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RESEARCH

Probiotic Strain *Limosilactobacillus reuteri* 29B is Proven Safe and Exhibits Potential Probiotic Traits in a Murine Vaginal Model

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Abstract

Lactobacilli, the most common group of bacteria found in a healthy vaginal microbiota, have been demonstrated to act as a defence against colonisation and overgrowth of vaginal pathogens. These groups of bacteria have sparked interests in incorporating them as probiotics aimed at re-establishing balance within the urogenital ecosystem. In this study, the safety characteristics of *Limosilactobacillus reuteri* 29B (L29B) strain were evaluated through whole genome sequencing (WGS) and animal study. Cell culture assay and 16S rDNA analysis were done to evaluate the ability of the strain to colonise and adhere to the mouse vaginal tract, and RAST analysis was performed to screen for potential genes associated with probiotic trait. The histological study on the mice organs and blood analysis of the mice showed there was no incidence of inflammation. We also found no evidence of bacterial translocation. The cell culture assay on HeLa cells showed 85% of adhesion, and there was a significant reduction of *Candida* strain viability in displacement assay. As for the 16S rDNA analysis, there was a significant amount of L29B colonisation of the vaginal microflora. Taken together, the intravaginal administration of L29B significantly reduced the number Enterobacteriaceae and Staphylococcaceae that were present in mouse vaginal tract. It also improved and promoted a balanced vaginal microflora environment without causing any harm or irritation to mice. *Limosilactobacillus* 29B (L29B) is safe to be administered intravaginally.

Keywords Probiotic · Safety · Colonisation · Adherence · Intravaginal

Introduction

Probiotics are known as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [1]. To consider a live microorganism as probiotic, there must be sufficient scientific evidences of safety, survivability, and efficacy, which distinguishes them from other live microorganisms with probiotic potential [1]. The human vaginal environment is in dynamic balance with a broad range of microbiota, which is primarily made up of lactic acid bacteria (LAB) [2]. A decrease in the protective LAB is the result of an imbalance in vaginal environment, making the host susceptible to vaginal infections [2, 3]. Many studies also have shown a correlation between loss

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and disruption of the normal vaginal microflora, specifically LAB and increased occurrence of vaginal infections [4].

Lactobacilli confer beneficial effects on vaginal health through their ability to colonise mucous membranes and compete with other microorganisms to adhere on vaginal epithelium, production of antimicrobial compounds (e.g., hydrogen peroxide, organic acids, and bacteriocin-like substances), and/or modulation of the host immune response against causative agents of vaginal diseases [5, 6]. Numerous in vitro studies demonstrated that vaginal LAB protect the vaginal environment against pathogenic microorganisms through several mechanisms that include colonising and production of antimicrobial substances [5, 7, 8]. Hereof, probiotics provide a great health benefit to women and are often promoted as an alternative choice of treatment and prevention in the management of vaginally acquired infections [9].

Probiotics are generally considered safe, with minimal adverse effects. Most species of the *Limosilactobacillus* genus are "generally regarded as safe" (GRAS) [10]. Lactobacilli have been successfully administered to a wide range of population, including newborns, immunocompromised individuals, and critically ill patients, with no apparent adverse effects [11–13]. It is not easy to assess the safety of probiotics. Even though most probiotics effects are positive, there are a few concerns to consider before incorporating them into food or medicinal applications. However, factors that can be determined in vitro are relatively easy to assess [14]. To date, there are few reports that linked lactobacilli to bacteraemia and endocarditis in patients with severe illnesses such as short gut syndrome, heart valve transplantation, and severe ulcerative colitis [15–18]. Other factors, such as pre-existing underlying disorders or strain- or species-specific differences, are thought to be the cause of these uncommon occurrences [19].

Lact. 29B was previously isolated from a healthy premenopausal woman from the Obstetrics and Gynaecology Department, Hospital Serdang, Selangor, Malaysia [20]. Previously, we demonstrated that L29B shows potential probiotic qualities such as the ability to produce potent antimicrobial activity against many pathogenic microorganisms and the ability to withstand and grow at pH as low as 3.5. Moreover, this strain also displayed in vitro properties relevant to colonisation, i.e., high hydrophobicity, strong self-aggregation and coaggregation with pathogenic microorganisms. In vitro safety assessment was also done where the strain produced low level of D-lactic acid. Since the human body lacks the ability to effectively metabolise D-lactic acid isomer due to absence of the D-lactate dehydrogenase enzyme [21, 22]; thus, it will cause D-lactate poisoning [23]. The strain also did not exhibit α- or β-haemolytic activities [20, 22].

In comparison to antibiotic therapy, probiotic therapy is considered a natural approach of inhibiting pathogen growth in a non-invasive manner that is free of unwanted side effects and is preventive in nature. Disruption of these beneficial bacteria would lead to overgrowth of pathogenic bacteria; therefore, re-establishing the normal flora colonisation with the supplementation of probiotics would counter the pathogenesis and prevent the development of infections. It can be used as a stand-alone treatment or as a supplement. Hence, in this present study, genomic analyses and murine model were employed to evaluate the safety characteristics of L29B for intravaginal administration, as well as its ability to colonise and induce restoration of the vaginal microflora in mouse vaginal tract.

Materials and Methods

Isolation of Genomic DNA for WGS

Genomic DNA of L29B was extracted according to the manufacturer's protocol using the Wizard® Genomic DNA Purification Kit (Promega, USA). The DNA concentration and purity were measured; then it was stored in − 20 °C until further usage.

Genome Sequencing, Assembly, and Annotation

Using the Illumina Novaseq platform, the WGS de novo sequencing was carried out for the safety analysis of L29B. Two hundred micrograms of genomic DNA was randomly fragmented by Covaris (Covaris, Wolburn, MA) to an average size of 300–350 bp. End Prep Enzyme Mix was used to repair the ends of the fragments, and adapters were added to both ends by 5′ phosphorylation and 3′ adenylation. DNA Clean-up beads were used to select the adaptor-ligated DNA based on size. Using P5 (AGATCGGAAGAGCGTCGT GTAGGGAAAGAGTGT) and P7 (AGATCGGAAGAG CACACGTCTGAACTCCAGTCAC) primers, each sample was amplified by PCR for 8 cycles, which both contain sequences that can anneal with flow cells to perform bridge PCR. The P7 primer also has a six-base index that enables multiplexing. The PCR products were cleaned up and validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

Identification of L29B Genes for Safety Assessment and Potential Probiotic Trait

The NCBI Prokaryotic Genome Annotation Pipeline (PGAP) and Rapid Annotations Using Subsystems Technology (RAST) were used to generate functional annotation, which was used to identify putative coding sequences for significant probiotic genes. The Comprehensive Antibiotic Resistance Database (CARD) was used to quickly

identify protein-encoding sequences that may represent antimicrobial resistance genes acquired within the draft genome. The BLASTn method was used to identify potential virulence factors using the virulence factor database (VFDB). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated genes (Cas) were detected using CRISPRCasFinder. Through the WGS annotations and RAST method, additional associated genes to probiotic traits including adhesion, antitoxin, and antibacterial activity of L29B were manually screened.

Animal and Housing

The Animal Resource Unit, Faculty of Veterinary Medicine, Universiti Putra Malaysia, (UPM) provided twenty (20) female BALB/c mice, aged 6 to 8 weeks, weighing between 20 and 22 g, which were housed in metal wire-topped plastic cages under standard conditions (temperature of 22 ± 2 °C, humidity of $55 \pm 2\%$, and 12-h light/dark cycle). Throughout the trial, the mice had unlimited access to water and a standard rodent feed (Specialty Feeds, Glen Forest, WA, Australia). Laboratory animals used in this study were cared for in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (Council, 2010). The experiment protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) (approval reference: UPM/IACUC/AUP–R030/2020).

Preparation of L29B for the Intravaginal Administration

For the preparation of the mouse vaginal inoculum sample, the L29B strain culture was prepared by incubating it for 48 h in MRS broth at 37 °C anaerobically. The L29B cells were harvested after 48 h of incubation by centrifugation at $9000 \times g$ for 10 min. They were then washed twice with phosphate-buffered saline (PBS, pH 7.4). The pellet was resuspended in fresh PBS and adjusted to an approximate concentration of 1×10^8 number of cells (OD600nm). Daily preparation of the inoculum was done.

Experimental Design

The mice were randomly divided into two experimental groups ($n = 10$ per group, the probiotic and control groups). All the mice had their vaginal lavage collected before the experiment. Using a pipette, a single dosage of 20 µL of L29B (1×10^8 cells) was administered intravaginally to the mice in the probiotic group for seven days in a row. On the contrary, the mice from the control group were administered only with equal volume of PBS for seven days in a row. Mice behaviour, activity, pain threshold, and general health were all monitored and recorded once every two days. Body weight (BW) of mice were recorded three days once throughout the experiment. All the mice were humanely euthanised at the end of the study (day 8) by administering xylazine (10 mg/kg) and ketamine (80 mg/kg) intraperitoneally. For haematological and blood biochemistry investigation, blood samples were taken via cardiac puncture. For the Gram staining preparation and DNA extraction for 16S rDNA, mouse vaginal lavage was collected. For histological examination, the liver, kidney, and vagina of the mice were carefully removed under sterile conditions and transferred to 10% neutral buffered formalin.

Haematology and Blood Biochemistry

An automated haematology analyser (CELL-DYN® 3700 system, Abbott Laboratories, Abbott Park, Illinois) was used to measure hematologic parameters, and for the clinical biochemical parameters measurements, an automated biochemical analyser (BioLis 24i premium Boeki machinery, Tokyo, Japan) was used.

