# CHAPTER 3

To assess the microbial flora from vaginal swabs of healthy reproductive-aged women (21-45 y).

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## 3.1. Introduction

Microbial flora of a particular niche is established by the survival of the microorganism from predation and environmental selection [370]. The vaginal canal (VC), the opening of the FRT harbours a unique microbial flora post puberty governed by multiple factors like: menstruation, copulation, parturition, and habitual behaviour. These factors occasionally influence the vaginal microflora negatively, making it prone to disturbance [194]. Vaginitis or vaginal infection is inflammation caused by microbes through invasion and/or multiplication. Pathogenic microbes either straight away cause infection behaving as agents or maintaining commensalism within the host escalating only under suitable circumstances as opportunistic pathogens [371]. These microorganisms attach and enter the vaginal epithelium cells eventually causing lysis. Through quorum sensing they produce biofilm and toxins [263]. They also evade the primary and secondary immune cells and induce the production of interleukins (IL6 and IL8) in the reproductive tract causing inflammation [372]. The residential pathogens of VC can be divided into two broad classes: aerobic and anaerobic [373]. Pathogens like *Atopobium vaginae* and *Gardnerella vaginalis* are causative organisms of bacterial vaginosis (BV) and grow only under anaerobic conditions [374]. On the other hand, pathogens that proliferate in both aerobic and anaerobic environments may enter the VC from the external environment initiating vaginal infections like aerobic vaginitis (AV) [106] and vulvovaginal candidacies (VVC) [375]. The quantitative overgrowth of these residential urogenital organisms causes vaginitis, which under healthy conditions is controlled by the high population of LAB in VC [376].

LAB encompasses a range of microorganisms that colonize soil, plants, animals, and humans [377]. Microbial study on LAB commonly known as “lactis” started around 1850 for its unique feature of adding flavour to edibles like food and beverage [378]. Further, their role in maintaining or improving human health has been studied since the early

1900's. These bacteria are abundantly found in the oral, gastrointestinal, and VC of humans and have been given the status of generally regarded as safe (GRAS) [379]. In 1890 Albert S Doderlein first discovered the presence of *Lactobacillus* in VC of women and thereafter their role has been studied thoroughly. LAB inhabit the VC post puberty, accounting for a minimum 70-95 % of the microbial population [380]. *Lactobacilli* sp. is generally acidophilic and halophilic in nature as a course of their evolution. The species that solely produce lactic acid through fermentation are referred to as homo-fermenters, whereas organisms that produce aldehydes and di-acetyl with lactic acid are called hetero-fermenters [381]. The absence of heme and menaquinone synthesizing genes hampers the respiratory pathway of these microorganisms, although few species have evolved oxygen tolerance over the centuries. One of the known mechanisms to tolerate oxygen is to use elements like zinc, selenium, and manganese for oxygen scavenging. Thus, these microbes range from being anaerobic to microaerophiles preferring the pathway of fermentation against respiration [382]. The fermentation pathway generates low energy in these microorganisms compelling them to live as symbionts or parasites [383].

## 3.2. Materials and Methods

### 3.2.1. Chemicals Used

- Cotton swab (Human Diagnostics and Surgichem an ISO certified Company, India)
- Luria Bertani Agar/ Broth (HiMedia, India)
- MacConkey agar (HiMedia, India)
- Potato dextrose agar (HiMedia, India)
- Yeast malt agar (HiMedia, India)
- Nutrient Agar/Broth (HiMedia, India)
- Lactic acid bacteria selective agar base (HiMedia, India)
- *Lactobacillus* MRS Agar/ Broth (HiMedia, India)
- Sodium hydroxide (HiMedia, India)
- Phenol: Chloroform: Isoamyl alcohol (HiMedia, India)
- RNase and Proteinase K (HiMedia, India)
- NaCl, Tris-EDTA, Triton X, SDS (HiMedia, India)
- Agarose (HiMedia, India)
- Primers: 27F and 1392R (IDT, India)

- PCR components: Reaction buffer (10X), dNTP (0.5 mM), Taq DNA polymerase (1 unit) (HiMedia, India)
- Gene JET gel extraction kit (ThermoFisher, India)

