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A novel double-layer mucoadhesive tablet containing probiotic strain for vaginal administration: Design, development and technological evaluation

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ABSTRACT

Vulvovaginal candidosis caused by *Candida* spp. is the most prevalent vaginal infection in Europe and the second one in EE.UU, so it has become a major female concern. Probiotics bacteria have been proposed as an alternative treatment with the aim of avoiding the adverse effects associated with conventional therapies including antibiotics and other aggressive drugs for the vaginal mucosa and microbiota. The purpose of this work was to design and develop a novel vaginal tablet that contained *Lactobacillus* spp. bacteria as a treatment against vulvovaginal infections. A total of 21 two-layers vaginal tablets, which contained different polymeric ratios, were proposed. However, formulation F4 (20 mg Na-CMC; 50 mg Carbopol® 934; 20 mg chitosan) was selected as optimal according to its swelling index and dissolution/erosion capability. F4 tablets showed suitable technological properties for vaginal administration as well as mucoadhesion time (24.36 ± 0.88 h) and force (0.0941 N). Disintegration assay in simulated vaginal fluid (SVF, pH 5.5) showed that effervescent layer disappeared in 27.48 ± 0.05 s whilst matrix layer was totally gelled in 1 h. Two different release profiles were achieved; on the one hand, a promptly release due to the dissolution of both effervescent layer and matrix layer's surface (1.10×10^8 CFU/g), on the second hand, a prolonged released of the remaining bacteria until 24 h (5.48×10^7 CFU/g). For stability and storage study, it was found that bacteria viability was constant until 90 days in both ways of storage, in a desiccator and at room temperature, with a final dosage of 10^8 CFU/g which was considered appropriate for vaginal therapy (10^8–10^{10} CFU/g).

1. Introduction

Vulvovaginal infections are so prevalent that they represent about 20% of women reason to seek medical assistance and gynecological consultation according to the Spanish Society of Obstetrics and Gynecology (Cancelo et al., 2013). They are a consequence of an imbalance in the vaginal ecosystem, caused by different exogenous or endogenous factors that affect the indigenous microbiota and produce a decrease in protective lactobacilli. Lactobacilli are the most numerous dominant microorganism, at 10^7 to 10^8 CFU of vaginal fluid in healthy premenopausal women, they are called Döderlein's bacilli or complex (Döderlein, 1982) They have the function of maintaining an environment that limits the growth of pathogenic microorganisms by the production of acids, hydrogen peroxide, bacteriocins and modulating local immunological response (IL-8, IL-10). The commonest vaginal infections are caused by bacteria, typically *Gardnerella vaginalis* (bacterial vaginosis –BV–), or by fungi such us *Candida* spp. (vulvovaginal candidosis –VVC–). About 75% of women experience an episode of vulvovaginal candidosis once in their lifetime and a 40–50% of them usually suffer a second episode. It is the most prevalent vaginal infection in Europe and the second one in EE.UU. Although they are not associated with high mortality, their symptoms are related with anxiety and depressive mood, even affect to sexual relationships and quality of life (Palmeira-de-Oliveira et al., 2015). In accordance with the guidelines of the Spanish Society of Obstetrics and Gynecology and the American College of Obstetricians and Gynecologists (ACOG, 1996) the usual therapy for BV is based on oral or intravaginal metronidazole or clindamycin whilst VVC is commonly treated with topic and systemic azoles or nystatin. Conventional therapies can produce adverse effects, emergence of drug-resistant strains and recurrence of symptoms. To minimize these undesirable effects it is very important try to develop different alternative treatments. With this aim, the use of specific probiotic products containing lactobacilli is the first choice to restore the physiological balance of vaginal ecosystem.

Probiotic bacteria are defined 'live microorganisms which, administered in adequate amounts, confer a beneficial physiological effect on the host' (FAO/WHO, 2001). Numerous authors have previously reported the beneficial effects that several probiotic lactobacilli strains

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have shown on BV and VVC (Abad and Safdar, 2009; Barrons and Tassone, 2008; Ehrström et al., 2010; Falagas et al., 2006; Kovachev and Vatcheva-Dobrevska, 2015; Petricevic et al., 2008; Pirotta et al., 2004). Up to date, probiotic formulations have been tested orally and locally, however the intravaginal route so far appears to be the first choice for probiotic administration. Some of its advantages are: a reduction in the severity of gastrointestinal enzymatic and acid effects, it overcomes the pain and tissue damage by other parenteral routes and it offers the possibility of self-insertion and removal of de dosage form (Vermani and Garg, 2000). These advantages allow a lower dosage, a less frequent administration and a high absorption of the bacteria not affected by gastrointestinal drawbacks. The dosage form and strategy adopted to deliver probiotic to the vaginal environment represent a detrimental point. The main technological challenge is to maintain the viability of lactobacilli until the end of their shelf life since probiotic bacteria should be delivered on the target side in a viable form. It is well known that moisture has a negative effect on bacteria survival; so vaginal probiotics have been traditionally commercialized in form of tablets, capsules, powders and suppositories/pessaries because of their low moisture contents (Nader-Macias et al., 2008; Pande et al., 2012). Tablets are comparatively more advantageous than other dosage forms: precise dosing, ease of storage, handling and administration as well as low costs thanks to large-scale production are some of their advantages. Furthermore, tablets can improve stability of bacteria at extremes of temperature and humidity (Garga et al., 2010). An assay carried out in India to study the acceptability of different vaginal formulations, tablets acceptability score was 4.35 out of 5, thus 95% of the women reported that the product was easy to use and did not affect sexual pleasure (Joglekar et al., 2006).

