Heliyon 10 (2024) e30495

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

From whole genomes to probiotic candidates: A study of potential lactobacilli strains selection for vaginitis treatment

Jinli Lyu [a,b], Mengyu Gao [b,c], Shaowei Zhao [b,c], Xinyang Liu [a], Xinlong Zhao [d], Yuanqiang Zou [b,c], Yiyi Zhong [e], Lan Ge [e], Hiafeng Zhang [e], Liting Huang [a], Shangrong Fan [a,**], Liang Xiao [b,c,***], Xiaowei Zhang [a,b,*]

[a] Department of Obstetrics and Gynecology, Peking University Shenzhen Hospital, Shenzhen, 518036, China
[b] BGI-Shenzhen, Shenzhen, 518083, China
[c] Shenzhen Engineering Laboratory of Detection and Intervention of Human Intestinal Microbiome, BGI-Shenzhen, Shenzhen, 518083, China
[d] College of Life Sciences, University of Chinese Academy of Sciences, Beijing, 100049, China
[e] BGI Precision Nutrition (Shenzhen) Technology Co., Ltd, Shenzhen, 518083, China

ARTICLE INFO

Keywords:
Adhering ability
Antimicrobial ability
Vaginitis
Lactic acid production
Lactobacilli
Whole genomes

ABSTRACT

Vaginitis, characterized by pathogenic invasion and a deficiency in beneficial lactobacilli, has recognized lactobacilli supplementation as a novel therapeutic strategy. However, due to individual differences in vaginal microbiota, identifying universally effective *Lactobacillus* strains is challenging. Traditional methodologies for probiotic selection, which heavily depend on extensive *in vitro* experiments, are both time-intensive and laborious. The aim of this study was to pinpoint possible vaginal probiotic candidates based on whole-genome screening. We sequenced the genomes of 98 previously isolated *Lactobacillus* strains, annotating their genes involved in probiotic metabolite biosynthesis, adherence, acid/bile tolerance, and antibiotic resistance. A scoring system was used to assess the strains based on their genomic profiles. The highest-scoring strains underwent further *in vitro* evaluation. Consequently, two strains, *Lactobacillus crispatus* LG55-27 and *Lactobacillus gasseri* TM13-16, displayed an outstanding ability to produce D-lactate and adhere to human vaginal epithelial cells. They also showed higher antimicrobial activity against *Gardnerella vaginalis, Escherichia coli*, *Candida albicans*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* compared to reference *Lactobacillus* strains. Their resilience to acid and bile environments highlights the potential for oral supplementation. Oral and vaginal administration of these two strains were tested in a bacterial vaginosis (BV) rat model at various doses. Results indicated that combined vaginal administration of these strains at 1×10^6 CFU/day significantly mitigated BV in rats. This research offers a probiotic dosage guideline for vaginitis therapy, underscoring an efficient screening process for probiotics using genome sequencing, *in vitro* testing, and *in vivo* BV model experimentation.

* Corresponding author. Department of Obstetrics and Gynecology, Peking University Shenzhen Hospital, Shenzhen, 518036, China.
** Corresponding author. Department of Obstetrics and Gynecology, Peking University Shenzhen Hospital, Shenzhen, 518036, China.
*** Corresponding author. BGI-Shenzhen, Shenzhen, 518083, China.
E-mail addresses: 13603025114@163.com (S. Fan), xiaoliang@genomics.cn (L. Xiao), zhangivy726@163.com (X. Zhang).

https://doi.org/10.1016/j.heliyon.2024.e30495
Received 11 December 2023; Received in revised form 12 March 2024; Accepted 29 April 2024
Available online 30 April 2024

1. Introduction

The vagina is a intricate and unique micro-ecosystem characterized by diverse microorganisms [1]. Ravel et al. clustered the vaginal microbial community into five groups, four dominated by *Lactobacillus* species and termed community state type (CST) I, II, III, and V. The dominant species for these CSTs were *Lactobacillus crispatus, Lactobacillus iners, Lactobacillus gasseri,* and *Lactobacillus jensenii* [2]. However, CST IV is colonized by more anaerobic bacteria instead of *Lactobacillus* and exhibited higher microbial diversity. Although women with vaginal CST IV could persist in this community state for a long time without any symptoms, they are exposed to a higher risk of female reproductive diseases [3]. Hence, a considerable abundance of *Lactobacillus* is regarded as the indicator of a healthy vaginal environment. The protective functions of *Lactobacillus* mainly include the following: (1) producing the metabolites capable of inhibiting the pathogens, such as D/L-lactic acid, H_2O_2, and bacteriocins [4]; (2) releasing lactic acid to keep a low vaginal pH ($\leq$4.5) by fermenting glucose [5]; (3) maintaining the vaginal mucous membrane barrier by adhering to the vaginal epithelial cells; and (4) regulating mucosal immune response by producing short-chain fatty acids to alleviate inflammatory injury [6].

The lack of *Lactobacillus* species lead to the dysbiosis of the vaginal microbiota, resulting in a higher risk of vaginitis, which is one of the most common gynecological diseases that frequently occur in women, including bacterial vaginosis (BV), aerobic vaginitis (AV), and vulvovaginal candidiasis (VVC). They are directly related to many serious complications, such as preterm birth, postpartum endometritis, cervicitis, and pelvic inflammatory disease [7]. Antibiotic intervention, a traditional therapy, has been the clinical standard of care (SOC) for vaginitis for decades [8]. Although the short-term cure rate of metronidazole therapy for BV treatment was up to 80%–90 %, the recurrence rate was 52 % after 6 months of follow-up and 68 % after 12 months. Prolonged metronidazole treatment could not reduce the recurrence rate after drug withdrawal but increased the incidence of VVC [9]. The traditional antimicrobial azole derivatives could cure most of the primary acute VVC but failed in preventing recurrent attacks [10]. This was also true for using clindamycin against AV [11]. Therefore, a novel vaginitis treatment approach is urgently needed.

Although pathogens can be eliminated using antibiotics, the abundance of *Lactobacillus* is still suppressed after treatment, which may be the main reason for the high recurrence rate after SOC [12]. Hence, the supplementation of *Lactobacillus*-containing probiotics adjunct to antibiotic treatment has been developed as a novel biotherapy and has shown beneficial effects. Actually, *Lactobacillus* has been used for the treatment of many diseases including gastrointestinal disorders, metabolic diseases, infectious diseases, and even assisting in cancer treatment [13–15]. However, they exihibited heterogeneous outcomes obtained in clinical trials. Achieving a probiotic *Lactobacillus* strain that is widely effective in all patients with vaginitis is difficult because the *Lactobacillus* strains colonized in the vagina vary among different ethnic women. Moreover, few *Lactobacillus* strains have been developed as live biotherapeutic products (LBPs) explicitly marketed for female reproductive health. Hence, more potential *Lactobacillus* strains should be discovered.

In 2001, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) defined probiotics as live microorganisms that, when given in sufficient amounts, can provide health benefits to the host. *Lactobacillus*, one of the most significant probiotic genera, is widely found in various natural sources [16–19] and human organs [17,20,21]. The species, origins and viabilities of *Lactobacillus* used in treatment significantly influences the therapeutic outcomes of diseases [22]. To ensure the probiotics' viability during storage and intake, various encapsulation techniques such as hydrogels and microcapsules have been developed [23–26]. For the treatments of vaginitis, the dominant *Lactobacillus* species in the vaginal microbiota should be used.

This study aimed to select the potential female probiotic strains from a pool of *Lactobacillus* strains from a human commensal bacteria collection constructed by BGI-Research, China. A high-throughput pre-screening pipeline for probiotics was built based on the whole genome of the strains. Subsequently, an *in vitro* test profile of preferred product characteristics, including growth rate, probiotic metabolite–producing ability, adhesion to vaginal epithelial cells, gastral acid/bile tolerance, antibiotic resistance, and antimicrobial capacity, was established and carried out to further identify the best-performing strains.

2. Materials and methods

2.1. Lactobacillus isolation and culturing

Samples from the fecal samples were collected in 2 mL self-standing screw cap tubes (Axygen, USA) with storage buffer (MGI, China), gradient diluted in phosphate-buffered saline (PBS, HyClone, USA), spread on sterile de Man, Rogosa, Sharpe (MRS, Solarbio, China) medium, and incubated at 37 °C under anaerobic conditions within a anaerobic chamber Bactron 300-2 (Bactron, USA) for 24 h. Individual colonies were picked based on different morphologies and transferred into MRS broth.

2.2. Whole genomes characterization of isolates

A total of 2 mL culture was sent for whole-genome sequencing using Illumina Hiseq 2000 platform. The reads were assembled using SOAPdenovo 2.0441 to form scaffolds. Single genomes were splited from the scaffolds of multi-genomes using an in-house developed pipeline. The taxonomic assignment was determined by the taxonomic information of correlated NCBI-available prokaryotic genomes according to average nucleotide identity (ANI) and percentage of conserved proteins (POCP) values. The whole-genome sequencing workflow was same as previous describe [27]. All strains belonging to *Lactobacillus* species were selected to build our candidate pool.