Histological Examination

According to standard staining procedures, the liver, kidney, and vagina of the mice were paraffin-embedded, sectioned at a thickness of 4 µm, and stained with haematoxylin and eosin (H&E) stain. An automated Leica TP 1020 tissue processor (Leica, USA) was used to process the tissues. DinoCapture 2.0 Microscope Imaging Software was used to perform histological measurements after microscopically examining histological samples under the Leica DM 2500 microscope.

Bacterial Translocation

The blood, liver, and kidney of the mice were examined for signs of bacterial translocation. Fifty microliters of the blood was cultured onto the MRS agar and incubated at 37 °C for 48 h under anaerobic condition. Following homogenising tissue samples in PBS (1 g/mL), 100 µL of the homogenates were cultured onto MRS agar. Colonies were observed and counted after incubation. The results were expressed as the incidence of translocation (the number of mice where translocation was detected/total number of mice).

Vaginal Smear Preparation

Vaginal lavage from mice was taken twice: once before the inoculation and once on the eighth day following the inoculation. The mice were held by the base of the tail with two fingers after anaesthesia in order to expose the vaginal opening. A micropipette was used to repeatedly aspirate

the lavage following administration of 100µL of PBS into the vaginal lumen. This process was done gently and meticulously to avoid any discomfort or injury to the mice. Ten microlitres of the vaginal lavage were placed on a glass slide and fixed for Gram staining as part of the preparation for the vaginal smear. The prepared slides were observed at 200 × and 400 × magnification by light microscopy (Optical microscopes Leica DM2500, Mannheim, Germany). The remaining vaginal lavage was kept in − 20 °C for further analysis for 16S rDNA sequencing.

16S rDNA Library Preparation and Illumina Miseq Sequencing

Genomic DNA of mouse vaginal lavage was extracted according to the manufacturer's protocol using the DNeasy® Blood and Tissue Kit (Qiagen, USA). Using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), the DNA sample concentration was determined, and the DNA quality was checked on an agarose gel with a 0.8% (w/v) concentration. A total amount of 30–50 ng DNA was used to generate amplicons using MetaVx™ Library Preparation Kit (Genewiz, Inc., South Plainfield, NJ, USA). V3 and V4 hypervariable regions of prokaryotic 16S rDNA were selected for generating amplicons and following taxonomy analysis. The V3 and V4 hypervariable regions of bacteria are bordered by a panel of proprietary primers that GENEWIZ created specifically for these regions. The V3 and V4 regions were amplified using forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGA CTACNVGGGTWTCTAATCC." Products from the first round of PCR were utilised as templates for the second round of amplicon enrichment PCR. In parallel, indexed adapters were attached to the ends of 16S rDNA amplicons to generate indexed libraries prepared for a later round of NGS sequencing on an Illumina Miseq.

Adhesion Assay

The adhesion ability of L29B to epithelial cells was evaluated as previously described with slight modifications [24]. HeLa cells were grown in 25-cm^2 tissue culture flasks with Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS), 1% (v/v) L-glutamine, and 1% (v/v) antibiotic (penicillin/streptomycin) in 5% carbon dioxide (CO_2) at 37 °C. Trypsin was used to detached the HeLa cells, and the cell number was determined by cell counting using a haemocytometer and Trypan blue.

In a 12-well tissue culture plate, the cells were seeded at a density of 1×10^3 and grown to 80% confluence over the course of overnight. After washing the cell monolayer with PBS, 1 mL of L29B in DMEM (1×10^8 cells) was added into the well with HeLa cells and incubated for 4 h at 37 °C. By washing with PBS three times, the non-attached bacteria were removed. The CFU plate counting method was used to count the number of L29B colonies present on the petri dish, and then the average L29B adherence percentages to HeLa cells were calculated. The percentage of adhesion was expressed as the percentage of the L29B adhered divided by the total of L29B added.

Percentage of adhesion (%) = [total L29B adhered/total L29B added] × 100%

Exclusion Assay

Exclusion assay was carried out to evaluate the ability of L29B in preventing adhesion of vaginal fungal pathogens, i.e., *Candida* isolates, *C. albicans* 81, and *C. glabrata* 95670, to HeLa cells. Briefly, HeLa cells (1×10^3) were incubated with L29B (1×10^8 cells) at 37 °C in 5% CO_2 for 1 h. The non-adhered L29B was removed by washing with PBS. Subsequently, *C. albicans* 81 (1×10^6 cells) [25] was added and incubated for another 1 h. Extensive washing was done with PBS to remove the non-adhered cells (L29B and *Candida* cells). HeLa cells were detached by trypsinisation, and the *Candida* cell number was determined by CFU plate counting. The assay was carried out in triplicates with three biological replicates. Similar steps were performed on *C. glabrata* 95670.

Displacement Assay

This test demonstrates the ability of L29B to remove pathogens that have adhered to HeLa cells. The sequence of inoculation was reversed compared to exclusion assay. For this assay, *C. albicans* 81 was added to the well with HeLa cells (1×10^3) and incubated at 37 °C for 1 h. Non-adhered *Candida* cells were removed by washing with PBS. L29B was added to the HeLa cells with pre-adhered *Candida* cells and incubated for another 1 h at 37 °C. Non-adhered L29B was washed, and the HeLa cells, adhered *Candida*, and L29B were detached with trypsin. The *Candida* cell number was determined through colony counting. The assay was carried out in triplicates with three biological replicates. Like above, the steps were performed on *C. glabrata* 95670.

Competition Assay

For the competition assay, 1 mL of L29B suspension (1×10^8) and *C. albicans* 81 (1×10^6) was inoculated to HeLa cells (1×10^3) simultaneously and incubated for 2 h at 37 °C with 5% CO_2. *C. albicans* 81 and L29B that were not adhered were washed with PBS three times, and trypsin was used to detach them. Colony counting was used to evaluate the competitiveness between the *C. albicans* 81 strain and

the L29B strain. The assay was carried out in triplicates with three biological replicates. Like the above exclusion and displacements assays, the steps were also performed on *C. glabrata* 95670.

Statistical Analyses

GraphPad Prism 8 was used for the statistical analysis (GraphPad Software, San Diego, CA, USA). Tukey's test and Student's *t*-test results showed that there were significant differences between mean values. All experiments were done in triplicate. Data were expressed as mean ± SD. A p value < 0.05 was considered statistically significant. For the analysis of 16S rDNA data, the QIIME (Quantitative Insights into Microbial Ecology) data analysis package and the R programming language were applied. The forward and reverse reads were combined, and the samples were assigned based on the barcode. Following quality screening on joined sequences, sequences that did not meet the following requirements were removed: sequence length < 200 bp, no ambiguous bases, and mean quality score ≥ 20. The UCHIME algorithm was used to compare the sequences to the reference database [Ribosomal Database Project (RDP) Gold database, 2.2] in order to find any chimeric sequences, which were subsequently eliminated.

Results

Safety Assessment of L29B Strain

The draft genome of L29B consists of 31 contigs (2,150,467 bp) with a G + C content of 45.72% and N50 values of 175,873 bp and L50 value is 4. Genome annotation indicated the presence of 2050 protein coding sequences (CDS). Of the 2094 predicted genes, 1991 (95.08%) were protein-coding genes and 103 (4.92%) were RNAs (63 transfer RNA genes and 9 ribosomal RNA genes and 31 transfer-messenger RNA genes). The properties and statistics of the genome are summarised in Table 1. Figure 1 shows the genome map generated in a CGViewer server.

The virulence factor database (VFDB) was used to identify known virulence factors and toxin genes that may exist in the L29B strain genome. After screening, one virulence gene was detected by the database which is the tufA gene (elongation factor Tu) with the coverage of 91% and identity of 74%. This gene is responsible in mediating the attachment of *Lactobacillus* to human intestinal cells and mucin [26, 27]. This gene can be considered in providing benefits to the bacteria itself. This was further confirmed by using the VirulenceFinder tool hosted by the Centre for Genomic Epidemiology (https://bio.tools/virulencefinder). There was no hit for virulence determinants detected.

Table 1 Nucleotide content and gene count levels of the genome of L29B strain

Feature	Value	% of total
Size (bp)	2,150,467	100
G + C content (bp)	983,100	45.72
Coding region (bp)	1,915,467	89.07
Total genes	2094	100
RNA genes	103	4.92
Protein-coding genes	1991	95.08
Protein coding genes with enzymes	777	37.11
Genes assigned to COGs	1619	77.32
COG clusters	1101	68
Genes with signal peptides	66	3.15
Genes with transmembrane helices	503	24.02

Comprehensive Antibiotic Resistance Database (CARD) for acquired antimicrobial resistance encoding genes was also done, and it revealed that L29B is resistant to the top 10 classes of antibiotics which are macrolide, peptide, fluoroquinolone, glycopeptide, lincosamide, penam, triclosan, carbapenem, aminoglycoside, and tetracycline as shown in Fig. 2.

In the genomes of L29B, genes related to the CRISPR-Cas type I-B system were discovered. It is believed that CRISPR-Cas system works as adaptive immunity in bacterial cells to fend off external infections like bacteriophages. In addition, CRISPR-Cas systems are involved in the regulation of gene activity, DNA repair, genome reorganisation, and the translation of a bacterial cell into an inactive (anabiosis) state [28]. In the genomes of L29B, six blocks of CRISPR arrays were detected as shown in Table 2. The recognition of foreign genetic material relies greatly on the nucleotide sequences of the CRISPR spacers. Upon contacting marker sites, they contribute to the formation of CRISPR-Cas complexes and subsequent degradation of foreign genetic material. The gene that was detected in the *Cas* system was *cas3* (evidence level 1) (Table 2). In this regard, when using the CRISPRCasFinder, programme sequences with evidence level below 3 should be disregarded, as they indicate potentially invalid CRISPR arrays.