Currently, two categories of tablets for vaginal application are described in literature: fast disintegrating tablets and controlled release systems.

These concepts of drug delivery systems can be used in conjunction to design tablets which could both be dispersed rapidly and have a longer residence time in the vaginal cavity. On the one hand, disintegrating tablets usually contain an effervescent mixture, which allow getting dispersion or a gel in a quite short of time. On the other hand, controlled release tablets or matrix tablets formulation includes retarding polymers, which are able to hydrate and gel very slowly leading to a prolonged release of bacteria. It must be taken in account mucoadhesive properties of these polymeric excipients in order to get longer residence times. With this purpose, polyacrylic acid derivatives (carbomer), cellulose derivatives and chitosan are one of the most common retarding and mucoadhesive polymers preferred (Valenta, 2005). In addition, it has been reported an intrinsic antifungal and antibacterial activity of chitosan (Kim et al., 2003; Palmeira-de-Oliveira et al., 2010) as well as the acid-buffering capacity of Carbopol® that allow the correction and maintenance of the vaginal pH in BV (Donders et al., 2014; Wilson, 2004). Similar beneficial effects have also been reported on ascorbic acid (Krasnopolsky et al., 2013; Petersen and Magnani, 2004; Zodzika et al., 2013) and lactose (Reid et al., 1998), which have showed to be an effective treatment and prevention of recurrences in vaginal infections. Thus, different excipient could be added to vaginal dosage forms in order to enhance lactobacilli beneficial properties.

The objective of this work was to develop a vaginal tablet that contains probiotic lactobacilli bacteria previously microencapsulated and freeze-dried. As far as we are aware of are no studies available on bi-layered vaginal tablets with microencapsulated probiotic bacteria obtained *via* emulsification/internal gelation. This tablet was divided into two layers: a fast- and slow-release layers. Both of them contain the same number of lactobacilli bacteria. The purpose of the first layer was to disperse the bacteria before 5 min after its contacts with vagina fluid whilst the second one should release lactobacilli by matrix diffusion as long as possible. Technological properties, vaginal behaviour as well as storage viability of tablets have been tested.

2. Materials and Methods

2.1. Materials

Lactobacillus cells were kindly provided by Biotmicrogen S.L. (Granada). Lactose monohydrate, maize starch, ascorbic acid, stearic acid, sodiumcarboxymethyl-cellulose (Na-CMC 1500–4500), sodium citrate dehydrate, glucose anhydrous, talc, magnesium stearate, sodium chloride (NaCl) and urea were purchased from Guinama S.L.U. (Valencia, Spain). Adipic acid, sodium bicarbonate, Chitosan medium molecular weight, acetic acid, Carbopol® 934 and Bovine Serum Albumin were supplied by Sigma-Aldrich Co. (MO, USA). Potassium hydroxide (KOH), calcium hydroxide Ca $(OH)_2$, glycerol were obtained from Fagron Ibérica S.A. (Barcelona, Spain). Lactic acid was purchased from Roig Farma S. A. (Terrasa, Spain).

2.2. Microencapsulation and Freeze-Dry Methods

Lactobacillus spp. *was* encapsulated into alginate microparticles by a modified emulsification/internal gelation technique, which was previously reported by our research group (Sánchez et al., 2017). Microparticles were then freeze-dried to assure bacteria survival during its storage.

2.3. Tablets Design and Preparation

The formulation designed for a fast-release of the bacteria contains a granulated base and an effervescent mixture to accelerate layer disintegration. The granulated base was prepared by mixing 74% (*w*/w) lactose, 11% (w/w) adipic acid and 15% (w/w) total maize starch. Previously, 2% (w/w) of the maize starch was taken apart to prepare a 10% (m/v) paste that was used to wet the granulate mixture. This wetted mass was forced though 710 μm mesh screen. Granules were dried in an oven at 37° C until a constant weight. Once granules have dried, they were forced though 1000 μm mesh screen to make any agglomeration disappear. Sodium bicarbonate was added to enhance the effervescent quality.

Slow-release formulation contains retarding polymers, which assure a controlled bacteria release.

Tablets were prepared by using a manual hydraulic press (SPECAC SC-15011, Spain) with a 13 mm diameter die on a single-punch and a compression force of 1 tons for 30 s.

Single-layer tablets were designed for slow- and fast-release formulation to study them on separately. Then a double-layer tablet was produced as final dosage form by layering in sequence both formulations (slow-, fast-release) in press matrix before compression.