2.3. Probiotic metabolism databases

The scaffold sequences of the *Lactobacillus* strains were annotated against seven metabolism databases to comprehensively choose

the suitable strains, which included the genes involved in the metabolic pathway related to acid resistance, bile resistance, lactate production, hydrogen peroxide production, butyrate synthesis, propanoate synthesis, and adhesive property. The dominant vaginal species were considered the key objects of selection. Further, the virulence factor database (VFDB) [28] and the Comprehensive Antibiotic Research Database (CARD) [29] were also employed to investigate the safety of strain for human use. The databases were originally built from KEGG [30], in which the enzymes were selected based on KO information. BLAST [31] was used in the annotation process, and the e-value was set as 0.01; the identity value should be greater than 60 %. We used subject length to calculate the coverage of aliment length to improve the selection quality; the coverage was greater than 80 %, and the length was greater than 100 bp. A scoring system was established after all genomes of the *Lactobacillus* strains were blasted against selected datasets. The highest numbers were scaled to 100 as the standard, and the remaining numbers were standardized based on this scale.

Three commercial vaginal probiotics were selected as control in our study. *Lacticaseibacillus rhamnosus* GR-1 and *Limosilactobacillus reuteri* RC-14 were purchased from Chr Hansen, Denmark. *Lactobacillus delbrueckii* DM8909, purchased from the Guangdong culture collection center, Guangzhou, China.

2.4. Growth assessment

A volume of 1 mL of a preselected candidate strain at a concentration of 1.7×10^6 CFU/mL was added to 10 mL of fresh MRS broth in an anaerobic chamber at 37 °C for 24 h. A volume of 200 μL was sampled in a 96-well plate with 3 replicates per group at 0, 2, 4, 5, 7, 9, 12, and 24 h' incubation to assess the growth state of the strains. The culture density was measured at a wavelength of 595 nm (OD_{595}). The growth rate was calculated based on the logarithmic phase of the growth curve.

2.5. Determination of D- and L-lactic acid production

The candidate strains were cultivated at 37 °C in MRS broth for 48 h in an anaerobic chamber. Then, they were centrifuged at 7104×*g* for 5 min. The supernatants were collected. The concentrations of D/L-lactic acid were measured using D/L-lactic acid assay kits (Megazyme, Ireland) following the manual assay procedure using ultraviolet spectrophotometry (Jinghua, Shanghai, China). The experiments were performed in triplicates.

2.6. Semi-quantitive analysis of hydrogen peroxide production

Next, 100 μL of each strain incubated at 37 °C for 24 h was plated on MRS agar with 0.25 mg/mL tetramethyl benzidine (TMB) and 0.01 mg/mL horseradish peroxidase (HRP) and incubated at 37 °C for 48 h under anaerobic conditions [32,33]. After exposure to ambient air, the colonies turned blue within 0–30 min depending on the concentration of hydrogen peroxide.

2.7. Evaluation of auto-aggregation

The auto-aggregating ability of *Lactobacillus* strains was evaluated using the spectrophotometer at 595 nm (OD_{595}). 10 mL of 10^8 CFU/mL *Lactobacillus* strains cultured for 24 h were added to a 15 mL test tube. The culture's top layer were tested separately after 0, 30, and 60 min. Auto-aggregation was assessed using the following equation: auto-aggregation rate (%) = $[1 - (OD_{60\ min}/\ OD_{0\ min})] \times 100$ [34].

2.8. Ability to adhere to vaginal epithelial cells VK2/E6E7

The vaginal epithelial cells VK2/E6E7, with a growth density of up to 80 % in complete DMEM (Gibco, USA), were harvested following trypsin digestion and resuspended in fresh medium at a concentration of 2×10^5 cells/mL. Then, 1 mL of the suspension was dispensed to each well in 6-well plates and incubated at 37 °C in a 5 % CO_2 environment until reaching 80 % confluence.

Lactobacillus strains were cultured in MRS broth at 37 °C for 24 h. Then, 1 mL of the strains suspension was added to 6-well plates with VK2/E6E7 and co-incubated at 37 °C for 90 min in 5 % CO_2. Following co-incubation, the cells were washed five times with 1 mL of PBS to remove unbound bacteria, after which 1 mL of trypsin was added to the wells for cell digestion. Subsequently, DMEM with fetal bovine serum was then added to terminate digestion. Further, 1 mL of 0.05 % Triton X-100 was added for 5 min to cause cell lysis. Tenfold gradient dilution was applied, and the dilution culture was plated on MRS agar. The colonies were counted after 24 h incubation at 37 °C under anaerobic conditions. The cell counting was performed using an automatic cell counter.

2.9. Antimicrobial activity testing

The antimicrobial activity primarily tested the inhibitory effect of five strains on the growth of *Gardnerella vaginalis* ATCC14018, *Escherichia coli* ATCC25922, *Candida albicans* ATCC10231, *Pseudomonas aeruginosa* ATCC9027, and *Staphylococcus aureus* ATCC6538, all purchased from the Guangdong culture collection center, Guangdong province, China. *Lactobacillus* strains were incubated in MRS broth for 24 h under anaerobic conditions. The suspension was filtered using a 0.2 μm filter membrane when the number of *Lactobacillus* peaked 1×10^8 CFU/mL. Then, 100 μL of *G. vaginalis, E. coli, C.albicans, S. aureus, and P. aeruginosa* suspension incubated in MRS broth for 24 h was added to 1.5 mL *Lactobacillus* supernatant. The absorbance at OD_{595} was then measured after 24 h co-incubation. The same procedure was followed for *E. coli* and *C. albicans.*

2.10. Antibiotic susceptibility testing

The Kirby–Bauer test was applied to assess antibiotic susceptibility. A total of 16 types of antibiotic susceptibility test discs (Bkmam, China) were chosen in this evaluation, including ampicillin (AM,10 μg/disk), bacitracin (B, 0.04 μg/disk), penicillin (P, 10 μg/disk), kanamycin (K, 30 μg/disk), tetracycline (TE, 30 μg/disk), piperacillin (PIP,100 μg/disk), erythromycin (E, 15 μg/disk), chloramphenicol (C, 30 μg/disk), oxacillin (OX, 1μg/disk), vancomycin (VAN, 30 μg/disk), azithromycin (AZI, 15 μg/disk), amoxicillin (AMX, 25 μg/disk), ceftriaxone (CRO, 30 μg/disk), gentamicin (GM, 10 μg/disk), clindamycin (CM, 2 μg/disk) and metronidazole (MNZ, 5 μg/disk). Further, 100 mL of *Lactobacillus* culture was added to fresh MRS broth and incubated for 24 h at 37 °C under anaerobic conditions. Then, it was spread on the MRS agar. The antibiotic disks were put onto an MRS medium. The radius of the inhibition zone was measured after 24 h.

