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ORIGINAL ARTICLE

Therapeutic effects of orally administration of viable and inactivated probiotic strains against murine urinary tract infection

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Abstract

Urinary tract infections (UTIs) are highly prevalent bacterial infections that pose significant health risks. Specific probiotic strains have been recommended for UTI control and management of antibiotic resistance. Otherwise, paraprobiotics, defined as inactivated probiotic cells, offer potential advantages by minimizing risks associated with live microorganisms. However, the effectiveness of heat-killed probiotic strains against UTIs remains uncertain. Additionally, lactoferrin (LF), an iron-binding glycoprotein, exhibits immunomodulatory, antimicrobial, and anti-inflammatory properties. Recently, we had developed recombinant LF-expression probiotics, which can display considerate antibacterial activities against select food-borne pathogens *in vitro*. Thus, the present study aimed to evaluate the antibacterial activities of heat-killed natural and recombinant LF-expressing probiotics against UTIs *in vitro* and *in vivo*. Firstly, using *in vitro* assays, we assessed the antibacterial activity of heat-killed natural and recombinant LF-expressing probiotics against uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*. Among the tested probiotics, 10 heat-killed LF-expressing strains displayed superior antibacterial efficacy compared to 12 natural probiotics. Based on their potent *in vitro* activity, selected probiotics were formulated into three probiotic mixtures: viable probiotic mixture (LAB), heat-killed probiotic mixture (HK-LAB), and heat-killed LF-expressing probiotic mixture (HK-LAB/LF). To further evaluate the therapeutic potential of these probiotic mixtures *in vivo*, we established a murine model of UTIs by intraurethral administration of *E. coli* to 40 female C57BL/6JNarl mice on day 0. Subsequently, mice received oral gavage of placebo, LAB, HK-LAB, or HK-LAB/LF for 21 consecutive days (n = 8 per group). An additional control group (n = 8) received ampicillin treatment for 7 days. To assess protective effects against re-infection or UTI relapse, all mice were challenged with *E. coli* on day 22 and *E. coli* plus *K. pneumoniae* on day 25. Results from the murine UTI model demonstrated that placebo administration did not reduce bacteriuria throughout the experiment. Conversely, supplementation with ampicillin, HK-LAB/LF, HK-LAB, or LAB significantly ($p < 0.05$) reduced daily bacteriuria by 10^3 to 10^4-fold on days 1, 3, 5, and 14, respectively. Furthermore, all four therapeutic treatments improved the bacteriological cure rate (BCR) with varying levels of efficacy. For the 7-day treatment course, the BCR was 25% (placebo), 62.5% (ampicillin), 37.5% (LAB), 37.5% (HK-LAB), and 62.5% (HK-LAB/LF). For the 21-day treatment course, the BCR was 25% (placebo), 75% (ampicillin), 37.5% (LAB), 37.5% (HK-LAB), and 75% (HK-LAB/LF). Notably, HK-LAB and HK-LAB/LF demonstrated superior therapeutic efficacy compared to viable LAB in treating UTIs. Overall, regarding BCR, the three probiotic mixtures can provide benefits against UTI in mice, but ampicillin therapy remains the most efficient among the four treatments. Furthermore, there was no significant difference between pre- and post-challenge courses for the two instances of re-challenging uropathogens in all mice groups, as bacteriuria levels remained below 10^3 CFU/mL, implying that adaptive responses of mice may help reduce the risk of recurrent UTIs. In conclusion, our results provide new evidence that oral administration of heat-killed probiotic mixtures can confer significant therapeutic efficacy against UTIs in a murine model.

Keywords: Infectious disease, Lactoferrin, Murine model, Probiotic, Urinary tract infection

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1. Introduction

Urinary tract infections (UTIs) are a type of infection that affects the urinary system, including kidneys, ureters, the bladder, and urethra. While UTIs can involve the upper urinary tract, they typically occur in the lower urinary tract [1]. Nowadays, UTIs are among the most frequent bacterial infections globally and are the second most common type of community-acquired and nosocomial infections [2]. Studies estimate that nearly one out of every two women will experience at least one UTI in their lifetime [3,4]. UTIs are quite common infections predominantly caused by bacteria, with *Escherichia coli* or uropathogenic *E. coli* (UPEC) strains being the most prevalent causative agent, responsible for approximately 80–85% of UTIs [1,3,5]. As such, the first line of treatment for UTIs is antibiotics. However, the development of resistance to traditional and even third-generation antibiotics by UPEC [2,6,7] is becoming increasingly challenging due to routine administration by many countries [5,8,9]. Furthermore, administering antibiotics to treat UTIs may also cause ecological disturbances in the normal microflora, and prolonged antibiotic use often results in the emergence of multidrug-resistant organisms [10].

As described above, the increasing incidence and global emergence of multi-drug resistant uropathogens has accentuated the requirement for alternative, non-antibiotic therapeutic and prophylactic strategies for UTIs [11]. Among the various non-antibiotic approaches, probiotic-containing remedies have garnered attention as the most promising alternative to traditional antibiotics [11–14]. Nonetheless, there exists considerable strain-level difference in probiotic potential against UTIs [15], necessitating further research to discover and develop novel probiotic treatments for UTIs.

In recent years, the field of probiotics has seen the emergence of new concepts or composites, such as "postbiotics" and "paraprobiotics," which pertain to non-viable microorganisms or bacterial-free extracts that could provide health benefits for the host by offering additional bioactivities to probiotics [16,17]. With regards to the utilization of the cell components and metabolites of probiotics, various terms have been suggested, such as "paraprobiotics," "ghost probiotics", "inactivated probiotics," "non-viable microbial cells," "metabolic probiotics," "postbiotics," etc. In general, "paraprobiotics" is often defined as the use of inactivated microbial cells or cell fractions that confer health benefits to the host [18]. Meanwhile, "postbiotics" encompasses the soluble products or metabolites secreted by probiotics that could confer physiological benefits to the host [19]. To date, studies have demonstrated the diverse and potent beneficial functions of inactivated probiotics, including paraprobiotics and postbiotics, such as anti-tumor effect, immunomodulation, preservation of epithelial barrier, and disease prevention or treatment [16,17,20]. An early study by Shirota demonstrated that heat-killed preparation of *Lactobacillus casei*, when administered intraurethrally, exerted significant antimicrobial effects against uropathogenic *E. coli* via single pretreatment or with multiple daily treatments during the post-infection period. However, the same study revealed that several other *Lactobacillus* strains, including *Lactobacillus fermentum* ATCC 14931^T, *Lactobacillus jensenii* ATCC 25258^T, *Lactobacillus plantarum* ATCC 14917^T, and *Lactobacillus reuteri* JCM 1112^T, did not exhibit significant antimicrobial activity against UTIs [21]. This previous report supports the notion of significant strain-level difference in probiotic potential against UTIs.

Lactoferrin (LF), an 80 kDa iron-binding protein predominantly present in mammalian milk and exocrine fluids, has exhibited multiple functions, including antimicrobial, anti-inflammatory, and immune-modulating properties [22–24]. Additionally, the pronounced antimicrobial activities of LFs against a wide spectrum of pathogens, particularly at mucosal surfaces [25,26] has made it a topic of considerable interest within the scientific community. A recent study had yielded a novel *L. casei* strain capable of secreting bovine lactoferrin (BLF) encoded by a secretion vector plasmid designated pPG612.1. The study further revealed that recombinant *L. casei*/pPG612.1-BLF can act as a prophylactic agent with enhancer properties, fortifying the immunity of vaginal mucosa against *Candida albicans*-induced vulvovaginal candidiasis in a murine model [27]. In our recent report, we developed and expressed recombinant human lactoferrin (rHLF), bovine lactoferrin (rBLF), and porcine lactoferrin (rPLF) in several lactobacillus or bifidobacterial strains resistant to BLF. Of particular significance, inactivated probiotic cell lysates containing functional rLFs can help considerably enhance antibacterial activity of host lactobacillus or bifidobacterial strains against food-borne pathogens *in vitro* [28]. Collectively, the present study was set up to determine whether oral intake of natural or recombinant LF-expressing probiotic strains could be a promising therapeutic intervention for UTIs. To validate this speculation, we subjected natural and

recombinant LF-expression probiotic strains to *in vitro* and *in vivo* murine UTI model assessments.