2.4. Technological Physical Characterization

Vaginal tablets were studied technologically in terms of diameter, thickness, mass uniformity and crushing strength.

Mass uniformity and crushing strength were analysed according to United States Pharmacopeia (USP 29). Mass uniformity was evaluated using a precision balance (A&D Europe GmbH, Germany), data is reported as an average of 20 measurements ± SD. Crushing strength was measured using a hardness tester (PharmTest, Germany), data shown is an average of 10 measurements ± SD.

2.5. Swelling Study

In order to evaluate swelling capacity, expressed as hydration percentage, single-layer tablets constituted by matrix polymers were weighted (W_1) and placed into a pre-weighed stainless steel basket with 200-mesh aperture. Then, mesh containing each tablet was submerged into 25 mL simulated vaginal fluid (SVF) at 37 ± 0.1 °C in a glass beaker allowing tablets to swell. Baskets were removed from beakers

and reweighed at predetermined times (W_2) after removing the excess water with a filter paper.

The experiment was carried out in triplicate and the final data for swelling index (%) and matrix dissolution (DS) were calculated as follows:

$$\text{Swelling}\ (\%) = (W_2 - W_1) \times 100/W_2.$$

$DS = (W_1 - W_3) \times 100/W_1$.where, W3 is the swollen tablet weighed after drying at 60° C for 24 h in an oven and then 24 h in a desiccator.

Simulated vaginal fluid was prepared as described Owen and Katz (Owen and Katz, 1999): 3.5 g/L NaCl; 1.4 g/L KOH; 0.2 g/L $Ca(OH)_2$; 2.0 g/L lactic acid; 1.0 g/L acetic acid; 0.2 g/L glycerol; 0.4 g/L urea; 5.0 g/L glucose; 0.02 g/L bovine serum albumin. The mixture was adjusted to pH 5.5.

2.6. In vitro Mucoadhesion Study: Mucoadhesion Force and Residence Time

2.6.1. In vitro Mucoadhesion Force

Porcine vaginal tissues were obtained from a local slaughterhouse immediately after sacrifice of animals. They were cleaned with phosphate buffer solution (PBS, pH 6.8), taking care to maintain integrity of mucosa, and frozen at − 20° C until their use.

Tablets' mucoadhesive strength (force required for tablets detachment from pig vaginal mucosa) was measured by using a dynamometer (Perioli and Pagano, 2013).

Vaginal tissues were thawed, washed with PBS and cut into pieces (2 cm × 2 cm). Vagina pieces were fixed with cyanoacrylate glue to the internal side of a beaker which contained SVF pH 5.5 to keep mucosal membrane moist. Tablets were attached on a support, connected to the dynamometer, by the use of cyanoacrylate glue. Then, the free side of the tablets were wetted with SVF and brought into contact by their matrix layer with the vaginal mucosa surface by applying a fingertip force for 20 s. A time of 2 min was required to allow formation of adhesive bond. Dynamometer measurements showed the bioadhesive strength of vaginal tablets in grams (g). Bioadhesive strength, force of adhesion (N) as well as the averages of three measurements was calculated.

$$\text{Adhesion Force (N)} = \text{bioadhesive strength (g)} \times 0.0098.$$

2.6.2. Residence Time

In vitro residence time was determined by using a locally modified USP 29 disintegration apparatus. Disintegration medium was 600 ml of SVF pH 5.5 at 37 ± 0.1 °C. Pig vagina was tied vertically to the disintegration apparatus. Tablets were wetted with SVF and brought in contact with vagina mucosa. The disintegration apparatus was started allowing mucosa moving down and up, so tablets were completely immersed in SVF and then were out respectively (Fig. 4). Tablets behaviour was monitored until their complete detachment (Biswal et al., 2014).

2.7. Vaginal Dosage Forms Disintegration

Disintegration assay was carried out according to Real Farmacopea Española (RFE) (5ª ed.) in a properly modified suppositories and ovules disintegration apparatus (Pharmatest, Germany). Double-layer tablets were studied in order to determined time of effervescency and time required for tablets disintegration.

The experiment consisted of placing tablets on a perforated plate that allowed their contact with the medium at 37 ± 0.1 °C and covered them in the top by a glass plate. The tablets will be found inside a “chamber” that would keep the moisture.

2.8. In vitro Lactobacillus Release Study

The release of *Lactobacillus* bacteria from vaginal tablet was studied using the USP 29 dissolution apparatus II with a paddle speed of 100 rpm. Dissolution medium consisted of 600 ml SVF (pH 5.5) at 37 ± 0.1 °C. *Lactobacillus* cells released along 8 h was determined by microbiological cultures in MRS Agar plates at established times (2 h, 4 h, 6 h and 8 h). Bacterial colonies were counted and converted to log CFU (colony forming units). This plating procedure was carried out in triplicates.