### 3.2.2. Instruments Used

- Thermo Multiskan Go, Thermo Scientific, India
- PCR thermal cycler, T100 Thermal Cycler, Bio-Rad, India
- Sanger sequencing (Eurofins, Bengaluru)

### 3.2.3. Collection of vaginal swabs

Tezpur University Ethical Committee (TUEC), Tezpur University (Central), Assam, India had given the approval for sample collection from consented women with Registration No: DORD/TUEC/PROP/2022/02-R2. Vaginal swabs were collected from healthy women who visited Tezpur University Health Center on request or for routine checkup. Women of age group (21-45 y), provided written informed consent after reading the patient information sheet in accordance with ICMR ethical guidelines. Medical history of volunteers was recorded; participants who were not under any antibiotic regime were selected. Aseptic swab (5 cm) length was used by the gynecologist to collect vaginal swabs from the external genitalia. The samples were placed on sealed dry closed tubes in melting ice.

### 3.2.4. Isolation of aerobic microbes

The isolation of microbes was performed by serial dilution method (up to $10^{-5}$ dilution). The swabs were immersed in sterile distilled water (1 ml) in a falcon tube and homogenized by vortexing for 1 min. The homogenate was spread on plates of Luria Bertani agar and nutrient agar for growth of general organisms, MacConkey agar for growth of coliforms, potato dextrose agar and yeast malt agar for growth of yeast and fungi. The plates were incubated aerobically for 24-48 h at 37 ℃. The morphologically distinct bacterial colonies were picked using a sterile tooth pick and grown on nutrient broth for 24 h at 37 ℃ to acquire pure culture [384].

### 3.2.5. Selection of potential aerobic microbes

3.2.5.1. *Selection of acidophilic microbes*

The pH of nutrient broth was reduced 6>5>4 by adding aliquots of hydrochloric acid (0.1 N). Fresh inoculum of the isolates were added to the acidic broth and incubated for 24 h at 37 ℃ [385].

3.2.5.2. *Selection of acid producing microbes*

Litmus milk broth with litmus is an indicator for acid production was used to check the acid producing ability of the microbes. Fresh inoculum of the isolates was added and incubated for 12-24 h at 37 ℃. Changing color of media from deep purple to white indicated acid production by the isolates [386].

3.2.5.3. *Morphological and biochemical characterization*

Biochemical characterization was done by performing Gram's, Oxidase, Catalase, Methyl red, Voges Proskauer, Indole, Hydrogen sulfide, and Citrate tests. The morphology of the isolates was also studied and recorded [387].

## 3.2.6. Isolation of genomic DNA from aerobic microbes

For genomic DNA isolation, all isolates were grown in 2 ml of nutrient broth for 24 h at 37 ℃. Bacterial DNA isolation method by [388] was improvised: cell lysis solution (100 mM NaCl, 10 mM Tris, 1 mM EDTA, 2% triton X, 1% SDS) was used to lyse the bacterial cell wall for 30 min. RNase and proteinase K, were added to the lysate and incubated at room temperature for 10 min each. This was followed by adding phenol: chloroform: isoamyl alcohol in 1:1 ratio to the lysate and centrifuged at 13,000 rpm. The organic phase was collected and precipitated with 100% ethanol overnight. The DNA was pelleted by centrifugation at 13,000 rpm. The pellet was washed with 70% ethanol and then was dissolved in TE buffer. The purity and concentration of the genomic DNA was checked spectrophotometrically at 260/280 nm and the samples were run in 0.8% agarose gel to check the quality of DNA [389].

## 3.2.7. PCR for amplification of universal gene

The universal bacterial primers 27F and 1392R and fungal primer NL1 and NL4 were used for amplification of 16s and 18s rDNA gene region [390]. The PCR mix was prepared and a program was run (Table 1 and 2). The PCR product was visualized using 1.2% agarose gel [391].

## 3.2.8. Collection of vaginal swabs

Aseptic swab of (12 cm) length was used by the gynecologist to collect vaginal swabs from healthy women of age group (21-45 y). Care was taken to avoid contact of the external genitalia. The samples were kept in sealed dry closed tubes on melting ice.

