Original Article

Vaginal delivery of protein drugs in rats by gene-transformed *Lactococcus lactis*

Gagan Kaushal[1,*], Jun Shao[2]

[1] *School of Pharmacy, University of Charleston, Charleston, WV, USA;*
[2] *Biotechnology and Drug Delivery Laboratory, Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, NY, USA.*

ABSTRACT: A probiotic bacterium, *Lactococcus lactis* subsp. *lactis* (*L. lactis*) transformed with plasmid ss80, which made it capable of synthesizing and secreting β-lactamase, a 29 kDa protein, was used to deliver β-lactamase *via* vaginal route. The vaginal absorption of β-lactamase in rats was studied when delivered by this *L. lactis* system and compared to the β-lactamase solution with or without the untransformed *L. lactis*. The vaginal administration of 1.2×10^7, 3×10^7, and 8×10^7 colony forming units (cfu) of *L. lactis* resulted in the amount absorbed of 77, 194, and 216 mU, with the respective doses. C_{max}, mean retention time and mean absorption time of β-lactamase were also increased with the increase in the cfu of *L. lactis* administered. These results have demonstrated that *L. lactis* can significantly increase ($p < 0.01$) the β-lactamase vaginal absorption as compared to the β-lactamase solution, which is probably due to the adhesion of *L. lactis* to and continuous synthesis and delivery of β-lactamase directly to the vaginal mucosa. In conclusion, transformed normal flora may be an efficient method to deliver protein drugs through the vaginal route.

Keywords: *Lactococcus lactis*, β-lactamase, normal flora, protein delivery, vaginal, pharmacokinetics

1. Introduction

Protein drugs are generally administered by parenteral route because of their low bioavailability through the other routes. There is a great need for an alternate non-invasive means for the delivery of the protein drugs. The non-invasive delivery routes that have been explored are oral, nasal, buccal, rectal and vaginal routes. The vagina has been focused as a favorable alternative site for the systemic delivery of protein drugs because of the relatively high permeability of the vaginal epithelium, by passage of the hepatic first-pass metabolism, large surface area and rich blood supply. In addition, a prolonged contact of a delivery system with the vaginal mucosa may be achieved more easily than at other absorption sites like rectum or intestinal mucosa. In a study where absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) from various routes of administration was compared, vaginal route showed the greatest potency as compared to the other non-parenteral routes (*1*). In post menopausal women, the reduced epithelial thickness may further increase the absorption (*2*).

We have proposed that normal flora may be used as a delivery system for the vaginal protein delivery. Normal flora consists of the non-pathogenic bacteria that exist in the open tracts of the human body such as intestine, nostril, and vagina. By recombinant DNA technology, the normal flora can be genetically engineered to synthesize and secrete the protein drugs. Their natural tendency to adhere tightly to the epithelial cell surface (*3*) of the channels where they normally reside will result in delivering sufficient amount of protein at the site of absorption and will also minimize the enzymatic and bacterial degradation of the protein drugs. This will result in the concentrate of protein drugs on the absorption surface to improve the bioavailability.

Lactobacillus is the most prevalent organism in the vaginal environment together with many other facultative and obligate aerobes and anaerobes. The acidic pH of 4-5 of healthy women of reproductive age is maintained by the lactobacilli (*4*). When the vaginal pH becomes alkaline it leads to various kinds of infections as the protective barrier provided by acidic layer becomes less effective.

L. lactis, one of the safest strains in the LAB (Lactic acid bacteria) family, is used in the present study. This strain has been transformed with plasmid ss80 (*5*).