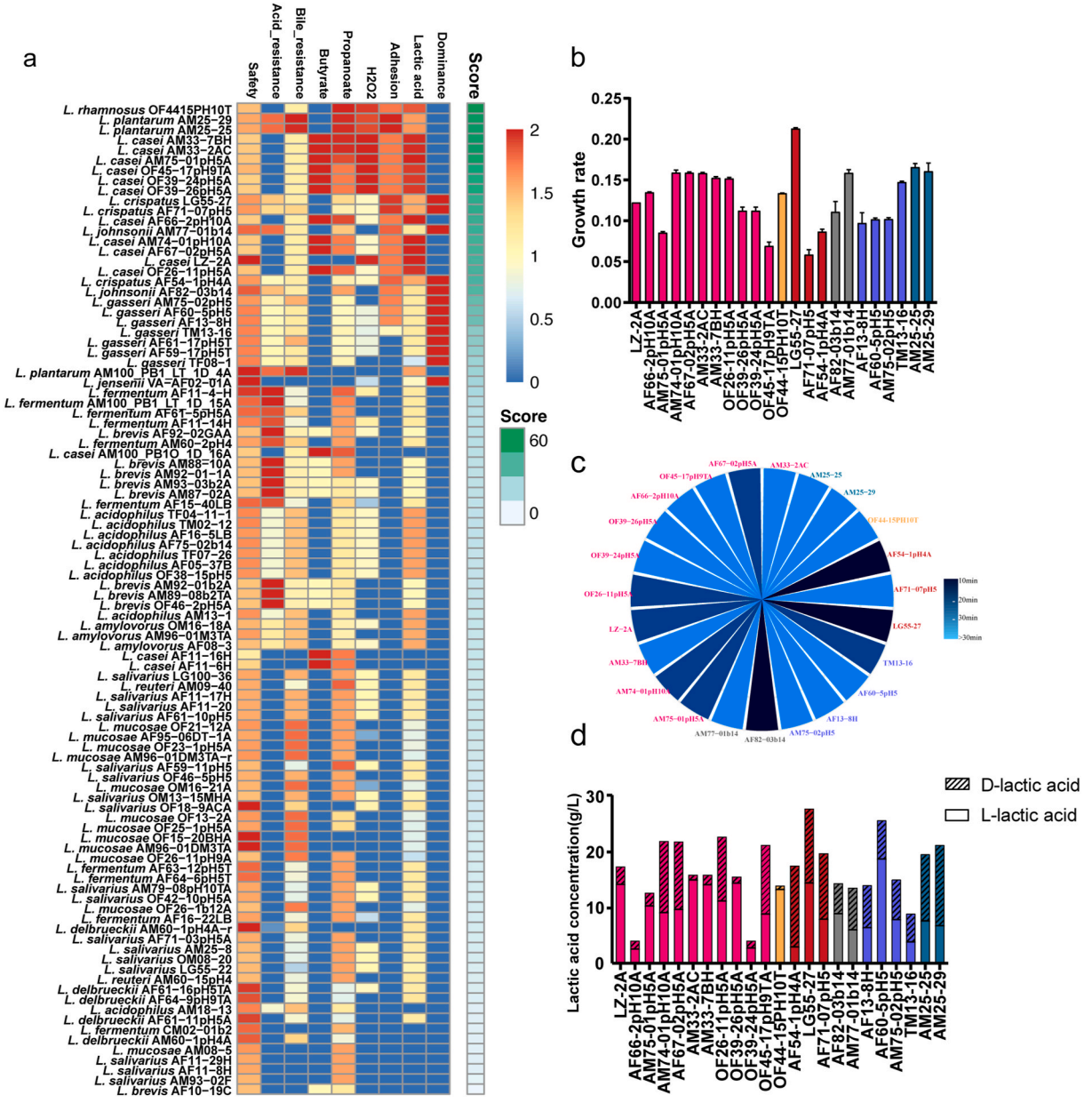

Fig. 1. ***Lactobacillus*** **strains were selected based on the whole-genome annotation results and** ***in vitro*** **examination**. (a) the total number of annotated enzymes for each strain and the final score of each strains showed in the last column; (b) growth rate of 23 pre-screened candidate strains; (c) H_2O_2 production assessed via semi-quantitive analysis; (d) The concentration of D-/L-lactic acid.

2.11. Acidic and bile salt tolerance measurement

A volum of 100 μL of *Lactobacillus* suspension incubated at 37 °C for 24 h under an anaerobic environment was added to MRS broth with pH = 2, 3, 4, 4.5, and 7 to investigate the influence of low pH on growth of *Lactobacillus* strains. Then, the culture was plated on MRS agar after 24 h incubation. The colonies counting was then performed. The same process was repeated to assess the influence of high concentrations of bile salt on the growth of *Lactobacillus* strain using MRS broth with 0.05 %, 0.1 %, 0.2 %, and 0.3 % of bile salt.

3. Administration of *Lactobacillus* strains on BV model rats

3.1. Modeling

Seven-week-old female Sprague–Dawley rats, weighed 222–264 g, were obtained from Hunan SJA Laboratory Animal Co. (Hunan, China). A total of 220 female rats were examined during the experimental period. The rats were housed in dry, stainless-steel cages accommodating two to five rats per cage. They were maintained in a standard animal care facility with controlled environment condition, including a 12 h light/dark cycle, temprature ranging from 20.0 °C to 24.4 °C, and humidity levels maintained between 40 % and 70 %. The experiment procedures was performed in a pathogen-free conditions to prevent inadvertent infections.

After inducing pseudoestrus in the rats by administered intramuscularly with 0.5 mg of estradiol benzoate (Full Woo Biotechnology Co., Ltd., Shanghai, China) three days, 150 μL of a 1×10^8 CFU/mL/day suspension of *G. vaginalis* strains was vaginally administered to each rat for three consecutive days. Gram staining was performed to evaluate the efficacy of the modeling process.

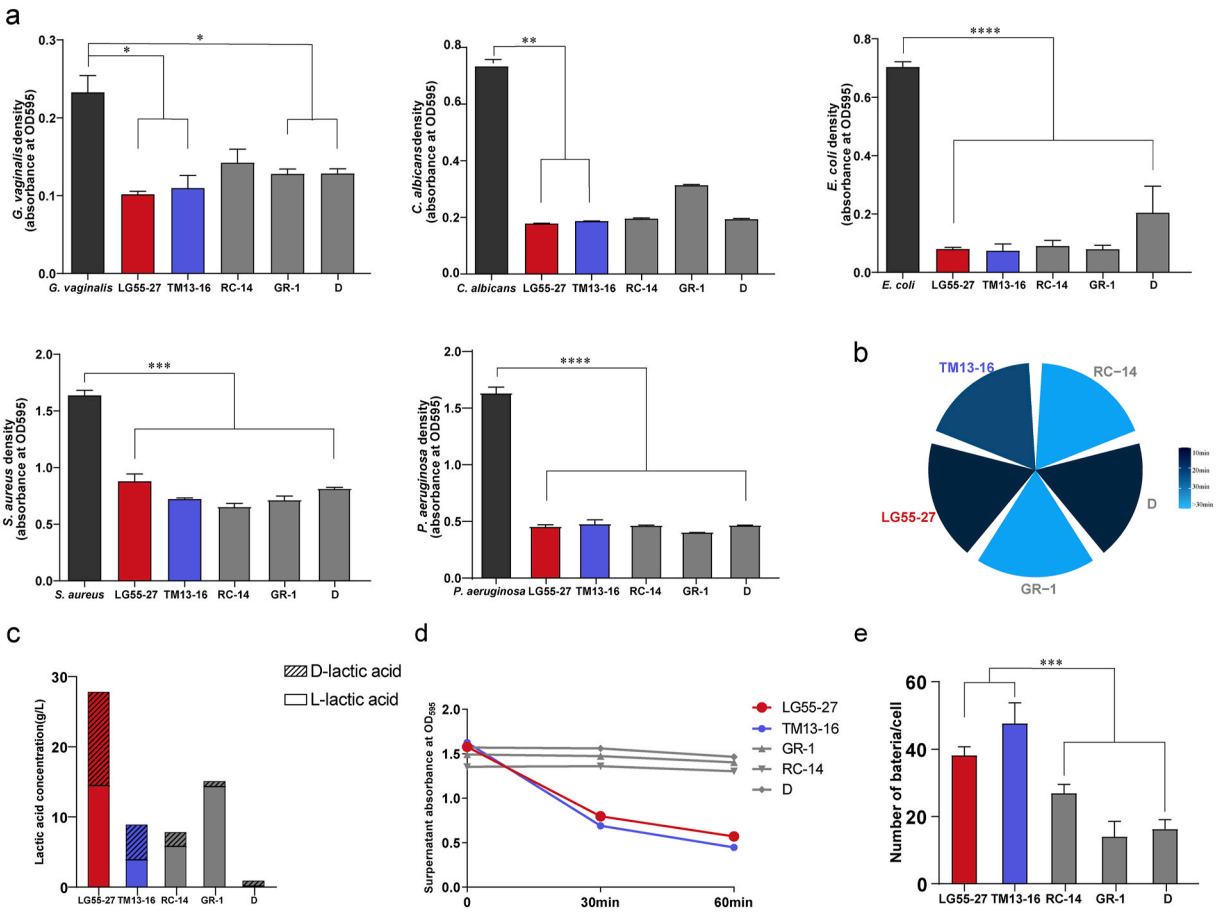

Fig. 2. Functional verification of candidate strains. (a) Antimicrobial characteristics of *Lactobacillus* strains; (b) H_2O_2 production assessed via semi-quantitive analysis; (c) concentration of D-/L-lactic acid for candidate and reference strains; (d) auto-aggregation rate was calcuted after 0, 30, and 60 min at OD_{595}; (e) Adhesion ability of candidate and reference strains to vaginal epithelium VK2/E6E7 cells. Statistical analysis was performed using the Kruskal–Wallis test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. LG55-27: *L. crispatus* LG55-27; TM13-16: *L. gasseri* TM13-16; GR-1: *L. rhamnosus* GR-1; RC-14: *L. reuteri* RC-14; D: *L. delbrueckii* DM8909.

3.2. Grouping and treatment

The successfully modeled rats were randomly allocated into 16 groups, each comprising 10 rats (Fig. 4a). Rats in the model group were served as negative control with no treatment. Rats in the metronidazole group were served as positive control with metronidazole intragastrically adiministrated (10 mL/kg body weight per day) for 2 days and remained untreated thereafter. The rats in the remaining 14 groups were subjected to 7 intragastrical (I) groups and 7 vaginal (V) groups. The intervention strains for each treatment methods were as follows: *L. crispatus* LG55-27 (LG, 1×10^8 CFU/day for 6 days); *L. gasseri* TM13-16 (TM, 1×10^8 CFU/day for 6 days); low dose of LG55-27 + TM13-16 (LC, 1×10^8 CFU/day for 6 days); medium dose of LG55-27 + TM13-16 (MC, 1×10^8 CFU/day for 6 days); high dose of LG55-27 + TM13-16 (HC, 1×10^8 CFU/day for 6 days); high dose of LG55-27 + TM13-16 in combination with metronidazole (HCM, metronidazole for 2 days and LG55-27 + TM13-16 1×10^8 CFU/day for 6 days); inactivated LG55-27 + TM13-16 (SC, 6 days).

