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# Probiotics with cranberry extract and vitamin C in women with recurrent urinary tract infections: a randomized controlled trial

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# Abstract

**Introduction and Hypothesis**: Recurrent urinary tract infections (rUTIs) pose a significant clinical burden in women and contribute to antibiotic use. This study investigates the probiotic properties of *Lactiplantibacillus plantarum* KABP062 and KABP063, and their efficacy combined with cranberry extract and vitamin C in reducing rUTIs recurrence.

**Methods**: Probiotic functionality and safety were assessed using *in vitro* and *in silico* approaches, including antimicrobial activity against uropathogens, gastrointestinal tolerance, enzymatic safety, and genomic screening for bacteriocin and biogenic amine biosynthesis genes. Clinical efficacy of the multi-component formulation was investigated in a double-blind, randomized, placebo-controlled trial involving women with active or recent rUTIs. Participants received daily capsules containing probiotic strains (≥ $10^8$ colony forming units, CFUs), cranberry extract (240 mg) and vitamin C (40 mg), or placebo for six months. The primary endpoint was the proportion of women with physician-confirmed recurrences. Secondary outcomes included number of recurrences, antibiotic use (days), vaginal microbiota, and safety.

**Results**: Both *L. plantarum* strains inhibited multiple uropathogens, including *Escherichia coli*, and showed gastrointestinal tolerance and a favorable safety profile. A total of 106 women were randomized. After six months, the proportion of participants experiencing at least one UTI did not differ between groups (p = 0.689). However, the active group was associated with a lower number of UTIs compared to control group (p = 0.013). Antibiotic use was also significantly reduced (p = 0.031) in active group vs control, alongside decreases in seven UTI-associated genera in the vaginal microbiota, including *Escherichia/Shigella*. Adverse events were comparable between groups.

Conclusions:

The multi-component formula was safe. Although the primary endpoint was not met, the intervention was associated with a lower number of UTIs and reduced antibiotic use, together with potential reductions in the vaginal uropathogen reservoir.


# Introduction

Recurrent urinary tract infections (rUTIs) are defined as at least three episodes within the previous 12 months or two episodes within 6 months and may result from either a relapse after treatment or reinfection with a different microorganism [1, 2]. Although incidence is difficult to determine, about half of women experience a urinary tract infection (UTI) in their lifetime, and 30–40% have a recurrence within six months [3]. rUTIs impair patients´ quality of life, pose clinical challenges, and create a significant socioeconomic burden on healthcare systems [4].

Most rUTIs (70–95%) are caused by uropathogenic *Escherichia coli* (UPEC), although other aerobic bacteria such as *Klebsiella* spp., *Enterococcus* spp, *Staphylococcus* spp., *Proteus* spp., and *Pseudomonas*

spp. are also common. A smaller proportion of cases involve anaerobes often undetected by routine diagnostics, including genera *Peptoniphilus*, *Parvimonas*, *Anaerococcus*, *Finegoldia*, *Prevotella* or *Actinotignum* among others [5–9]. Uropathogens may migrate from the intestine or vagina to the urinary tract. Although less commonly acknowledged, the vagina also serves as a reservoir for urogenital pathogens. This is supported by studies that have identified identical strains of *E. coli* in both the urinary tract and the vaginal microbiota of women with a history of UTIs [10, 11].

Long-term antibiotic prophylaxis remains widely used to prevent rUTIs; however, evidence for its sustained effectiveness after treatment ends is limited [12], and prolonged exposure may lead to antimicrobial resistance, adverse events, and microbiota disruption due to the non-selective action [13, 14]. These limitations have stimulated interest in functional ingredients as preventive strategy against rUTIs [15]. Probiotics are defined as “live microorganisms that when administered in adequate amounts, confer a health benefit on the host” [16]. Although certain probiotic strains show promise for UTI treatment and prevention [1, 17, 18], the overall evidence remains limited, likely due to heterogeneity in strains, dosing regimen and patient populations [23]. Specifically, we previously showed strains *Lactiplantibacillus plantarum* KABP062 and KABP063 may inhibit uropathogens [19]. Evidence also supports the use of cranberry for reducing recurrence risk in women with rUTIs [1, 20, 21], and it is known to inhibit UPEC *E. coli* adhesion to urinary epithelial cells [22]. Vitamin C acidifies urine, inhibiting pathogenic bacterial growth, and contributes to immune defense [23]. Its intake has been associated with a decreased incidence of UTIs in women [24]. Combining multiple components may enhance clinical benefits, as suggested by previous studies and by the concept that combining agents with complementary mechanisms of action may improve the prevention of recurrent UTIs [25, 26]. Nevertheless, well-designed randomised controlled trials are needed to achieve definitive evidence of specific combinations.

This study aimed to investigate the probiotic potential of *L. plantarum* KABP062 and KABP063, and to evaluate a formula combining these strains with cranberry extract and vitamin C – ingredients that might exert additive or synergistic effects – for rUTI prevention in women.