L29B strain also was predicted to be a non-human pathogen by the Pathogen-Finder tool hosted by the Centre for Genomic Epidemiology (https://bio.tools/pathogenfinder). The probability of being a human pathogen was calculated as 0.061, indicating a low probability for L29B to present pathogenicity, and the estimated matched pathogenic families were 0. One prophage region was identified within the entire genome, and analysis using the PHASTER tool (https://phaster.ca/) revealed that the sequences were incomplete. After running an analysis using the PlasmidFinder web-tool (https://bio.tools/PlasmidFinder),

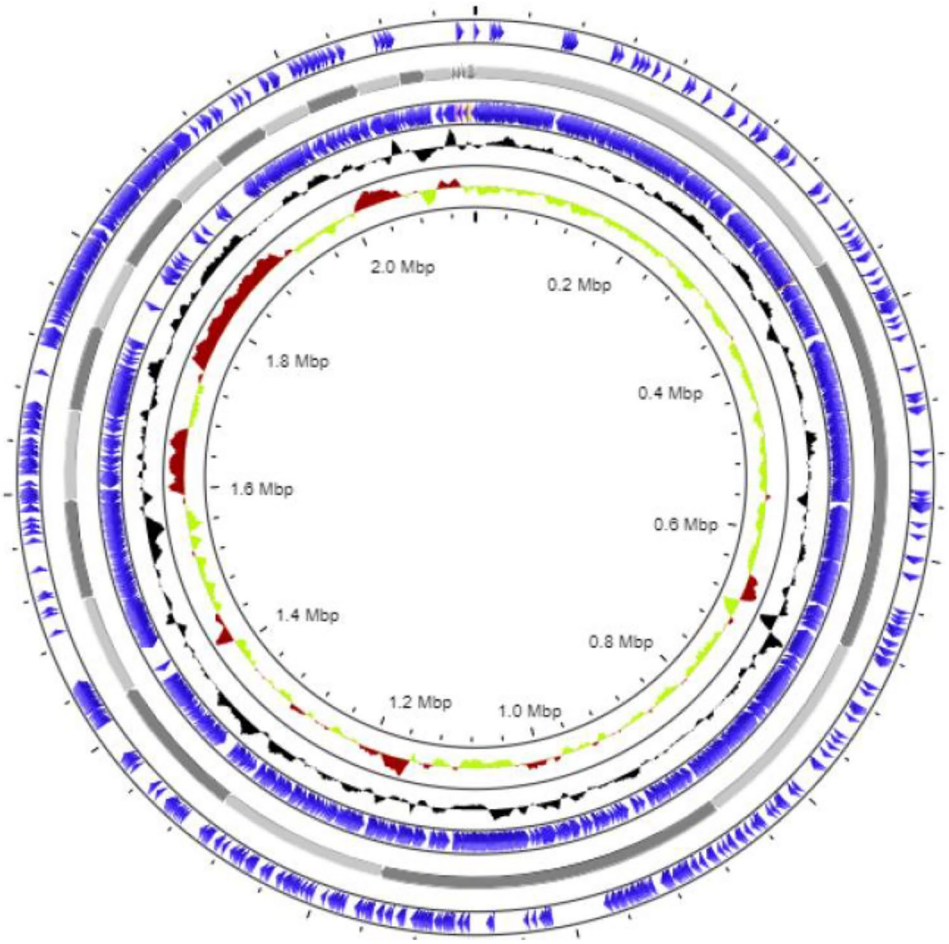


Fig. 1 L29B genome map generated using the CGView Server (https://js.cgview.ca/), showing a full view of the genome. The blue arrows represent the CDSs, and the grey arrows are the contigs. The black plot shows the GC content, whilst the red and yellow plots show CG skew + and −, respectively

no plasmids were found in the L29B draft genome. This result is to some extent a positive probiotic characteristic, as plasmids can often carry antimicrobial resistance and virulence factor genes. One of the most important characteristics for a bacterial strain to be proposed as a safe microorganism and, eventually as a probiotic, is the absence of transmissible antibiotic resistances, as they pose a threat to both animal and human health. Although LAB are generally classified with GRAS and/or QPS status, it is of utmost importance to screen all potential LAB probiotic candidates for transferable antimicrobial resistances, as they can still act as reservoirs for antimicrobial resistance genes.

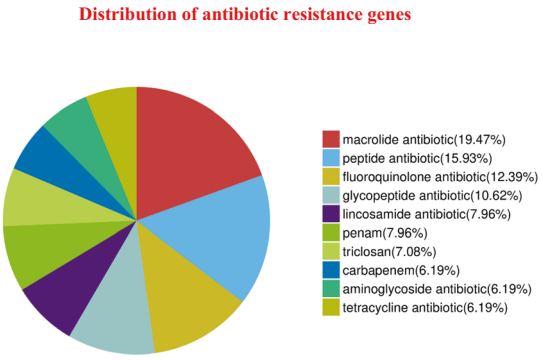


Fig. 2 CARD resistance gene distribution map. The different colours of the pie chart represent different antibiotic resistant organism (ARO) categories, and the area size indicates the relative proportion of genes in the category

Table 2 Genes of L29B that encode CRISPR-Cas system

Gene	Contig	Range in contig	Gene translation product
CRISPR 1	8	14,897–15,171	First CRISPR array
CRISPR 2	8	27,793–28,144	Second CRISPR array
CRISPR 3	8	35,859–35,945	Third CRISPR array
CRISPR 4	8	35,874–36,154	Fourth CRISPR array
CRISPR 5	12	32,688–32,890	Fifth CRISPR array
CRISPR 6	19	13,397–13,764	Sixth CRISPR array
Cas3	8	67,178–68,479	Type I–B CRISPR-associated helicase/cas3 endonuclease

Table 3 Body and organ weight of mice between the control and probiotic groups

Groups (CFU/mouse/day)		
	Control (PBS only)	**Probiotic (1×10^8)**
Initial weight (g)	22.8 ± 1.32	23.4 ± 1.62
Final weight (g)	23.7 ± 1.42	23.5 ± 1.5
Liver (g)	1.106 ± 0.004	1.105 ± 0.003
Kidney (g)	0.258 ± 0.04	0.257 ± 0.03

Table 4 Haematological and blood biochemistry parameters of mice between the control and probiotic groups

Groups (CFU/mouse/day)	Control (PBS only)	Probiotic (1×10^8)
Parameters		
Haematology		
RBC (10^{12}/L)	7.686 ± 0.92	7.698 ± 1.41
Hb (g/L)	117 ± 14.4	117.9 ± 22.56
MCV (fL)	47.29 ± 1.86	47.05 ± 1.40
MCHC (g/L)	322.29 ± 13.57	325.01 ± 9.27
WBC (10^9/L)	1.95 ± 0.72	2.19 ± 1.477
PLT (10^9/L)	201.9 ± 180.5	248 ± 209.7
PP (g/L)	50.8 ± 2.22	50.8 ± 1.32
Blood biochemistry		
ALT (U/L)	180.06 ± 146.4	62.02 ± 35.16
AST (U/L)	254.11 ± 173.06	139.38 ± 63.34*
TP (g/L)	57.16 ± 1.38	60.73 ± 1.7
ALB (g/L)	30.09 ± 0.83	30.97 ± 0.74
GLUC (mmol/L)	13.62 ± 3.82	11.71 ± 2.0
CHOL (mmol/L)	2.416 ± 0.08	2.446 ± 0.07

Data are presented as means ± SD of measurements per group ($n = 10$) for each parameter

RBC red blood cells, *Hb* haemoglobin, *MCV* mean cell volume, *MCHC* mean corpuscular haemoglobin concentration, *WBC* white blood cells, *PLT* platelet, *PP* plasma proteins, *ALT* alanine transaminase, *AST* aspartate aminotransferase, *TP* total protein, *ALB* albumin, *GLUC* glucose, *CHOL* cholesterol

Thereafter, the analysis of safety via WGS, the investigation was performed in a murine model where it was shown that there was no significant difference in body and organ weights of BALB/c mice between the control and probiotic groups (Table 3). There was also no evidence of inflammation or injury to the visceral organs upon macroscopic inspection. At the time of necropsy, there were no discernible variations in the size and appearance of the organs. Furthermore, no significant changes were found in haematological and blood biochemical parameters between the control and probiotic groups, respectively, except for aspartate aminotransferase (AST), but its level continues to stay within the normal range of values (Table 4). In short, the vaginal administration of L29B does not appear to have any negative impact on the mice physiological status. This further elucidated that L29B is safe to be administered.

Histological examination of the mouse vagina evidenced that the characteristics (non-keratinised squamous epithelium) of the epithelium and lamina propria (rich in elastic fibre) were similar in both the control and probiotic groups. Figure 3a, b shows that vaginal histology from both experimental groups has similar numbers of layers composing the stratified squamous epithelium. There were no signs of inflammation, degeneration, or necrosis observed. Similarly, no signs of inflammation were observed in the histology of kidney and liver of the mice (Fig. 4a, b and Fig. 5a, b respectively). There was no evidence of translocation of L29B to blood in both the experimental groups. Similarly, bacterial translocation was not detected in the liver and kidney of mice in both groups.

