

# **DEVELOPMENT OF BIOPOLYMERIC SUPPOSITORIES FOR THE TREATMENT OF BACTERIAL VAGINOSIS**

---

Thesis submitted in partial fulfillment of the requirements for the degree of

**Master of Technology**

in

**BIOTECHNOLOGY**

By

**NIKHIL KUMAR**

**ROLL NO: 208BM207**



**DEPARTEMENT OF BIOTECHNOLOGY AND MEDICAL ENGINEERING  
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA**

**2010**

# **DEVELOPMENT OF BIOPOLYMERIC SUPPOSITORIES FOR THE TREATMENT OF BACTERIAL VAGINOSIS**

---

Thesis submitted in partial fulfillment of the requirements for the degree of

**Master of Technology**  
in  
**BIOTECHNOLOGY**  
By

**NIKHIL KUMAR**

Under the Guidance of

**Dr. KUNAL PAL**



**DEPARTMENT OF BIOTECHNOLOGY AND MEDICAL ENGINEERING  
NATIONAL INSTITUTE OF TECHNOLOGY, ROURKELA**

**2010**



# National Institute Of Technology Rourkela

## C E R T I F I C A T E

This is to certify that the thesis entitled “**DEVELOPMENT OF BIOPOLYMERIC SUPPOSITORIES FOR THE TREATMENT OF BACTERIAL VAGINOSIS**” by Mr. **Nikhil Kumar** submitted to the **National Institute of Technology**, Rourkela for the Degree of Master of Technology in Biotechnology, is a record of bonafide research work, carried out by him in the Department of Biotechnology and Medical Engineering under my supervision. I believe that the thesis fulfils part of the requirements for the award of Master of Technology. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any Degree or Diploma.

Date:

Place:

**Dr. Kunal Pal**

Department of Biotechnology and Medical Engineering

NIT Rourkela

## Acknowledgement

This project is by far the most significant accomplishment in my life and it would have been impossible without people who supported me and believed in my calibers.

I would like to extend my gratitude and sincere thanks to my honorable supervisor **Dr. Kunal Pal**, Assistant Professor, Department of Biotechnology and Medical Engineering. He is not only a great lecturer with deep vision but also most importantly a kind person. I sincerely thank for his exemplary guidance and encouragement. His trust and support inspired me in the most important moments of making right decisions and I am glad to work under his supervision.

I also express my sincere gratitude to Dr. B. P. Nayak, Dr. S. S. Ray and Dr. S. Paul who permitted me to carry out experiments in their laboratories. I am very thankful for their relentless help in various ways for providing me with an utmost homely and amicable atmosphere to work. I am thankful to them for showering their love and affection.

I would like to thanks Dr. S. Paria, Assistant Professor, Department of Chemical Engineering to allow me to use their laboratory facilities. I express my sincere gratitude to Mr. Naveen for his continuous support during my visit in the department.

I am also thankful to my lab mates and colleagues Mrs. Charulata Bhattacharya, Mr. Sai Sateesh and Ms. Swagatika Sahoo, who were a great moral and practical support during my work. I cannot forget to thank my friends at NIT, Rourkela especially Mr. Saurabh Chaudhary, Mr. Shrinivas Kapparapu, Mr. Viraj Krishna Mishra, Ms. VS Praveena Ch, Mr. Chandan Kumar, Mr. Himanshu Sinha, Mr. Ashwini Kumar, Mr. Prabhash Dadheech and Mr. Ashutosh Shukla. They made my stay at NIT Rourkela worth living.

Last, but not the least, I would thank the Almighty and my parents, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

**Nikhil Kumar**

# CONTENTS

LIST OF TABLES	I
LIST OF FIGURES	II
ABBREVIATIONS	III
ABSTRACT	IV

	Page no.
1. INTRODUCTION AND OBJECTIVES	1-3
1.1. Introduction	2
1.2. Objectives	3
2. REVIEW OF LITRATURE	4-15
2.1. Vaginal system	5
2.2. Microflora of vagina	9
2.3. Bacterial vaginosis	10
2.4. Treatment of BV	12
2.4.1. Antimicrobials in the treatment of BV	12
2.4.2. Probiotics in the treatment of BV	13
2.4.2.1. Selection criteria for probiotic for the treatment of BV	14
2.4.2.2. Probiotic treatments available for BV	15
3. METHODS AND MATERIALS	16-21
3.1. Materials	17
3.2. Methods	17
3.2.1. Preparation of MRS broth and agar	17
3.2.2. Preparation of nutrient broth and agar	18
3.2.3. Culturing of <i>Lactobacillus</i>	18
3.2.4. Preparation of suppositories	19
3.2.5. Antimicrobial study	19
3.2.6. <i>In vitro</i> drug release studies	20
3.2.7. Mucoadhesion testing by <i>in vitro</i> wash off method	21

<b>4. RESULTS AND DISCUSSION</b>	<b>22-34</b>
<b>4.1. <i>L. acidophilus</i> culture</b>	<b>23</b>
<b>4.2. Preparation of suppositories</b>	<b>24</b>
<b>4.3. Antimicrobial study against <i>E. coli</i></b>	<b>25</b>
<b>4.4. Antimicrobial study against <i>L. acidophilus</i></b>	<b>30</b>
<b>4.5. Drug release study</b>	<b>32</b>
<b>4.6. Mucoadhesive analysis</b>	<b>34</b>
<b>5. CONCLUSION</b>	<b>35-36</b>
<b>6. REFERENCES</b>	<b>37-44</b>

## LIST OF TABLES

<b>Table No.</b>	<b>Title</b>	<b>Page No</b>
<b>T1</b>	<b>Composition of the Suppositories prepared</b>	<b>20</b>
<b>T2</b>	<b>Antimicrobial activity against <i>E. coli</i></b>	<b>27</b>
<b>T3</b>	<b>Antimicrobial activity against <i>L. acidophilus</i></b>	<b>32</b>

## LIST OF FIGURES

<b>Figure no.</b>	<b>Title</b>	<b>Page no.</b>
<b>Figure 1</b>	<b>Method of serial dilution</b>	<b>18</b>
<b>Figure 2</b>	<b><i>L. acidophilus</i> culture plate</b>	<b>23</b>
<b>Figure 3</b>	<b>Micrograph of gram stained <i>L. acidophilus</i></b>	<b>24</b>
<b>Figure 4</b>	<b>Gelatin-based suppository and micrograph of the sliced lactobacilli loaded suppository, after incubation.</b>	<b>25</b>
<b>Figure 5</b>	<b>Antimicrobial activity against <i>E. coli</i></b>	<b>26</b>
<b>Figure 6</b>	<b>Antimicrobial activity of <i>L. acidophilus</i> based products against <i>E. coli</i> culture.</b>	<b>29</b>
<b>Figure 7</b>	<b>Biodegradability of control sample by <i>E. coli</i></b>	<b>30</b>
<b>Figure 8</b>	<b>Comparative antimicrobial study on <i>E. coli</i></b>	<b>31</b>
<b>Figure 9</b>	<b>Antimicrobial activity against <i>L. acidophilus</i></b>	<b>32</b>
<b>Figure 10</b>	<b>Release profile of metronidazole from MZ-cGG sample</b>	<b>33</b>
<b>Figure 11</b>	<b>Release kinetics of metronidazole from MZ-cGG sample.</b>	<b>34</b>
<b>Figure 12</b>	<b>b-cGG sample adhered to the intestinal mucosa of goat</b>	<b>35</b>

## ABBREVIATIONS

%	Percentage
µl	Microlitre
b-cGG	Blank suppository
C	Degree Centigrade
CF-cGG	Suppository containing ciprofloxacin
GS	Gelatin solution
h	Hour
L	Litre
LA-cGG	Suppository containing lactic acid
LALB-cGG	Suppository containing lactic acid plus <i>L. acidophilus</i>
min	Minute
MRS	de Man Rogosa and Sharpe
MZ-cGG	Suppository containing metronidazole
MZLALB-cGG	Suppository containing metronidazole plus lactic acid plus <i>L. acidophilus</i>
MZLB-cGG	Suppository containing metronidazole plus <i>L. acidophilus</i>
Psi	Per Square Inch

## ABSTRACT

The current work deals with the development of the biopolymer based suppositories for the treatment of bacterial vaginosis (BV). BV is characterized with a reduction in the *Lactobacillus* count in the vaginal lumen with the subsequent reduction in pH and growth of the pathogenic microorganisms. The mode of treatment of BV by alternative therapies includes either restoration of *Lactobacillus* colonies or restoration of the acidic environment in the vaginal lumen. In this study, attempts were made to develop gelatin-based biopolymeric suppositories. Further attempts were made to entrap *Lactobacillus acidophilus* within the suppositories, which may help in the release of lactic acid for a prolonged period to restore the acidic pH of the vaginal lumen. Microscopic examination of the section showed a homogeneous distribution of the viable bacteria within the polymeric matrix indicating its probable use in the treatment of BV. Thereafter, various bioactive agents were also incorporated either in combination of *Lactobacillus acidophilus* or as separate entities. The combination of *Lactobacillus acidophilus* and metronidazole, *Lactobacillus acidophilus* and lactic acid showed the most promising results.

**Keywords:** Bacterial vaginosis, gelatin, suppositories, metronidazole, ciprofloxacin, lactobacilli.

# *Chapter 1*

## **Introduction and objectives**

## 1.1.Introduction

The inner genital system in women is a lumen of length 7.5 cm (approx) called vagina. It has a pH of ~3.5-4.5. It may be defined as a dynamic ecosystem because of its capability to host a diverse microflora (Kale, Trivedi et al. 2005) consisting of anaerobic and aerobic bacterial genera and species dominated by the facultative, microaerophilic, anaerobic genus *Lactobacillus*. The activity of *Lactobacillus* is essential to protect women from genital infections and to maintain the natural healthy balance of the vaginal flora (Donati, Di Vico et al. 2010). Lactic acid, secreted by *Lactobacillus*, is responsible in maintaining the acidic pH within the vaginal lumen. In addition to the lactic acid, *Lactobacillus* also produces hydrogen peroxide and antimicrobials (lactocin and bacitracin) which prevent the growth of anaerobic pathogenic microorganisms within the lumen (Aroutcheva, Gariti et al. 2001). Any compromise with the micro-flora of the vagina may lead to the drastic decrease of the *Lactobacillus* population. Various scientific reports reveal that this disease affects 25% (approx) of the women population in India. The women in the urban setup are more prone to this disease. According to a recent study, 33% of women population in Delhi were found to be affected by this syndrome (Bhalla 2007). Every one out of three women is affected by this syndrome at some point of their lives. This condition may be treated with the local application and/or delivery of lactic acid, probiotics (Reid 2001) and antibiotics either singly or in various combinations using suppositories (Kale, Trivedi et al. 2005). The therapy regimes mainly concentrate on the restoration of the acidic pH or *Lactobacillus* dominated micro-flora. Suppositories often suffers with a disadvantage of migration within the vaginal tract after it is inserted resulting in an improper delivery of the active agent. The use of mucoadhesive polymers for the development of the suppositories may help in preventing the migration of the suppositories within the vaginal tract in addition to the controlled release of the bioactive agent (Fontaine and Taylor-Robinson 1990). In the current study, attempts will be made to develop suppositories using generally regarded as safe (GRAS) materials. Several approaches will be considered to develop gelatin-based suppositories containing *Lactobacillus*, antimicrobials and lactic acid, either alone or in combination. Lactic acid being a small molecule will be released almost instantaneously and will lower the pH of the vaginal tract which might inhibit the bacterial proliferation in the initial phases (Petrova, Petrov et al. 2007) whereas the antimicrobials may inhibit the growth of the pathogenic microbes for prolonged period.

## 1.2 Objectives

1. To develop biopolymer-based suppositories containing *Lactobacillus*, antimicrobials and lactic acid, either alone or in combination.
2. To analyze the efficacy of the suppositories by *in vitro* techniques.

# *Chapter 2*

## **Review of Literature**

## 2. Review of literature

### 2.1. Vaginal system

Vagina may be defined as an elastic lumen, which is 7.5 cm (approx) long. The elastic nature of the vagina may be attributed to the fibromuscular structure. Vaginal lumen has got three layers, viz. an outer layer of areolar tissue, a middle layer of smooth muscle and an inner lining of stratified squamous epithelium. The lumen arises from the cervix and extends up to vaginal orifice in the perineum. The vagina runs obliquely upwards and backwards at an angle of 45° and lies in between the urethra and rectum. Urethra is anteriorly positioned while rectum is posteriorly positioned to the vagina. Vagina acts as a connector between the external and internal organs of reproduction in women. In normal healthy women, the length of the anterior wall is 3 inches (approx) while its posterior wall is 3.5 inches (approx) long. The vaginal opening is at the caudal end of the vulva. It is usually partially covered by the hymen, a thin membrane of connective tissue. The lubricating fluid of the vagina is secreted by the Bartholin's glands, which is located near the vaginal opening and the cervix. The pH of the vagina lies in between 3.5 and 4.5 during the period between puberty and menopause. It harbors a number of microorganisms and *Lactobacillus* is the predominant species.