2. Materials and methods

2.1. Bacterial strains and growth

Two common uropathogens associated with UTIs, *E. coli* (BCRC 10675) and *Klebsiella pneumoniae* (BCRC 10694), were purchased from Bioresource Collection and Research Center (BCRC) in Taiwan. Additional pathogenic strains, including *E. coli* (HER 1255), *Staphylococcus aureus* (ATCC 25953), and *Salmonella* Typhimurium (ATCC 14028), were also acquired from BCRC Taiwan. All pathogenic strains were cultured aerobically at 37 °C for 16–18 h in Nutrient broth (Difco TM Nutrient Broth, BD, USA). A collection of probiotic strains, including *Lactobacillus casei* (BCRC 10358), *L. casei* (BCRC 10697), *Pediococcus pentosaceus Mees* (BCRC 11064), *Lactobacillus paracasei* (BCRC 12193), *L. fermentum* (BCRC 12194), *Bifidobacterium breve Reuter* (BCRC 12584), *Lactobacillus coryniformis* (BCRC 12935), *Lactobacillus elbrueckii* (BCRC 14008), *Lactobacillus acidophilus* (BCRC 14065), *C. tyrobutyricu*m (BCRC 14535), *Bifidobacterium angulatum* (BCRC 14605), *Bifidobacterium bifidum* (BCRC 14615), *L. reuteri* (BCRC 14625), *Lactobacillus rhamnosus* (BCRC 16000), *L. paracasei* (BCRC 17483), and *Lactobacillus paraplantarum* (BCRC 17971) were obtained from BCRC Taiwan as well. In addition, this study utilized several recombinant LF-expression probiotic strains developed in our prior report [28], including *Lactobacillus delbrueckii*/HLF, *L. delbrueckii*/BLF, *B. angulatum*/BLF, *B. angulatum*/PLF, and *Lactobacillus gasseri*/BLF, as well as three strains of *L. gasseri* (HM-1, HM-3 and HM-4; laboratory stock) isolated from human milk. All probiotic bacterial strains were activated and cultured anaerobically in MRS broth (Lactobacilli MRS Broth, Difco, BD, USA) at 37 °C without agitation. The expression of recombinant LF was induced by supplementing cultures of recombinant LF-expression probiotics with nisin at a concentration of 1 ng/mL (supplemented in fresh medium) for 16 h at 30 °C, as previously described in our report [28].

2.2. Preparation of heat-killed probiotic and pathogenic cells

The preparation of heat-killed probiotic solutions involves cultivation and activation of probiotic strains. Centrifugation was used to harvest approximately 5×10^{10} CFU bacterial cells, resulting in the formation of bacterial pellets. The pellets underwent two rounds of washing with 25 mL of 1x PBS (Phosphate 0.1M, NaCl 0.15M, pH 7.2) using vortex homogenization for 15–30 s. The suspension was then subjected to a final centrifugation at 10,000×*g* for 10 min to remove supernatant. Resultant bacterial pellets were collected and resuspended in fresh MRS broth prior to autoclaving at 121 °C for 15 min. The same method was also employed to harvest individual heat-killed pathogenic cell solutions from strains such as *E. coli* (BCRC 10675), *K. pneumoniae* (BCRC 10694), *E. coli* (HER 1255), *S. aureus* (ATCC 25953), and *Salmonella* Typhimurium (ATCC 14028). Finally, the prepared heat-killed probiotic or pathogenic preparations were subjected to *in vitro* antibacterial analysis against two uropathogens, including *E. coli* (BCRC 10675) and *K. pneumoniae* (BCRC 10694) as indicated below.

2.3. Antibacterial activities of inactivated probiotic composite in vitro

To evaluate *in vitro* efficacy of heat-killed probiotic preparations in the inhibition of two uropathogens, *E. coli* (BCRC 10675/ATCC 11775) and *K. pneumoniae* (BCRC 10694), agar well diffusion assay was employed with some modifications [29,30]. The two uropathogens were grown in NB overnight and adjusted to 10^7 CFU/mL. Subsequently, the pathogens were spread onto the NA plates by a sterile cotton-tipped swab. Eight mm diameter holes were then created in the inoculated plates using a sterilized tip. As blank control, the MRS medium was autoclaved following the same procedures as probiotic preparations. Then, aliquots (120 μL) of the sterilized MRS and heat-killed probiotic or pathogenic preparations, were each loaded into individual agar wells. Additionally, positive inhibitory controls, ampicillin (100, 200 or 400 μg/mL; positively inhibitory control), or chloramphenicol (25, 50 or 100 μg/mL; positively inhibitory control) were also introduced into individual agar wells. The plates were incubated at 37 °C for 18–24 h, and the inhibition zone (mm) was then quantified by measuring its diameter around each well. Three independent experiments were conducted, each performed in duplicate.

2.4. Preparation of probiotic composite for animal study

Results of the *in vitro* antibacterial activities on inactivated-probiotic strains informed the selection of several natural or recombinant probiotic strains, which were combined to form three probiotic mixers (composites): LAB, HK-LAB, and HK-LAB/

LF. The LAB composite was formulated from a blend of selected viable and natural probiotic strains. HK-LAB composed of selected heat-killed natural probiotic strains, while the HK-LAB/LF consisted of selected heat-killed recombinant probiotic strains expressing the LF gene. Ultimately, the therapeutic efficacy of the three probiotic composites against UTIs were determined through examination in a murine model.

2.5. Establishing the murine UTI model and reinfection experiment

All animal experiments and protocols were reviewed and approved by the Institutional Animal Care and Use Committees at National Chung Hsing University (NCHU IACUC number 111−014). Mice 7 weeks of age (National Laboratory Animal Center, Taipei, Taiwan) were housed under a constant temperature of 22 ± 2 °C with a 12-h light-dark cycle. These mice were provided ad libitum access to food and water and acclimated to the environment for a period of one week prior to initiation of experiments.

In the preliminary analysis, we had tried to evaluate the amount of bacteria in the urine of three groups of mice: healthy mice (no treatment), mice treated with PBS (vehicle control), and mice infected with UPEC bacteria. To do this, we collected urine samples from healthy mice every day for a week (12 mice in total) and immediately analyzed the bacteria in their urine using established methods [33]. After that, we divided the mice into two groups (6 mice in each group) and instilled either PBS or UPEC bacteria into their urinary bladders. We then monitored the amount of bacteria in their urine every day for 21 days [33]. The way we introduced the bacteria or PBS followed previous studies [31,32]. Briefly, for the mice infected with UPEC bacteria (UPEC infection model), we anesthetized them by injecting tiletamine-zolazepam (Zoletil®; Virbac) into their abdomens and sterilized the area around the urethra with 70% ethanol. Then, we inserted a sterile 24-gauge catheter (0.47 mm inner diameter, 19 mm length; Terumo Corporation, Philippines) into their bladders through the urethra (transurethral catheterization) and slowly introduced 50 μL of UPEC *E. coli* (ATCC 11775) at a concentration of 8×10^8 colony forming units (4×10^7 CFU/mice). Similarly, for the group of mice treated with PBS, we followed the same procedures and anesthesia as the UPEC group, but instead of introducing bacteria, we used sterile PBS. After the mice were treated with either PBS or UPEC, we collected their urine samples daily for 21 days, and we determined the amount of bacteria using the SP-SDS method [33]. Notably, we conducted serial dilutions of the urine samples to also count the number of Gram-negative bacteria and total bacterial cells. For reference, we used MacConkey and nutrient agar plates for this purpose, respectively. All the plates were incubated aerobically for 24 h, and the results were reported as Log10 CFU/mL.

To test the effectiveness of different probiotics composites (LAB, HK-LAB, and HK-LAB/LF) in treating or preventing urinary tract infections (UTIs) in mice, we conducted an experiment using 40 female C57BL/6 mice. They were randomly divided into five groups, each containing eight mice: placebo group, antibiotic-treated group (treated with ampicillin), viable probiotic-treated group (LAB), heat-killed probiotic-treated group (HK-LAB), and heat-killed recombinant probiotic-treated group (HK-LAB/LF). The experiment consisted of two courses: the treatment course and the re-infection course. During the treatment course (as further explained in Fig. 2), each probiotic mixers was given orally through oral gavage once a day for 21 days after UPEC bacteria were introduced into the mice's bladders using a transurethral approach according to previous reports [31,32]. Notably, the antibiotic group received ampicillin (200 mg/kg/day) through oral gavage for seven days, followed by autoclaved MRS for another 14 days. Importantly, the duration of antibiotic treatment for uncomplicated urinary tract infections in clinical practice varies depending on the site of infection and the choice of medication. Generally, antibiotic treatment for cystitis lasts for 3−7 days, and thus, in the present study, we chose a 7-day course of antibiotics for bladder infection. Moreover, the placebo group received autoclaved MRS through oral gavage once a day for 21 days. Urine samples were collected from all mice on various days throughout the experiment, including day 0, 1, 3, 5, 7, 14, and 21. On day 22, during the re-infection course, all mice were challenged with 4×10^7 UPEC *E. coli* using the transurethral method. On day 25, the mice were additionally infected with UPEC *E. coli* and *K. pneumoniae* BCRC 10694 using the transurethral method. Collection of urine samples was performed at alternating intervals until day 10 of the reinfection phase (reinfection course), and TBCs in urine were promptly enumerated in accordance with the previous methodology [33].