L29B Potential Probiotic Trait Characterisation

Potential mechanisms of colonisation and adhesion properties of L29B were tested for the adherence capability on HeLa cells. As indicated in Fig. 6a, it could be seen

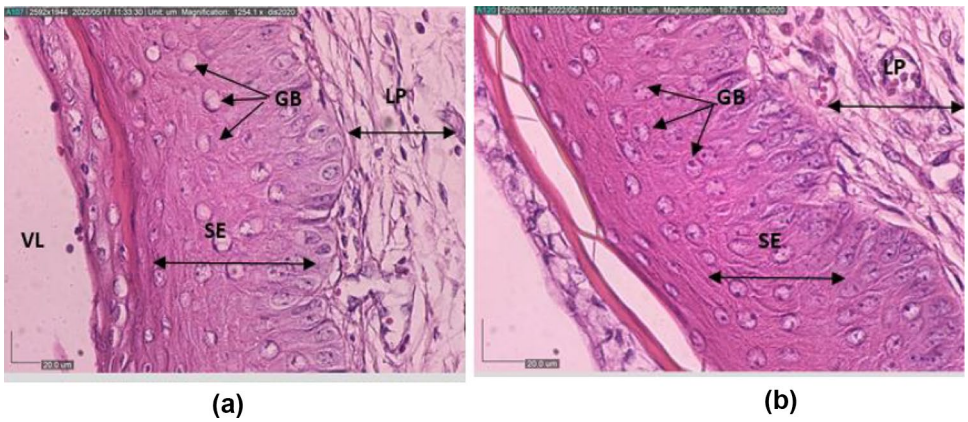


Fig. 3 Photomicrograph of H&E-stained sections of mouse vagina from the **a** control and **b** probiotic groups. SE, stratified squamous epithelial layer; LP, lamina propria; GB, goblet cells; VL, vaginal lumen. Magnification 400x

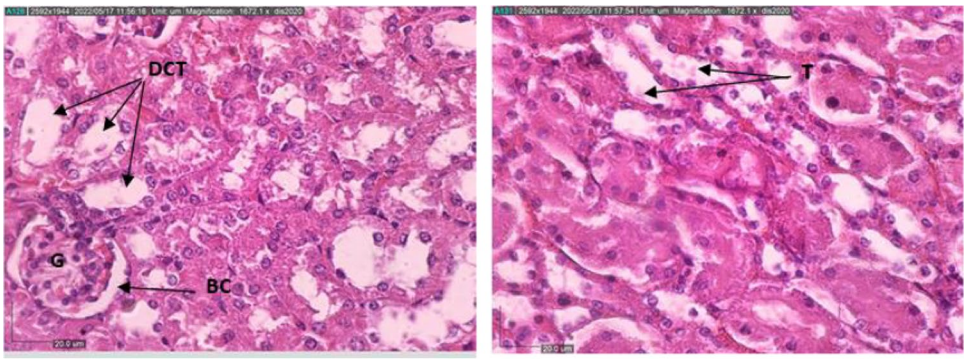


Fig. 4 Photomicrograph of H&E-stained sections of mouse kidney from the **a** control and **b** probiotic groups. DCT, distal convoluted tubules; G, glomeruli; T, tubules; BC, Bowman's capsule. Magnification 400x

that 85.5% of L29B adhered to HeLa cells after 4 h of coincubation. In addition to that, L29B was able to demonstrate probiotic-mediated competition, exclusion, and displacement mechanisms against *Candida* species tested in this study. As shown in Fig. 6b, displacement of *C. albicans* 81 and *C. glabrata* 95670 by L29B was highly significant. More than 90% of both *Candida* isolates on HeLa cells were displaced, and only 8–9% of them were able to remain attached to HeLa cells following introduction of L29B. Although L29B was able to moderately compete and exclude *C. albicans*, the effects were not significant (Fig. 6b). However, it is worth mentioning that the readings were quite varied and mixed. However, L29B was able to significantly compete and exclude *C. glabrata* (Fig. 6b). In general, it was demonstrated that L29B has strong pathogen-displacement ability, as well as competition and exclusion potential to a certain degree. The in vitro data obtained strongly indicates that L29B is likely to adhere and colonise human vaginal epithelium and assist in warding off yeast pathogens through displacement mechanism.

To further confirm the result of the cell culture assay in which to elucidate the potential mechanisms of colonisation and adhesion properties of L29B, the mouse vaginal lavage that was collected from the safety study was screened. These vaginal lavages collected from pre- and post-administered mice were utilised. Prior to the administration of L29B, vaginal lavage from the mice comprised only of cornified epithelial cells, with noticeable absence of any microflora (Fig. 7a). Following administration of L29B, many rod-shaped bacilli were detected in the mouse vaginal lavage (Fig. 7B), suggesting that the probiotic strain L29B was able to successfully adhere and colonise the mouse vaginal tract.

To further investigate the properties of L29B probiotic trait in conferring vaginal health, 16S rDNA was performed on the DNA extracted from the mouse vaginal lavage, to look at the shifting of the mouse vaginal microflora. To explore the variations in the structural diversity of vaginal microbiota between mice pre- and post-administrated with L29B, 16S rDNA sequencing was used to unravel the distinctive spectra of vaginal secretions. A total of 1,672,780 paired-end (PE) reads were generated. After quality filtering and chimaera check, approximately 9% of sequences were removed and 1,521,090 sequences were further processed for operational taxonomic unit (OTU) picking. The average sample length was 463.44 bp. A total of 241 OTUs were observed from the pre- and post-administered groups. About 96 OTUs were shared between the two groups (Fig. 8). Rarefaction curve-based OTU abundance prediction, following clustering of sequences at 97% nucleotide identity, was used to standardise and compare observed taxon richness amongst samples and

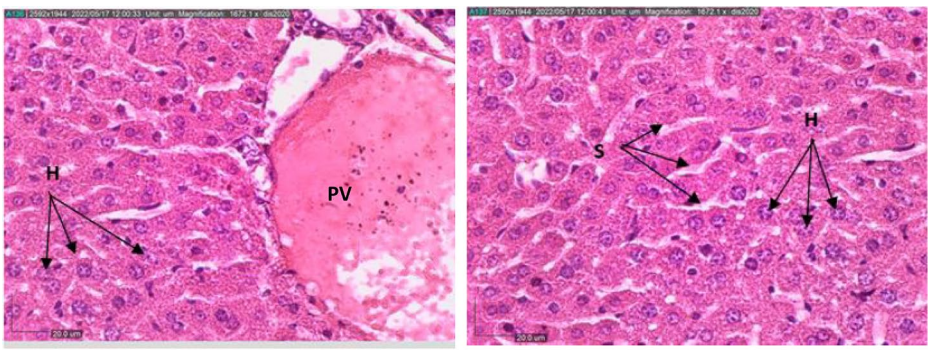
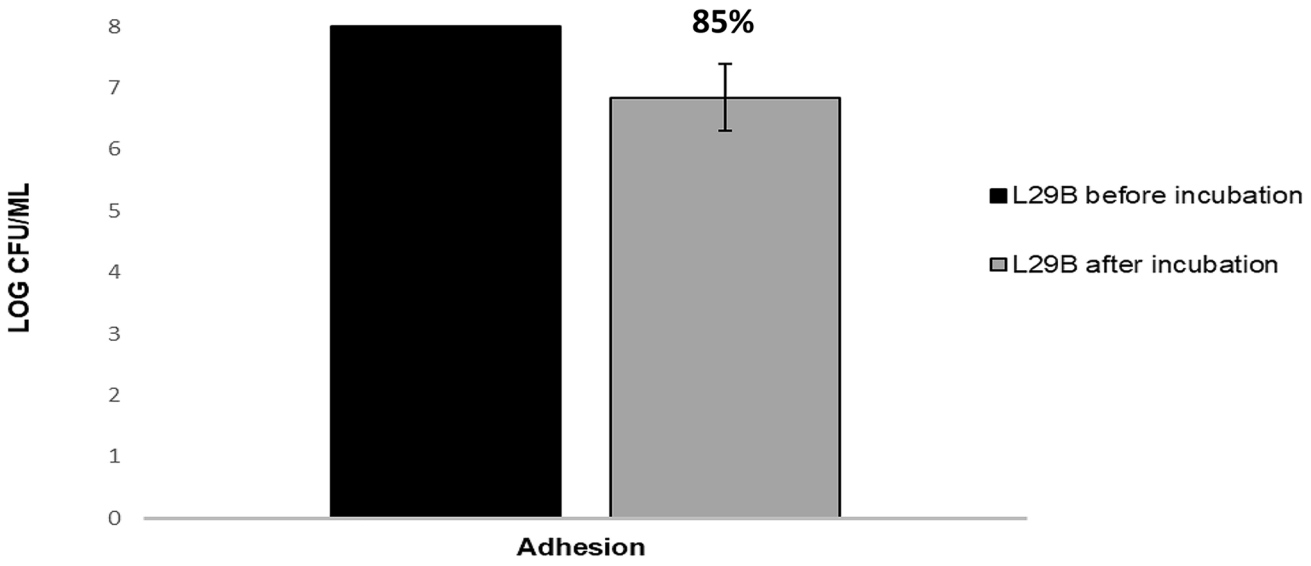
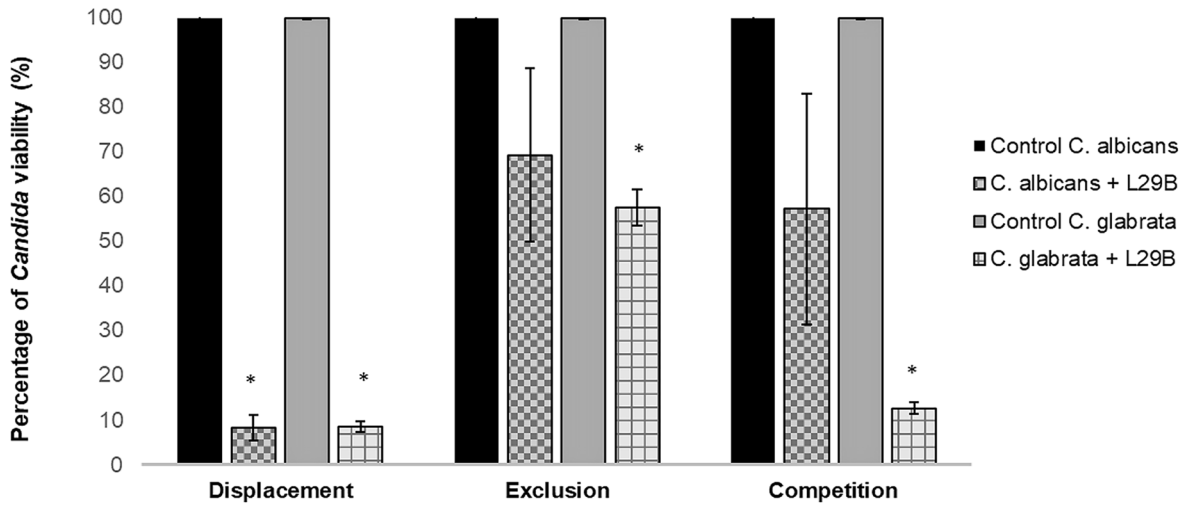