### 3.2.9. Isolation of LAB

Lactic acid bacteria selective agar base was used for specific isolation of LAB. Cycloheximide was added to the media for inhibition of fungal growth. The cotton head of the swab was rubbed on the agar plates and incubated at 37 ℃ aerobically and anaerobically for 48-72 h [392].

### 3.2.10. Selection of LAB

Selection of LAB was done through gram test and catalase test [393]. The colony morphology of the isolates was noted as well.

### 3.2.11. PCR for amplification of universal gene

The LAB isolates were grown in *Lactobacillus* MRS agar anaerobically for 48 h at 37 ℃. Colonies were picked up with sterile loop and mixed with sodium hydroxide (20 mM). A thermal PCR cycle of 10 min at 95 ℃ was run. Universal primers 27F and 1392R was used to amplify 16s rDNA. PCR was performed following (Table 1 and 2) [390] [391].

### 3.2.12. Sequencing and Identification

The amplified PCR products were run in 1.2% agarose gel. Gene JET gel extraction kit was used to purify the PCR product from the agarose gel and sent for sanger sequencing. The forward and reverse sequences were aligned using BLASTn software and the isolates were identified [394].

**Table 1:** Thermal Cycle of PCR for Universal 16s and 18s rDNA gene

| **Steps** | **Temperature (°C)** | **Time** | **Cycle** |
|---|---|---|---|
| Initial Denaturation | 95 | 300 | 1 |
| Denaturation | 95 | 30 | 35 |
| Annealing | 54 | 30 | |
| Extension | 72 | 105 | |
| Final Extension | 72 | 300 | 1 |

**Table 2:** Components of PCR master mix

| **Components** | **Total Volume (50 µl)** |
|---|---|
| Buffer with $MgCl_2$ (10x) | 5 µL |
| Forward Primer (0.5 µM) | 2.5 µL |
| Reverse primer (0.5 µM) | 2.5 µL |
| DNA Pol (I Unit) | 0.5 µL |
| Genomic DNA (100 ηg/ µL) | 2 µL |
| Nuclease free water | 37.5 µL |

## 3.3. Results

Seventy-four isolates were obtained, using the serial dilution method from the vaginal swabs. Forty-five isolates out of seventy-four were able to grow at low pH (Fig 1). All forty-five isolates were able to ferment litmus milk broth and produce acid (Fig 2). The genomic DNA of the isolates were isolated and the 16s and 18s rDNA PCR products were visualized (Fig 3) The isolates were identified as *Enterobacter cloacae, Escherichia fergusonii, Enterococcus faecalis, Staphylococcus epidermidis, Shigella* sp., and *Candida albicans* (Fig 4). A total of fifteen isolates were isolated on the selective lactic acid agar media, among which only five (LC, LG, LG2, LV, and LV2) isolates were gram-positive and catalase negative bacilli (Fig. 5). These isolates were strict anaerobes and specifically grew in *Lactobacillus* MRS broth. The colony morphology of the isolates were recorded (Table 3). Colony PCR of the isolates using 27F and 1392R primers produced a band of ~ 1500 bp, (Fig. 6). Post sequencing the isolates were identified as *L. crispatus*, *L. gasseri*, and *L. vaginalis* (Table 4) (Fig 7).

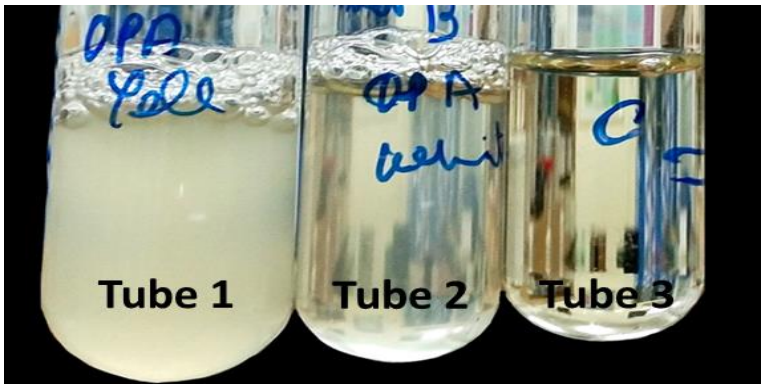


**Fig 1:** Nutrient broth of pH 4 inoculated with potential pathogens (Tube 1- growth, Tube 2- No growth, and Tube 3- Blank)

