

Preparation and evaluation of Novel Vaginal Pessaries of Lactobacilli

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Abstract

The objective of this study was to develop Multifunctional vaginal Pessary containing Probiotic and Prebiotic. The novel Multifunctional Bilayer Pessary with Probiotic and Prebiotic in separate layers was developed using blends of PEGs as base. Solubility of Blend of PEG used for preparation of Prebiotic layer was greater than that used for Probiotic layer. That makes Prebiotic to release first and create favorable condition for growth of Probiotic that would be released subsequently. Prepared Multifunctional Bilayer Pessaries were evaluated for viability and stability. Also, plain and hollow type Pessary containing Probiotic alone were prepared and comparatively evaluated with Multifunctional Bilayer Pessary. The effect of Prebiotic on growth of Probiotic was studied for Bilayer Pessaries and results indicated that growth rate of Probiotic increases in presence of Prebiotic. The selected *lactobacillus sp.* was tested for membrane adherence and for production of antimicrobial substances. Result showed the ability of selected *lactobacillus sp.* for membrane adherence and production of antimicrobial substances like bacteriocins. The stability studies demonstrated that the hollow-type and Bilayer Pessaries with mixed PEGs as the base are stable after storage at 2-8°C over the period of 4 weeks.

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Introduction

Bacterial vaginosis and yeast vaginitis are affecting a large number of women and these ailments are major public health concerns. Generally within these patients the normal vaginal microflora is disturbed and the natural protection against infectious microorganisms is lost. The use of probiotic microorganisms to restore the normal vaginal flora is gaining interest as an alternative to conventional antibiotic treatment [1].

However, to efficiently deliver the probiotic to the vagina the probiotic must be formulated in a dosage form that maintains the viability of these

microorganisms during manufacturing and shelf life. In addition the carrier formulation of the probiotic must ensure that residence time and distribution of viable microorganisms is sufficient to colonize the vaginal epithelium and restore the microflora [2].

Nowadays, there are many products for vaginal delivery of lactobacillus for examples yogurt, acidophilus milk, powders and gels [4]. These products often have poor patient compliance for several reasons such as irritation, discomfort, and leakage at the application site. Therefore, a vaginal pessary containing probiotic would be more suitable. A vaginal pessary has some advantages such as dose uniformity can be maintained, insertion into the vagina without irritation is possible, and a large volume of dissolution fluid is not required for the release of active substance.

In this work organic acids were incorporated into Bilayer Pessaries formulation, which restores normal vaginal pH and creates favorable condition for growth of probiotic.

Another aim of this study was to evaluate the viability of the selected *lactobacilli sp* in function of organic acid as Prebiotic to maximize viability. Three types of vaginal Pessaries containing probiotic lactobacilli were prepared; a) Plain Pessaries with Probiotic alone, b) Hollow type of Pessaries with Probiotic alone and c) Bilayer Pessaries containing Probiotic (*lactobacillus sp.*) and Prebiotic (organic acid) in separate layer.

Material

The lyophilized *Lactobacillus sp.* as Probiotic was obtained as a gift from Uni Sankyo Ltd., Chiplun, India. Organic acid as Prebiotic was synthesized in our laboratory. DeMan–Rogosa–Sharpe (MRS) medium was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Polyethylene glycols (PEG600, PEG6000, and PEG1500) were purchased from S. D. Fine Chemicals, Mumbai, India. All other chemicals were analytical grade and, where required, bacteriological grade.

Methods

Preparation of Multifunctional Bilayer Pessaries

Two types of Pessary bases were prepared from blends of PEGs with different solubility. The Multifunctional Bilayer Pessaries as shown in Figure 1 were prepared using above bases as follows; first, a base mixture for the front layer with higher solubility was melted over the water bath. Then, appropriate quantity of organic acid as Prebiotic was added and mixed in molten mass. The mixture was poured into the mold, cooled, removed from the mold, and the top of 15 mm of the base was cut off and used. The front layer was put into the mold again and the second layer containing Probiotic was poured next to it at 45°C and cooled at room temperature [1, 2]. Prepared Pessaries were kept in the refrigerator for further studies.



Figure 1 Multifunctional Bilayer Pessaries

Preparation of Plain and Hollow type of Probiotic Pessaries

Plain and Hollow type of Pessaries containing Probiotic alone were prepared for comparative study with Multifunctional Bilayer Pessaries.