Metronidazole tablets (Shanghai Sine Wanxiang Pharmaceuticals Co., Ltd., China) were dissolved in pure water to achieve a concentration of 0.0108 g/mL. The pellet of strains was resuspended in PBS, and various concentrations were prepared. Mixed strains were prepared with a total concentration of 1×10^8 CFU/mL (ratio of 1:1). Prior to administration, inactivated strains were autoclaved at 121 °C for 15 min.

3.3. Detection of the levels of inflammation cytokines, pH value, and colonization of Lactobacillus

On days 0, 3, and 5, the pH value of the vagina of rats was measured. The vaginal discharge and the fecal sample were collected on days 0 and 5. Genomic DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen Biotech, China), and the abundance of *L. crispatus* and *L. gasseri* was examined using primers LcrisF (AGCGAGCGGAACTAACAGATTTAC), LcrisR

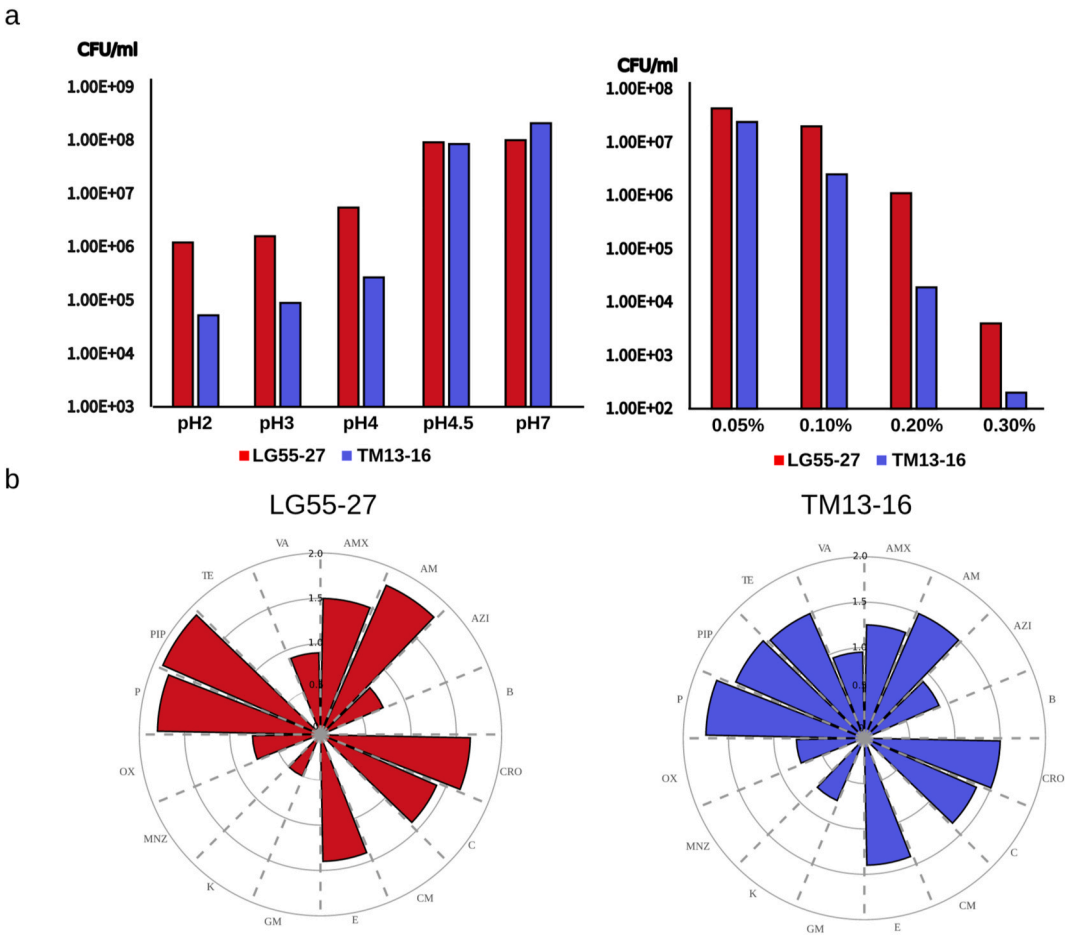

Fig. 3. Growth ability of strains under extreme conditions. (a) Growth ability of candidate strains under different pHs and concentrations of bile salt. (b) Antibiotic susceptibility of candidate strains. AM: Ampicillin; B: Bacitracin; P: Penicillin; K ： Kanamycin; TE: Tetracycline; PIP: Piperacillin; E: Erythromycin; C:chloramphenicol; OX：oxacillin; VAN: vancomycin; AZI: Azithromycin; AMX: Amoxicillin; CRO: ceftriaxone; GM: Gentamicin; CM: Clindamycin; MNZ: Metronidazole.

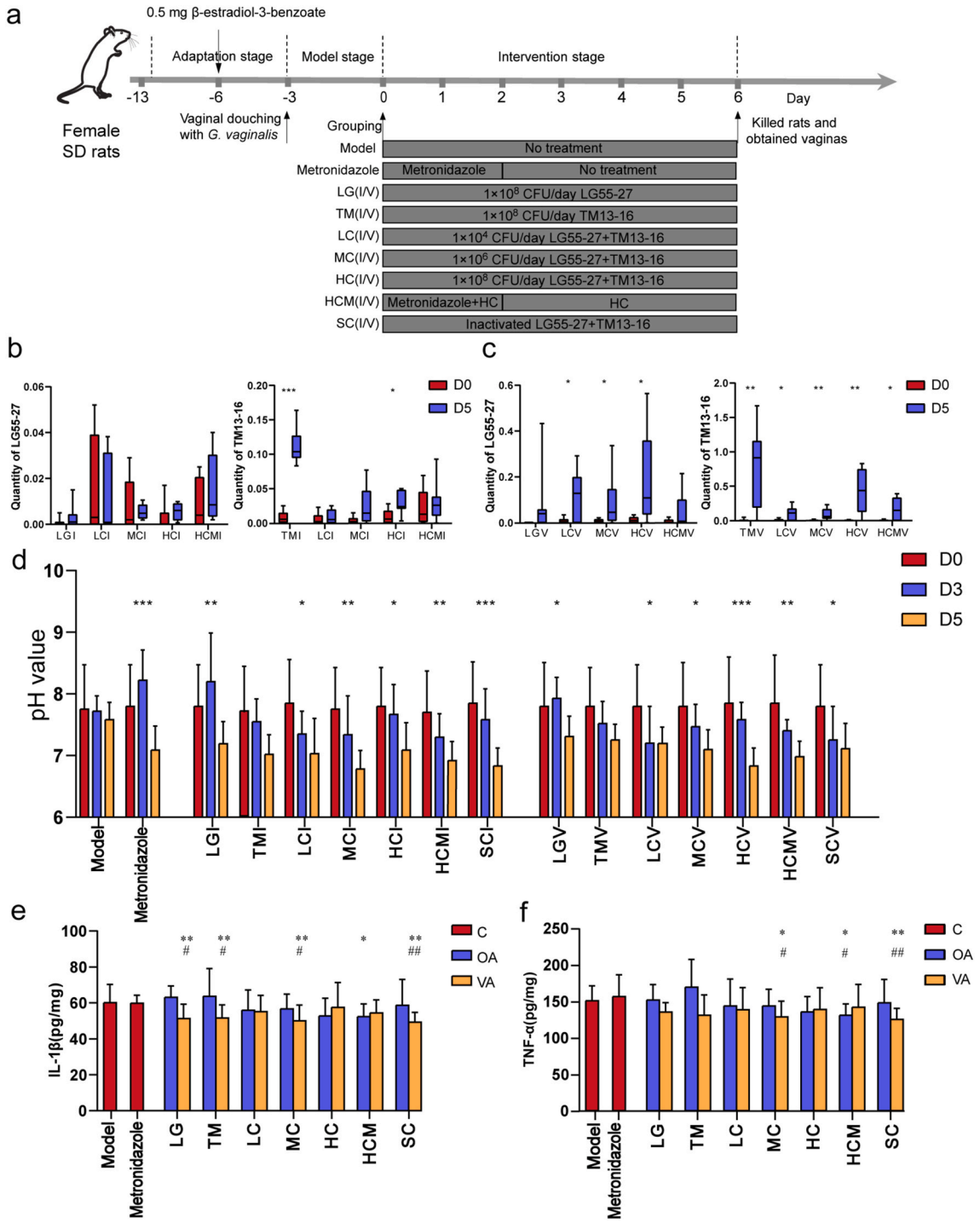

Fig. 4. Design and result of the BV model. (a) intervention of rats and grouping after the BV model were established; (b) the quantity of LG55-27 and TM13-16 in fecal samples after oral administration; (c) the quantity of LG55-27 and TM13-16 in vaginal discharge after vaginal administration; D0 and D5 represent the fecal and vaginal samples collected after 0 and 5 days, respectively; (d) pH value in the vagina was measured after 0, 3, and 5 days; (e) the levels of IL-1β in vagina were measured after a 6-day intervention; (f) the level of TNF-α in the vagina were measured after a 6-day intervention. The Wilcoxon test was used to perform the statistical analysis in each of the two independent groups, and the analysis of variance was used for statistical analysis in multiple groups. C represents the control group; OA represents the oral administration group; and VA represents the vaginal administration group. * represents comparison to the metronidazole group ($^{*}P < 0.05$; $^{**}P < 0.01$). $^{\#}$ represents comparison with the model group ($^{\#}P < 0.05$; $^{\#\#}P < 0.01$).