2.6. Histologic analysis and severity scoring

Upon conclusion of the experiment, mice were dissected, and select tissues, such as the bladder and kidney, were excised and fixed in a 10% neutral buffered formalin overnight for assessment of inflammation status between different treatment

groups. Paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E), later examined, and scored by a blinded pathologist. The extent of lesions was scored on a scale ranging from one to five according to a previous report [34], reflecting the severity of inflammation: 1 = minimal (<1%); 2 = slight (1–25%); 3 = moderate (26–50%); 4 = moderate/severe (51–75%); 5 = severe/high (76–100%).

2.7. Statistical analysis

The statistical significance of differences was evaluated using the Student's *t*-test to compare benchmark results. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. In vitro antibacterial activities of heat-killed probiotic against uropathogens

In the present study, we determined and compared antibacterial activities between heat-killed probiotics (natural or LF-expression) and pathogenic strains. The representative inhibitory zones of probiotic or pathogenic preparations against uropathogens are shown in Fig. 1. We found that no heat-killed pathogenic preparations, including *E. coli* (ATCC 11775 and 13084), *K. pneumoniae*, *Salmonella* Typhimurium, and *S. aureus*, can display antibacterial activities. In contrast, some probiotic preparations displayed relatively potent antibacterial activities in comparison to antibiotics, as summarized in Tables 1 and 2. The tables show that all natural and recombinant LF-expression probiotic strains exhibited antibacterial activity against two pathogens, with inhibition zones ranging from 10 to 11 mm (weak), 12–14 mm (moderate), to greater than 15 mm (strong). In general, heat-killed recombinant probiotic strains displayed stronger antibacterial activity than natural probiotic strains against *E. coli* and *K. pneumoniae*. For example, the inhibitory zones of LF-expressing probiotic strains ranged from 13.3 mm to 20.7 mm (Table 2), while the inhibitory zone of natural probiotic strains was between 10.7 mm and 17.2 mm (Table 1). In addition, it was observed that the volume of medium used to resuspend probiotic pellets can impact the potency of their parabiotic counterparts. Larger medium volumes reduce antibacterial activities, while smaller volumes enhance it. Previously, we had demonstrated that inactivated recombinant LF-expression probiotics, which were inactivated through sonication, display potent antibacterial activities against important food-borne pathogens, surpassing those of their original host strains [28]. In the present study, we extend these findings to demonstrate the *in vitro* antibacterial activities of selected, inactivated probiotic strains against two uropathogens. Of importance is the inactivated probiotic cells in this study were processed via autoclaving, which presents an easily scalable inactivation method for the preparation of large amounts of probiotics. This could help the transition of these inactivated probiotic cells to clinical settings. In contrast, no antibacterial activities (no inhibitory zones) were observed in the solvent control (MRS medium) or any heat-killed pathogenic bacterial strains, including *E. coli* HER 1255, *S. aureus* ATCC 25953, and *Salmonella* Typhimurium ATCC 14028.

For the positive inhibitory control, chloramphenicol, at a concentration of 100 μg/mL, always demonstrated the strongest antibacterial activities against UPEC (30.3 ± 2.87) and *K. pneumoniae* (22.3 ± 2.22). However, the efficacy of chloramphenicol at a concentration of 50 μg/mL appeared relatively weaker or comparable to that of 25 μg/mL. It is possible that the discrepancy could be due to the experiments being completed at different times. Nevertheless, chloramphenicol at 100 μg/mL consistently contributed to strongest activities against both uropathogens than those at 25 and 50 μg/mL. Conversely, ampicillin at 100 μg/mL failed to display antibacterial activities against two uropathogens, and only a concentration of 400 μg/mL blocked the growth of both uropathogens. Altogether, these findings have supported that heat-killed probiotic preparation could be a good alternative therapy in managing UTIs, particularly in cases of antibiotic-resistant infections. Several probiotic strains were selected based on their relatively strong antibacterial activities, as demonstrated by inhibitory zones >10.7 mm against *E. coli* or >12 mm against *K. pneumoniae*. These strains were then used to prepare three probiotic mixtures, which were further used in the treatment of mice with UTIs as described in subsequent experiments.

3.2. Establish a murine UTI model: variations of bacteriuria between healthy and UPEC-challenged mice

It is widely acknowledged that urine culture is the gold standard diagnostic test for UTIs [35]. Thus, in our preliminary analysis, we employed urine cultures to determine bacterial load in urine samples from healthy mice (without inoculation) and UPEC-inoculated mice (Tables 3 and 4) to uncover the

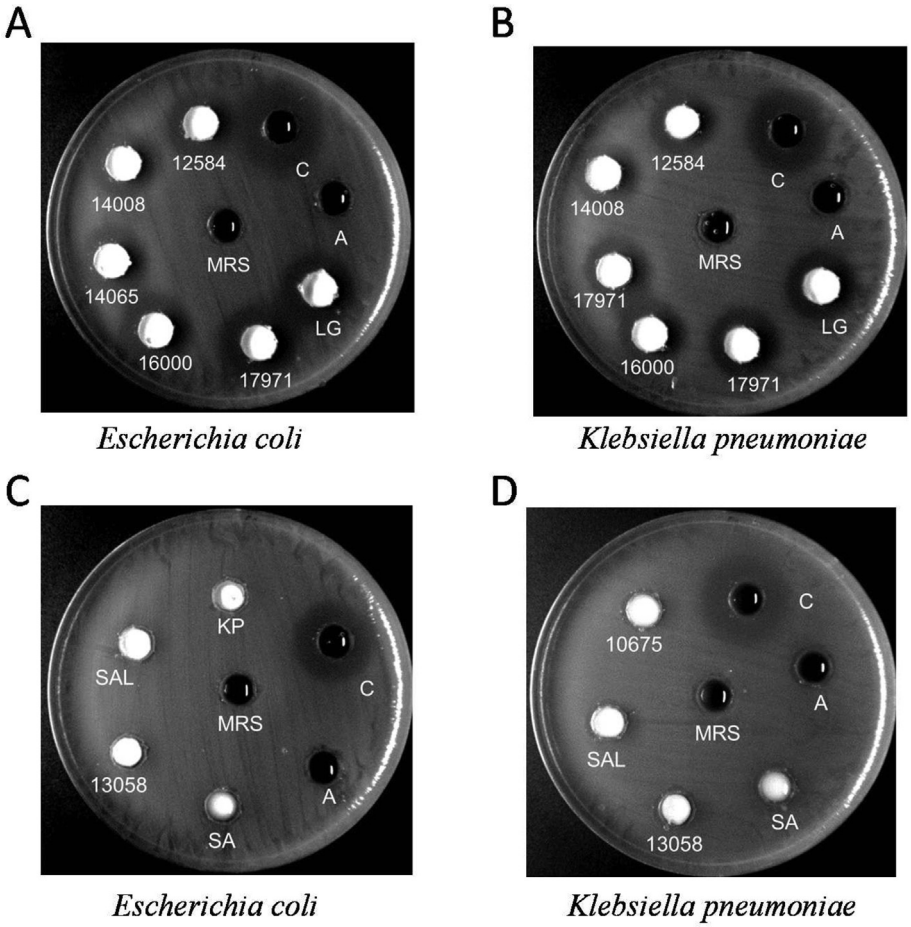

Fig. 1. ***Representative inhibitory zone (mm) against uropathogenic Escherichia coli BCRC 10694 or Klebsiella pneumoniae BCRC 10694 for individual heat-killed probiotic strains (A and B) and pathogenic preparations (C and D).*** *Uropathogenic bacteria was spread onto the NA plate, and individual heat-killed probiotic strains, the medium control (MRS), and antibiotics were inoculated into the pre-cut wells. 12584: Bifidobacterium breve Reuter (BCRC 12584); 14008: Lactobacillus delbrueckii sub sp. (BCRC 14008); 16000; Lactobacillus rhamnosus (BCRC 16000); 17971: Lactobacillus paraplantarum (BCRC 17971). LG: Lactobacillus gasseri; KP: K. pneumoniae; SAL: Salmonella Typhimurium ATCC 13311; 13058: Escherichia coli BCRC 13058; SA: Staphylococcus aureus ATCC 25923; MRS: deMan-Rogosa-Sharpe medium (solvent control); A: Ampicillin 100 μg/mL; C: Chloramphenicol 25 μg/mL.*

background of bacteriuria levels between healthy and UPEC-challenged mice. For this purpose, we conducted an enumeration of bacterial cells in urine by using NA or MCK plates to dissect TBCs or Gram-negative bacterial colonies, respectively. In Table 3, TBCs in urine of healthy mice (without any inoculation) were lower than 10^2 CFU/mL, and no Gram-negative bacterial cells were detected. However, in UPEC-challenged mice (Table 4), levels of Gram-negative bacterial cells and TBCs in urine were more than 10^6 CFU/mL 24 h post-inoculation, and bacteriuria persisted above 10^3 CFU/mL until 7 days post-inoculation, before both Gram-negative bacterial cells and TBCs declined from day 7–21. Furthermore, statistical analysis revealed that bacterial burden remained similar between days 1–7 ($p > 0.05$). These findings support that a single inoculation of UPEC into the bladder can contribute to a higher bacterial burden in urine for at least one week. In contrast, control mice (only inoculated with PBS) were found to be free of Gram-negative bacterial cells throughout the experimental course, with bacteriuria continually lower than the 10^3 CFU/mL criteria.