Plain Pessaries containing Probiotic alone were prepared by fusion method [3, 4]. Mixed PEG as the pessary base was melted over the water bath. Then, lyophilized Probiotic was added in the melted base at the temperature about 40-45°C with gentle stirring until a homogeneous mass was produced. The mixture was poured into a metal pessary mold at a

temperature just above the congealing point of the pessary base and cooled. Each pessary contained 10^8 CFU of Probiotic.

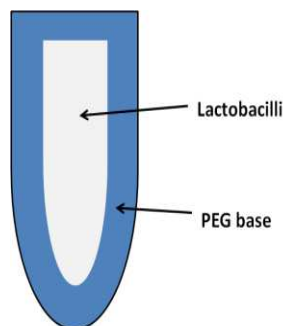


Figure 2 Schematic illustration of Hollow-type Pessary

Hollow type Pessaries, shown in Figure 2, were prepared by the method reported by Watanabe *et al*. [5, 6]. In brief, mixed PEGs base was melted at approximately 40°C , poured into the pessary mold equipped with cylindrical tube in the center of the mold and allowed to solidify for 1 hour at room temperature. After construction of a hollow cavity of the solidified base, the lyophilized Probiotic was added to each cavity. The opening at the hind part of the pessary was sealed with the melted base. Each pessary contained 10^8 CFU of Probiotic.

Evaluation of physical properties of prepared vaginal Pessaries

The prepared Pessaries were evaluated for uniformity of weight according to the BP 1988 and content uniformity. The mechanical strength test was carried out on a Monsanto hardness tester.

The disintegration test of vaginal Pessaries was modified from the method described by BP [7], by using tablet disintegrator. The Pessary to be tested was placed in a cylindrical glass container with perforated ends and immersed in 1,000 ml of citric acid/phosphate buffer solution pH 4.4 maintained at $37 \pm 0.5^\circ\text{C}$. The cylindrical glass container was moved up and down in the buffer. The time for disintegration was noted when the Pessary was completely dissolved in the medium. The mean

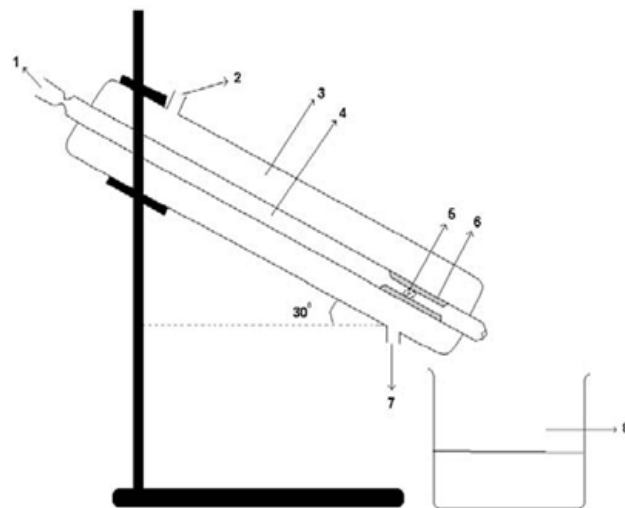
values were calculated from six parallel measurements.

Effect of Prebiotic on growth of Probiotic

Effect of Prebiotic on growth of Probiotic was determined for Multifunctional Bilayer Pessaries [8]. Multifunctional Pessary containing Probiotic and Prebiotic in separate layer was made to dissolve in 9 ml sterile nutrient broth media to release the bacteria and organic acids. The resulting media was then incubated for 48 hr at 37°C . A series of dilution was prepared from this grown culture and was plated out using MRS agar as growth medium. After 48 hr incubation at 37°C the colony forming units were counted and cfu/Pessaries were calculated from colony count. Similar experiment was performed on Plain Pessary containing Probiotic alone. Effect on growth was established by comparing viable count after incubation for Multifunctional Bilayer Pessaries and Plain Pessaries.

Membrane adherence test

Membrane adherence test was performed on prepared vaginal formulation using dissolution test apparatus (Figure 3) proposed by Vinita kale *et.al*.



- 1- Inlet for MRS medium (5 mL), 2- Inlet for warm water (37°C), 3- Outer tube (4 cm), 4- Inner Tube (1.5 cm), 5- Vaginal tablet, 6- Cellophane membrane,
- 7- Outlet for warm water, 8- Collecting vessel.