## Materials and Methods

**Characterization of strains Lactiplantibacillus plantarum KABP062 and KABP063**

# In vitro assays

*L. plantarum* KABP062 and KABP063, both of human origin, belong to Kaneka-AB-Biotics’ culture collection. Strains used as controls and indicator strains as well as growth conditions are listed in Supplementary Table S1. *In vitro* and *in silico* assays were performed to evaluate gastrointestinal tolerance, lactate production, antimicrobial activity, and safety as described previously [27]. Briefly, resistance to simulated gastric stress and bile salts was evaluated by exposing the strains to simulated gastric medium at pH 3 for 90 min or de Man, Rogosa and Sharpe (MRS) medium supplemented with

0.3% bile salts for 180 min, respectively, and viable counts were determined before and after incubation. *Lacticaseibacillus rhamnosus* GG was used as reference. Lactate production was determined in filtered supernatants from 3-day MRS cultures by High-Performance Liquid Chromatography (HPLC) [28], and the L-lactate isomer was quantified enzymatically.

Antimicrobial activity against uropathogens was assessed by a microplate growth inhibition assay using neutralized supernatants from probiotic–pathogen co-cultures [27]. Uropathogens included 6 enterobacteria, staphylococci and enterococci strains (Supplementary Table S1). Cell-free supernatants from 24 h co-cultures were adjusted to pH 6 and filtered to assess organic-acid-independent inhibition. Pathogen growth in conditioned supernatants was monitored spectrophotometrically for 16 h. Medium was used as control. Activity was quantified by the area under the growth curve normalized for baseline optical density.

Strain safety was assessed by measuring biogenic amine production, hemolytic activity, and gelatinase activity as described previously [27]. Biogenic amines biosynthesis was evaluated by Ultra HPLC in overnight cultures with 5 precursors at 1mM [29]. Hemolysis was determined on Columbia Blood Agar plates supplemented with 5% defibrinated sheep blood using *Streptococcus pyogenes* CECT191 as positive control [30] and gelatinase activity on nutrient gelatin agar plates using *Bacillus cereus* CECT 5144 as positive strain [31].

## In silico analysis

The genomes of *L. plantarum* KABP062 and KABP063 (GenBank accession numbers JBUPYW000000000 and JBUPYV000000000, respectively) [32] were inspected for the presence of genes of interest. DNA sequences for the four types of bile salt hydrolase encoding genes in *L. plantarum*: *bsh1, bsh2, bsh3* and *bsh4* [33] were retrieved from the NCBI with the accession numbers ACL98168.1, KC197710, KC197711, and KC197712, respectively, and analyzed using BLAST [34]. Gene sequences of key enzymes involved in biogenic amine metabolic pathways within diverse lactic acid bacteria were sourced from the NCBI database and subjected to BLAST analysis. These sequences included *tdcA* (tyrosine decarboxylase; GenBank accession number JX204286.1), *cadA* (lysine decarboxylase; AE014075.1), *hdcA* (histidine decarboxylase; AJ749838.1), *aguA* (agmatine deiminase; AF446085.5), and *speF* (ornithine decarboxylase; EF633730.1). Probiotic genomes were analyzed with BAGEL 4 webserver [35] to detect bacteriocin genes.

## Clinical study

# Study design and participants

This multicentric, randomized, double-blind, placebo-controlled, parallel-group study was conducted at six Spanish sites with 1:1 allocation. The study was performed in accordance with the Declaration of

Helsinki, Good Clinical Practice, and applicable legislation, and was approved by the Institutional Review Board at each site. All participants provided written informed consent. The study protocol and statistical analysis plan were established before participant enrolment, and no changes were made to the primary or secondary endpoints after recruitment commenced. The trial was retrospectively registered at ClinicalTrials.gov (NCT06192966) on 21 December 2023.

Women aged ≥ 18 years with active UTI or at least one episode in the previous four months, together with a documented history of rUTIs (≥ 2 in the last 6 months or ≥ 3 in the last 12 months, with at least two positive urine cultures), were eligible. Included participants were already under clinical follow-up, facilitating monitoring and adherence, had received antibiotic treatment for their most recent infection and confirmation of recurrence due to reinfection (not relapse), defined as episodes separated by at least 2 weeks or, if closer, by a negative urine culture between them. Exclusion criteria included pregnancy or lactation; relevant comorbidities such as uncontrolled diabetes, kidney failure, cancer, spinal cord injury, or polycystic ovary; use of sodium-glucose cotransporter-2 inhibitors; urethral catheter or renal lithiasis; intake of probiotics or cranberry products in the 2 weeks before recruitment; high consumption of phenolic-rich fruits, especially berries; berry allergy; and UTI vaccination within the previous 12 months.