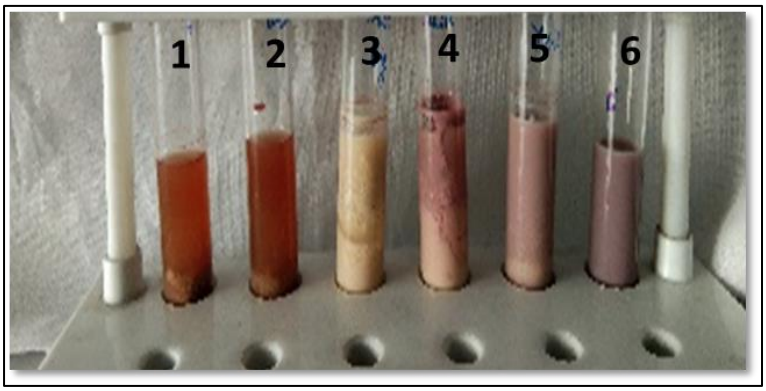


**Fig 2:** Litmus milk broth inoculated with potential pathogens (Tube 1 and 2 Complete Fermentation), (Tube 3 Fermentation), (Tube 4 Partial fermentation) (Tube 5 No fermentation), (Tube 6 Control).

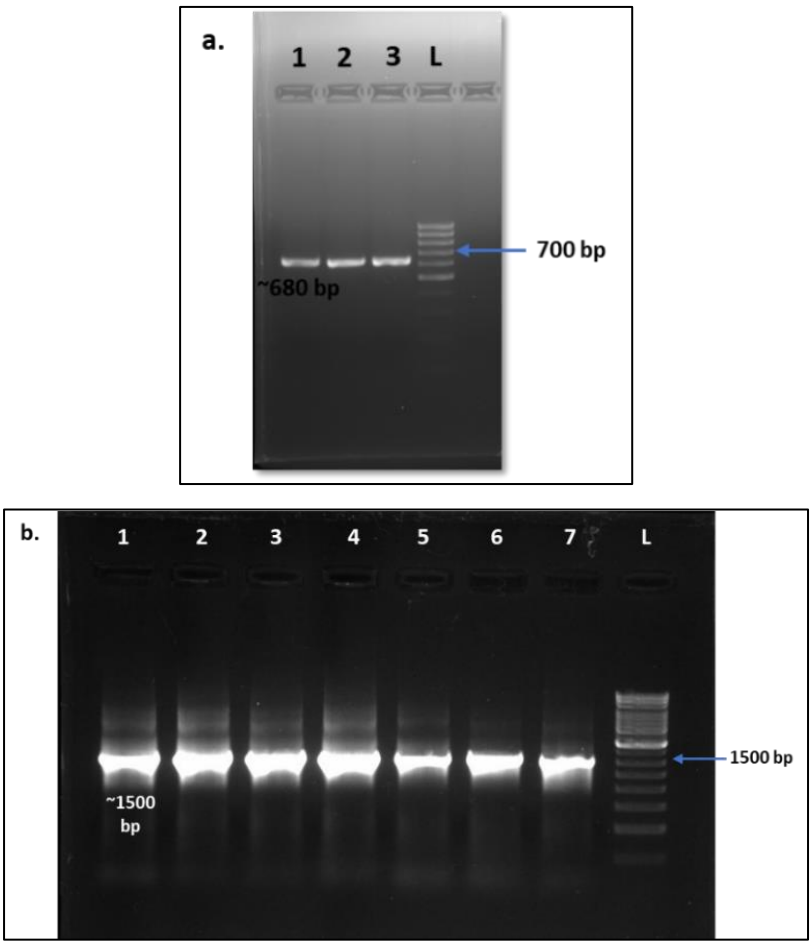


**Fig 3: a.** 18s rDNA of fungal isolates (Lane 1: Fungal isolate S3, Lane 2 and 3: *Candida albicans* MTCC 3017) **b.** 16s rDNA of bacterial isolates (Lane 1 to 5: Selected bacteria strain (B1, J3, T4, R2, and X3), Lane 6 and 7*: E. coli* MTCC 40) (L- Ladder)