Figure 3: Design of a dissolution apparatus for vaginal formulations containing Probiotics.

The experimental set up of apparatus was done as shown in Figure 3.3. The vaginal physiology was simulated by means of a 1.3-cm diameter (inner) cellophane tube tied to both ends of the apparatus and partially filled with the MRS broth corrected to pH 4 with an 85% (v/v) solution of lactic acid. Water at $37 \pm 1^\circ\text{C}$ was circulated through the openings of the apparatus (the directions are shown). Once the temperature of the circulating water was equilibrated, the Pessary containing Probiotic agent was dropped into the cellophane tube; after 10 min, 5 ml of MRS broth was added over the formulation. A container of sterile water was placed under the discharge outlet to collect the discharged liquid. During 90 min of adherence test, discharge fluid was allowed to leak out from cellophane membrane. After completion of the test, cellophane membrane was removed, and placed over solidified MRS agar medium for incubation. After incubation at 37°C for 24 hr, membrane was removed and washed with sterile water. A section of 1cm was cut, mounted on microscopic slide and stained with methylene blue. The prepared slide was observed under motic microscope for cell adherence.

Antimicrobial activity of *Lactobacillus* inoculum

The production of antimicrobial substances by *lactobacillus sp.* was detected using agar diffusion method described by Tag and McGivney [11]. 1 cm diameter disc of filter paper was soaked in culture filtrate of *lactobacillus sp.* and was placed on surface of solidified agar plate, which was inoculated with pathogenic bacteria *E. coli* as indicator strain. After 24 hr incubation at 37°C , plate was examined for zone of inhibition on indicator lawn.

Viability and Stability Studies

All three types of vaginal Pessaries containing Probiotic were kept in glass containers at ambient temperature ($30 \pm 2^\circ\text{C}$) and $2-8^\circ\text{C}$ for 4 weeks. At appropriate time intervals, 0, 1, 2, 3 and 4 weeks the

survival of Probiotic was determined by plate count method using MRS agar medium [8].

Results and Discussion

Physical characteristics of prepared vaginal Pessaries

The hollow-type pessary was developed by Watanabe *et al.* in order to evaluate the effectiveness of the drug when administered rectally. This pessary has a hollow cavity in which drugs in the form of powder, liquid, or solid could be placed. The advantage of using the hollow-type pessary in this study is that it can eliminate the effect of the heating process on the survival of lactobacillus during preparation and interactions between lactobacillus and the pessary materials can be essentially eliminated.

Bilayer Pessaries were prepared for the purpose of separating Probiotic and Prebiotic in two different layers. Prebiotic releases first and creates favorable condition for growth of probiotic in vagina. The physical properties of prepared Pessaries and the viability of microorganism were evaluated. The effect of Prebiotic on growth of Probiotic was studied for Bilayer Pessaries. Also the selected *lactobacillus sp.* was tested for membrane adherence and for production of antimicrobial substances.

All Pessaries were found to satisfy the BP 1988 for weight uniformity. The results are given in Table 1.

The Plain Pessaries and Bilayer Pessaries prepared by fusion method were found to be the strongest, than the hollow type Pessaries, against breaking forces. Since a loose structure is formed in Hollow type of Pessaries, that have much lower resistance to breaking forces.

The disintegration test determines whether Pessaries soften or disintegrate within a prescribed time when placed in an immersion fluid. According to the BP requirement, disintegration occurs in not more than 60 minutes. The results of disintegration test are shown in Table 1. All Pessaries were found to satisfy the BP requirement for disintegration.

Table 1 Results: physical characteristics of prepared vaginal Pessaries

Formulation	Weight variation (gm)		Hardness (kg)		Disintegration time (min)	
	Mean value	±SD	Mean value	±SD	Mean value	±SD
Plain Pessaries	3.88	0.021	3.6	0.51	12.67	0.51
Hollow type Pessaries	3.821	0.018	1.5	0.54	7.33	0.81
Multifunctional Bilayer Pessaries	2.91	0.075	2.3	0.39	10.33	0.52