## Procedures

Participants were randomized centrally using a computer-generated list with a block size of 4 (SAS/SAT) into an active (AG) or control group (CG) (Fig. 1). The list was generated by an unblinded statistician and kept by personnel not otherwise involved in the study. The AG received a daily capsule containing *L. plantarum* KABP062 and KABP063 (minimum $1x10^8$ colony forming units, CFUs), cranberry extract (240 mg) and vitamin C (40 mg) (Gyntima® Cyscare, AB-Biotics S.A., KANEKA Corporation) for 6 months. The rationale for including *L. plantarum* KABP062 and KABP063 in the formula is based on previous results showing significant *in vitro* inhibition of uropathogens by the two strains [19]. Cranberry extract was included because of its documented use in the prevention of rUTIs [21]; while vitamin C was incorporated as a complementary component because of its recognized role in supporting normal immune function [23]. The probiotic dose was chosen to provide a viable daily amount consistent with doses commonly used in oral probiotic products, whereas the amount of cranberry extract and vitamin C were selected to provide a practical and feasible formulation within a single oral dosage form. The investigational product was a commercially available food supplement notified to the competent authorities prior to being placed on the market, in accordance with the requirements applicable to food supplements in Spain. The CG received an identical-looking placebo capsule containing the same excipient. Capsules were packaged in anonymous coded boxes. Participants, caregivers and data analysts remained blind to allocation.

The study included two on-site visits (baseline and month 6) and five monthly phone contacts to foster adherence and ask for symptoms and potential adverse events. At all on-site visits, either scheduled or

unscheduled, the investigator recorded urinary symptoms including dysuria, higher urinary frequency, enuresis, abdominal or lower back pain or strong urine smell and performed a urine dipstick test (SIEMENS multistix 8SG). If nitrites or leukocyte esterase were positive (> 0.05 mg nitrites/deciliter and > 15 leukocytes/microliter, respectively), UTI diagnosis was confirmed by urine culture in a certified laboratory, considering a threshold of ≥ $10^3$ CFU/mL. Additionally, participants were instructed to contact the investigator and arrange an unscheduled visit if UTI symptoms occurred. In those visits, the same procedure for UTI diagnosis was followed, involving dipstick evaluation and if indicative of urine infection, confirmation by urine culture. For symptomatic women with an initial culture-negative result, urine culture was repeated. If the repeat culture remained negative, the result was considered negative. Confirmed UTIs were treated with antibiotics according to national clinical practice guidelines. [36] No prophylactic antibiotics for rUTIs were used in the present study. Participants recorded medication intake and adverse events in a diary. Vaginal swabs for microbiota analysis were collected at baseline and month 6.

# Study endpoints

The primary endpoint was the proportion of women experiencing recurrences, defined as at least one UTI diagnosed by a specialist physician during the 6-month intervention. Secondary endpoints were number of recurrences, time to first UTI among participants presenting an episode, number of days of antibiotic use, incidence of adverse events, and differences in vaginal microbiota composition and diversity from baseline to month 6.

For vaginal microbiota analysis, DNA extraction, library preparation, sequencing and bioinformatics were performed as previously described [37]. Briefly, DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen), and the V3-V4 16S rRNA gene region was sequenced on an Illumina MiSeq platform (2×300 bp). Human reads were removed by alignment to the GRCh38 reference genome using bowtie2. Samples were rarefied to 4290 reads; eight samples were excluded due to insufficient DNA. Amplicon Sequence Variants (ASVs) and phylogenetic data were used to calculate alpha-diversity (Observed ASVs and inverse Simpson index), and beta-diversity (Bray-Curtis). Taxonomic assignment was performed using SILVA v138 database [38] and the Qiime feature-classifier classify-sklearn method [39].

# Statistical analysis

*In vitro* data were expressed as mean ± SD and analyzed by one-way ANOVA followed by Bonferroni *post hoc* test (GraphPad Prism Software 5.0).

For the clinical study, given the limited evidence available for comparable multi-component formulations and the absence of previous clinical studies evaluating this specific formulation, the recurrence rates used for the sample size calculation were selected empirically. The calculation assumed recurrence rates of 50% for the control group and 25% for the active group, with two-sided $\alpha = 0.05$ and 80% power, yielding 55 participants per group. The total recruitment target was increased to 120 to account for

dropouts. Clinical analyses were performed on the full analysis set according to randomized allocation, without imputation for missing data, using SAS version 9.4.

Continuous variables were presented as mean ± SD, and categorical variables as absolute and relative frequencies. Categorical comparisons were assessed using Fisher's exact test while continuous variables were compared using Student's *t* test or permutation Mann–Whitney test depending on distribution. Number of UTIs were initially compared by ordinal regression. Upon finding a major violation of the proportional odds assumption, a two-part hurdle model was used combining a logistic regression on the presence/absence part and an ordinal regression on the number of UTIs. The sum of likelihood ratios was compared to a Chi-squared distribution with two degrees of freedom. All clinical analyses were performed with R packages STATS and ORDINAL (R Core Team, Austria).