Glycogen, an analogue of starch found in animals, is the main source of nutrients for the microbial flora residing in the lumen of the vagina. The metabolism of glycogen in the vaginal system is mediated by the estrogen hormone via estrogen receptors located in the epithelial cells covering the vaginal lumen. The activity of the estrogen receptors is dependent on the ovarian hormonal cycle. The increase in the proliferation of the epithelial cells and subsequent increase in the glycogen content during the midcycle stage of the menstrual cycle has been attributed to the increase in the estrogen levels (Owen 1975). Due to the increased proliferation rate of the epithelial cells, there is an increase in the thickness of the epithelial cell layers (Wagner and Ottesen 1982; Patton, Thwin et al. 2000). It has been observed that the quantity of the mucus, which lines the vaginal epithelia, increases as the estrogen level increases. The increased estrogen level results in the decrease in the viscosity of the mucus, which results in a watery discharge. As the menstrual cycle enters into the latter half of the follicular phase, the production of the mucus increases by 30 folds (Owen 1975). Apart from the change in the epithelial and mucosal layers, the physiology of the vagina is also dependent on the menstrual cycle. At the time of menstruation, there is an increase in the pH of the vagina to pH=6 at day 2 with the subsequent decrease of the same to pH=4 at day 4 (Eschenbach, Thwin et al. 2000). Due to the dynamic changes in the environment of the

vagina during the menstrual cycle leads to the drastic changes in the ecology of the vaginal microflora. In general, it is regarded that Lactobacilli species is the predominant microflora responsible for maintaining the pH of the vaginal lumen (Eschenbach, Thwin et al. 2000). A recent study on Chinese women has also supported the same (Shi, Chen et al. 2009). But the analysis of the DNA of the microflora from the vagina of women indicate that some women harbor *Atopobium*, *Megasphaera* and *Leptotrichia* which produces the lactic acid and not the lactobacilli species (Zhou, Bent et al. 2004).

The mucus secreted in the vagina is mainly composed of glycoprotein, mucopolysaccharides (i.e. glycogen), electrolytes and a larger fraction of water. The mucosal layer not only provides nutrients to the vaginal microflora but also acts as receptors for them. For example, Tamm Horsefall protein (THP) which is present both in the human urine and vaginal region promotes adhesion of *E. coli* to these regions. Though THP is not secreted by the vaginal epithelia, their presence in the vagina has been attributed to the regular contact of the external vaginal structures with the urine (Hawthorn, Bruce et al. 1991; Otero and Nader-Macías 2007). Lactobacilli undergoes physicochemical interaction with the vaginal epithelia, which helps in the colonization of the lactobacilli and biofilm formation within the mucosal and the epithelial layer of the vagina (Busscher and Weerkamp 1987; Otero and Nader-Macías 2007). The biofilm consists of the bacterial cell layer(s) and the secretory components from the vagina (Bibel, Aly et al. 1987; Otero and Nader-Macías 2007).

The dynamic nature of the microflora is dependent on the age of the women. As the women approaches the age of 45 yrs, the menstrual cycle may either pauses or ceases forever. The condition of permanent cessation of the menstrual cycle is regarded as menopause, where there is a complete loss of follicular activity (Farage and Maibach 2006). Quite often, menopause is associated with a condition where there is a remarked decrease in the vaginal secretions, especially mucus, known as post-menopausal atrophic vulvo-vaginitis. This results in the decrease in the lubrication of the vagina causing discomfort during coitus (Kamarashev and Vassileva 1997). Also, there is a decrease in the thickness of the epithelial layer thereby increasing the susceptibility of the vaginal tissue towards infection and associated irritation (Kamarashev and Vassileva 1997; Farage and Maibach 2006). As a result of the above conditions, there is a subsequent increase in the vaginal discomfort, dryness, burning, itching and dyspareunia (Castelo-Branco, Cancelo et al. 2005). The frictional damage of the vaginal epithelia increases due to the exposure of the vaginal tissue to the moisture of the urine and is quite a common phenomena. Additionally, there is a subsequent

increase in the vaginal pH in the presence of the urinary ammonia which further complicates the condition and provides an open invitation for the growth of the pathogenic microorganisms (Andersen, Bucher et al. 1994).

The presence of moisture is a necessity for the of microorganisms on any surface and the vaginal system is not an exception (Warren, Bauer et al. 2005). Sweat and transepidermal water loss is the major source of hydration of the epithelial cells. Transepidermal water loss plays an important role in the hydration of the vulva region (Warren, Bauer et al. 2005). The vaginal lumen is kept moist mainly by the vaginal secretion and to a certain extent by the urine (Faergemann, Aly et al. 1983). In normal healthy women, there is an increase in the dynamic nature of the microflora with an increase in the hydration of the epithelial layer (Freinkel and Shen 1969). This results in the decreased permeability of the mucosal layer for the pathogenic organisms. If due to pathophysiological conditions, there is a decrease in the moisture content within the vaginal lumen then there is a corresponding decrease in the microbial count resulting in the increase in the permeability of the barrier layer (Elias 2007). Similarly, it was found that with the increase in the moisture content around the urogenital tracts there is a corresponding increase in the microbial activity in and around the interface of the urogenital organs and the skin (Farage, Bramante et al. 2007).

Most microbes have an optimal pH range in which they show an improved activity. Any intervention with the pH of the system may result in the growth of other microbes. The same phenomenon is also applicable for the vaginal system, which is widely populated with the lactobacilli species. The pH of the normal healthy vagina is within 3.5-4.5. The pH is mainly maintained by the production of lactic acid by the lactobacilli. Any compromise with the pH (due to pathophysiological conditions or physical activities like insertion of contraceptive devices and intercourse) may result in the increase of the pH within the vaginal lumen, which in turn may result in the lactobacilli population and a subsequent increase in the growth of other microbe (Aroutcheva, Gariti et al. 2001). As the lactobacilli count decreases, there is a decrease in the production of lactic acid. Lactic acid has a potent anti-microbial property which helps in preventing the growth of the pathogenic microbes (Nieman 1954; Kabara, Swieczkowski et al. 1972). Lactobacilli also produce antimicrobial products like bacitracin and hydrogen peroxide which further help in the prevention of the proliferation of the pathogenic microorganisms (Brook 1999; Reid and Burton 2002). Lactobacilli have the capability to excrete the substances, which hinders their multiplication, from the vaginal lumen (Aroutcheva, Gariti et al. 2001). Additionally, bacterial interference also plays an

important role in checking the growth of the pathogens. Bacterial interference is a phenomena by which helpful bacteria utilizes the space, resources and nutrients thereby not allowing the pathogens to proliferate (Lina, Boutite et al. 2003). This concept has been successfully used for the treatment/ prevention of infections in vaginal region. Similar approaches have been used to decrease the infection rate in the nasal mucosa (Lina, Boutite et al. 2003).

The nutrient for the microbes is supplied by the vaginal secretions in addition to the secretions from the sweat, apocrine and sebaceous glands. These secretions are composed of glycoproteins, polysaccharides, electrolytes, amino acids, nucleic acids and fatty acids (Pommerenke and Taylor Jr 1953). The metabolic products secreted by the microbes may influence the availability of the nutrients (Cavallo 1987). Fatty acids have shown antimicrobial activity (against *Streptococcus pyogenes*, *Staphylococcus aureus* and skin micrococci) and may help in fine tuning the composition of the microbial flora (Kabara, Swieczkowski et al. 1972). They failed to produce substantial activity against gram negative bacteria (Ouattara, Simard et al. 1997). Apart from this, some peptides have also shown antimicrobial activity against various pathogenic bacteria, fungi, viruses and protozoa (Zasloff 2002).

From the above discussion, it is now clear that the interaction amongst the host and the microbe may either create a mutually beneficial relationship (e.g. *Lactobacillus*) or may produce a deleterious effect on the host, which will lead to diseased condition and even death in severe conditions (e.g. *Candida*, *Gardnerella* and/or *trichomonas*) (Casadevall and Pirofski 2000). The adhesion of the pathogenic microbes to the epithelial cells is an important factor governing the colonization and/or biofilm development. This has been attributed to the competition amongst the natural and pathogenic microflora for the receptor sites present in the vaginal lumen (Boris, Suarez et al. 1998; Otero and Nader-Macías 2007). Unfortunately, there is lack of clear understanding of the molecular basis of adherence of the lactobacilli to the epithelial cells of vagina. Some reports suggests that the adherence is mediated by the proteins present on the cell surface (Conway and Kjelleberg 1989; Coconnier, Klaenhammer et al. 1992; Bernet, Brassart et al. 1993) while some researchers claim that the adherence is either mediated by lipoteichoic acid (Sherman and Savage 1986; Granato, Perotti et al. 1999) or carbohydrates (Neeser, Granato et al. 2000).

## 2.2 Microflora of the vagina

Vagina provides a biosynthetic environment which has the capability to sustain a diverse ecosystem. The microflora of the vagina can be categorized as transient and resident microflora and can easily be distinguished by newer molecular methods. The transient microflora are not capable of competing with the resident microflora in establishing a permanent residence in the vagina (Lowbury, Lilly et al. 1964). Knowledge about the transient and resident microflora of vagina is meager as the interpretation of the available data is very difficult. Apart from this, a large number of transient organisms continuously migrate from the exogenous source (e.g. anus and urethra).

The composition of the vaginal microflora was first described by Döderlein during the year 1892. He described the vaginal microflora as gram positive, pleomorphic and asporogenic bacteria, which were homogeneously distributed throughout the vaginal lumen and are often referred to as bacillus Döderlein (Lepargneur and Rousseau 2002). In a study, where the samples were collected from college going students and volunteers throughout the menstrual cycle indicates that the vagina is inhabited with the anaerobic and facultative microflora (Bartlett, Onderdonk et al. 1977). Similar results were also obtained from the studies on children having age varying from 2 months to 15 yrs (Hammerschlag, Alpert et al. 1978). Recent studies have found that lactobacilli are the dominant microflora of the vaginal system. The other microbes include Peptococcus species, Bacteroides species, *Staphylococcus epidermidis*, *Corynebacterium* species, *Peptostreptococcus* species, and *Eubacterium* species (Bartlett 1977). The lactobacilli species is prevalent throughout the lifespan of women and has also been confirmed with molecular identification tools (Stoyancheva, Danova et al. 2006). The predominance of lactobacilli is lost during various vagina diseases (e.g. bacterial vaginitis) as determined both by conventional and molecular identification techniques (Stoyancheva, Danova et al. 2006). Amongst the *Lactobacillus* species, *Lactobacillus acidophilus* was considered to be the dominant microbe present in the vaginal microflora (Lachlak, Ageron et al. 1996). With the advent of the use of molecular identification techniques, it was found that other species (e.g. *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus jensenii*) may also dominate the ecosystem in the microflora (Vasquez, Jakobsson et al. 2002). The molecular identification techniques have the ability to specify the species easily in case of diverse microflora. As described earlier, vagina is a dynamic ecosystem and is a complex ecosystem having diversified species of *Lactobacillus* and other microbes. Even though the molecular identification techniques (viz.

16s r-RNA analysis, RFLP, heat shock protein) have made the life of the researchers and clinician easy, our knowledge is quite limited on the vaginal microflora. The main advantage of molecular identification techniques is its fast and efficient methods as compared to the conventional culture based methods.

### **2.3 Bacterial vaginosis (BV)**

BV is a clinical syndrome associated with a group of pathogenic microorganisms rather than a specific pathogen. It is a very common manifestation amongst the women population. Though the exact causative pathogen has not been figured out, it has been observed that there is a corresponding decrease in the population of the lactobacilli species. This results in the increase in the pH of the vaginal lumen due to the reduction in the lactic acid production. Apart from the lactic acid, the production of lactocin and H<sub>2</sub>O<sub>2</sub> also receives a setback. In general, the lactobacilli is replaced with the increased population of pathogenic gram negative anaerobic bacteria like *E. coli*, *G. vaginalis*, *M. hominis* and *M. curtisii* (Hill 1993; Hillier 2005). This condition may lead to several complications, which include continuous vaginal discharge, high HIV risk, malodor (fishy smell), stomach pain, abortion, infertility, preterm birth, chorioamnionitis and urinary tract infection (Hillier, Nugent et al. 1995; Afrakhteh and Mahdavi 2007; Darwish, Elnshar et al. 2007).