**Address correspondence to:*
Dr. Gagan Kaushal, School of Pharmacy, University of Charleston, 2300 MacCorkle Ave. SE, Charleston, WV 25304, USA.
e-mail: gagankaushal@ucwv.edu

Therefore it can synthesize and secrete β-lactamase, a 29 kDa protein, which is the non-therapeutic model protein used in our study. β-Lactamases, produced by some of the gram-positive as well as gram-negative bacterium, are the enzymes that catalyze the hydrolysis of β-lactam ring and are responsible for the bacterial resistance to penicillin, cephalosporin and many other antibiotics. Previously, we have reported that the oral delivery of β-lactamase by this *L. lactis* could significantly increase the β-lactamase oral bioavailability by 2~3 folds ($p < 0.01$), and the mean transit time (MTT) by 3~4 times ($p < 0.01$), as compared to the solution form with/without the untransformed *L. lactis* (*6*). In our another study (*7*) we have found that *L. lactis* could significantly increase the transportation of β-lactamase through C-33A monolayer (human cervical cell monolayer) and almost double the transportation rate as compared to the solution form. The present study was carried out to examine the feasibility of this *L. lactis* to secrete and deliver β-lactamase *in vivo* and its bioavailability through vaginal route in rats. In addition, the effect of different doses of *L. lactis* on the plasma profile of β-lactamase was also investigated.

2. Materials and Methods

2.1. *Materials*

Lactococcus lactis subsp. *lactis*, transformed with plasmid ss80 (thereafter referred as *L. lactis*) encoding for β-lactamase and its secretion signal was generously provided by Dr. Soile Tynkleynen (Valio Ltd. Helsinki, Finland). M17 broth and agar were purchased from Becton Dickinson (Sparks, MD, USA). Ampicillin, β-lactamase (from Bacillus cereus EC 3.5.2.6), ascorbic acid, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the chemicals were of analytical grade.

Female Sprague-Dawley rats weighing 300 ± 25 g, purchased from Taconic Farms, Germantown, NY, USA, were used in these experiments. The rats were acclimated to their surrounding for at least one week before the experiment. The micro-isolator cages were used with two animals in each cage. They were housed on a 12-h light/dark cycle and a relative humidity of 40 to 60%. All the experiments were performed under a vertical Laminar Flow Hood.

2.2. *Vaginal delivery of β-lactamase in rats*

A total of 36 female Sprague-Dawley rats were evenly randomized into six groups. The rats were restrained from food and water for 12 h prior to the dosing. The rats were given 0.2 mL of β-lactamase free solution or *L. lactis* in log-growing phase according to the schedule in Table 1. For the intravaginal administration, the solution was deposited deep into the vagina, using a 1 mL disposable syringe attached to a mouse gavaging needle. The blood samples (about 0.4 mL of each) were collected in an Eppendorf tube containing 25 mg of EDTA at the predetermined time points. The research protocol was approved by Institutional Animal Care and Use Committee (IACUC) at the St. John's University.

2.3. *β-lactamase HPLC assay*

β-Lactamase concentration in the plasma was assayed by an HPLC method reported previously (*8*). To a 0.2 mL of the plasma sample, 0.4 mL of 6.25 mM ampicilllin (substrate) was added. The reaction mixture was incubated at 37°C for 30 min and then 0.1 mL of 60% TCA at 4°C was immediately added to cease the reaction. The solution was centrifuged at 9,000 × g for 5 min and 0.5 mL of the supernatant was added to 2 mL

Table 1. Experimental design and pharmacokinetic parameters of β-lactamase in rats after vaginal administration (mean ± S.D., *n* = 6)

Group	Dose and route	$AUC_{0-\infty}$ mU•h•mL^{-1}	C_{max} mU	T_{max} h	MRT h	MAT h	AB[1] mU
I	1,008 mU of β-lactamase, vaginal	24.8 (8.9)	1.08 (0.09)	4-8	14.1 (2.2)	9.0 (2.2)	78.7 (28.2)
II	1,008 mU of β-lactamase and 3×10^7 cfu of the untran-*L. lactis,* vaginal	25.5 (4.0)	1.32 (0.2)	2-8	14.8 (1.1)	9.7 (1.1)	81.0 (12.6)
III	1.2×10^7 cfu of *L. lactis,* vaginal	24.4 (5.8)	1.3 (0.2)	4-8	14.7 (3.0)	9.6 (3.0)	77.4 (18.6)
IV	3×10^7 cfu of *L. lactis*, vaginal	61.0* (17.0)	2.8* (0.7)	4-8	20.0* (4.4)	14.9 (4.4)	193.6* (53.9)
V	8×10^7 cfu of *L. lactis*, vaginal	68.1* (18.5)	4.1* (0.6)	6-8	22.5* (4.1)	17.4* (4.1)	216.1* (58.7)
VI	252 mU of β-lactamase, *i.v.*	79.4 (12.4)	-	-	5.1 (0.9)	-	100