For vaginal microbiota analyses, alpha-diversity was assessed using Generalized Linear Mixed Models (GLMM) with R package NBZIMM v1. 0 for Observed ASVs and betareg v3.1-4 for inverse Simpson index [40, 41]. Community structure was evaluated by Bray–Curtis-based principal coordinates analysis, with group differences tested by two-way PERMANOVA and dispersion by PERMDISP. Differential taxon abundances was analyzed using negative binomial GLMM. Statistical significance was set at two-sided $p < 0.05$.

# Results

### Functional and safety characterization of L. plantarum KABP062 and KABP063

Both strains showed strong resistance to gastric stress and bile salts with minimal loss of viability in both tests (< 0.2 log CFU/mL loss), comparable to control *L. rhamnosus* GG (Table 1). Consistently, the four *bsh* genes described in *L. plantarum* were identified in both genomes.

Table 1
Tolerance of *L. plantarum* KABP062 and KABP063 to simulated gastric stress and bile salts.

| Strain | Gastric medium pH 3 | | | Bile salts medium | | |
|---|---|---|---|---|---|---|
| | Count | | Loss | Count | | Loss |
| | T0 | T90 min | T90-T0 | T0 | T180 min | T180-T0 |
| **L. plantarum KABP062** | 7.49 ± 0.02 | 7.38 ± 0.09 | 0.11 ± 0.06 | 7.06 ± 0.07 | 7.06 ± 0.08 | 0.00 ± 0.01 |
| **L. plantarum KABP063** | 7.28 ± 0.18 | 7.12 ± 0.07 | 0.15 ± 0.09 | 7.00 ± 0.06 | 6.99 ± 0.06 | 0.01 ± 0.02 |
| **L. rhamnosus GG** | 6.92 ± 0.24 | 7.09 ± 0.19 | -0.17 ± 0.16 | 7.01 ± 0.42 | 6.85 ± 0.26 | 0.15 ± 0.24 |

Values are presented as means and standard deviations of log CFU/mL. Statistical differences between probiotic strains and control strain *L. rhamnosus* GG were determined by one–way ANOVA followed by Bonferroni's Multiple Comparison test. No significant differences were observed.

Both strains produced lactate, with KABP062 accumulating 194.6 ± 15.7 mM (16% L-lactate) and KABP063 203.1 ± 15.6 mM (70% L-lactate), and other antimicrobial molecules. Neutralized supernatants from probiotic–pathogen co-cultures significantly inhibited the growth of Gram-positive and Gram-negative uropathogens, including *Escherichia coli* (both UPEC and β-lactam resistant strains), *Klebsiella pneumoniae*, *Proteus mirabilis*, *Staphylococcus saprophyticus*, and *Enterococcus faecalis* (all $p < 0.001$ vs control) (Fig. 2). Genomic analysis supported these findings by identifying bacteriocin-related genes including 2 Plantaricin J and 1 Enterocin_X in KABP062, and 3 PlnS, 1 PlnH, 1 PlnG and 1 Plantaricin_E in KABP063.

No biogenic amines were produced, consistent with the absence of related biosynthetic enzymes in their genomes. Additionally, strains did not exhibit hemolytic or gelatinase activity, supporting their safety profile.

# Clinical study

# Baseline characteristics

The trial was conducted between January 2018 and May 2022. A total of 106 women were randomized (55 AG, 51 CG) (Fig. 3). Two participants discontinued the intervention but were retained in the full analysis set. In addition, two participants (one per group) were excluded from the analysis due to missing infection history data as their compliance with inclusion criteria could not be verified. Therefore, analysis set consisted of 54 participants in AG and 50 in CG.

Baseline demographic and clinical characteristics were comparable between groups (Table 2). The proportion of women with physician-diagnosed active UTI at baseline was similar in both groups (61.1%

in AG and 62.0% in CG), with 96.9% showing symptoms - most commonly dysuria and strong urine odor.

Table 2
Baseline characteristics of the study population.

| **Age in years, mean ± SD** | **Active group (N = 54)** | **Control group (N = 50)** |
|---|---|---|
| | **58.8 ± 14.7** | **56.9 ± 16.2** |
| **Family history of UTI (yes), N (%)** | 12 (22.2%) | 8 (16.0%) |
| **Sexual activity in the last month (yes), N (%)** | 31 (60.8%) | 22 (48.9%) |
| **Active UTI at baseline (yes), N (%)** | 33 (61.1%) | 31 (62.0%) |

SD, standard deviation; UTI, urinary tract infection.

# Efficacy

The proportion of women experiencing at least one specialist-confirmed UTI during the 6-month period was similar in both groups (63.0% AG vs 58.0% CG, p = 0.689; OR = 1.23, 95%CI 0.56–2.71; Fig. 4A and Supplementary Table S2). Therefore, the prespecified primary endpoint was not met.