4.2 Effect of Prebiotic on growth of Probiotic

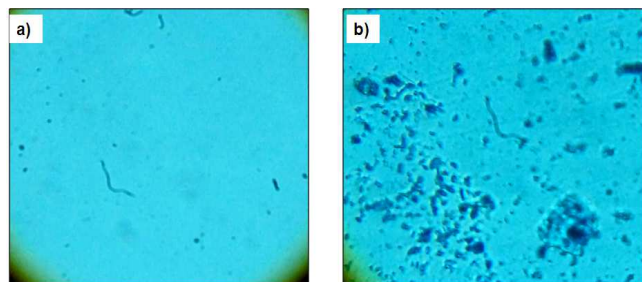
Effect of Prebiotic on growth of Probiotic was determined. Viable count of *lactobacillus sp.* after 48 hr of incubation at 37°C for Multifunctional Bilayer and Plain Pessaries containing probiotic alone were shown in Table 2. Bilayer Pessary showed greater viable count after 48 hr of incubation than Plain Pessary. This suggests that Prebiotic present in Bilayer Pessaries after melting creates favorable environment for growth of lactobacilli by acidifying media to normal vaginal pH 4.

Table 2 Effect of Prebiotic on growth of Probiotic from Pessary formulation

Formulation	Plain Pessaries	Multifunctional Bilayer Pessaries
Viability after 48 hr of incubation	$5.8 \pm 0.19 \times 10^6$ CFU	$6.4 \pm 0.27 \times 10^{10}$ CFU

4.3 Membrane adherence test

The adhesiveness of selected *lactobacillus sp.* to the cellophane membrane was investigated. Microscopic examinations (Figure 4) showed that this species strongly adhered to selected membrane.

**Figure 4** Membrane adherence test with lactobacilli on cellophane membrane a) before and b) after test

4.4. Antimicrobial activity of *Lactobacillus* inoculum

Culture filtrate of selected *lactobacillus sp.* was studied for antimicrobial activity. The result showed that selected *lactobacillus sp.* has inhibitory activity against selected indicator strain. Figure 5 shows zone of inhibition by culture filtrate in the indicator lawn. There could be two reasons for the inhibiting activity of *lactobacillus sp.* first the production of lactic acid; and second production of substances such as H₂O₂ or bacteriocins.

**Figure 5** Antimicrobial activity of culture filtrate of *lactobacillus sp.* against *E. coli*

Viability and Stability Studies

The survival of Probiotic in prepared vaginal Pessaries, stored at ambient temperature at 2-8°C during 4 weeks was determined and the results are shown in Table 3. Marked reduction of Probiotic in all Pessaries stored at ambient temperature was observed because of the temperature effect.

Table 3 Viability of Probiotic in prepared vaginal Pessaries

Formulation	Storage time (Weeks)	CFU	
		Ambient temperature	2-8°C
Plain Pessaries	0	$5.2 \pm 0.20 \times 10^5$	$5.2 \pm 0.20 \times 10^5$
	1	$4.2 \pm 0.17 \times 10^5$	$4.6 \pm 0.28 \times 10^5$
	2	$1.3 \pm 0.35 \times 10^4$	$3.3 \pm 0.18 \times 10^5$
	3	$4.4 \pm 0.21 \times 10^4$	$1.2 \pm 0.15 \times 10^5$
	4	$5.4 \pm 0.15 \times 10^3$	$6.5 \pm 0.11 \times 10^4$
Hollow type Pessaries	0	$5.3 \pm 0.27 \times 10^8$	$5.3 \pm 0.31 \times 10^8$
	1	$4.8 \pm 0.22 \times 10^8$	$3.4 \pm 0.24 \times 10^8$
	2	$2.8 \pm 0.24 \times 10^7$	$2.2 \pm 0.16 \times 10^8$
	3	$1.3 \pm 0.11 \times 10^7$	$8.3 \pm 0.34 \times 10^7$
	4	$6.9 \pm 0.17 \times 10^6$	$7.1 \pm 0.18 \times 10^7$
Multifunctional Bilayer Pessaries	0	$5.8 \pm 0.21 \times 10^7$	$5.8 \pm 0.36 \times 10^7$
	1	$4.6 \pm 0.28 \times 10^7$	$4.4 \pm 0.25 \times 10^7$
	2	$8.8 \pm 0.22 \times 10^6$	$9.2 \pm 0.19 \times 10^6$
	3	$6.3 \pm 0.28 \times 10^6$	$8.1 \pm 0.31 \times 10^6$
	4	$4.9 \pm 0.34 \times 10^5$	$7.8 \pm 0.32 \times 10^6$

The plain vaginal Pessaries containing Probiotic alone prepared by fusion method showed reduction in viable count to the extent of 3 log. This may be attributed to the heating process during the preparation. Hollow-type Probiotic vaginal Pessaries, showed decrease in the viable counts by 2 log. However all the Pessary formulations were stable when stored at 2-8°C over the period of this study.