2.9. Storage and Viability Assay

Probiotic vaginal tablets were stored both in hermetic containers and in a desiccator at room temperature (RT) for 90 days. Silica gel was used as drying agent to preserve bacteria from humidity. Viability of cells inside tablets was evaluated along that period of time by microbiological cultures.

3. Results and Discussion

3.1. Tablets Pharmaceutical Formulation

With the aim of incorporating lyophilized microparticles into vaginal solid dosage forms, single- and double-layer tablets were formulated. The composition of fast- and slow-release layer of vaginal tablets is set forth in Table 1.

As we can see in Table 1, we studied 4 formulations of slow-release layer which were selected from a total of 21 formulations (data not shown) based on technological properties required for vaginal administration. It contains the mucoadhesive polymers (chitosan, Carbopol® 934, Na-CMC) in different proportions. Furthermore, chitosan had another important role in formulation due to its intrinsic antifungal and antibacterial activity against some of the most prevalent vaginal pathogens as *Candida* spp. (Rabea et al., 2003; Seyfarth et al., 2008). Along with chitosan, Vitamin C (ascorbic acid) also plays an important role since an amount of 250 mg of ascorbic acid contained in that formulation, is related to a reduction of pathogens bacteria and an increase of *Lactobacillus* cells (Petersen and Magnani, 2004). Other substances as lactose could be defined as prebiotics since it has the ability to promote growth of protective *Lactobacillus* microorganism (Reid et al., 1998).

Fast-release layer composition was the same for each formulations studied. Double-layer tablets were obtained for formulations 1 to 4, each one with a final weight of 1000 mg.

3.2. Swelling study

Because of the presence of swellable bioadhesive polymers in formulations, it was necessary to perform hydration studies in order to know tablets swelling capability, which is directly involved in mucoadhesion mechanism. Result for water-absorbing capacity of each formulation with the time is showed in Fig. 1. Besides Fig. 2 shown DS % tablets' matrix dissolution.

Swelling capacity of tablets and matrix dissolution made easy to select the formulation that presents an optimum technological behaviour for a vaginal administration. As selection criteria it was established that an ideal formulation should not lead to the detachment of small fragments of tablets whilst it should form an aqueous gel, which would have to disappear over time. Furthermore, its swelling capacity should be adequate to a comfortable stay in the vaginal cavity and to allow its mucoadhesion. It is necessary to mention here that once the optimum hydration point has been reached, an overhydrating leads to a decrease in the adhesion force due to disentanglement at the polymer/tissue interface.

In this assay it became apparent how chitosan/Carbopol® ratio is related to tablets' swelling property leading to the formation of a spongy matrix or a fluid gel. F1 and F2 with chitosan/Carbopol® ratio of 1/1 and 1/1.6 respectively showed an increase in volume as the

Table 1
Tablet formulation (1–4) based on each slow- and fast-release layer composition.

	Formulation (mg)			
Slow- release layer	**1**	**2**	**3**	**4**
Lyophilized *Lactobacillus spp* microparticles	22	22	22	22
Ascorbic acid	125	125	125	125
Lactose	255	255	255	255
Na- CMC	30	25	40	20
Carbopol® 934	30	40	40	50
Chitosan	30	25	10	20
Talc	12	12	12	12
Magnesium stearate	12	12	12	12
Fast- release layer	**1**	**2**	**3**	**4**
Lyophilized *Lactobacillus spp* microparticles	22	22	22	22
Granulated base	300	300	300	300
Sodium bicarbonate	30	30	30	30
Ascorbic acid	125	125	125	125
Stearic acid	2	2	2	2
Magnesium stearate	5	5	5	5
Weight of double- layer tablet	1000			

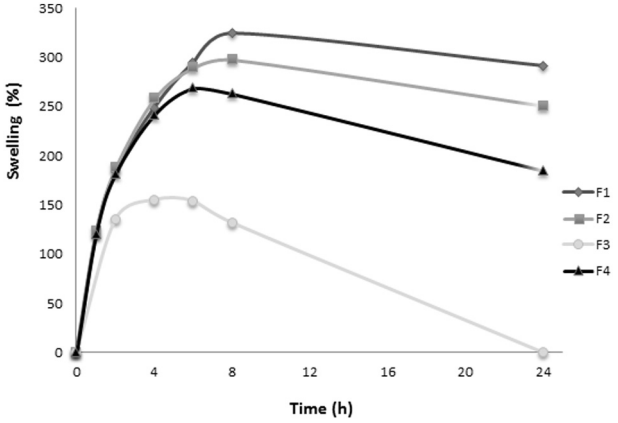

Fig. 1. Swelling index (%) of Formulations F1 to F4 (SVF, 37 °C, 24 h).