Fig. 5 Photomicrograph of H&E-stained sections of mice liver from the **a** control and **b** probiotic groups. H, hepatocytes; S, sinusoids; PV, portal vein. Magnification 400x



(a)



(b)

Fig. 6 **a** Adhesion of L29B to HeLa cells. **b** Inhibition of *C. albicans* 81 and *C. glabrata* 95670 strains by L29B probiotic strain on HeLa cells tested under the condition of displacement, exclusion, and competition. The data presented are the mean ± SD of three independent assays with $p < 0.05$

to determine whether the contents of vaginal lavage were unequally sampled.

We found a total of 17 phyla in the vaginal lavage samples that were analysed. Figure 9a shows that Firmicutes (59.14%), Proteobacteria (34.09%), and Actinobacteria (6.19%) are the most common phyla found in mouse vaginal tract, whilst *Proteus* (32.86%, phylum Proteobacteria), *Staphylococcus* (25.82%, phylum Firmicutes), *Sporosarcina* (11.38%, phylum Firmicutes), and *Atopostipes* (8.56%, phylum Firmicutes) are the most dominant genus. Following administration of L29B, the relative abundance of Firmicutes increased drastically from 59.14 to 98.50%. *Lactobacillus*, which belongs to the phyla Firmicutes, is the most dominant bacteria at the genus level (96.15%) in the mouse

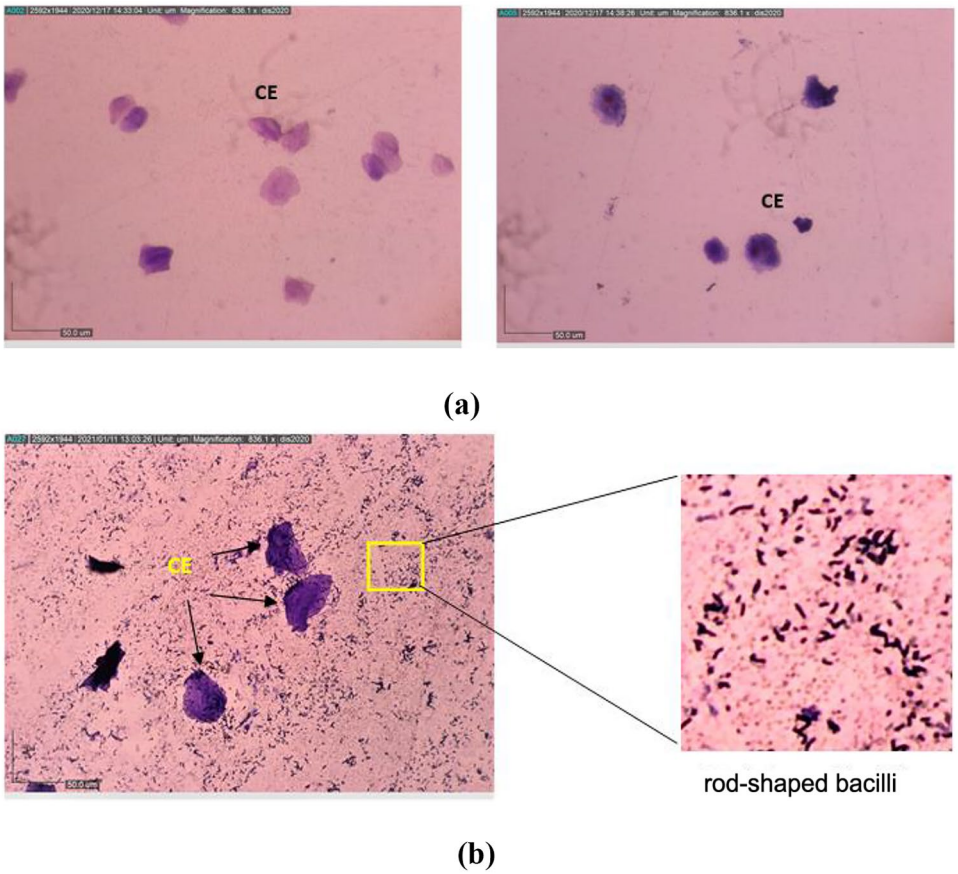


Fig. 7 **a** Photomicrograph of Gram-stained mouse vaginal lavage for pre-administered group. **b** Post-administered group. CE, cornified epithelial cells. Magnification 200x

vagina post-administered with L29B, followed by *Staphylococcus* (2.23%) and *Proteus* (1.23%) (Fig. 9b).

Chao1 and Shannon indices are important components of alpha and beta diversities of a microbial profiles [29]. Chao1 index is used to estimate the total number of OTU contained in a sample, whilst Shannon index is used to estimate the diversity of the species given in a community in the sample [30]. The pre-administered group has a higher Shannon index (2.542) compared to the post-administered group (0.2319) (Fig. 10). Hence, the microbial distribution differed significantly between the two groups. It can be observed that the diversity microorganisms present in the pre-administered group were significantly reduced by the presence of L29B. As a result, the Simpson index confirmed that species richness and evenness are high in the pre-treatment group (0.5867) compared to the post-treatment group (0.068) (Table 5). The good coverage value was > 0.99, indicating that a high degree of sequencing coverage, with all microbiota in each group, was represented by the number of OTUs identified. This data confirmed the number of species present in the vaginal sample as well as their abundance, allowing for a comparison of diversity between the two groups.

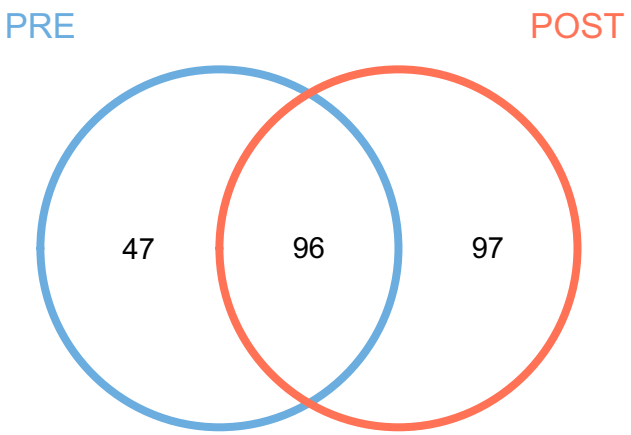


Fig. 8 The Venn diagram generated to describe the common and unique operational taxonomical units (OTUs) between the pre- and post-administered groups

On the other hand, Linear Discriminant Analysis Effect Size (LEfSe) was utilised to see if there was any difference between the two groups in terms of bacterial group enrichment. Significant variations can be seen in the vaginal microbiota between the pre- and post-administered groups. Figure 11a shows distinct groups are indicated by nodes with different background colours (red or green). The red nodes represent microorganism groups that are present in the post-treatment group; the green nodes represent microorganism groups that are present in the pre-treatment group;

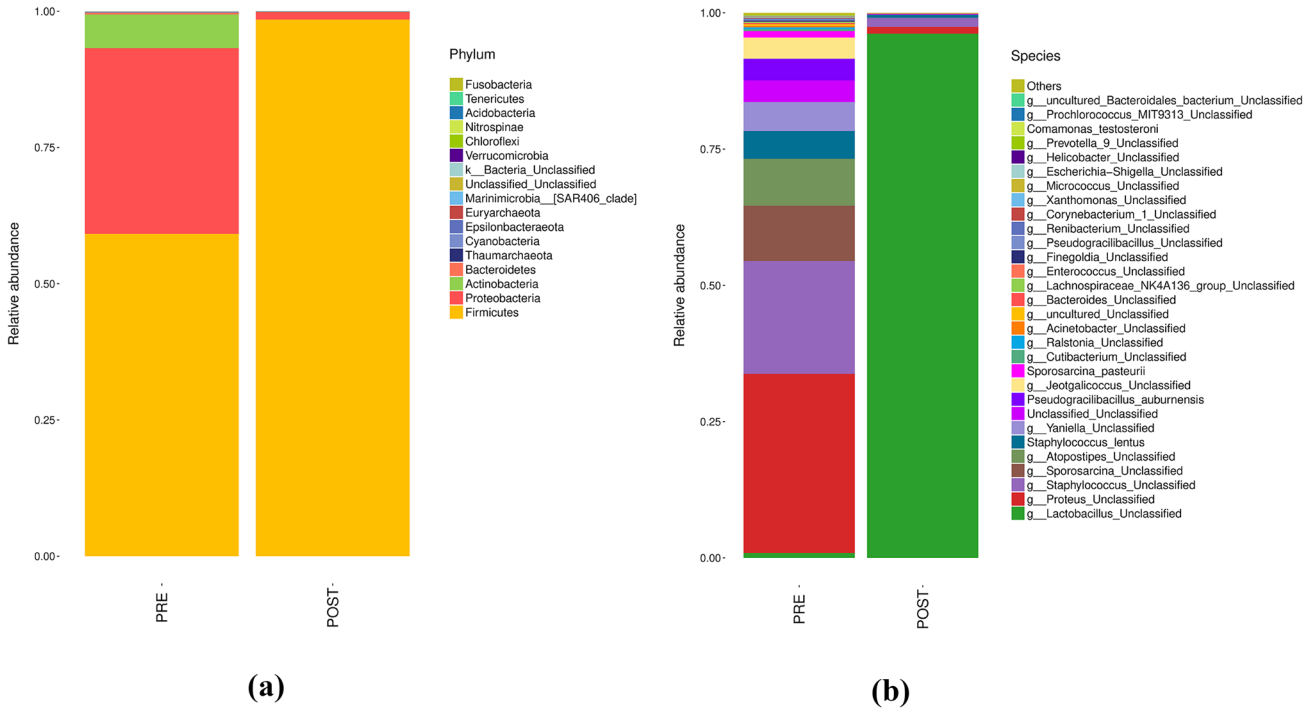


Fig. 9 **a** Microbial composition abundance at phylum level. **b** Microbial composition abundance at species level between the pre- and post-administered groups

