CHAPTER 4

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4.1. Introduction

The microbial flora of VC proliferates maintaining a subtle symbiotic relationship with the holobiont. The 16s rRNA studies from the VC have reported microbes like: *Clostridiales, Bacteroidalesllus, Clostridium, Lactococcus, Lachnospiraceae, Actinomyces, Segniliparus, Campylobacter, Anaerococcus, Finegoldia, Peptostreptococcus, Fusobacterium, Peptoniphilus, Porphyromonas, Mobiluncus, Prevotella, Dialister, Atopobium, Megasphaera, Sneathia, Eggerthella, Anaerotruncus, Parvimonas, Prevotellaceae, Anaeroglobus, Ruminococcaceae, Prevotellaceae, Gemella, Coriobacteriaceae, Aerococcus, Gardnerella, Coriobacteriaceae, Bacteroidetes, Bacteroidetes, Ruminococcaceae, Bulleidia, Proteobacteria, Proteobacteria, Proteobacteria, Lachnospiraceae, Bacteroidetes, Clostridiales, Mycoplasmataceae, Arcanobacterium,* and *Moryella* [408, 409]. Among these microbe's opportunistic pathogens causes variable symptoms in the vulval region, vaginal canal, and even in the uterine environment. The most common signs of mild vaginitis are: itching, redness, and swelling in the genital area. The expulsion of unusual vaginal discharge, dysuria, dyspareunia, irritation, odour, and burning sensation are severe signs of vaginitis [410]. These infections may also lead to critical outcomes such as: genital sores, STI, PROM, and PTL [411]. Healthy women with no vaginal infections tend to maintain a high population of *Lactobacilli* sp. (~10^7-10^8 CFU per gram) [412] in the cervical vaginal fluid. The production of glycogen by the vaginal epithelial cells has a proportional relationship with the population density of LAB [413]. The LAB maintains a mutualistic symbiotic relationship in the VC post puberty [412]. Around eight dominant and different species of LAB have been reported from the VC of healthy women [414], among which *L. crispatus* and *L. iners* are the most prevalent species [406]. LAB either inhibits the binding of pathogens by forming a heavy biofilm on vaginal epithelium or by secreting extracellular metabolites [265, 415]. Thus, the inhibitory effect of LAB on

pathogens helps to understand the dynamics maintained in the vaginal microenvironment. This expands the scope of using these microbes for the improvement of vaginal health.

4.2. Materials and Methods

4.2.1. Chemicals Used

- Nutrient Agar/Broth (HiMedia, India)
- *Lactobacillus* MRS Agar/Broth (HiMedia, India)
- Yeast Potato Dextrose Broth (HiMedia, India)
- Starch agar (HiMedia, India)
- Tryptic soy broth (HiMedia, India)
- Skim milk agar (HiMedia, India)
- Phenol red agar (HiMedia, India)
- Tributyrin oil (HiMedia, India)
- Glycogen (from oyster) (HiMedia, India)
- Antibiotic disc: Augmentin (30 Mcg), Nitrofurantoin (30 Mcg), Nalidixic Acid (30 Mcg), Cefuroxime (30 Mcg), Co-Trimoxazole (25 Mcg), Norfloxacin (10 Mcg), Gentamicin (10 Mcg), Cefixime (5 Mcg), Cefotaxime (30 Mcg), Augmentin (30 Mcg), Erythromycin (10 Mcg), Chloramphenicol (30 Mcg), Ofloxacin (5 Mcg), Co-Trimoxazole (25 Mcg), Kanamycin (30 Mcg), Tetracyclin (10 Mcg), Ceftazidine (30 Mcg), Erythromycin (10 Mcg), Clindamycin (10 Mcg), Itraconazole (10 Mcg), and Fluconazole (10 Mcg) (HIMEDIA, India)
- 1 N and 4N Hydrochloric acid
- Sodium chloride salt
- Bile salt
- Sugar Disc: Maltose, Lactose, Sucrose, Melibiose, Raffinose, Xylose, Arabinose, Galactose, and Fructose (HiMedia, India)
- Horse radish peroxidase (HiMedia, India)
- O-dianisidine reagent (HiMedia, India)
- Syringe filter 0.22 μm (Sigma-Aldrich, India)
- Iron heptahydrate (HiMedia, India)
- Lactic acid (85%) (HiMedia, India)
- C-18 column (ThermoFisher, India)
- Phosphoric acid (Sigma-Aldrich, India)

- Acetonitrile (Sigma-Aldrich, India)
- Propidium iodine (HiMedia, India)
- Spider Agar (HiMedia, India)
- RPMI 1640 (HiMedia, India)
- SDS, amylase, trypsin, proteinase K (HiMedia, India)
- Sodium acetate (HiMedia, India)
- Crystal violet (HiMedia, India)
- Menadione Salt (HiMedia, India)
- XTT salt (SRL, India)
- Super Reverse Transcriptase MuLV Easy Kit, (Bio Bharati Life Sciences Pvt.Ltd.)
- GSP1, BCR1, TEC1, CPH1, ALS3, HYR1, ECE1, and HWP1 (IDT, India)

4.2.2. Instruments Used

- Thermo Multiskan Go, Thermo Scientific, India
- Varioskan Lux, ThermoScientific, India
- NIKON Inverted Microscope, India
- JEOL, Scanning Electron Microscopy imaging, Japan
- Q-Exactive Plus Biopharma-High Resolution Orbitrap, Thermo Fiseher Scientifi, India
- Freeze Dryer, Labconco, USA

4.2.3. Characterization of potential aerobic pathogens

Identified aerobic isolates were freshly grown in nutrient broth aerobically for 12-24 h at 37 ℃ and used in the following experiments for further characterization:

4.2.3.1. *Amylolytic activity*

Amylolytic activity was assessed with starch agar plates. Freshly grown isolates were streaked on plates and incubated for 12-24 h at 37 ℃. A zone of clearance (ZOC) around the streak was checked after flooding the plates with Lugol's iodine. Amylase producing *Bacillus cereus* was used as the positive control [416].

4.2.3.2. *Hemolytic activity*

Hemolytic activity was assessed with tryptic soy broth with 5% sterile human blood. The isolates were streaked on plates and incubated anaerobically for 24-48 h at 37 ℃. β-hemolytic *Staphylococcus aureus* was used as the positive control [417].

4.2.3.3. *Proteolytic activity*

Proteolytic activity was assessed with skim milk agar was. The isolates were streaked on the plates and incubated for 24-48 h at 37 ℃. Zone of clearance was checked. Proteolytic *Pseudomonas aeruginosa* was used as the positive control [418].

4.2.3.4. *Lipolytic activity*

Lipolytic activity was assessed with phenol red agar with tributyrin oil as a substrate. The isolates were streaked on plates and incubated for 24-48 h at 37 ℃. A change in color from red to yellow was checked. Both *Staphylococcus aureus* and *Bacillus cereus* were used as positive control [419].

4.2.3.5. *Biofilm formation*

Biofilm formation by the potentially pathogenic isolates was analyzed using the tissue culture plate method. The isolates were grown in tryptic soya broth with 1% glucose at for 24 h 37 ℃. The fresh culture was diluted (1:1) ratio with sterile PBS and 100 µl of the diluted concoction was added to a sterile 96 well plates with fresh media 200 µl. The plate was incubated at 37 ℃ for 24 h. Post incubation, the liquid media was discarded and the wells were gently washed with sterile PBS twice. The bacterial cells in the wells were fixed using 2% sodium acetate for 15 min followed by staining with crystal violet for 30 min. The excess stain was removed with PBS wash thrice. The plates were allowed to dry at 40 ℃ for 30 min. The OD was measured spectrophotometrically at 600 nm. *Staphylococcus aureus* a pathogenic biofilm producing strain was used as the positive control and no organism i.e., broth with no inoculum was used as blank [420].

4.2.3.6. *Ability to grow anaerobically*

Isolates were inoculated in nutrient broth and the tubes were incubated in an anaerobic chamber for 24 h at 37 ℃. The turbidity was observed after 24 h and OD was measured spectrophotometrically at 600 nm [421].

4.2.3.7. Ability to utilize glycogen

Isolates were inoculated to a basal yeast nitrogen broth with glycogen as the only source of carbon and grown for 12-24 h at 37℃ aerobically. The turbidity was observed for growth [422].

4.2.3.8. *Antibiotic susceptibility*

Effect of twenty commercially available antibiotic disc (HIMEDIA, India) was tested on the isolates. Inoculum of 10 µl (1×10^4) number of cells was added to nutrient agar just before solidification. Post solidification, antibiotic disc was placed on the plate and incubated aerobically for 24 h at 37 ℃. Appearance for zone of inhibition was noted [423].

4.2.4. Characterization of lactic acid bacteria

The *Lactobacillus* isolates were grown in *Lactobacillus* MRS broth for 48 h to reach (1×10^8) number of cells. The culture tubes were centrifuged at 8,000 rpm for 5 mins, the supernatant was discarded and the microbes were resuspended in sterile PBS and used as an inoculum for the experiments below:

4.2.4.1. Tolerance to bile salt

Bile tolerance was tested by adding bile salt to *Lactobacillus* MRS broth at an increasing concentration of (0.05, 0.1, 0.2, 0.3, and 0.6%). The inoculum was added and the tubes were incubated anaerobically for 48-72 h at 37 ℃. The growth was monitored by observing the turbidity and measuring the OD spectrophotometrically at 600 nm [133].

4.2.4.2. Tolerance to sodium chloride salt

Sodium chloride salt tolerance was tested by adding the salt at increasing concentration of (2, 3, and 4%) to *Lactobacillus* MRS broth. The inoculum was added and the tubes were incubated anaerobically for 48-72 h at 37 ℃. The growth was monitored by observing the turbidity and measuring the OD spectrophotometrically at 600 nm [424].

4.2.4.3. Tolerance to low pH

The LAB isolates were studied for their tolerance to low pH. The pH of *Lactobacillus* MRS broth was reduced to 4 by adding 0.1 N hydrochloric acid. The inoculum was added and the tubes were incubated anaerobically for 48-72 h at 37 ℃. The growth was monitored by observing the turbidity [424].

4.2.4.4. Antibiotic susceptibility and resistance

Effect of twenty commercially available antibiotic disc was tested on the LAB isolates. Inoculum was added to *Lactobacillus* MRS agar prior solidification. Post solidification, antibiotic disc was placed on the plate and incubated anaerobically for 48 h at 37 ℃. Appearance of ZOI was noted [425].

4.2.4.5. Sugar fermentation assay

Sugar fermentation of disaccharides, trisaccharide's, pentose, and hexose sugar was added to *Lactobacillus* MRS broth with slight modification (replacement of ammonium citrate with ammonium di-phosphate and removal of dextrose sugar). Sugar discs of nine different sugars were added to the culture tubes with the inoculum, and then grown anaerobically for 24-48 h at 37 ℃. Phenol red was used as an indicator, the change of color from red to yellow indicated fermentation. A slight change of color to orange indicated less fermentation and no change in color was considered as no fermentation [426].

4.2.4.6. Hydrophobicity assay

Hydrophobicity of LAB isolates were studied, organic solvents (Hexane/xylene) were added in a 1:1 ratio with LAB cells resuspended in PBS and vortexed for 1 min. The OD of the aqueous phase and non-aqueous phase was checked after 1 hr of incubation at room temperature spectrophotometrically at 600 nm when the phases settled. The formula $H=(1-H_t/H_0) \times 100$ % was used to calculate the hydrophobicity, where H_0 is the hydrophobicity at 0 h and H_t is the hydrophobicity at any time point [427].

4.2.4.7. Aggregation assay

Aggregation of LAB isolates resuspended in PBS was measured at 0, 6, and 24 h, by measuring the OD of the supernatant PBS spectrophotometrically at 600 nm. The formula $A=(1-A_t/A_0) \times 100$ % was used to calculate the aggregation, where A_0 was the OD at 0 h and A_t the aggregation at any time point [427].

4.2.4.8. Co-aggregation assay

Shigella sp., Candida albicans, Escherichia coli, and Staphylococcus aureus were grown in nutrient broth for 24 h to reach (1×10^8) number of cells. The cultures were centrifuged at 8,000 rpm for 5 mins, the supernatant was discarded and the microbes were resuspended in sterile PBS. Co-aggregation assay was performed to check the aggregation between the LAB isolates and these potential pathogenic microbes. Resuspended LAB

isolate in PBS was mixed in equal volume (1:1) with the pathogens and vortexed for 15 secs and kept still thereafter. OD of the supernatant was measured spectrophotometrically at 0, 6, and 24 h at 600 nm. The formula $C=(1-C_t/C_0) \times 100$ % was used to calculate the co-aggregation where C_0 was the OD at 0 h and C_t the aggregation at any time point. *Lactobacillus crispatus* was used as a positive control. [428].

4.2.4.9. Detection of hydrogen peroxide

The LAB isolates were grown in *Lactobacillus* MRS broth for 48 h, spined down, washed twice with PBS and resuspended in 5 mM glucose. Supernatant samples were taken for assessing hydrogen peroxide production after 24 h. An aliquot of 5 ml LAB culture was added to 1ml of 0.001% horse radish peroxidase aqueous solution followed by adding 0.1 ml of aqueous O-dianisidine reagent and incubated for 10 min at 37 ℃. 200 µl of 4N hydrochloric acid was added to the mixture to stop the reaction. The formation of brown colour indicated presence of hydrogen peroxide. Glucose 5 mM with no microorganism was taken as blank [429].

4.2.4.10. Detection and quantification of lactic acid spectrophotometrically

Lactic acid detection and quantification in the culture free supernatant (CFS) of LAB was done. LAB culture tubes were grown in *Lactobacillus* MRS broth, centrifuged at 13,000 rpm. The supernatant was passed through 0.22 µm syringe filter. 200 µl of (0.3%) iron heptahydrate was added to 250 µl CFS, the OD was measured spectrophotometrically at 390 nm [430]. For quantitative estimation of lactic acid, a standard graph was prepared by serial dilutions of raw lactic acid (85%). *Lacticaseibacillus rhamnosus* CFS was used as control.

4.2.4.11. Detection and quantification of lactic acid through RP-HPLC

The lactic acid in CFS was also estimated through RP-HPLC using C18 column at 200 nm, with isocratic elution and flow rate of 1 ml/min. Two solvents, 0.03 M phosphoric acid (Solvent A) and 80% acetonitrile (Solvent B) were used. Serially diluted lactic acid (85%) was run through the RP-HPLC machine followed by CFS of the isolates. A standard graph was prepared with the AU value representing amount of lactic acid. The quantification of lactic acid in CFS was done [431].

4.2.5. Effect of LAB on pathogens

4.2.5.1. Overlay Assay with LAB

The LAB, *L. crispatus* (LC), *L. gasseri* (LG), and *L. vaginalis* (LV) were streaked on *Lactobacillus* MRS agar at the centre in a round motion and grown anaerobically for 48 h at 37 ℃. Soft nutrient agar (0.8%) inoculated with 10 µl potential pathogens (1×10^7/100 µl), was overlayed on the *Lactobacillus* MRS agar with the LAB colonies and incubated anaerobically for 12 h at 37 ℃. Inhibition of pathogens around the LAB colonies was observed [432].

4.2.5.2. Cup assay with LAB

Potentially pathogenic isolates were freshly grown with OD (1×10^7/100 µl). Nutrient agar plates were inoculated with 10 µl of the freshly grown culture prior to solidification and wells were bored on the agar aseptically. The Lactobacillus strains were also grown fresh anaerobically in *Lactobacillus* MRS broth for 48 h. The LAB was then centrifuged at 13,000 rpm and supernatant was discarded, the pellet was washed and resuspended in PBS to obtain the cell density of (1×10^8/100 µl). The cells (100 µl) were resuspended in *Lactobacillus* MRS media and added on to the bored wells. The plates were incubated anaerobically for 12 h at 37 ℃. Appearance for the zone of clearance was checked [433].

4.2.6. Inhibitory effect of Culture Free Supernatant (CFS) on potential bacterial pathogens

The LAB (C, G, and V) was grown in *Lactobacillus* MRS broth anaerobically for 48 h at 37 ℃. The tubes were spined at 13,000 rpm and the supernatant was filtered through 0.22 µM syringe filter. The culture free supernatant (CFS) was used fresh:

4.2.6.1. Bacteriostatic effect of CFS

The cup assay technique mentioned in Methodology 1.3.2. was repeated. An aliquot of 100 µl of the filtered (100%) CFS was added to the wells and the plates were incubated aerobically for 12 h at 37 ℃. Appearance for zone of clearance was checked for inhibition of the pathogens [434].

4.2.6.2. Bactericidal effect of CFS

Pathogens were freshly grown to reach OD of ($1 \times 10^{7/}$100 µl), the tubes were centrifuged at 13,000 rpm and the supernatant was discarded. The microbial cells were washed twice with PBS and resuspended in 1ml CFS of (C, G, and V). PBS was used as control. The

potential pathogens were treated with the CFS for 12 and 24 h at 37 ℃. Post treatment the bacterial cells were pelleted, washed with PBS twice and resuspended in 1ml PBS. 50 µl propidium iodine (15 µM) was added to the control and treated tubes followed by an incubation for 15 - 20 min in dark. Post incubation, the tubes were centrifuged, after which the supernatant was discarded and the excess propidium iodine was washed with PBS twice. The bacterial cells were resuspended in PBS and fluorescence spectrophotometric reading was taken at 535/615 (excitation/ emission) [435, 436].

4.2.6.3. Minimum Inhibition Volume (MIV) of CFS

The method of (Yang et al., 2021) was adopted with some modifications. Fresh 2 µl of the potential pathogens were inoculated in 200 µl nutrient broth in a 96 well plate. 100% filtered CFS of variable volumes (100 µl, 50 µl, and 25 µl) from LAB (C, G, and V) were added to the 96 well plate. *Lactobacillus* MRS broth was used as the positive control and autoclaved distilled water used as control. The growth of the potential pathogens was checked after 12 h of incubation at 37 ℃. The volume of CFS inhibiting growth of the pathogens completely was measured spectrophotometrically at 600 nm [437].

4.2.6.4. Minimum Inhibition Concentration (MIC) of CFS

The filtered CFS (C, G, and V) was lyophilized and the powdered CFS was resuspended in sterile distilled water. The ability of lyophilized CFS to inhibit the potential pathogens was checked. Cup assay technique mentioned in Methodology 1.3.2. was repeated. The MIC of the lyophilized CFS from (LC, LG, and LV) was determined [286, 438].

4.2.7. Inhibitory effect of Culture Free Supernatant (CFS) on *C. albicans*

4.2.7.1. Effect of CFS on budding of C. albicans

Fresh YPD broth 200 µl was inoculated with 2 µl *C. albicans*. Increasing volume of 50 µl, 100 µl and 150 µl of CFS from (C, G, and V) were added into wells of a sterile 96 well plate. Fresh MRS broth was used as the positive control and sterile distilled water as the control. The OD was measured spectrophotometrically at 600 nm to monitor the effect of CFS on the growth of *C. albicans* after 12 and 24 h [439].

4.2.7.2. Effect of treated CFS on budding of C. albicans

CFS of (LC, LG, and LV) were treated with: SDS detergent (1 mg/ml); amylase (0.5 mg/ml), trypsin (0.5 mg/ml) and proteinase K (0.5 mg/ml) enzymes; 1M NaOH to neutralize the pH and heat treatment at 70 ℃ for 20 mins. YPD broth 200 µl was inoculated with 2 µl of *C. albicans* and 100 µl of pre-treated CFS were added. The growth of *C. albicans* was monitored by measuring the OD spectrophotometrically at 600 nm after 12 h. Untreated CFS was used as the control [439, 440].

4.2.7.3. *Effect of CFS in hyphae formation of C. albicans in solid media*

Spider agar plates were laced with fresh 1 ml CFS from (C, G, and V) and allowed to dry for 60 mins. *C. albicans* was spread on the plates and incubated at 37 ℃ and grown for 5 days. CFS of (LC, LG, and LV) was sprayed around the colonies post 24 h of growth. The hyphal growth of the colonies was monitored for 5 days [441].

4.2.7.4. Effect of CFS on hyphae formation of C. albicans in liquid media

C. albicans was freshly grown in YPD broth for 12 h at 37 ℃, the tubes were centrifuged at 5,000 RPM and the worn medium was discarded. The pellet was washed with sterile PBS twice and resuspended in RPMI 1640 media for an OD of (~1×10^{8}/100 µl). Fresh RPMI 1640 media 600 µl was pipetted into a 6 welled sterile cell culture plate and inoculated with 100 µl of *C. albicans*. CFS of (LC, LG, and LV) was inoculated onto the plates in 1:2 ratio (CFS: Volume of media with inoculum) and incubated at 37 ℃ for 3 h. The plates were observed under at 40x magnification for hyphae formation [442, 443].

4.2.7.5. Effect of CFS on biofilm formation of C. albicans

Biofilm formation of *C. albicans* was checked at two stages viz: (i) Secondary Colonization (SC) (3+24 h) the first stage of biofilm formation and (ii) Tertiary Colonization (TC) (3+24+24 h) the second stage of mature/dense biofilm formation:

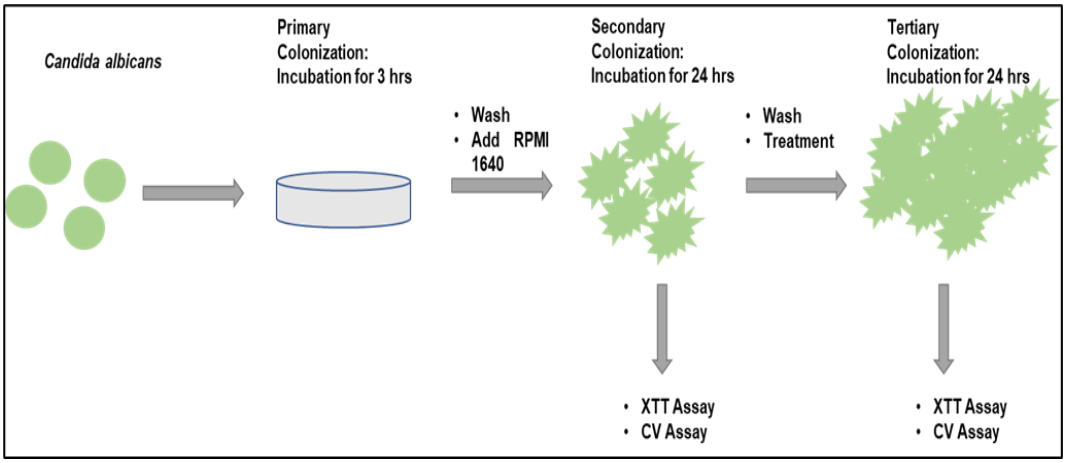


Fig 8: Schematic representation of biofilm formation in polystyrene well

C. albicans was pipetted onto a 96 well plate and incubated at 37 ℃ for 3 h to allow primary colonization [444], the non-adhered were pipetted out and washed with sterile PBS twice to remove the loosely adhered cells. Fresh RPMI 1640, 200 µl was added to the wells with the adhered cells. CFS of (LC, LG, and LV) 50 µl, 100 µl and 150 µl was added and its effect on SC phase of biofilm formation was checked. The plate was incubated at 37 ℃ for 24 h, post incubation the aliquot was carefully discarded and the wells were washed with sterile PBS twice. Fresh RPMI 1640 was used as the control and sterile distilled water as the positive control.

On the other hand, fresh RPMI 1640 300µl was added to the wells post PC and incubated at 37 ℃ for 24 h to complete SC phase of biofilm formation. After incubation the aliquot was carefully discarded and the wells were washed with PBS twice. Fresh RPMI 1640, 200 µl was added to the wells and CFS of (LC, LG, and LV) 50 µl, 100 µl and 150 µl were added into the wells to check its effect on TC phase of biofilm formation. The 96 well plate was incubated at 37 ℃ for another 24 h, post incubation the media was carefully discarded, the wells were washed with sterile PBS twice. RPMI 1640 was used as the control and sterile distilled water as the positive control [445] [446].

a. **Crystal Violet Assay for biofilm detection**: After formation of biofilm post TC phase, 300 µl sodium acetate (2%) was used for fixing the biofilm for 30 mins. Post fixation the wells were washed with PBS (2x) and crystal violet was used to stain the biofilm for 30 mins. Excess stain was washed with PBS (2x), the OD of the biofilm was measured spectrophotometrically at 600 nm to measure the density of biofilm [447].

b. **XTT Assay for biofilm detection**: The biofilm formation post SC and TC phase was also measured using 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide or XTT a tetrazolium salt. XTT (1mg/ml) solution was prepared in sterile PBS and filtered through a 0.22 µM syringe filter. Menadione salt (0.4 mM) was prepared fresh and used to enhance the activity of XTT. Fresh PBS 157 µl was added to the well post SC and TC phase followed by addition of 3 µl menadione and 40µl XTT solution. The 96 well plate was stored in dark at 37 ℃ for 5 h, post incubation the spectrophotometric reading of the plate was measured at 490 nm to measure the intensity of orange/red colour indicating the density of the biofilm formed [448].

c. **SEM of biofilm formation**: *C. albicans* was primarily colonized on a sterile 10 mm glass cover slip inside a six welled cell culture plate. Post SC phase the wells were washed with PBS twice. RPMI 1640 was used as control and sterile distilled water as the positive control for the TC phase [449]. Four treatments were done: **a.** Only CFS **b.** CFS + RPMI 1640 in 1:1 ratio **c.** Only LAB **d.** LAB + RPMI 1640 in 1:1 ratio. Post TC phase the biofilm was washed with PBS (2x) and fixed with 1000 µl glutaraldehyde solution (3 %). The plate was stored in dark overnight at 4℃ for fixation. Excess fixative was washed with sterile distilled water twice followed by removal of excess water by serial dehydration using (60 %, 70 %, 80 % and 95 %) ethyl alcohol. Post dehydration the wells were air dried for 3 h in sterile condition and the glass cover slips were taken for Scanning Electron Microscopy imaging (SEM) [450].

