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Efficacy of lyophilized *Lactobacillus sakei* as a potential candidate for preventing carbapenem-resistant *Klebsiella* infection

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Abstract

Background Antimicrobial resistance is considered one of the greatest threats to human health, according to the World Health Organization (WHO). Gram-negative bacteria, especially carbapenem-resistant *Enterobacteriaceae* (CRE), have become a significant concern in antimicrobial-resistant bacteria's global emergence and spread. Among CRE pathogens, carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has recently been reported as a highly infectious strain associated with high mortality and morbidity in adults and immunocompromised patients. Additionally, CRKP-related infections are challenging to treat, as carbapenems are the last resort of antibiotics. Therefore, developing novel drugs with different mechanisms of action from existing drugs is urgently required to defeat this lethal menace. Under such circumstances, probiotics can be therapeutic candidates for inhibiting pathogens. Thus, our research team has been focusing on probiotics for a long time to develop potential anti-CRKP drug agents.

Methods After extensive efforts, we finally found a novel probiotic strain, *Lactobacillus sakei* PMC104, suitable for treating CRKP infection. It was isolated from kimchi. As part of our expansion into therapeutic development, we evaluated the *L. sakei* strain effect against CRKP pathogens in both in vitro and in vivo experiments. Moreover, we conducted media optimization at food grade and then established a scale-up process on a pilot scale. Subsequently, a lyophilizate was obtained and used in a mouse model infected with CRKP.

Results Data demonstrated that the *L. sakei* strain has an inhibitory effect against CRKP infection both in in vitro and in vivo experiments and also increases the level of short-chain fatty acids in the feces of mice after receiving *L. sakei* strain treatment for 10 days. Furthermore, treatment with *L. sakei* powder remarkably diminished body weight loss, mortality, and illness severity in CRKP-infected mice, showing a preventive effect of our PMC 104 against CRKP infection.

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Discussion Our results demonstrate the potential therapeutic effect of our candidate probiotic strain against CRKP, suggesting that *L. sakei* PMC 104 could be considered an antimicrobial candidate for treating CRKP infections. However, extensive studies, including additional toxicity tests and clinical trials, are still essential to develop it as a new anti-CRKP therapeutic agent.

Keywords Carbapenem-Resistant *Klebsiella*, *Lactobacillus sakei*, Probiotics, Pilot-scale production, Food-grade medium

Introduction

Antimicrobial resistance (AMR) caused by the global excess use of antibiotics has emerged as a public health threat in the 21st century (Hu et al. 2020). According to 2022 published data, bacterial AMR was associated with 4.95 million deaths in 2019, including 1.27 million deaths directly attributable to bacterial AMR (Murray et al. 2022). Among AMR infections, carbapenem-resistant *Enterobacteriaceae* (CRE) has been identified as one of the primary pathogens associated with high mortality and mobility, particularly in clinical settings over the last two decades (Munoz-Price et al. 2013; Ventola 2015; Bologna et al. 2024; Wang et al. 2024). Infections due to CRE in healthcare settings are on the rise. They are classified as "urgent threats" by the Centers for Disease Control and Prevention (CDC). In 2017, an estimated 13,100 CRE infections were reported among hospitalized patients, resulting in 1,100 deaths in the USA (Control and Prevention, 2019).

Enterobacteriaceae includes several Gram-negative bacteria that have progressively developed resistance to nearly all last-resort antibiotics (Atterby et al. 2019; Janda and Abbott 2021). *Klebsiella pneumoniae* is an opportunistic pathogen responsible for most CRE infections and deaths worldwide (Xu et al. 2017; Centers for Disease Control and Prevention, 2019; Vaez et al. 2019). According to the China Antimicrobial Surveillance Network (CHINET), *K. pneumoniae* resistance to meropenem and imipenem had increased rapidly from 2.9% to 3.0% in 2005 to 26.3% and 25% in 2018, respectively (Han et al. 2020). Moreover, studies have shown that combination therapy with antibiotics is ineffective against this deadly pathogen (Xiao et al. 2020). Therefore, it is urgently necessary to develop antimicrobial agents with different action mechanisms to overcome current carbapenem drug limitations (Demiankova et al. 2023). Researchers are now focusing on novel probiotics as potential candidates to combat this deadly menace effectively (Davies et al. 2013; Lawrence and Jeyakumar 2013; Harikumar and Krishanan 2022).