[1]: absolute bioavailability; *: significant difference from Group II and III ($p < 0.05$).

solution of 0.5 M acetate buffer at pH of 5 containing ascorbic acid (0.5 mg/mL) and EDTA (50 mM). The resulting solution was heated at 100°C for exactly 30 min. The processed samples were then analysed by an HPLC method after cooling them to the room temperature.

The HPLC system consisted of a Waters 600E system controller, a Waters 717 Autosampler, and a Waters 470 Scanning fluorescence detector. The separation was done on a μ Bondapak C18 cartridge column (300 × 3.9 mm I.D.). The injection volume was 10 μL. The mobile phase was 80% of 0.1 M phosphate buffer (pH = 5.0) and 20% of acetonitrile with a flow rate of 1.5 mL/min. The column effluents were monitored at excitation and emission wavelengths of 410 nm and 475 nm, respectively, for a run time of 11 min, and the peak of interest was seen at the retention time of 9.2 min. Two standard curves were constructed by analysis of the peak area against the concentration of the β-lactamase in 2 different concentration ranges of 0.252-1.26 mU/mL and 1.26-12.6 mU/mL, which were prepared by spiking the blank plasma with β-lactamase standard solution. The concentration of β-lactamase in the rat plasma samples was determined by the standard curve method.

2.4. *Pharmacokinetic (PK) parameters and statistics*

The PK parameters were calculated by the noncompartmental analysis (*9*). The area under the plasma concentration versus time curve from beginning to the last measurable concentration time point, $AUC_{0\text{-last}}$, was determined by the linear trapezoidal method, AUC from the last measurable concentration time point to infinite, $AUC_{last\text{-}\infty}$, was calculated as C_{last}/k, where C_{last} is the last measurable concentration and k the elimination rate constant. The area under the first moment curve (AUMC) was computed from time zero to infinity. MRT (Mean Residence Time) and MAT (Mean Absorption Time) were also calculated. The amount absorbed (AB) vaginally were determined as:

$$AB = \frac{AUC_{(vaginal)}}{AUC_{(i.v.)}} \times Dose_{(i.v.)}$$

The maximum plasma concentration reached (C_{max}) and the time at which it was reached (T_{max}) were observed from the β-lactamase plasma concentration-time profile. Statistical analysis (ANOVA) was performed with $\alpha = 0.05$ as the minimal level of significance.

3. Results and Discussion

3.1. *Vaginal absorption of β-lactamase in rats*

In the present experiment the feasibility of *L. lactis* to secrete β-lactamase in the cervico-vaginal ecosystem and investigate the absorption efficiency of β-lactamase into the systemic circulation by the delivery of *L. lactis* to the rat's vagina was studied. Figures 1 and 2 show the plasma concentration profile of β-lactamase after the vaginal administration. The pharmacokinetic parameters are listed in the Table 1.

For this study the free solution of β-lactamase was used as control. The vaginal administration of 1,008 mU of β-lactamase in free solution resulted in the mean C_{max} of 1.98 mU/mL, T_{max} between 4-8 h, MRT of 14 h, MAT of 9 h and no β-lactamase was detected at 48 h (Figure 1). The co-administration of the untransformed *L. lactis* with β-lactamase free solution showed similar ($p > 0.05$) β-lactamase plasma concentration profile as the free solution alone, which indicates that the untransformed *L. lactis* did not affect the absorption of β-lactamase *via* vaginal route.