4.2.7.6. Effect of CFS on hyphal/ biofilm gene expression of C. albicans

Freshly grown *C. albicans* was resuspended in PBS and inoculated to RPMI 1640 medium, 4 ml for hyphae stimulation. CFS of (LC, LG, and LV) was added to the above aliquot in 1:1 ratio (i.e., 4ml). Similarly, 1:2 ratio (i.e., 8ml) treatment was also given. RPMI 1640 was used as control and MRS broth as the negative control. The tubes were incubated at 37℃ for 5-6 h [451]. Post Incubation the tubes were centrifuged at 5,000 RPM for 10 mins, the supernatant was discarded and the cells were washed with PBS twice. The total RNA of the cells was isolated following Cold Spring Harbor Laboratory Protocol [452]. Quantity of RNA was measured spectrophotometrically by measuring the ratio of absorbance at 260/280 nm, quality of RNA was checked by running the total RNA in 1.5% agarose gel. Semi-Quantitative Real Time PCR was performed, the total mRNA was converted to cDNA using Super Reverse Transcriptase MuLV Easy Kit. The cDNA

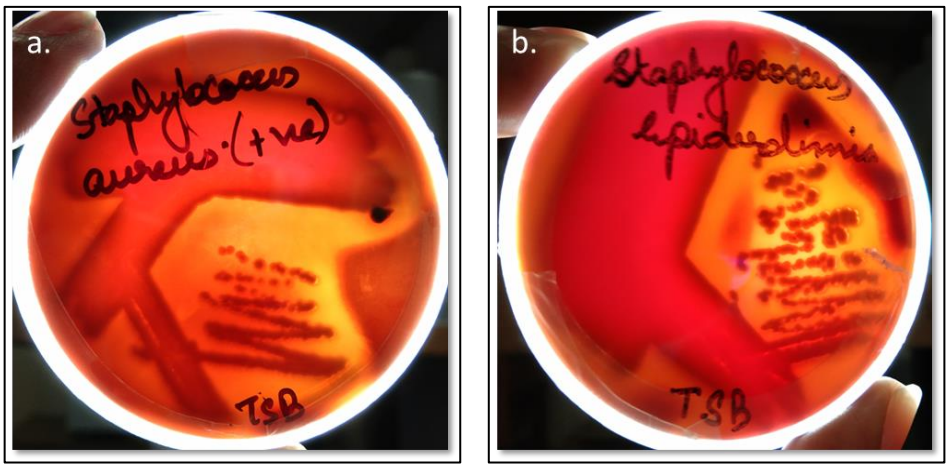
was used to quantify the expression of hyphae/biofilm specific genes through PCR [453] using the primers for the following genes: transcription factor gene (CPH1) (TEC1), agglutin like protein (ALS3) gene, biofilm, and cell wall regulator (BCR1) gene, hyphally regulated cell wall protein (HYR1) gene, hyphal cell wall protein (HWP1) gene and extent of cell elongation protein (ECE1) gene. The GTP binding nuclear protein (GSP1) a constitutive gene was used to compare the expression profile of hyphal genes [454].

4.2.8. LC-MS/MS of CFS for identification of antimicrobial compounds

L. crispatus, *L. gasseri* and *L. vaginalis* were grown in *Lactobacillus* MRS broth anaerobically for 48 hours at 37 ℃. The CFS (LC, LG, and LV) of the isolates were studied through LC-MS/MS analysis. The extracellular metabolites produced by the isolates anaerobically were identified [444, 455].

4.3. Results

E. cloacae, *E. faecalis*, and *S. epidermidis* were hemolytic (Fig 9); *E. cloacae* was proteolytic (Fig 10); *E. faecalis*, *S. epidermidis*, and *C. albicans* were lipolytic in nature (Fig 11); *Escherichia fergusonii*, *Shigella* sp., and *C. albicans* were amylolytic in nature (Fig 12). All the isolates grew anaerobically. Itraconazole (IT-10 mcg) was the most potent antibiotic against all the potential pathogens (Fig 13). *C. albicans* formed the heaviest biofilm followed by *S. epidermidis* and *E. faecalis* (Fig 14). The aerobic pathogens were able to grow properly in anaerobic/microaerophilic conditions. Only, *C. albican*s utilized glycogen when grown on a minimal nitrogen base media (Table 5).



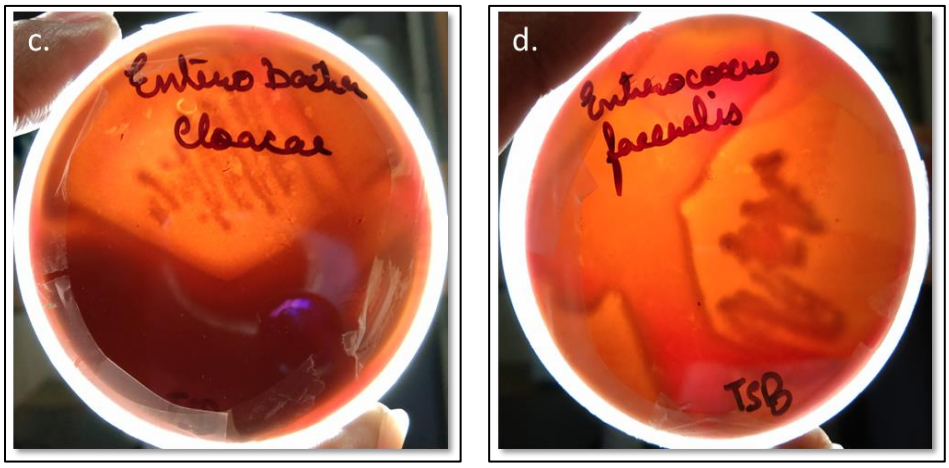


Fig 9: Hemolysis assay of potential pathogens (**a.** *S. aureus* as positive control, **b.** *S. epidermidis*, **c.** *E. cloacae,* and **d.** *E. faecalis*)

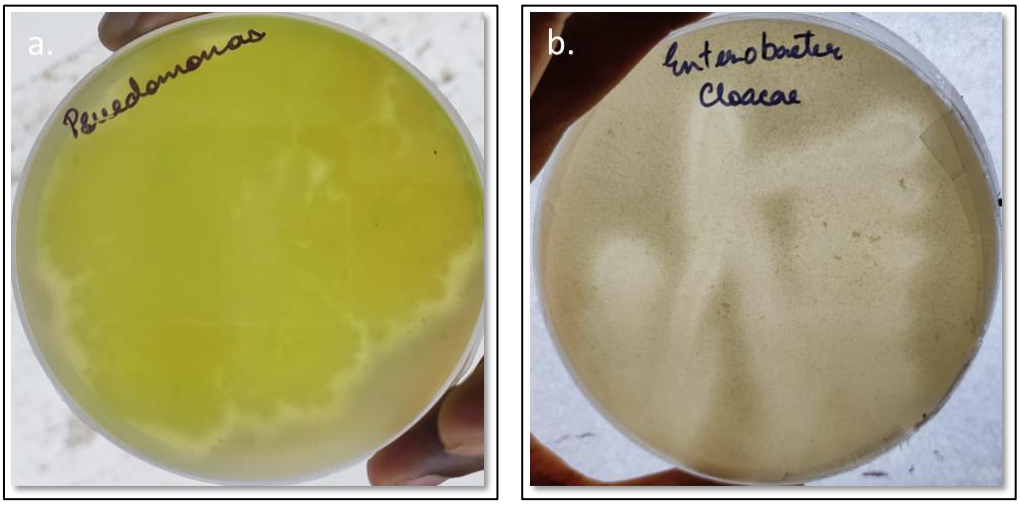
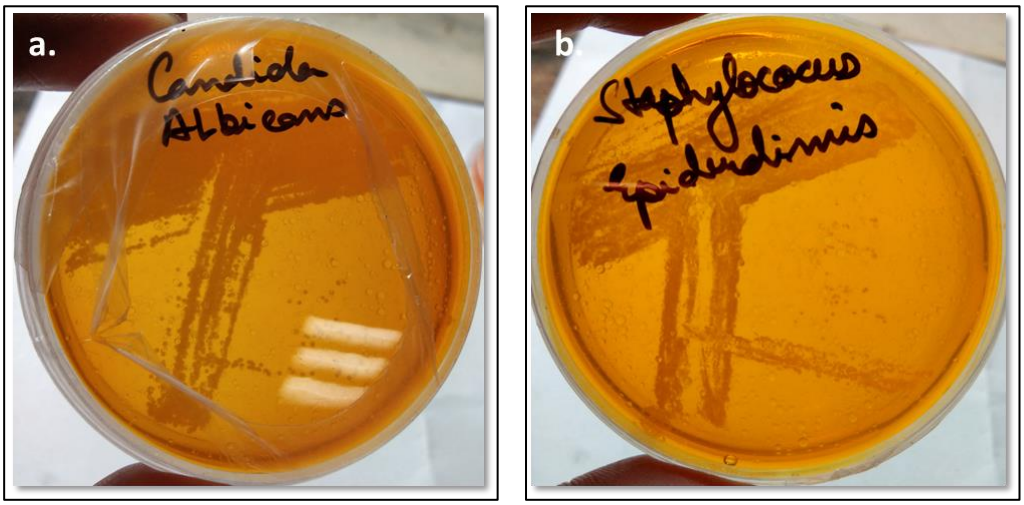


Fig 10: Proteolytic assay of potential pathogens (**a.** *P. aeruginosa* as positive control and **b.** *E. cloacae*)



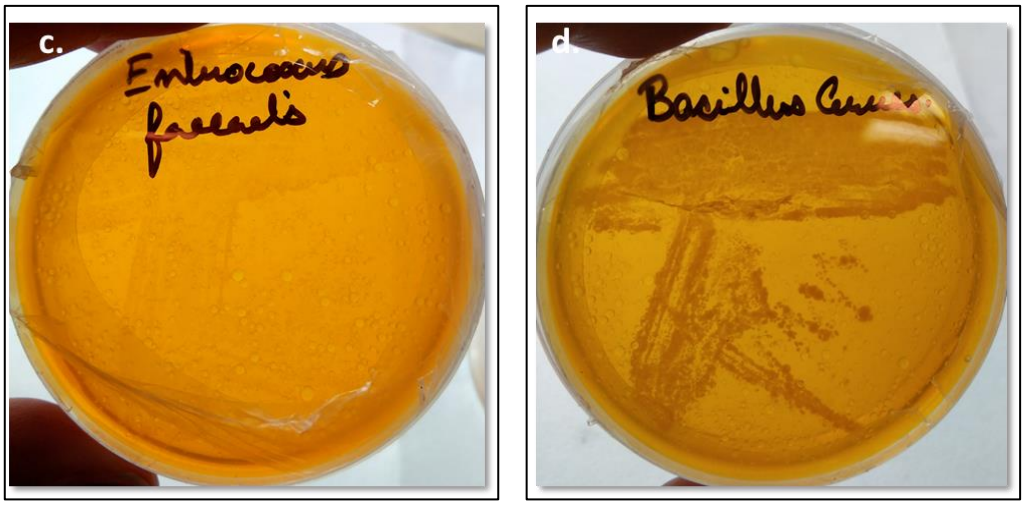


Fig 11: Lipolytic assay of potential pathogens (**a.** *C. albicans,* **b.** *S. epidermidis,* **c**. *E. faecalis* and **d**. *B. cereus* as positive control)

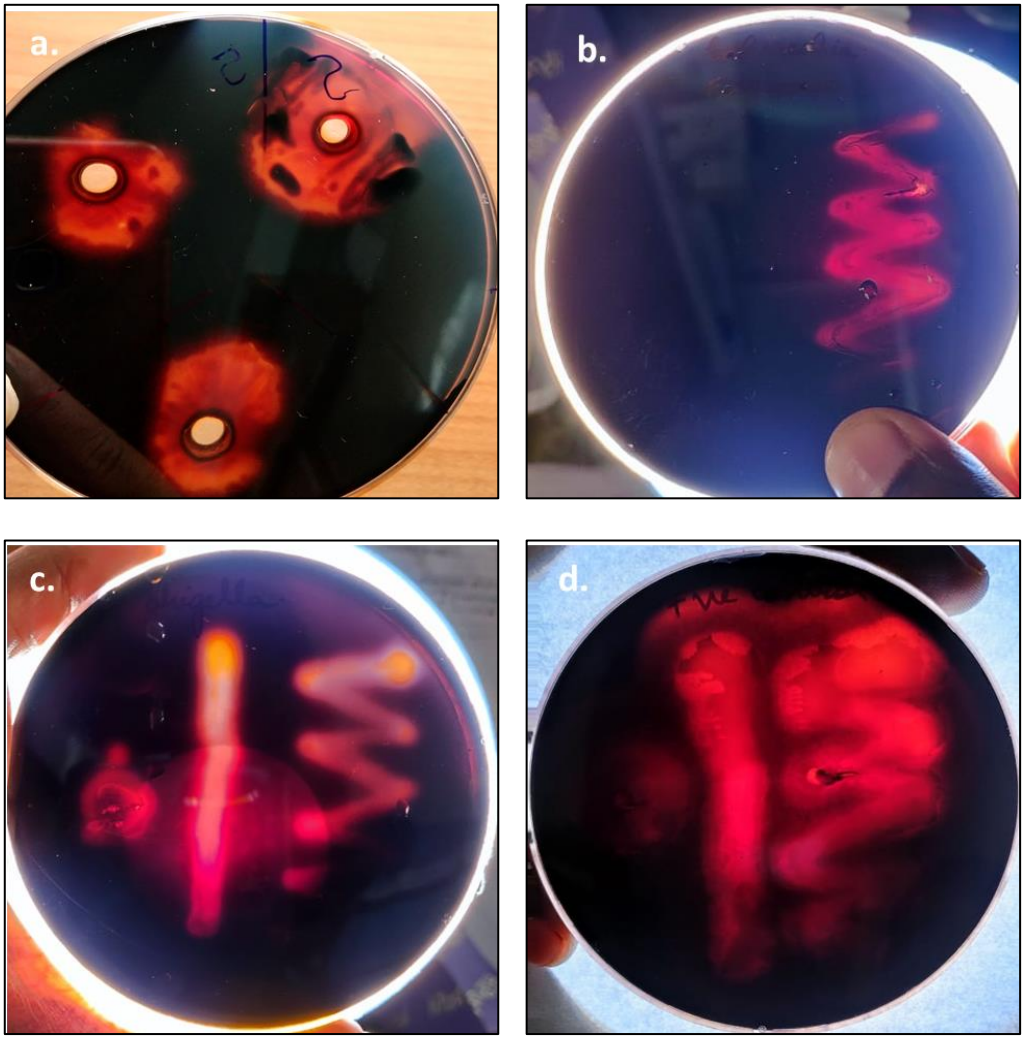


Fig 12: Amylolytic assay of potential pathogens (**a.** *C. albicans* **b.** *E. fergusonii* **c.** *Shigella* sp. and **d.** *B. cereus* as positive control)

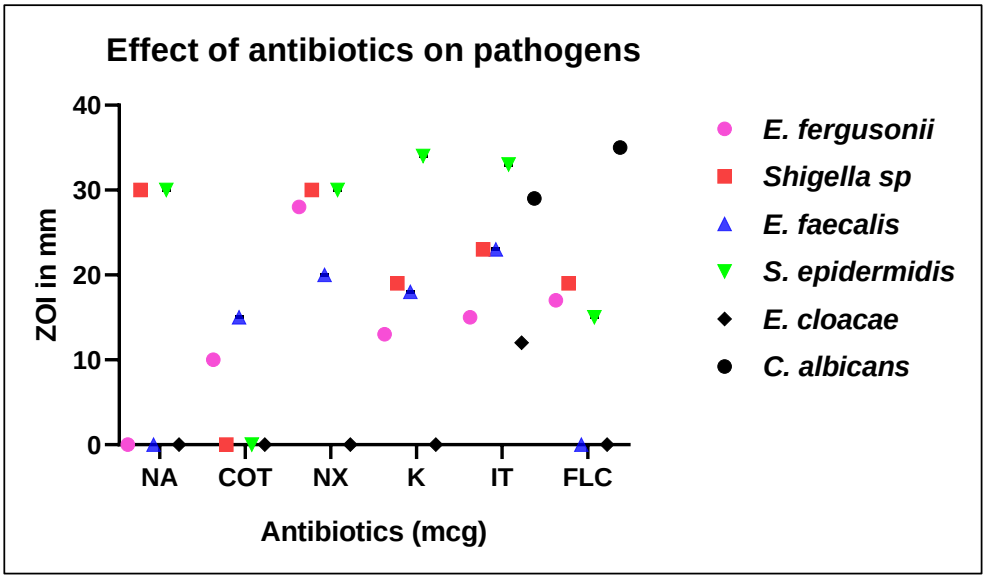


Fig 13: Effect of LAB resistant antibiotics on potential pathogens

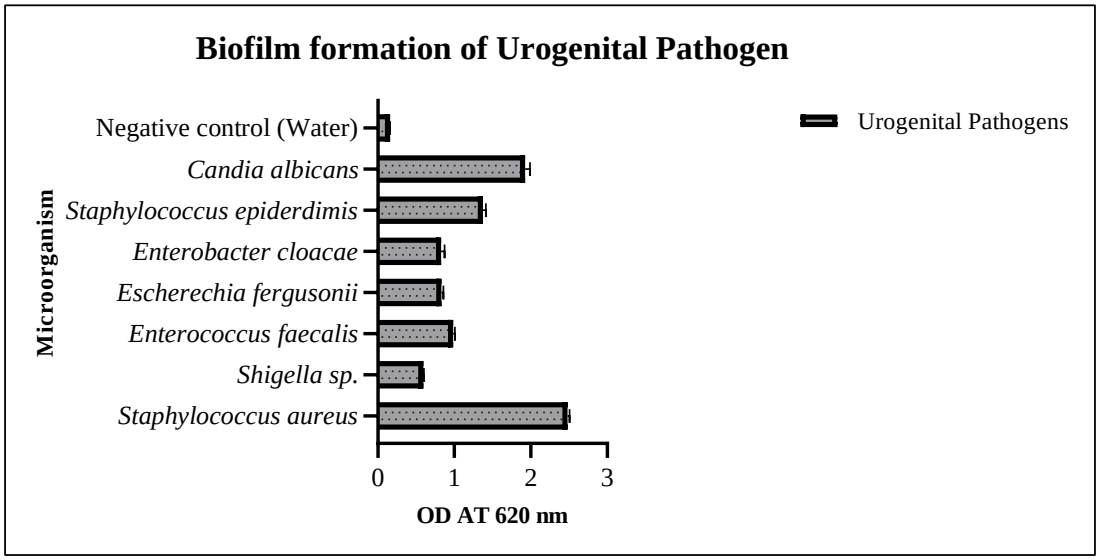


Fig 14: Biofilm formation by the potential pathogens

Table 5: Characterization of potential pathogens

	E. cloacae	*E. faecalis*	*E. fergusonii*	*S. epidermidis*	*Shigella sp*	*C. albicans*
Amylolytic	-	-	+	-	+	+
Haemolytic	+	+	-	+	-	-
Proteolytic	+	-	-	-	-	-
Lipolytic	-	+	-	+	-	+
Biofilm Formation	-	+	-	+	-	+
Anaerobic Growth	+	+	+	+	+	+
Glycogen Utilization	-	-	-	-	-	+

L. crispatus (LC), *L. gasseri* (LG), and *L. vaginalis* (LV) were significantly ($p \leq 0.0001$) tolerant to 0.05% bile salt, whereas only LG and LV showed minimal tolerance to 0.1% bile salt (Fig. 15). The LAB isolates were significantly ($p \leq 0.0001$) tolerant to 2% NaCl, showing growth within 24 h of incubation; whereas at 3% NaCl growth was observed post 48 h of incubation (Fig. 16). The growth of the isolates was significantly ($p \leq 0.0001$) reduced in high salt conditions in comparison to the control. 4% NaCl completely inhibited the growth of all the LAB's. All the isolates were tolerant to low pH 4 (Table 6). Nalidixic acid (NA) 30, Co-Trimoxazole (COT) 25 mcg, Kanamycin (K) 10 mcg, Norfloxacin (NX) 10 mcg, Fluconazole (FLC) 10 mcg, showed no zones of clearance for LC, LG, and LV. Itraconazole (IT) 10 mcg only inhibited the growth of LG and LV (Table 7). The saccharides fermented by LC, LG, and LV were listed in (Table 8), melibiose and xylose was slightly fermented by LC, whereas arabinose was not fermented by any of the isolates. Only LC showed the production of hydrogen peroxide utilizing 5 mM glucose as a substrate post 24 h of incubation and produced a brown pigment (Fig. 17). LC, LG, and LV exhibited hydrophobic nature preferring organic solvents hexane and xylene over aqueous PBS. LC showed 2.5% and 0% hydrophilicity with hexane and xylene. LG and LV showed hydrophilicity of 21.6% and 23.6% with hexane and 25.83% and 22% with xylene (Fig. 18). LC, LG, and LV showed significantly ($p \leq 0.0001$) increasing auto-aggregation with increasing time. LC and LV showed 41.6% aggregation and LG showed 52.6% aggregation post 6 h. Whereas, post 24 h LG showed 92% aggregation, LC showed 85% aggregation and LV showed 77% aggregation (Fig. 19). LC, LG, and LV showed

significant ($p \leq 0.0001$) co-aggregation with pathogens (gram-positive bacteria, gram-negative bacteria and yeast) post 6 h of incubation. LC showed significantly ($p \leq 0.0001$) higher aggregation with all these potential pathogens in comparison to the control. LG and LV aggregated better with *C. albicans*, rather than gram-positive bacteria and gram-negative bacteria (Fig. 20).

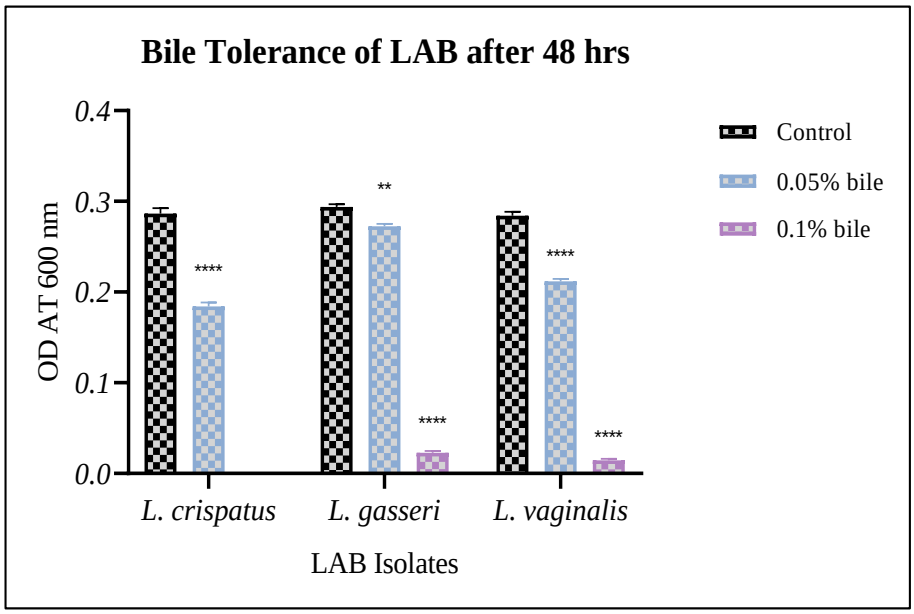


Fig 15: Bile salt tolerance of LAB isolates

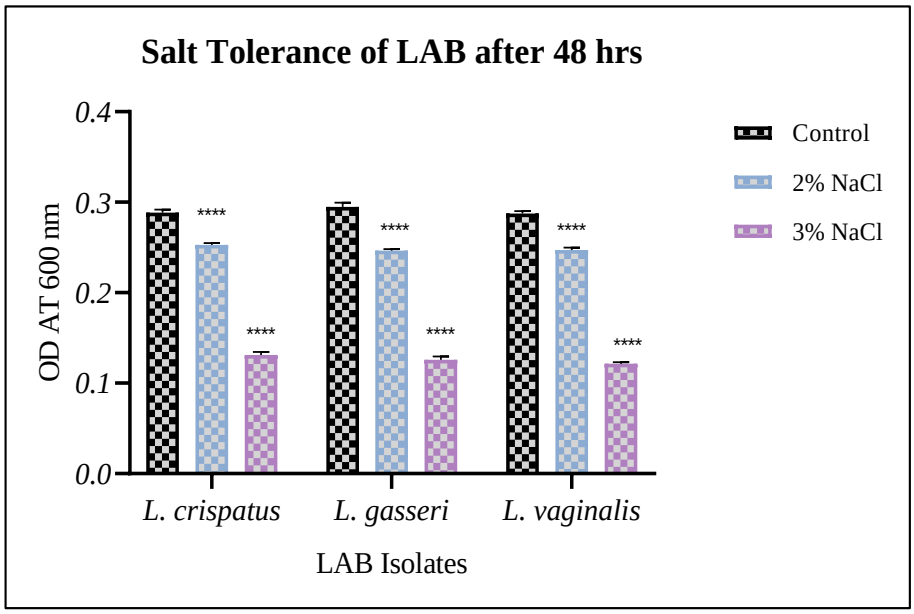


Fig 16: Sodium chloride salt tolerance of LAB isolates

Table 6: Ability of LAB isolates to grow at neutral and acidic pH

Isolate	pH 7	pH 4
L. crispatus	+	+
L. gasseri	+	+
L. vaginalis	+	+

(+ = growth)

Table 7: a. Antibiotic resistance of LAB; **b.** Antibiotic Susceptibility

a.

	L. crispatus	*L. gasseri*	*L. vaginalis*
Norfloxacin (NX) 10mcg	+	+	+
Kanamycin (K) 10mcg	+	+	+
Fluconazole (FLC) 25mcg	+	+	+
Itraconazole (IT) 10mcg	+	-	-
Co-Trimoxazole (COT) 25mcg	+	+	+
Nalidixic acid (NA) 30µg	+	+	+

b.