Previous studies have shown that kimchi, the Korean traditional fermented vegetable food, has been known as one of the five top healthiest foods due to its health-promoting effects in humans(Lee et al. 2011, 2016; Park et al. 2014; Kim et al. 2023a). Kimchi-derived lactic acid bacteria have demonstrated probiotic properties inhibiting human disease by exhibiting antimicrobial, anti-inflammatory, and antioxidant activities(Jeong et al. 2021). The abundant lactic acid bacteria found in kimchi are valuable potential sources of probiotics, as they are known to be the dominant microorganisms in kimchi(Won et al. 2020).

According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), probiotics are nonpathogenic living microbes that can confer a health benefit to the host organism when they are administered in adequate amounts (Food and Food 2002). Probiotics greatly benefit the host by stimulating different immunomodulatory responses, lymphocyte proliferation, and production of anti-inflammatory regulatory cytokines (Ashraf et al., 2014; Manuel et al. 2017). They also possess medicinal properties. Thus, they are widely used as therapeutic candidates (Ishibashi and Yamazaki 2001; Ukeyima et al. 2010; Ozyurt and Ötles 2014; Owaga et al. 2015). Several studies have demonstrated the effectiveness of probiotics in treating cutaneous inflammation, managing diabetes, mediating metabolic disease, and treating gastrointestinal disorders (Bekkali et al., 2007; Hacini-Rachinel et al. 2009; Aggarwal et al. 2013; Homayouni-Rad et al. 2017). Additionally, previous studies have shown potential activities of probiotics against urgent threats, including tuberculosis, HIV, and COVID-19 (Bolton et al. 2008; Olaimat et al. 2020; Liu et al. 2021; Rahim et al. 2022). Furthermore, they possess antibacterial effects against superbugs such as CRE, vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA), making the use of probiotics a potential alternative treatment for nearly all pathogens (Manley et al. 2007; Mirnejad et al. 2013; McFarland 2015; Kumar et al. 2016; Sidjabat et al. 2017; Moghadam et al. 2018). Previous studies using in-vivo animal models have indicated that *Lactobacillus sakei*, a dominant species among lactic acid bacteria in kimchi produced at -1 °C, can prevent pathogenic bacteria by reducing septic shock and body weight loss in mice and improve illness symptoms (Hong et al. 2014; Lee and Jeon 2015; Lim et al. 2016; Kwon et al. 2018; Ji et al. 2019; Jang and Min 2020). Therefore, *L. sakei* has attracted particular attention as a good candidate for developing novel probiotics as an alternative to conventional antibiotics to

treat CRE-related infections (Penchovsky and Traykovska 2015).

This study aimed to determine the efficacy of *L. sakei*, isolated from Korean kimchi, as a new pharmabiotic alternative among probiotic strains to treat infections caused by CRKP. We also performed a scale-up study and novel food-grade medium development.

Materials and methods

CRKP clinical strain

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) clinical strains were collected from the Infectious Diseases Department of Soonchunhyang University Hospital (South Korea). Upon receiving, the strain was streaked onto MacConkey agar plates (BD Difco, USA) and incubated at 37 ℃ for 18 h. Subsequently, single colonies were selected and cultured aerobically overnight at 37 ℃ in MacConkey broth (BD Difco, USA). Afterward, bacteria were grown to OD 1.0 (2×10^9 CFU/mL) at 600 nm. CRKP colonies were isolated as described in a previous work (Tajdozian et al. 2021a). Antimicrobial susceptibilities of our CRKP strain were obtained using a standard broth dilution method according to the CLSI guideline. Minimum inhibitory concentrations (MICs) were 1,025-512.5 mg/l for vancomycin (Sigma-Aldrich), >1,025 mg/l for imipenem (Sigma-Aldrich), >1,025 mg/l for metronidazole (Sigma-Aldrich) and 1,025-512.5 mg/l for kanamycin (SigmaAldrich).