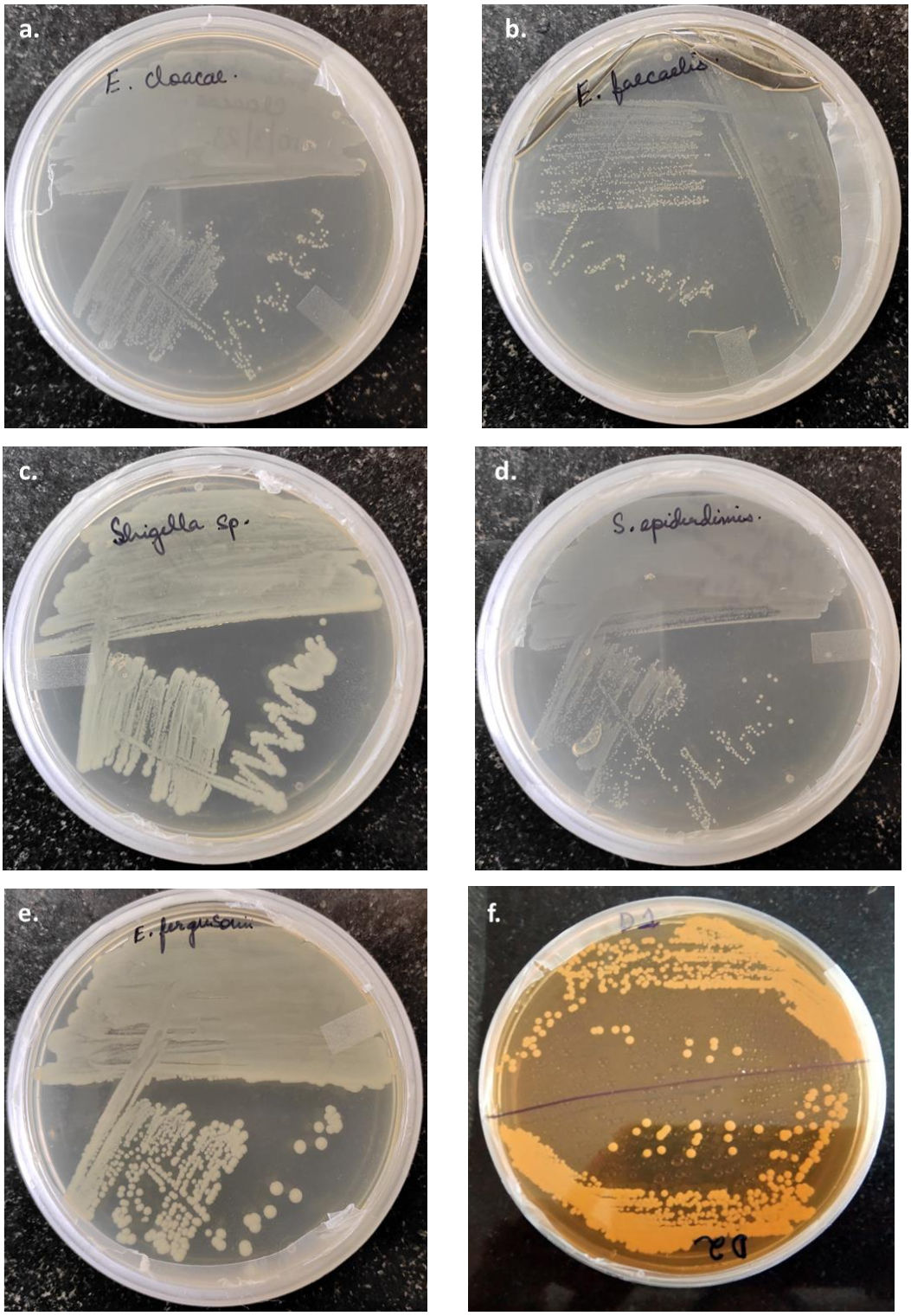


**Fig 4:** Pure culture of isolates **a.** *Enterobacter cloacae,* **b.** *Enterococcus faecalis,* **c.** *Shigella sp.,* **d.** *Staphylococcus epidermidis,* **e.** *Escherichia fergusonii,* and **f.** *Candida albicans*