Antibiotic Susceptibility	
Tetracycline (TET) 10mcg	Nitrofurantoin (NIT) 30mcg
Erythromycin (E) 10mcg	Cefuroxime (CXM) 30mcg
Gentamicin (GEN) 10mcg	Gentamicin (GEN) 10mcg
Clindamycin (CD) 10mcg	Cefixime (CFM) 5mcg
Augmentin (AMC) 30mcg	Cefotaxime (CTX) 30mcg
Ofloxacin (OF) 5mcg	Augmentin (AMC) 30mcg
Erythromycin (E) 10mcg	Erythromycin (E) 10mcg
Chloramphenicol (C) 30mcg	

Table 8: Fermentation of sugars by lactic acid bacteria isolates

	L. crispatus	*L. gasseri*	*L. vaginalis*
Maltose	++	++	++
Lactose	++	++	++
Sucrose	++	++	++
Melibiose	+	-	-
Raffinose	++	++	++
Xylose	+	-	-
Arabinose	-	-	-
Galactose	++	++	++
Fructose	++	++	++

(++ Fermentation, + Partial fermentation, -No Fermentation)

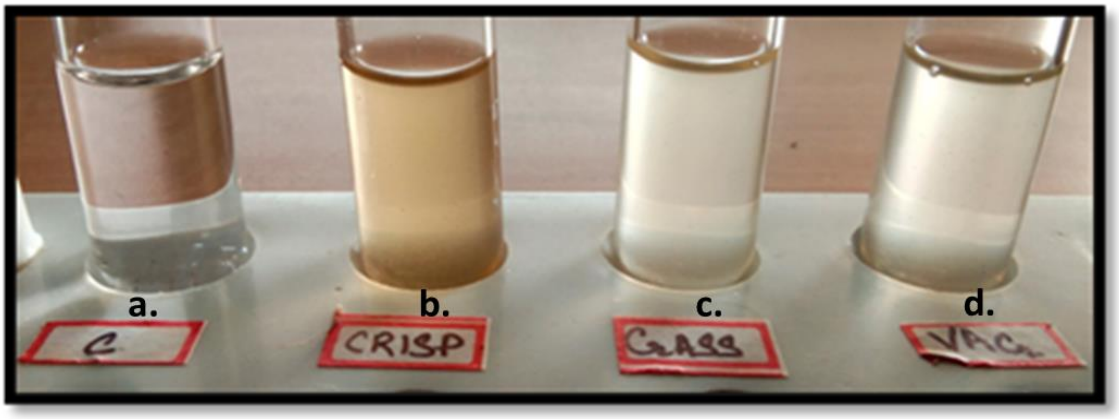


Fig 17: Detection of hydrogen peroxide production by LAB isolates (**a.** Blank **b.** *L. crispatus*, **c.** *L. gasseri*, and **d.** *L. vaginalis*)

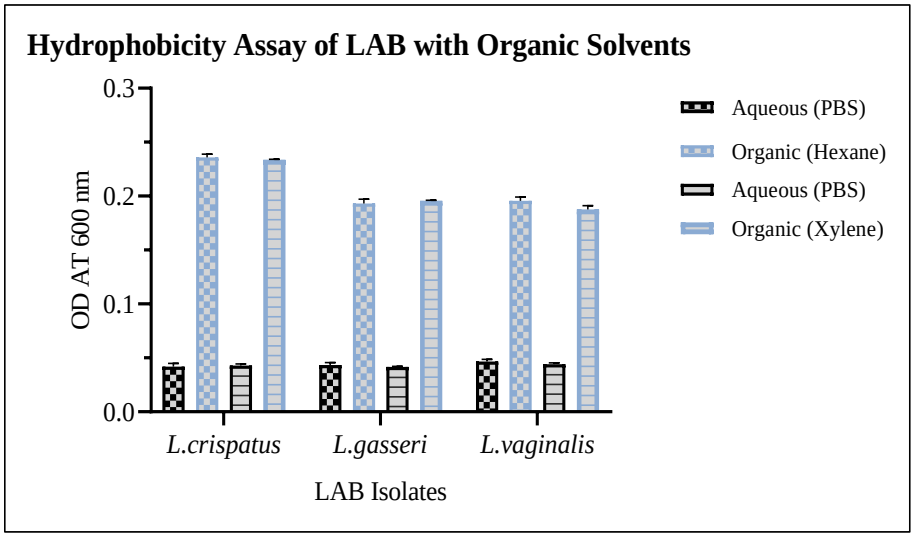


Fig 18: Hydrophobicity assay of LAB isolates

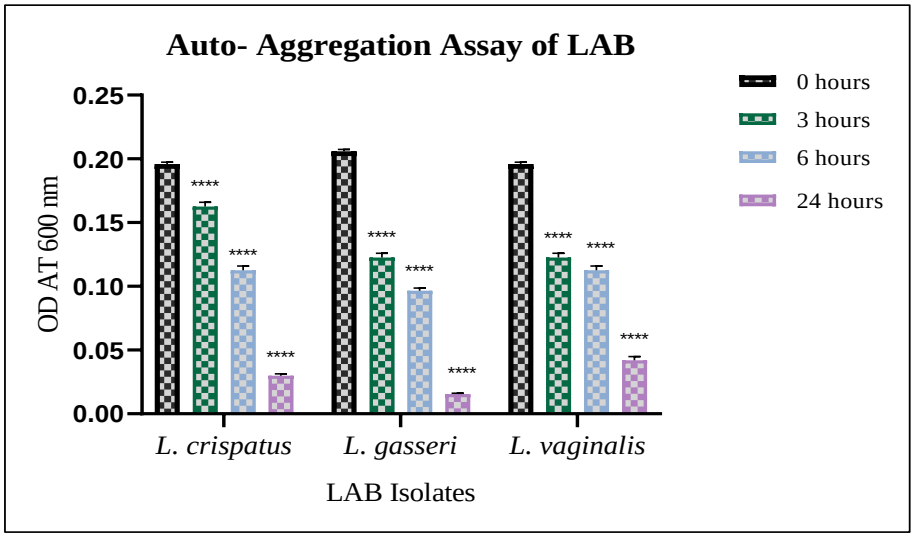


Fig 19: Auto- aggregation assay of LAB with increasing time

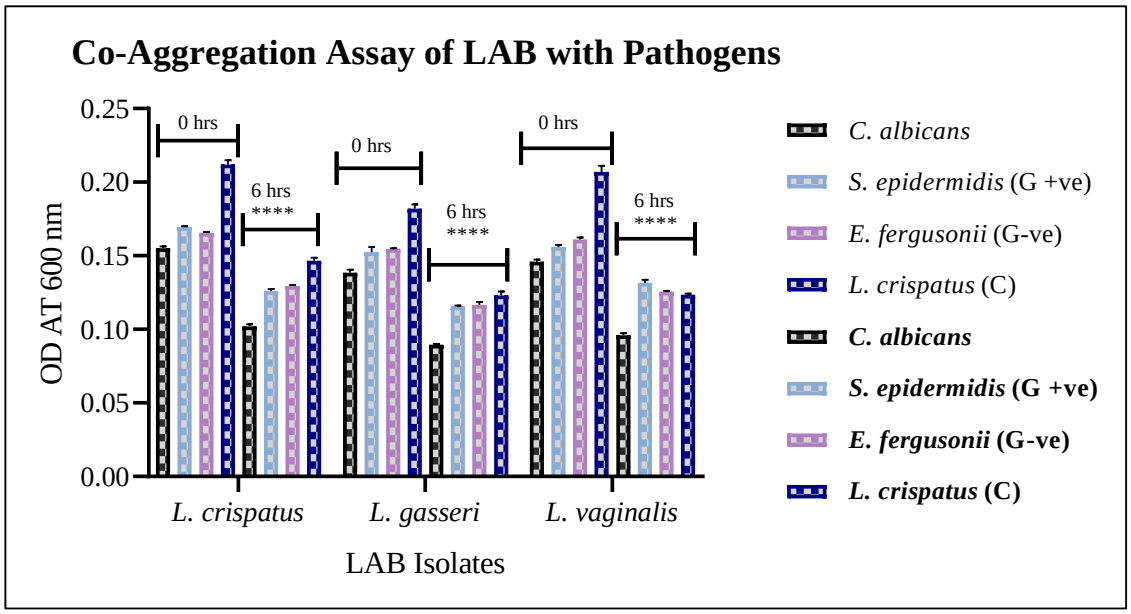


Fig 20: Co- aggregation assay of LAB with increasing time

The spectrophotometric method was used to estimate the amount of lactic acid produced by LC, LG, and LV. The standard graph was prepared with (R^2 value- 0.9815) (Fig. 21), and the estimation of lactic acid in CFS was done (Table 9). The chromatogram of serially diluted lactic acid (85%) showed a peak at 4 min. The absorbance unit (AU) decreased with increasing dilution and was used to make a standard graph with (R^2 value- 0.9971) (Fig 22). Similarly, the CFS of LC, LG, and LV also showed the presence of lactic acid with a peak at the same time point. The AU values of LC, LG, and LV were plotted on the graph to estimate the concentration of lactic acid produced by the isolates (Table 10) (Fig 23).

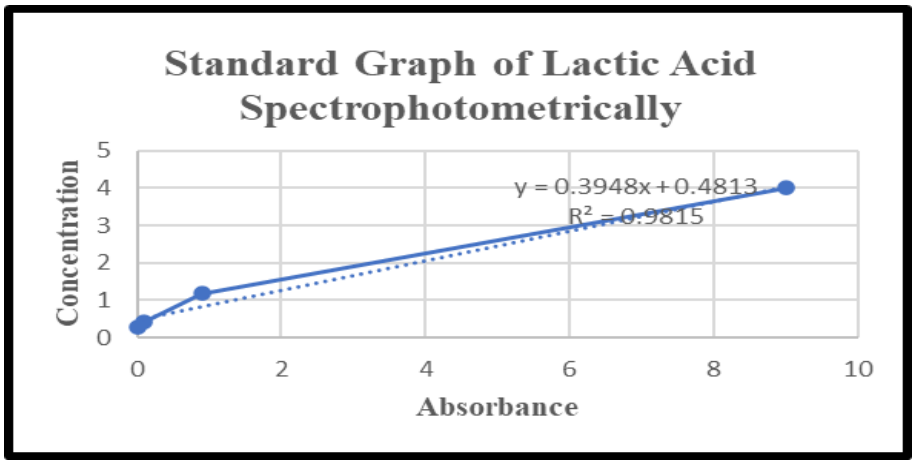


Fig 21: Standard graph of serially diluted lactic acid (85%) measured spectrophotometrically

Table 9: Estimation of lactic acid in culture free supernatant of LAB through RP-HPLC

Isolate	Lactic Acid (mg/ml)
L. crispatus	7.23
L. gasseri	7.81
L. vaginalis	6.65

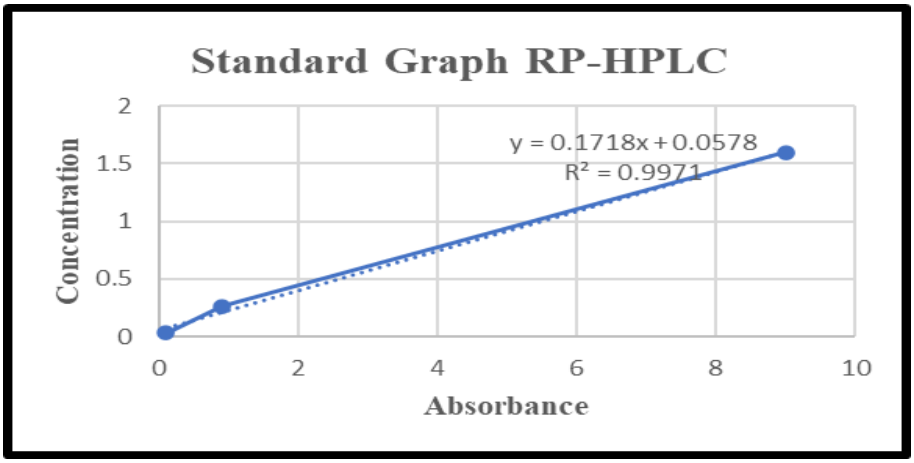
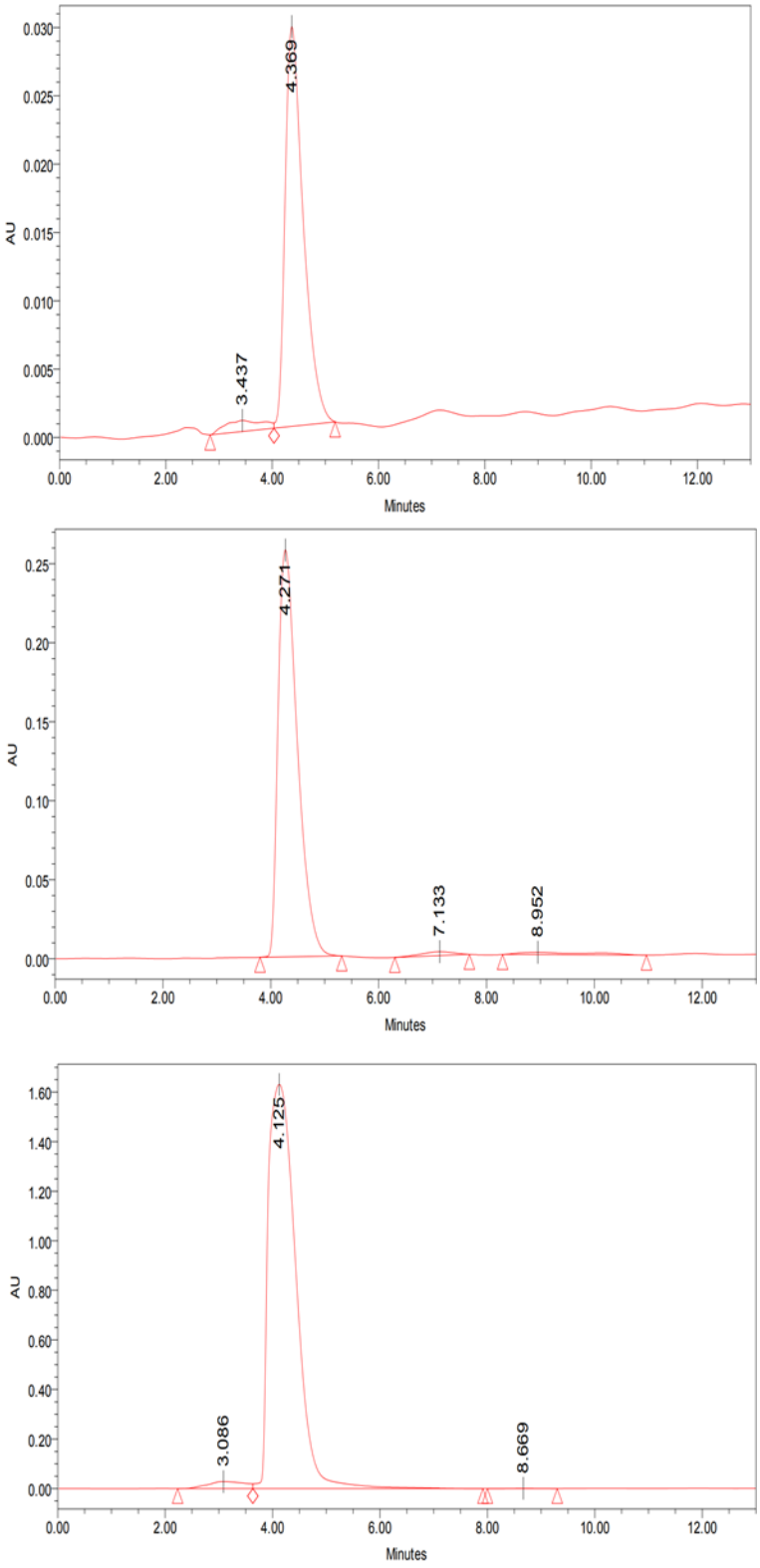


Fig 22: Standard graph of serially diluted lactic acid (85%) measured from AU value of chromatogram

Table 10: Estimation of lactic acid in culture free supernatant of LAB measured spectrophotometrically

Isolate	Lactic Acid (mg/ml)
L. crispatus	12.54
L. gasseri	11.89
L. vaginalis	9.77

a.


4.369
3.437
AU
Minutes
4.271
7.133
8.952
AU
Minutes
4.125
3.086
8.669
AU
Minutes

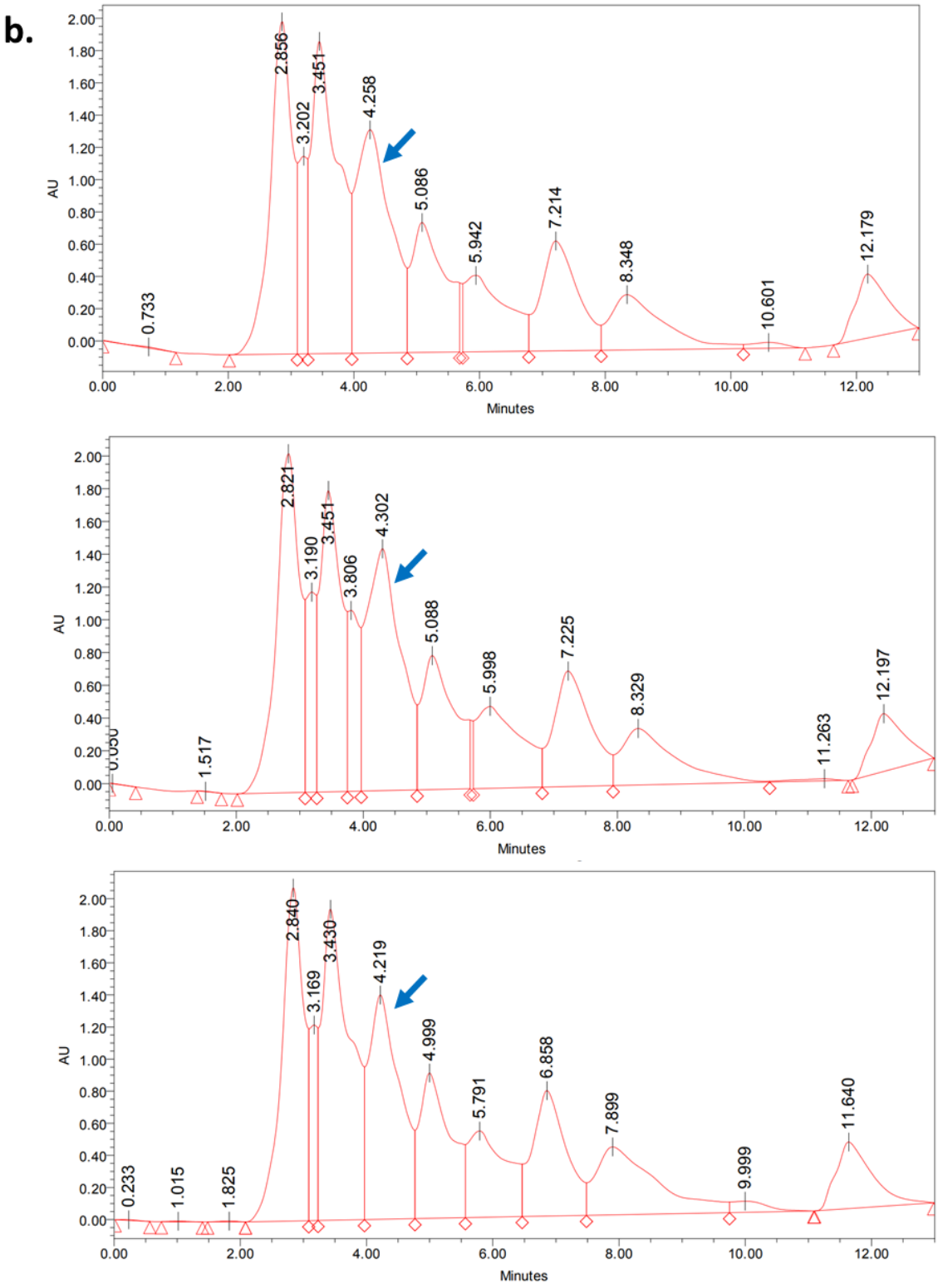


Fig 23: Chromatogram of RP-HPLC **a.** Serially diluted lactic acid (85%) **b.** Culture free supernatant of LAB *L. crispatus, L. gasseri* and *L. vaginalis* (Top to bottom) with blue arrow representing peak for lactic acid.

The ZOI in (mm) for the overlay assay was calculated (Table 11). LC was found to be more potent against *E. cloacae* and *Shigella* sp.*;* whereas LG effective against *E. fergusonii*, *E. faecalis*, and *S. epidermidis* (Fig 24.A., 24.B., and 24.C.). The cup assay demonstrated that LC was potent against *E. Cloacae* and *Shigella sp.;* LG and inhibited *E. faecalis* minimally (Table 12) (Fig. 25).

Table 11: Zone of clearance (mm) by vaginal LAB on potential urogenital pathogens by overlay assay

	***L. crispatus* (mm)**	***L. gasseri* (mm)**	***L. vaginalis* (mm)**
E. cloacae	**7.1 (± 0.160)**	**5 (± 0.115)**	**7 (± 0.152)**
E. fergusonii	**13 (± 0.2)**	**14.3 (± 0.360)**	**5.7 (± 0.251)**
E. faecalis	**6 (± 0.152)**	**7 (± 0.2)**	**6.2 (± 0.173)**
S. epidermidis	**10.2 (± 0.2)**	**13.7 (± 0.264)**	**7.1 (± 0.217)**
Shigella sp.	**11.2 (± 0.251)**	**8 (± 0.20)**	**5.9 (± 0.152)**
C. albicans	**0**	**0**	**0**

Data are mean values; ± SD of independent experiments (n = 3)

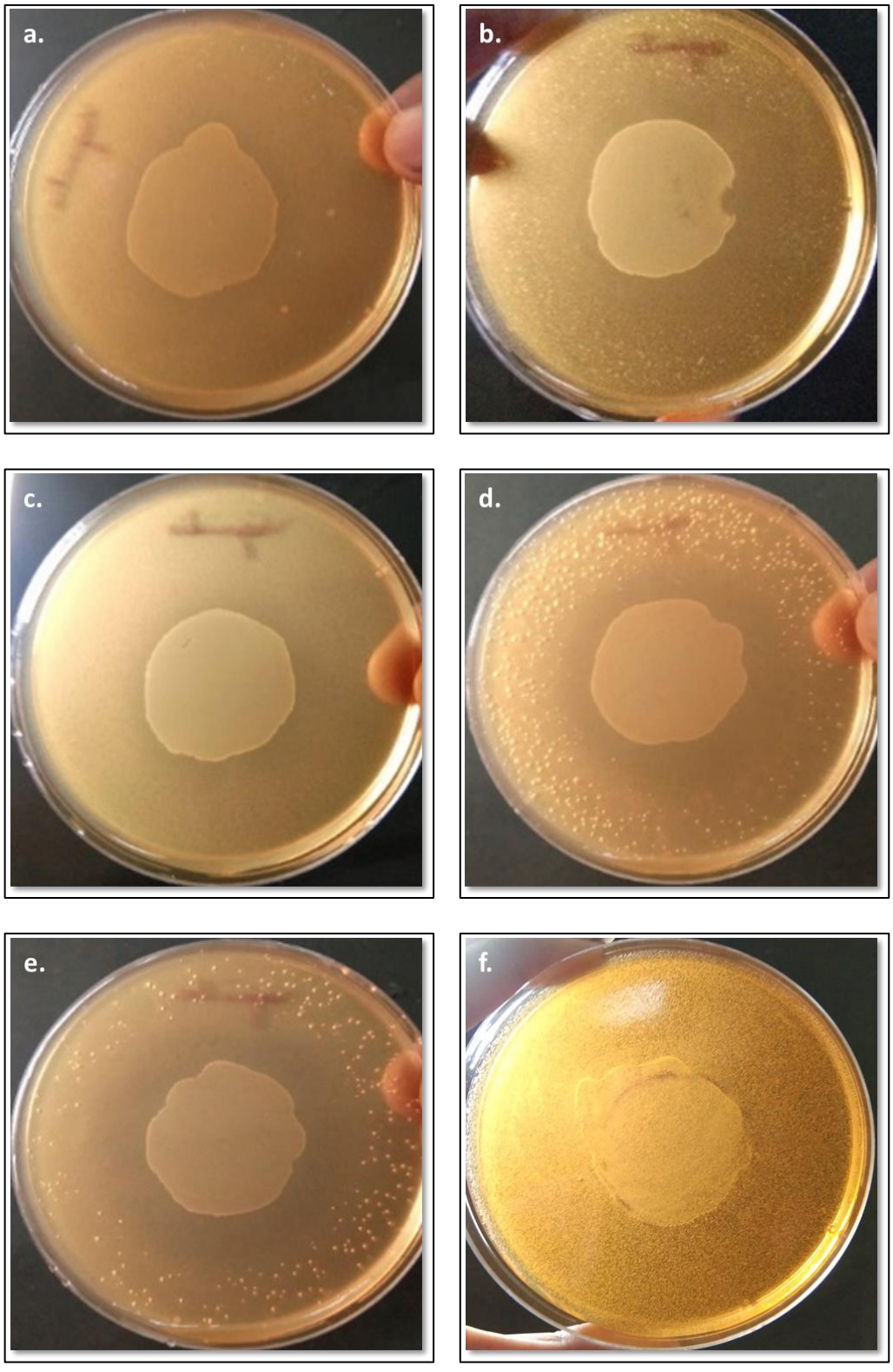


Fig 24.A.: Inhibition of potential urogenital pathogens by LAB through overlay assay using *L. crispatus* (**a.** *E. cloacae,* **b**. *S. epidermidis,* **c.** *E. faecalis,* **d.** *E. fergusonii* and **e.** *Shigella sp* **f.** *C. albicans*)

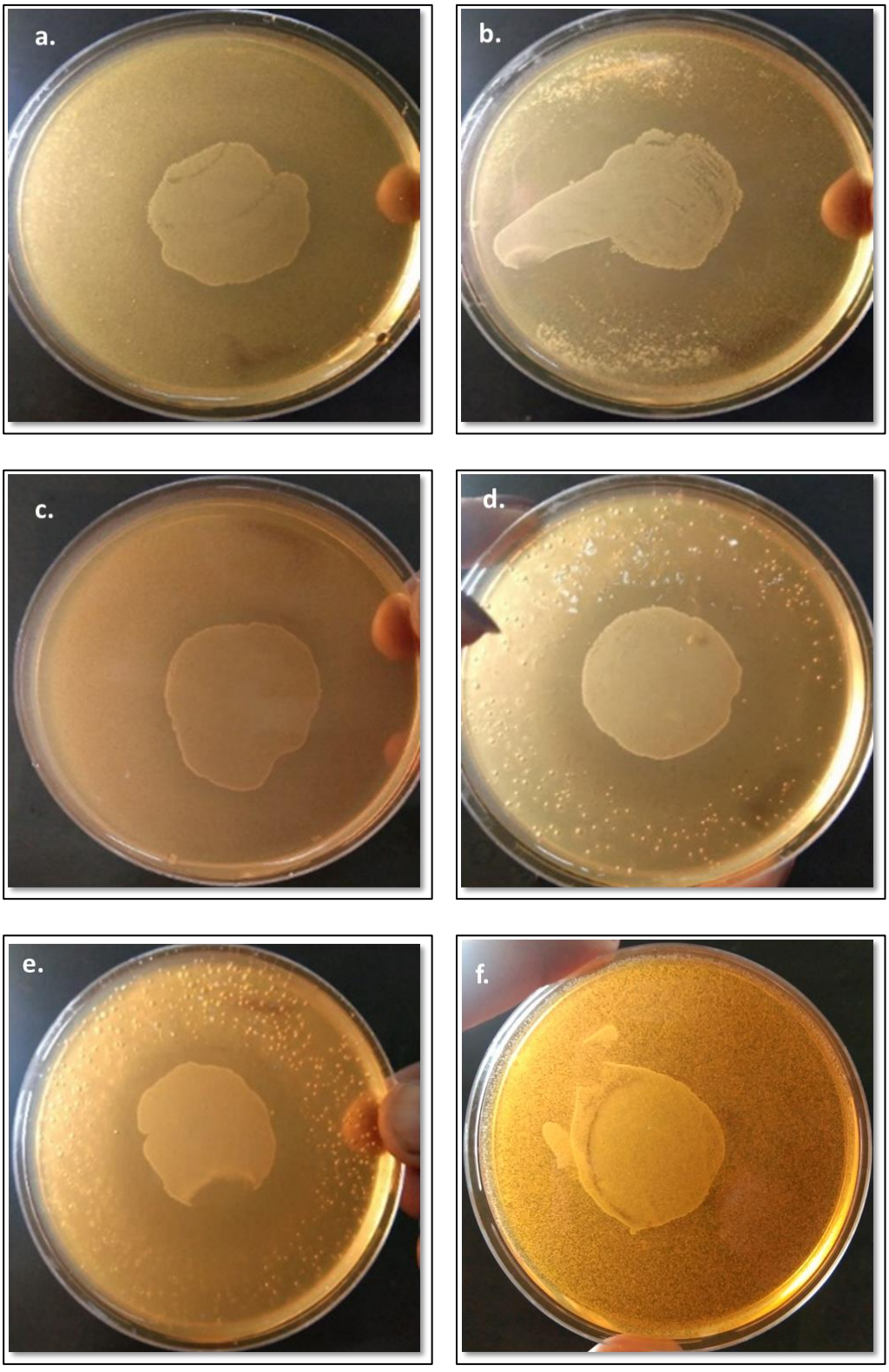


Fig 24.B.: Inhibition of potential urogenital pathogens by LAB through overlay assay *L. gasseri* (**a**. *E. cloacae,* **b.** *S. epidermidis,* **c.** *E. faecalis,* **d**. *E. fergusonii* and **e.** *Shigella sp* **f.** *C. albicans)*