The microflora diversity of the vagina from the patients of BV was first reported during the year 1921 by Schröder. Till recent past, the exact causative microorganism was not known and has eluded the scientists for long, newly discovered vaginal microbes during BV were often considered as BV causing microbes (Hillier 2005). Currently, BV is characterized by Amsel's criteria (Wang 2000). Amsel's criteria enumerates four criteria out of which at least three criteria has to be met before a woman can be declared as a patient of BV. The criteria include 1) watery discharge, 2) pH greater than 4.5, 3) positive amine test, and 4) the presence of clue cells (Wang 2000).

Scientists and researchers have associated BV with the following factors

1. Vaginal douching by the use of scented soaps or perfumed bubble bath and antiseptics during bath (Klebanoff, Nansel et al. 2010)
2. Women having multiple sex partners or a new sex partner.

3. Women with smoking habit are more prone to BV (Ryckman, Simhan et al. 2009).
4. Use of contraceptives (e.g. spermicides).

The microflora found in the case of BV do not follow the Koch's postulates and are resistant to that approach (Hillier 2005). Since the pathogenesis of BV is not clear, the management of BV is a challenging aspect for the clinicians and throughout the world, it is becoming a very common syndrome in women in the reproductive age (Spiegel 1991; Fredricks, Fiedler et al. 2005). If proper care is not taken in the early phase of BV, then it may lead to secondary complications. In pregnant women, it may either cause preterm delivery or choriamnionitis (Hillier, Nugent et al. 1995; Aruna and Jyoti 2007). The low weight of the infants (from BV patients) may also be accounted to the preterm birth (Hillier, Nugent et al. 1995). The risk of contracting sexually transmitted diseases (e.g. HIV, like syphilis, gonorrhoea and trichomoniasis) is higher in BV patients as compared to the healthy individual (Taha, Hoover et al. 1998).

If the patients fulfill the Amsel's criteria, various diagnostic tests are used for the confirmation of the syndrome. The **whiff test** is the most common test which involves the addition of potassium hydroxide solution to the vaginal discharge. The presence of a strong fishy smells indicates that the patient is suffering from BV. The microscopic examination of the vaginal smear, which is analyzed for the presence of bacteria, white blood cells and clue cells, the presence of clue cells indicates BV. The gram staining method, introduced by Dunkelberg in the year 1965, is also a simple method for the diagnosis of BV (Dunkelberg Jr 1965). The method helps in the confirmation of the presence of gram positive and negative bacteria in the vaginal discharge. Recently, a scoring system (on a scale of 10) based on the presence of large gram-positive rods, small gram negative or variable rods and small curved gram negative to variable rods have been reported. If the score lies in between 7 and 10, then the patient is suffering from BV (Joesoef, Schmid et al. 1999). The various modern analytical techniques involve the use of polymerase chain reaction and oligonucleotide probes for the determination of BV. These methods are fast, reliable and accurate (Fredricks, Fiedler et al. 2005). Unfortunately, these techniques are not available in all the labs and the clinician is forced to accept the results of the afore-mentioned diagnostic tests.

## **2.4 Treatment of BV**

Many strategies for the treatment of BV are being used by the clinicians all over the world. The treatment protocol varies from the use of synthetic drugs to the use of probiotics. In the current section, an attempt will be made to explore the different modes of treatment of BV.

### **2.4.1 Antimicrobials in the treatment of BV**

A lot of antimicrobial agents (e.g. ampicillin, penicillin, and metronidazole) have been used in the treatment of bacterial vaginitis (Spiegel 1991). Metronidazole have evolved as a drug of choice for the treatment of BV and is the widely prescribed drug. It is a nitroimidazole derivative, having activity against anaerobic microbes and protozoans. It has been administered either orally or locally. Tablets of metronidazole are easily available for oral administration. Formulations for the local administration of the drug include gels and suppositories (Adegboye and Itiola ; Decena, Co et al. 2006; Sobel, Ferris et al. 2006; Mitchell, Hitti et al. 2009). Metronidazole and tinidazole (a chemical analogue of metronidazole) are preferred for the treatment of BV as against ampicillin. Tinidazole has a better pharmacokinetics and longer half-life than metronidazole and its recommendation for the treatment of BV is on the rise (Dickey, Nailor et al. 2009). The use of ampicillin is avoided due to the emergence of ampicillin resistant bacteria in patients with BV. It also inhibits the growth of lactobacilli (Spiegel 1991). The acceptance of suppositories is lower than the oral administration of the drug as they might cause irritation (Adegboye and Itiola). The insertion of suppositories into the vagina also creates problem with the patient's compliance and is worse in working women. In a recent study, it has been reported that the mode of administration of metronidazole, either orally or locally, do not have a significant difference in the eradication of the pathogenic bacteria (Mitchell, Hitti et al. 2009). The release of the metronidazole from the suppositories is dependent on the composition of the formulation. The release rate may be tailored by the incorporation of adjuvant. Amongst the metronidazole gels and lactic acid gels, for local application, lactic acid gels have been found to be more efficient and safer. The gel formulation containing a combination of both lactic acid and metronidazole has shown superior ability to recolonize the vaginal lumen with lactobacilli. The recurrence of BV is less common in patients treated with lactic acid gel when compared with patients treated with metronidazole gels (Decena, Co et al. 2006). This may be attributed to the hindrance and/ or inhibition in the growth of the lactobacilli when metronidazole is used for the treatment and depends on the concentration of the lactobacilli (Simoes, Aroutcheva et al. 2001). Studies on the treatment of the BV have also been done

with tinidazole, clindamycin, polystyrene sulfonate, and cellulose sulfate, polycarbophil-carbopol acidic vaginal gel (Simoës, Citron et al. 2002; Nyirjesy, McIntosh et al. 2006; Otero and Nader-Macías 2007; Dickey, Nailor et al. 2009). Reports on clindamycin have suggested that it can be used in the treatment of BV and may be administered either orally or locally (McGregor, French et al. 1994). Intravaginal deliveries of clindamycin and metronidazole for the treatment of BV have shown that there was an improvement in the clinical symptoms of the patients within 21 to 30 days of the starting of the treatment. Unfortunately, the vagina was not recolonized with lactobacilli within the stated period (Sobel, Ferris et al. 2006). The use of formulation consisting polycarbophil-carbopol and lactic acid-chitosan mucoadhesive vaginal gels have also been reported (Zhou, Bent et al. 2004; Bonferoni, Giunchedi et al. 2006). The polycarbophil-carbopol gels have been found to be safe (Zhou, Bent et al. 2004). Similarly chitosan based lactic acid delivery gels have also been found to be a safe (Bonferoni, Giunchedi et al. 2006).

Unfortunately, there is an increased number of reoccurrence of BV when the synthetic antimicrobials are used and may be attributed to the development of antimicrobial resistance mechanism within the microbes (Beigi, Austin et al. 2004). Hence, the researchers and clinicians are looking for alternative methods for the treatment of BV.

#### **2.4.2 Probiotics in the treatment of BV**

The importance of probiotics in maintaining a normal health in human and animals was described by Dr. R. Fuller during the year 1989 (Ouweland, Salminen et al. 2002). It has recently been described as “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” by the WHO (Reid, Jass et al. 2003). The lactobacilli, present in curd and different milk products, have also been categorized under probiotics. From the discussions in the previous sections, it is quite clear now that lactobacilli plays an important role in the inhibition of growth, adhesion and spread of pathogenic microbes. This has been accounted to its ability to form biofilms over the mucosal layer of the vagina and thereby compete for the nutrients and receptors with the pathogenic microbes (Lepargneur and Rousseau 2002). In addition to this, they secrete lactic acid, H<sub>2</sub>O<sub>2</sub>, bacteriocins and biosurfactants which have good antimicrobial property. Apart from the antimicrobial property of lactic acid, they help in maintaining the pH of vagina within 3.5-4.5 thereby not allowing a conducive environment for the growth of the pathogenic microbes (Aroutcheva, Gariti et al. 2001). *Lactobacillus fermentum*, *Lactobacillus casai*, *Lactobacillus*

*acidophilus* and *Lactobacillus iners* are some of the species which have been found in vagina (Burton, Cadieux et al. 2003).

#### **2.4.2.1 Selection criteria for probiotic for the treatment of BV**

The absence of *Lactobacillus* from the vagina is the specific feature of BV (Hillier 1993). The major question in the treatment of BV with the use of lactobacilli as probiotics is whether it can cure BV and can inhibit the reoccurrences. Also, which species of lactobacilli can effectively inhibit the pathogens (e.g. *Escherichia coli* and *Gardnerella vaginalis*) and at the same time allow recolonization of the (Reid and Bocking 2003). The exact species of *Lactobacillus* for the treatment of BV may be selected based on the antipathogenic activity of the species. The consideration of following factors should also be taken into consideration before selecting the *Lactobacillus* species (McClean and Rosenstein 2000):

- 1) Production of lactic acid (Juarez Tomas, Ocana et al. 2003),
- 2) Production of H<sub>2</sub>O<sub>2</sub> (Eschenbach, Davick et al. 1989),
- 3) Adhesion of the species to the epithelial layer (Boris, Suarez et al. 1998)
- 4) Production of antimicrobials (Hamdan and Mikolajcik 1974; Abee, Klaenhammer et al. 1994).

#### **2.4.2.2 Probiotic treatments available for BV**

Sour milk and fermented milk were conventionally used for various health benefits. Elie Metchnikoff proposed the use of lactic acid producing bacteria as probiotic, with scientific reasons, during the year 1907 (Anukam, PhD et al. 2008). He was the first person to propose the use of *Lactobacillus* in the restoration of gastrointestinal flora (Sieber and Dietz 1998; Anukam, PhD et al. 2008). Various commercially available *Lactobacillus*-based products for the treatment of BV include yoghurt, acidophilus milk and available *Lactobacillus* powder and tablets (Hughes and Hillier 1990).

Two types of treatment methodologies are available for the treatment of BV using probiotics. The first one includes the oral administration of the *Lactobacillus* whereas the second method includes the local administration of the same in the vagina of the patient. The probiotics, when orally administered, have the natural ability to migrate to the vaginal region from the intestine via perineal and vulval skin. Studies using *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 have confirmed the phenomena (Reid, Bruce et al. 2001; Anukam, Osazuwa et al. 2006). The treatment of pregnant women, with symptoms with BV,

was carried out with yoghurt containing *Lactobacillus acidophilus*. Vaginal douching of the patients were done with the yoghurt, continuously for a week and was repeated after every 1 week's interval. The treatment helped in improving the conditions of the patients (Tasdemir, Tasdemir et al. 1996). Similar results were observed when yoghurt was locally applied in the vaginal lumen. At the cessation of the study, the vaginal pH was within the normal range and the presence of *Lactobacillus* within the lumen was also observed (Neri, Sabah et al. 1993). This kind of treatment may result in the decrease of the watery vaginal apart from its bacterial recolonization effects (Chimura, Funayama et al. 1995). In a recent study, two groups of women were treated with *Lactobacillus*- based products. The first group was treated with *L. acidophilus* suppositories whereas the second group was treated received a combination of *L. acidophilus* suppositories and oral dose of *L. paracasai* for a period of 3 months. Both the groups showed an increase in the colonies of *Lactobacillus* with a concurrent restoration of the pH. In addition to this, the group which received combination therapy, showed an increase in the restoration of the intestinal microflora (Delia, Morgante et al. 2006).

# *Chapter 3*

## **Methods and Materials**

### **3. Method and Materials**

#### **3.1 Materials**

*Lactobacillus* MRS (de Man, Rogosa and Sharpe) agar, *Lactobacillus* MRS broth and gelatine (extra pure) were procured from Hi-Media, Mumbai, India. Beef Extract, peptone, sodium chloride, nutrient broth, crystal violet solution, glutaraldehyde (25%, analytical grade) and hydrochloric acid (HCl, 35%) were purchased from Merck Mumbai, India. Gram's iodine and safranin solution were purchased from Nova biotech, Kolkata, India and SD'S chemical, Mumbai, India, respectively. Becelac Forte containing *Lactobacillus acidophilus* was purchased from Dr. Reddy lab purchased. The composition of Becelac Forte is biotin (100 µg), calcium pantothenate (50 mg), cyanocobalamin (15 µg), folic acid (1.5 mg), *Lactobacillus acidophilus* (2000 lacs) and niacinamide (100 mg). The bacterial strains of *E. coli* (NCIM strain no.-5051) and *Bacillus subtilis* was procured from NCIM Pune. Ciprofloxacin was purchased from fluka biochemika (Sigma-aldrich) and metronidazole was received as gift from Aarti drugs, Mumbai, India. Metrozyl® dental gel, Lekar Pharma Ltd. Khatraj, India, was purchased from local market. Lactic acid was procured from loba chemie. Rectified spirit and pure ethanol analytical grade were purchased from Trimurthy Chemical Industries, Cuttack, India and Changshu Yangyuan chemical, China respectively. Fresh goat intestine were procured from the local butcher shop. Unionized mili-Q water used throughout the study.

