Abstract

Healthy vaginal microbiota post puberty constitutes a consortium made up of bacteria, fungi, and virus [1]. *Lactobacilli* species account for 70% of the vaginal microbiome followed by enteric gram-negative bacteria, gram-positive/negative coccis, and mollicutes. Ascomycota, Basidiomycota, and Oomycota comprise the fungal population of the vaginal microbiome [2]. Genetic diversity, monthly hormonal changes, hygiene, and sexual practices determine the microbiome diversity in the vaginal canal (VC) of women [3].

Normal vaginal microbiome harbors species of lactic acid bacteria (LAB) like *L. crispatus, L. gasseri*, and *L. jensenii* which are known to produce both chiral forms of lactic acid. On the other hand, other commonly found species of LAB in VC are *L. vaginalis* and *L. iners* which produce only the L form of lactic acid. The LAB is known to deprive the overgrowth of other opportunistic pathogens, which in high populations cause inflammation and infection in the VC [4].

Vaginitis or vaginal infection causing microbes that reside in the VC are: *Atopobium vaginae, Gardnerella vaginalis*, *Staphylococcus* sp., *Streptococcus* sp., *Escherichia* sp., and *Candida* sp., etc. These microbes are known to cause diseases like Bacterial Vaginitis (BV), Vulva-Vaginal Candidiasis (VVC), and Aerobic Vaginitis (AV). These infections cause itching, redness, swelling, dysuria, dyspareunia, pain during intercourse, pain during menstruation, malodor, and discharge from the VC. The opportunistic pathogens may overgrow in the moisture filled VC unless an elevated population of healthy LAB is maintained [5, 6].

Probiotics are live microorganisms that are safe to consume, provide health benefits, and maintain beneficial microbial flora in humans. Whereas, postbiotics are bi-products or metabolites produced by beneficial bacteria with anti-inflammatory, anti-oxidant, and anti-microbial potential [7]. Studying the impact of these beneficial microbes and their extracellular metabolites *in-vitro* deepens our knowledge of their antimicrobial aspect. This also extends the medical-industrial usage of these compounds for maintaining and improving vaginal health [8].

Swab samples collected from the VC (~2-inch depth) of healthy women showed presence of a few LAB isolates. Three species of gram-positive, catalase-negative, bacilli

were isolated from the swabs on specific Lactic Acid Bacteria Selective Agar Base. They were identified as viz- *L. crispatus, L. gasseri,* and *L. vaginilis* and were studied further. These LAB were strict anaerobes exhibiting the potential to be used as probiotics. The *L. gasseri* strain (LG) was the most potent and survived for ~90 days at 12 ℃. LG produced the highest amount of lactic acid (i.e., 7.81 mg/ml), and showed the best bile and sodium chloride tolerance. This isolate also showed the best hydrophobicity, aggregation, and co-aggregation property. On the other hand, *L. crispatus* (LC) strain produced hydrogen peroxide and the second highest amount of lactic acid (i.e., 7.23 mg/ml). This strain had a short life span of ~30 days at 12 ℃. *L. vaginalis* (LV) strain showed fair potential to be used as a probiotic and had a life span of ~60 days at 12 ℃. Further in the study, the potency of these isolates was tested against the isolated aerobic opportunistic pathogens to evaluate their antimicrobial/ antifungal potential.

Vulval swabs collected from healthy women showed the presence of several potential aerobic microbes. Thirty-eight isolates among these showed tolerance to low pH of 4 and produced acid on utilization of carbohydrates showing the potential to overpopulate the VC in the absence of LAB. These isolates were identified as: *Enterococcus faecalis, Enterobacter cloacae, Shigella, Staphylococcus epidermidis, Escherichia fergusonii,* and *Candida albicans*. The microbes were further characterized as haemolytic: *S. epidermidis, E. cloacae,* and E. *faecalis*; lipolytic: *E. faecalis, C. albicans*, and *S. epidermidis*; amylolytic: E. *fergusonii* and *Shigella* sp; and proteolytic: *E. cloacae* in nature. *C. albicans* showed the strongest biofilm formation ability among the isolates and it could also utilize the complex carbohydrate glycogen. They were all able to grow in anaerobic conditions and had the potential to cause pathogenicity. The identified isolates are generally known to cause Urinary Tract Infection (UTI), AV, and VVC. Therefore, the inhibitory effect of LAB on these potential pathogens was studied further.