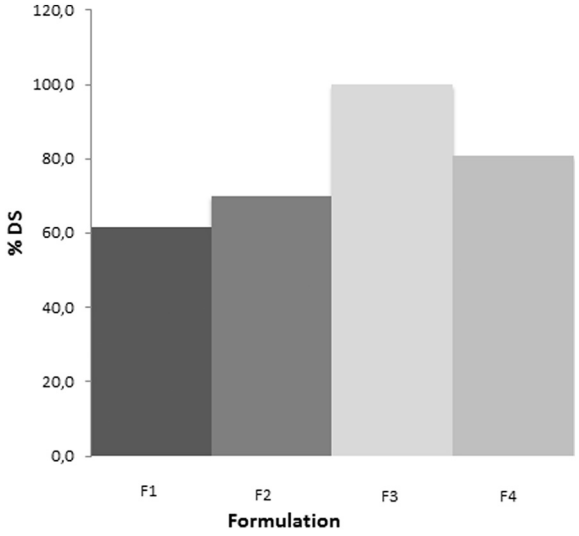

Fig. 2. Matrix dissolution/erosion (%) of Formulations F1 to F4.

hydration increased in the first 8 h of the assay, reaching there the maximum swelling and remaining until 24 h. Similar results were founded by Rossi et al. (2003), who reported that these ratios close to the chitosan/Carbopol® interaction product stoichiometry would present poor solubility properties. Furthermore, it is important to highlight the role of Carbopol® due to its high sensibility to the pH of the medium. According to other authors (Perioli et al., 2011), it was recognized that tablets hydration and erosion ability was also related and proportionally to Carbopol® content. So, as the amount of Carbopol® increased from F1 to F4, tablets hydration took place faster (Fig. 3) consequently tablets cannot keep further water and easily loses fragments because of hydrodynamic pressure effect exerted on the polymer network by water. Taking into account the selection requirements, F3 was discarded because its high rate of dissolution/erosion (Fig. 2) which was not enough to guarantee its mucoadhesion for an extended time. On the contrary, F1 and F2 were rejected because of their large increase in volume and space required. All in all, F4 was selected as ideal to following studies.

3.3. Technological Physical Characterization

Tablets F4 were technologically evaluated for diameter, thickness, mass uniformity and crushing strength. Results are given in Table 2.

In order to promote patient compliance, diameter and thickness of vaginal tablets were taken in special care and measured. Results showed in Table 2 allowed to hypothesize that this formulation could be easily inserted into the vagina without the need for an additional applicator, which facilitates patient. Furthermore, it would not cause pressure on vaginal walls or discomfort to the patient since vagina presents approximately 7–10 cm in length and 4 cm in width.

Tablets were submitted to hardness test because taking in account the application site, the formulation must show compactness and hardness to resist mechanical stress. As final result tablets had a hardness of 23.3 N ± 4.42, which allowed them a high resistance during their handling throughout this research work. According to USP29 mass uniformity test, 20 tablets were weighted and mean were calculated 0.99 ± 0.01 g; any tablet weighted showed a deviation greater than 5% of the mean. Therefore, we could affirm that our selected formulation meets all the technological requirements for vaginal tablets.

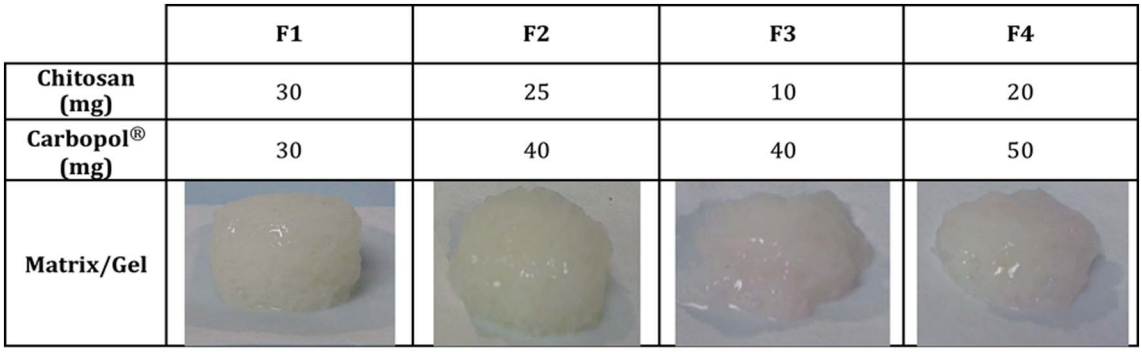

Fig. 3. Influence of chitosan and Carbopol® on tablets' behaviour. Images taken at 8 h of swelling study.

Table 2
Tablets technological evaluation.

	Diameter (mm) $n = 10$	Thickness (mm) $n = 10$	Weight variation (g) $n = 20$	Crushing Strength force (Newton, N) $n = 10$
F4	13.10 ± 0.06	5.94 ± 0.02	0.99 ± 0.01	23.3 ± 4.42

Values are expressed as mean ± SD.

3.4. Mucoadhesion Study: Mucoadhesion Force and Residence Time

Tablets' mucoadhesion is a very important aspect for maintaining high *Lactobacillus* levels at the site of administration and prevents from formulation expulsion.