#### **3.2 Methods**

##### **3.2.1 Preparation of MRS broth and agar**

MRS broth was prepared by mixing 55.15 g of MRS *Lactobacillus* broth powder into 1 L of mili-Q water followed by autoclaving at 121 °C and 15 psi pressure for 15 min. Autoclaving solved two purposes. It enhanced the dissolution of the broth media and simultaneously sterilized the broth.

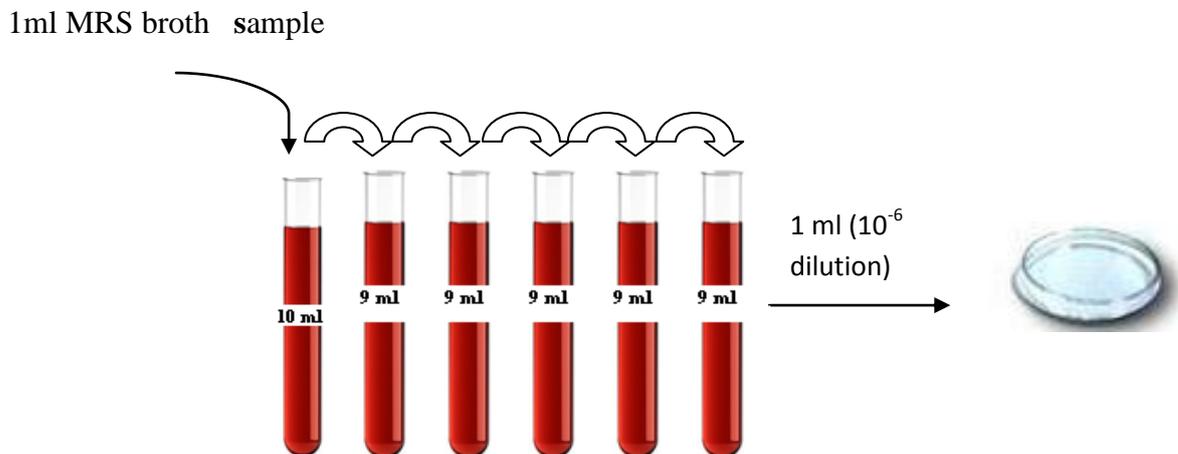
MRS agar was prepared by the addition of the 66.33 g of MRS agar powder into 1 L of mili-Q water followed by autoclaving at 121 °C and 15 psi pressure for 15 min. Autoclaved MRS agar solution were immediately poured into the petriplates and allowed to solidify.

### 3.2.2 Preparation of nutrient broth and agar

Nutrient broth was prepared by dissolving 10 g of beef extract, 10 g of peptone and 5 g of sodium chloride into 1 L of mili-Q water. The above solution was subsequently autoclaved. Nutrient agar media was prepared by mixing 28 g of nutrient agar powder in 1 L of mili-Q water and was subsequently autoclaved. The hot media solution was immediately poured into sterile petriplates and was allowed to solidify.

### 3.2.3 Culturing of *Lactobacillus*

*Lactobacillus* cultures were obtained from the Becelac forte® capsule. The culture was prepared by inoculating the freshly prepared MRS broth with the contents of the Becelac forte® capsule. Thereafter, the inoculated broth was incubated at 35 °C for 36 hours in an orbital incubator shaker. Serial dilution method was used to obtain a single colony culture from the broth culture. The broth was diluted 10<sup>-6</sup> fold (illustrated in figure 1) and was mixed with freshly prepared warm MRS lactobacillus agar solution in a petriplate and left to solidify under laminar hood. Thereafter the plate was incubated for 18 h at 35 °C in an orbital shaker incubator.



**Figure 1: Method of serial dilution**

Streak plate method was used to prepare a culture from the single colony obtained from the spread culture. After streaking, the petriplate was incubated at 35 °C for 18 h. After the incubation for 18 h, *L. acidophilus* culture was stored at 4 °C. The culture can be stored up to 2 weeks under the given condition. The culture can be maintained by repeated inoculation at regular intervals of time, which should be less than 2 weeks. To be on the safer side, the cultures were re-inoculated every week. The gram staining of the *L. acidophilus* culture were

done using standard gram staining technique(Xu 1997) and the microscopic pictures were obtained from the microscope (Hund Wetzlar, Model # H600, Germany) to characterize the bacterium.

### **3.2.4 Preparation of the suppositories**

Twenty per-cent (w/v) gelatin solution (GS) was prepared by dissolving 8 g of gelatin in 30 ml of warm water and the final volume was adjusted to 40 ml. To the above solution, a mixture of 350 µl of glutaraldehyde and 250 µl of hydrochloric acid was added with continuous stirring and were subsequently poured into 15 ml falcon tube (used as mold). The suppositories were isolated after 10 min, which allowed the crosslinking of gelatin. The prepared suppositories were measured and weighted for physical characterization. Pictures were taken with the help of a Sony 10 MPixel camera.

*Lactobacillus* loaded suppositories were prepared by suspending 40 mg of bacteria into the warm gelatin solution, having 8 g of gelatine in 30 ml water. The rest of the procedure was same as above. The biopolymeric suppositories were incubated in MRS broth to analyse the viability of the entrapped bacteria. The MRS broth containing *Lactococcus acidophilus* based suppositories were incubated period of 18 h. After 18 h of incubation, the suppositories were harvested and a thin section of the same was cut. Gram staining was done for the sliced section and was observed under microscope.

### **3.2.5 Antimicrobial study**

Samples loaded with ciprofloxacin, metronidazole, *Lactobacillus acidophilus*, lactic acid, lactic acid + *Lactobacillus acidophilus*, metronidazole + *Lactobacillus acidophilus*, and metronidazole + lactic acid + *Lactobacillus acidophilus* were developed to study the antimicrobial activity of the suppositories. Blank samples served as control. Hydrochloric acid was not added to the samples containing lactic acid. The compositions of the samples have been tabulated in Table 1. The method of preparation is similar to the development of *Lactobacilli*-loaded suppositories. The only exception being the crosslinking reaction was carried out in petriplates instead of falcon tubes.

**Table 1- Composition of the suppositories prepared**

sample	Components				
	Gelatin solution (20% w/v; ml)	Ciprofloxacin (g/ml)	Metronidazole (g/ml)	<i>Lactobacillus acidophilus</i> (g/ml)	Lactic acid (g/ml)
<b>b-cGG</b>	<b>40</b>		-	-	-
<b>CF-cGG</b>	<b>40</b>	<b>0.005</b>	-	-	-
<b>MZ-cGG</b>	<b>40</b>	-	<b>0.005</b>	-	-
<b>LA-cGG</b>	<b>40</b>	-	-	-	<b>0.036</b>
<b>LB-cGG</b>	<b>40</b>	-	-	<b>0.001</b>	-
<b>MZLB-cGG</b>	<b>40</b>	-	<b>0.005</b>	<b>0.001</b>	
<b>LALB-cGG</b>	<b>40</b>	-	-	<b>0.001</b>	<b>0.036</b>
<b>MZLALB-cGG</b>	<b>40</b>		<b>0.005</b>	<b>0.001</b>	<b>0.036</b>

Circular discs of 12 mm were cut using a borer from the bioactive agent loaded samples and blanks. These discs were put into the wells, having a diameter of 13 mm, prepared in the nutrient agar plates. 500 µl of *E. coli* suspension, from the culture broth, was added on the nutrient agar culture plate and was spread over the surface of the plate with the help of L-shaped spreader. The plates were subsequently incubated at 35 °C for 12 h. The comparative antimicrobial efficacy of the antimicrobials was studied on the basis of the zone of inhibition. The study was continued for 48 h for the blank samples so as to check the biodegradability of the crosslinked gelatine gel. Pictures were taken at 12, 24 and 48 h of incubation.

The effect of the bioactive active agents on *L. acidophilus* was studied in a similar manner as described above, where *E. coli* was replaced with 500  $\mu$ l of *Lactobacillus* culture. The samples were incubated for 36 h and observations were made after 18 and 36 h.

A comparative study was done amongst the MZ-cGG and Metrozyl® with *E. Coli* as the challenging microorganism. The concentration of metronidazole used for the development of MZ-cGG in this study was 10 mg/ml and was named as MZ-cGG\_1. This was done to have same amount of drug concentration in both the formulations. 0.7 g of both the samples was used and the study was continued for a period of 12 h. At the end of the study, zone of inhibition for *E. coli* was measured.

### **3.2.6 *In vitro* drug release studies**

Accurately weighed 6 g of the MZ-cGG were dipped into a beaker containing 50 ml of water. For the first hour, the MZ-cGG were taken out from the beaker and were put into a beaker containing fresh 50 ml of water at every 10 min interval. After 1 h, the changeover was done at an interval of 30 min. After 2 h, the changeover was done at an interval of 60 min. 2 ml of the media containing the drug was collected, at the above-mentioned intervals, in a test-tube and the rest was discarded. The experiment was carried out for a period of 8 h. The amount of metronidazole released was measured at  $\lambda_{\max}$  of 255 nm using UV-visible spectrophotometer (Shimadzu UV 1601 r).

### **3.2.7 Mucoadhesion testing by *In vitro* wash off method**

The mucoadhesive properties of b-cGG were evaluated by *in vitro* wash off method. A freshly excised piece of goat intestine mucosa (2.5 cm  $\times$  2.5 cm) was mounted on a glass slide (7.5 cm  $\times$  2.5 cm) with the help of Fevi Kwik®. The b-cGG sample (2.5 cm  $\times$  2 cm) was placed over the intestine mucosa and weight of 2 gm was applied over it for 5 minutes. Then the prepared specimen was transferred to the beaker and placed on an angle of 60 °C from the surface beneath. The beaker was filled up by 1 L mili-Q water and was kept under stirring at 150 rpm using an overhead stirrer. The test was carried out until the gel was removed from the mucosal surface.

# *Chapter 4*

## **Results and Discussion**

## 4. Results and discussion

### 4.1 *L. acidophilus* culture

*L. acidophilus* was isolated from Becelac forte® capsules by serial dilution method using MRS broth. The isolated bacteria were cultured at 35 °C and were subsequently maintained at 4 °C on MRS agar media (Nighswonger, Brashears et al. 1996). **Figure 2a** shows the colonies obtained by serial dilution method while **figure 2b** shows the culture obtained by streaking the spread plate colonies for maintenance. MRS broth and agar helps in selective growth of lactobacilli species due to the presence of sodium acetate and salt of manganese and magnesium (Hartemink, Domenech et al. 1997).



(a)



(b)

**Figure 2. (a) *Lactobacillus acidophilus* culture plate obtained after serial dilution (b) *Lactobacillus acidophilus* culture obtained by streak plate method for a single colony of (a).**

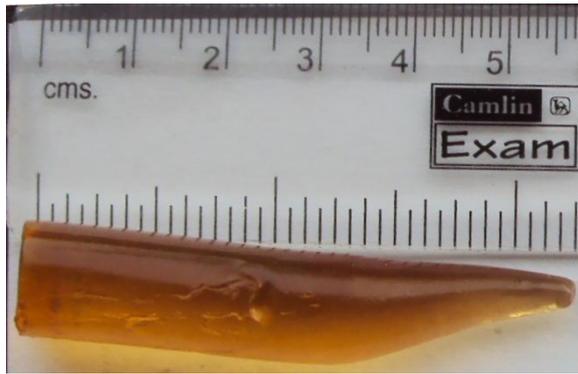
The isolated bacteria were morphologically characterized by gram staining. The micrographs of the gram stained bacteria, isolated from the capsules have been shown in **figure 3**. The results indicate the presence of rod shaped purple colored gram positive bacteria (characteristics of lactobacilli species) and absence of any other contaminants.



**Figure 3. Micrograph of gram stained *L. acidophilus***

#### **4.2 Preparation of suppositories**

The developed gelatin-based b-cGG suppositories have been shown in **Figure 4a and 4b**. The lactobacilli loaded suppositories were prepared in a similar manner. For checking the viability, the lactobacilli loaded suppositories were incubated overnight in MRS media. The MRS media was turbid after the incubation and the gram staining of the thin slice of the suppositories confirmed the presence of live *L. acidophilus* (**Figure 4c**). The result indicates that the bacteria were able to overcome the glutaraldehyde treatment shock and proliferated with ease.