Among secondary endpoints, we found a statistically significant difference in the number of UTIs favoring the AG (p = 0.013) using two-part modeling. As a sensitivity analysis, the model was adjusted for age, presence of active UTI at study entry and baseline symptoms (dysuria, hematuria and malodorous urine), yielding similar results (treatment effect p = 0.009). As seen in Fig. 4A, the effect was driven by the reduction in multiple infections in the AG compared to the CG (OR = 0.125, 95%CI 0.018–0.534) among the participants experiencing at least one UTI (N = 63). Of note, baseline demographic and clinical characteristics of this subpopulation were consistent with those observed in the overall study population (Supplementary Table S3).

The time from the last documented UTI to the first recurrence during the intervention did not differ significantly between groups (123.6 ± 61.8 days in AG vs 116.4 ± 66.0 days in CG; p = 0.783, Mann–Whitney test). Conversely, the number of antibiotic treatment days differed significantly between groups, being lower in the AG, with a mean reduction of 3.8 ± 1.7 days compared with CG (p = 0.031, Fig. 4B). Regression analysis showed a significant association between the number of UTI episodes and antibiotic-treatment days (Spearman *rho* = 0.40, p < 0.001, Supplementary Fig. S1). Although a comparable number of participants reported antibiotic use (22 AG vs 19 CG, p = 0.842), stratification by treatment duration (1 day, 2–7 days, and 8–20 days) revealed significant differences (p = 0.017), with more women in the CG requiring longer courses (8–20 days: 5 AG vs 12 CG) (Fig. 4C). Of note, one-day antibiotic treatment consisted of fosfomycin, whereas longer regimens comprised ciprofloxacin, cefuroxime, cefixime, nitrofurantoin, and trimethoprim/sulfamethoxazole. The most frequently used antibiotic was fosfomycin, followed by cefuroxime and cefixime.

For vaginal microbiota analysis, paired samples from 86 women (46 AG, 40 CG) were analyzed. No significant differences were observed in alpha-diversity indexes across treatment groups, visits, or their interaction (observed ASVs, inverse Simpson index; both $p > 0.05$; Supplementary Fig. S2A and S2B). Community structure remained stable with no effect of the intervention (Permanova $p > 0.05$), although a time effect was detected (Permanova $p < 0.001$) but deemed unreliable due to high dispersion (Permdisp < 0.002) (PCoA in Supplementary Fig. S2C).

Six genera showed comparable baseline relative abundances but differed significantly between groups at the final visit (Supplementary Table S4). Notably, *Facklamia* and *Anaerococcus*, both associated with UTIs [5, 42], were significantly lower in the AG compared to the CG (FDR-adjusted $p = 0.025$ and $p = 0.042$, respectively; Fig. 5). Although no significant intergroup differences were observed for other genera, intragroup analysis identified 17 genera that changed significantly from baseline in one group only and showed no baseline differences between groups (Supplementary Table S4). Five uropathogen-associated genera [5, 43–45] decreased significantly in AG but not in CG: *Escherichia/Shigella* (AG baseline vs final FDR-adjusted $p < 0.001$), *Actinotignum* ($p = 0.002$), *Cutibacterium* ($p = 0.042$), *Finegoldia* ($p = 0.025$), and *Peptoniphilus* ($p = 0.003$) (Fig. 5). Notably, *Escherichia/Shigella* decreased from 6.74% to 0.81% in AG compared with 4.23% to 1.02% in CG. Conversely, UTI-related pathogens [5, 6] *Prevotella* ($p = 0.001$) and *Varibaculum* ($p = 0.028$) decreased significantly only in CG, although the reduction in *Varibaculum* was greater but non-significant in AG (Fig. 5).

# Safety of the multi-component formulation

Mild adverse events were reported by 3 women (5.6% of participants) in AG and 2 women (4% of participants) in CG. In the AG, the reported events included transient dermatitis, pain in the renal fossa and mastalgia. The first two events were rated as mild, whereas mastalgia was rated as mild to moderate. In the CG, the reported adverse events were constipation and abdominal pain, rated as mild and severe, respectively. All adverse events were considered as possibly related to the study products.

## Discussion

In this study, we characterized the probiotic properties and safety of *L. plantarum* KABP062 and KABP063 strains, and evaluated the clinical efficacy and safety of a multi-component formulation combining the strains with cranberry extract and vitamin C, in women with rUTIs.

The comprehensive characterization confirmed that both strains fulfill essential probiotic requirements, including gastrointestinal resistance and safety. The dual *in vitro* and *in silico* approach validated their adaptability to the gastrointestinal tract and the absence of undesired enzymatic activities, reinforcing their safety profile and aligning with recent findings reporting no phenotypic or genotypic antimicrobial resistance [32]. Building upon previous evidence demonstrating inhibitory activity of non-pH-adjusted supernatants against uropathogens [19], we confirmed lactate biosynthesis as contributor to pathogen inhibition. In addition, using neutralized co-culture supernatants, we observed broad-spectrum

antimicrobial activity against relevant uropathogens, including UPEC and β-lactam-resistant *E. coli*, indicating the involvement of additional inducible antimicrobial molecules beyond organic acids. *In silico* analysis identified genes encoding putative bacteriocins, suggesting mechanisms based on combined production of lactate and other antimicrobial molecules.