(AGCTGATCATGCGATCTGCTT), and LactoF (TGGAAACAGRTGCTAATACCG), LgassR CAGTTACTACCTCTATCTTTCTTCACTAC [35]. Vaginal tissues were collected on day 6. The levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α, were quantified using cytokine kits (Elisa Biotech Co.,Ltd, Shanghai, China) in accordance with the manufacturer's protocols.

4. Results

4.1. Pre-screening based on the whole genomes of Lactobacillus strains

After comparing the whole genomes of 98 *Lactobacillus* strains with the database using BLAST, the total number of enzymes annotated by each strain was calculated as one of the factors in our scoring system. The function in the right part of Fig. 1a had more weight than that on the left because they contributed more to the environment in the future (Table S1). The scores of safety were individually calculated because we believed that they contributed negatively. Hence, they were given negative weight in our score-calculating process. Moreover, 23 *Lactobacillus* strains were preliminary, as shown by the final score in the last column of Fig. 1a.

4.2. Further screening based on in vitro examination of preselected Lactobacillus strains

The examination focused on growth rate, hydrogen peroxide (H_2O_2) production, and D/L-lactate levels. Among all tested strains, *L. crispatus* LG55-27 exhibited the highest growth rate, while *L. crispatus* AF71-01pH5 had the lowest, a difference that was statistically significant ($P < 0.0001$). *L. jensenii* AM77-01b14 and *L. gasseri* TM13-16 demonstrated the top growth rates within their respective species. For the *Lacticaseibacillus casei* species, *L. casei* OF45-17pH9TA showed the lowest growth rate (Fig. 1b). In terms of H_2O_2 production assay, *L. crispatus* LG55-27, *L. crispatus* AF54-1pH4A, and *L. jensenii* AF82-03b14 colonies turned blue within 10 min, indicating a higher H_2O_2 production. Conversely, the colonies from the other 14 strains failed to turn blue within 30 min. There was a marked variation in the ability to produce D/L-lactate across different strains within the same species (Fig. 1c). *L. crispatus* LG55-27 produced the highest total amount of (D + L) -lactate compared to all other species (Fig. 1d). Given these findings, *L. crispatus* LG55-27 and *L. gasseri* TM13-16 were selected as prime candidates for further *in vitro* functional evaluation.

5. Functional validation of the candidate probiotic strains

5.1. Antimicrobial properties

The antimicrobial efficacy of culture supernatants from two selected *Lactobacillus* strains (*L. crispatus* LG55-27 and *L. gasseri* TM13-16) alongside three reference strains (*L. reuteri* RC-14, *L. rhamnosus* GR-1, and *L. delbrueckii* DM8909) was assessed. All evaluated strains effectively suppressed the growth of *G. vaginalis*, with the sole exception of *L. reuteri* RC-14, which did not achieve statistical significance ($P = 0.0603$). Both L. *crispatus* LG55-27 and *L. gasseri* TM13-16 significantly hindered the growth of *C. albicans*, and the other strains demonstrated no significant effect. Moreover, all five *Lactobacillus* strains considerably inhibited the growth of *E. coli*, *S. aureus*, and *P. aeruginosa* (Fig. 2a). These findings highlight the superior antimicrobial capabilities of our selected strains against the pathogens tested.

5.2. Measurement of hydrogen peroxide

The strains L. *crispatus* LG55-27 and *L. delbrueckii* DM8909 exhibited the highest hydrogen peroxide (H_2O_2) production, as evidenced by turning blue within 10 min (Fig. 2b). In contrast, *L. gasseri* TM13-16 turned blue after 20 min, indicating a moderate level of H_2O_2 production. Conversely, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 failed to turn blue within 30 min, demonstrating a reduced capability to produce H_2O_2.

5.3. Measurement of D/L-lactate production

L. crispatus LG55-27 exhibited elevated production of both D-lactate and L-lactate, particularly D-lactate, significantly surpassing the levels produced by other strains ($P < 0.0001$, Fig. 2c). In contrast, *L. rhamnosus* GR-1 was characterized by a greater concentration of L-lactate relative to its production of D-lactate. Additionally, a mixture of *L. crispatus* LG55-27 and *L. gasseri* TM13-16 generated a high level of total lactate, exceeding that of the combined reference strains, with a notably higher quantity of D-lactate (Fig. S1).

5.4. Colonization ability

Self-aggregation of the bacteria serves as a foundation for biofilm formation, which further allows the bacteria to adhere more effectively to epithelial cells. So we assessed the self-aggregation and adherence abilities to vaginal epithelial cells to evaluate the colonization potential of *L. crispatus* LG55-27 and *L. gasseri* TM13-16. Both strains demonstrated rapid clumping leading to sedimentation and a clear supernatant within 30 min. This self-aggregation was more pronounced in the first 30 min than in the subsequent period. Furthermore, a significant decrease in OD_{595} in the culture's top layer was observed for these two strains ($P < 0.0001$, Fig. 2d), a pattern not seen in the other strains. In terms of adhesion, The adhesion capability of both *L. crispatus* LG55-27 and *L. gasseri* TM13-16 was significantly superior compared to other strains ($P < 0.01$). *L. gasseri* TM13-16 showed the highest capacity to adhere to VK2/E6E7

cells among all tested strains. The elevated self-aggregation levels of *L. gasseri* TM13-16 likely contribute to its enhanced adhesion capacity (Spearman rho = 0.759, $P < 0.01$). No significant differences in adhesion were noted among the remaining strains (Fig. 2e).

5.5. Resistance to gastric acid and bile salt

Initially, we inoculated the media with 10^8 CFU/mL of two candidate strains across various pH levels. After 24 h of incubation at pH 7, both *L. crispatus* LG55-27 and *L. gasseri* TM13-16 showed no significant decrease in viability. At pH 2, *L. crispatus* LG55-27 maintained a survival rate of 10^6 CFU/mL, and *L. gasseri* TM13-16 had 10^4 CFU/mL, demonstrating the acid resistance of both candidate strains (Fig. 3a). Similarly, after 24 h of incubation in media with a high bile salt concentration (0.3 %), *L. crispatus* LG55-27 sustained a survival rate of 10^3 CFU/ml, whereas *L. gasseri* TM13-16 exhibited a lower viability (Fig. 3a).

5.6. Antibiotic resistance

The candidate *Lactobacillus* strains exhibited comparable susceptibility to penicillin, piperacillin, erythromycin, and chloramphenicol. *L. gasseri* TM13-16 demonstrated the highest susceptibility to tetracycline, in contrast to *L. crispatus* LG55-27, which showed the least susceptibility. Both L. *gasseri* TM13-16 and *L. crispatus* LG55-27 were resistant to bacitracin and metronidazole, with *L. crispatus* LG55-27 also exhibiting resistance to tetracycline. Additionally, *L. gasseri* TM13-16 displayed resistance to clindamycin and gentamicin (Fig. 3b).

5.7. Safety analysis of selected Lactobacillus strains

The scaffold of two selected strains TM13-16 and LG55-27 were used for safety analysis. We used the scaffolds to perform with the CARD, Resfinder, VFDB, prophage, plasmid, and mobile genetic element databases.

The antibiotic resistance of two strains was predicted using CARD and Resfinder [36]databases. The annotation of the CARD database showed the tetracycline antibiotic and macrolide antibiotic genes in the strain LG55-27, and carbapenem, lincosamide, and tetracycline antibiotic genes in TM13-16. The results of Resfinder illustrated the macrolide and tetracycline resistance of LG55-27 and tetracycline resistance of TM13-16.