Traditionally, the threshold for bacterial counts in urine to be diagnostic was set at 10^5 CFU/mL for dominant bacterial species. However, this criterion could result in a large number of false negatives, potentially overlooking many relevant infections [36]. Thus, depending on the types of bacteria detected, some laboratories recommended the diagnosis of UTIs from a count of 10^3 CFU/mL as the established threshold [37]. Therefore, according to the threshold for UTIs, our preliminary findings have confirmed our successful establishment of UTIs in mice. Moreover, our results agree with prior reports

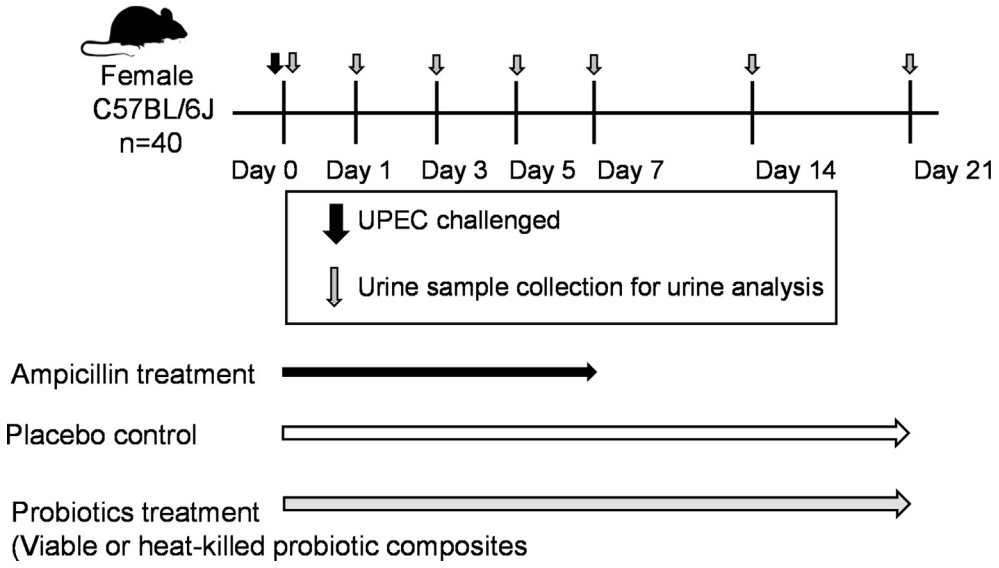

Fig. 2. ***Animal experiment design.*** *Eight-week-old mice BALB/c mice were inoculated into the urinary bladder with 4×10^7 colony forming units of uropathogenic E. coli by transurethral catheterization on day 0. Following infection, mice were randomized into five groups (8 mice in each group), and subjected to a daily oral gavage of placebo control (MRS broth), viable probiotic composite (LAB), or heat-inactivated probiotic composites (HK/LAB or HK/LAB/LF) supplements for three weeks, or a 7-day treatment of ampicillin. Urine samples were collected on days 0, 1, 3, 5, 7, 14, and 21 and subjected to bacterial culture analysis to enumerate bacterial colonies in urine.*

indicating a spontaneous decline of bacterial load in urine following bacterial challenge into the bladder of mice, especially 7 days post-infection [31].

3.3. Efficacies of orally administered composites of probiotics against mice suffered from UTI

We demonstrated that several heat-killed strains of lactobacillus and bifidobacterial could confer relatively strong antibacterial activities *in vitro*. Then, we further evaluated these antibacterial activities *in vivo* using a mice UTI model. Conventionally, each potential probiotic strain should be tested in animal experiments to determine its efficacy against infection, but we had at least 10 potential probiotic strains that needed to be evaluated *in vivo* (Table 1). Therefore, we explored antibacterial activities of mixtures between viable and heat-

Table 1. Average inhibitory zone of heat-killed lactobacillus or bifidobacterial strains against uropathogens via the agar diffusion test. The average inhibitory zones of ampicillin in three concentrations are also shown for antibiotic control. Each value represents the mean ± SD value calculated from three independent replicates.

Probiotic preparation	Inhibitory zone (mm) against pathogen	
	Escherichia coli BCRC 10675	*klebsiella pneumoniae* BCRC 110694
Lactobacillus casei IAM 10475	15.5 ± 1.4	17.2 ± 1.6
Lactobacillus casei ATCC 393	12.5 ± 0.5	13.5 ± 1.22
Lactobacillus paracasei ATCC 25598	14.8 ± 1	16 ± 0.63
Lactobacillus fermentum ATCC 11739	11.8 ± 0.8	13.8 ± 0.98
Bifidobacterium breve Reuter NCDO 1452	11.7 ± 0.8	13.5 ± 1.05
Lactobacillus delbrueckii subsp.*subsp.* NCDO 2394	11.8 ± 0.4	13.5 ± 1.38
Bifidobacterium angulatum ATCC 29521	11.8 ± 0.4	13.2 ± 0.41
Lactobacillus rhamnosus ATCC 53103	10.7 ± 0.8	12.7 ± 1.03
Lactobacillus paraplantarum ATCC 700210	11.5 ± 0.5	12.7 ± 0.52
Lactobacillus gasseri (laboratory stock; HM-1	11.8 ± 0.4	13 ± 0.63
Lactobacillus gasseri (laboratory stock; HM-2)	11.7 ± 0.8	12.7 ± 0.82
Lactobacillus gasseri (laboratory stock; HM-3)	11.8 ± 1.0	12.8 ± 0.98
Solvent control	No zone	No zone
Ampicillin (100 μg/mL)	No zone	No zone
Ampicillin (200 μg/mL)	12.8 ± 0.96	No zone
Ampicillin (400 μg/mL)	22 ± 1.83	13 ± 0.82
Chloramphenicol (25 μg/mL)	19.5 ± 0.58	17.8 ± 0.5
Chloramphenicol (50 μg/mL)	18 ± 0.82	14.5 ± 0.58
Chloramphenicol (100 μg/mL)	30.3 ± 2.87	22.3 ± 2.22

Table 2. Average inhibitory zone of heat-killed recombinant lactoferrin-expression probiotics against uropathogens via the agar diffusion test. The average inhibitory zones of ampicillin in three concentrations are also shown for antibiotic control. Each value represents the mean ± SD value calculated from three independent replicates.

Inhibitory zone (mm) against pathogen	*Escherichia coli* BCRC 10675	*klebsiella pneumoniae* BCRC 110694
Probiotic preparation		
Human Lactoferrin (HLF) expression probiotic		
L. delbrueckii/HLF	13.8 ± 1.0	14.7 ± 1.0
L. paraplantarum/HLF	13.8 ± 0.4	14.3 ± 1.2
L. rhamnosus/HLF	14.8 ± 1.3	16.0 ± 1.0
Bovine Lactoferrin (BLF) expression probiotic		
P. pentosaceus Mees/BLF	13.3 ± 1.2	13.8 ± 0.8
B. breve Reuter/BLF	17.3 ± 0.5	17.0 ± 0.9
L. delbrueckii/BLF	16.2 ± 1.0	16.2 ± 1.7
L. paraplantarum/BLF	19.0 ± 1.2	20.7 ± 1.2
L. gasseri/BLF	14.4 ± 1.8	14.7 ± 1.5
Porcine Lactoferrin (PLF) expression probiotic		
B. angulatum/PLF	13.5 ± 0.8	14.7 ± 1.0
B. catenulatum/PLF	16 ± 1.2	17 ± 1.0

Table 3. Quantified bacterial load (Log 10 CFU/mL) in urine of healthy mice (n = 12). Urine samples were collected from healthy mice twice a day over 7 days. The bacterial colonies in urine were enumerated by counting bacterial colonies on nutrient agar (NA) and MacConkey agar (MCK) plates. Mean ± SD value is shown.

