Table 5
Effect of HPMC[a] on lactobacilli adhesion to HeLa cells[b]

Strains	% Cells with adherent bacteria	Mean number of adherent bacteria/ infected cell	Mean number of adherent bacteria/cell
CD2	96.1	28.8	27.7
+ HPMC-LV	98.6	42.5	41.9
+ HPMC-HV	92.0	30.0	27.6
FV2	28.1	4.1	1.1
+ HPMC-LV	33.2	5.2	1.7
+ HPMC-HV	32.9	8.2	2.7
FV3	16.3	7.7	1.2
+ HPMC-LV	19.6	5.9	1.1
+ HPMC-HV	22.9	4.0	0.9
FV4	30.5	7.0	2.1
+ HPMC-LV	11.2	2.5	0.3
+ HPMC-HV	19.5	8.7	1.7
FV5	97.0	22.5	21.8
+ HPMC-LV	95.3	26.2	24.9
+ HPMC-HV	84.8	14.1	11.9
FV6	73.2	10.4	7.6
+ HPMC-LV	92.8	13.9	12.9
+ HPMC-HV	72.8	14.4	10.5
FV7	39.8	6.5	2.6
+ HPMC-LV	20.1	5.1	1.0
+ HPMC-HV	27.0	2.7	0.7
FV8	88.0	22.5	19.8
+ HPMC-LV	88.6	33.7	29.8
+ HPMC-HV	98.8	27.9	27.6
FV9	85.4	23.2	19.8
+ HPMC-LV	98.4	32.1	31.6
+ HPMC-HV	91.0	27.3	24.8
FV10	59.5	10.0	5.9
+ HPMC-LV	42.2	6.9	2.9
+ HPMC-HV	16.8	4.5	0.7

[a] The polymers, HPMC-LV and HPMC-HV, at a concentration of 1 mg/ml, were present during the adhesion step.
[b] See footnotes to Table 4.

compounds caused a consistent reduction of FV7 and FV10 adhesiveness, while FV3 adhesion was not affected by either molecule.

CP is one of the bioadhesive compounds mostly employed to confer adhesive properties to vaginal tablets. The effect of the addition of CP (0.3 mg/ml) to the adhesion medium is reported in Table 6. Unexpectedly, the presence of this polymer produced a great reduction in the adhesion of all lactobacilli strains, with the exception of FV2 (slight enhancement) and FV3, which was unaffected. The reduction concerned either the percentage of cells with adherent bacteria or the number of adherent bacteria/infected cell.

3.5. Strain selection and evaluation of the final batch production

HPMC-LV was the retarding polymer selected for the final formulation of the slow-release layer because it is able to enhance the adhesion of lactobacilli to the cell surface, and, at the same time, can suitably control the dissolution rate of the slow-release layer.

On the basis of viability and adhesive properties (Tables 3–6), the three strains, CD2, FV2 and FV9, were chosen as components of the mixtures to be tested for the final formulation production.

A larger batch of single- and double-layer tablets was then prepared, starting from a mixture of freeze-dried CD2, FV2 and FV9. The mixture was processable, and tabletting was easy and reproducible.

Fig. 2 shows the survival of the three strains, CD2, FV2 and FV9, in the freeze-dried powder and in the slow- and

Table 6
Effect of CP[a] on lactobacilli adhesion to HeLa cells[b]

Strains	% Cells with adherent bacteria	Mean number of adherent bacteria/ infected cell	Mean number of adherent bacteria/cell
CD2	96.1	28.8	27.7
+ CP	64.5	7.0	4.5
FV2	28.1	4.1	1.1
+ CP	26.5	7.0	1.8
FV3	16.3	7.7	1.2
+ CP	29.5	5.5	1.6
FV4	35.7	5.9	2.1
+ CP	23.4	6.2	1.4
FV5	96.0	19.9	19.1
+ CP	57.7	8.8	5.1
FV6	69.8	12.5	8.7
+ CP	31.2	6.2	1.9
FV7	35.2	6.0	2.1
+ CP	9.7	5.7	0.5
FV8	87.0	21.0	18.3
+ CP	32.3	6.5	2.1
FV9	85.4	23.2	19.8
+ CP	59.3	6.1	3.6
FV10	64.6	11.0	7.1
+ CP	40.6	5.5	2.2

[a] CP was added to the adhesion medium at a concentration of 0.3 mg/ml.
[b] See footnotes to Table 4.

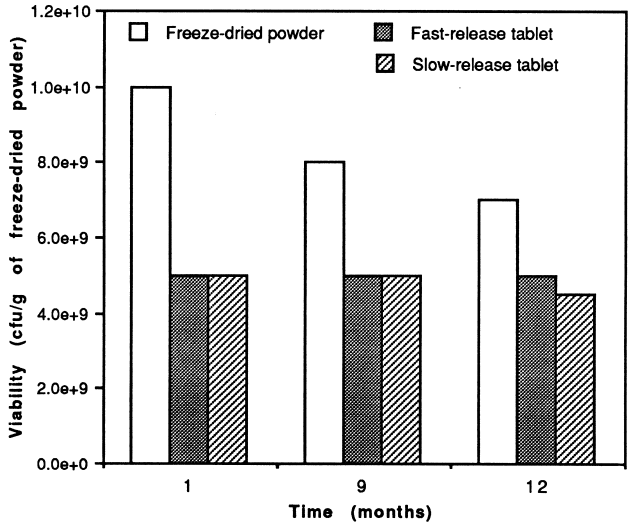

Fig. 2. Strain viability of the CD2 + FV2 + FV9 freeze-dried mixture and fast- or slow-release tablets during 1 year storage.

fast-releasing single-layer tablets. The results show that, starting from a freeze-dried powder that contains about 10^{10} cells/g, the mixture remained highly viable even after 1 year of storage at 4°C. Both fast- and slow-release tablets presented a very stable viability, lower than the freeze-dried powder, during the experiment period. This behaviour is probably due to the loss of viability of the cells present on the surface of the tablets which are in close contact with the outer environment, and consequently, anaerobic conditions are not maintained, whilst inside the tablet, the bacteria are probably better protected from contact with oxygen and their viability can be maintained for longer.

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