All three species of LAB and their extracellular metabolites/ culture free supernatant (CFS) inhibited the growth of the bacterial isolates. Among the LAB, *L. crispatus* showed the best potential against *E. cloacae* and *Shigella sp*. Whereas, *L. gasseri* was more potent against *E. fergusonii, E. facaelis,* and *S. epidermidis*. The LCCFS showed the best bacteriostatic property against all the potential bacterial pathogens with a Minimum Inhibition Concentration (MIC) of 0.1mg/μL and a Minimum Inhibition Volume (MIV) of 50 μL. Whereas, LGCFS and LVCFS showed better bactericidal properties.

Candida albicans was the only fungi isolated from the vulval swabs. *L. crsipatus* LC, *L. gasseri* LG and *L. vaginilis* LV were not fungistatic *in vitro*. Nevertheless, LCCFS and LGCFS showed relative inhibition in the budding of *C. albicans* till 24 hrs. LGCFS highly suppressed the hyphae formation of the yeast on stimulatory solid and liquid growth conditions. The genes of hyphal cell wall protein viz: HWP1, ALS3, ECE1, and HYR1; and hyphal transcription factors BCR1 and CPH1 were downregulated by CFS treatment. Moreover, LGCFS also most efficiently inhibited biofilm formation and eradicated established fungal biofilm. The LAB's transited the fungal *C. albicans* cells to their yeast form. Thus, the LAB and their metabolites prevented budding, hyphae formation, and biofilm development of *C. albicans* reducing their overall pathogenicity.

The identification of metabolites in CFS through LC-MS/MS revealed the presence of various antibiotics, antimicrobial metabolites, and antimicrobial peptides of therapeutic value. LCCFS had twenty-eight metabolites with antimicrobial properties, LGCFS had thirty-five, and LVCFS had twenty-three. *L. gasseri* produced the highest anti-fungal metabolites and antibiotics showing the highest potential against *C. albicans*. Whereas, *L. crispatus* produced the highest antibacterial compound showing the best potential against opportunistic bacterial pathogens. *L. vaginalis* produced the highest antimicrobial peptides and antiviral compounds. The LAB also produced toxins, anti-inflammatory compounds, anti-cancer metabolites, anti-oxidants, sedatives, and other medicinal compounds.

L. crispatus was selected for its potential and was preserved for commercial usage. LC was successfully encapsulated into Carboxy-Methyl Cellulose (CMC)-Alginate beads. These beads exhibited ~2600 % absorptive capacity and preserved the LAB for ~30 days. The beads could serve as an eco-friendly alternative to superabsorbent polymers used in sanitary napkins with a potential probiotic nature. The embedded LAB in the beads grew ideally in MRS *Lactobacillus* broth at a variable pH range of (4-7) producing ~$2.4*10^8$ number of cells within 48 hrs. Efficaciously lyophilized LC also exhibited a shelf life of 36 months, with the potential revival of ~$1.42*10^8$ number of cells within 48 hrs. On the other hand, the LCCFS showed the potential to be used as a spray *in-vitro* for complete inhibition of bacterial consortium on agar plate. LCCFS when used as an additive on sanitary fabric showed imbibition of extracellular-acidic-antimicrobial metabolites without compromising the solvent absorption or retention capacity of the fabric. The characterization of the treated fabric showed a changed nature post treatment without

affecting the tenacity of the fabric in comparison to the control. The treated NWF with fresh LCCFS showed an antimicrobial effect as an additive.

The metabolites produced by the LAB had a range of known antimicrobial compounds that could be exploited commercially as potential antimicrobial agents. Thus, the LAB and their CFS could be used in sanitary/menstrual products to improve hygiene standards and could also be incorporated as a hygiene habit among reproductive aged women.

Keywords: Female Reproductive Tract, Vaginal Canal, Vaginal Microbiome, Lactic acid bacteria, Oppurtunistic Vaginal Pathogens, Candidiasis, Aerobic Vaginitis, Potential Probiotics, Potential Postbiotics, Metabolites.

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