Mucoadhesion force test was performed on triplicate and three tablets randomly selected were submitted to study each time, so a total of nine tablets were studied. Results of mucoadhesion strength and force of adhesion are expressed as the mean of three tablets measurements for each test realized (Table 3). In order to check whether the adhesion force achieved ensured the permanence of tablets inside the vagina until the complete release of the probiotic load, time of mucoadhesion was also studied; the assay was carried out with three different tablets and results were expressed as hours of adhesion (*h*) for each tablet individually and as mean of the three measurement (Table 3). Fig. 4 shows a schematic representation of the assay developed.

As can be seen in Table 3, similar results were obtained in the adhesion force test for each tablet studied. The second tablet tested showed a slightly lower adhesion force, this fact could be related to the state of the vaginal mucosa used since, in this case, it presented a more uniform and smooth surface. These results obtained did not differ much from those reported by Biswal et al. (2014) with a maximum of 0.0754 N.

With regard to the adhesion time assay, a mean of 24.36 ± 0.88 h was achieved. This result suggested that this dosage forms could be administrated once daily, it supposed an advantage and therapeutic compliance by patients which was our main goal.

F4 tablets included a high Carbopol® 934 content and it is well known that this polymer has good mucoadhesion properties (Baloglu et al., 2003; Valenta, 2005; Wang and Tang, 2008). Its presence in formulation provided higher bioadhesive strength. This may be due to fact that carboxylic groups on surface of Carbopol® 934 could lead to form hydrogen bonds with mucin chains. Even there are some authors like Perioli et al. (2011) came to affirm that Carbopol®, included together with cellulose derivatives was the main responsible for mucosa adhesion. By the way, it has been reported that those formulation containing Na-CMC as a secondary polymer increased force of mucoadhesion. This could be attributed to the hydrophilic character of cellulose derivatives which favors water penetration into matrix promoting the relaxation of Carbopol® chains and increasing the availability of ionizable functional groups for mucoadhesion (Iman and Nidhal, 2014). Moreover, chitosan has also showed a surprisingly strong interaction with mucus glycoprotein or mucosal surface proportional to its concentration in formulation. Even, Rossi et al. (2003) demonstrated the importance of avoiding a chitosan/Carbopol® ratio close to 1/1 since this supposes the complete neutralization of charges involved in mucoadhesion.

Table 3
Mucoadhesion strength, force and time.

Test number	Mucoadhesion strength (g)*	Force of adhesion (N)	Mucoadhesion time (*h*)	Mucoadhesion time (*h*)*
1	9.60 ± 0.12	0.0941	23.20	24.36 ± 0.88
2	7.80 ± 0.23	0.0764	24.36	
3	9.60 ± 0.31	0.0941	24.92	

* ($n = 3$; values are expressed as mean ± SD).

It is important taking on account the negative effect that effervescence could have on mucoadhesion. The influence of effervescent on adhesion has been previously reported by different authors who related the appearance of tiny bubbles during the effervescence with a decrease of the adhesion force (Wang and Tang, 2008). This negative effect was accentuated as the effervescent mixture content increased.

So, we performed a previous study in order to select that amount of optimum effervescent mixture included in the fast-release layer of the tablet (data not shown) that would allow the dissolution of this layer before 5 min without affecting tablets' mucoadhesion properties.

All in all, we could affirm that the obtained adhesion force was optimal to ensure the permanence of the tablets inside the vagina until the total release of the probiotic load, mainly thanks to the contribution of each of the polymers included in F4.

3.5. Disintegration

Double-layer tablets of F4 were submitted to disintegration assay, results are shown in Fig. 5. As expected, the disintegration time of the fast-release layer was less than 5 min; exactly it was 27.48 ± 0.05 s (s).

After 30 min, it was possible to see the formation of a gel on the surface of the matrix tablets. It was possible to notified two different parts in these tablets: an external gelled layer and a non-hydrated inner zone containing polymers in a glassy state. When matrix tablets were placed in contact with the medium, it was created a diffusion/erosion front that made possible the release of the lyophilized bacteria to the medium.

As the test progressed, we could observe how this compact gelled layer was dissolved gradually in medium without evident fragment loss.

Finally, after 1 h, medium penetration was evident. As can be seen in Fig. 5D, tablets were pierced with a punch in the central zone in order to verify that there was no inner core and the whole tablets were

Fig. 4. Experimental device used to evaluate residence time (600 mL SVF pH 5.5 at 37 ± 0.1 °C).

in a gelled state. At this point, the tablets were completely gelled and had lost their original shape.

3.6. In vitro Lactobacillus Release Study

The release of *Lactobacillus* cells from vaginal tablet was described as a function of time (Fig. 6). Viability of initial tablets was 1.65×10^8 CFU/g considering *Lactobacillus* bacteria included in both tablets' layers. Analysing results obtained, at 2 h in SVF it had been released a total amount of 1.10×10^8 CFU/g. This was a result of the total release of probiotic bacteria contained in fast-release layer, which disintegrated in few seconds as we have seen in disintegration test, and a large amount of probiotic content of the slow-release layer. It still remained in tablets approximately 5.48×10^7 CFU/g, which would be released slowly in small amounts. At 8 h, it had been released 1.49×10^8 CFU/g. The release of these bacteria would continue progressively until 24 h, when we found residues of a gel completely depleted. Analysing results obtained, we could verify a prompt release at 2 h and then a sustained release until 24 h reaching our proposed objectives. Borges et al. (2013) reported similar results for vaginal tablets with *Pediococcus pentosaceus* SB83, with a maximum release of 10^8 CFU/g that occurred in the early hour of contact with SVF.