Given the antimicrobial potential of the strains, we sought to enhance their protective potential by combining them with cranberry extract and vitamin C targeting complementary mechanisms, an approach proposed for rUTI prevention [25]. The clinical evaluation of this multi-component formula showed no differences in the proportion of women experiencing ≥ 1 UTI over 6 months and thus the primary endpoint was not met. However, analysis of the number of UTIs as secondary endpoint showed a significant treatment effect, which remained significant in sensibility analysis after adjustment for age, active UTI at study entry, and baseline symptomatology. This effect was driven by a lower likelihood of multiple infections in the active group among participants who experienced at least one UTI. Although these findings suggest a potential reduction in recurrence burden, they should be interpreted with caution given the negative primary endpoint. Considering the mean age of 57.9 ± 15.5 years, the cohort in our study included pre-, peri-, and postmenopausal women. As hormonal stage is known to influence gut and urogenital microbiota profiles and the predominant uropathogens involved in UTIs [46], these findings suggest that the potential benefits of the formula may extend across different age groups and hormonal stages, although hormonal status was not directly assessed.

Other studies have investigated multi-component formulas for UTIs prevention. Koradia et al [47] demonstrated in a randomized, double-blind, placebo-controlled trial that supplementation with *Lactobacillus acidophilus* PXN 35 and *L. plantarum* PXN 47, cranberry extract and vitamin A significantly reduced the percentage of women experiencing UTIs and prolonged the time to first infection over a 26-week period. In contrast, we did not observe differences in the time to first infection, which may be partly explained by variations in baseline infection status. While their study enrolled women without active UTIs, more than half of our participants presented an active infection and received antibiotic treatment, which may have disrupted their microbiotas [13] and consequently delayed the product's effect. Nevertheless, our intervention showed potential effect in reducing number of recurrences, which remained significant after adjustment for baseline infection status, suggesting that the formula influenced recurrence patterns over time rather than postponing the first recurrence. Another clinical study reported efficacy of the use *L. plantarum* LP01 and *L. paracasei* LPC09, cranberry extract and D-mannose for 3 months in preventing rUTIs [48], although the lack of a control group limits interpretation. In contrast, our randomized, double-blind, placebo-controlled design strengthens the validity of our findings.

Another noteworthy finding from our study is that, consistent with the observed reduction in recurrences, the formula significantly decreased antibiotic use by an average of 3.8 days. However, the positive correlation between the number of UTIs and antibiotic-treatment days indicates that these outcomes are closely related; therefore, reduced antibiotic exposure should not be interpreted as an effect independent of UTI frequency. The supplement also lowered the proportion of women requiring longer treatment

courses (> 7 days), suggesting a reduction in the need of prolonged treatments. Similarly, Koradia et al showed their formula shortens median duration of antibiotic treatment [47]. The reduction in antibiotic exposure may be clinically relevant as it may decrease adverse effects and help mitigate the growing global concern about antimicrobial resistance, particularly in women with rUTIs [14, 49]. These findings suggest that the formulation may have potential as a complementary strategy for managing recurrence burden and antibiotic use.

In addition, 16S rRNA gene sequencing analysis of vaginal samples revealed a significant reduction in the relative abundance of the genus *Escherichia/Shigella* in the AG group, an effect not observed in the placebo group. The high baseline abundance of *Escherichia/Shigella* supports the concept that the vaginal microbiota may serve as a reservoir for uropathogens in women with rUTIs, as previously reported [10, 11]. Notably, the AG group also exhibited significant reductions in six additional anaerobic UTI-associated genera. Although these emerging uropathogens are less frequently cultured due to their anaerobic nature, they pose increasing challenges for diagnosis and treatment [5, 44]. The similar proportion of participants receiving antibiotics between groups over 6-month period reduces the likelihood that these differences are driven by antibiotic exposure. Nevertheless, some findings were not consistently observed as significant between-group differences and should therefore be interpreted cautiously. The absence of such differences in certain taxonomic endpoints is consistent with a modest intervention effect within a highly variable ecosystem, potentially obscured by limited statistical power and the inherently dynamic, non-linear nature of vaginal microbiota.

In this context, microbiota changes should be considered exploratory rather than definitive indicators of clinical efficacy. Although the observed changes may be compatible with the *in vitro* antimicrobial activity of the probiotic strains against uropathogens, including *E. coli*, they do not establish a mechanism of action or clinical efficacy. Furthermore, the specific contribution of the probiotic strains cannot be fully distinguished, as the formula also included cranberry and vitamin C, whose effects on vaginal microbiota cannot be excluded. While one study found no effect of their combination on the urinary microbiota of healthy women [50], evidence in the vaginal environment remains limited. Furthermore, whether the formula exerts the modulation by direct inhibition within the urogenital tract or through host-mediated mechanisms remains to be elucidated. Altogether, these findings suggest a potential effect of our multi-component formula on the vaginal ecosystem and its role as a reservoir of uropathogens in women with rUTIs, warranting further investigation.