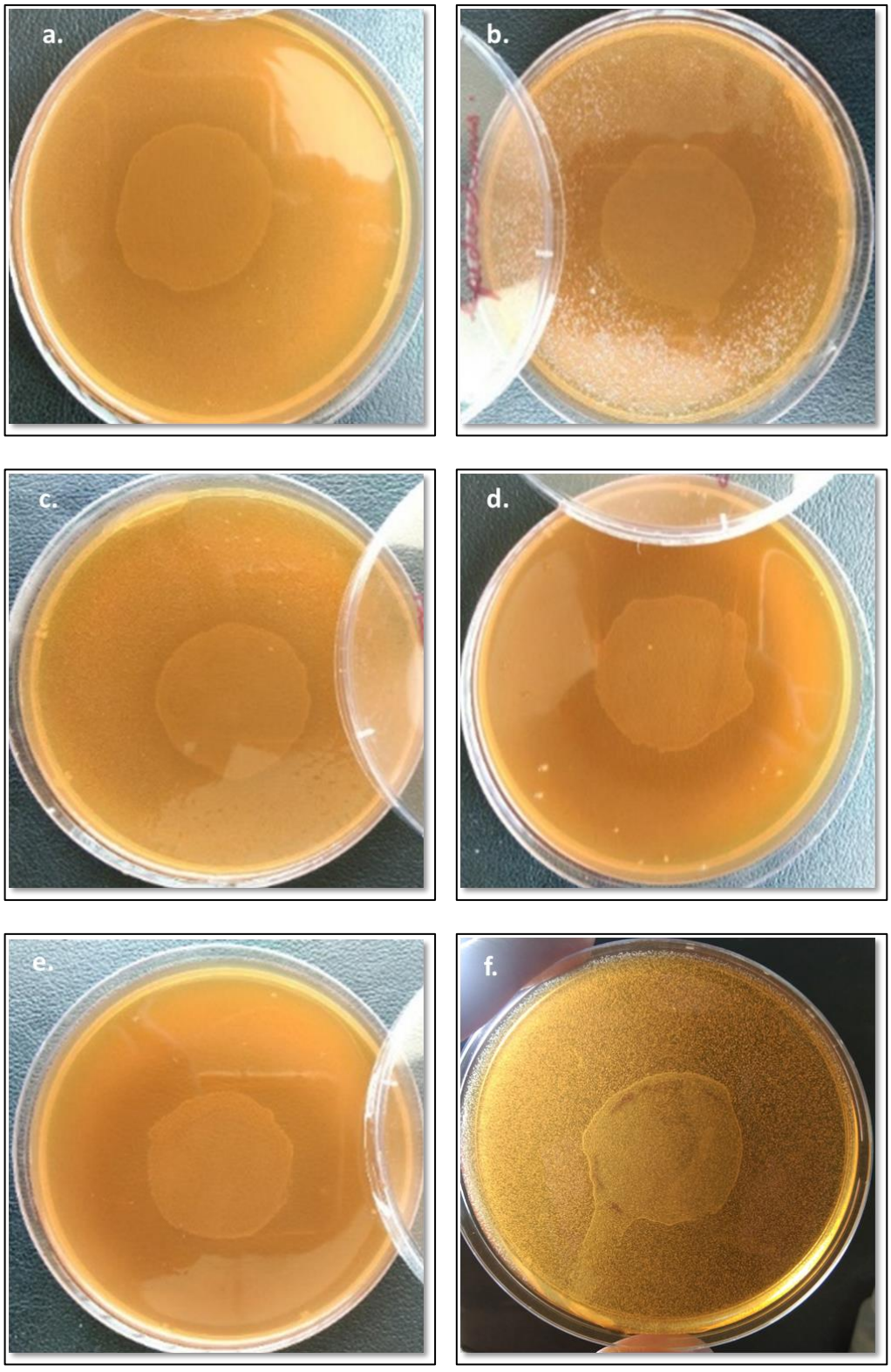


Fig 24.C.: Inhibition of potential urogenital pathogens by LAB through overlay assay using *L. vaginalis* (**a.** *E. cloacae,* **b**. *S. epidermidis, **c.** E. faecalis,* **d.** *E. fergusonii and* **e.** *Shigella* sp**. f.** *C. albicans*)

Table 12. Inhibition of potential urogenital pathogens by LAB through cup assay

	L. crispatus	*L. gasseri*	*L. vaginalis*
E. cloacae	++	-	-
E. fergusonii	-	-	-
E. faecalis	-	+	+
S. epidermidis	-	-	-
Shigella sp.	++	-	-
C. albicans	-	-	-

(++ clear ZOI, + Slight Inhibition and – as no inhibition)

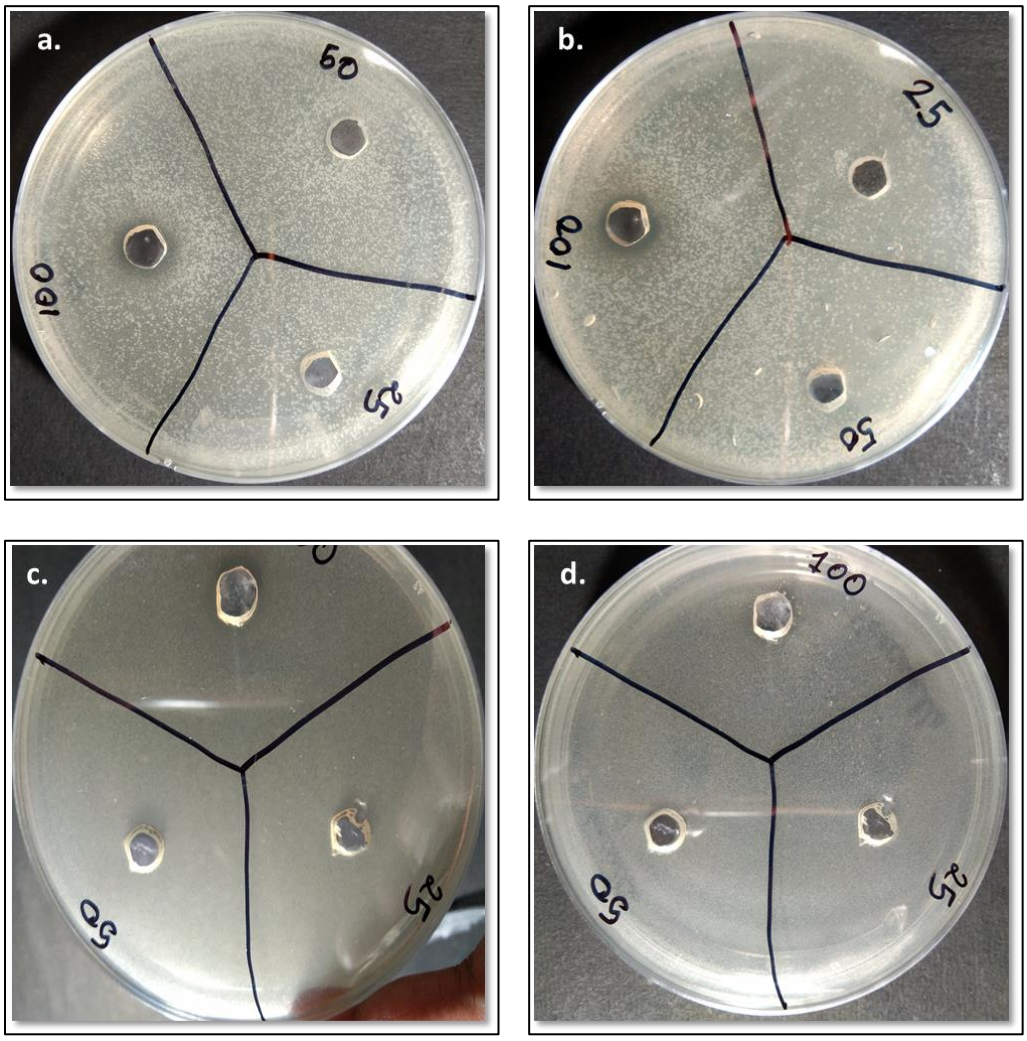


Fig 25: Inhibition of potential urogenital pathogens by LAB through cup assay **a.** *E. cloacae* and **b.** *Shigella* sp and *L. gasseri* **c.** *E. cloacae* and **d.** *Shigella* sp.

The LCCFS showed the highest bacteriostatic effect against all the potential pathogens, the ZOI in mm was measured and noted (Table 13) (Fig. 26). LVCFS showed better bactericidal activity against the *E. fergusonii*, *E. facaelis*, and *Shigella* sp.; whereas LGCFS was more effective against *E. cloacae* and *S. epidermidis*. The CFS showed bactericidal activity and there was significant ($p \leq 0.0001$) increase in bactericidal activity from 12 to 24 h (Fig. 27). LC and LV CFS 50 µl were able to completely inhibit the growth of all potential pathogens (Fig. 28.a.). LGCFS able to inhibit the growth of these aerobic microbes was significantly ($p \leq 0.0001$) in comparison to the negative control but had a MIV of 100 µL. Lesser volume of CFS (25 µl) of LC, LG, and LV was less potent in inhibiting the pathogens, although *S. epidermidis* was completely inhibited (Fig 28.b.). The lyophilized CFS of LC, LG, and LV inhibited the potential pathogens. LCCFS showed the best inhibition potential against all the aerobic isolates (Table 14). LVCFS showed the second-best potential against them (Fig 29).

Table 13: Zone of inhibition (mm) of potential urogenital pathogens by CFS LAB through cup assay

CFS	*L. crispatus*	*L. gasseri*	*L. vaginalis*
E. cloacae	**12.7 (± 0.256)**	**11.8 (± 0.152)**	**0**
E. fergusonii	**13.9 (± 0.152)**	**13 (± 0.154)**	**12 (± 0.251)**
E. faecalis	**14.1 (± 0.157)**	**11.7 (± 0.251)**	**12 (± 0.173)**
S. epidermidis	**14 (± 0.115)**	**11.2 (± 0.251)**	**11 (± 0.1)**
Shigella sp.	**13.8 (± 0.152)**	**12 (± 0.3)**	**11 (± 0.208)**
C. albicans	**0**	**0**	**0**

Data are mean values; ± SD of independent experiments (n = 3)

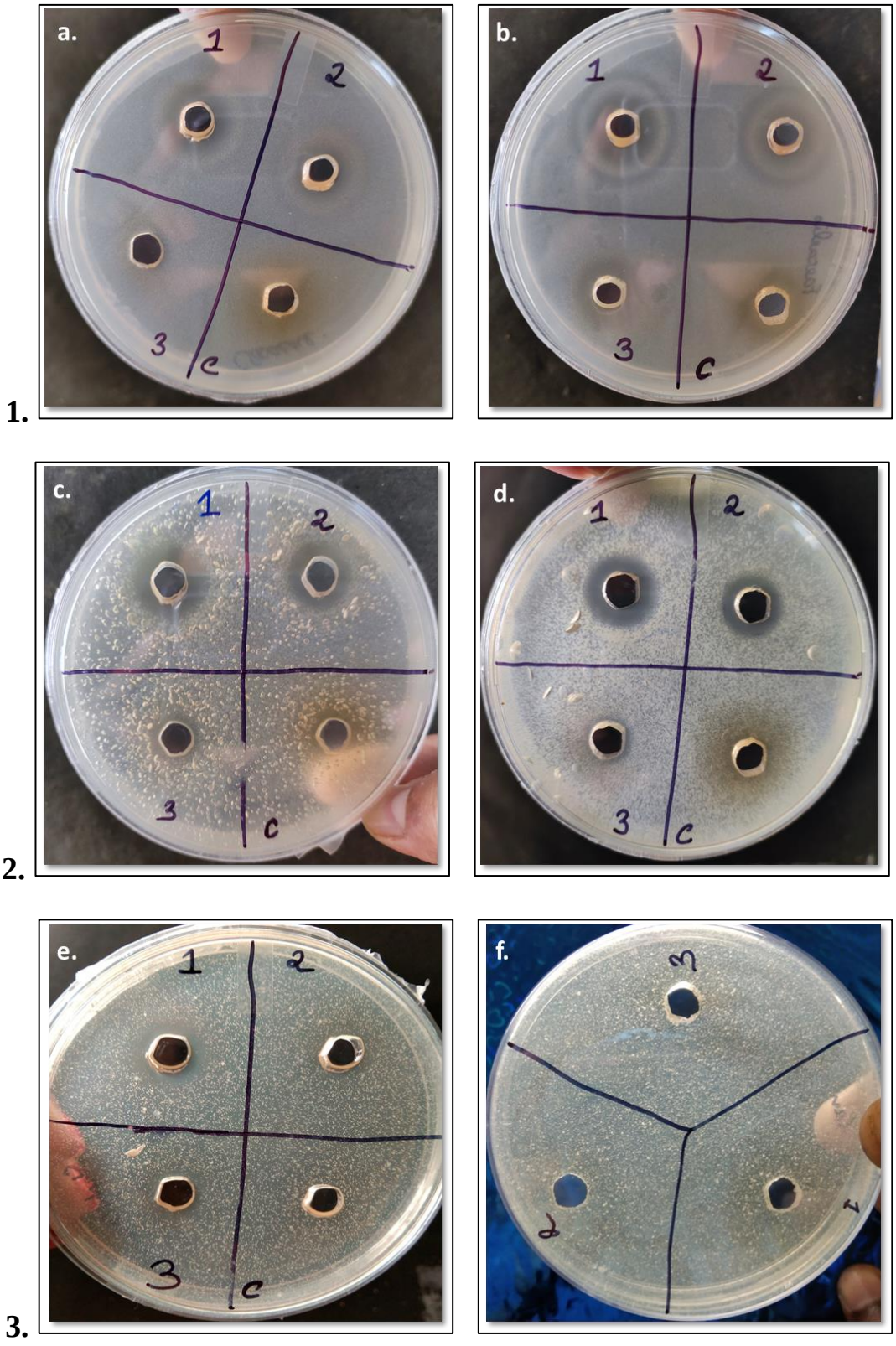


Fig 26: Inhibition of potential urogenital pathogens by LAB CFS through cup assay. **a.** *E. cloacae* and **b.** *E. faecalis* **c.** *E. fergusonii* **d.** *Shigella* sp. **e.** *S. epidermidis* **f.** *C. albicans*; **1.** *L. crispatus*, **2.** *L. gasseri*, **3.** *L. vaginalis* C Blank

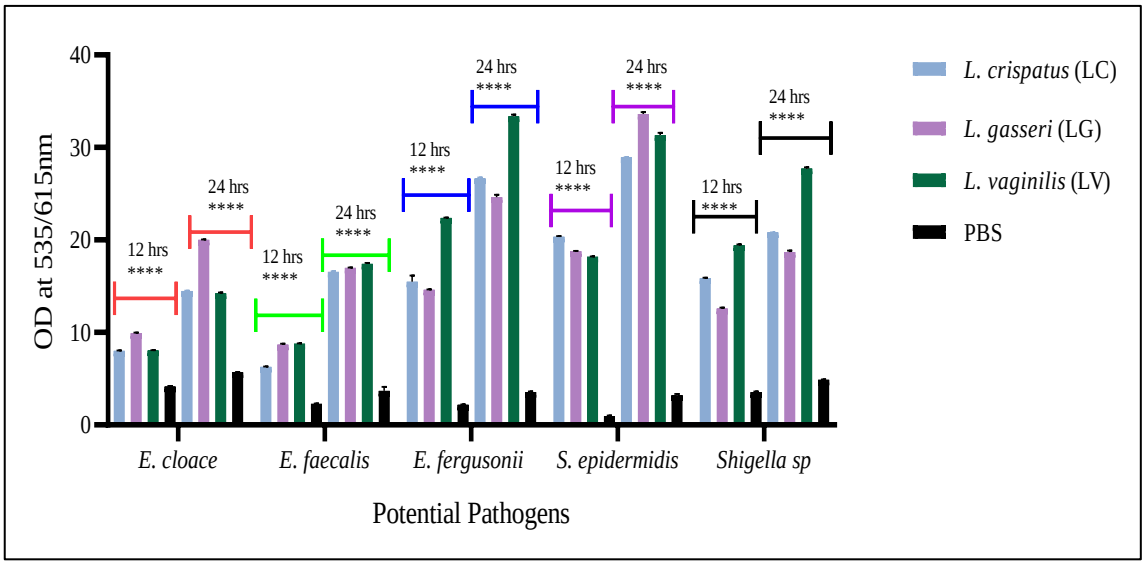


Fig. 27: Bactericidal effect of vaginal LAB CFS on potential urogenital pathogens (PBS- Phosphate Buffer Saline) (****=p≤0.0001)

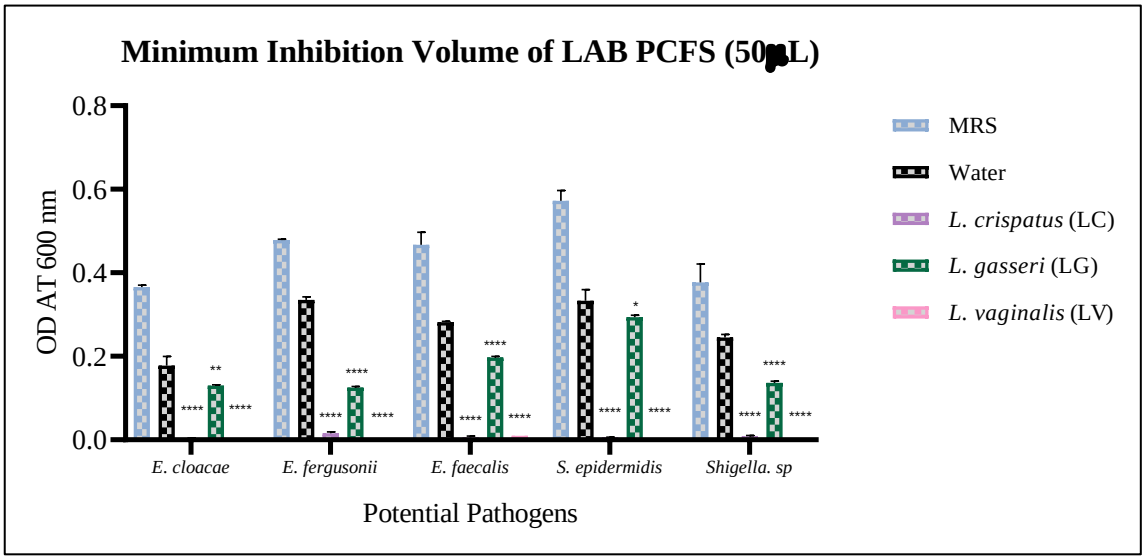


Fig. 28.a.: Minimum inhibition volume of vaginal LAB CFS on potential urogenital pathogens (MRS- De-Man Ragosa Sharpe broth) (**** = p≤0.0001, ** = p≤0.01 and * =p≤0.05)

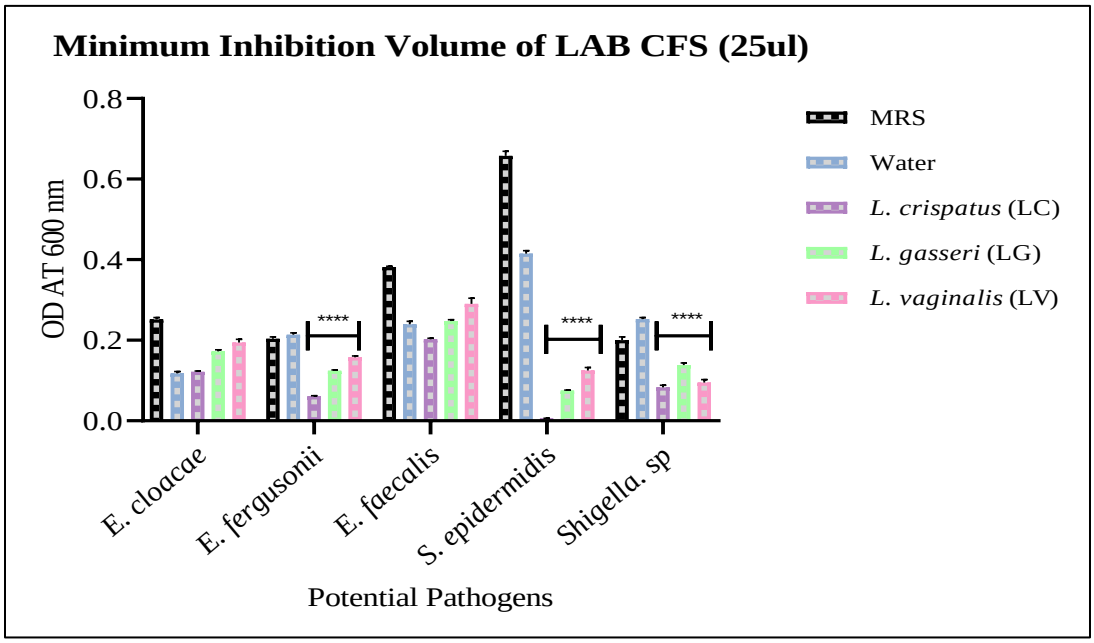


Fig 28.b.: Minimum inhibition volume of LAB CFS (25µL) on potential urogenital pathogens. (MRS- De-Man Ragosa Sharpe Broth) (**** = p≤0.0001)

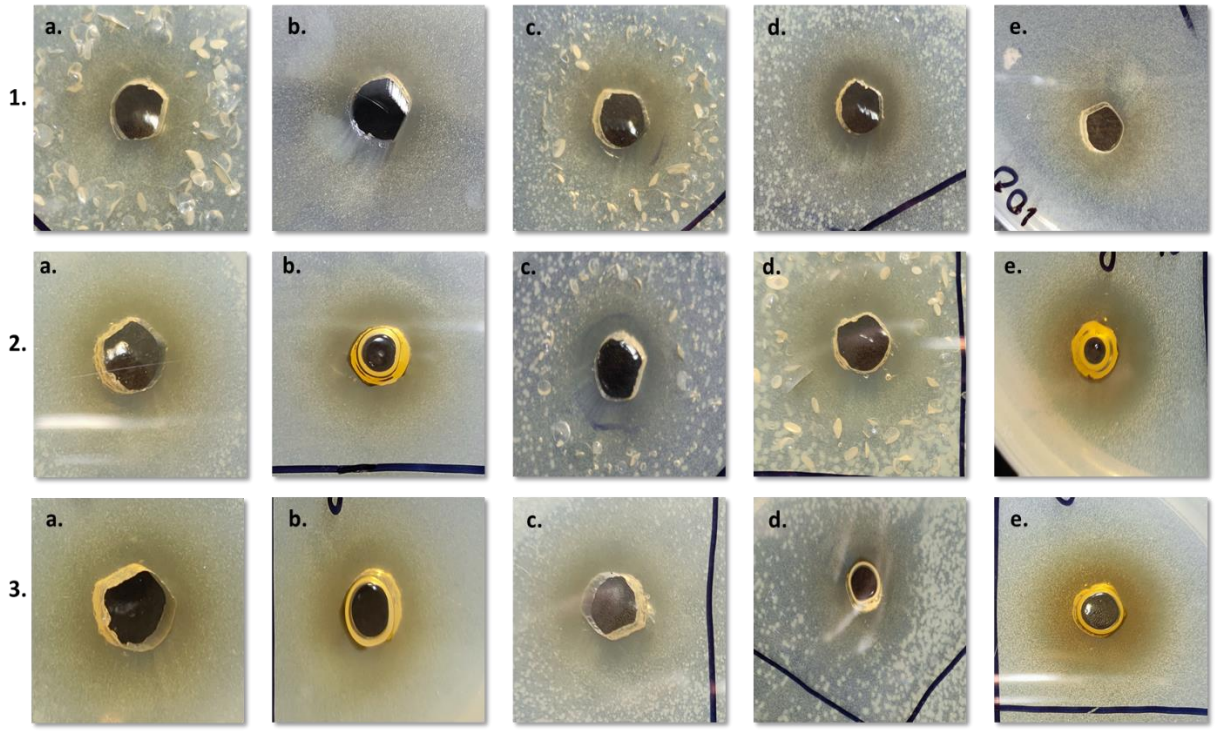


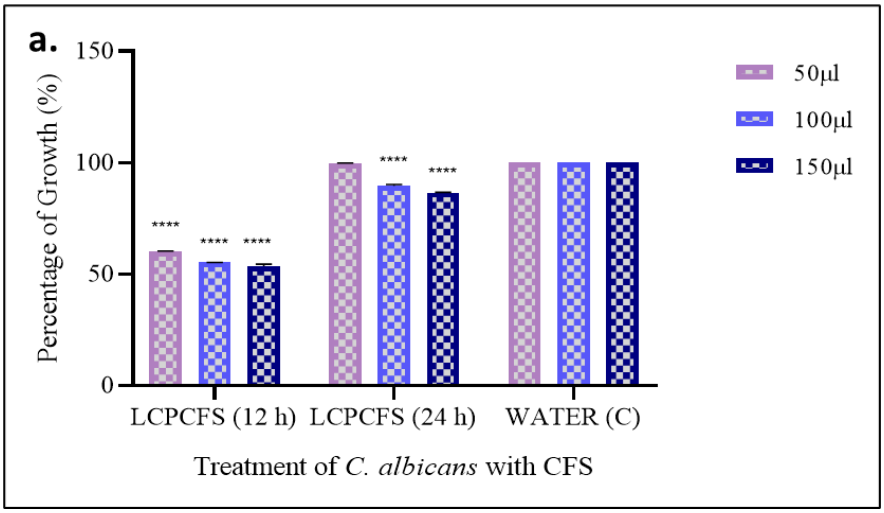
Fig. 29: Inhibitory effect of lyophilized CFS of LAB on potential urogenital pathogens through cup assay. (**a**. *E. cloacae*, **b.** *E. faecalis*, **c.** *E. fergusonii*, **d.** *Shigella sp*, **e.** *S. epidermidis* Lyophilized CFS; **1.** *L. crispatus*, **2.** *L. gasseri*, **3.** *L. vaginalis*.)