The potential prophage sequences were identified using PHAST [37] database. None of the two strains contained the prophage gene under the threshold identity >90 %, e-value >0.01, and minimum aligned length >5 kbps. The mobile genetic elements were also analyzed using BLAST search against the corresponding database. Islander [38] was used to analyze genomic islands, and ICEberg database [39] was used to identify the integrative and conjugative elements. The insertion sequence was annotated against the ISfinder database [40]. We did not find any genomic islands or integrase and conjugative elements in the scaffolds of our two strains. Regarding the result of insertion sequences, the annotation demonstrated two hits in TM13-16. These hits existed in different scaffolds and might not be capable of triggering the transposition activity. In the strain LG55-27, 22 hits were found similar to insertion sequences. Although some hits were located on the same scaffold, possibly leading to transposition activity, we believe that no harmful genes in LG55-27 needed to be transposed. The results of virulence factors showed that the cutoff was e-value >0.01, coverage >70 %, and identity >30 %. Under this threshold, 151 potential virulence genes were identified in TM13-16, and 175 potential virulence genes were identified in LG55-27. The annotation function mainly included secretion and transport system, structure-forming systems such as capsule and cell wall, regulation molecules, and cell surface proteins for better adherence and motility. Most of these genes were defensive or nonclassical virulence factors, helping improve cell survivability in a complex environment. The two strains contained genes encoding adherence and lipopolysaccharides, which helped colonize cells. The toxic gene *lpxM* was not found in their scaffolds. Notably, we found cytolysin/hemolysin-related genes in the two strains. The product of this gene was a pore-forming structure, and it might cause infections in humans and animals. However, it was a bacteriocin which was harmful to a broad range of Gram-positive bacteria, maintaining the balance in the intestinal environment.

6. Evaluation of the effect of probiotic administration on BV rats

6.1. Lactobacillus was colonized in the vagina and gut

Gram staining showed *G. vaginalis* adherence to epithelial cells, indicating a successful of BV modeling (Fig. S2a). qPCR analysis of fecal samples from intragastrically administered groups revealed a significant increase in *L. gasseri* TM13-16 in the TM and HC groups after 5 days, but not for *L. crispatus* LG55-27 (Fig. 4b), indicating TM13-16's superior gut colonization. However, both strains showed increased vaginal colonization after intervention across various groups (Fig. 4c), demonstrating their more effective colonization in the vagina than the gut.

6.2. pH changes in the vagina after Lactobacillus intervention

pH levels in BV rats were tracked on days 0, 3, and 5 after *Lactobacillus* administration, showing a decline across all groups (Fig. 4d and Fig S2). By day 3, the HCMI and HCMV groups had significantly lower pH than the metronidazole group, indicating the combination of metronidazole and *Lactobacillus*, administered either intragastrically or vaginally, was more effective than metronidazole alone. By day 5, the HCV group exhibited significantly lower pH than the LGV, TMV, and LCV groups, highlighting the superior efficacy

of vaginal mixed *Lactobacillus* therapy over single-strain treatments (Fig. S2).

6.3. Inflammatory cytokine responses to Lactobacillus in rats

In single-strain treatments, only the vaginal administration (LGV and TMV) significantly reduced IL-1β levels, with no reduction observed in the oral group, indicating vaginal routes are more effective for single strains (Fig. 4e). The medium mixed-strain dose (MCV) notably decreased both IL-1β and TNF-α levels, suggesting it as the optimal vaginal treatment (Fig. 4e and f). While IL-1β and TNF-α levels significantly dropped in the HCMI group, they did not in the HCMV group, pointing to oral administration as the preferred method for probiotic-metronidazole combination therapy. Interestingly, even inactivated strains (SCV) reduced IL-1β and TNF-α levels, showing *Lactobacillus* metabolism mitigates inflammation in BV rats. Moreover, no marked difference was noted between the model and metronidazole groups, indicating metronidazole's delayed anti-inflammatory effect in BV rats.

7. Discussion

Traditional probiotic selection methods, relying on extensive *in vitro* experiments, are time-consuming and labor-intensive. This study established metabolism databases tailored to *Lactobacillus* probiotic traits and utilized whole-genome high-throughput screening annotated to these databases for more efficient and accurate selection. This approach also offers an effective method for identifying other functional strains. The study highlighted the lack of effective animal models for vaginal research, introducing a preliminary BV model in rats. This model, alongside selected clinical indicators, evaluated the impact of candidate strains on BV, with different strains' doses providing data for clinical intervention strategies.

Three well-developted *Lactobacillus* strains were selected as controls in this study. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 are well-developed vaginal health probiotics identified in the 1980s by Reid et al. and explored for decades [41]. To date, many clinical trials have been conducted on treating vaginitis and urethritis by administering the strains orally or vaginally [42,43]. Moreover, *L. delbrueckii* DM8909 served as the active ingredient of the unique IND-approved live biotherapeutic drug in China. These three strains have been recognized as excellent probiotics for female reproductive health.

Lactic acid is a main metabolite of *Lactobacillus*. Despite the controversial effects of its isomers D-lactate and L-lactate, a D/L-lactate ratio of 1 is likely to be more beneficial [44], matching the ratio found in our candidate strains. Specifically, *L. crispatus* LG55-27 and *L. gasseri* TM13-16 displayed D/L-lactate ratios of 1.00 ± 0.11 and 1.16 ± 0.19, respectively, distinguishing them from other strains. Considering that probiotics often mix strains to enhance efficacy, we evaluated the D-/L-lactic acid production of a combined culture of *L. crispatus* LG55-27 and *L. gasseri* TM13-16. This combination produced significantly more total lactic acid than any single strain or the reference strain duo GR-1 and RC-14 ($P < 0.001$), highlighting its potential as an effective LBP for vaginal health. Furthermore, lactic acid produced by *Lactobacillus* is the major antimicrobial compound, hindering the growth of *G. vaginalis* [45], *E. coli* [46], and *C. albicans* [47]. The supernatants of our candidate strains inhibited the growth of *E. coli*, *G. vaginalis*, *C. albicans*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* significantly, which indicated that the production of lactic acid may contribute to protecting the vagina against pathogen infections.

Generally, vaginal and oral probiotics are two key administration methods for treating vaginitis. Vaginally, the adhesion of probiotics to vaginal epithelial cells is crucial for outcompeting pathogens and enhancing protection [48,49], particularly for exogenous *Lactobacillus* strains like TM13-16 and LG55-27. These strains exhibit superior adhesion to VK2/E6E7 vaginal epithelial cells. Orally, these strains demonstrate enhanced survival in acidic conditions and high bile salt concentrations, suggesting oral probiotics' potential for supporting vaginal health. Oral lactobacilli can increase vaginal *Lactobacillus* abundance, helping maintain a healthy vaginal environment or aiding in vaginitis treatment [43,50,51].

Safety is critical for potential probiotics, including assessing the risk of antibiotic resistance genes that may transfer to pathogens [52]. Except for a tetracycline resistance gene, no antibiotic resistance genes were found in *L. crispatus* LG55-27 and *L. gasseri* TM13-16. Despite this, both strains remained sensitive to tetracycline and most other tested antibiotics *in vitro*, except for metronidazole and bacitracin. This sensitivity profile confirms the strains' safety and potential for use alongside metronidazole, a common treatment for BV. Additionally, the absence of virulence genes in *L. crispatus* LG55-27 and *L. gasseri* TM13-16 suggests a low risk of pathogenicity to humans.

The effectiveness of probiotics significantly depends on the appropriate formulation and dosage [53]. In our study, we explored various efficacy factors through oral and vaginal administrations, assessing different dosages and strain combinations. Our findings suggest that for oral administration, a combination of metronidazole with a high dose of probiotics is advisable. This approach can effectively decrease vaginal pH and lower pro-inflammation levels. Indeed, evidence confirms the higher cure rate of bacterial vaginosis by combining metronidazole with a high dose of probiotics administrated orally [54]. For vaginal administration, a high probiotic dose efficiently reduces vaginal pH, whereas a medium dose is more effective in alleviating the inflammatory response. Intravaginal probiotics supplementation was useful in decreasing the vaginal pH an hindering the pathogens' growth after metronidazole therapy [55]. Further exploration into the probiotics' colonization and their functional mechanisms should be conducted through microbiota analysis. Such insights will improve guidance on the appropriate dosages and methods of administering probiotics for vaginitis treatment.

8. Conclusion

This study developed metabolism databases for Lactobacillus probiotics and applied whole-genome high-throughput screening for

more precise selection. Two candidate strains were identified as superior, based on their effective pathogen inhibition, higher lactic acid and H_2O_2 production, improved adherence to vaginal cells, and resistance to bile and acid, compared to three well-established reference strains. Optimal dosages and methods for BV treatment were explored through animal studies. Findings recommend combining metronidazole with high dose probiotics for oral administration. A high dose probiotics lowers vaginal pH effectively, while a medium dose alleviates inflammation better for vaginal administration. This research presents two promising probiotics and strategies for vaginitis biotherapy.

Funding

This study was supported by the National Natural Science Foundation of China (82171676 and 82201793), the Science and Technology Planning Project of Shenzhen Municipality (JCYJ20220530160206014and JCYJ20190809101409603), and the Scientific Research Foundation of Peking University Shenzhen Hospital (KYQD202100Xand KYQD2022111).

Data avability statement

The whole genomes and taxonomic information of 98 *Lactobacillus* strains has been published [27]. The other datasets generated for this study are included in the manuscript and/or the Supplementary Files.

Ethics statement

Animal research was approved by the Institutional Animal Care and Use Committee, China, with approval number IA-PD2020013-01. All of the experimental procedures involving aminals were conducted in accordance with the Regulations of Guangdong Province on the administration of laboratory animals.