In case of *L. lactis*, three doses were used to study not only the delivery efficiency but also the dose-absorption relationship. As shown in the Figure 2, the administration of 1.2×10^7, 3×10^7, and 8×10^7 cfu, resulted in C_{max} of 1.28 mU/mL, 2.79 mU/mL, and 4.07 mU/mL, respectively; T_{max} in the range of 4-8 h; MRT of 14.7 h, 20.0 h, and 22.5 h, respectively; MAT of 9.6 h, 14.9 h, and 17.4 h, respectively; and there was still a quantifiable amount of β-lactamase in the plasma at 72 h after dosing. The T_{max} for all the three doses was between 4-8 h. After the oral administration of 1.2×10^7 *L. lactis* cfu, β-lactamase was still detectable in plasma at 24 h, while 48 h after the 3×10^7 and 8×10^7 *L. lactis* cfu administration. MRT of the β-lactamase when delivered by the three doses of *L. lactis* cfu was 14.7, 20.0, and 22.5 h, respectively; and the MAT 9.6, 14.9, and 17.4 h, respectively. The amount of β-lactamase absorbed when delivered vaginally by 1.2×10^7, 3×10^7, and 8×10^7 of *L. lactis* was 77.4, 193.6, and 216.1 mU of *i.v.* dose, respectively.

There was a 40% increase in the MRT and MAT when 3×10^7 *L. lactis* cfu were administered ($p < 0.05$) as compared to the free solution. This increase was most probably due to the ability of *L. lactis* to adhere to the vaginal mucosa, and continuously multiply and secrete β-lactamase right onto the absorption epithelium. Our previous *in vitro* study (*7*) has also demonstrated that there was a 50% increase in the β-lactamase transport across the C-33A monolayer when delivered by the *L. lactis* as compared to the free solution.

The concentration of β-lactamase on the absorption surface is the major factor for absorption enhancement. As the transformed *L. lactis* adhered to the epithelial layer, it secreted β-lactamase directly onto the absorption surface, resulting in a locally high concentration. Over the time, this localized β-lactamase would diffuse through these membranes. The transformed *L. lactis* is thus able to significantly enhance the β-lactamase absorption *in vivo*. First,

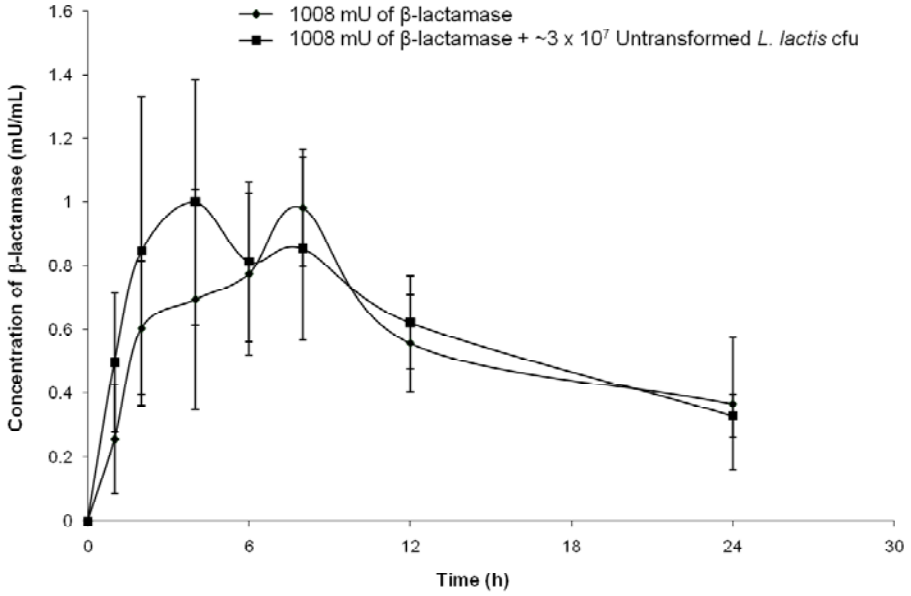

Figure 1. β-Lactamase plasma concentration after the vaginal administration of 200 µL of β-lactamase solution with or without untransformed *L. lactis* to the rats (*n* = 6).