Isolation of PMC 104

Various fermented food products were used to isolate the candidate probiotic strain, including many fermented kimchi and soybean products. Fermented foods were streaked onto De Man, Rogosa, and Sharpe (MRS) agar (BD Difco, USA) plates containing vancomycin (Colombo et al. 2014) at 4 µg/mL (Sigma-Aldrich, USA) followed by incubation at 37 ℃ for 18 h using a microaerophilic chamber (Daeiltech, Korea). Next, single colonies were cultured in MSR broth (Sigma-Aldrich, USA) and incubated under the same conditions. Subsequently, bacterial growth was measured. Bacteria were washed, centrifuged, and resuspended in 0.85% NaCl solution. Then, 16 S rRNA sequencing analysis was performed on the probiotic culture at Biofact Co, Korea.

16 S rRNA identification

BIOFACT Co, Korea, performed the 16 S rRNA gene sequencing for the candidate probiotic and analyzed as reported in a previous study (Tajdozian et al. 2021a). The most popular unique sequences were searched against the NCBI (National Center for Biotechnology Information) 16 S microbial database using NBLAST. The resulting hits for the sequence were sorted first by e-value and then by bit score. The taxonomy of the hit with the highest score was then reported for the sequence.

PCR identification of the candidate probiotic strain

PCR amplification and qPCR analysis were conducted to confirm 16 S rRNA sequencing results. Genomic DNA was extracted from the candidate probiotic culture using a bead-beating method, as described previously (Ritchie et al. 2010). We also extracted genomic DNAs from *L. sakei* KGMB05070 (used as a positive control), *Lactobacillus curvatus* KGMB 05016, and *Lactobacillus rhamnosus* KGMB 06348 (used as a negative control) obtained from Korean Gut Microbiome Bank (KGMB) as reference organisms. DNA quality and concentrations were analyzed using 0.8% agarose gel electrophoresis and Qubit-4 fluorometer (Thermo Fisher Scientific, UK), respectively. After that, PCR amplification was performed using species-specific primers (F: GATAAGCGTGAGGTCGA TGGTT, R: GAGCTAATCCCCCATAATGAAACTA T) (Justé et al. 2008; Andani et al. 2012) with a Thermal Cycler Applied Biosystems platform (Thermo Fisher Scientific, USA). The quality of each PCR product was then checked by agarose gel electrophoresis. Bands were visualized with a Molecular Imager ChemiDoc XRS+imaging system platform (Thermo Fisher Scientific, USA). Quantitative analysis of agarose gel was performed using Bio-Rad's image lab software (version, 6.0.1).

In addition, qPCR was carried out using a LightCycler 480® (BioRad Diagnostics, USA) based on SYBR Green detection (SYBR Green 2× Master mix; Qiagen, USA) with the same primers as described above and *curvatus* species-specific primer. Triplicate reactions were used for each sample (candidate stain, *L. curvatus* KGMB 05016, and *L. rhamnosus* KGMB 06348). PCR conditions were optimized as described previously (Seo et al. 2023).

Characterization of the biochemical profiles of *L. Sakei* PMC 104

API 50CH Biochemical test was used to determine the biochemical profiles of our candidate probiotic. A single colony of our candidate strain culture was transferred to 1 mL of suspension medium, included in the API kit, to achieve a turbidity corresponding to a 2.0 McFarland standard. Afterward, 1 mL of suspension was diluted in 10 mL CHB medium (bioMerieux, France). Finally, API kit strip (bioMerieux, France) wells were filled with the bacterial suspension and incubated at 37 ℃ for 48 h. An identification table with results of +or - was then assembled according to the color shifts of each well.