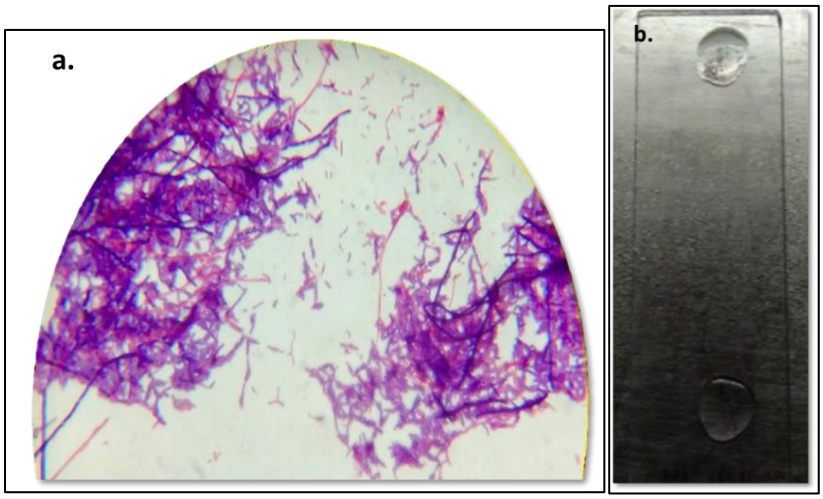


**Fig 5: a.** Grams Staining of lactic acid bacteria under 100x magnification and **b.** Catalase test of lactic acid bacteria

**Table 3: a.** Biochemical and **b.** Morphological characterization of lactic acid bacteria (LAB) isolates

| **a.** | **Biochemical Test** | **Result** |
|---|---|---|
| Oxidase Test | | -ve |
| Methyl Red Test | | -ve |
| Voges Proskauer Test | | -ve |
| Citrate Test | | -ve |
| Indole Test | | -ve |

| **b.** | **Morphological Characteristics** | **LAB Isolates** |
|---|---|---|
| Margin | | Entire |
| Colour | | White |
| Elevation | | Flat |
| Shape | | Punctiform |
| Texture | | Translucent |

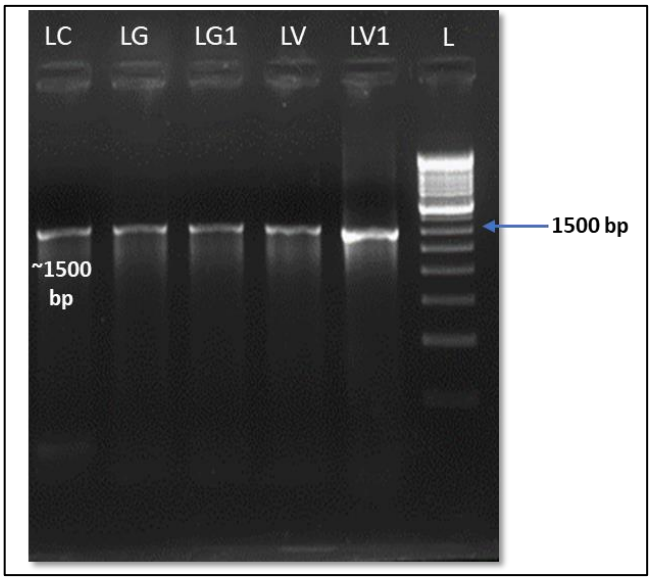


**Fig. 6:** Colony PCR of lactic acid bacteria isolates on 1% agarose gel (Lane 1 to 5- PCR product of 16s rDNA ~1500bp) Lane 6- Ladder (1kb)

**Table 4:** Identification of the isolates using BLASTn

| **Isolate** | **Identity Percentage (%)** |
|---|---|
| G1 | *L. crispatus* LC (99.85) |
| G2 | *L. gasseri* LG (99.71) |
| G3 | *L. gasseri* LG1 (99.89) |
| H1 | *L. vaginalis* LV (99.51) |
| H2 | *L. vaginalis* LV1 (99.10) |