(a)



(b)



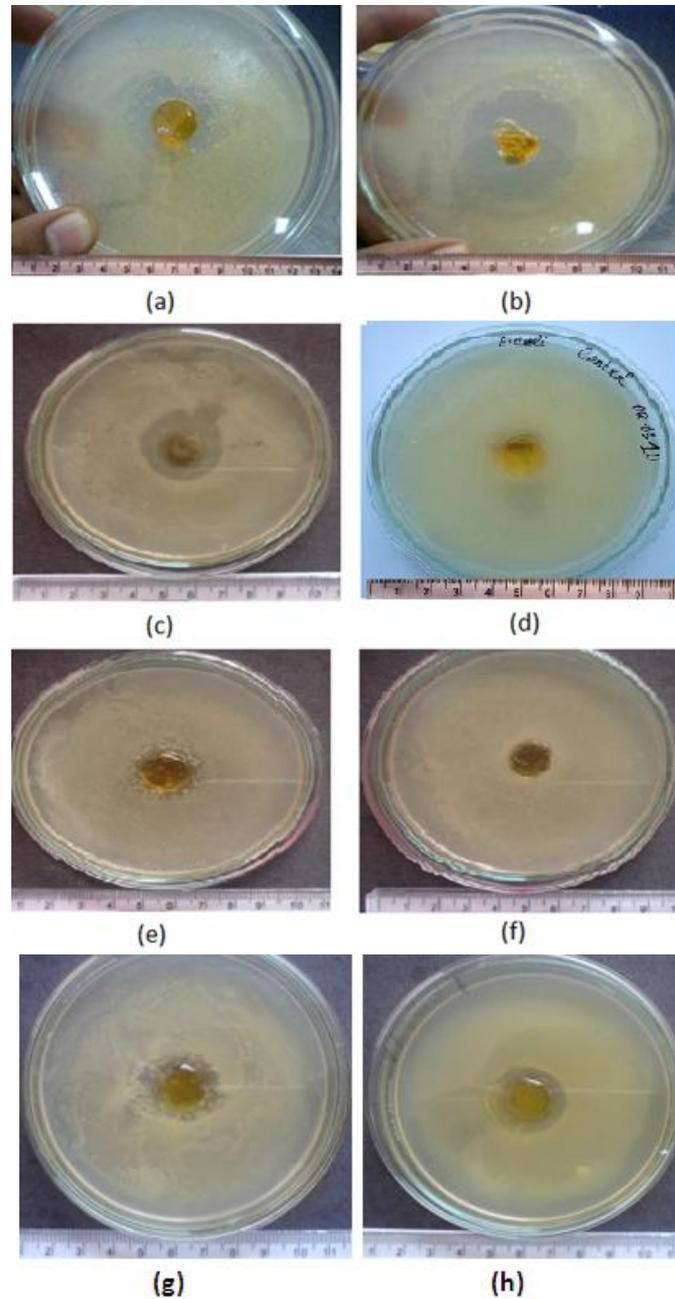
(c)

**Figure 4. (a) & (b) Gelatin-based suppository; (c) Micrograph of the sliced lactobacilli loaded suppository, after incubation.**

#### **4.3 Antimicrobial study against *E. coli***

*E. coli* is one of the main causative organisms for BV (Reid and Bocking 2003). Hence, it was used as a model microorganism to test the efficiency of the developed biopolymeric suppositories loaded with antimicrobials, lactic acid, *L. acidophilus* and combinations of all three. The antimicrobials used in the study include ciprofloxacin and metronidazole. Ciprofloxacin is a broad-spectrum antibiotic with activities against both gram +ve and gram –ve bacteria but its activity is higher against gram –ve bacteria. Most of the microbes responsible for BV are gram –ve bacteria. Metronidazole is the drug of choice for the treatment of bacterial vaginitis and hence was used in the study. Many metronidazole based tablets and gels are available in the market for the treatment of the BV. Lactic acid containing gels have also been used in the treatment of BV. *L. acidophilus* secretes lactic acid, H<sub>2</sub>O<sub>2</sub> and

antimicrobials (viz. lactocin and bacitracin), which allows them to combat the pathogenic bacteria. The weight and size of the all samples were 0.7 g and 12 mm, respectively. The results of the test are shown in **figure 5** and have been tabulated in **table 2**.



**Figure 5. Antimicrobial activity against *E. coli* (a) MZ-cGG suppository (b) CF-cGG suppository (c) LA-cGG suppository (d) b-cGG suppository, control (e) MZLB-cGG suppository (f) LB-cGG suppository (g) LALB-cGG suppository (h) MZLALB-cGG suppository**

**Table 2. Antimicrobial activity against *E. coli***

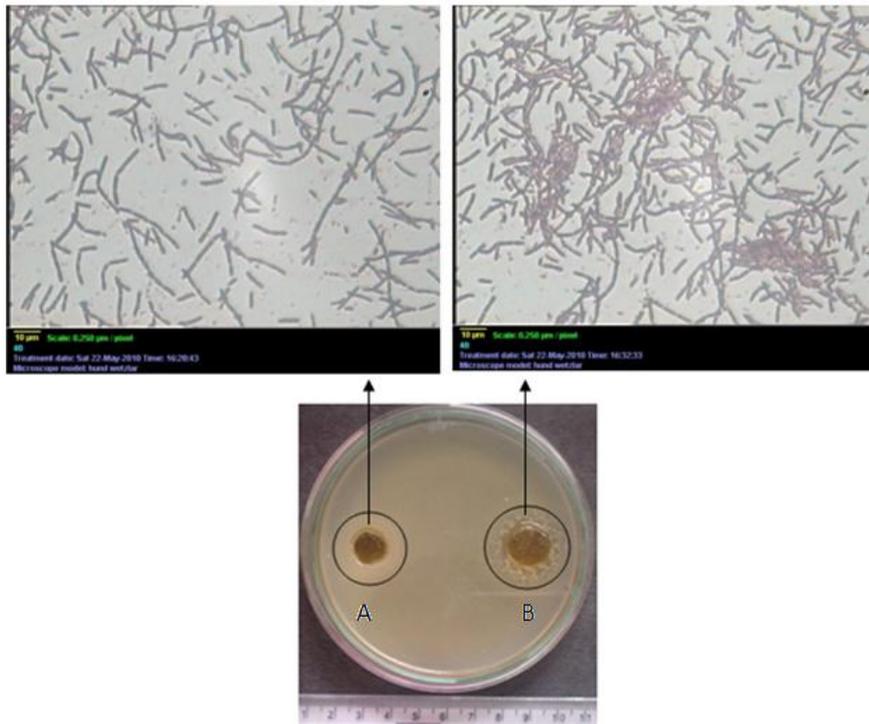
<b>Samples</b>	<b>Zone of inhibition (mm)</b>
<b>b-cGG</b>	<b>00 ± 0</b>
<b>MZ-cGG</b>	<b>35 ± 2</b>
<b>CF-cGG</b>	<b>50 ± 5</b>
<b>LA-cGG</b>	<b>25 ± 7</b>
<b>MZLB-cGG</b>	<b>25 ± 5</b>
<b>LB-cGG</b>	<b>20 ± 4</b>
<b>MZLALB-cGG</b>	<b>25 ± 4</b>
<b>LALB-cGG</b>	<b>22 ± 3</b>

The inhibition zones of the *E. coli* culture in the presence of the suppositories were in the following order: ciprofloxacin > metronidazole > lactic acid, metronidazole + *Lactobacillus*, metronidazole + lactic acid + *Lactobacillus* > lactic acid + *Lactobacillus* > *Lactobacillus*.

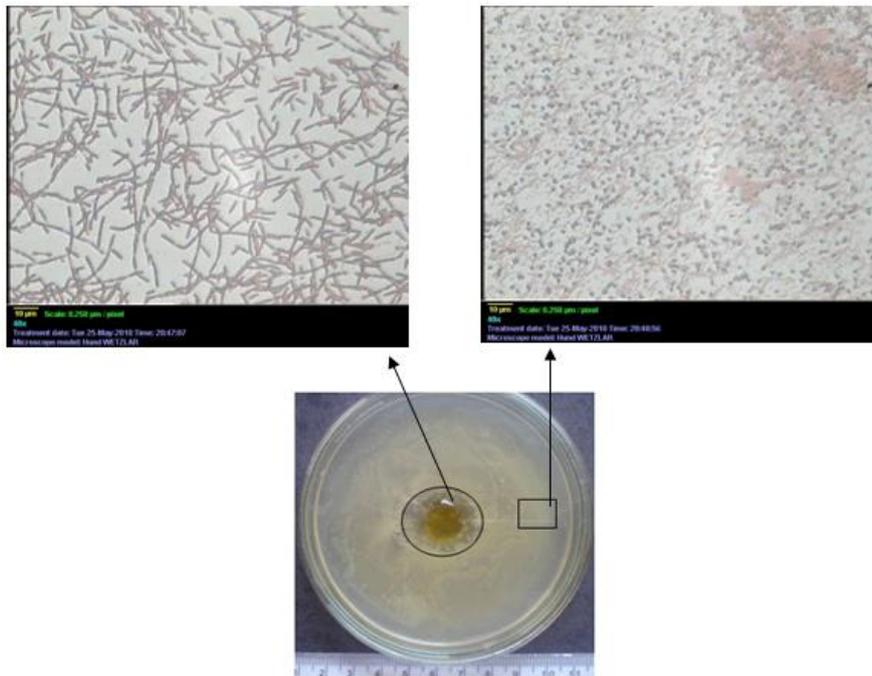
Ciprofloxacin, metronidazole and lactic acid containing suppositories showed a clear zone of inhibition around the wells for a period of 12 h (**figure 5**). These bioactive agents were able to inhibit the growth of *E. coli* for a prolonged period indicating the sustained action of the same and probable use in controlling the growth of *E. coli* during the treatment of BV.

The LB-cGG samples have shown a distinct circular zone adjacent to the wells containing the samples (**figure 6**). This zone was followed by a continuous culture of *E. coli*. The cells from the adjacent layer of the wells were collected and were subjected to gram staining. The results showed the presence of purple colored rod shape gram-positive bacteria and not *E. coli*. This can only happen when the metabolic products of lactobacilli (viz. lactic acid, H<sub>2</sub>O<sub>2</sub>, lactocin and bacitricin) have inhibited the growth of *E. coli*, which have allowed the lactobacilli to migrate out from the LB-cGG samples and colonize the vicinity of the wells. This indicates that *Lactobacillus* not only has the ability to inhibit the growth of *E. coli*, it can even proliferate in the presence of *E. coli*. The result explains the conventional use of lactobacilli based products (e.g. gels and yoghurt) for the treatment of BV (Neri, Sabah et al. 1993)

The MZLB-cGG and LALB-cGG samples have shown several colonies in the inhibition zone (**figure 6**). The morphologies of the colonies were different from the morphology of *E. coli*. Gram staining of the colonies showed purple colored rod shape gram-positive bacteria indicating that the lactobacilli from the suppository migrated out of the suppository. The finding is quite interesting and may allow the simultaneous delivery of metronidazole and lactobacilli using a same delivery system. Reported literatures suggest that BV treated with metronidazole, both by oral and local administration, do not show any colonization of lactobacilli within the vaginal lumen, though there is an improvement in the conditions of the BV patients (Anukam, Osazuwa et al. 2006). Until the vagina is rehabilitated with *Lactobacillus*, there are every chances of reoccurrence of the condition. Hence, the use of MZLB-cGG delivery system may allow recolonization of the vagina with *Lactobacillus* apart from the eradication of the pathogenic microorganisms. Similar results were also obtained with LALB-cGG samples indicating that LALB-cGG samples have the ability to effectively inhibit the growth of *E. coli* and may help in reducing the use of metronidazole for the treatment of BV. Though MZLB-cGG have shown slightly better inhibition zone as compared to LALB-cGG, the use of LALB-cGG may be preferred as there are increased chances of the development of bioactive agent resistant pathogenic microbes against metronidazole as against lactic acid or *Lactobacillus* (Beigi, Austin et al. 2004).