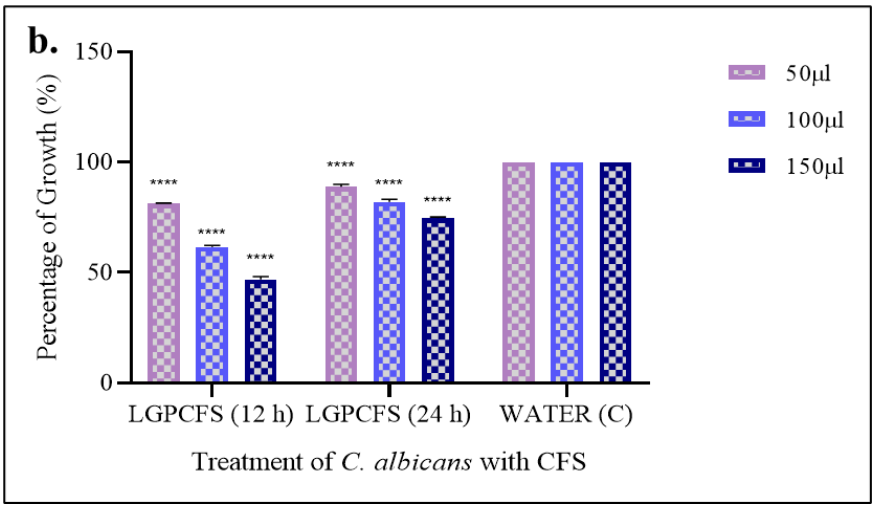
Table 14: Minimum inhibition concentration (mm) of lyophilized CFS of LAB

PCFS	*E. cloacae*		*E. faecalis*		*E. fergusonii*		*Shigella sp.*		*S. epidermidis*	
	Conc (mg/µL)	ZOI (mm)	Conc (mg/µL)	ZOI (mm)	Conc (mg/µL)	ZOI (mm)	Conc (mg/µL)	ZOI (mm)	Conc (mg/µL)	ZOI (mm)
***L. crispatus* (mm)**	**0.2**	**12**	**0.1**	**11**	**0.1**	**13**	**0.1**	**13**	**0.1**	**12**
***L. gasseri* (mm)**	**0.3**	**13**	**0.3**	**15**	**0.2**	**14**	**0.2**	**13**	**0.4**	**18**
***L. vaginalis* (mm)**	**0.2**	**12**	**0.2**	**11**	**0.1**	**12**	**0.1**	**13**	**0.3**	**18**

Data are mean values; ± SD of independent experiments (n = 3)

The LAB (LC, LG, and LV) showed no zone of inhibition (ZOI) against *C. albicans* in the overlay assay and the cup assay.The variable volumes of CFS from (LC, LG, and LV) significantly ($p \leq 0.0001$, $p \leq 0.01$) minimalized the growth of *C. albicans* till 12 h of incubation. After 24 h all volumes of LPCFS inhibited the growth of *C. albicans* significantly ($p \leq 0.0001$) in comparison to the control. On the other hand, 50µl of LCCFS showed non-significant inhibition, but increasing volume of 100µl and 150µl LCCFS showed significant ($p \leq 0.0001$) inhibition. LVCFS was unable to inhibit the growth of *C. albicans* till 24 h of incubation. Increasing volume of CFS showed escalating inhibition (Fig. 30). Treatment of (LC, LG, and LV) CFS with sodium hydroxide showed significant ($p \leq 0.0001$) decrease in its efficiency to inhibit *C. albicans*, whereas heat treatment affected the efficiency of LGCFS only. Potency of LCCFS was affected by proteinase K and amylase treatment. The efficiency of LGCFS decreased on treatment with proteinase K; whereas efficiency of LVCFS was slightly affected by every enzyme treatment (Fig. 31). Spider agar plates laced with LCCFS and LGCFS showed no hyphae or pseudo-hyphae formation post incubation of 24 h to 5 days at 37℃. In comparison the plates laced with LVCFS showed minimal formation of hyphae around single colonies post 5 days of incubation; whereas the control untreated plates showed prominent hyphal growth around the colonies of *C. albicans* post 24 h and 5 days (Fig. 32). RPMI 1640 basal medium promotes hyphae formation of *C. albicans*. The control plates showed heavy formation of hyphae. Whereas the treatment with (LC, LG, and LV) CFS inhibited the hyphae formation which was supposed to be promoted by RPMI 1640. LGCFS was most effective in inhibition of hyphae formation, it completely inhibited hyphal growth. LCCFS showed least potency in inhibiting hyphae formation and LVCFS inhibited the hyphae formation to some extent (Fig. 33). The ratio 2:1 of RPMI: CFS was capable in inhibiting the hyphae formation of *C. albicans*.





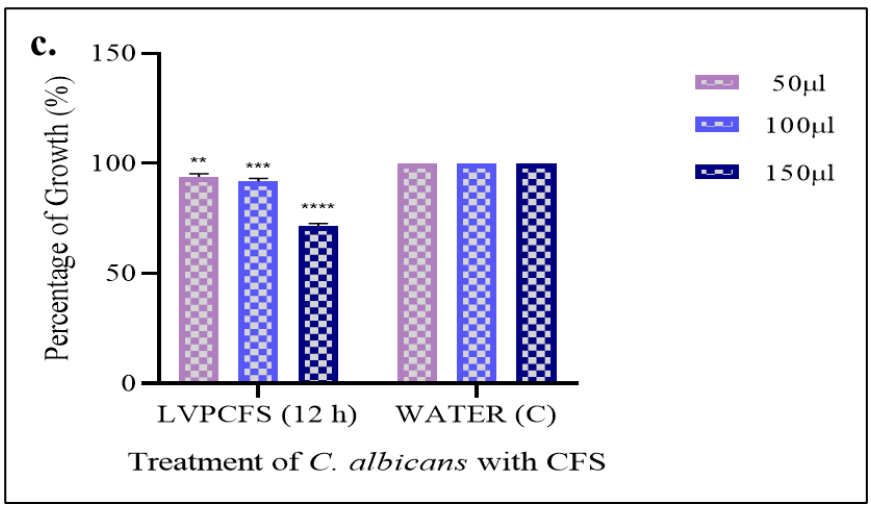


Fig 30: Effect of CFS on budding of *C. albicans*. **a.** CFS of *L. crispatus*, **b.** *L. gasseri* and **c.** *L.* (****=p≤0.0001) (***=p≤0.001) (**=p≤0.01)

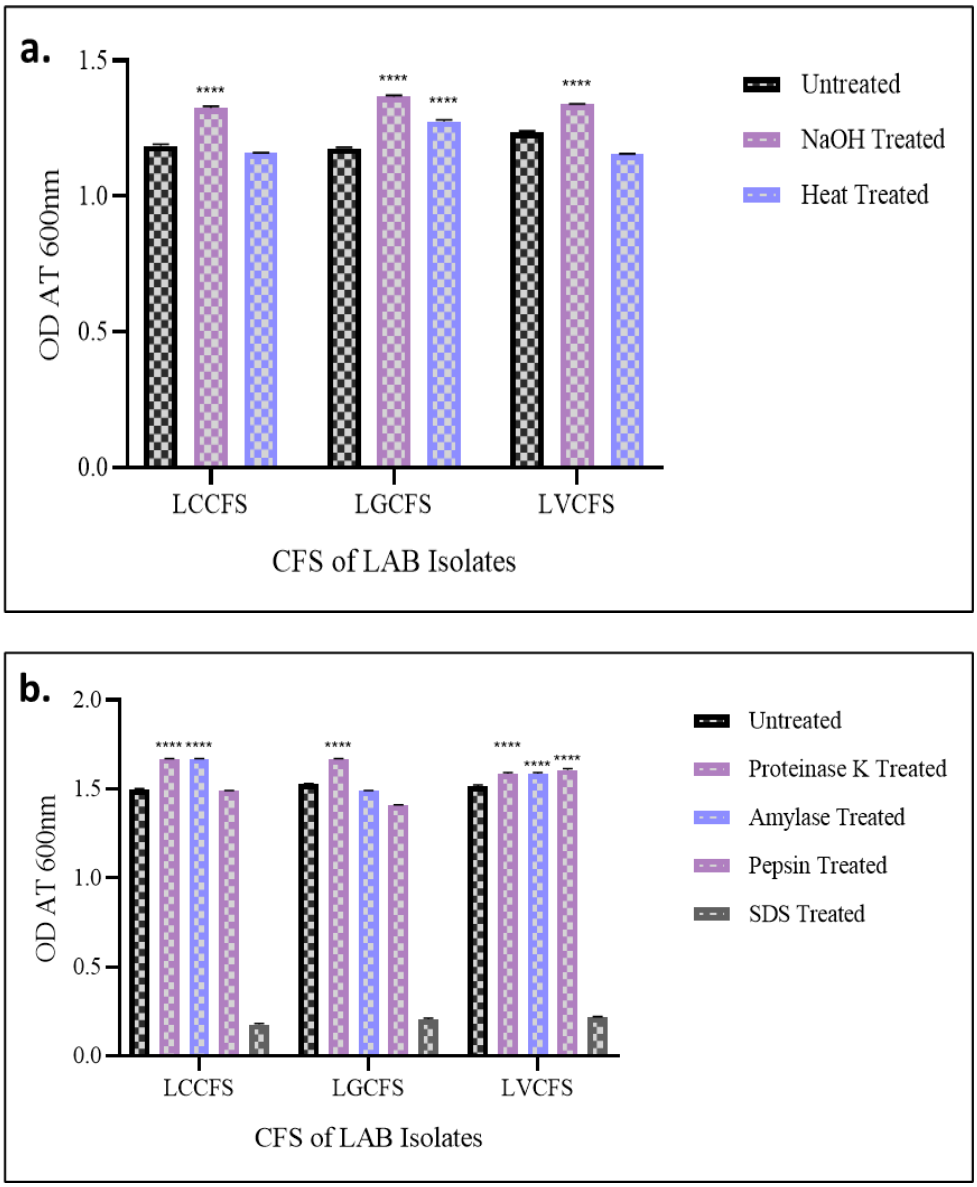
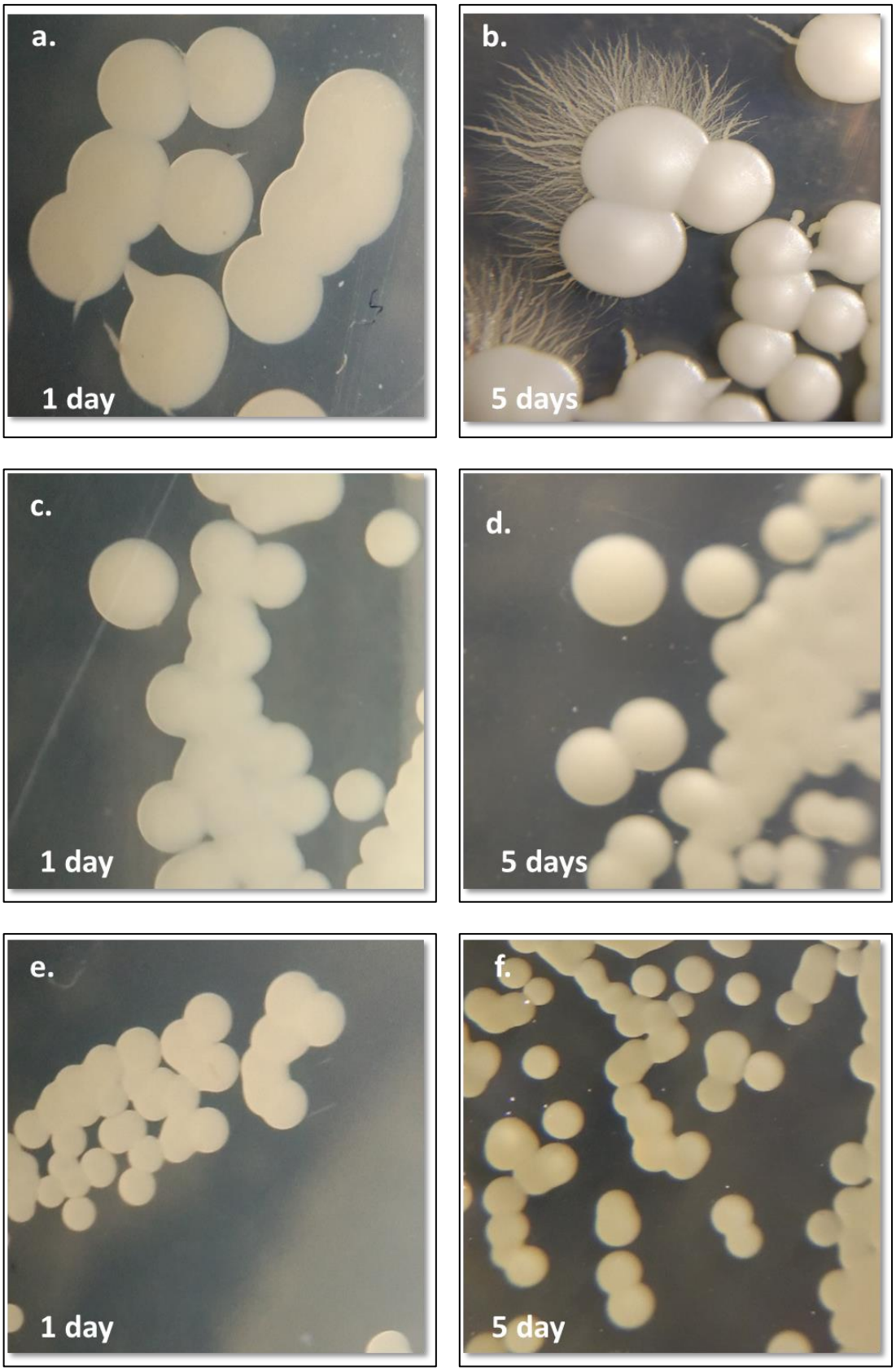


Fig. 31: Effect of treated CFS on budding of *C. albicans* Treatment of LCCFS, LGCFS, and LVCFS with **a.** alkali- sodium hydroxide and heat, **b**. protease enzymes, amylase enzyme, pepsin, and detergent (****=p≤0.0001)


a.
1 day
b.
5 days
c.
1 day
d.
5 days
e.
1 day
f.
5 day

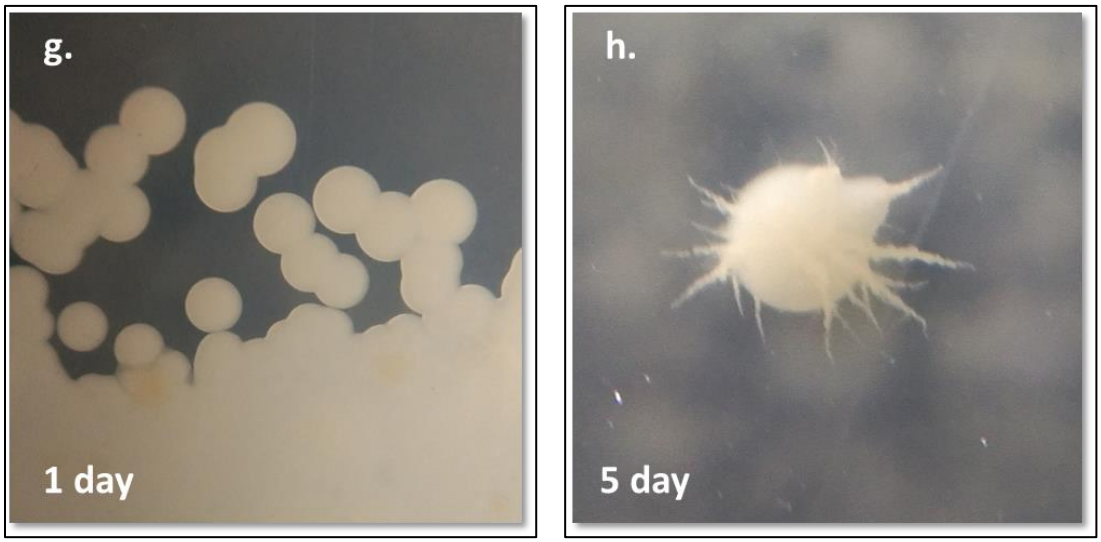
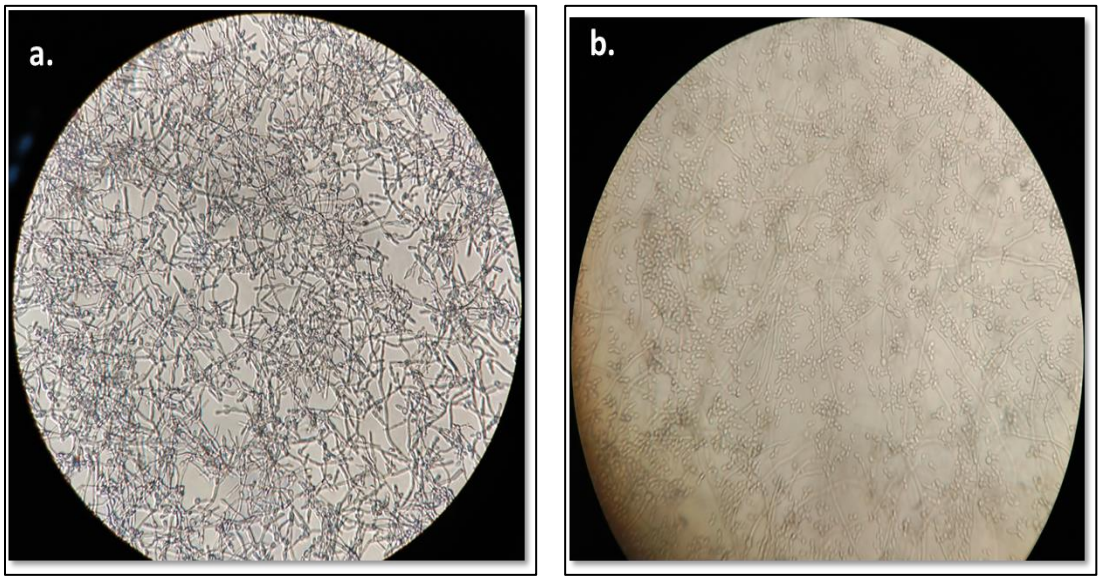


Fig. 32: Effect of CFS on hyphae formation of *C. albicans* on spider agar plate. **a. and b.** Control Day 1 and Day 5, **c. and d.** LCCFS Day 1 and Day 5, **e. and f.** LGCFS Day 1 and Day 5, and **g. and h.** LVCFS Day 1 and Day 5



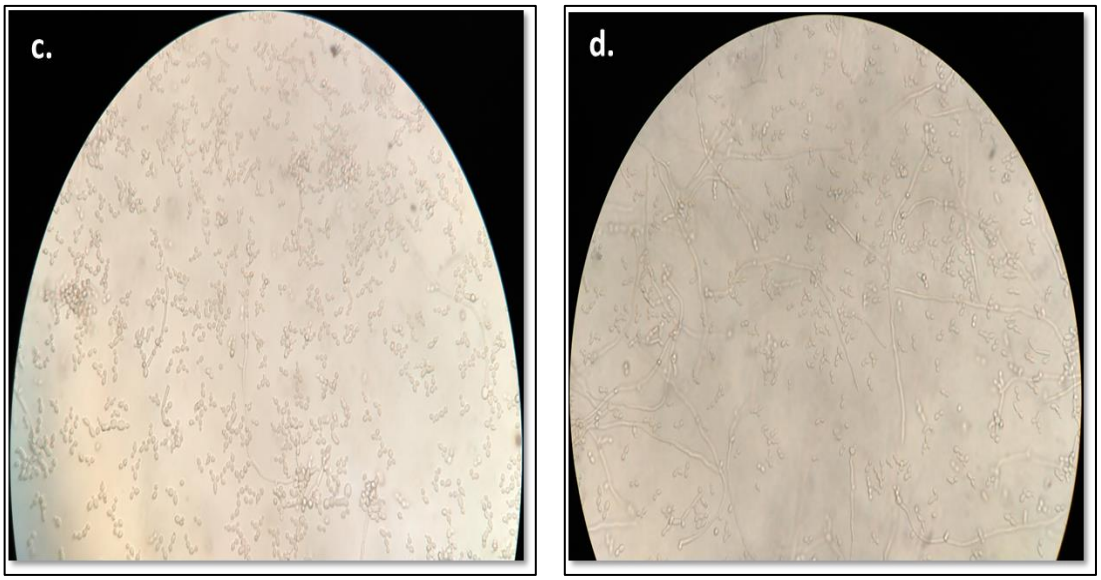


Fig. 33: Effect of CFS on hyphae formation of *C. albicans* on RPMI 1640 media. **a.** RPMI 1640, **b.** RPMI 1640 + LCCFS, **c.** RPMI 1640 + LGCFS, and **d.** RPMI 1640 + LVCFS under 40x magnification.

The effect of CFS on biofilm formation was checked post tertiary phase using crystal violet dye. The biofilm formation was significantly less on treatment with 100µL and 150µL of CFS in comparison to the control. Higher volume of CFS showed significantly better inhibition of biofilm. LCCFS showed the best inhibition of *C. albicans* biofilm among the LAB with the crystal violet dye stain (Fig. 34). The biofilm formed by the fungi was estimated by estimating counting the live fungal cells through coulometric method using XTT dye. Biofilm formation was significantly less when treated with 100µL and 150µL of CFS on the secondary colonization stage. Increasing volume of CFS showed higher resistance to biofilm formation (Fig. 35.a.). Post-tertiary colonization stage only LGCFS and LCCFS at 100µL volume showed significant suppression of biofilm in comparison to the control. Whereas, 150µL CFS of (LC, LG, and LV) showed better and significant inhibition of biofilm formation (Fig 35.b.). LGCFS treatment showed the best inhibition of live fungal cells in the biofilm formed by *C. albicans*. Growth of *C. albicans* in RPMI1640 till tertiary stage of biofilm formation, showed clumping of the fungal cells with the formation of dense hyphae (Fig. 36); whereas treatment with RPMI 1640 and sterile distilled water in 1:1 ratio post-secondary stage showed denser and more aggressive formation of hyphae (Fig. 37). Treatment with LGCFS completely inhibited biofilm formation and reduced adhesion of *C. albicans* cells

(Fig. 38.a.); whereas the treatment with RPMI 1640 and LGCFS in 1:1 ratio resulted in suppressed hyphal formation (Fig. 38.b.). On treatment with *L. gasseri*, fungal cells of *C. albicans* reverted back to their yeast form and lost their adhering and biofilm forming capacity (Fig. 38.c.); whereas in the presence of RPMI 1640, *L. gasseri* proliferated in large amount inhibiting the growth of *C. albicans* and reverting the fungus back to its yeast form (Fig. 38.d.).