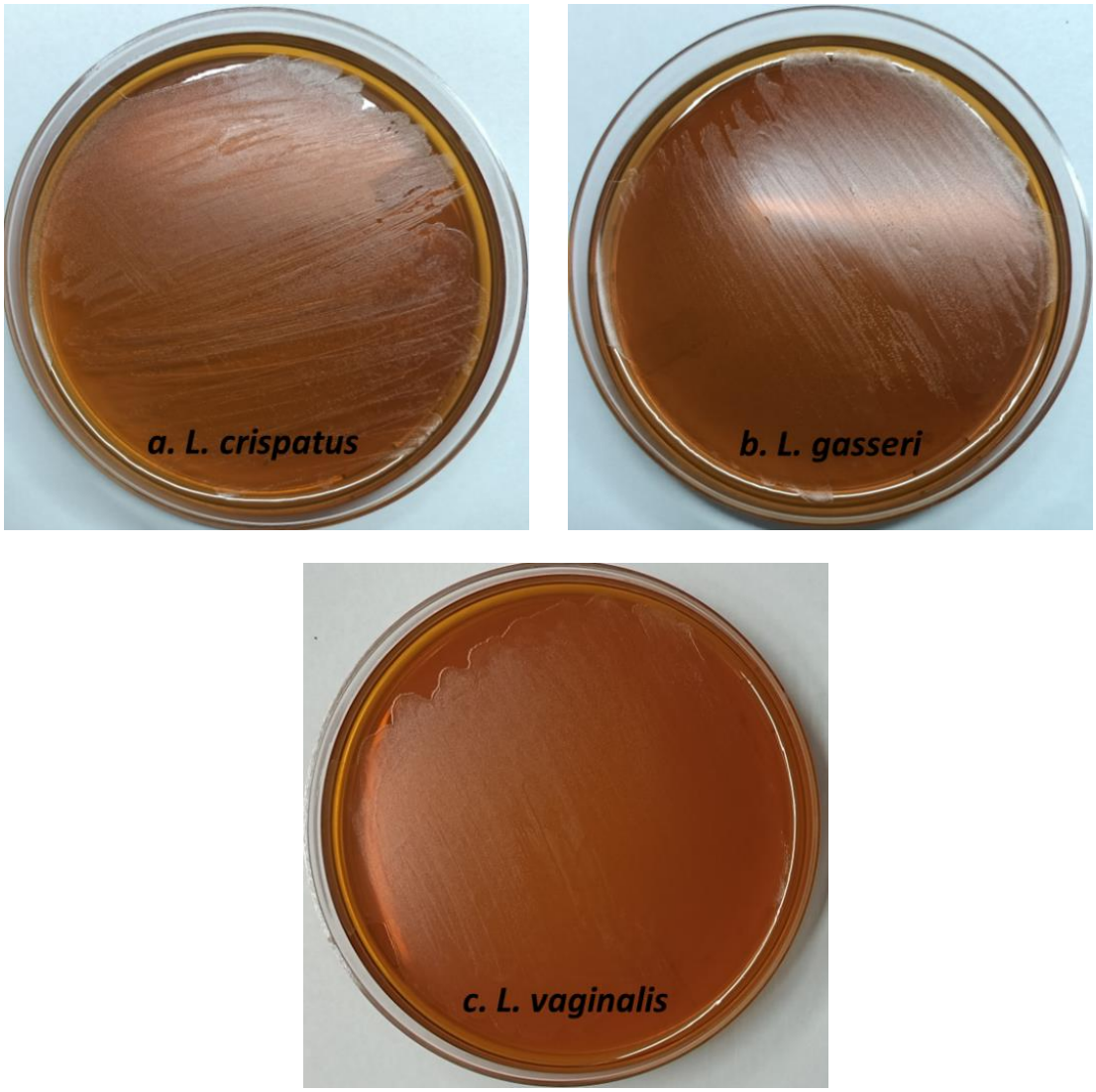


**Fig 7:** Pure culture of LAB isolates (**a.** *L. crispatus* LC, **b.** *L. gasseri* LG, and **c.** *L. vaginalis* LV)

## 3.4. Discussion

Urogenital infections result from the overpopulation of residential microflora. The microbes isolated from vaginal swabs were able to grow at a low pH of 4.5, which resembles the healthy physiological pH of VC. This manifests the potential of these opportunistic pathogens to coexist in the VC with LAB as opportunistic pathogens [395].