	Bacteria colonies in urine (Log 10)			
	Day 1	Day 3	Day 5	Day 7
Nutrient Agar (NA)[a]	1.5 ± 1.1	1.2 ± 1.3	1.0 ± 1.4	1.0 ± 1.2
MacConkey agar (MCK) [b]	0	0	0	0

[a] Nutrient agar plates used for determining the total bacterial counts in urine.
[b] MCK agar plates employed for determining the number of viable Gram-negative bacterial cells in urine.

killed probiotics, as the use of multi-strain probiotic supplements in clinics is widely accepted globally. In Fig. 3, the effects of oral placebo, ampicillin, and three probiotic composites on UTI mice are shown. Three therapeutic indicators were analyzed to compare treatments, including the degree of reducing bacteriuria, the time required for treatments to take effect, and the bacteriological cure rate (no bacterial colonies in urine). The results indicated that viable bacteria count slightly decreased over time in the placebo group (Fig. 3A), but this reduction was not statistically significant throughout the experimental course ($p > 0.05$), demonstrating the inefficacy of placebo treatment in reducing bacteriuria of UTI mice. In contrast, all four treatments significantly improved the bacterial burden in urine by approximately 10^3–10^4 fold, albeit with a different course. For example, in the ampicillin-treated group (Fig. 3A; treated with 200 mg ampicillin/kg/day), although UPEC-challenged mice were administered with ampicillin for only 7 days, the treatment significantly ($p < 0.05$) reduced bacteriuria of every mouse throughout the experiment. In this group, bacteriuria was reduced from 10^7 CFU/mL (day 0) to 10^{1-3} CFU/mL (day 1 to day 21). In contrast, although LAB, HK-LAB, and HK-LAB/LF supplementation also reduced the bacteriuria significantly, the administration of probiotics could not improve bacteriuria in every mouse. Additionally, compared to ampicillin therapy, probiotic therapies also took longer to improve bacteriuria. For example, LAB treatment significantly ($p < 0.05$) decreased bacteriuria between days 14–21 for about 10^{3-4} folds, and HK-LAB treatment significantly ($p < 0.05$) reduced bacteriuria on days 5–21. Importantly, HK-LAB/LF treatment significantly ($p < 0.05$) reduced bacteriuria on days 3–21.

We also observed that bacteriuria could disappear in some mice of all mice groups and calculated the bacteriological cure rates (no bacteria colonies in urine culture) between five treatment groups. As

Table 4. The variations of Escherichia coli and total bacterial cells in urine samples between PBS- and UPEC-challenged mice. Female C57BL/6J mice were divided into: UPEC-challenged group (+) and PBS-inoculated group (−). In the UPEC-challenged group, mice were infected via transurethral catheterization with 4 × 10^7 CFU/mice of uropathogenic Escherichia coli. In PBS-challenged group, mice were injected with sterile PBS. Urine samples were collected for 21 days. All data are presented as mean ± SD, with n = 6 for each group.

Days post-infection	Total bacterial count [a]		Gram-negative bacterial counts [b]	
	UPEC-inoculated mice	PBS-inoculated mice	UPEC-inoculated mice	PBS-inoculated mice
Day 1	6.35 ± 0.44[d]	1.15 ± 1.59	6.08 ± 0.90	0.00
Day 3	5.36 ± 1.67	2.98 ± 0.19	5.81 ± 1.22	0.00
Day 5	5.38 ± 1.99[d]	2.34 ± 0.96	4.99 ± 2.87	0.00
Day 7	4.86 ± 1.66[d]	1.70 ± 1.25	3.13 ± 3.46	0.00
Day 14	2.92 ± 1.51[d,c]	1.17 ± 1.30	1.14 ± 1.76[c]	0.00
Day 21	2.21 ± 1.75[d,c]	1.01 ± 1.09	1.08 ± 1.68[c]	0.00

[a] Nutrient Agar Plates used for determining the total bacteria count in urine.
[b] MCK Agar Plates used for determining the number of viable *E. coli* in urine after challenging of UPEC.
[c] Indicates the number of viable bacteria is statistically different ($p < 0.05$) when compared with day 1 (24 h post-infection).
[d] Indicates the number of viable bacteria enumerated via NA plate is statistically different ($p < 0.05$) to blank control (non-infected group) on the same day.

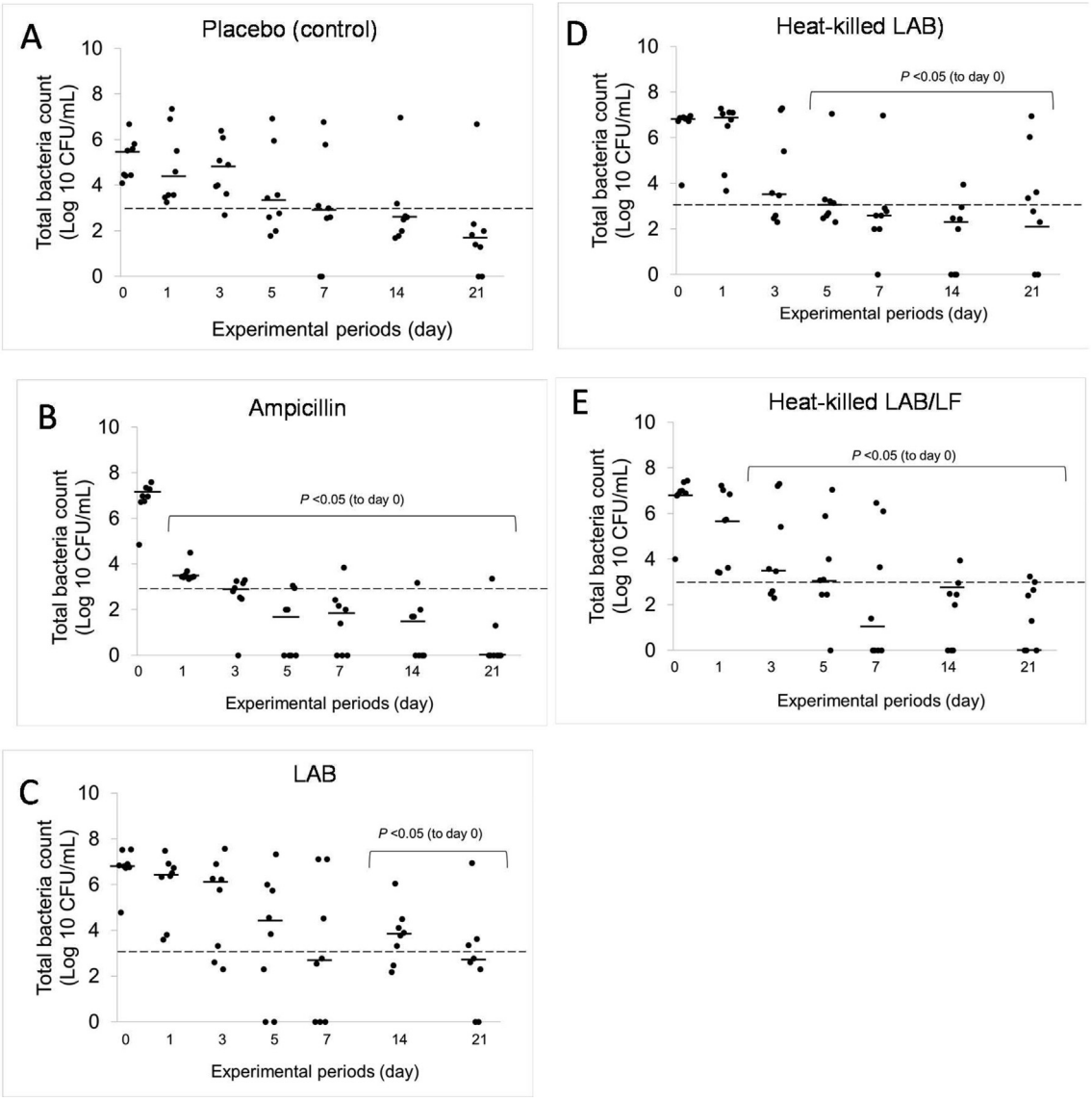

Fig. 3. ***Total bacterial counts in urine across five treatments****. Urine was collected from mice following UTI induction (day 0) and subsequent administration of placebo (MRS), probiotic composite, or two heat-killed probiotic composites (HK/LAB or HK/LAB/LF) via oral gavage. Mice in the ampicillin-treated group received ampicillin for 7 days (day 1 to day 7), while mice in the other four groups received placebo or probiotic preparations for 21 days (day 1 to day 21). At predetermined time intervals (days 0, 1, 3, 5, 7, 14, and 21), aliquots of urine were collected, serially diluted, and plated on agar (37 °C, 18–24 h) to determine the total bacterial colony. Each symbol represents a single mouse, with median values represented by bars. *p < 0.05: indicates the total bacterial count significantly different from the day 0 control. In the Log10 scale, the absence of bacteriuria (0 CFU/mL) was arbitrarily assigned a value of 1 CFU/mL, and the dotted line within the figures represents a bacterial concentration of 10^3 CFU/mL.*

shown in Fig. 4, although mice had received ampicillin for only 7 days, this led to a 13%, 50%, 38%, 50%, and 75% bacterial cure rate on days 3, 5, 7, 14, and 21, respectively. Although ampicillin treatment reduced the bacterial load in urine (Fig. 3B) immediately and by a considerable amount, only half of the mice (50%) displayed no bacteriuria during the treatment course. Nevertheless, there were sustained benefits until day 21 after cessation of antibiotics on day 7, and the highest cure rate was recorded on day 21 (75%). As for LAB-treated mice, about 25%, 38%, and 25% of mice had no bacteriuria on days 5, 7, and 21, respectively. In HK-LAB mice groups, 13%, 38%, and 38% of mice had no bacteriuria on days 7, 14, and 21, respectively. Finally, in HK-LAB/LF mice group, 13%, 50%, 50% and 75% of mice had no bacteriuria on days 5, 7, 14, and 21, respectively. In contrast, the placebo-treated mice group, bacteriuria was absent in only 25% of mice on days 7 and 21 (Fig. 4). It is important to note that in our preliminary analysis, even healthy mice can harbor limited bacterial cells in urine, usually below 100 CFU/mL. Thus, all the supplements administered, including ampicillin and three probiotic