Preparation of food-grade media (FGM) and edible FGM (EFGM)

According to a previous study, seven types of FGMs were prepared based on MRS medium to culture our

probiotic strain using a lab-scale fermenter (Sawatari et al. 2006). FGM compositions containing the yeast-peptone standard type of F (MB cell, Korea), Tween 80 (Sigma-Aldrich, USA), d-glucose (Sigma-Aldrich, USA), magnesium sulfate heptahydrate (Sigma-Aldrich, USA), sodium acetate 3 M (Biosesang, Korea), sodium acetate (Sigma-Aldrich, USA), and trisodium citrate (MB cell, Korea) mentioned in Table 1 were used to develop FGM medium. After preparation, pH values of all FGMs except FGM 1 were adjusted to 6.4 using NaOH 10 M (Sigma Aldrich, USA). Initial pHs of FGM 1, FGM 2, FGM 3, FGM 4, FGM 5, FGM 6, and FGM 7 were 6.4, 6.3, 6.2, 6.5, 6.1, 5.8, and 6.0, respectively. Next, all FGMs were sterilized at 121 ℃ for 15 min. The probiotic strain was then inoculated into the prepared FGMs and incubated at 37 ℃ for 18 h to reach endpoint growth. All the cultures started at the same OD600 (0.043±0.01) at time 0 h. Subsequently, the OD was checked with a spectrophotometer, and the bacterial culture with the highest growth rate (OD_{600}=1.0, 2×10^8 CFU/mL) was picked and stored at -80 ℃ using 20% glycerol. All the cultures started at the same OD600 (0.043±0.01) at time 0 h.

We also prepared edible FGM (EFGM) for pilot plant fermentation by optimizing the selected FGM with the highest growth rate. The EFGM consisted of glucose (Dong-A Chemical Co., Ltd, Korea), yeast extract (Sensient Flavors, USA), sodium (hanjusalt, Korea), soy peptone A2 SC (Organotechnie, France), magnesium sulfate (Bittersalz chemisch rein, Germany), and Tween-80 (Kao corporation, Japan). Detailed compositions of EFGM are listed in Table S1. After preparing EFGM, the pH was adjusted using NaOH 10 M.

Inoculum preparation for a scale-up process

We prepared L. sakei inoculum from a lab scale to an industrial scale for a scale-up process. First, 1 mL of our probiotic bacterial culture was inoculated into 10 mL of EFGM and incubated at 37 ℃ for 18 h in a microaerophilic chamber. After that, 3 mL of grown culture was transferred into 500 mL of EFGM and incubated overnight under the abovementioned conditions. Next, for preparing industrial-scale inoculum, the previously grown whole culture was transferred into 5 L of fresh EFGM and incubated at 37 ℃ for 24 h in a shaking incubator at 200 rpm.

Fermentation, freeze-drying, and packaging

For an industrial-scale pilot plant fermentation, the grown culture inoculum was transferred to a 500 L pilot plant scale-up facility fermenter (Kobiotec, Korea) containing 200 L of autoclaved EFGM. Compositions of EFGM included glucose (5 kg), yeast extract (2.5 kg), sodium (1.25 kg), soy peptone A2 SC (2.5 kg), magnesium sulfate (50 g), Tween 80 (250 mL), and distilled water. The fermentation process was performed at 37 ℃ with only agitation at 25 rpm (no sparging of air or N_2 gas). The pH was maintained at 6.0 using 28% (w/v) NH_4OH. The culture broth was collected aseptically during fermentation at 0, 8, 16, 18, and 22 h of incubation. The residual glucose concentration was analyzed using an Agilent 1200 series HPLC (high-performance liquid chromatography) system (Agilent Technologies, USA). The growth rate of the probiotic strain was also determined turbidimetrically with a spectrophotometer at 600_{nm}. After fermentation, centrifugation was performed with a disc centrifugal separator (DHC-400, Hanil, Korea) at 7,000 rpm. Cells were then collected and homogenized using reconstituted skim milk (RSM) (5% (w/v), 1.5 kg).