CRediT authorship contribution statement

Jinli Lyu: Writing – original draft, Methodology, Investigation. **Mengyu Gao:** Methodology, Data curation. **Shaowei Zhao:** Resources, Investigation. **Xinyang Liu:** Resources, Methodology. **Xinlong Zhao:** Formal analysis. **Yuanqiang Zou:** Investigation. **Yiyi Zhong:** Methodology, Resources. **Lan Ge:** Investigation, Resources. **Hiafeng Zhang:** Investigation. **Liting Huang:** Methodology. **Shangrong Fan:** Investigation, Supervision. **Liang Xiao:** Investigation, Supervision. **Xiaowei Zhang:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This study was supported by the BGI Precision Nutrition (Shenzhen) Technology Co., Ltd., China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30495.

References

[1] V. Buchta, Vaginal microbiome, Ceska Gynekol. 83 (5) (2018) 371–379.
[2] X. Zhou, M.A. Hansmann, C.C. Davis, H. Suzuki, C.J. Brown, U. Schütte, J.D. Pierson, L.J. Forney, The vaginal bacterial communities of Japanese women resemble those of women in other racial groups, FEMS Immunol. Med. Microbiol. 58 (2) (2010) 169–181, https://doi.org/10.1111/j.1574-695X.2009.00618.x.
[3] S. Gupta, V. Kakkar, I. Bhushan, Crosstalk between vaginal microbiome and female health: a review, Microb. Pathog. 136 (2019) 103696, https://doi.org/10.1016/j.micpath.2019.103696.
[4] G. Tachedjian, M. Aldunate, C.S. Bradshaw, R.A. Cone, The role of lactic acid production by probiotic Lactobacillus species in vaginal health, Res. Microbiol. 168 (9–10) (2017) 782–792, https://doi.org/10.1016/j.resmic.2017.04.001.
[5] W.A. Wilson, P.J. Roach, M. Montero, E. Baroja-Fernández, F.J. Muñoz, G. Eydallin, A.M. Viale, J. Pozueta-Romero, Regulation of glycogen metabolism in yeast and bacteria, FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 34 (6) (2010) 952–985, https://doi.org/10.1111/j.1574-6976.2010.00220.x.
[6] M. Aldunate, D. Srbinovski, A.C. Hearps, C.F. Latham, P.A. Ramsland, R. Gugasyan, R.A. Cone, G. Tachedjian, Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis, Front. Physiol. 6 (2015) 164, https://doi.org/10.3389/fphys.2015.00164.
[7] P.L. Carr, D. Felsenstein, R.H. Friedman, Evaluation and management of vaginitis, J. Gen. Intern. Med. 13 (5) (1998) 335–346, https://doi.org/10.1046/j.1525-1497.1998.00101.x.

[8] A.R. Oduyebo Oo, F.T. Ogunsola, The effects of antimicrobial therapy on bacterial vaginosis in non-pregnant women, Cochrane Database Syst. Rev. 3 (2009) CD006055.
[9] P. Bagnall, D. Rizzolo, Bacterial vaginosis: a practical review, Jaapa 30 (12) (2017) 15–21, https://doi.org/10.1097/01.JAA.0000526770.60197.fa.
[10] C.H. Kang, S.H. Han, Y. Kim, N.S. Paek, J.S. So, In vitro probiotic properties of Lactobacillus salivarius MG242 isolated from human vagina, Probiotics Antimicrob Proteins 10 (2) (2018) 343–349, https://doi.org/10.1007/s12602-017-9323-5.
[11] G.G. Donders, K. Ruban, G. Bellen, Selecting anti-microbial treatment of aerobic vaginitis, Curr. Infect. Dis. Rep. 17 (5) (2015) 477, https://doi.org/10.1007/s11908-015-0477-6.
[12] J.D. Sobel, D. Ferris, J. Schwebke, P. Nyirjesy, H.C. Wiesenfeld, J. Peipert, D. Soper, S.E. Ohmit, S.L. Hillier, Suppressive antibacterial therapy with 0.75% metronidazole vaginal gel to prevent recurrent bacterial vaginosis, Am. J. Obstet. Gynecol. 194 (5) (2006) 1283–1289, https://doi.org/10.1016/j.ajog.2005.11.041.
[13] B. Haghshenas, A. Kiani, S. Mansoori, E. Mohammadi-Noori, Y. Nami, Probiotic properties and antimicrobial evaluation of silymarin-enriched Lactobacillus bacteria isolated from traditional curd, Sci. Rep. 13 (1) (2023) 10916, https://doi.org/10.1038/s41598-023-37350-3.
[14] B. Haghshenas, Y. Nami, A. Kiani, N. Moazami, O. Tavallaei, Cytotoxic effect of potential probiotic Lactiplantibacillus plantarum KUMS-Y8 isolated from traditional dairy samples on the KB and OSCC human cancer cell lines, Heliyon 9 (9) (2023) e20147, https://doi.org/10.1016/j.heliyon.2023.e20147.
[15] A. Badgeley, H. Anwar, K. Modi, P. Murphy, A. Lakshmikuttyamma, Effect of probiotics and gut microbiota on anti-cancer drugs: mechanistic perspectives, Biochim. Biophys. Acta Rev. Canc 1875 (1) (2021) 188494, https://doi.org/10.1016/j.bbcan.2020.188494.
[16] Z. Qian, D. Zhao, Y. Yin, H. Zhu, D. Chen, Antibacterial activity of Lactobacillus strains isolated from Mongolian yogurt against Gardnerella vaginalis, BioMed Res. Int. 2020 (2020) 3548618, https://doi.org/10.1155/2020/3548618.
[17] B. Haghshenas, A. Kiani, Y. Nami, Probiotic potential and safety evaluation of lactic acid bacteria isolated from colostrum, Journal of Biosafety 14 (3) (2021) 37–60.
[18] J. He, Y. Liu, F. Li, H. Wu, H. Yang, Complete genome sequence of Lactobacillus nenjiangensis SH-Y15, isolated from sauerkraut, Microbiol Resour Announc 9 (23) (2020), https://doi.org/10.1128/mra.01473-19.
[19] A. Kiani, Y. Nami, S. Hedayati, D. Elieh Ali Komi, F. Goudarzi, B. Haghshenas, Application of tarkhineh fermented product to produce potato chips with strong probiotic properties, high shelf-life, and desirable sensory characteristics, Front. Microbiol. 12 (2021) 657579, https://doi.org/10.3389/fmicb.2021.657579.
[20] Y. Nami, B. Haghshenas, A. Javanmard, M. Samari, N. Mohammadi, F. Oroojalian, A. Mokhtarzadeh, A critical review of the recent concept of artificial mechanical uterus design in relation to the maternal microbiome: an Update to past researches, J. Reprod. Immunol. 156 (2023) 103828, https://doi.org/10.1016/j.jri.2023.103828.
[21] R. Sengupta, E. Altermann, R.C. Anderson, W.C. McNabb, P.J. Moughan, N.C. Roy, The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract, Mediators Inflamm 2013 (2013) 237921, https://doi.org/10.1155/2013/237921.
[22] H.N. Youn, D.H. Lee, Y.N. Lee, et al., Intranasal administration of live Lactobacillus species facilitates protection against influenza virus infection in mice, Antiviral Res 93 (1) (2012) 138–143, https://doi.org/10.1016/j.antiviral.2011.11.004.
[23] M. Kahieshesfandiari, Y. Nami, G. Lornezhad, A. Kiani, A. Javanmard, M. Jaymand, B. Haghshenas, Herbal hydrogel-based encapsulated Enterococcus faecium ABRIINW.N7 improves the resistance of red hybrid tilapia against Streptococcus iniae, J. Appl. Microbiol. 131 (5) (2021) 2516–2527, https://doi.org/10.1111/jam.15098.
[24] Y. Nami, M. Kahieshesfandiari, G. Lornezhad, A. Kiani, D. Elieh-Ali-Komi, M. Jafari, M. Jaymand, B. Haghshenas, Administration of microencapsulated Enterococcus faecium ABRIINW.N7 with fructo-oligosaccharides and fenugreek on the mortality of tilapia challenged with Streptococcus agalactiae, Front. Vet. Sci. 9 (2022) 938380, https://doi.org/10.3389/fvets.2022.938380.
[25] Y. Nami, A. Kiani, D. Elieh-Ali-Komi, M. Jafari, B. Haghshenas, Impacts of alginate–basil seed mucilage–prebiotic microencapsulation on the survival rate of the potential probiotic Leuconostoc mesenteroides ABRIINW.N18 in yogurt, Int. J. Dairy Technol. 76 (1) (2023) 138–148, https://doi.org/10.1111/1471-0307.12909.
[26] A. Kiani, Y. Nami, S. Hedayati, M. Jaymand, H. Samadian, B. Haghshenas, Tarkhineh as a new microencapsulation matrix improves the quality and sensory characteristics of probiotic Lactococcus lactis KUMS-T18 enriched potato chips, Sci. Rep. 11 (1) (2021) 12599, https://doi.org/10.1038/s41598-021-92095-1.
[27] Y. Zou, W. Xue, G. Luo, et al., 1,520 reference genomes from cultivated human gut bacteria enable functional microbiome analyses, Nat. Biotechnol. 37 (2) (2019) 179–185, https://doi.org/10.1038/s41587-018-0008-8.
[28] B. Liu, D. Zheng, Q. Jin, L. Chen, J. Yang, Vfdb 2019: a comparative pathogenomic platform with an interactive web interface, Nucleic Acids Res. 47 (D1) (2019) D687–d692, https://doi.org/10.1093/nar/gky1080.
[29] B.P. Alcock, A.R. Raphenya, T.T.Y. Lau, et al., Card 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database, Nucleic Acids Res. 48 (D1) (2020) D517–D525, https://doi.org/10.1093/nar/gkz935.
[30] M. Kanehisa, S. Goto, KEGG: kyoto encyclopedia of genes and genomes, Nucleic Acids Res. 28 (1) (2000) 27–30, https://doi.org/10.1093/nar/28.1.27.
[31] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2.
[32] M.S.J. Tomás, E. Bru, M.E. Nader-Macías, Comparison of the growth and hydrogen peroxide production by vaginal probiotic lactobacilli under different culture conditions, Am. J. Obstet. Gynecol. 188 (1) (2003) 35–44, https://doi.org/10.1067/mob.2003.123.
[33] H.Y. Xu, W.H. Tian, C.X. Wan, L.J. Jia, L.Y. Wang, J. Yuan, C.M. Liu, M. Zeng, H. Wei, Antagonistic potential against pathogenic microorganisms and hydrogen peroxide production of indigenous lactobacilli isolated from vagina of Chinese pregnant women, Biomed. Environ. Sci. 21 (5) (2008) 365–371, https://doi.org/10.1016/s0895-3988(08)60056-2.
[34] K. Angmo, A. Kumari, Savitri, T.C. Bhalla, Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh, LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 66 (2016) 428–435, https://doi.org/10.1016/j.lwt.2015.10.057.
[35] E. De Backer, R. Verhelst, H. Verstraelen, M.A. Alqumber, J.P. Burton, J.R. Tagg, M. Temmerman, M. Vaneechoutte, Quantitative determination by real-time PCR of four vaginal Lactobacillus species, Gardnerella vaginalis and Atopobium vaginae indicates an inverse relationship between L. gasseri and L. iners, BMC Microbiol. 7 (2007) 115, https://doi.org/10.1186/1471-2180-7-115.
[36] E. Zankari, H. Hasman, S. Cosentino, M. Vestergaard, S. Rasmussen, O. Lund, F.M. Aarestrup, M.V. Larsen, Identification of acquired antimicrobial resistance genes, J. Antimicrob. Chemother. 67 (11) (2012) 2640–2644, https://doi.org/10.1093/jac/dks261.
[37] Y. Zhou, Y. Liang, K.H. Lynch, J.J. Dennis, D.S. Wishart, PHAST: a fast phage search tool, Nucleic Acids Res. 39 (suppl_2) (2011) W347–W352, https://doi.org/10.1093/nar/gkr485.
[38] C.M. Hudson, B.Y. Lau, K.P. Williams, Islander: a database of precisely mapped genomic islands in tRNA and tmRNA genes, Nucleic Acids Res. 43 (Database issue) (2015) D48–D53, https://doi.org/10.1093/nar/gku1072.
[39] M. Liu, X. Li, Y. Xie, D. Bi, J. Sun, J. Li, C. Tai, Z. Deng, H.-Y. Ou, ICEberg 2.0: an updated database of bacterial integrative and conjugative elements, Nucleic Acids Res. 47 (D1) (2018) D660–D665, https://doi.org/10.1093/nar/gky1123.
[40] P. Siguier, J. Perochon, L. Lestrade, J. Mahillon, M. Chandler, ISfinder: the reference centre for bacterial insertion sequences, Nucleic acids research 34 (2006) D32–D36, https://doi.org/10.1093/nar/gkj014.
[41] G. Reid, R.L. Cook, A.W. Bruce, Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract, J. Urol. 138 (2) (1987) 330–335, https://doi.org/10.1016/s0022-5347(17)43137-5.
[42] R.C. Martinez, S.A. Franceschini, M.C. Patta, S.M. Quintana, B.C. Gomes, E.C. De Martinis, G. Reid, Improved cure of bacterial vaginosis with single dose of tinidazole (2 g), Lactobacillus rhamnosus GR-1, and Lactobacillus reuteri RC-14: a randomized, double-blind, placebo-controlled trial, Can. J. Microbiol. 55 (2) (2009) 133–138, https://doi.org/10.1139/w08-102.
[43] M. Ho, Y.Y. Chang, W.C. Chang, H.C. Lin, M.H. Wang, W.C. Lin, T.H. Chiu, Oral Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 to reduce Group B Streptococcus colonization in pregnant women: a randomized controlled trial, Taiwan. J. Obstet. Gynecol. 55 (4) (2016) 515–518, https://doi.org/10.1016/j.tjog.2016.06.003.