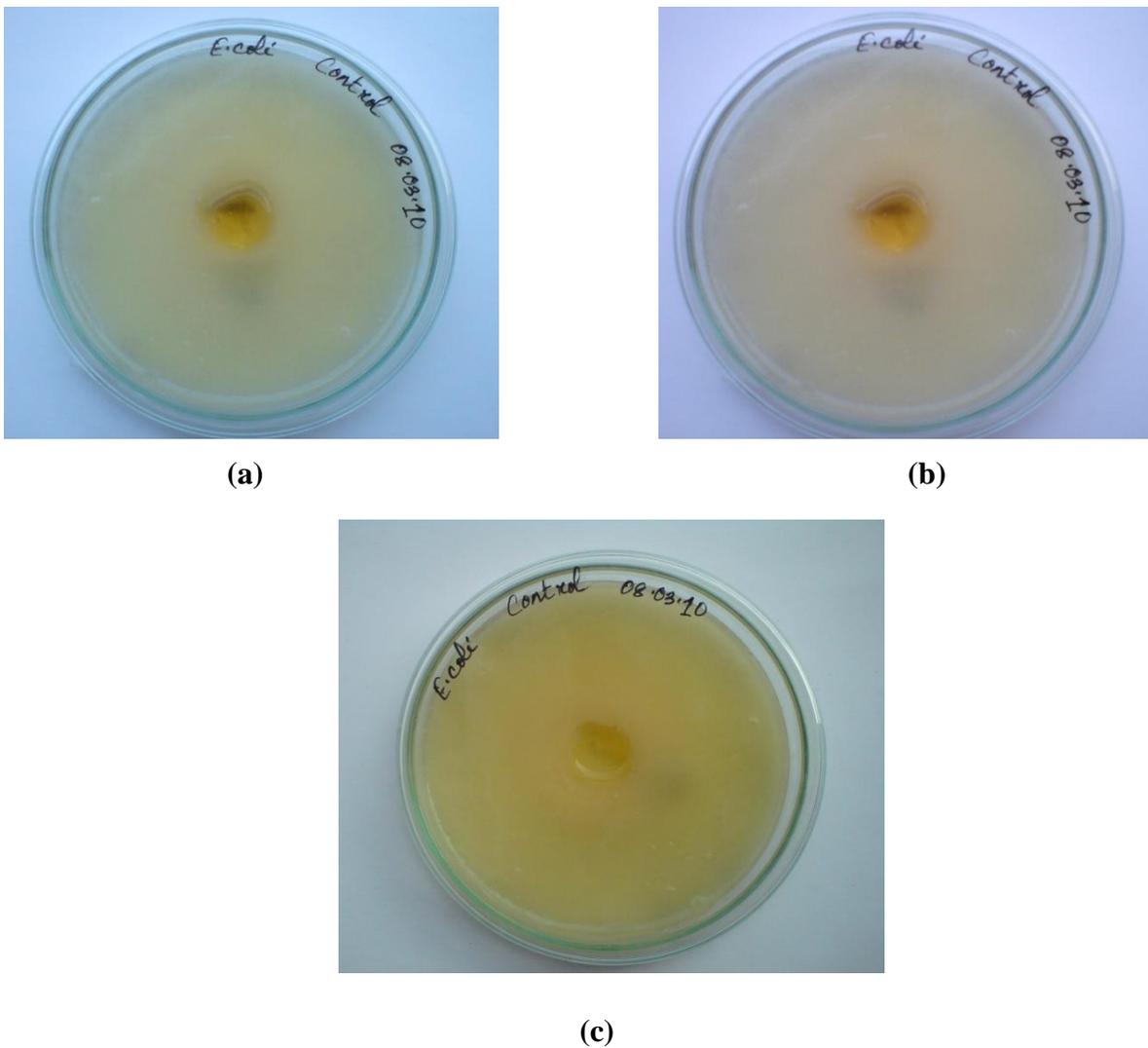
(a) (A) LB-cGG sample (B) MZLB-cGG sample



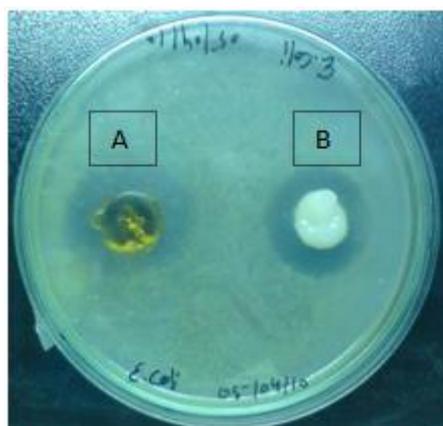
(b) LALB-cGG sample

Figure 6. Antimicrobial activity of *Lactobacillus acidophilus* based products against *E. coli* culture.

The samples of b-cGG (control) did not show any zone of inhibition indicating that the blank suppository did not show any antimicrobial activity (**figure 7**). The results confirm that glutaraldehyde did not exert any toxic response and the sample may be regarded as biocompatible. It was also found that *E. coli* brought a change in the physical property of the b-cGG samples, which became liquid at the end of 48 h indicating its complete degradation. This change in the physical property can be attributed to the corresponding change in the chemical properties of the samples. The physical property of the b-cGG changed during the course of the study indicating that the metabolites produced by the *E. coli* was able to bring some chemical change in the b-cGG network. The results indicate that the gelatin-based suppositories may be used for the treatment of BV.



**Figure 7. (a), (b) and (c) showing the biodegradability of control sample by *E. coli*, (a) after 12 hours, (b) after 24 hours and (c) after 48 hours**



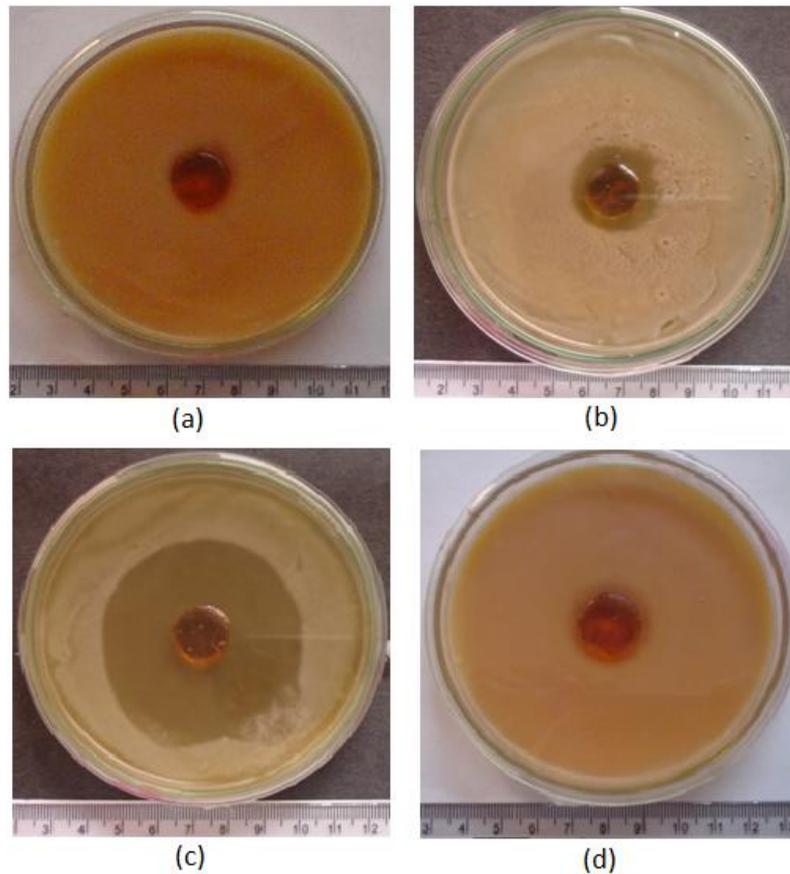
**Figure 8. Comparative antimicrobial study on *E. coli*. (A) MZ-cGG\_1 and (B) Metrozyl®**

An antimicrobial comparative study was carried against *E. coli* so as to judge the activity of the prepared metronidazole loaded suppository as against metrozyl®, a marketed metronidazole formulation. Metrozyl® consists of metronidazole at a concentration of 10 mg/gm of gel. To carry out the comparative study, gelatin based samples were prepared which contained metronidazole at the same concentration and were named as MZ-cGG\_1. (The experimental results showed that MZ-cGG\_1 created greater zone of inhibition as against metrozyl® (**figure 8**). This indicated that MZ-cGG\_1 is more effective compared to metrozyl®.

#### **4.4 Antimicrobial study against *L. acidophilus***

The effect of suppositories on lactobacilli were also analyzed (**figure 9, table 3**). The results showed that b-cGG samples do not have any detrimental effect on lactobacilli and may be used for the development of biopolymeric suppositories. This result is also a support for the previous results in which lactobacilli was entrapped within the crosslinked gelatin samples and did not show any detrimental effect on lactobacilli. The zone of inhibition in the presence of MZ-cGG sample was found to be  $25 \pm 3$  mm at the end of 18 h. On further incubation, the zone of inhibition was found to be  $14 \pm 4$  mm at the end of 36 h. This might be attributed to the improved resistance of the *Lactobacillus* to metronidazole. To confirm the same, the old samples of MZ-cGG were replaced with fresh samples and were further incubated for 12 h. It was found that *Lactobacillus* was able to grow towards the sample and finally showed no zone of inhibition. This confirmed that the *Lactobacillus* became resistant to metronidazole

and were able to grow even in the presence of metronidazole, when administered along with the antimicrobial. CF-cGG samples showed an inhibition zone of 55 mm, which did not recover from the ciprofloxacin shock indicating that the ciprofloxacin samples cannot be used for the treatment of BV, where the restoration of *Lactobacillus* colonies within the vagina is a must.



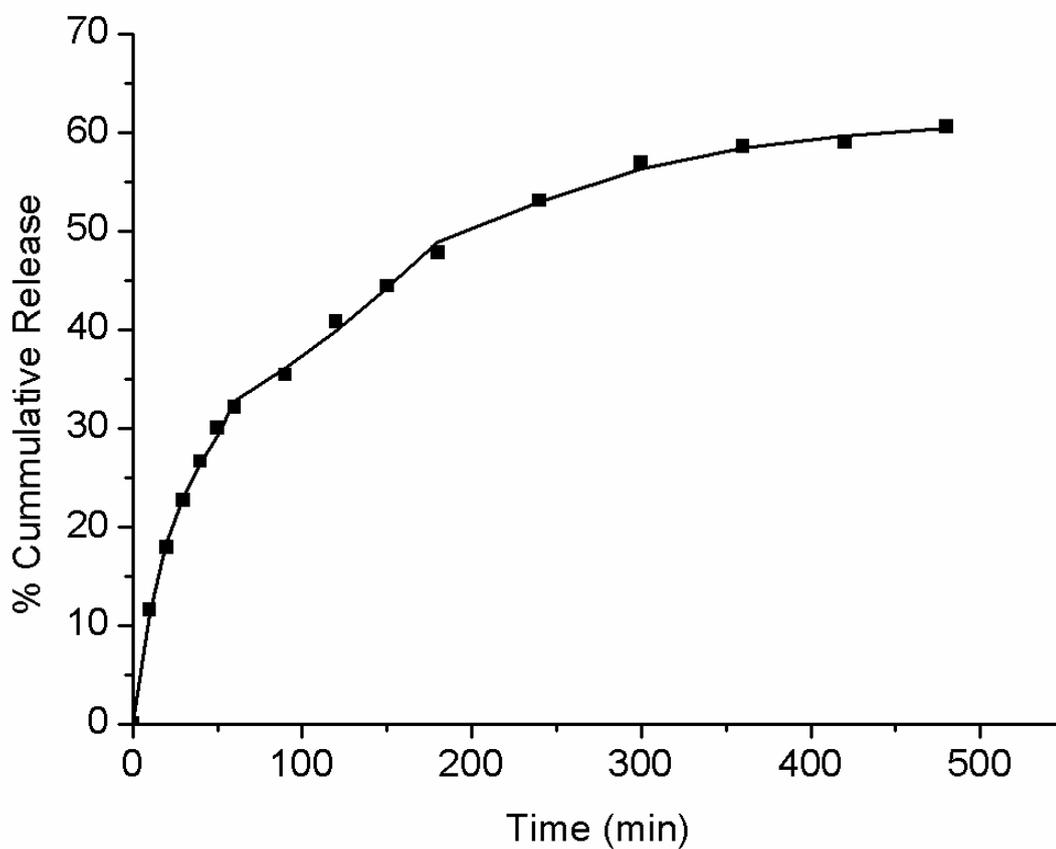
**Figure 9. Antimicrobial activity against *Lactobacillus acidophilus* (a) b-cGG sample (control) (b) MZ-cGG samples (18 h incubation) (c) CF-cGG samples (18 h incubation) (d) MZ-cGG samples (36 h incubation)**