Bi-layered tablets without probiotic bacteria were used as negative control in order to discard bacterial growth due to contamination of the medium. Furthermore, as positive control, freeze-dried bacteria were studied, showing no viability after the first hour in SVF (data not shown). This result allowed us to corroborate the protection granted by the designed tablet.

Although Carbopol® has many advantages as good mucoadhesion properties and high gelling ability; it has a disadvantage due to its high sensibility to the pH of the medium as it has been announced above. This fact supposed a difficulty to control *Lactobacillus* bacteria release and to assure an extended-release from tablets and was taken into account to design and select the formulations under study, so we considered the formation of an interpolymer complex (IPC) formed by the interaction of Carbopol® and chitosan. This IPC solved the problem of the pH dependency of Carbopol® because of its carboxyl group are complexed with protonated amine group of chitosan, consequently there is a reduction on pH-dependent drug release and it let us to get this controlled release.

Even, it should be taken into account that this bioadhesive formulation proposed in our research work could be used without a therapeutic agent to treatment dry vagina thanks to its moisturizing properties.

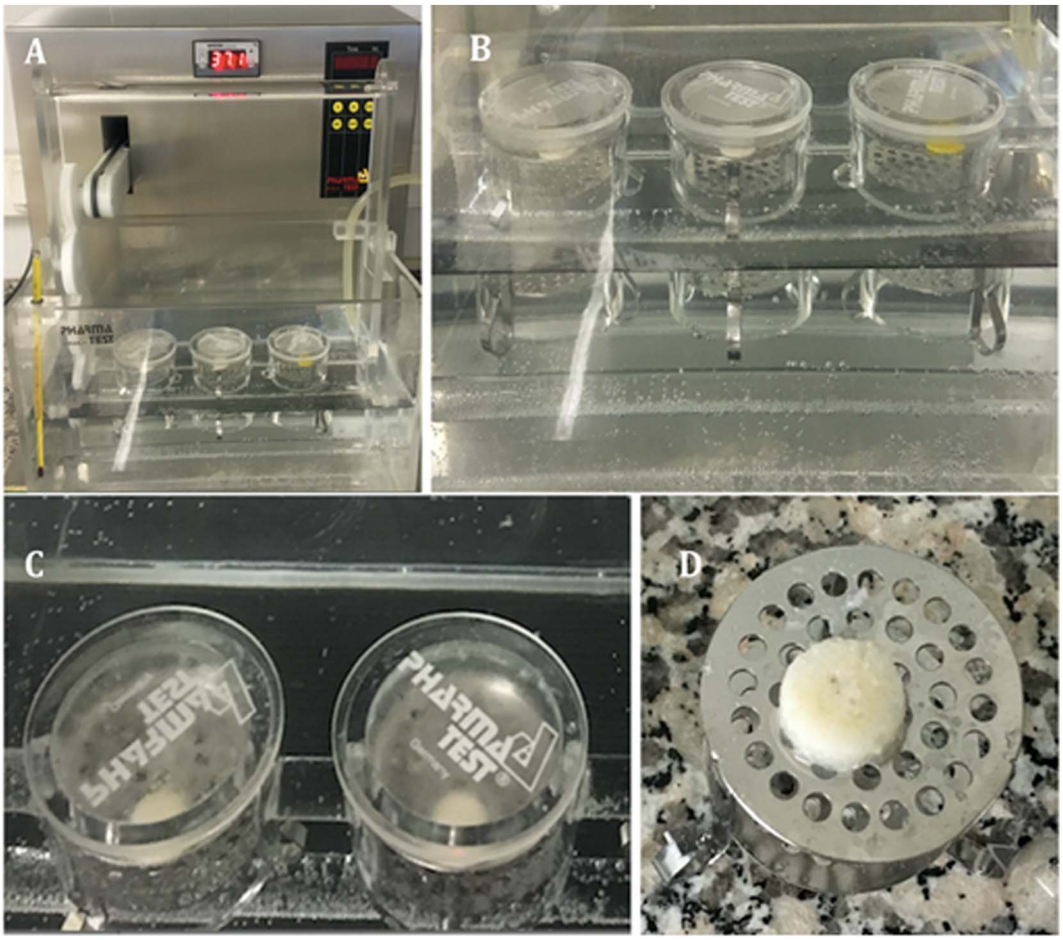

Fig. 5. Experimental device used to evaluate tablets' disintegration. (A) Suppositories and ovules disintegration apparatus. (B) Three tablets submitted to test inside each "chamber". (C) Glass plates that covered each tablet and prevented from medium evaporation. (D) Tablet at the end of the assay constituted by a soft matrix with marks on its surface of having been pierced by punch.