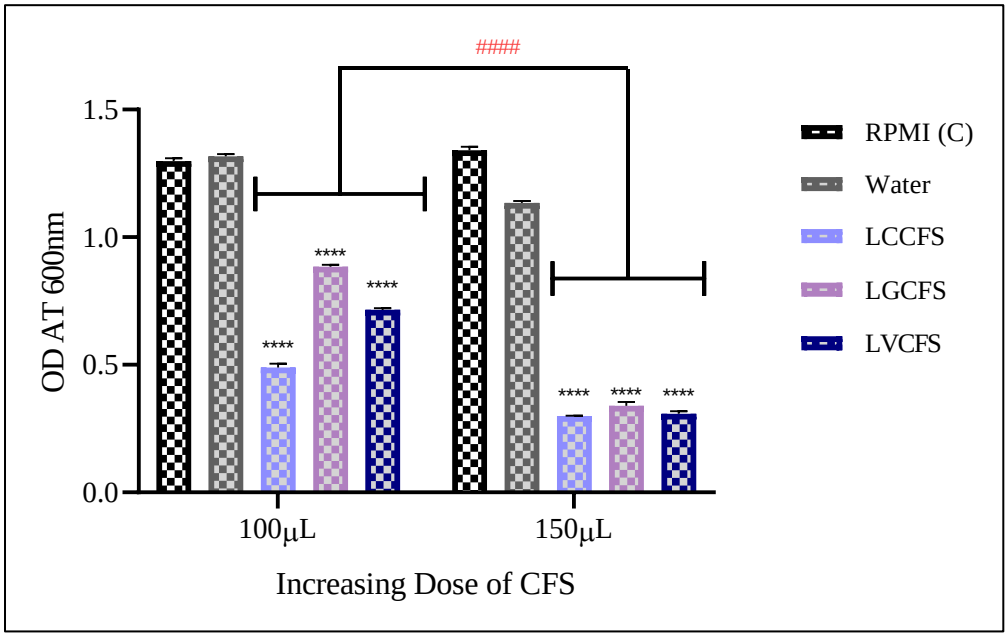


Fig. 34: Crystal violet assay to assess the effect of CFS on biofilm formation of *C. albicans*. (****=p≤0.0001, ####=p≤0.0001)

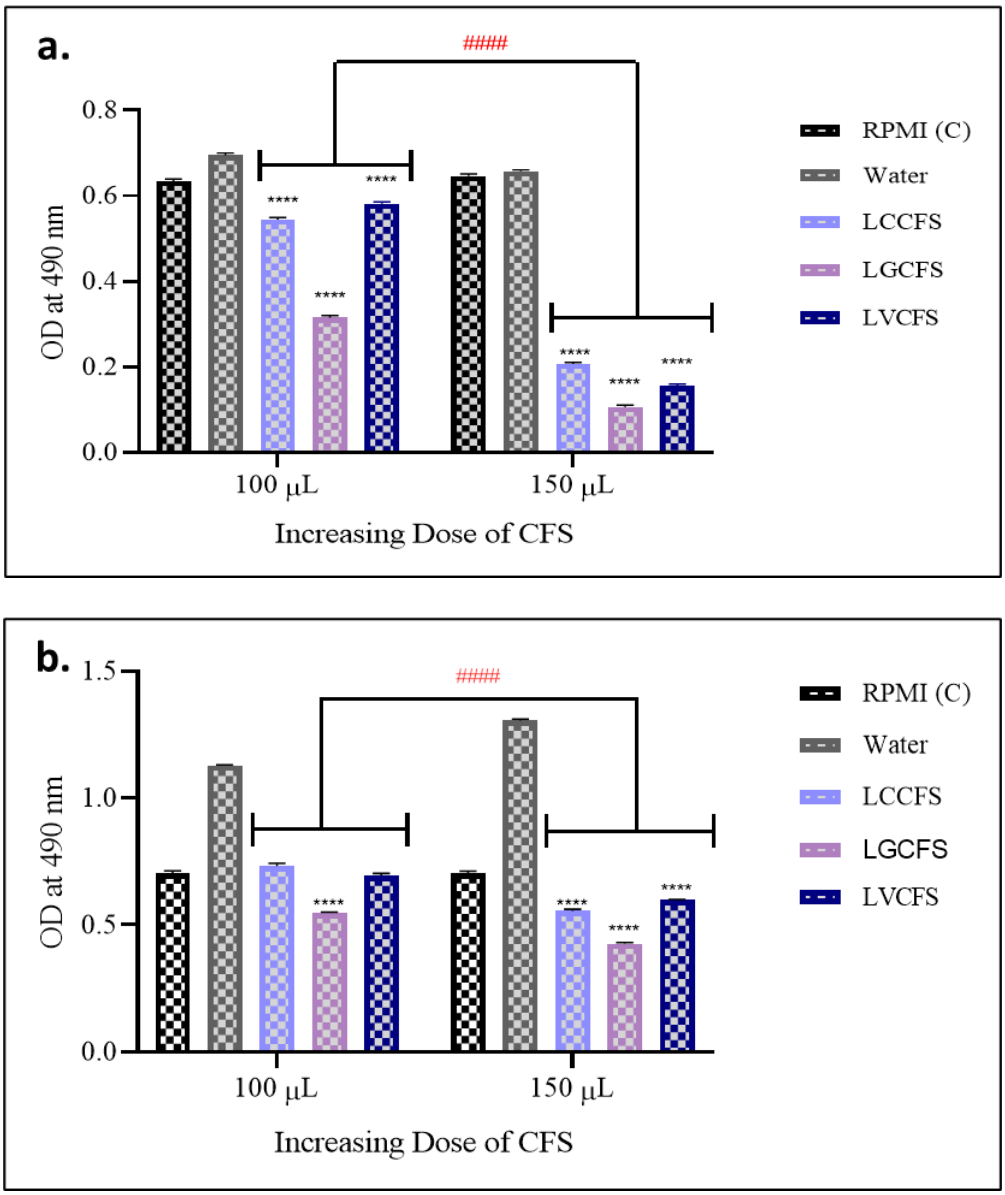


Fig. 35: XTT assay to assess the effect of CFS on biofilm formation of *C. albicans*. **a.** Secondary Colonization, **b.** Tertiary Colonization . (****=p≤0.0001, ####=p≤0.0001)

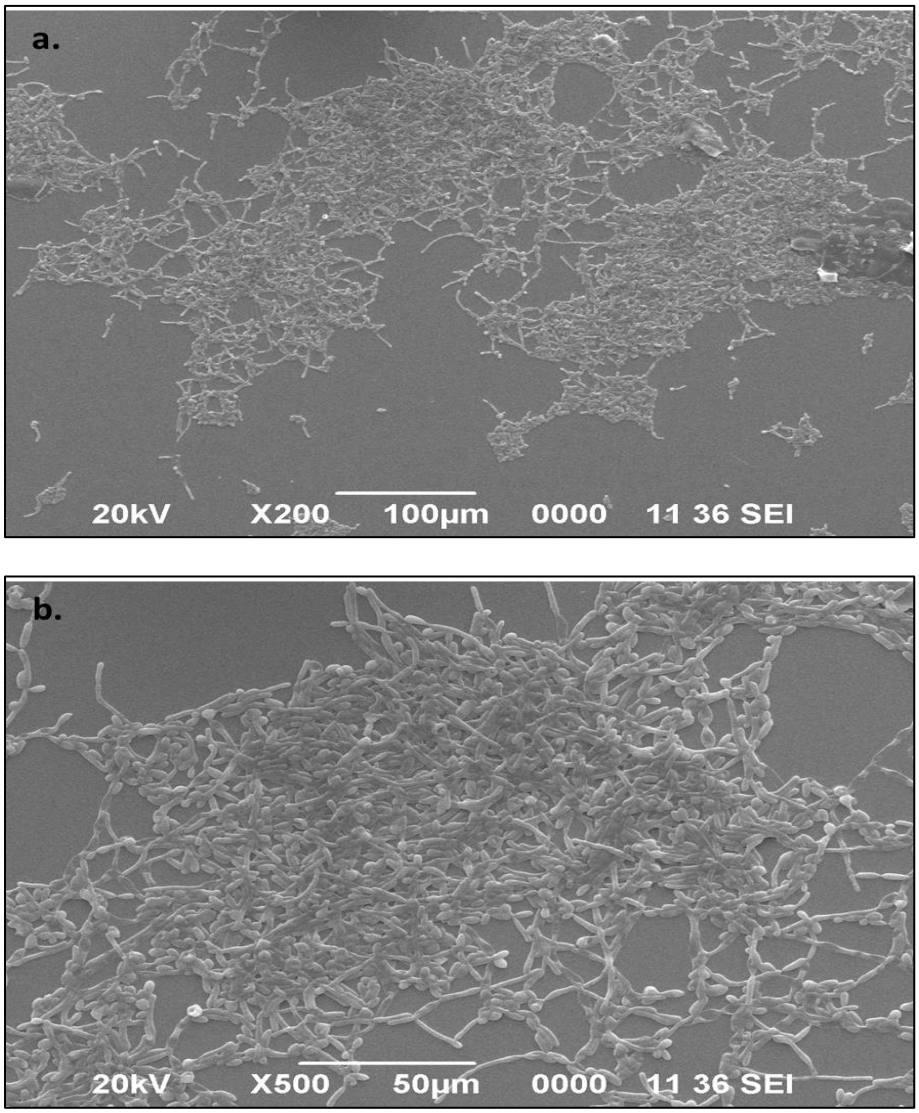
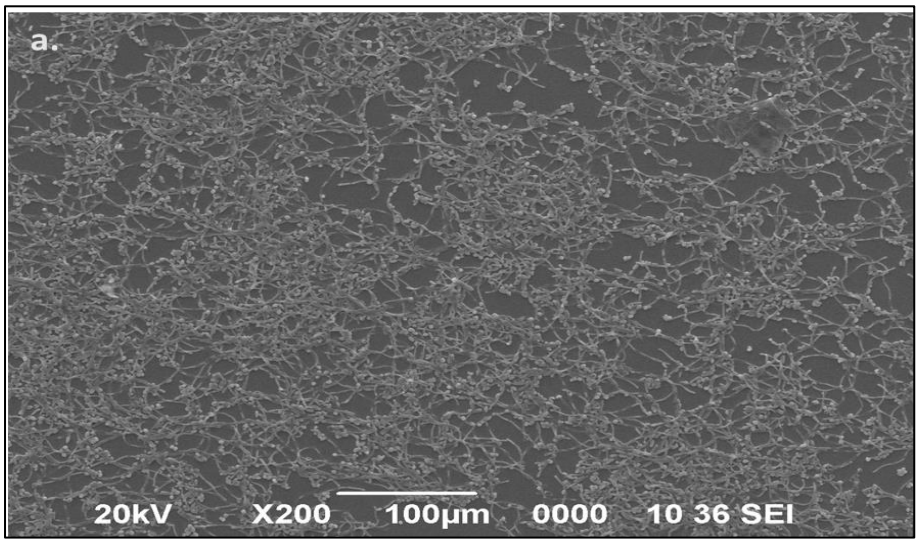


Fig. 36: Biofilm formation by *C. albicans* with RPMI 1640 under scanning electron microscope. **a.** 200x magnification, **b**. 500x magnification



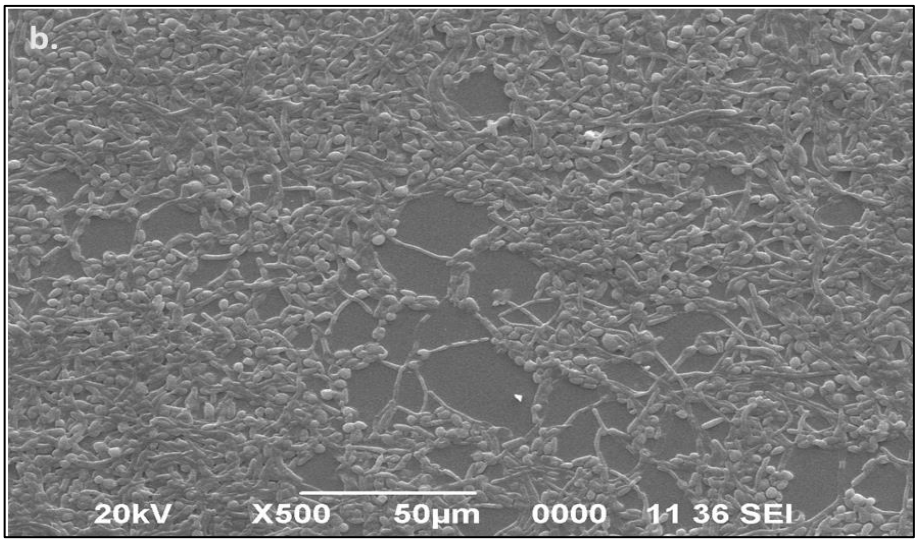
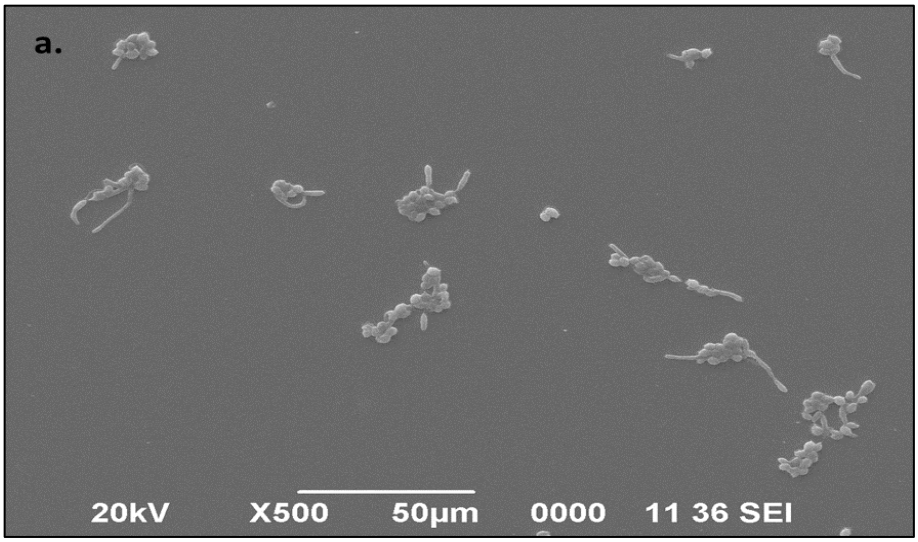
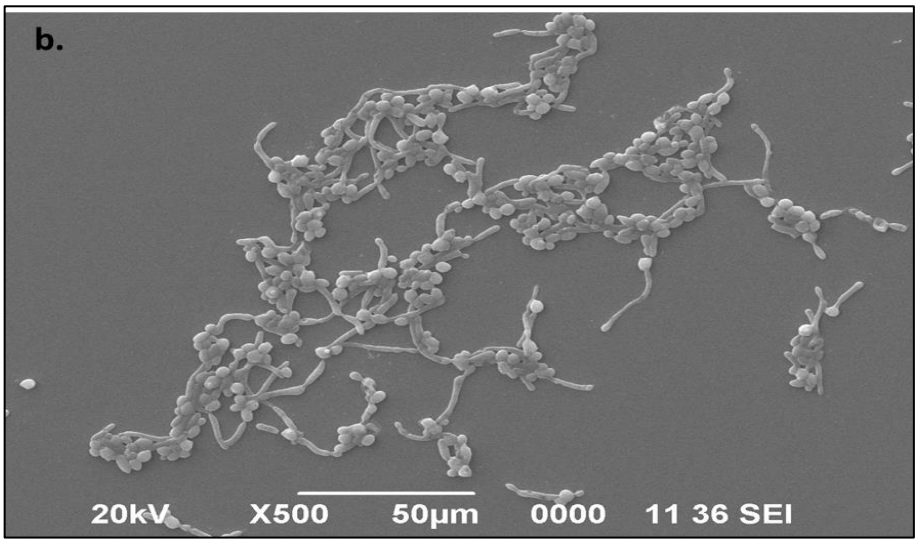


Fig. 37: Biofilm formation by *C. albicans* with RPMI 1640 and distilled water under scanning electron microscope. **a.** 200x magnification, **b.** 500x magnification





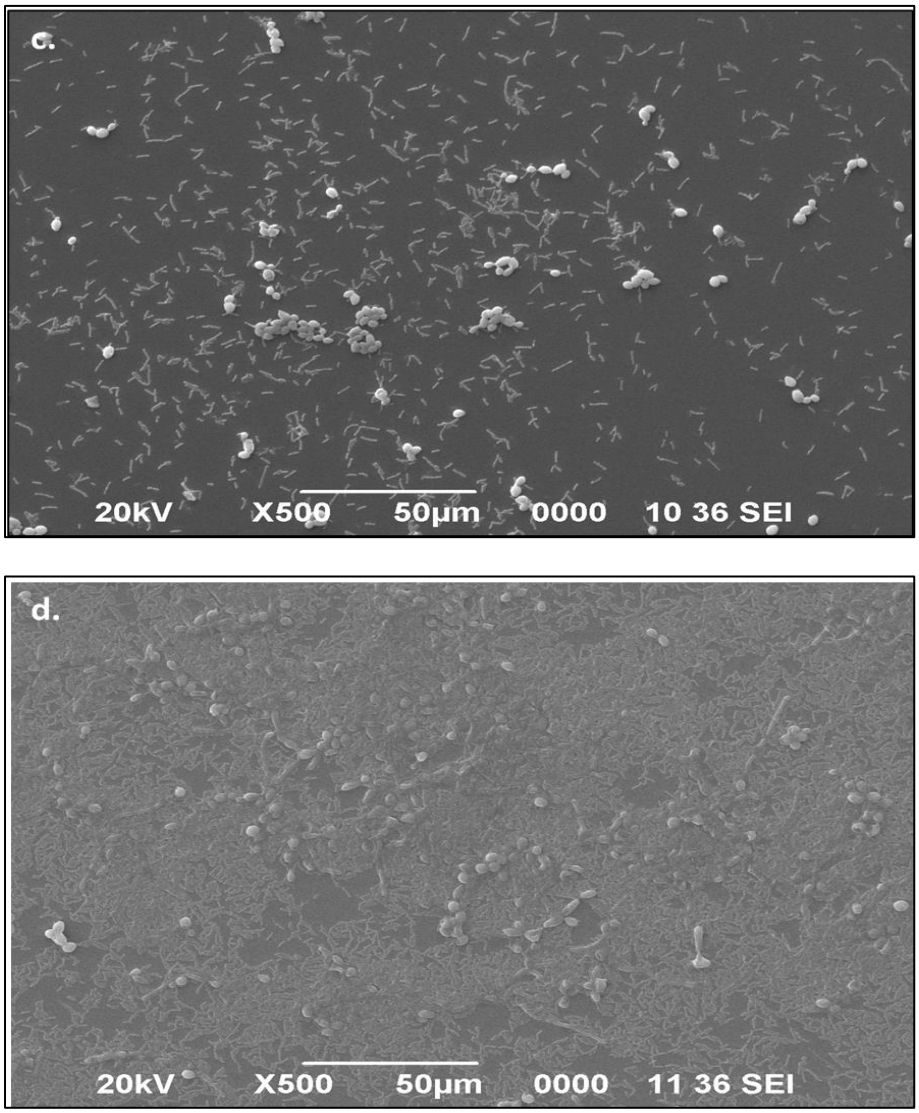
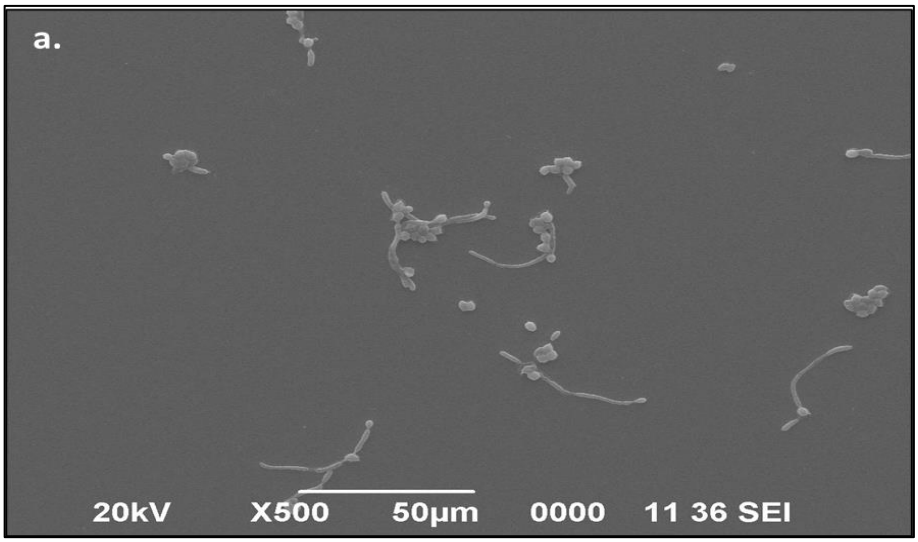
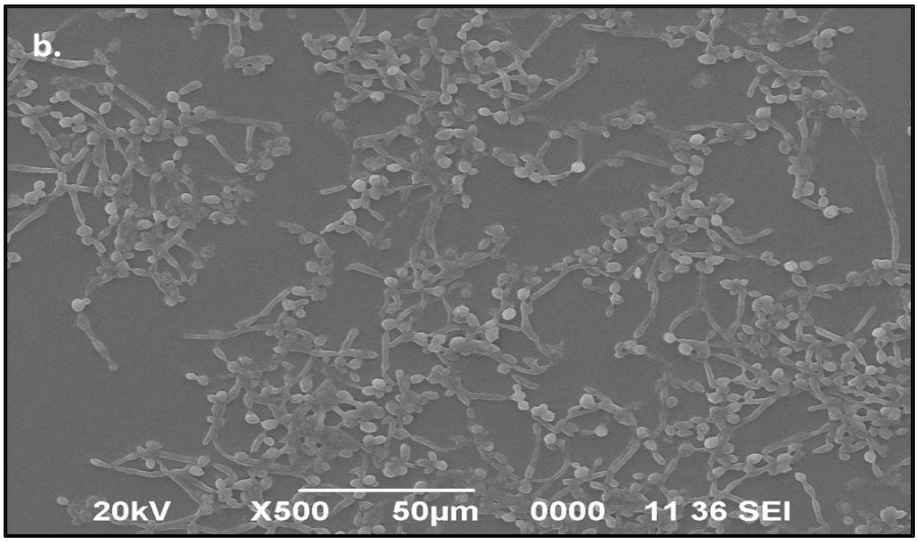
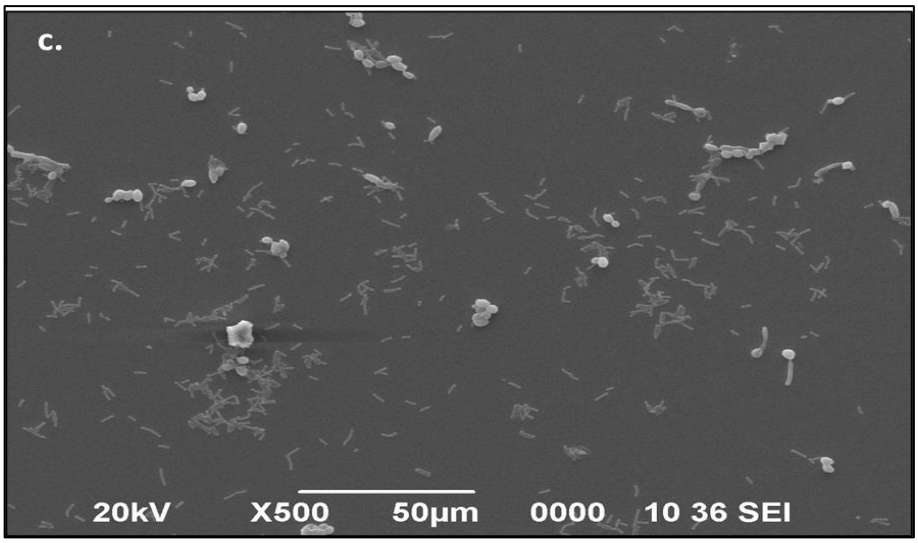


Fig. 38: Biofilm formation by *C. albicans* on treatment with **a.** LGCFS, **b.** RPMI 1640 + LGCFS (1:1) ratio, **c.** *L. gasseri*, and **d.** 1640 + *L. gasseri* (1:1) ratio under scanning electron microscope

Treatment with LCCFS completely inhibited biofilm formation and reduced adhesion of *C. albicans* cells (Fig. 39.a.).; whereas treatment with RPMI 1640 and LCCFS in 1:1 ratio initiated minimal formation of hyphae and biofilm due to the presence of a stimulator (Fig. 39.b.). On treatment with *L. crispatus*, *C. albicans* cells reverted back to their yeast form and lost their adhesive and biofilm formation capacity (Fig. 39.c.).; whereas in the presence of RPMI 1640, *L. crispatus* retarded growth of *C. albicans* with less hyphae formation (Fig. 39.d.).


a.
20kV X500 50µm 0000 11 36 SEI


b.
20kV X500 50µm 0000 11 36 SEI


c.
20kV X500 50µm 0000 10 36 SEI

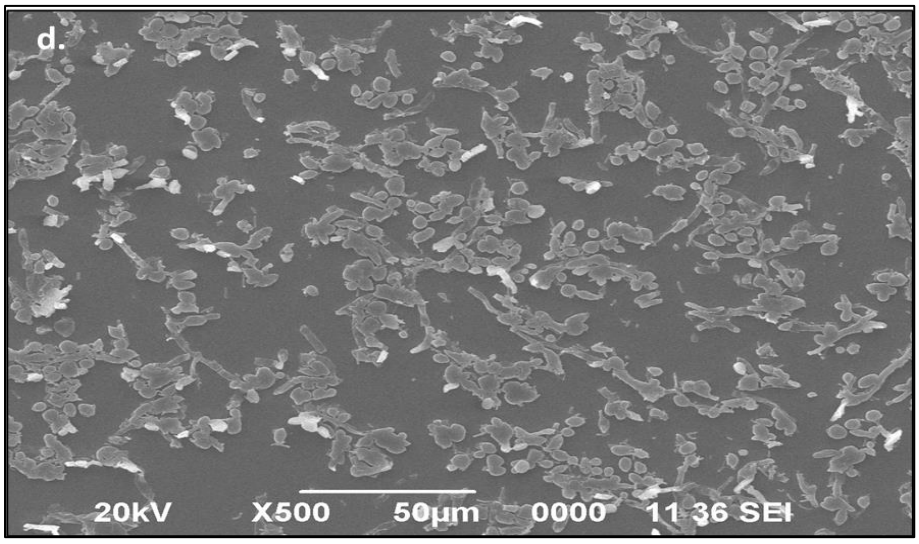
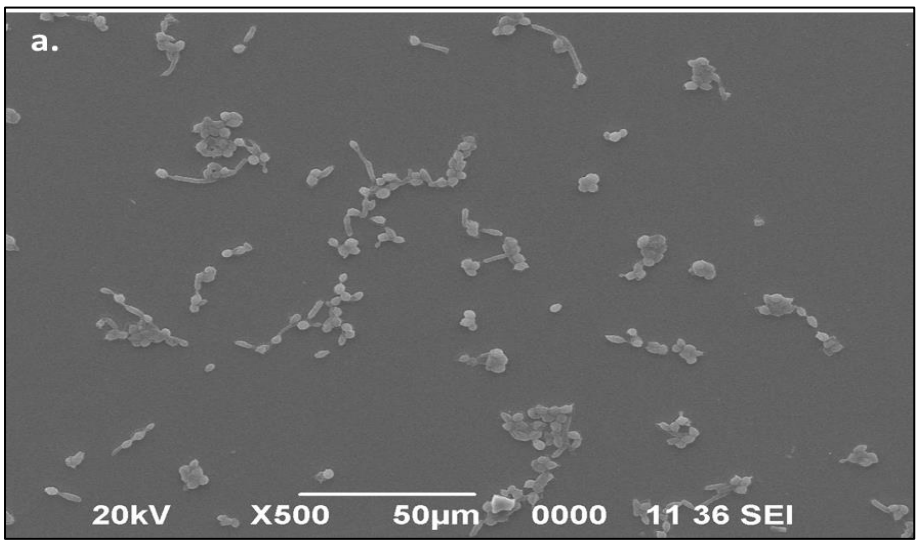


Fig. 39: Biofilm formation by *C. albicans* treatment with **a.** LCCFS, **b.** RPMI 1640 + LCCFS (1:1) ratio, **c.** *L. crispatus*, and **d.** 1640 + *L. crispatus* (1:1) ratio under scanning electron microscope

Treatment with LVCFS completely inhibited the hyphae and biofilm formation (Fig. 40.a.); whereas the treatment with RPMI 1640 and LVCFS in 1:1 ratio minimalized dense hyphae and biofilm formation (Fig. 40.b.). On treatment with *L. vaginalis*, *C. albicans* reverted back to their yeast form and lost their adhering and biofilm forming capacity (Fig. 40.c.); whereas in the presence of RPMI 1640, both the microbe's showed hyphae like network and biofilm formation (Fig. 40.d.). *L. gasseri* and GCFS showed the best inhibition of *C. albicans* biofilm.