Table 1 Ingredients of optimized food-grade media (FGM)

Ingredients	Concentration (g/L)							
	MRS media	FGM 1	FGM 2	FGM 3	FGM 4	FGM 5	FGM 6	FGM 7
Glucose	-	20	20	20	20	20	20	20
Yeast peptone standard type F	-	25	25	25	25	25	25	25
Proteose peptone No. 3	10	-	-	-	-	-	-	-
Beef extract	10	-	-	-	-	-	-	-
Yeast extract	5	-	-	-	-	-	-	-
Dextrose	20	-	-	-	-	-	-	-
Tween 80	1*	1*	1*	1*	1*	1*	1*	1*
Triammonium citrate	2	-	-	-	-	-	-	-
Trisodium citrate	-	2.1	-	2	-	2	2.1	2
Sodium acetate	5	5	-	5	5	-	-	-
Sodium acetate 3 M	-	-	-	-	-	-	5	-
magnesium sulfate	0.1	0.1	0.2	0.3	0.2	0.3	0.1	0.2
Manganese (II) sulfate	0.05	-	-	-	-	-	-	-
Dipotassium phosphate	2	-	-	2	-	-	-	2

*mL

Next, to produce probiotic powder from harvested cells, freeze-drying was performed at -60 ℃ for 72 h under a pressure of 6.7×10^{-2} mbar using a 50 L freeze-dryer (PVTFD 50R, Korea). Out of the 3.0 kg powder produced, 2.5 kg was packed for further experiments. Before packaging, some food additive ingredients, including D-glucose (5 kg), dextron (dextran?) (1 kg), and cornmeal (1 kg), were added into the powder and blended with a pin crusher machine at 380.3-volt Power (Sung Chang Machinery, Korea). Subsequently, the mixed powder was packed using polyethylene material with an auto packaging machine (Sae Han F&B, Korea) in Techno pack, Cheonan, Korea. During 3 h of the packaging process, 1,672 probiotic powder bags were produced and stored at room temperature until use for further experiments.

Acute toxicity of candidate probiotics

To conduct a primary oral toxicity assay of *L. sakei*, a two-week toxicity assay was performed using four-year adult male guinea pigs 1,000 g to 1,280 g, according to previous studies (Lee et al. 2021) after it was approved by the IACUC (IACUC approval number: SCH-22-0111). Animals were separated into a control group and a probiotic treatment (4 animals per group). *L. sakei* powder was diluted with sterile water. Guinea pigs in the treatment group received *L. sakei* powder at 2×10^8 CFU/animal (200 µL per animal) orally for 14 days, whereas the control group received just saline. Body weight and mortality were measured, and clinical symptoms were monitored and recorded during the study. Later, for comprehensive acute toxicological studies, nine-week-old mice (weight 19.5±0.2 g) were randomly divided into three groups ($n=5$ mice per group) and treated with saline drinking water (control), *L. sakei* strain (first treatment group) or *L. sakei* powder (second treatment group) based on previous studies (Tajdozian et al. 2021b). *L. sakei* strain and *L. sakei* powder treatment groups received *L. sakei* at a concentration of 3×10^9 CFU/ml through drinking water daily and the single dose administration of 3×10^9 CFU/animal (200 µL per animal) once a day using NaCl solution from day 1 to day 14. Animals were kept in individual cages under a 12-h light-dark cycle with a temperature ranging from 20 to 25 °C and relative humidity of 30–70%. They received the *L. sakei* treatment based on cfu/mL body weight. Moreover, the animals' clinical signs, mortality, and body weights were observed for 14 days following treatment. This study was performed Following the drug safety evaluation test guidelines provided by the Ministry of Food and Drug Safety (Notice No. 2015-82) and according to the OECD Test Guidelines 423 with some modifications to test acute oral toxicity of *L. sakei* in the guinea pigs and mouse models.