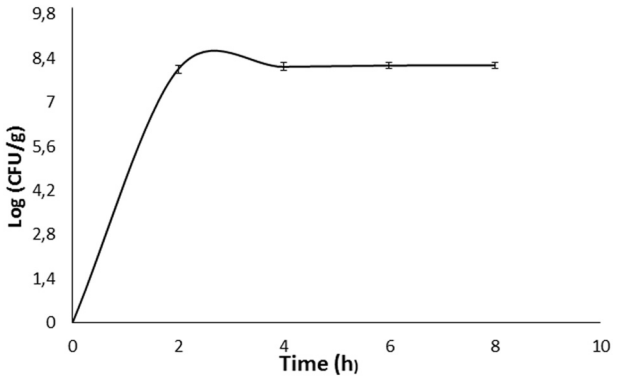

Fig. 6. Release profile of *Lactobacillus* bacteria from vaginal tablets tested in SVF pH 5.5 at 37 ± 0.1 °C for 8 h (n = 3, all points are expressed as mean ± SD).

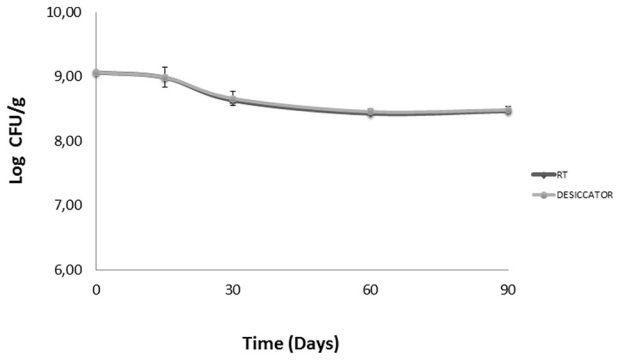

Fig. 7. Storage and viability assay for 90 days at Room Temperature (RT) and in a desicator. (n = 3, all points are expressed mean ± SD).

3.7. Storage and Viability Assay

The evolution of *Lactobacillus* viable cells in vaginal tablets throughout 90 days at RT and in a desiccator is displayed in Fig. 7.

Initial viability of the vaginal tablets containing the encapsulated bacteria was.

9.04 ± 0.02 log CFU/g. Similar results of bacteria viability were obtained from both storage conditions. Viability decreased barely 0.4 log units until 30 days of storage. At 60 days, viability of tablets at RT and in desiccator was 8.42 ± 0.01 log CFU/g and 8.43 ± 0.05 log CFU/g respectively. This bacteria viability remained stable from then on with a viability decrease of 0.62 log units over the period of storage tested (90 days). Despite the loss of 0.62 log units, vaginal tablets assured high survival rates throughout the 90 days of storage with viable numbers above 10^8 CFU/g which supposed an optimal probiotic quantity for vaginal therapy (10^8–10^{10} CFU/g) (Borges et al., 2013).

Lactobacillus microparticles incorporated into these vaginal tables, which were previously reported (Sánchez et al., 2017), showed no reduction in bacteria viability throughout the 150 days stability assay under the same conditions. However, encapsulated bacteria survival in acid medium was lower with a loss of 5 log units after 2 h in acid, hence

our aim of conditioning them into a secondary pharmaceutical form in order to avoid that inconvenience. If we analyze vaginal tablets results (Section 3.6), we can detect a decrease of only 1 log unit bacteria that was released to the medium after 2 h in acid, ensuring the achievement of our objective. In the same way, Stadler and Viernstein (2003) considered as optimal a bacteria decrease of 1 log unit after being immersed 2 h in acid environment.

4. Conclusions

In this research work, double-layers vaginal tablets that contain microencapsulated *Lactobacillus* spp. bacteria have been developed. Formulation F4 was selected as optimal for further studies. It becomes clear the importance of chitosan/Carbopol® 934 ratio in formulation, since hydration capability depended on it. F4 met both technological and mucoadhesion requirements of the vaginal dosage forms. Furthermore, disintegration assay showed that effervescent layer disappeared in 27.48 ± 0.05 s whilst matrix layer was totally gelled in 1 h. This gelled phase was homogeneous and solubilized in SVF progressively with no fragment loss. That fact together with long period of residence time contributed to a greater acceptance of the patient and greater therapeutic compliance. Dissolution study confirmed two different release profiles according to the proposed tablet design, a promptly bacteria released of the effervescent layer, and a prolonged release until 24 h associated to the matrix layer. Stability study revealed that bacteria viability was constant until 90 days in both ways of storage, in a desiccator and at RT, with a final dosage optimal for vaginal therapy (10^8–10^{10} CFU/g).

The overall results of this investigation suggested that this vaginal tablets designed had ideal technological and pharmaceutical properties, making them a novel dosage form for effective and convenient treatment of vaginal infections for improving women's health.

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