**Table 3. Antimicrobial activity against *L. acidophilus***

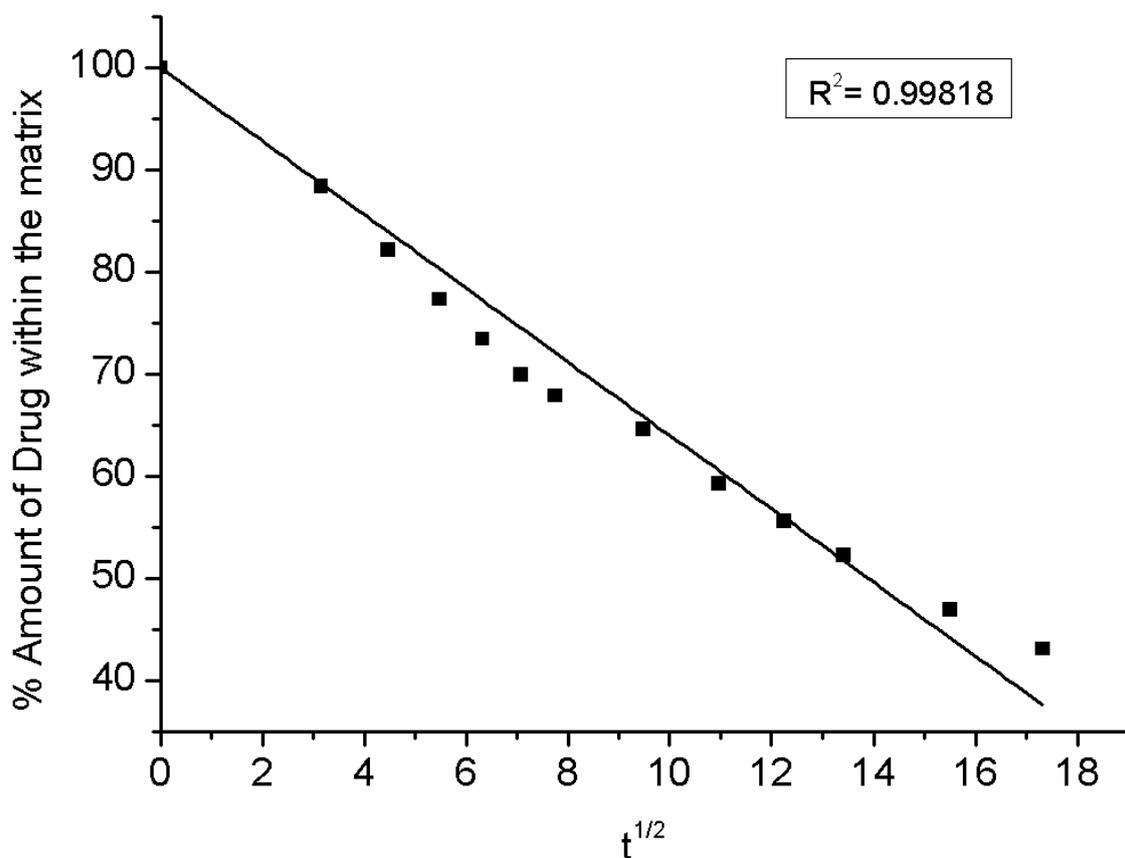
<b>Sample</b>	<b>Zone of inhibition: 18 h incubation (mm)</b>	<b>Zone of inhibition: 36 h incubation (mm)</b>
<b>b-cGG</b>	<b>0</b>	<b>0</b>
<b>CF-cGG</b>	<b>55 ± 8</b>	<b>55 ± 6</b>
<b>MZ-cGG</b>	<b>25 ± 3</b>	<b>14 ± 4</b>

#### 4.5 Drug release study

The release profile of metronidazole from MZ-cGG is shown in **figure 10**. The study was carried out for a period of 8 h. The results indicate an initial burst release of metronidazole up to 1 h. Thereafter, the rate of release of the drug became slower. At the end of 8 h, it was found that only 60 % drug was released from the MZ-cGG sample indicating a sustained release of metronidazole from the crosslinked gelatin matrix.



**Figure 10. Release profile of metronidazole from MZ-cGG sample.**



**Figure 11. Release kinetics of metronidazole from MZ-cGG sample.**

The release kinetics of the metronidazole from the gelatin matrix showed Higuchian kinetics indicating diffusion controlled release of metronidazole (**figure 11**). This model of release kinetics is followed by the matrix type delivery system in which the drug is homogeneously distributed throughout the polymer matrix of the delivery system. When the delivery matrix is bathed in the dissolution solution, the drug present in the outermost layer is dissolved and subsequently diffuses out of the delivery matrix. As the outermost layer gets depleted, the interface between the dissolution solution and the solid drug moves towards the core of the delivery system until the entire drug diffuses out of the delivery system

#### 4.6 Mucoadhesive analysis-



**Figure 12. b-cGG sample adhered to the intestinal mucosa of goat**

The mucoadhesive study (**figure 12**) showed that the b-cGG sample was adhered to the intestinal mucosa for a period of 5 h. The result indicates that the developed products may be used successfully as a mucoadhesive delivery system. The vagina, which has a mucosal lining, may serve as an excellent biological substrate for the b-cGG and may be used successfully for delivering the drug into the vaginal lumen for a prolonged period.

# *Chapter 5*

## **Conclusion**

## 5. Conclusion

In the current study, successful attempts were made to develop biopolymeric suppositories for the treatment of BV. The formulations were tested under *in vitro* conditions on *E. coli*, taken as a model causative organism. The suppository were loaded with various bioactive agents (viz. lactic acid, metronidazole, ciprofloxacin and lactobacilli), either alone or in combination. Suppositories without any bioactive agents served as control. Ciprofloxacin containing suppositories showed to inhibit lactobacilli to a greater extent and were not found to be suitable for the treatment of BV. All other bioactive agents, when used alone or in combinations, showed beneficial effects to different extents and may be tried in the treatment of BV. The delivery system was found to be biodegradable in the presence of *E. coli* and may be used biodegradable delivery system. Suppository samples containing lactobacilli cells, except the sample containing a combination of lactic acid, metronidazole and lactobacilli, showed the growth of lactobacilli in the inhibition zone indicating their ability to recolonize the vaginal lumen. The release of metronidazole from the suppository indicated sustained delivery for a prolonged period and the release was found to be diffusion controlled. The suppository samples showed sufficient mucoadhesive property to be regarded as mucoadhesive delivery system. In short, the developed delivery systems may be used as a mucoadhesive delivery system for the delivery of bioactive agents in the vaginal lumen for the treatment of BV.

# References

## References

Abee, T., T. Klaenhammer, et al. (1994). "Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane." Applied and Environmental Microbiology **60**(3): 1006.

Adegboye, T. and O. Itiola "Physical and Release Properties of Metronidazole Suppositories." Tropical Journal of Pharmaceutical Research **7**(1): 887-896.

Afrakhteh, M. and A. Mahdavi (2007). "Bacterial vaginosis and urinary tract infection." J Obstet Gynecol India **Vol. 57**(6): 513-516.

Andersen, P. H., A. P. Bucher, et al. (1994). "Faecal enzymes: in vivo human skin irritation." Contact Dermatitis **30**(3): 152-158.

Anukam, K., E. Osazuwa, et al. (2006). "Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial." Microbes and Infection **8**(6): 1450-1454.

Anukam, K., M. PhD, et al. (2008). "Probiotics: 100 years (1907-2007) after Elie Metchnikoff's Observation." Kingsley C Anukam: 21.

Aroutcheva, A., D. Gariti, et al. (2001). "Defense factors of vaginal lactobacilli." American Journal of Obstetrics and Gynecology **185**(2): 375-379.

Aruna, K. and K. Jyoti (2007). "**Role of bacterial vaginosis in preterm labor.**" J Obstet Gynecol India **57**(5): 413-416.

Bartlett, G., J. , Onderdonk , B, A., et al (1977). "Quantitative Bacteriology of the Vaginal Flora." The Journal of Infectious Diseases **136**(2): 271-277.

Bartlett, G. J., B. A. Onderdonk, et al. (1977). "Quantitative Bacteriology of the Vaginal Flora." The Journal of Infectious Diseases **136**(2 ): 271-277

Beigi, R., M. Austin, et al. (2004). "Antimicrobial resistance associated with the treatment of bacterial vaginosis." American journal of obstetrics and gynecology **191**(4): 1124-1129.

Bernet, M. F., D. Brassart, et al. (1993). "Adhesion of human bifidobacterial strains to cultured human intestinal epithelial cells and inhibition of enteropathogen-cell interactions." Appl. Environ. Microbiol. **59**(12): 4121-4128.

Bhalla, P. C., R. Garg, S. (2007). "Prevalence of bacterial vaginosis among women in Delhi, India." Indian J Med Res **125**(February): 167-172.

Bibel, D. J., R. Aly, et al. (1987). "Microbial adherence to vulvar epithelial cells." J Med Microbiol **23**(1): 75-82.

Bonferoni, M., P. Giunchedi, et al. (2006). "Chitosan gels for the vaginal delivery of lactic acid: Relevance of formulation parameters to mucoadhesion and release mechanisms." AAPS PharmSciTech **7**(4): E141-E147.

Boris, S., J. Suarez, et al. (1998). "Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens." Infection and Immunity **66**(5): 1985.

Boris, S., J. E. Suarez, et al. (1998). "Adherence of Human Vaginal Lactobacilli to Vaginal Epithelial Cells and Interaction with Uropathogens." Infect. Immun. **66**(5): 1985-1989.

Brook, I. (1999). "Bacterial Interference." Critical Reviews in Microbiology **25**(3): 155-172.

Burton, J. P., P. A. Cadieux, et al. (2003). "Improved understanding of the bacterial vaginal microbiota of women before and after probiotic instillation." Applied and Environmental Microbiology **69**(1): 97-101.

Busscher, H. J. and A. H. Weerkamp (1987). "Specific and non-specific interactions in bacterial adhesion to solid substrata." FEMS Microbiology Letters **46**(2): 165-173.

Casadevall, A. and L.-a. Pirofski (2000). "Host-Pathogen Interactions: Basic Concepts of Microbial Commensalism, Colonization, Infection, and Disease." Infect. Immun. **68**(12): 6511-6518.

Castelo-Branco, C., M. J. Cancelo, et al. (2005). "Management of post-menopausal vaginal atrophy and atrophic vaginitis." Maturitas **52**(SUPPL. 1): S46-S52.

Cavallo, G. (1987). "Vaginal microbial flora and infectious pathology." La flora microbica vaginale e la patologia infettiva. **80**(1-12): 277-295.

Chimura, T., T. Funayama, et al. (1995). "Ecological treatment of bacterial vaginosis." The Japanese journal of antibiotics **48**(3): 432.

Coconnier, M. H., T. R. Klaenhammer, et al. (1992). "Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture." Appl. Environ. Microbiol. **58**(6): 2034-2039.

Conway, P. L. and S. Kjelleberg (1989). "Protein-mediated Adhesion of *Lactobacillus fermentum* Strain 737 to Mouse Stomach Squamous Epithelium." J Gen Microbiol **135**(5): 1175-1186.

Darwish, A., E. M. Elnshar, et al. (2007). "Treatment options for bacterial vaginosis in patients at high risk of preterm labor and premature rupture of membranes." Journal of Obstetrics and Gynaecology Research **33**(6): 781-787.

Decena, D., J. Co, et al. (2006). "Metronidazole with Lactacyd vaginal gel in bacterial vaginosis." Journal of Obstetrics and Gynaecology Research **32**(2): 243-251.

Delia, A., G. Morgante, et al. (2006). "Effectiveness of oral administration of *Lactobacillus paracasei* subsp. *paracasei* F19 in association with vaginal suppositories of *Lactobacillus acidophilus* in the treatment of vaginosis and in the prevention of recurrent vaginitis." Minerva ginecologica **58**(3): 227.

Dickey, L., M. Nailor, et al. (2009). "Guidelines for the treatment of bacterial vaginosis: focus on tinidazole."

Donati, L., A. Di Vico, et al. (2010). "Vaginal microbial flora and outcome of pregnancy." Archives of Gynecology and Obstetrics **281**(4): 589-600.

Dunkelberg Jr, W. (1965). "DIAGNOSIS OF HEMOPHILUS VAGINALIS VAGINITIS BY GRAM-STAINED SMEARS." American Journal of Obstetrics and Gynecology **91**: 998.

Elias, P. (2007). "The skin barrier as an innate immune element." Seminars in Immunopathology **29**(1): 3-14.

Eschenbach, D. A., P. R. Davick, et al. (1989). "Prevalence of hydrogen peroxide-producing Lactobacillus species in normal women and women with bacterial vaginosis." J. Clin. Microbiol. **27**(2): 251-256.

Eschenbach, D. A., S. S. Thwin, et al. (2000). "Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora." Clinical Infectious Diseases **30**(6): 901-907.

Faergemann, J., R. Aly, et al. (1983). "Skin occlusion: Effect on Pityrosporum orbiculare, skin, pH, transepidermal water loss, and water content." Archives of dermatological research **275**(6): 383-387.