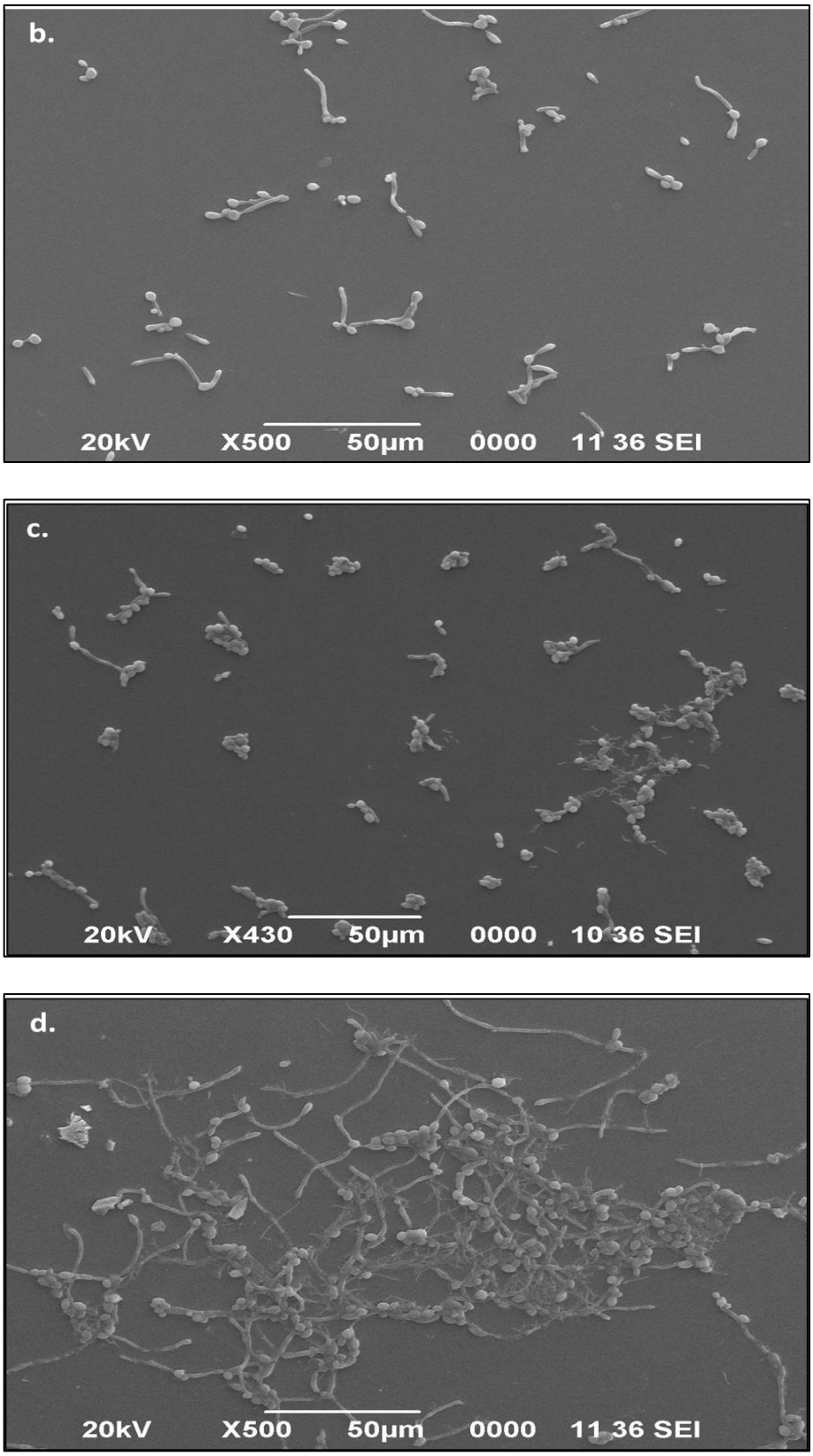
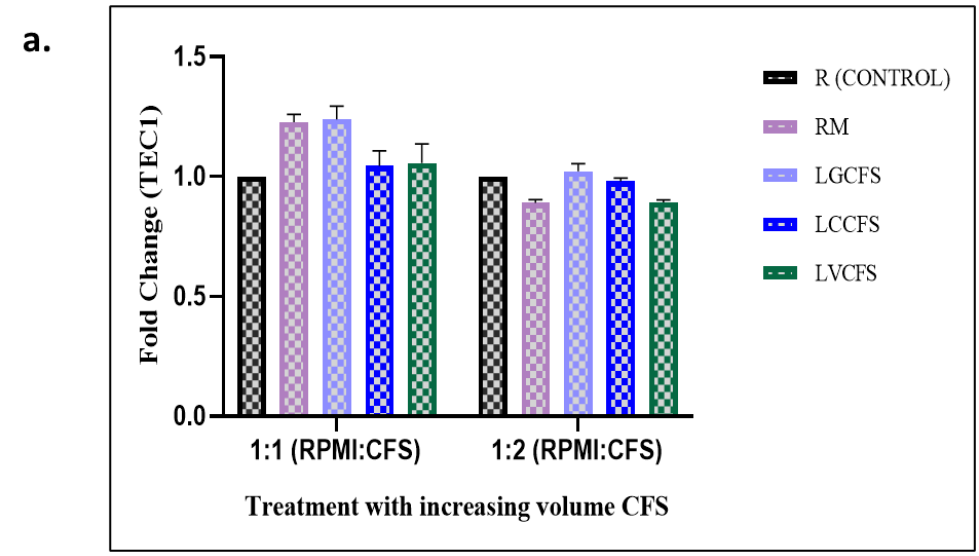
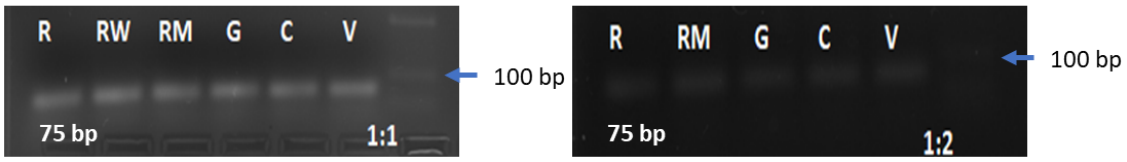


Fig. 40: Biofilm formation by *C. albicans* with **a.** LVCFS, **b.** RPMI 1640 + LVCFS (1:1) ratio, **c.** *L. vaginalis*, and **d.** 1640 + *L. vaginalis* (1:1) ratio under scanning electron microscope

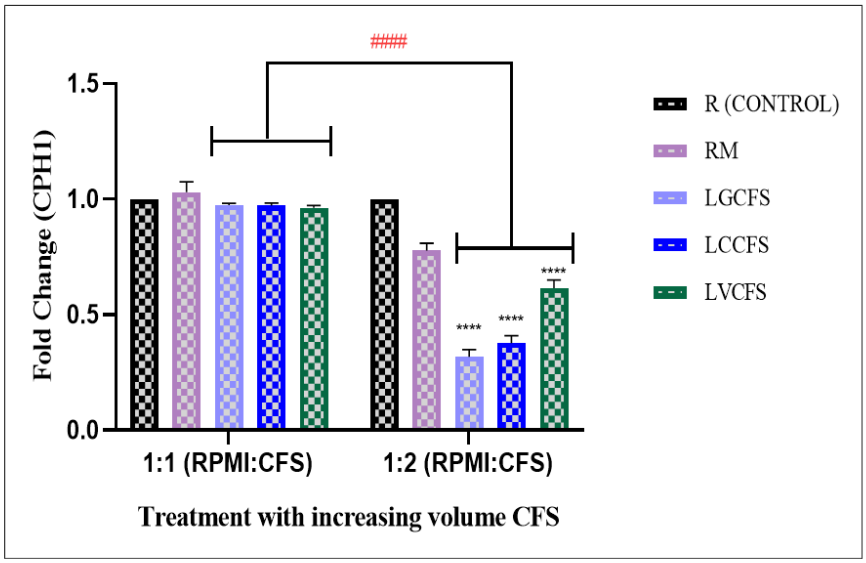
The freshly grown *C. albicans* was inoculated to RPMI 1640 which triggered hyphae formation at the transcriptional level. The expression profile of facultative genes responsible for the initiation of hyphae/pseudo-hyphae and biofilm formation were compared. All the variably treated cells (R=RPMI, RM= RPMI + MRS broth, RW= RPMI + Water, G= RPMI+ LGCFS, C= RPMI + LCCFS, V= RPMI + LVCFS) showed similar expression profile for GSP1 the constitutive gene. TEC1 a transcription factor responsible for the initiation of hyphae formation showed no visible difference among the control and the treated samples in both (1:1) and (1:2) ratio treatments (Fig. 41.a.). Another transcription factor CPH1 showed no visible difference among the control and treated samples in (1:1) ratio treatment, but higher volume of CFS treatment i.e., (1:2) ratio showed lower expression of CPH1 in treated *C. albicans* cells in comparison to the control (R) (Fig. 41.b.). BCR1 another transcription factor showed significantly lower expression for all CFS treated samples in both (1:1) and (1:2) ratio treatments (Fig. 41.c.). ALS3 and HWP1, genes responsible for hyphal wall proteins showed significantly lower expression for all CFS treated samples in both (1:1) and (1:2) ratio treatments (Fig. 41.d., 41.e.). HYR1 showed significantly lower expression for GCFS in (1:1) ratio treatment and ECE1 showed significantly lower expression for GCFS and VCFS in (1:1) ratio treatment. Whereas a significant lower expression of the HYR1 and ECE1 gene was observed in the all samples treated with higher volume (i.e., 1:2) of CFS (Fig. 41.f. and 41.g.) (Fig. 42 and 43).

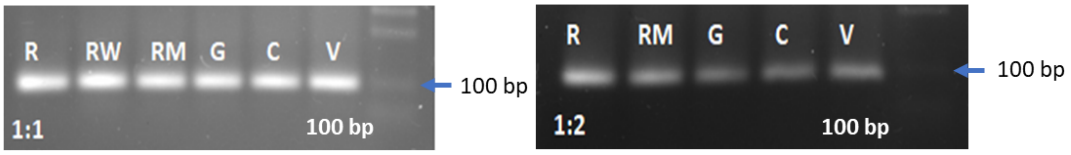
a.


Fold Change (TEC1)
1.5
1.0
0.5
0.0
1:1 (RPMI:CFS)
1:2 (RPMI:CFS)
Treatment with increasing volume CFS
R (CONTROL)
RM
LGCFS
LCCFS
LVCFS

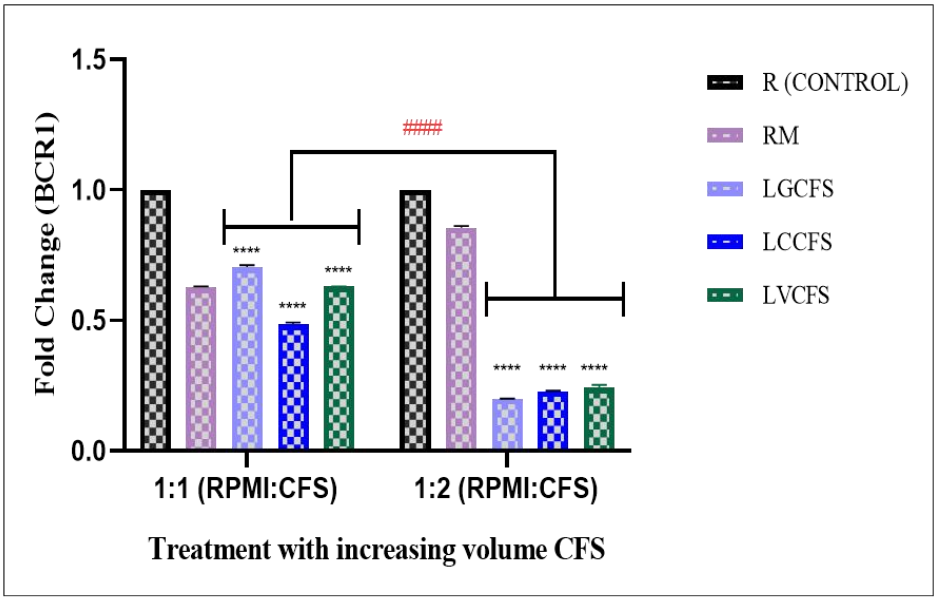

R RW RM G C V
75 bp
1:1
100 bp
R RM G C V
75 bp
1:2
100 bp

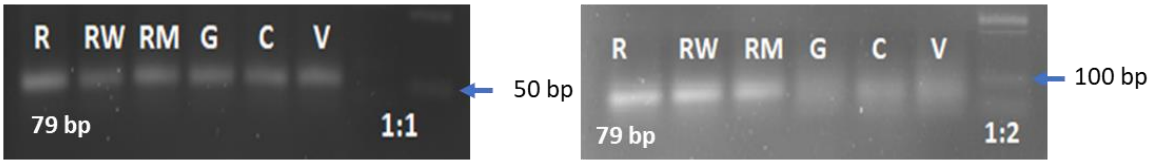
b.


####
Fold Change (CPH1)
1.5
1.0
0.5
0.0
****
****
****
1:1 (RPMI:CFS)
1:2 (RPMI:CFS)
Treatment with increasing volume CFS
R (CONTROL)
RM
LGCFS
LCCFS
LVCFS

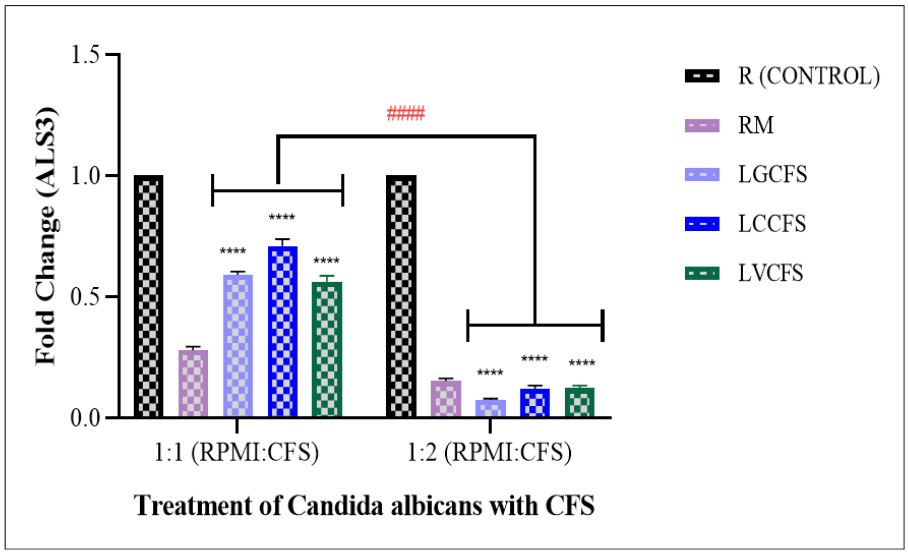

R RW RM G C V
1:1
100 bp
100 bp
R RM G C V
1:2
100 bp
100 bp

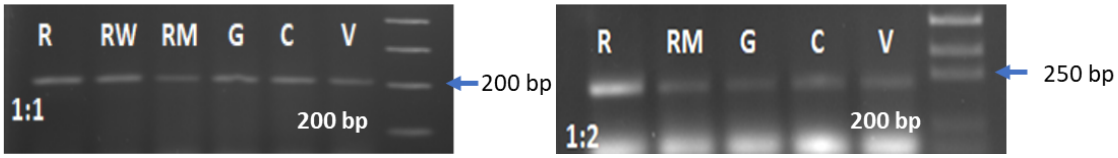
c.


1.5
1.0
0.5
0.0
Fold Change (BCR1)
####
****
R (CONTROL)
RM
LGCFS
LCCFS
LVCFS
1:1 (RPMI:CFS)
1:2 (RPMI:CFS)
Treatment with increasing volume CFS

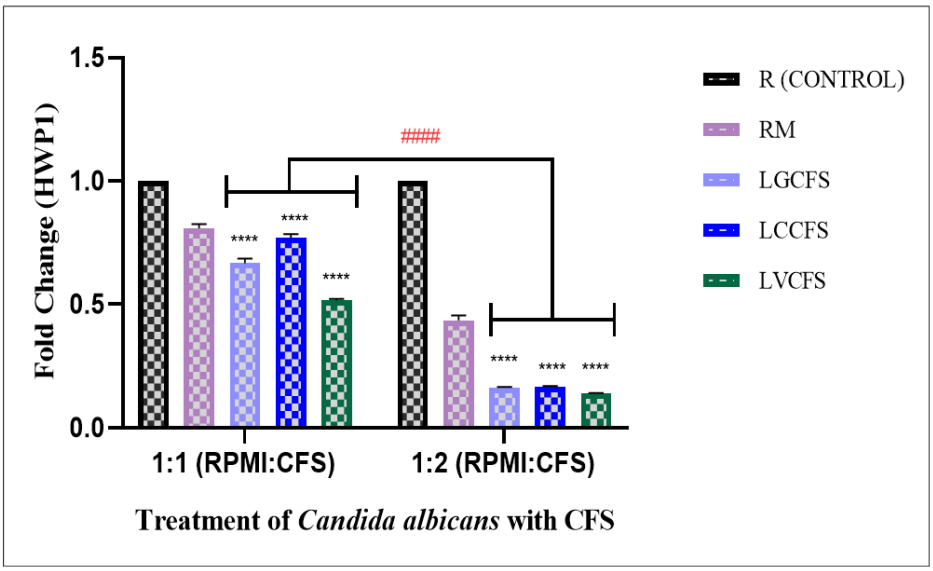

R RW RM G C V
79 bp
1:1
50 bp
R RW RM G C V
79 bp
1:2
100 bp

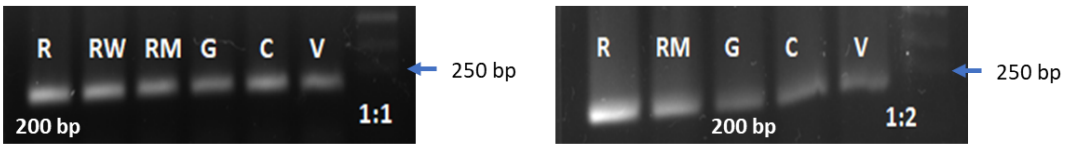
d.


1.5
1.0
0.5
0.0
Fold Change (ALS3)
####
****
R (CONTROL)
RM
LGCFS
LCCFS
LVCFS
1:1 (RPMI:CFS)
1:2 (RPMI:CFS)
Treatment of Candida albicans with CFS

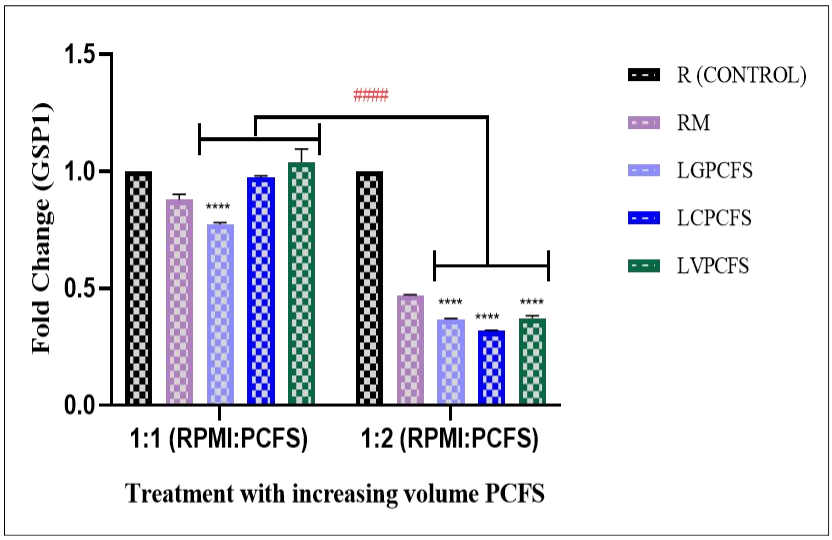

R RW RM G C V
1:1
200 bp
200 bp
R RM G C V
1:2
200 bp
250 bp

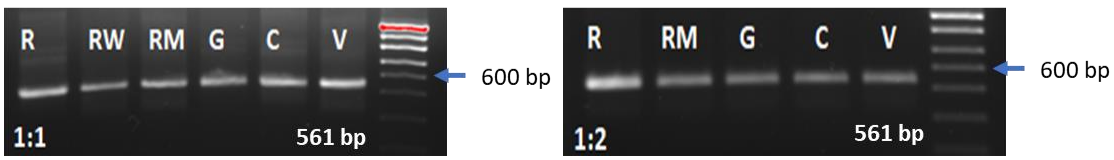
e.


Fold Change (HWP1)
1.5
1.0
0.5
0.0
####
****
R (CONTROL)
RM
LGCFS
LCCFS
LVCFS
1:1 (RPMI:CFS)
1:2 (RPMI:CFS)
Treatment of *Candida albicans* with CFS


R
RW
RM
G
C
V
200 bp
1:1
250 bp
R
RM
G
C
V
200 bp
1:2
250 bp

f.


Fold Change (GSP1)
1.5
1.0
0.5
0.0
####
****
R (CONTROL)
RM
LGPCFS
LCPCFS
LVPCFS
1:1 (RPMI:PCFS)
1:2 (RPMI:PCFS)
Treatment with increasing volume PCFS


R
RW
RM
G
C
V
1:1
561 bp
600 bp
R
RM
G
C
V
1:2
561 bp
600 bp

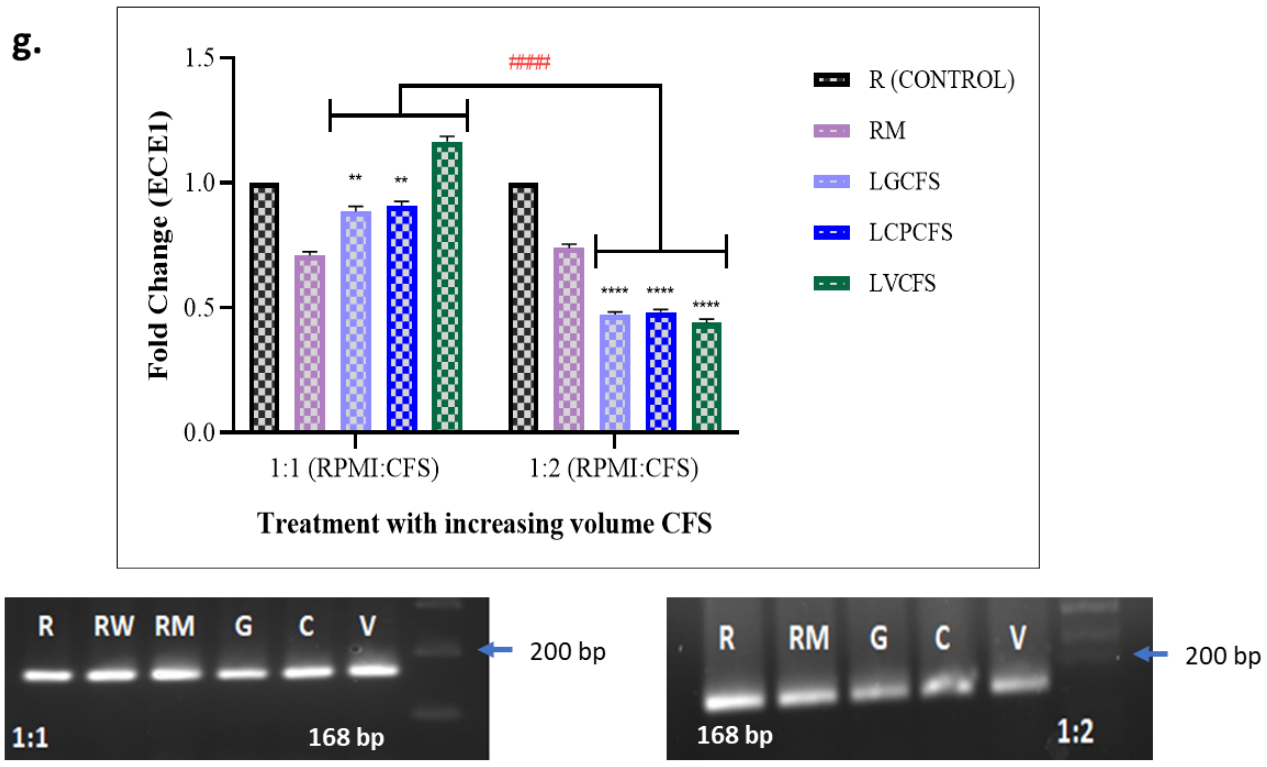


Fig. 41: Differential regulation of hyphal genes in *C. albicans*

a. TEC1, **b.** CPH1, **c.** BCR1, **d.** ALS3, **e.** HWP1, **f.** HYR1, and **g.** ECE1; (****=p≤0.0001) (####=p≤0.0001); *L. crispastus* (LC) *L. gasseri* (LG) *L. vaginalis* (LV) Probiotic culture free supernatant (CFS); RPMI1640 (R); RPMI1640+MRS broth (1:1 ratio) (RM)

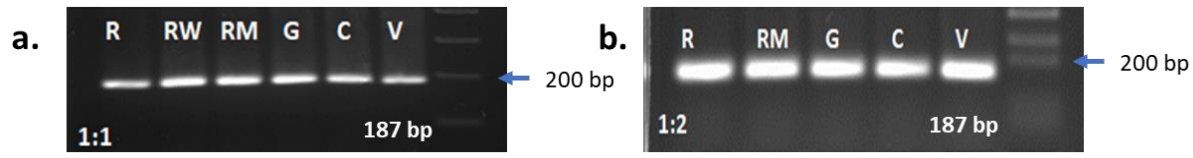


Fig 42: PCR product of GSP1 gene in 1.5% agarose gel. **a.** 1:1 ratio treatment and **b.** 1:2 ratio treatment showing similar gene expression for variably treated cells of *C. albicans*

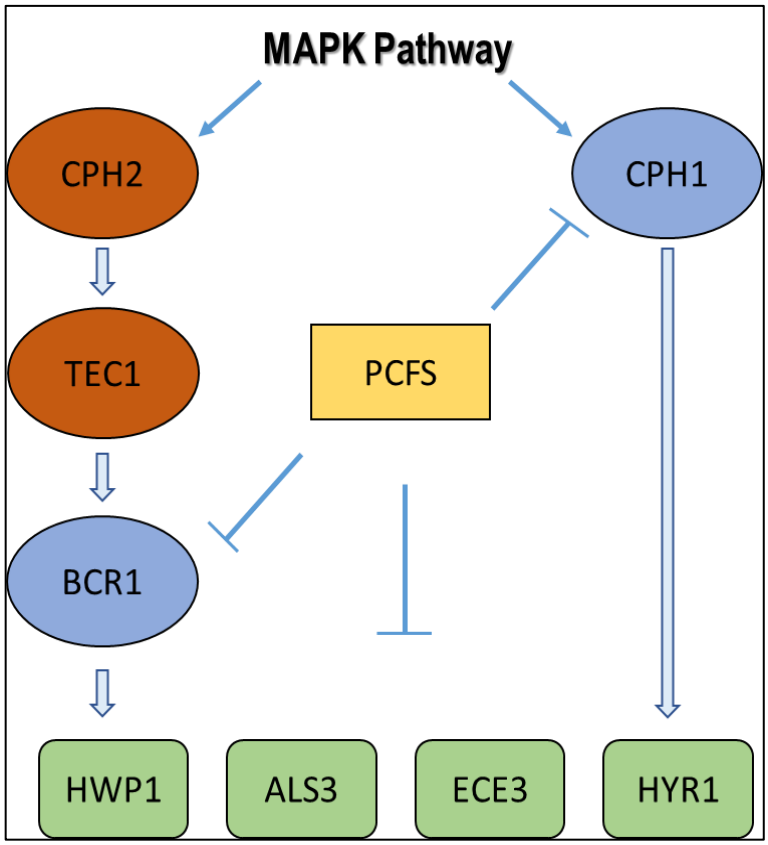


Fig 43: Proposed mechanism of hyphal gene inhibition on treatment with CFS (LC, LG, and LV). Transcription factors showing similar expression on treatment with CFS (Brown colour). Transcription factor showing differential expression on treatment with CFS (Blue colour). Genes of cell wall proteins showing differential expression on treatment with CFS (Green Colour).