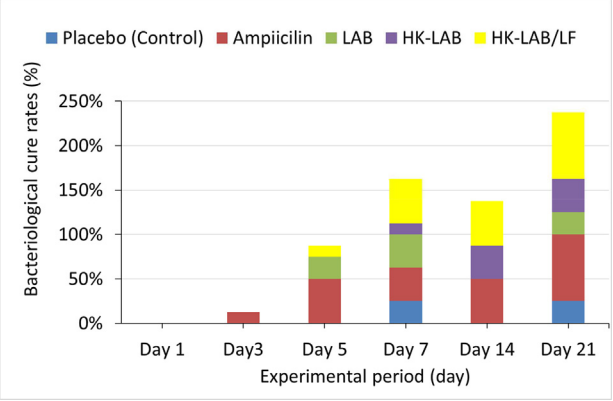

Fig. 4. ***Bacteriological cure rates between treatment mice groups.*** *See Fig. 2 for experimental design. Briefly, on day 0, the urinary bladders of mice were inoculated with UPEC. After that, one group of mice received ampicillin for 7 days (positive treatment control); the remaining four groups received placebo or probiotic mixtures (LAB, HK-LAB, or HK-LAB/LF) for 21 days. Urine from mice was collected on days 0, 1, 3, 5, 7, 14, and 21, and total bacterial counts in urine were determined. In addition, the ratio of mice with no bacteriuria (0 bacterial cells in urine) was counted.*

preparations, were capable of further reducing the bacterial load in urine, albeit with varying degrees of efficacy. Overall, regarding "bacteriological cure rates", the three probiotic mixtures can provide benefits against UTI in mice, but ampicillin therapy remains the most efficient among the four treatments; however, even ampicillin therapy cannot completely cure every UTI in mice. Intriguingly, HK-LAB/LF therapy provides equal long-term therapeutic effects to ampicillin (from days 7–21). In light of these findings, we believe that these probiotic mixes, either viable or heat-killed probiotic compounds, can be used as an alternative therapy, especially in prophylaxis, to replace prophylactic antibiotic therapy. These could mitigate the spread of antibiotic-resistant bacteria as well as avoid interfering with the composition of normal flora. Furthermore, given the varying degrees of efficacy demonstrated by the three probiotic composites against UTIs *in vivo*, it is of interest to further explore the mechanisms between inactivated probiotics and their viable composites against UTIs.

The present study aims to investigate the potential benefits of especially "inactivated probiotic mixture" for the treatment of UTIs. It should be noted that several clinical studies have provided support for the use of specific "viable probiotics" as a safe and effective intervention in preventing recurrent UTIs [38–41]. However, conflicting reports have emerged that do not endorse the use of probiotics as prophylaxis for UTIs [42]. The divergence in conclusions among studies may be attributed to the varying efficacy of probiotics, which heavily depends on the strain, dosage, and timing of administration. Currently, there is no consensus regarding the optimal dosing and duration of viable probiotic use for combating UTIs [40]. Notably, it may not be appropriate to directly compare the findings of our study with previous reports, as most of the existing literature focuses on viable probiotic supplementation for the prevention of recurrent UTIs, whereas our study primarily concentrates on the treatment of UTIs. A notable differentiation between the prevention and treatment models resides in the sequential order of interventions. In the prevention experiment, the initial step entails the consumption of specific probiotics, followed by the subsequent observation of potential recurrent UTIs in patients. Similarly, within the animal prophylaxis model, the prescribed course involves the initial consumption of specific probiotics, succeeded by the deliberate infection of animals with uropathogenic UPEC or similar pathogens. In contrast, the treatment model encompasses the infection of animals with pathogenic bacteria, followed by the subsequent administration of therapeutic agents. Nevertheless, given that long-term or low-dose prophylactic antibiotics have been utilized in clinical settings to prevent recurrent UTIs [43,44], it is plausible that our therapeutic probiotic mixers could also exhibit prophylactic effects against recurrent UTIs. This speculation warrants further investigation in our subsequent study.

On the other hand, exogenous HLF exhibited a protective effect against UTIs caused by UPEC in a co-culture of human bladder epithelial cells and neutrophils [45]. Additionally, it has been reported that BLF could aid in reducing the invasion of *E. coli* into urinary bladder epithelial cells [46]. In this previous study, an observational survey was conducted on 33 patients with recurrent cystitis who received oral treatment with BLF alone or in combination with antibiotics and/or probiotics. The results showed a significant decrease in cystitis episodes ($p < 0.001$) compared to the 6-month period prior to BLF treatment. While acknowledging certain limitations in this recent report, it has suggested that BLF could be a valuable and safe treatment option for recurrent cystitis [46]. Furthermore, in a murine UTI model, a single intravesicular dose of HLF was found to significantly reduce bladder bacterial burden and neutrophil infiltration. This indicates that HLF plays a crucial role as an innate immune response modulator in the urinary tract and holds potential for novel therapeutic interventions in UTI [45]. However, it is important to note that direct comparison between our study and the aforementioned reports may not be appropriate