Farage, M., M. Bramante, et al. (2007). "Do panty liners promote vulvovaginal candidiasis or urinary tract infections?: A review of the scientific evidence." European Journal of Obstetrics & Gynecology and Reproductive Biology **132**(1): 8-19.

Farage, M. and H. Maibach (2006). "Lifetime changes in the vulva and vagina." Archives of Gynecology and Obstetrics **273**(4): 195-202.

Fontaine, E. A. and D. Taylor-Robinson (1990). "Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacilli." Journal of Applied Bacteriology **69**(3): 326-331.

Fredricks, D., T. Fiedler, et al. (2005). "Molecular identification of bacteria associated with bacterial vaginosis." The New England journal of medicine **353**(18): 1899.

Freinkel, R. K. and Y. Shen (1969). "The origin of free fatty acids in sebum. II. Assay of the lipases of the cutaneous bacteria and effects of pH." Journal of Investigative Dermatology **53**(6): 422-427.

Granato, D., F. Perotti, et al. (1999). "Cell Surface-Associated Lipoteichoic Acid Acts as an Adhesion Factor for Attachment of *Lactobacillus johnsonii* La1 to Human Enterocyte-Like Caco-2 Cells." Appl. Environ. Microbiol. **65**(3): 1071-1077.

Hamdan, I. and E. Mikolajcik (1974). "Acidolin: an antibiotic produced by *Lactobacillus acidophilus*." The Journal of Antibiotics **27**(8): 631-636.

Hammerschlag, M. R., S. Alpert, et al. (1978). "Anaerobic microflora of the vagina in children." American Journal of Obstetrics and Gynecology **131**(8): 853-856.

Hartemink, R., V. R. Domenech, et al. (1997). "LAMVAB--A new selective medium for the isolation of lactobacilli from faeces." Journal of Microbiological Methods **29**(2): 77-84.

Hawthorn, L. A., A. W. Bruce, et al. (1991). "Ability of uropathogens to bind to Tamm Horsfall protein-coated renal tubular cells." Urological Research **19**(5): 301-304.

Hill, G. B. (1993). "The microbiology of bacterial vaginosis." American Journal of Obstetrics and Gynecology **169**(2 II SUPPL.): 450-454.

Hillier, S. (2005). "The complexity of microbial diversity in bacterial vaginosis." New England Journal of Medicine **353**(18): 1886.

Hillier, S. L. (2005). "The complexity of microbial diversity in bacterial vaginosis." New England Journal of Medicine **353**(18): 1886-1887.

Hillier, S. L., Krohn, A. M., Rabe, K. L., Klebanoff, J. S., Eschenbach, A. D (1993). "The Normal Vaginal Flora, H<sub>2</sub>O<sub>2</sub>-Producing Lactobacilli, and Bacterial Vaginosis in Pregnant

Women." Clinical Infectious Diseases, Proceedings of the First North American Congress on Anaerobic Bacteria and Anaerobic Infections **16**(4): S273-S281

Hillier, S. L., R. P. Nugent, et al. (1995). "Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant." New England Journal of Medicine **333**(26): 1737-1742.

Hillier, S. L., R. P. Nugent, et al. (1995). "Association between Bacterial Vaginosis and Preterm Delivery of a Low-Birth-Weight Infant." N Engl J Med **333**(26): 1737-1742.

Hughes, V., L. and S. Hillier, L. (1990). "Microbiologic Characteristics of Lactobacillus Products Used for Colonization of the Vagina." Obstetrics & Gynecology **75**(2): 244-248.

Joesoef, M., G. Schmid, et al. (1999). "Bacterial vaginosis: review of treatment options and potential clinical indications for therapy." Clinical Infectious Diseases **28**(S1): 57-65.

Juarez Tomas, M. S., V. S. Ocana, et al. (2003). "Growth and lactic acid production by vaginal Lactobacillus acidophilus CRL 1259, and inhibition of uropathogenic Escherichia coli." J Med Microbiol **52**(12): 1117-1124.

Kabara, J. J., M. D. Swieczkowski, et al. (1972). "Fatty Acids and Derivatives as Antimicrobial Agents." Antimicrobial Agents and Chemotherapy, American Society for Microbiology **Vol. 2**, (1): 23-28.

Kale, V. V., R. V. Trivedi, et al. (2005). Development and Evaluation of a Suppository Formulation Containing Lactobacillus and Its Application in Vaginal Diseases, ANNALS NEW YORK ACADEMY OF SCIENCES. **1056**: 359-365.

Kamarashev, J. A. and S. G. Vassileva (1997). "Dermatologic diseases of the vulva." Clinics in Dermatology **15**(1): 53-65.

Klebanoff, M. A., T. R. Nansel, et al. (2010). "Personal hygienic behaviors and bacterial vaginosis." Sexually Transmitted Diseases **37**(2): 94-99.

Lachlak, N., E. Ageron, et al. (1996). "Composition of the *Lactobacillus acidophilus* complex isolated from vaginal flora." New Microbiologica **19**(2): 123-132.

Lepargneur, J. P. and V. Rousseau (2002). "Protective role of the Doderlein flora." Rôle protecteur de la flore de Doderlein **31**(5): 485-494.

Lina, G., F. Boutite, et al. (2003). "Bacterial Competition for Human Nasal Cavity Colonization: Role of Staphylococcal agr Alleles." Appl. Environ. Microbiol. **69**(1): 18-23.

Lowbury, E. J. L., H. A. Lilly, et al. (1964). "Disinfection of Hands: Removal of Transient Organisms." Br Med J **2**(5403): 230-233.

McGregor, J., J. French, et al. (1994). "Bacterial vaginosis is associated with prematurity and vaginal fluid mucinase and sialidase: results of a controlled trial of topical clindamycin cream." American Journal of Obstetrics and Gynecology **170**(4): 1048.

Mclean, N. and I. Rosenstein (2000). "Characterisation and selection of a *Lactobacillus* species to re-colonise the vagina of women with recurrent bacterial vaginosis." Journal of medical microbiology **49**(6): 543.

Mitchell, C., J. Hitti, et al. (2009). "Comparison of oral and vaginal metronidazole for treatment of bacterial vaginosis in pregnancy: impact on fastidious bacteria." BMC Infectious Diseases **9**(1): 89.

Neeser, J.-R., D. Granato, et al. (2000). "Lactobacillus johnsonii La1 shares carbohydrate-binding specificities with several enteropathogenic bacteria." Glycobiology **10**(11): 1193-1199.

Neri, A., G. Sabah, et al. (1993). "Bacterial vaginosis in pregnancy treated with yoghurt." Acta Obstetrica et Gynecologica Scandinavica **72**(1): 17 - 19.

Nieman, C. (1954). "Influence of trace amounts of fatty acids on the growth of microorganisms." Microbiology and Molecular Biology Reviews **18**(2): 147.

Nighswonger, B. D., M. M. Brashears, et al. (1996). "Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in Fermented Milk Products During Refrigerated Storage." J. Dairy Sci. **79**(2): 212-219.

Nyirjesy, P., M. McIntosh, et al. (2006). "The effects of intravaginal clindamycin and metronidazole therapy on vaginal lactobacilli in patients with bacterial vaginosis." American journal of obstetrics and gynecology **194**(5): 1277-1282.

, et al. (2001). "Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells." Journal of Infectious Diseases **183**(3): 485-491.

Otero, C. M. and E. M. Nader-Macías (2007). "Lactobacillus adhesion to epithelial cells from bovine vagina." Communicating Current Research and Educational Topics and Trends in Applied Microbiology **Vol. 2**: 749-757.

Ouattara, B., R. E. Simard, et al. (1997). "Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms." International Journal of Food Microbiology **37**(2-3): 155-162.

Ouwehand, A., S. Salminen, et al. (2002). "Probiotics: an overview of beneficial effects." Antonie van Leeuwenhoek **82**(1): 279-289.

Owen, J., Jr (1975). "Physiology of the menstrual cycle." Am J Clin Nutr **28**(4): 333-338.

Patton, D. L., S. S. Thwin, et al. (2000). "Epithelial cell layer thickness and immune cell populations in the normal human vagina at different stages of the menstrual cycle." American Journal of Obstetrics and Gynecology **183**(4): 967-973.

Petrova, P., K. Petrov, et al. (2007). "Probiotic properties of Bulgarian vaginal lactobacillus isolates." Comptes Rendus de L'Academie Bulgare des Sciences **60**(8): 871-878.

Pommerenke, W. T. and P. W. Taylor Jr (1953). "Antibacterial properties of vaginal and cervical secretions and amniotic fluid." Annali di ostetricia e ginecologia **75**(6): 891-899.

Reid, G. (2001). "Probiotic agents to protect the urogenital tract against infection." American Journal of Clinical Nutrition **73**(2 SUPPL.): 437S-443S.

Reid, G. and A. Bocking (2003). "The potential for probiotics to prevent bacterial vaginosis and preterm labor." American journal of obstetrics and gynecology **189**(4): 1202-1208.

Reid, G., A. W. Bruce, et al. (2001). "Oral probiotics can resolve urogenital infections." FEMS Immunology and Medical Microbiology **30**(1): 49-52.

Reid, G. and J. Burton (2002). "Use of Lactobacillus to prevent infection by pathogenic bacteria." Microbes and Infection **4**(3): 319-324.

Reid, G., J. Jass, et al. (2003). "Potential Uses of Probiotics in Clinical Practice." Clinical Microbiology Reviews **16**(4): 658-672.

Ryckman, K. K., H. N. Simhan, et al. (2009). "Predicting risk of bacterial vaginosis: the role of race, smoking and corticotropin-releasing hormone-related genes." Mol. Hum. Reprod. **15**(2): 131-137.

Sherman, L. A. and D. C. Savage (1986). "Lipoteichoic acids in Lactobacillus strains that colonize the mouse gastric epithelium." Appl. Environ. Microbiol. **52**(2): 302-304.

Shi, Y., L. Chen, et al. (2009). "Preliminary characterization of vaginal microbiota in healthy Chinese women using cultivation-independent methods." Journal of Obstetrics and Gynaecology Research **35**(3): 525-532.

Sieber, R. and U. Dietz (1998). "Lactobacillus acidophilus and yogurt in the prevention and therapy of bacterial vaginosis." International Dairy Journal **8**(7): 599-607.

Simoës, J., A. Aroutcheva, et al. (2001). "Effect of metronidazole on the growth of vaginal lactobacilli in vitro." Infectious Diseases in Obstetrics & Gynecology **9**(1): 41-45.

Simoes, J., D. Citron, et al. (2002). "Two novel vaginal microbicides (polystyrene sulfonate and cellulose sulfate) inhibit Gardnerella vaginalis and anaerobes commonly associated with bacterial vaginosis." Antimicrobial agents and chemotherapy **46**(8): 2692.

Sobel, J., D. Ferris, et al. (2006). "Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis." American Journal of Obstetrics and Gynecology **194**(5): 1283-1289.

Spiegel, C. (1991). "Bacterial vaginosis." Clinical Microbiology Reviews **4**(4): 485.

Stoyancheva, G., S. Danova, et al. (2006). "Molecular identification of vaginal lactobacilli isolated from Bulgarian women." Antonie van Leeuwenhoek **90**(3): 201-210.

Taha, T., D. Hoover, et al. (1998). "Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV." Aids **12**(13): 1699.

Tasdemir, M., I. Tasdemir, et al. (1996). "Alternative treatment for bacterial vaginosis in pregnant patients; restoration of vaginal acidity and flora." Archives of AIDS research **10**(4): 239.

Vasquez, A., T. Jakobsson, et al. (2002). "Vaginal Lactobacillus Flora of Healthy Swedish Women." J. Clin. Microbiol. **40**(8): 2746-2749.

Wagner, G. and B. Ottesen (1982). "Vaginal physiology during menstruation." Annals of Internal Medicine **96**(6 II): 921-923.

Wang, J. (2000). "Bacterial vaginosis. Primary Care Update for OB/GYNS." Primary Care Update for OB/GYNS **7**(5): 181-185.

Warren, R., A. Bauer, et al. (2005). "Transepidermal water loss dynamics of human vulvar and thigh skin." Skin Pharmacology and Physiology **18**(3): 139-143.

Xu, G. (1997). "Gram stain." Retrieved 18 oct, 2009, from [http://www.ups.upenn.edu/bugdrug/antibiotic\\_manual/gram.htm](http://www.ups.upenn.edu/bugdrug/antibiotic_manual/gram.htm).

Zasloff, M. (2002). "Antimicrobial peptides of multicellular organisms." Nature **415**(6870): 389-395.

Zhou, X., S. J. Bent, et al. (2004). "Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods." Microbiology **150**(8): 2565-2573.