Forty-eight documented anti-microbial metabolites, antimicrobial peptides (AMP) and antibiotics were produced by the LAB (LC, LG, and LV) extracellularly among the hundred and nine identified metabolites (Fig 44) (Table 15). *L. crispatus* produced twenty-eight, *L. gasseri* produced thirty-five, and *L. vaginalis* produced twenty-three antimicrobial metabolites. LG produced highest anti-fungal, anti-candidal, anti-microbial metabolites and antibiotics. *L. gasseri* CFS showed the best anticandidal effect and bacteriostatic effect. Whereas, *L. crispatus* CFS was the most bacteriostatic in nature and showed second best anti-candidal activity.

Table 15: Antimicrobial metabolites identified in the CFS of *L. crispatus, L. gasseri* and *L. vaginalis*

Sl. No.	Compound	*L. crispatus*	*L. gasseri*	*L. vaginalis*	Function
1	Fenapanil	(+)	(+)	(+)	Anti-fungal, Fungicide [456]
2	L-isoleucyl-L-proline			(+)	Cyclopeptide, Anti-candidal and bacterial [457]
3	Chivosazole F		(+)		Anti-fungal and cancer [458]
4	4-Phenylpyridine	(+)	(+)		Anti-fungal and microbial [459]
5	Erythrophleguine	(+)	(+)	(+)	Anti-fungal and bacterial [460]
6	Calycanthine		(+)		Anti-fungal, microbial, convulsant, tumour [461]
7	Chamazulene	(+)	(+)		Anti-fungal, inflammatory, allergic for skin [462]
8	Mansonone C	(+)	(+)	(+)	Anti-fungal and Anti-candidal [463]
9	Borrelidin	(+)			Antifungal, candidal, bacterial viral, insecticidal, malarial [464]
10	Europine	(+)	(+)		Antifungal [465]
11	S-Japonin	(+)	(+)		Antifungal [466]
12	Enniatin B4		(+)		Antifungal, cancer, bacterial and cytotoxic [467]
13	Fistuloside C		(+)		Antifungal [468]
14	Sethoxydim	(+)	(+)	(+)	Antifungal and Herbicide [469]

15	Reboxetine		(+)	(+)	Antifungal and Antidepressant [470]
16	Cycloxydim		(+)		Antifungal and Herbicide [471]
17	Guaiazulene	(+)	(+)	(+)	Anti-fungal, oxidant, cancer and bacterial [472]
18	2-Phenylethyl benzoate	(+)	(+)	(+)	Anti- Candidal, Anti-fungal [473]
19	Reserpine		(+)		Biofilm Inhibitor, Anti-fungal [474]
20	Linalool	(+)	(+)		Anti- Candidal [475]
21	Taurallocholic acid	(+)			Anti-fungal, bacterial, viral and candidal [476]
22	Pravastatin		(+)		Azole enhancer and anticandidal [445]
23	Benzo[α]fluorene		(+)	(+)	Anti candidal and microbial [477]
24	Vignatic acid A	(+)	(+)	(+)	Cyclopeptide and insecticidal [478]
25	Blasticidin S	(+)	(+)		Antibiotic and Anti-candidal [479]
26	Netilmicin	(+)	(+)	(+)	Antibiotic [480]
27	Mycinamicin IV	(+)	(+)		Antibiotic [481]
28	Istamycin C1	(+)	(+)	(+)	Antibiotic [482]
29	Troleandomycin		(+)	(+)	Antibiotic [483]
30	Mupirocin	(+)	(+)	(+)	Antibiotic and Anti-candidal [480]
31	Gentamicin	(+)	(+)	(+)	Antibiotic [484]
32	Chalcomycin	(+)	(+)	(+)	Antibiotic [485]
33	Arbekacin		(+)		Antibiotic and Anti-candidal [480]
34	Leucomycin A7	(+)	(+)		Antibiotic [486]

35	Asukamycin	(+)			Antibiotic [487]
36	Tartaric Acid	(+)			Antimicrobial [488]
37	7-Hydroxyflavanone		(+)		Anti-candidal [489]
38	Oxoglaucine			(+)	Antimicrobial [490]
39	(S)-Norreticuline			(+)	Antifungal [491]
40	Forskolin	(+)			Anti- candidal [492]
41	Kolanone	(+)	(+)		Antimicrobial and Anticandidal [493]
42	1alpha-O-Methylquassin			(+)	Antimicrobial [494]
43	2-methyl-3-oxo-propanoic acid	(+)			Anticandidal and Antimicrobial [495]
44	(R)-Juziphine	(+)	(+)	(+)	Antimicrobial [496]
45	Uzarigenin			(+)	Antibacterial [497]
46	Maclurin 3		(+)		Antibacterial, anticancer and antioxidant [498]
47	23-trans-p-Coumaroyloxytormentic acid			(+)	Antiviral [499]
48	Theophylline			(+)	Antimicrobial and Anticancer [500]
49	Citreoviridin	(+)	(+)	(+)	Mycotoxin, Antiviral [501]
50	Janthitrem E	(+)	(+)		Mycotoxin
51	Janthitrem F	(+)		(+)	Mycotoxin
52	3'-Hydroxy-HT2	(+)			Mycotoxin
53	Betamethasone	(+)	(+)	(+)	Anti-inflammatory
54	Flurandrenolide	(+)	(+)	(+)	Anti-inflammatory
55	Thalidasine		(+)		Anti-inflammatory

56	Indoleacrylic acid	(+)	(+)	(+)	Anti-inflammatory
57	Ophiopogonin B	(+)			Anticancer
58	Evodiamine		(+)	(+)	Anticancer
59	8beta-Angeloyloxy-15-hydroxy-1alpha,10R-dimethoxy-3-oxo-11(13)-germacren-12,6alpha-olide		(+)		Anticancer
60	Physapubenolide		(+)		Anticancer
61	Guaiazulene	(+)	(+)	(+)	Anticancer and Antioxidant
62	Dihydrocumambrin A	(+)	(+)	(+)	Anticancer, Blood pressure
63	Vindoline			(+)	Antioxidant and Anticancer
64	Thalidasine		(+)		Antitumour
65	Capsoside A	(+)			Antioxidant
66	Kanzonol M			(+)	Antioxidant
67	Promazine sulfoxide	(+)		(+)	Sedative
68	Tetrahydropentoxyline	(+)	(+)	(+)	Sedative
69	Midazolam		(+)	(+)	Sedative
70	Methylprednisolone succinate	(+)			Corticosteroid
71	6alpha-Hydroxycortisol	(+)	(+)	(+)	Steroid
72	3beta-Hydroxy-16-phosphonopregn-5-en-20-one monoethyl ester		(+)		Corticosteroid
73	Decoside	(+)	(+)	(+)	Steroid
74	Paraxanthine		(+)		Nerve stimulator
75	Dictyoquinazol C		(+)	(+)	Neurological Drug
76	Fasoracetam			(+)	Neurological Drug
77	Terazosin			(+)	Blood Pressure Drug
78	Ketotifen	(+)	(+)	(+)	Asthmatic Drug
79	Mycosporine	(+)	(+)	(+)	Anti UV formula
80	Pyrilamine			(+)	Anti-histamine

81	Tripelennamine			(+)	Anti allergen
82	Isoamyl nitrite		(+)	(+)	Vasodilator
83	Hydratopyrrhoxanthinol			(+)	Vasodilator
84	Vindoline			(+)	Antidiabetic and Antioxidant
85	Pioglitazone			(+)	Antidiabetic
86	Isoproterenol			(+)	Bradycardia Drug
87	Ganodosterone			(+)	Anti hepatoxic
88	Ibutilide			(+)	Antiarrhythmic Drug
89	Dehydrodieugenol			(+)	Antiparasitic
90	Kalihinol A	(+)			Antimalaria
91	Avermectin		(+)		Antiparasitic
92	Licarin A			(+)	Antiparasitic
93	Pendimethalin	(+)	(+)	(+)	Herbicide
94	Isouron	(+)			Herbicide
95	Clofentezine			(+)	Insecticide
96	Cartap	(+)			Herbicide and Insecticide
97	Erythrophleguine	(+)	(+)	(+)	Insecticidal
98	Ursiniolide A			(+)	Cytotoxic
99	Deoxyloganin	(+)			Medicinal
100	Valtratum		(+)		Medicinal
101	Hemibrevetoxin B		(+)		Neurotoxin
102	Neosaxitoxin	(+)		(+)	Neurotoxin
103	3,3'-Dimethylbenzidine	(+)	(+)	(+)	Carcinogen
104	Leucine enkephalin		(+)		Opioid Dipeptide (Pain Killing)
105	Armillane				Antimicrobial Ester
106	Zalcitabine			(+)	Antiviral Drug (HIV)
107	Tosyllysine Chloromethyl Ketone			(+)	Toxin
108	Pentosidine	(+)	(+)	(+)	Toxin
109	Pfaffoside A			(+)	Toxin

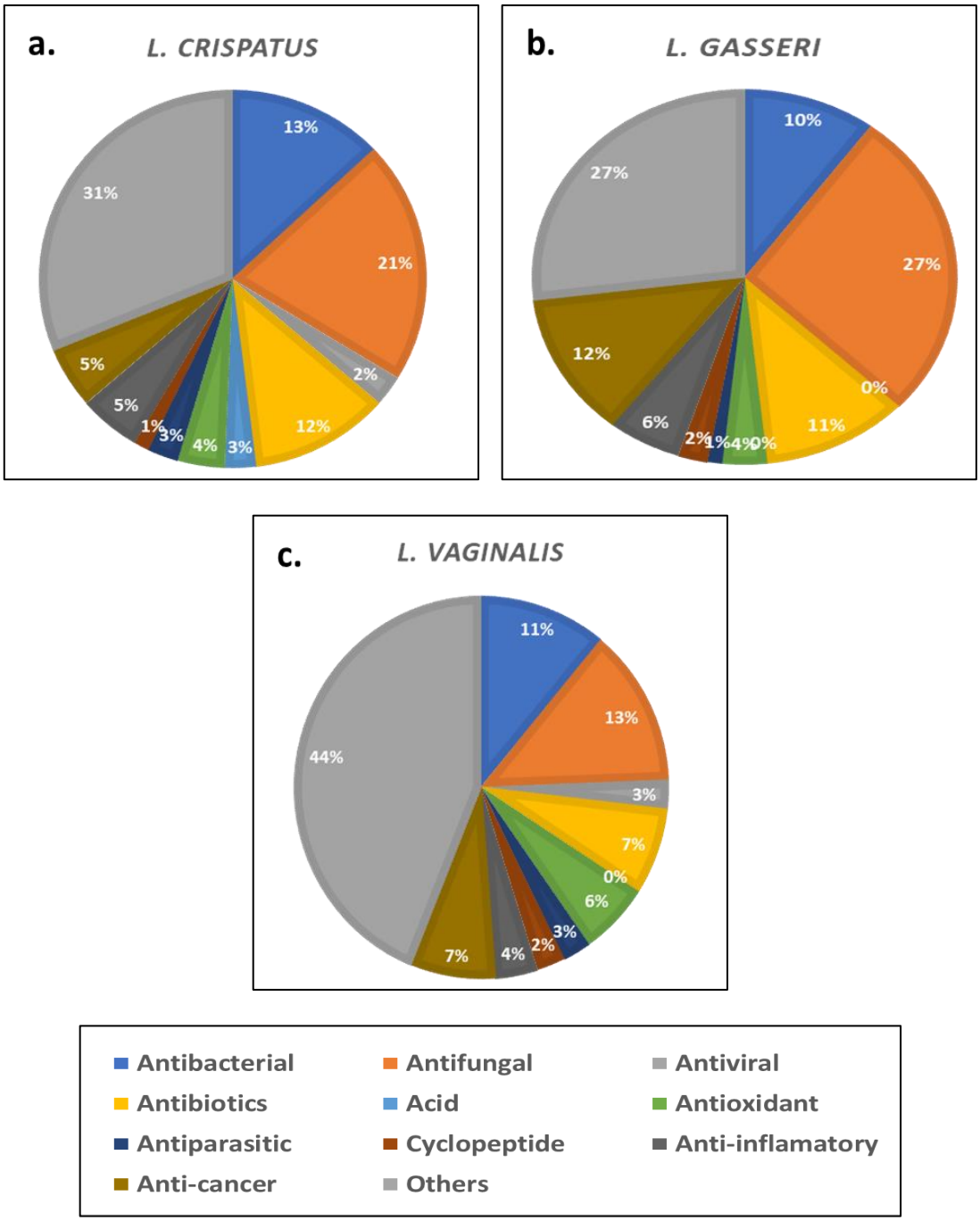


Fig 44: Extracellular metabolome of CFS from LC, LG, and LV with antimicrobial compounds.

4.4. Discussion

The potential pathogens from vulval swabs could easily grow in aerobic as well as anaerobic conditions, manifesting their ability to grow on the outer parts of vagina such as the labia major and minor as well as inside the urogenital system. These organisms mostly maintain commensalism in the VC, however, they can ably manifest their

pathogenicity under a disturbed vaginal microenvironment [502, 503]. The production of proteolytic, hemolytic, and lipolytic toxins allow them to interact with the primary/ secondary immune cells and epithelial cells of the host. These interactions triggers: coagulation, activation of complement system, influx of reactive oxygen/nitrogen species, and cytolytic cytokines leading to inflammation [504]. Hemolysins produced by pathogenic microbes like *S. aureus* lyses neutrophils and lysosomes. The release of intracellular proteins from these immune cells, in turn injure the host cells [505]. Bacterial proteases from pathogens behave as exotoxins, by conforming host protein to a non-functional state and dysregulating the signaling pathway of the host and immune cells [506]. PAR receptors on epithelial cells, leukocytes, and endothelial cells are also affected by bacterial proteases [507]. Lipolytic enzymes cleave lipids to form fatty acids helping in the adhesion and colonization of pathogens to cause infection. The ability of pathogens to utilize lipids also provides them an edge to grow in adverse conditions [508]. The lipolytic enzymes breakdown triacylglycerols, influencing the chemotaxis of immune cells (granulocytes, T-cells, macrophages, and platelets) and eventually causing structural damage to them as well.

Although most strains of *S. epidermidis* are non-hemolytic [509], the strain isolated from this study showed hemolytic property indicating its emergence as a potential pathogen [402]. *S. epidermidis* has also been previously reported to produce extracellular proteolytic enzymes that degrade AMP dermcidin, present on the skin [507]. Lipolytic activity of the pathogen is detectable post reaching the stationary growth phase in the host, allowing the pathogenicity of the microorganism to spread [508]. *E. cloacae* strain from our study showed proteolytic activity with high antibiotic resistance to a range of antibiotics indicating heavy pathogenicity [510]. The *E. facaelis* strain from our study was both hemolytic and lipolytic in nature. *E. facaelis* has been previously reported to release a protease that disrupts AMPs (LL-37) and activates the complement system as well [507]. *C. albicans* the 4th most virulent nosocomial pathogen, grew at varying range of temperatures (28°C - 40°C) and pH levels (3-9). The transformation of *C. albicans* from yeast to fungal state has shown a deep correlation with the lipase producing ability of the yeast [508]. Lipase producing *C. albicans* exert their virulence on vaginal epithelial cells and cause cell lysis [511]. *C. albicans* was the only isolate that grew in minimal media supplemented with glycogen as the carbon source. Glycogen is the primary carbohydrate found in VC prior its digestion by α-amylase enzyme. The ability of the

isolate to utilize stored glycogen exhibits its potential to grow on a substrate that could not support the growth of beneficial LAB or other microbes [422, 512]. Although, the enteric isolates produced amylolytic enzymes they were unable to breakdown complex carbohydrate glycogen, suggesting only intracellular production of amylase enzymes [513].

LAB are the most prevalent species in the vaginal microflora and are beneficial for vaginal health. Contrary to the LAB isolated from the human gut [514], the isolated vaginal LAB demonstrated low bile and sodium chloride salt tolerance. Therefore, when administered orally it stands as a challenge for the vaginal LAB to withstand the adverse intestinal environment and provide their benefits to the VC [515]. On the other hand, vaginal LAB isolates exhibited strong hydrophobicity and auto-aggregation properties. This shows their capacity to adhere firmly to the epithelial cells and prevent them from passing through the vaginal discharge. Therefore, vaginal administration of LAB is recommended for optimum repopulation of LAB in the VC [346]. Additionally, these LAB isolates demonstrated high co-aggregation property. This highlights the ability of the LAB to interact potently with the aerobic potential pathogens and create an antagonistic environment around them [516]. Moreover, these LAB also showed specific co-aggregation to these aerobic isolates; this manifests strain specificity for optimal pathogen elimination [517, 518].

In our study, *L. crispatus, L. gasseri*, and *L. vaginalis* were unable to cease the growth of *C. albicans*, rest all the potential bacterial pathogens were inhibited. *L. gasseri* and *L. crispatus* are consistent candidates of healthy vaginal microflora [123, 519]. The auto-aggregation property of *L. crispatus* has been previously highlighted by [520], establishing the bacteria as an effective probiotic against colitis. Furthermore, studies on *L. crispatus* strains have showed that 60% of its sequence is conserved translating orthologous proteins with antibacterial properties [521]. Likewise, hydrogen peroxide and acids produced by this LAB have proven to be efficient against a wide range of fungal and bacterial pathogens. Additionally, *L. crispatus* also modulates the secretion of inflammatory (IL-8) and pro-inflammatory (IL-2) cytokines by immune cells and positively regulates the vaginal health [522]. *L. gasseri* is known to produce bacteriocins like gassericin, which has antibacterial potential [523]. Similarly, this LAB also downregulates the production of cytokines TNF-α and IL-10 [524] and helps improving

vaginal immunity. LAB, *L. vaginalis* has also been strongly associated with healthy vaginal microflora [173]. Although, the population of *L. vaginalis* in VC showed a negative correlation with multiple sexual encounters and multiple pregnancy loses, indicating its instability in the vaginal microbiota [525]. Moreover, *L. vaginalis* was reportedly more susceptible to toxins and ceased in growth in absence of proper growth conditions unlike *L. crispatus* and *L. gasseri* [117]. Regardless of the availability of suitable growth conditions, it was observed that *L. crispatus* potently inhibited *E. cloacae* and *Shigella sp in-vitro.*

The extracellular metabolites (CFS) produced by LAB are antimicrobial in nature [526] displaying low toxicity to humans [527]. The CFS from the LAB demonstrated both bacteriostatic and bactericidal properties. LCCFS showed the best bacteriostatic effect, this can be correlated with the lactic acid and hydrogen peroxide producing ability of *L. crispatus* [528]. On the other hand, LGCFS and LVCFS showed better bactericidal effect with lactic acid as one of the major component of their CFS as well [529, 530]. Studies, on *L. gasseri* and *L. jensenii* isolated from VC, [531] and *L. crispatus, L. gasseri, and L. vaginalis* also isolated from VC, [307] have also reported the presence of biosurfactants in the CFS. [532]. These surfactants disrupt the cell membrane of microbial pathogens behaving as microbicidal agents. Moreover, compounds like cyclic peptides [533], bacteriocin [534], secondary metabolites, and reactive oxygen scavenging metabolites have also been reported in the CFS of LAB, all of which are antimicrobial in nature.

Budding of *C. albicans* was inhibited by the CFS demonstrating the suppressive effect of the metabolites produced by the LAB on budding of the yeast. The LAB in the VC not only competes with the yeast but also, to some extent, inhibits their proliferation as well [535]. Proteinase K, α-amylase enzyme, and sodium hydroxide treatment of the CFS had a negative impact on the inhibitory action of CFS against *C. albicans*. This confirms the presence of organic acids, proteins/peptides, glycolipoproteins, glycoproteins, and glycolipids in the CFS with anticandidal activity [536] [537]. Pathogenicity of *C. albicans* is promoted by hyphal growth [80], and the morphogenesis of the yeast to fungal state encourages virulence by targeting host immune cells (i.e., macrophage and complement proteins) [538]. The fungal form also has a higher chance to penetrate the host cell and escape detection by the immune cells [539]. The media composition of spider agar induces hyphae formation in *C. albicans* [540], but spraying the agar with CFS had a

severe negative impact on the hyphae formation of the yeast. Similarly, RPMI 1640 also stimulates hyphae/biofilm formation at 37 ℃ temperature and neutral pH [541]. But regardless of the stimulation, concurrent treatment with CFS resulted in restricted hyphae formation and resulted in severe depletion of biofilm with a sparse population of fungal cells as compared to the control treated with RPMI 1640 only [542] [543]. Candida biofilm was also completely inhibited by live LAB, through competitive exclusion in the presence of nutritional supplement. The LAB adhered rigidly and grew vehemently itself forming a biofilm as suggested by *Ventolini* [544], preventing the biofilm formation of *C. albicans*. Among the numerous transcription factors regulating hyphae/biofilm formation in *C. albicans*, the expression of three transcription factors i.e., CPH1, TEC1, and BCR1 were studied. TEC1 gene is regulated by transcription factor CPH2, which is regulated by the MAPK pathway [545]. In the current study, TEC1 gene was constitutively regulated. However, BCR1 gene which is induced by TEC1 showed differential regulation on treatment with CFS. The expression of BCR1 gene induces adhesion, hyphae formation, and transcription of hyphal cell wall proteins in *C. albicans* [546]. A similar study was reported highlighting the effect of *L. rhamnosus* ATCC 53103, *L. plantarum* ATCC 8014, and *L. acidophilus* ATCC 4356 against *C. albicans*. This study also reported the inhibitory effect of CFS from the LAB at the transcriptional level [547]. This study showed no differential regulation for TEC1 gene which was in accordance with our data. Although, expression of BCR1 was downregulated in our study unlike their report showing a stronger inhibitory effect of the vaginal specific LAB CFS. CPH1 gene which is also regulated by the MAPK pathway [548], was also found to be affected by the CFS treatment. The damage of cell wall causes downregulation of this gene in *C. albicans* inhibiting hyphal maturation [549]. Hyphal cell wall proteins expressed by ALS3 and HWP1 gene express adhesins that help in the strong adhesion and invasion of *C. albicans* to the vaginal epithelial cells [550]. HWP1 protein forms a covalent bond with the epithelium layer ensuring adhesion. Similarly, ALS3 protein helps in interaction with epithelial cells, endothelial cells, and extracellular matrix proteins. ALS3 protein also helps in sequestering iron from human ferritin and produces invasin protein on hyphal stimulation that helps in endocytosis of the *C. albicans* [543]. Thus, these proteins are highly responsible for pathogenesis and antigen production by *C. albicans* [551]. These hyphal genes are highly expressed during the formation of the first layer of anchor cells via glycosylphosphatidylinositol anchor proteins [552]. Likewise, expression of

ECE1 gene is essential for elongation and maintenance of hyphal morphology [553], this protein is required for proper adherence of *C. albicans* post hyphae formation [552]. ECE1 expresses a protein candidalysin a cytolytic peptide toxin, that causes epithelial membrane disruption causing calcium influx into the cells and triggers immune response [554]. HYR1 gene codes for virulent proteins which is not expressed by other non-albican species, this protein provides evasion to the pathogen from neutrophils [555]. The CFS treatment in our study, downregulated the expression of ALS3 by 0.9-fold, HWP1 by 0.85-fold, HYR1 by 0.65-fold, and ECE1 by 0.5-fold in comparison to the control. Hyphal cell wall comprises of glucans, chitin, and glycoproteins which are negatively affected by the metabolites produced by the probiotic LAB [556, 557]. Downregulated expression of hyphal protein at transcriptional level curtails the pathogenesis of *C. albicans* in VC. Therefore, the metabolites produced by vaginal LAB's persistently keeps *C. albicans* in a non-pathogenic, avirulent state [536, 558].

The availability of nutrients in the vaginal microenvironment post puberty, promotes the overgrowth of LAB over any microorganism [183, 559]. Subsequently, the LAB adheres more rigidly to the vaginal epithelial cells and produces metabolites that inhibit adherence, overgrowth, and biofilm formation of microbial pathogens [560]. Additionally, the outflow of vaginal fluid with high LAB and their exudates washes off loosely adhered pathogens limiting the chance of pathogenesis [561].

Previous spectrophotometric analysis (GC-MS) of the CFS from LAB have shown the presence of organic acids, amino acids, alkanes, alcohols, fatty acids, aldehyde alkaloids, and phenol [562]. The metabolomic analysis of the CFS through LC-MS/MS in our study, revealed the presence of several documented antimicrobial metabolites. *L. crispatus* showed the best antimicrobial activity against microbial pathogens. Both lactic acid and H_2O_2 produced by the isolate possess strong antimicrobial potential [563], moreover, LCCFS had the highest antimicrobial metabolites as well. The population of H_2O_2 producing *Lactobacillus* is higher among healthy women and has been strongly correlated with inhibition of BV and UTI causing organisms [245]. The LAB producing H_2O_2 have long term colonization and exhibits a healthy vaginal microenvironment [253]. H_2O_2 converts to reactive hydroxyl free radical species that either inhibit the growth of microbes or cause damage to the cell membrane and DNA of microbes [564]. The *L. gasseri* strain showed the best inhibitory action against *C. albicans*. LGCFS produced the

highest amount of lactic acid [565], it also reported the highest anti-candidal metabolites and antibiotics. Although *L. crispatus* strain of our study produced hydrogen peroxide[565], it showed relatively less activity against *C. albicans*. This deduces the inferior role of H_2O_2 in inhibition of *C. albicans,* as it has been previously reported by Takano *et. al* [566]. LVCFS reported the highest antiviral metabolites and AMP's. Other metabolites in the CFS with established anti-microbial activity were antibiotics; macrolides; terpenoids; hydroxy flavanone; cyclic hydrocarbons; benzoate esters; and alkaloids. The CFS had anti-inflammatory, anti-cancer, and anti-oxidant metabolites as well. The identification of extracellular metabolites through LC-MS/MS has been previously suggested [448]. According to *Kumar et al.* metabolites are capable of tuning cell signaling pathways and may be used as a potential therapeutic postbiotic agent [255]. Thus, the CFS produced by these LAB can be studied further as a prophylactic postbiotic for the prevention of vaginal infections.

No LAB were isolated from the swabs of menstruating women, indicating a decline in their population during menstruation [567]. This presents the need for menstrual hygiene products that may provide protection to the VC and assist repopulation of LAB to maintain vaginal health [568]. Among the vaginal LAB isolates, *L. crispatus* produced hydrogen peroxide and lactic acid, thereby presenting itself as a potent probiotic and postbiotic candidate. They may be further studied in mammalian cell lines and mice model to comprehend their potential.