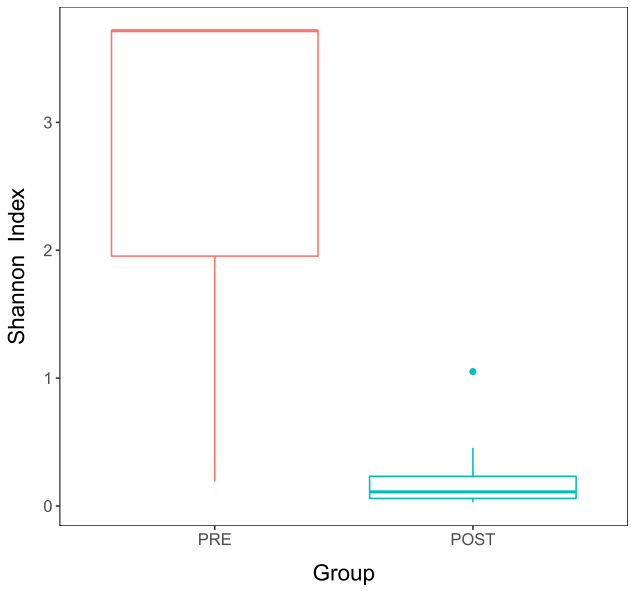


Fig. 10 Shannon index of between pre- and post-administered group

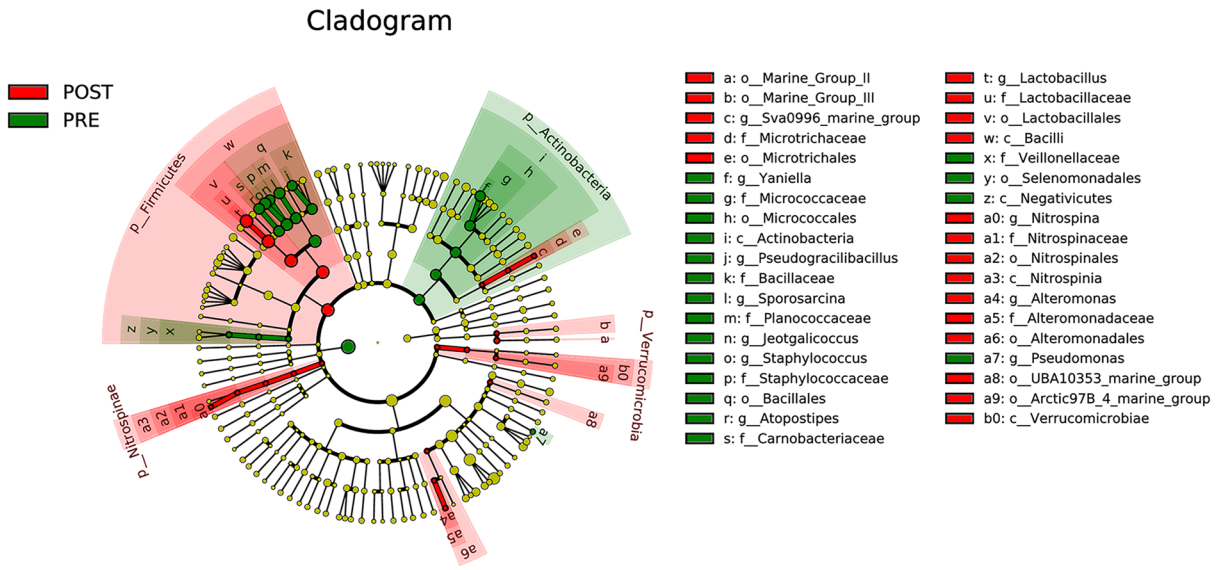
and the yellow nodes show groups that are not present in either group. The findings revealed a significant variation in vaginal microbiota distribution between the two groups.

The effect size distribution histograms of the two groups (Fig. 11b) revealed that 20 genera, which are mainly Bacillales and Staphylococcaceae, were the dominant microorganisms in the pre-administered group and 22 genera with *Lactobacillus* species being the most crucial microorganism in the post-administered group (LDA Core ≥ 2).

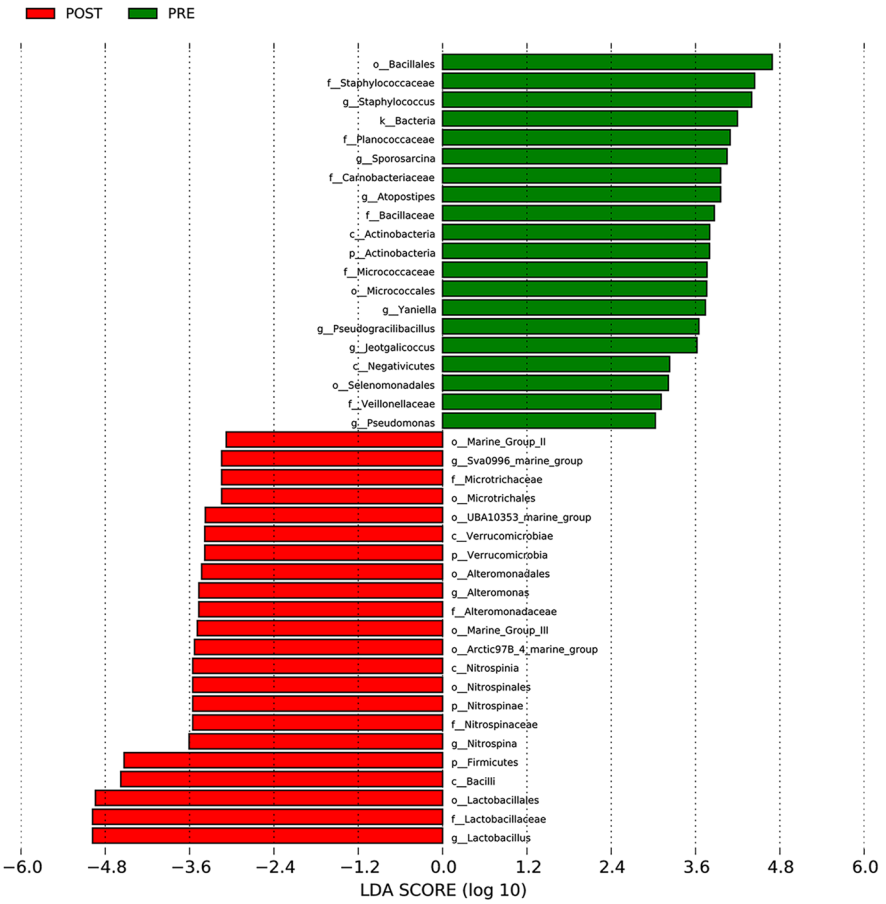
Lastly, we screened the WGS annotations for the potential genes that could be responsible for the above L29B probiotic trait. Various probiotic-related genes were identified in the genomes of the L29B suggesting their potential probiotic properties. These identified genes could be associated in the mechanisms of adhesion, antimicrobial, antitoxin system, quorum sensing, and biofilm formation (Table 6). The Rapid Annotations using Subsystems Technology (RAST) analysis showed the presence of 2015 coding sequences distributed in 223 subsystems, of which 1354 were related to non-hypothetical

Table 5 The diversity and abundance indices: Chao1, Shannon diversity index, and ACE and Simpson index with good coverage

Sample	Ace	Chao1	Shannon	Simpson	Good coverage
Pre-administered group	94.22567	93.90467	2.542	0.586667	1
Post-administered group	112.1744	109.1095	0.2319	0.068	1


Cladogram
POST
PRE
p__Firmicutes
p__Actinobacteria
p__Verrucomicrobia
p__Nitrospinae
a: o__Marine_Group_II
b: o__Marine_Group_III
c: g__Sva0996_marine_group
d: f__Microtrichaceae
e: o__Microtrichales
f: g__Yaniella
g: f__Micrococcaceae
h: o__Micrococcales
i: c__Actinobacteria
j: g__Pseudogracilibacillus
k: f__Bacillaceae
l: g__Sporosarcina
m: f__Planococcaceae
n: g__Jeotgalicoccus
o: g__Staphylococcus
p: f__Staphylococcaceae
q: o__Bacillales
r: g__Atopostipes
s: f__Carnobacteriaceae
t: g__Lactobacillus
u: f__Lactobacillaceae
v: o__Lactobacillales
w: c__Bacilli
x: f__Veillonellaceae
y: o__Selenomonadales
z: c__Negativicutes
a0: g__Nitrospina
a1: f__Nitrospinaceae
a2: o__Nitrospinales
a3: c__Nitrospinia
a4: g__Alteromonas
a5: f__Alteromonadaceae
a6: o__Alteromonadales
a7: g__Pseudomonas
a8: o__UBA10353_marine_group
a9: o__Arctic97B_4_marine_group
b0: c__Verrucomicrobiae