Multifunctional Bilayer Pessaries are as stable as Hollow-type Pessaries. Previous study suggested the effect of Prebiotic on viability and growth of Probiotic. The greater count of Probiotic in Multifunctional Bilayer Pessaries than plain Pessaries containing Probiotic alone may be attributed to presence of Prebiotic in formulation.

Conclusion

The results suggest that the Multifunctional Bilayer Pessary with Prebiotic in separate layer supports the growth of Probiotic by maintaining pH 4. The Multifunctional Bilayer Pessary may be useful for delivery of chemically incompatible drugs/substances with varying release order.

References

- 1) Neri A., Sabah G. and Samra Z. Bacterial vaginosis in pregnancy treated with yoghurt. *Acta Obstetrica et Gynecologica Scandinavica*. 1993; 72(1):17-19.
- 2) Heinemann C and Reid G. Vaginal microbial diversity among postmenopausal women with and without hormone replacement therapy. *Canadian Journal of Microbiology*. 2005; 51(9): 777-781.
- 3) Reid G. Probiotic agents to protect the urogenital tract against infection. *The American Journal of Clinical Nutrition*. 2001; 73(2): 437S-443S.
- 4) Oakley BB, Fiedler TL, Marrazzo JM, and Fredricks DN. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Applied and Environmental Micro*. 2008; 74(15): 4898-4909.
- 5) Yahagi R., Onishi H., Machida Y. 1999. "Preparation and evaluation of double-phased mucoadhesive suppositories of lidocaine utilizing Carbopol and white beeswax," *Journal of Controlled Release*. 61 (2), 1-8.
- 6) Huang C.C., Tokumura T., Machida Y., Nagai T. 1987. "Formulation of double-layered suppository for prolonged stay in lower rectum," *Yakuzaigaku*. 47 (1), 42-48.
- 7) Kale V.V., Trivedi R.V., Sanjay P. 2005. "Development and evaluation of a suppository formulation containing lactobacillus and its application in vaginal diseases," *Ann. N.Y. Acad. Sci*. 1056, 359-365.
- 8) Hosny E.A., Abdeel-Hady S.S., El-Tahir K. 1996. "Formulation, in vitro release and ex vivo spasmolytic effects of mebeverine hydrochloride suppositories containing polycarbophil or polysorbate 80," *Int. J. Pharm*. 142, 163-168.
- 9) Watanabe Y. 1986. "Pharmaceutical evaluation of hollow type suppositories. IV. Improvement of bioavailability of propranolol in rabbits after rectal administration," *J. Pharmacobiodyn*. 9, 526-531.
- 10) Watanabe Y. and Matsumoto M. 1984. "Pharmaceutical evaluation of hollow type suppository. I. Brilliant blue FCF release characteristics of oleaginous hollow type

suppository,” *Journal of the Pharmaceutical Society of Japan*. 104(5), 479-484.

- 11) British Pharmacopoeia. London: The Stationary Office; 2001: p. 2015, A235R.
- 12) Maggi L., Mastromaria P., Macchia S., Brigid P., Pirovano F., Matteuzzi D., Conte U. 2000. “Technological and biological evaluation of tablets containing different of lactobacilli for vaginal administration,” *Eur J Pharm Biopharm*. 50, 389-395.
- 13) Kale V., Trivedi R., Muley P. 2008. “Proposed Design of a Dissolution Apparatus for Vaginal Formulations Containing Probiotics” *Dissolution Technologies*. 6, 27-29.
- 14) Kos B., Frece J., Motosic S. 2003. “Adhesion and aggregation ability of Probiotic strain lactobacillus acidophilus M92,” *journal of applied microbiology*. 94, 981-987.
- 15) Tagg J.R., McGiven A.R. 1971. “Assay system for bacteriocins,” *journal of applied microbiology*. 21 (5), 943.