[44] H. Verstraelen, C. Vervaet, J.P. Remon, Rationale and safety assessment of a novel intravaginal drug-delivery system with sustained DL-lactic acid release, intended for long-term protection of the vaginal microbiome, PLoS One 11 (4) (2016) e0153441, https://doi.org/10.1371/journal.pone.0153441.
[45] F. Atassi, D. Brassart, P. Grob, F. Graf, A.L. Servin, Lactobacillus strains isolated from the vaginal microbiota of healthy women inhibit Prevotella bivia and Gardnerella vaginalis in coculture and cell culture, FEMS Immunol. Med. Microbiol. 48 (3) (2006) 424–432, https://doi.org/10.1111/j.1574-695X.2006.00162.x.
[46] E.V. Valore, C.H. Park, S.L. Igreti, T. Ganz, Antimicrobial components of vaginal fluid, Am. J. Obstet. Gynecol. 187 (3) (2002) 561–568, https://doi.org/10.1067/mob.2002.125280.
[47] P.R. De Gregorio, J.A. Silva, A. Marchesi, M.E.F. Nader-Macias, Anti-Candida activity of beneficial vaginal lactobacilli in in vitro assays and in a murine experimental model, FEMS Yeast Res. 19 (2) (2019), https://doi.org/10.1093/femsyr/foz008.
[48] A.C. Vallor, M.A. A, S.E. Hawes, S.L. Hillier, Factors associated with acquisition of, or persistent colonization by, vaginal lactobacilli: role of hydrogen peroxide production, J. Infect. Dis. 184 (11) (2001) 1431–1436.
[49] S. Borges, J. Silva, P. Teixeira, The role of lactobacilli and probiotics in maintaining vaginal health, Arch. Gynecol. Obstet. 289 (3) (2014) 479–489, https://doi.org/10.1007/s00404-013-3064-9.
[50] D. De Alberti, R. Russo, F. Terruzzi, V. Nobile, A.C. Ouwehand, Lactobacilli vaginal colonisation after oral consumption of Respecta((R)) complex: a randomised controlled pilot study, Arch. Gynecol. Obstet. 292 (4) (2015) 861–867, https://doi.org/10.1007/s00404-015-3711-4.
[51] R. Russo, A. Edu, F. De Seta, Study on the effects of an oral lactobacilli and lactoferrin complex in women with intermediate vaginal microbiota, Arch. Gynecol. Obstet. 298 (1) (2018) 139–145, https://doi.org/10.1007/s00404-018-4771-z.
[52] S.D. Mitra, R. Shome, S. Bandopadhyay, S. Geddam, A.M.P. Kumar, D. Murugesan, A. Shome, B.R. Shome, Genetic insights of antibiotic resistance, pathogenicity (virulence) and phylogenetic relationship of Escherichia coli strains isolated from livestock, poultry and their handlers - a one health snapshot, Mol. Biol. Rep. 51 (1) (2024) 404, https://doi.org/10.1007/s11033-024-09354-3.
[53] N. Tian, L. Li, J.K. Ng, P.K. Li, The potential benefits and controversies of probiotics use in patients at different stages of chronic kidney disease, Nutrients 14 (19) (2022), https://doi.org/10.3390/nu14194044.
[54] K. Anukam, E. Osazuwa, I. Ahonkhai, M. Ngwu, G. Osemene, A.W. Bruce, G. Reid, Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14: randomized, double-blind, placebo controlled trial, Microbes Infect 8 (6) (2006) 1450–1454, https://doi.org/10.1016/j.micinf.2006.01.003.
[55] N. Recine, E. Palma, L. Domenici, et al., Restoring vaginal microbiota: biological control of bacterial vaginosis. A prospective case-control study using Lactobacillus rhamnosus BMX 54 as adjuvant treatment against bacterial vaginosis, Arch. Gynecol. Obstet. 293 (1) (2016) 101–107, https://doi.org/10.1007/s00404-015-3810-2.