(a)


POST
PRE
o__Bacillales
f__Staphylococcaceae
g__Staphylococcus
k__Bacteria
f__Planococcaceae
g__Sporosarcina
f__Carnobacteriaceae
g__Atopostipes
f__Bacillaceae
c__Actinobacteria
p__Actinobacteria
f__Micrococcaceae
o__Micrococcales
g__Yaniella
g__Pseudogracilibacillus
g__Jeotgalicoccus
c__Negativicutes
o__Selenomonadales
f__Veillonellaceae
g__Pseudomonas
o__Marine_Group_II
g__Sva0996_marine_group
f__Microtrichaceae
o__Microtrichales
o__UBA10353_marine_group
c__Verrucomicrobiae
p__Verrucomicrobia
o__Alteromonadales
g__Alteromonas
f__Alteromonadaceae
o__Marine_Group_III
o__Arctic97B_4_marine_group
c__Nitrospinia
o__Nitrospinales
p__Nitrospinae
f__Nitrospinaceae
g__Nitrospina
p__Firmicutes
c__Bacilli
o__Lactobacillales
f__Lactobacillaceae
g__Lactobacillus
−6.0
−4.8
−3.6
−2.4
−1.2
0.0
1.2
2.4
3.6
4.8
6.0
LDA SCORE (log 10)

(b)

◂**Fig. 11** **a** Cladogram generated from the LEfSe LDA analysis identifying the bacterial abundance between the two groups (LDA Core ≥ 2). Cladogram displays significantly enriched bacterial taxa (from the phylum to the genus level). **b** Phylogenetic distribution of vaginal microbiota from phyla to genera in each group via LEfSe analysis. The LDA scores represented the difference in relative abundance with exponent fold change of 10 between two groups

proteins and 661 to hypothetical proteins. These genes were found to be associated with several subsystems (Fig. 12). Through image analysis, it was seen that the subsystems with the highest number of genes are related to the production of cofactors, vitamins, prosthetic groups, pigments, protein metabolism, amino acids, and carbohydrates.

Discussion

In this study, genomic analysis combined with experimental studies provided an approach to study the characteristics and the safety aspect of L29B comprehensively. The safety of probiotic strains is of utmost importance, and guidelines for the safety assessment can be found in numerous publications [14, 31–33]. All microbes can be divided into three groups based on their relative safety: (1) non-pathogenic, (2) opportunistic pathogens, and (3) pathogens. Every viable microbe that is able to grow under the conditions encountered in a host can cause an infection under certain circumstances (e.g., in severely immunocompromised hosts) [34].

Whole genome sequencing (WGS) is a method for examining the functional traits of microorganisms by sequencing their entire genome and comparing it to a known gene [35]. Complete genome analysis and functional annotation of the L29B genome revealed the presence of several genes within the genome assembly, which are important for probiotic efficacy and safety.

When assessing the potential use of *Lactobacillus* strains as probiotics and additional traits, such as virulence factors, should also be screened, both in vitro and in silico [36]. In the screening of virulence factor database (VFDB), one virulence factor was detected which is elongation factor Tu (EF-Tu) (tufA) in L29B genome. This gene is responsible in mediating the attachment of *Lactobacillus* to human intestinal cells and mucin [27]. In addition to that, immunomodulation studies performed on HT29 cells showed that EF-Tu recombinant protein can induce a proinflammatory response in the presence of soluble CD14 [37]. A study conducted by Dhanani and Bagchi mentioned that the adhesion of *Lactobacillus* strains to mucin in the presence of recombinant EF-Tu showed strong adhesion inhibition of *Lact. delbrueckii* M and the strains of *Lact. plantarum* [26]. Hence, the presence of this gene can be considered essential for L29B strain in survivability and colonisation.

In addition to that, the BLASTn search on the VirulenceFinder v.2.0 database found no matchings, further confirming the lack of virulence factors in L29B genome. The L29B genome sequence was compared with the genomic sequences of four noted pathogens (*Escherichia coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus*). *Escherichia coli* shiga toxin gene and *Staphylococcus aureus* exoenzyme genes, host immune modification or evasion genes, and toxin genes were amongst the virulence factors examined. The L29B genomic sequencing revealed no virulence factors, toxic or pathogenic genes.

Resistance to antibiotics is a concern because of the possibility of transferring the plasmid containing these genes to other pathogenic bacteria, making infections difficult to treat. In modern medicine, antibiotics are the mainstay of defence against bacterial infections, but bacteria are versatile and can adopt mechanisms to counter the antibiotic action [38]. Antibiotic resistance has been developing at an alarming rate and has become a growing global public health concern [39]. Some *Lactobacillus* are resistant to one or multiple antibiotics. Antibiotic resistance may be naturally occurring or can be acquired by genetic mechanisms like horizontal gene transfer through plasmids or transposons [40, 41].

To identify this risk, mobile genetic elements (MGEs) such as plasmid need to be identified, since they are the most likely vehicles involved in inter-cellular genetic exchange through transformation/conjugation and transduction process, respectively [42]. L29B strain genome was analysed using the Plasmidfinder 2.0, and no plasmid was detected in the genome. Therefore, this result confirmed that L29B is safe and possesses a safe probiotic characteristic, as plasmids can often carry antimicrobial resistance and virulence factor genes. ResFinder tool v.4.1. database results of L29B genome too further confirmed the absence of transferable and acquirable antibiotic resistances.

Moreover, the genome of L29B harboured Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), with associated Cas-gene (type 1) and spacer (Table 2). The presence of CRISPR region within a genome limits the spread of antimicrobial resistant genes through obstruction of multiple pathways of horizontal gene transfer [43–45]. With the presence of effective CRISPR region in L29B genome, it equips the strain with sequence specific defence-line against plasmids, insertion sequences, and phages [46].

Alongside WGS analysis, an animal study was conducted to confirm the safety of L29B strain through intravaginal administration in the mouse model. The administration of L29B for seven days did not result in significant differences in body and organ weights between the pre- and post-administered groups of mice (Table 3). No observable changes in the mice activity, behaviour, or hair lustre were

Table 6 Genes possibly involved in the probiotic trait or defence mechanism of L29B strain

Genes/RAST description	Functions	Contig	Region in the contig
Exopolysaccharide biosynthesis protein (epsA)	Adhesion	3	15,821–16,699
Class A sortase (srtA)	Adhesion	12	61,909–62,616
Fibronectin-binding protein (fnbB)	Adhesion	16	2–1519
Anti-holin (lrgB)	Anti-microbial	5	79,375–80,115
CvpA family protein	Anti-microbial	1	22,781–23,308
Colicin imported membrane protein (tolA)	Anti-microbial	9	72,121–73,062
Hypothetical protein (vapB)	Anti-toxin system	13	21,059–21,301
Hypothetical protein (maze, chpAI)	Anti-toxin system	2	311,473–311,724
Type II toxin-antitoxin system Phd/YefM family antitoxin	Anti-toxin system	3	69,987–70,259
S-Rribosylhomocysteine lyase (LuxS)	Quorum sensing/biofilm formation	13	24,043–24,519
Glycosyltransferase (icaA, pgaC)	Quorum sensing/biofilm formation	1	276,491–277,792
Surface protein G (sasG)	Biofilm formation	19	5799–12,101

noted throughout the experiment. Additionally, no illnesses or deaths were observed throughout the experimental period. The current findings demonstrated that L29B does not exert or stimulate any toxicological effect on the growth and development of the mice. Examination of the haematological and blood biochemical parameters revealed no significant differences between the control and probiotic group mice (Table 4). Hence, this further reasserts that L29B is unlikely to cause any adverse effects in a healthy host.

Bacterial translocation is a crucial sign of probiotic infectivity since it is the first stage in opportunistic bacteria pathogenesis [47]. The present findings indicate that no bacterial translocation to the blood was observed in both the pre- and post-administered groups, which denotes that there was no translocation of L29B. The mesenteric lymph nodes, spleen, liver, and bloodstream of healthy animals are generally free from bacteria from the indigenous microflora [32].

Composition of the vaginal microbiota has significant influence on female urogenital health and control of infectious disease. The natural strategy to prevent or treat vaginal infections is to use probiotics to maintain and re-establish the human vaginal microbiota, respectively. Several studies have successfully used animal models to investigate the prevention and treatment of vaginal infection using LAB via intravaginal administration [48–50]. The composition of endogenous vaginal flora remains largely undefined with microbiome analyses, despite the widespread use of murine models to characterise host-pathogen interactions within the vaginal tract [51].