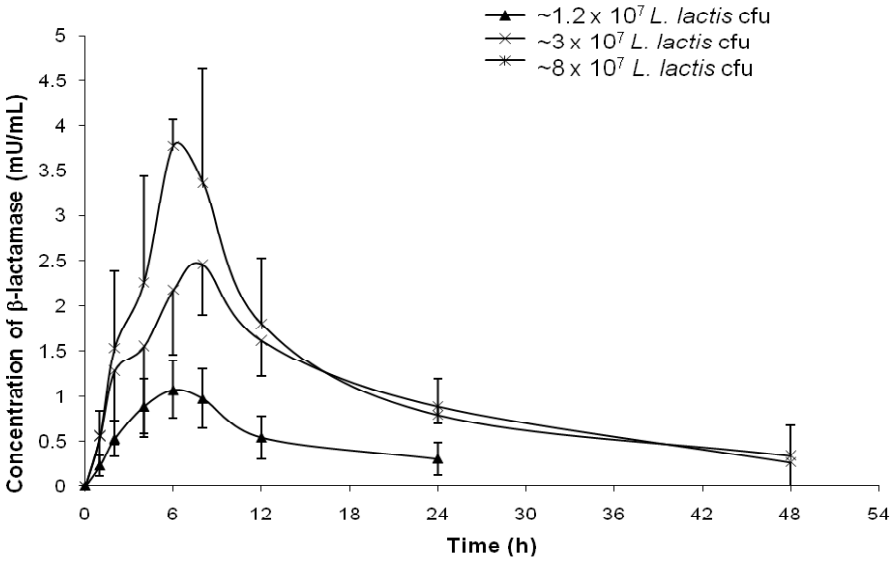

Figure 2. β-Lactamase plasma concentration after the vaginal administration of 200 µL of different doses of *L. lactis* to the rats (*n* = 6).

through the adherence to the vaginal epithelium, the transformed *L. lactis* will secrete β-lactamase onto the vaginal epithelium to concentrate the protein drug on the absorption surface and reduce the exposure of the protein drug to a hostile environment. Second, the transformed *L. lactis* will continuously produce and secrete β-lactamase, and due to its adhesive property it usually can stay in the vagina for a certain period before being eliminated, so that the transformed *L. lactis* can provide a prolonged delivery mechanism.

The amount of protein that can be delivered through this delivery system can be controlled by controlling the number of bacteria that is being delivered. Thus it would be of significant interest to compare the absorption profiles by the three different doses of the *L. lactis* (Figure 3). When the dose was increased from 1.2×10^7 cfu to 3×10^7 cfu (a 1.5-fold increase), the AUC and C_{max} were increased by 1.5 and 1.2 folds, respectively, showing a direct dose-absorption relationship. However, the further increase of the dose to 8×10^7 cfu (a 5.7-fold increase), did not resulted a proportional increase in absorption, although there was a 1.8-fold increase in AUC and a 2.2-fold increase in C_{max}. These results can be explained by the limited nutrients and space *in vivo*. In overall, the results demonstrate the relationship between the dose *L. lactis* and the protein drug entering into the systemic circulation, providing some guidance for dosing