Cytotoxicity test

To determine the cell viability of *L. sakei* extract against RAW 264.7 macrophage cell line, a water-soluble tetrazolium salt (WST) assay was performed. Cells were seeded (1.5×10^5 cells/mL) into a 96-well plate (SPL life science, Korea) and incubated at 37 ℃ overnight with 5% CO_2. After that, RAW cells were treated with *L. sakei* extract diluted to various concentrations using fresh media and incubated again at 37 ℃ for 24 h. Following incubation, 10 µl of WST solution was added to each well. After incubation for 30 min, absorbance was measured at 450 nm with a Victor Nivo Multiplate reader (Perkin Elmer, USA), and cell viability was checked by methylene blue staining. Cells were seeded onto 2-well cell culture slides (SPL life sciences, Korea) and treated with our *L. sakei* strain extract. Finally, cells were counted with a hemocytometer (Marienfeld) using an optical microscope (AX10, Carl Zeiss, Germany).

Experimental design for preventive in vivo model

Animal experiments using specific pathogen-free (SPF) BALB/c female mice (9 weeks old) purchased from Doo Yeol Biotech company, Korea were performed as described previously (Tajdozian et al. 2021c) after they were approved by our IACUC (approval number: SCH23-0004). Two preventive mice experiments were performed to estimate the efficacy of our candidate probiotic against CRKP infection. All mice were randomly divided into two groups (an infection group and a treatment group, $n=5$ mice per group) for both models. In the first model, we investigated long-term preventive effects by administering a probiotic culture before infection. In the second model, we estimated the efficacy of the probiotic powder instead of the culture in a preventive mouse model. The treatment group received *L. sakei* culture or *L. sakei* powder as probiotics in both models. In contrast, the infection group only received sterile saline through oral gavage similarly under the same conditions. Sodium bicarbonate ($NaHCO_3$) was administered before infecting both models with CRKP to increase the severity of infection by neutralizing stomach acidity (Czuprynski and Faith 2002). Additionally, neutropenia was induced by 200 µl of cyclophosphamide injection at 450 mg/kg intraperitoneally three days prior to infection (Sigma Aldrich, USA). Moreover, CRKP bacterial colonies obtained from the 18-hour culture were suspended in a sterile saline solution (0.9% sodium chloride (NaCl) to achieve the desired concentration of infection in each mice model infection. The survival rate, body weight, and illness score of mice in both models were obtained during the experimental period. The therapeutic dosage of *L. sakei* in mice models was determined according to previous studies (Tallon et al. 2003; Manzano et al. 2017; Mohammadian et al. 2022; Zulkhairi Amin et al. 2023).

Prior to the initiation of efficacy studies, successful infection of mice with *Klebsiella pneumoniae* was confirmed by comparing mortality rates between the control group (which only received sterile saline) and the infected group (200 µL of CRKP to mice orally at 4×10^{10} CFU/animal and 6×10^{9} CFU/animal on days 0 and 1) under the same conditions as efficacy studies (Tajdozian et al. 2021d).

Long-term treatment efficacy of *L. Sakei* in a CRKP-infected mice model

We estimated the long-term preventive effect of our probiotic strain, *L. sakei*, in CRKP-infected BALB/c mice. The probiotic culture was prepared using EFGM and adjusted to an approximate concentration of 3×10^{9} CFU/animal (200 µL/animal) using NaCl solution. The culture was administered twice a day by oral gavage from 3 days before infection to day 29. Three days after neutropenia, 200 µL of CRKP was administered to mice orally at 4×10^{10} CFU/animal and 6×10^{9} CFU/animal on days 0 and 1, respectively. $NaHCO_3$ (0.2 M, 200 µl) (Sigma Aldrich, USA) was administered before infection. Fecal samples of mice were collected from each cage on days 1, 4, 7, and 8.

Preventive effect of *L. Sakei* on a CRKP-infected mice model

We investigated the preventive potential of *L. sakei* powder in CRKP-infected BALB/c mice. The probiotic powder was first prepared using sterile water and administered to mice at 1×10^{8} CFU/animal (200 µL per animal) orally as a single dose from day −3 to day 7. At the same time, 3×10^{8} CFU/mL of probiotic powder was provided through drinking water. In this model, infection was induced orally using CRKP at 4×10^{10} CFU/animal (200 µL) on day 0 and 6×10^{9} CFU/animal on days 1 and 3 with pretreatment of $NaHCO_3$ (0.2 M, 200µL).