Several limitations should be considered. First, the 6-month intervention period may not be sufficient to assess the long-term sustainability of the effects. Second, the use of a multi-component formula prevented the identification of the individual contribution of each ingredient, although this approach aligned with the objective of evaluating the efficacy of the combination. Third, the planned recruitment target was not reached, which may have reduced the statistical power to detect between-group differences. Moreover, the study was powered for the primary endpoint but not specifically for the secondary outcomes, which should therefore be interpreted with caution. Finally, the trial was registered retrospectively after completion of recruitment and follow-up, which limits the independent verification

of the prospective specification of the study outcomes and analyses. Nevertheless, the study protocol and statistical analysis plan were established before participant enrolment, and no changes were made to the primary or secondary endpoints after recruitment commenced.

Despite these limitations, the study also presents strengths. Its randomized, double-blind, placebo-controlled design enhances the reliability of the findings, and UTI diagnosis by specialists following international guidelines adds clinical rigor. Furthermore, the inclusion of vaginal microbiota analysis provides valuable insights into its role as a reservoir for uropathogens and may help generate new hypotheses on the potential mechanisms underlying the observed clinical effects.

In conclusion, this study characterizing *L. plantarum* strains KABP062 and KABP063 strains demonstrated that both meet established probiotic and safety criteria and exhibit functional properties related to uropathogen inhibition. The administration of these strains, in combination with cranberry extract and vitamin C, was safe and may offer potential benefits for women with rUTIs, regardless of age or baseline infectious status. Although the primary endpoint was not met, secondary findings suggest that the multi-component formulation may reduce recurrence patterns and antibiotic use, while potentially contributing to a reduction in the vaginal reservoir of uropathogens. Overall, these findings support the potential of this multi-component formulation and warrant further confirmation.

## Declarations

### Financial Disclosure/Conflict of Interest

MP is employee of AB-Biotics S.A. (KANEKA Corporation). The remaining authors declare no conflict of interest. This work was sponsored by AB-Biotics, S.A. (KANEKA Corporation), which participated in the conceptualization, study design, data analysis, decision to publish and revision of the final manuscript.

# Ethics approval

The study adhered to the ethical principles outlined in the World Medical Association (WMA), Declaration of Helsinki for Medical Research involving Human Subjects (64th WMA General Assembly, Fortaleza, Brazil, October 2013), the local regulations and the Good Clinical Practice guidelines in the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use E6(R2). Ethical approval was obtained from Institutional Review Boards at reference study centers Hospital Virgen de la Arrixaca (approved on April 24, 2017), Fundación Jimenez Díaz (February 23, 2021) and Fundació Puigvert (March 12, 2021) under the protocol code AB-CYSCARE 2017. The study protocol was retrospectively registered in clinicaltrials.gov (NCT06192966) on December 21, 2023.

### Consent to participate

Signed informed consent was obtained from all individual participants included in the study.

# Funding

This study was fully funded by AB-Biotics S.A (KANEKA Corporation).

# Authors' contributions

MP performed *in vitro* and *in silico* assays. CG-E, LLM and MP designed the clinical study. LLM, VMB, JBS, GMG, JFEB, II-P, RG-B, JAC-N and CG-E recruited, assessed, and followed participants. MP, CG-E and LLM analyzed the data, contributed to the writing of the manuscript and provided critical edits. All authors reviewed the manuscript and approved the final version.

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# Availability of data and materials

All clinical data used in the current publication are available (duly anonymized) from the corresponding author upon reasonable request. The 16S rRNA sequencing data generated in this study are available in the Sequence Read Archive. The associated BioProject number is PRJNA1365838.