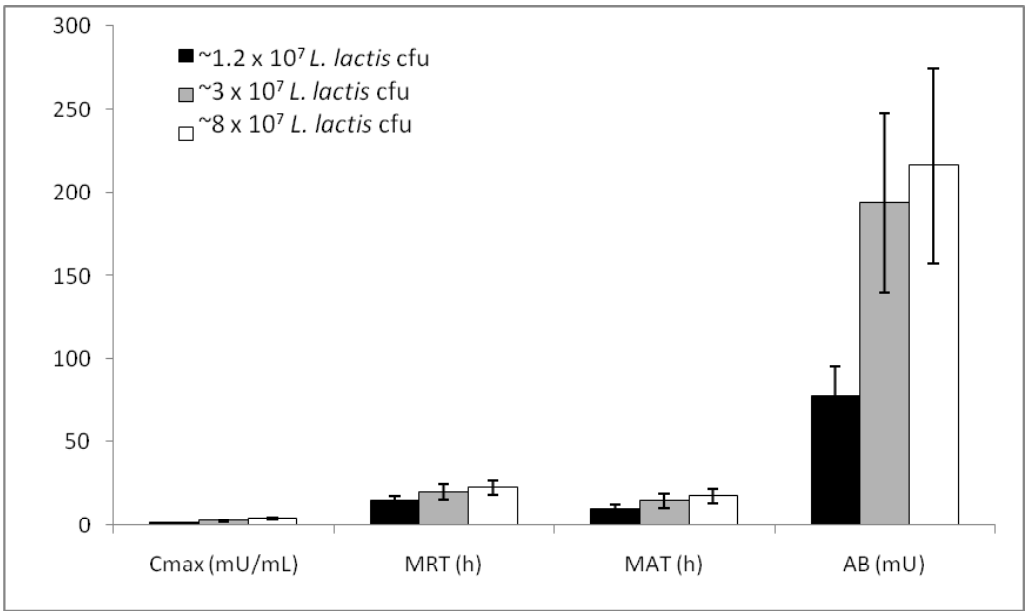

Figure 3. Relationship between *L. lactis* dose and some of the absorption PK parameters.

consideration in the future.

Since no β-lactamase was detected at 72 h after the administration of *L. lactis*, it can be assumed that most of *L. lactis* were either dead or eliminated out of the body by that time. This kind of phenomenon is actually desired in terms of drug delivery. Normal flora delivery system can provide a prolonged delivery mechanism, but to a certain degree, so that uncontrolled and undesired long-term actions can be avoided. Based on our previous study (*10*), we have observed that *L. lactis* are eliminated out of the body after oral administration. This phenomenon is supported by another report, which showed that *L. lactis* was a non-colonizing and was transient bacteria in the body (*11*). Thus this would terminate the drug delivery and also the possible risks of super bug development in the body after its administration.

The vagina extends from the vestibule to the uterus, and is situated behind the bladder and in front of the rectum; it is directed upward and backward, its axis forming with that of the uterus an angle of over 90°, opening forward. Its walls are ordinarily in contact, and the usual shape of its lower part on transverse section is that of an H, the transverse limb being slightly curved forward or backward, while the lateral limbs are somewhat convex toward the median line; its middle part has the appearance of a transverse slit (*12*). Drugs are transported across the vaginal membrane by the transcellular route, intracellular route or vesicular and receptor-mediated transport mechanisms (*13*). Its unique features in terms of secretion pH and microflora, and must be considered during the development and evaluation of vaginal delivery systems.

The vaginal route has been explored previously by many scientists for the delivery of various therapeutically active proteins such as insulin (*13*), calcitonin (*4*), and sex hormones (*4*). A very limited success has been achieved in the development of cervico-vaginal region as a potential systemic delivery site of these macromolecules. A safe and viable formulation is required to achieve a breakthrough in the field of this underutilized delivery route. One of the major concerns about vaginal delivery is the disturbance of the vaginal environment. For example, the depletion of vaginal lactobacilli can result in serious consequences which may lead to infection, thus maintenance of a normal microflora and the vaginal pH is important (*14*). In the complex vaginal ecosystem, lactobacilli are the most predominant bacterial species in healthy women. Delivery of the strains from lactobacilli family may be a choice from the safety view point. The adherence of normal flora to the mucosa provides a great advantage for the recombinant bacteria to deliver the protein drugs, since the bacteria will directly deliver the protein drugs onto the epithelial cell surface where the absorption takes place. The direct delivery of the protein drugs onto the epithelial surface will concentrate the protein drugs on the absorption surface to improve their absorption, and also minimize the exposure of the protein drugs to the degradation factors in the environment to reduce the pre-absorption degradation which usually is significant by other delivery methods.