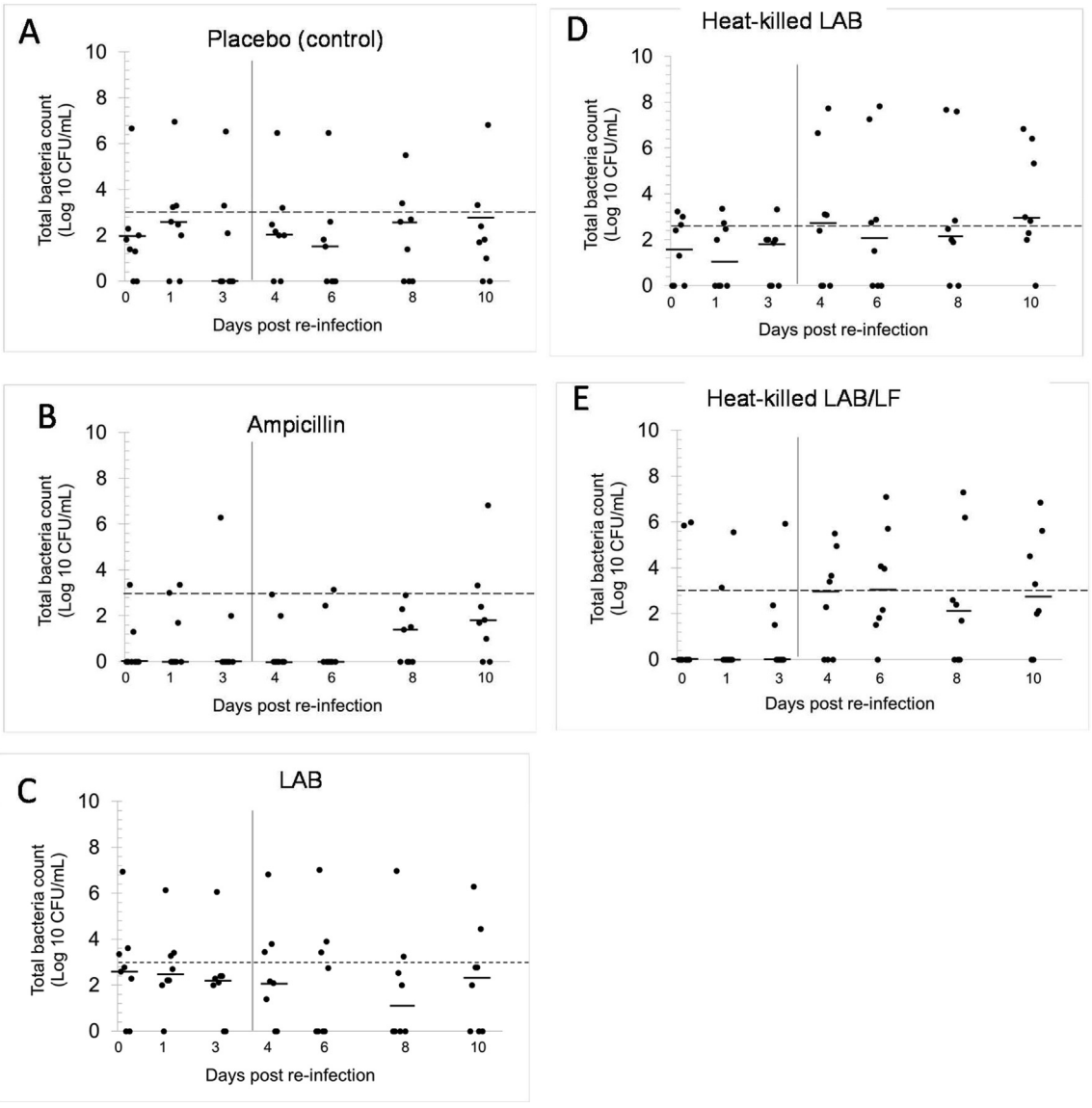

Fig. 5. ***Variations of bacterial load in urine during the reinfection course.*** *UPEC-challenged mice were divided into five experimental groups and orally administered placebo, ampicillin, or probiotic mixtures consisting of LAB, HK-LAB, or HK-LAB/LF (see Fig. 2 for experimental design). Then, as part of the reinfection regimen, mice were re-infected with UPEC at the end of the experiment (day 22) through transurethral catheterization, and urine samples were harvested every other day (day 1 and day 3; reinfection course). Additionally, the mice were re-infected with E. coli plus K. pneumonie on day 3 of re-infection course, also through transurethral catheterization. Urine samples were collected every other day (day 4 till day 10; reinfection course). The bacterial colonies in urine were enumerated. Each data point represents bacterial concentration from individual animals, with median values represented by bars. For the Log10 scale, the no bacteriuria of 0 CFU/mL was arbitrarily assigned as 1 CFU/mL. Dotted line indicate bacterial colonies in urine at 10^3 CFU/mL.*

due to the differing administration approaches. The previous report employed intravesicular administration, while our study utilized oral administration. Nonetheless, we believe that oral administration of probiotics offers greater convenience and acceptance in comparison to urethral instillation, and our findings provide further evidence supporting the ability of recombinant BLF compound (recombinant LF-expression probiotics) to inhibit UPEC growth both *in vitro* and in an animal model.

3.4. Preventative effect of orally probiotic against UTIs

The data presented above support the therapeutic effects of both viable and heat-killed probiotic composites in treating UTIs in terms of improving bacteriuria and BCRs. We therefore wondered whether previous interventions of ampicillin, probiotics, or paraprobiotics may also offer protective effects against re-infection or relapse of UTIs. In

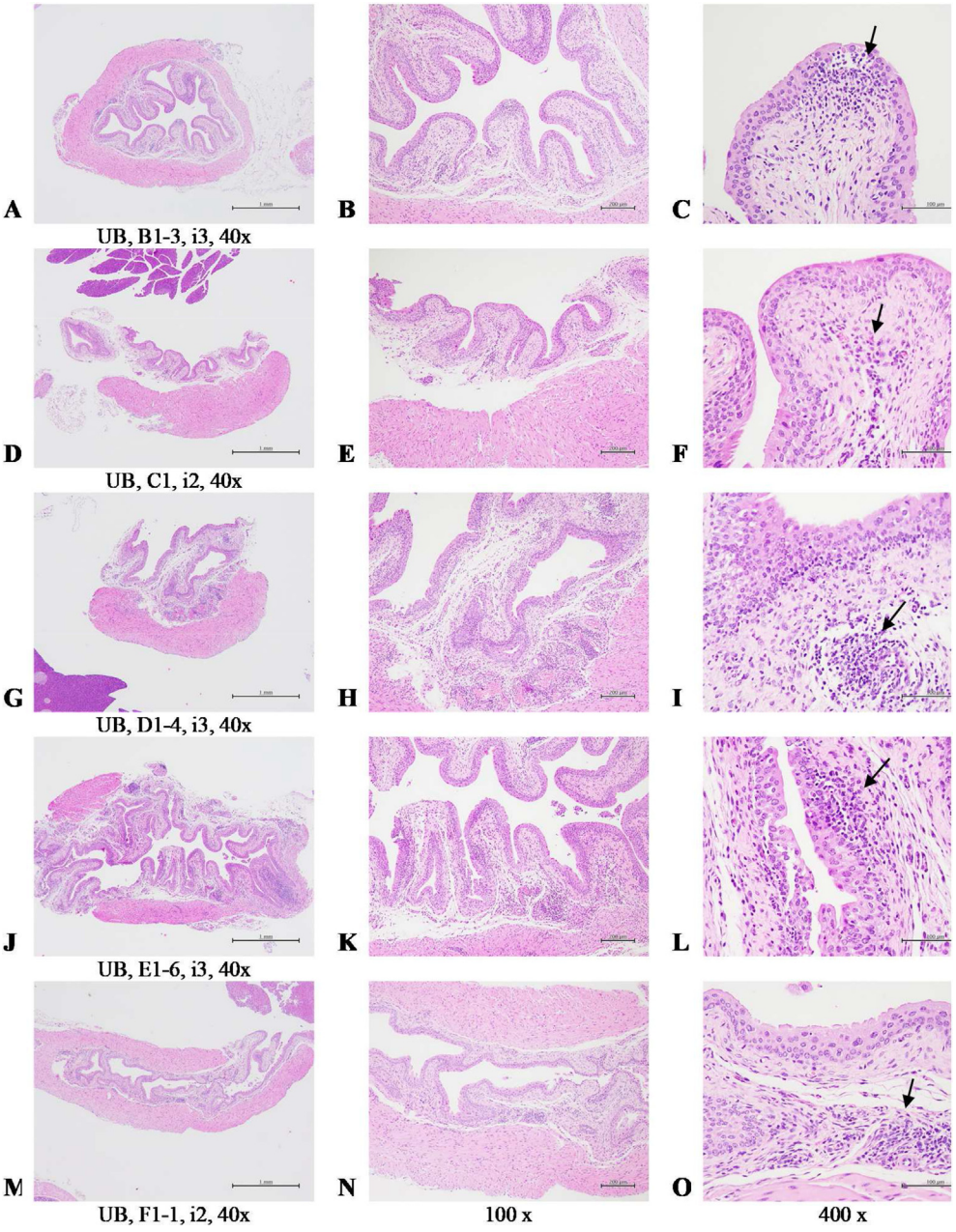

Fig. 6. ***Histopathological evaluation of the urinary bladder in uropathogen-induced urinary inflammation in female mice post one term of treatment and two terms of re-infection.*** *Multifocal inflammation in the urinary bladder was graded moderate (3) in the control group (A-C, B1/3), slight (2) in the Ampicillin group (D-F, C1), moderate (3) in the LAB group (G-I. D1/4), moderate (3) in the heat-killed LAB group (J-L. E1/6) and slight (2) in the heat-killed LAB/LF group (M-O. F1/1). H&E. 40 x, 100 x and 400 x.*

Fig. 5, following the first inoculation, a comparison of bacterial load in urine and statistical analysis was made. No significant difference between pre- and post-challenge courses was found in any mice group. Thus, re-infection of UTIs in mice failed to occur, even in the placebo mice group. Next, all mice were simultaneously re-inoculated with UPEC and *K. pneumoniae* on day 25. Still there was no significant difference between pre- and post-challenge courses among all mice groups. Therefore, based on our current experiment design, it is not possible to definitively determine whether our probiotic composites provide prophylactic effects against recurrent or relapsing UTIs. Our mice (11-week-old) may have been relatively older (stronger) and could resist the same-strain recurrent UTIs. For example, a previous study demonstrated that both age and parity are interrelated factors contributing to UTI susceptibility, with younger, nulliparous animals exhibiting 10 to 100-fold higher bacterial titer