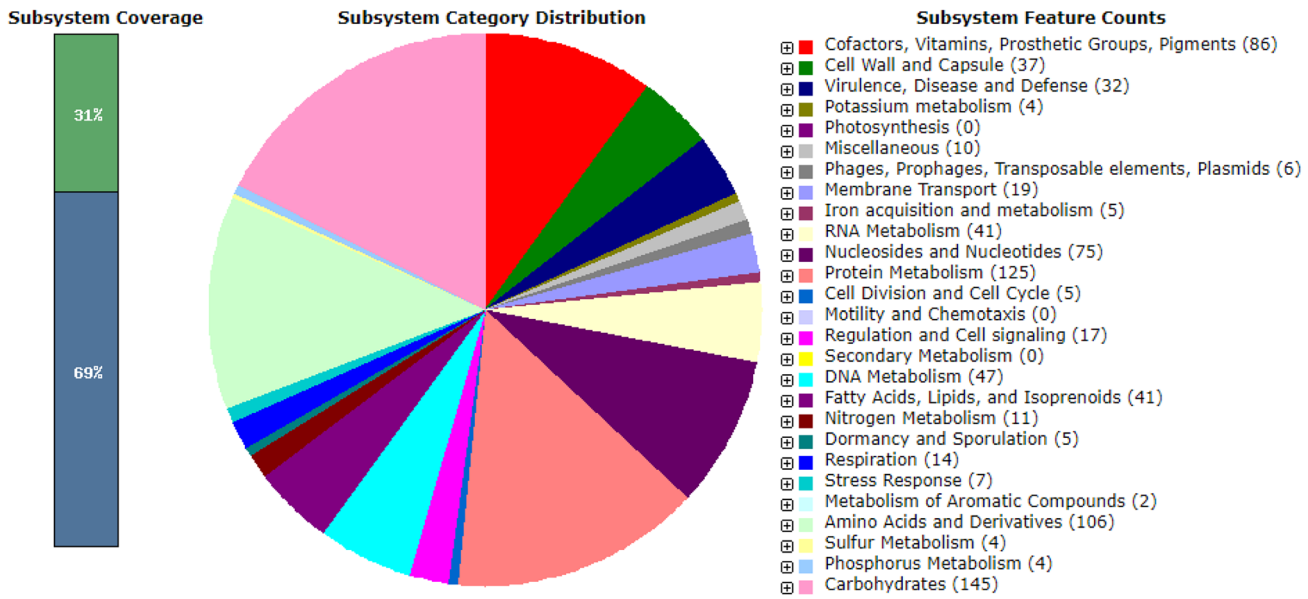


Fig. 12 Categories of subsystems of the L29B genome annotated by RAST

The bacterial microflora in an animal model could pose a challenge in determining the biotherapeutic effect of these beneficial microbes but at the same time; according to some research, it is reported that lactobacilli are only present in a very minute amount in mice [52]. This can be further confirmed in the pre-administered mice 16S rDNA result where *Proteus* (32.86%, phylum Proteobacteria), *Staphylococcus* (25.82%, phylum Firmicutes), *Sporosarcina* (11.38%, phylum Firmicutes), and *Atopostipes* (8.56%, phylum Firmicutes) are the most dominant genus. However, for human vaginal microflora, over 50 microbial species have been recovered from the vaginal tract [53, 54]. The microbial flora of the lower female vaginal tract is a dynamic, complicated example of microbial colonisation which is not fully understood. Much of what we know about the bacterial composition of the female genital tract is derived from qualitative, descriptive studies [55]. The normal flora content of the female genital tract is dependent on various factors such as age, hormones, and pH in the genital tract of the host, to name a few [56]. Hence, the mouse model was chosen for this study as it is less complex than human vaginal microflora, in analysing the capability of L29B colonisation.

Lactobacillus species in the female urogenital system act as an infection barrier, offer colonisation resistance, and contribute to the modulation of the vaginal microbiota by competing with other bacteria for epithelial cell adhesion, displacing pathogen biofilm [57, 58] and/or reducing pathogen growth [59]. Based on our previous studies evaluating the probiotic properties of L29B, we found the strain to be a great candidate for adherence [20]. In addition, the cell surface of L29B is moderately hydrophobic, and this strain is capable of self-aggregation to form biofilms. In this study, we observed that L29B strongly adhered on HeLa cells. The ability of L29B to adhere and colonise vaginal epithelia *in vitro* was further corroborated by the presence of L29B in the vaginal lavage collected from the mice seven days post-inoculation. These results further highlighted the probiotic properties of L29B in vaginal epithelia adhesion and colonisation. Furthermore, it was confirmed that L29B can survive in the mouse vaginal environment. Lactobacilli exhibit a strong adhesion to the stratified, nonkeratinizing vaginal epithelium, leading to exclusion and rejection of pathogenic microorganisms from attaching to vaginal epithelium [60]. Mastromarino et al. (2002) found that in the presence of LAB, *C. albicans* and *G. vaginalis* adhesion to vaginal epithelial cells were reduced by 50%, whereas exogenous lactobacilli displaced more than 60% of the *C. albicans* and *G. vaginalis* already attached to the epithelial cells [61]. Adhesion and colonisation are key determinants in bacterial competition and microbiome homeostasis [57, 62].

The ability of lactobacilli to attach to vaginal mucosa and exert antipathogenic action is unquestionably linked to probiotic therapy success. The FAO/WHO considers the ability of lactobacilli to attach to human epithelial cells, as well as inhibit pathogen colonisation, to be important factors in the *in vitro* evaluation of potential probiotic candidates [63]. Previously, L29B has shown a great antagonistic activity against selected pathogenic pathogens [22]. We assessed L29B capability of interfering with the adhesion of vaginal pathogens, with *Candida* strains in HeLa epithelium cell line. The indigenous lactobacilli in the vaginal tract of adult healthy women are thought to prevent harmful bacteria colonisation by occupying or obscuring (via stearic hindrance) their potential binding sites on the mucosa [64]. Exogeneous probiotic lactobacilli should also compete for the same receptors on the mucosa surface, displace, and remove pre-attached pathogens to replenish the normal vagina flora [65]. Hence, this further reasserts L29B in displacing the *Candida* strains from the HeLa cells as shown in Fig. 6B. Kaewscrichan et al. (2007) also reported the ability of lactobacilli to displace the adhesion of *Candida* strains that indicates the affinity of lactobacilli for the specific receptors is higher than that of the pathogenic strain tested. In general, probiotic work prevents the growth of harmful organisms, defends against infection, and, as a result, maintains the balance of microbial populations in the body's various tracts [61, 66].

We further analysed the mouse vaginal lavage for 16S rDNA sequencing to observe the ability of L29B to colonise and shift the vaginal microflora in mice after the administration of L29B. The result of the sequencing can be seen to correlate with the result of cell culture assay and the vaginal lavage smear in term of adhesion, colonisation, and replenishment of the vaginal microflora. We compared the vaginal microbial community between the pre- and post-administered groups. As shown in Fig. 9b, the pre-administered group has the highest number of *Proteus* followed by *Staphylococcus* and for post-treatment it could be observed that the *Lactobacillus* has fully occupied the vaginal environment of the mice by displacing most the other bacterial community that was present prior to the administration. This supports the in vitro data on cell culture and the vaginal lavage smear that L29B was able to significantly reduce the pathogens that were present in the vaginal environment without causing any inflammation or irritation to the mice.

The Shannon index is used to quantify the diversity of microbial communities, and Chao1 is used to determine the total number of species in ecology. The greater the Chao1 and Shannon values, the more species there are and the more diverse they are [67, 68]. As shown in Table 5, the Shannon value for the pre-treatment was higher compared to the post-administered group because of the high amount of microbial community that present. This correlate with the Fig. 10. L29B strain was able to balance the vaginal environment of the mice by inhibiting all the other microorganisms that were present. Interestingly, with only 1×10^8 number of cells of inoculation, it is more

than sufficient for the probiotic strain to establish a balanced microflora and eliminate most of the microorganisms in the vaginal tract of the mice. A similar significant degree of inhibitory activity can also be observed via this analysis as it was proven by *in vitro* assay in our previous study [22]. Although it is difficult to determine the number of various microorganisms in the vaginal population *in vivo*, evidence of their presence has been confirmed by microscopy analysis, and identification tests. Prior to the 16S rDNA part of the study, both Gram-staining and 16S rDNA analyses showed the absence of lactobacilli in the vagina lavages. It is worth mentioning that other studies have found 25% of lactobacilli in the vaginal microbiota of experimental mice prior to a introduction of probiotic [69, 70].

Despite the fact that probiotics have been used to colonise the vagina and prevent or treat vaginal infections for a long time, their efficiency has only been proved recently, and unlike antibiotics, very minimal adverse effects have been identified [69, 71]. Nonetheless, the safety of probiotic strains must be constantly evaluated and considered when conducting clinical trials. One thing to consider is the possibility for antibiotic resistance to spread, whilst it has yet to be established that probiotics have contributed in any manner to drug resistance or disease [72]. The use of probiotic could be an excellent alternative approach compared to antibiotic treatment and many studies have supported this concept [48, 49, 73–75]. In this present work, the intravaginal administration of L29B to healthy mice induced a significant reduction in Enterobacteriaceae and Staphylococcaceae. L29B replenished and promoted a healthy vaginal environment for the mice. It was also able to survive, adhere, and colonise the vaginal tract of the mice on day 8 as shown in Fig. 7b and safe to be administered without causing any inflammation or irritation.

Amongst the probiotic traits that are present in L29B, analysed through RAST and manual screening of the genes, few putative genes were identified to be involved in the mechanisms of adhesion, antimicrobial, antitoxin system, quorum sensing, and biofilm formation (Table 6). One of the most important traits for a potential probiotic strain is the ability to adhere to the epithelial cells of the host. In this regard, the genome analysis of L29B identified genes encoding fibronectin-binding protein (fnbB), exopolysaccharide (epsA) biosynthesis protein, and sortase A (srtA). Studies suggest that the health-promoting effects of probiotics could be related to their capacity to adhere to epithelial cells and/or mucus, as it can promote colonisation, pathogen exclusion, and interactions with the host [76, 77].

Taken together, with proven safety and probiotic efficacy shown in this study, further functional and mechanistic studies as well as clinical trials are warranted to reflect and translate it to practical and effective applications for human use.

Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Premmala Rangasamy, Leslie Thian Lung Than, and Hooi Ling Foo. The first draft of the manuscript was written by Premmala Rangasamy, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics Approval and Consent to Participate The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Universiti Putra Malaysia (UPM/IACUC/AUP-R030/2020).

Conflict of Interest The authors declare no competing interests.

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