The present study has further verified that the probiotics such as *L. lactis* when transformed by a special plasmid can be a living source for the protein drugs through vaginal route. This kind of delivery system provides a sustained delivery mechanism by which delivery period can be extended. It may be used for the delivery of suitable proteins which are capable of functioning locally or systemically.

3.2. *Conclusion*

There was an increase in the numerical value of the PK parameters, such as C_{max}, MAT, MRT, and AUC with the increase of dose of *L. lactis*.

Probiotics such as *L. lactis* when transformed by special plasmids can be a living source for efficient and sustained vaginal delivery of protein drugs. The amount delivered and the delivery period can be regulated by the number of the probiotics to be administered.

Acknowledgements

We would like to thanks Dr. Soile Tynkleynen, Valio Ltd., R&D (Helsinki, Finland) for generously providing the strain *Lactococcus lactis* subsp. *lactis* (ss80). The financial support from St. John's University is also acknowledged.

References

1. Okada H, Yamazaki I, Ogawa Y, Hirai S, Yashiki T, Mima H. Vaginal absorption of a potent luteinizing hormone-releasing hormone analog (leuprolide) in rats I: absorption by various routes and absorption enhancement. J Pharm Sci. 1982; 71:1367-1371.
2. Vermani K, Garg S. The scope and potential of vaginal drug delivery. Pharm Sci Tech Today. 2000; 3:359-364.
3. Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM, Fonden R, Saxelin M, Collins K, Mogensen G, Birkeland SE, Mattila-Sandholm T. Demonstration of safety of probiotics – a review. Int J Food Microbiol. 1998; 44:93-106.
4. Richardson JL, Illum L. The vaginal route of peptide and protein drug delivery. Adv Drug Del Rev. 1992; 8:341-366.
5. Sibakov M, Koivula T, von Wright A, Palva I. Secretion of TEM β-Lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* sussp. *lactis*. Appl Environ Microbiol. 1991; 57:341-348.
6. Kaushal G, Shao J. Oral delivery of β-lactamase by *Lactococcus lactis* subsp. *lactis* transformed with Plasmid ss80. Int J Pharm. 2006; 312:90-105.
7. Kaushal G, Trombetta L, Ochs RS, Shao J. Delivery of TEM β-lactamase by gene-transformed *Lactococcus lactis* subsp. *lactis* through cervical cell monolayer. Int J Pharm. 2006; 313:29-35.
8. Kaushal G, Shao J. Determination and pharmacokinetics study of β-lactamase in rat plasma by using a fluorimetric HPLC method (In Press).
9. Gibaldi M, Perrier D. Pharmacokinetics, 2nd ed., Marcel Dekker, New York and Basel, 1982.
10. Kaushal G, Shao J. Genetically engineered normal flora for oral polypeptide delivery: dose-absorption response, J Pharm Sci. 2009; 98:2573-2580.
11. Geoffroy MC, Guyard C, Quatannens B, Pavan S, Lange M, Mercenier A. Use of green fluorescent protein to tag lactic acid bacterium strains under development as live vaccine vectors. App Environ Microb. 2000; 66:383-391.
12. Gray H, Anatomy of the Human Body, 5th ed., Lippincott Williams and Wilkins, 2007.
13. Richardson JL, Illum L, Thomas NW. Vaginal absorption of insulin in the rat: effect of penetration enhancers on insulin uptake and mucosal histology. Pharm Res. 1992; 9:878-883.
14. Gardiner GE, Heinemann C, Bruce AW, Beuerman D, Reid G. Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. Clin Diagn Lab Immunol. 2002; 9:92-96.

(*Received September 2, 2009; Revised September 26, 2009; Accepted October 3, 2009*)