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# Figures

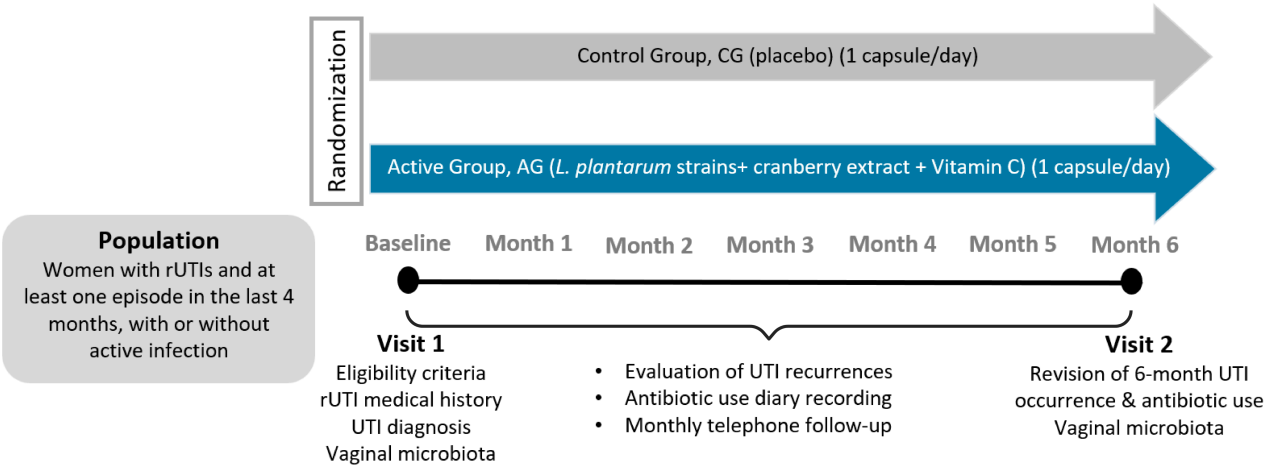



**Figure 1**

Schematic illustration of clinical trial design.

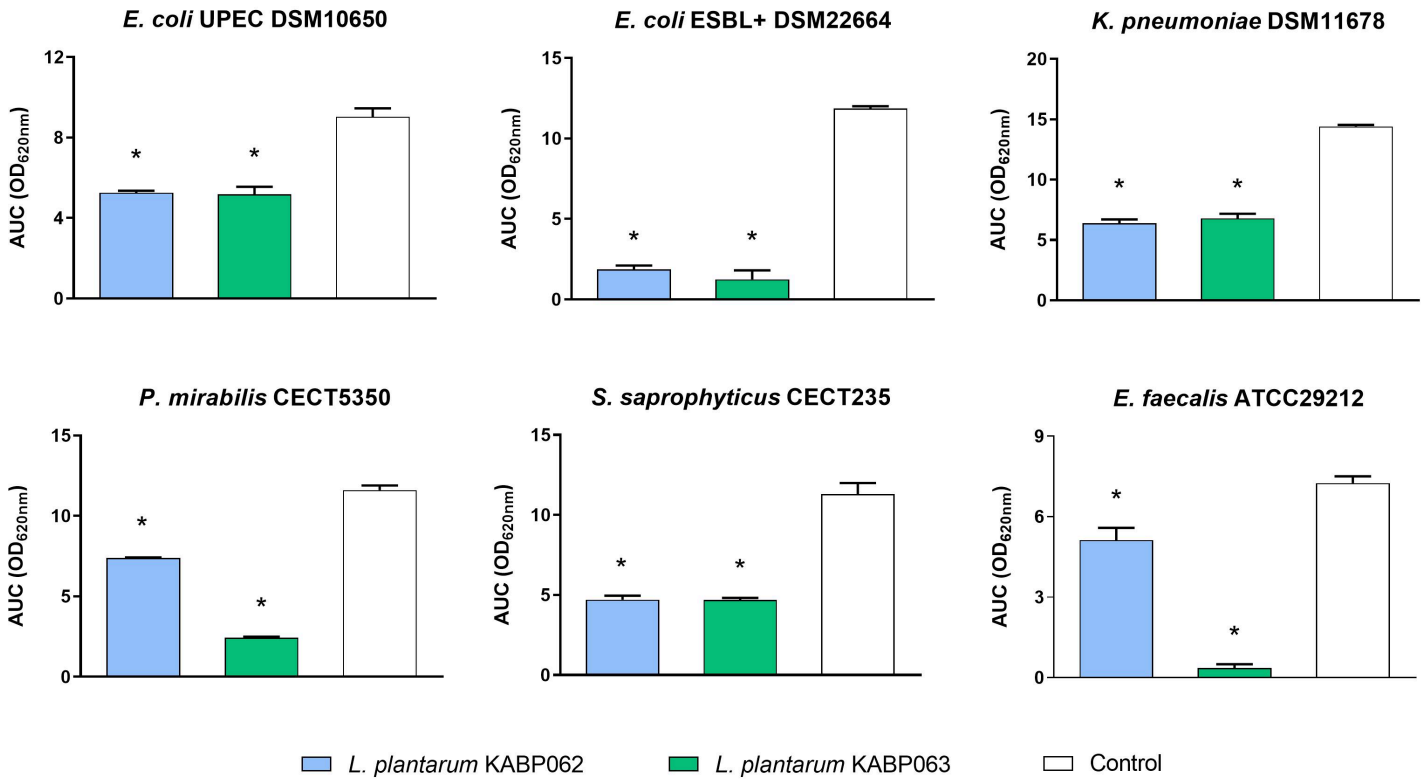



**Figure 2**

Antimicrobial activity of *L. plantarum* KABP062 and KABP063 against urogenital pathogens. Area under the curve (AUC) of 16 h-growth curves of indicator strains grown in neutralized supernatants obtained from co-culture of probiotic plus pathogen. Statistical analyses were performed by the one-way ANOVA with Bonferroni's Multiple Comparison Test. *$p < 0.0001$.

# Supplementary Files

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- ESM3SupplementaryTableS4rev.xlsx
- ESM2SupplementaryFigS2.pdf