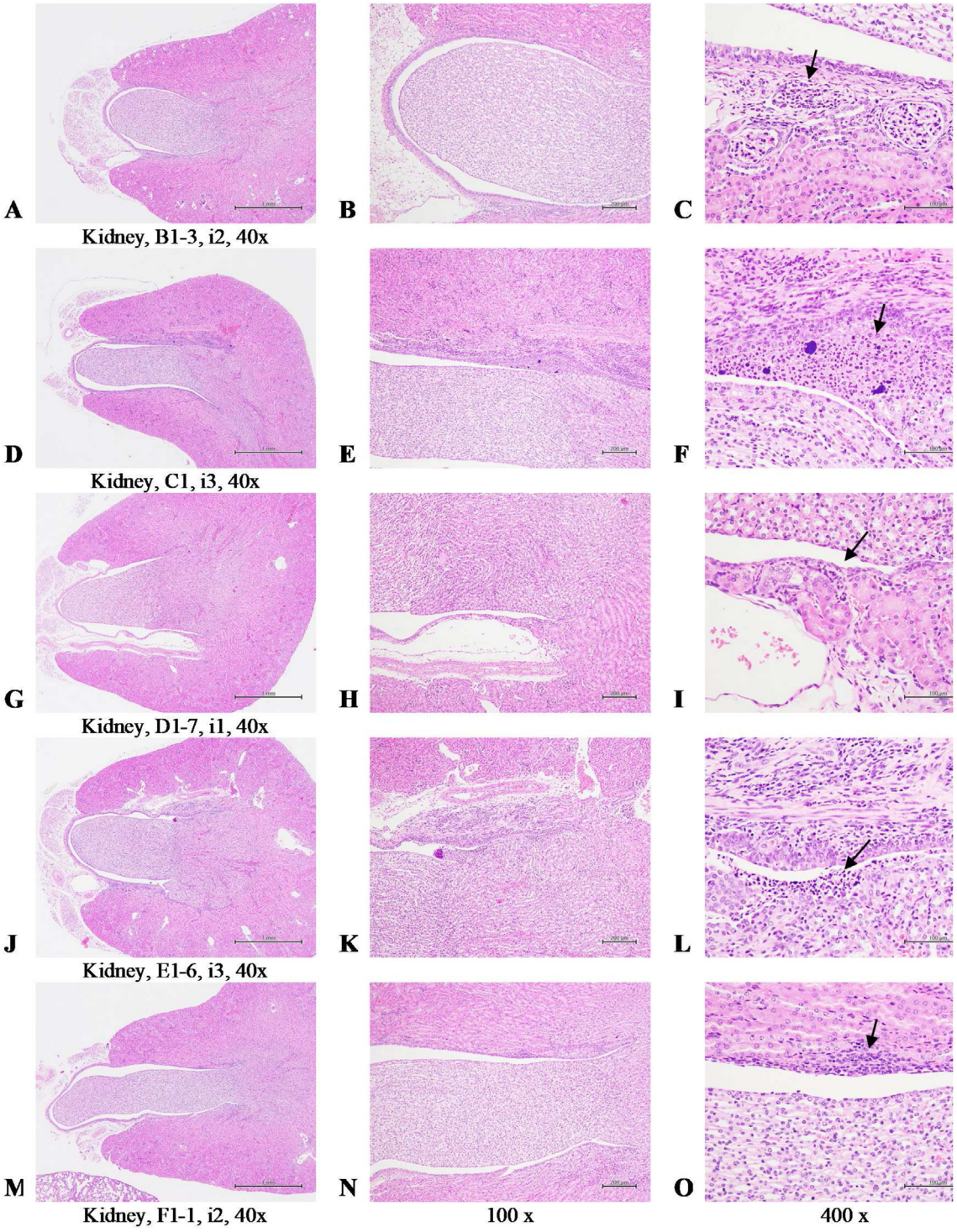

Fig. 7. ***Histopathological evaluation of the kidney in the uropathogen-induced urinary inflammation in female mice after one term of treatment and two terms of re-infection.*** *Multifocal inflammation in kidney pelvis was graded slight (2) in the control group (A-C, B1/3), moderate (3) in the Ampicillin group (D-F, C1), minimal (1) in the LAB group (G-I. D1/7), moderate (3) in the heat-killed LAB group (J-L. E1/6) and slight (2) in the heat-killed LAB/LF group (M-O. F1/1. H&E. 40 x, 100 x and 400 x.*

compared to older animals. Thus, mice become resistant to specific uropathogens with age [47]. Additionally, an initial *E. coli* UTI, whether chronic or self-limiting, leads to a long-lasting molecular imprint on the bladder tissue that changes the pathophysiology of subsequent infections, influencing both host susceptibility and disease outcome [48]. Furthermore, previous reports have observed

Table 5. Mean histopathological score of inflammation in the bladder and kidney of UTI mice following supplementation with placebo or three probiotic mixtures for three weeks, followed by a re-infection course for an additional 10 days. Degree of lesions was graded from one to five depending on severity: 1 = minimal (<1%); 2 = slight (1–25%); 3 = moderate (26–50%); 4 = moderate/severe (51–75%); 5 = severe/high (76–100%).

Organ		Urinary bladder	Kidney
Histopathology		Inflammation, multifocal	Inflammation, pelvis, multifocal
Treatment	Placebo	2.86 ± 0.9	1.75 ± 1.58
	Ampicillin	2 ± 1	1.43 ± 1.13
	LAB	3 ± 1.26	1.38 ± 1.19
	Heat-killed LAB	2.43 ± 0.98	1.25 ± 1.39
	Heat-killed LAB/LF	1.86 ± 1.21	1.5 ± 1.85

that C57BL/6J mice could resist chronic cystitis after a single infection, and thus, the adaptive immune responses would help protect against the same uropathogenic strain during the next UTI [49,50]. However, another study demonstrates that the clearance and susceptibility to recurrent UTIs is strain-dependent. For example, UTI89 and CFT073 both caused infections that persisted for at least two weeks in a murine UTI model. Nonetheless, UTI89 infections persist indefinitely, while CFT073 infections started to clear two weeks after inoculation and were uniformly cleared within eight weeks. This study highlights the complex interplay between the broad genetic diversity of UPEC and the host's innate and adaptive immune responses during UTI, which are indeed dependent on bacterial strain [51]. Collectively, these may explain why it was not easy in the present study, to establish a recurrent UTI infection in the same mice. Therefore, for our next study, healthy mice will be administered our probiotic supplements, then challenged with different uropathogens to determine the exact roles of viable or inactivated-probiotic mixtures in prophylactic therapy. Despite this, our probiotic compounds can already provide therapeutic activities against one UPEC in the murine UTI model. Hence, it can be reasonably inferred that these compounds may play a positive role in prophylactic therapy.

3.5. Histopathological evaluation of the bladder and kidney in the uropathogens-induced urinary inflammation

Our data indicated that re-infected with uropathogens were not successful in any mice groups in terms of bacteriuria. We further evaluated histopathological changes between mice groups after this re-infection course. The representative images of histopathological examination of the bladders and kidneys are indicated in Figs. 6 and 7, respectively. The images depict a relatively minor to medium inflammatory status in all five mice groups. This could be attributed to all mice having recovered or improved considerably from the bacteriuria, as the bacterial load in urine was lower than 10^3 CFU/mL by the end of the experiment (Fig. 5). In line with this, previous studies indicate that the bladder mucosa heals and returns to a non-inflamed state after a period of convalescence [48,52]. Subsequently, the mean histopathological scores of changes to kidney and bladder were also calculated and presented in Table 5. The cystitis score of the HK-LAB/LF mice group (1.86 ± 1.21) was relatively lower than that of other mice groups, but no statistical differences were discerned between treatment groups. Moreover, treatments of ampicillin, LAB, HK-LAB, and HK-LAB/LF all contributed to relatively lower bladder inflammatory scores when compared to the placebo mice group. Otherwise, the inflammatory scores of kidneys in HK-LAB/LF (1.5 ± 1.85), HK-LAB (1.25 ± 1.39), and LAB (1.38 ± 1.19) groups were found to be relatively lower than the placebo group (1.75 ± 1.58). However, no significant difference or diverse scores were observed between the five treatment groups. Collectively, the level of bacteriuria appears to be positively correlated with the inflammation status in bladder and kidney. Nevertheless, histopathological evaluation of the bladder and kidney all supports the notion that most mice recovered from UTIs and suggests that the adaptive immune responses could help protect from re-infection of UTIs.

4. Conclusion

In the present study, antibiotic treatments proved successful in improving UTI in mice; however, they may demonstrate reduced effectiveness or even complete ineffectiveness against antibiotic-resistant infections. Moreover, administering antibiotics to treat UTIs can disrupt the ecological balance of normal microflora, and prolonged antibiotic use often contributes to the emergence of multidrug-resistant organisms. Considering the growing concern of antimicrobial resistance worldwide, researchers are exploring alternative non-antibiotic

prophylaxis strategies for recurrent UTIs. Our results provide evidence that most tested probiotic strains display *in vitro* antibacterial activities against two uropathogens. Additionally, we further demonstrate that the oral administration of composites of selected heat-killed recombinant or natural probiotic strains can confer good therapeutic efficacy against UTI in a mice model. The use of inactivated probiotic composites could be a novel alternative approach to treating UTIs, but further investigation is necessary to understand the mechanisms by which individual probiotic stains inhibit or reduce uropathogenic infections *in vivo* and *in vitro*. Furthermore, the HK-LAB/LF composite appears to be the most promising candidate against UTIs among the three probiotic composites. Further testing is needed to evaluate its efficacy against UTIs caused by other bacterial strains. Finally, we posit that these heat-killed probiotic mixtures have the potential to serve as prophylactic therapy for the prevention of UTIs. However, it is imperative to conduct further experiments to validate this hypothesis.

Author contributions

Conceptualization, Po-Wen Chen; Data curation, Vo Thi Hong Van, Yueh-Jen Hsieh, Yu-We Ku, Wei-Chen Shiu, and Bo-Yuan Chen; Funding acquisition, Zhen-Shu Liu and Po-Wen Chen; Analysis and interpretation of data: Vo Thi Hong Van, Zhen-Shu Liu and Po-Wen Chen; Resources, Po-Wen Chen and Zhen-Shu Liu; Writing – original draft, Po-Wen Chen; Writing – review & editing, Zhen-Shu Liu, Yueh-Jen Hsieh, and Po-Wen Chen.

Conflict of interest

No conflict of interest exists.

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