A high abundance of nosocomial pathogens like *Enterobacter cloacae* and *Enterococcus faecalis*, indicated that both the isolates belong to the natural vaginal flora of healthy women. *E. faecalis* has shown to be a multidrug resistant pathogen that produces aggregation substrates and binding substrates which helps the pathogen to bind to epithelial cells. They harbor pili gene cluster that aids their adhesion and biofilm

formation. Horizontal transfer of virulence genes among the strains stimulates the production of bacteriocins (like AS-48), enterolysin, and cytolysin. These toxins are bactericidal, forming pores on epithelial cell membrane and cause tissue damage as well. *E. faecalis* produces proteinases and superoxidases conferring them pathogenicity [396]. On the other hand, *E. cloacae* from the urogenital origin are known to produce a high quantity of toxins and show antibiotic resistance. The pathogen adheres and invades the vaginal epithelial cells. It causes blebbing of cell membrane, forms apoptotic bodies, causes condensation and fragmentation of nucleic DNA, increases intracellular calcium, and induces oxidative stress by (ROS, NO, and $H_2O_2$); that ultimately induces apoptosis in healthy epithelial cells [397]. *E. cloacae* also produce enzymes like hydrolases and proteinases. Both pathogens are known to increase the secretion of TNF-α and IL-6 and are the leading cause of UTI and AV [105, 398, 399].

Isolation of enteric bacteria like *Escherichia* sp. and *Shigella* sp. from the urogenital tract indicates the close physiological proximity of VC to the anus. Microcins and colicins are a class of toxins produced by the enterobacteria family that can survive at low pH causing virulence in the VC [400]. *E. fergusonii* has been previously reported as a causative organism of UTI and AV [105, 398]. This bacterium is an emerging pathogen with high similarity to *E. coli*. The bacterium has been reported previously to form biofilm and has shown emerging antibiotic resistance. Contrastingly, *Shigella* sp. is associated with vaginitis only among prepubertal girls. The probability of this bacteria to cause vaginitis in adult women is slim. Nevertheless, it has the potential of producing toxins that may cause harmful effects on the VC in the absence of LAB. Endometriosis in women shows a high correlation with *Shigella* sp. and *Escherichia* sp. population in the stool, elucidating the interlink between the anal and vaginal microflora.

*S. epidermidis* is also a common inhabitant of mucosal membranes like the urogenital tract [401]. This species is also an emerging opportunistic pathogen. The gram-positive pathogen produces autolysins and adhesive proteins to attach to the mucosal surface. They form strong biofilm and evade the primary and secondary immune cells to cause pathogenicity [402].

*Candida albicans* was the only yeast isolated from the vaginal swab samples. *Candida* is one of the most prevalent fungal genera found in VC of reproductive women wherein *C. albicans* is the most abundant species [403, 404]. Overgrowth of *Candida sp.*

has been reported to cause inflammation, redness, itching, and rash in the infected vaginal areas leading to heavy discomfort [405]. *Candida sp* transits from yeast to hyphal form, initiates biofilm formation, and adheres as well as invades the vaginal epithelial cells causing pathogenicity [287].

The prevalence of LAB in VC has an extensive role in maintaining the vaginal health of reproductive women. In the current study, *L. crispatus, L. gasseri,* and *L. vaginalis* were isolated from vaginal swabs of healthy women. *L. crispastus* is one of the most prevalent species [406] of LAB in the VC. It forms the most stable microbial flora against pathogens and also produces a relatively high acidic environment [44]. All these isolates had indistinguishable colony morphology and grew particularly in anaerobic conditions. This indicates the inability of vaginal LAB to grow vehemently on the outer parts of the vagina (i.e, labia major/minor), making the vulval region more prone to infection [407]. Unless an optimum population density of LAB is maintained, pathogens may thrive ascending upward through the VC resulting in urogenital infection. Competitive exclusion by the LAB for nutrition and binding site to the epithelial cells, inhibit the inexorable proliferation of pathogens [44]. Hereafter, the strains *L. crispatus* (LC), *L. gasseri* (LG), and *L. vaginalis* (LV) were used for further experiments.