Whole genome sequencing

To better identify our candidate probiotic, whole-genome sequencing was done by a WGS service company (Chunlab Inc. Seoul, Korea). The Genomic DNA (gDNA) of the *L. sakei* strain was extracted using a QIAamp DNA Mini kit (Qiagen, Germany). PacBio sequencing results were obtained with an HGAP2 protocol using PacBio SMRT Analysis 2.3.0. Circlator 1.4.0 (Sanger Institute, UK) was performed, resulting in circulated contigs from PacBio sequencing. Protein coding sequences (CDSs) were predicted with Prodigal 2.6.2 (Hyatt et al. 2010) and grouped according to roles regarding orthologous groups (EggNOG; http://eggnogdb.embl.de). Genes encoding tRNAs were searched using tRNAscan-SE 1.3.1 rRNAs (Lowe and Eddy 1997), and other noncoding RNAs were searched by covariance model searches using the Rfam 12.0 database (Nawrocki et al. 2015). The OrthoANI values between prokaryotic genome sequences were calculated using the OrthoANIu algorithm-based Average Nucleotide Identity (ANI) calculator (https://www.ezbiocloud.net/tools/ani) (Yoon et al. 2017).

Determination of pH and SCFA levels in fecal samples during *L. sakei* treatment

Nine-week-old BALB/c mice were randomly divided into two groups ($n=5$ mice in each group). All mice had free access to food and water. The treatment group was given 100 mL saline containing 1×10^{8} CFU/mL of *L. sakei* strain and administered to a probiotic culture from day 0 to 10 using a zonde (3×10^{8} CFU/200 µL/mice). Control mice were exposed to saline under the same conditions as the treatment group. Fresh stool samples were collected on days 0, 3, 4, 5, 7, 9, and 10 during the study. Each 100 mg of stool was vortexed with 1 mL of phosphate-buffered saline (PBS) and analyzed using an electronic pH meter (Mettler Toledo, Korea). Additionally, blood was obtained intracardially from each mouse on day 10 to determine short-chain fatty acid (SCFA) levels in serum samples collected from both treatment and control groups. Serum levels of SCFAs were measured using a mouse short-chain fatty acids (SCFAs) ELISA kit (AMSBIO, UK). Later, to determine acetic acid, propionic acid, butyric acid, and valeric acid levels, fresh fecal samples (1 g) were homogenized in 5 mL of PBS distilled water. The homogenate was mixed with 1.2 g of NaCl and 1 mL of 2% sulfuric acid. Subsequently, the mixture was subjected to gas chromatography according to the previous studies with some modifications (Hung and Suzuki 2016; Le et al. 2019). This experiment received approval from the Institutional Animal Ethics Committee of Soonchunhyang University, Asan, Korea (SCH21-0019), and was conducted following the committee's guidelines.

Time-kill assay test

Time-kill assay was conducted against the CRKP pathogen using cell-free-culture supernatant (CFCS) of *L. sakei* following previous studies (Prabhurajeshwar and Chandrakanth 2017; Rastogi et al. 2021). The *L. sakei* strain was cultured overnight in MRS broth and EFGM to measure its antimicrobial effects on viable pathogen counts. To perform the assay, 300 µL of CRKP suspension (10^8 CFU/mL) was introduced into 15 mL of CFCS in MRS broth and CFCS in EFGM, respectively, and incubated at 37 °C. Control groups included CRKP pathogen suspended in MRS broth and EFGM without CFCS inocula. Aliquots were pipetted out from each tube at predetermined intervals and serially diluted to determine viable CFU of CRKP on MacConkey agar plates. The assay was performed in triplicate. Total surviving cells were recorded as mean log CFU/mL.

Statistical analysis

All data are presented as mean ± SD. They were log-transformed for each experiment. The statistical difference is indicated by asterisks (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$) using unpaired Student's t-test. A p-value of less than 0.05 was considered statistically significant.

Results

16 S rRNA-based identification of the isolated strain

Among colonies isolated from fermented products, a kimchi-derived strain turned out to be an effective candidate against CRKP infection. After 16 S rRNA gene sequencing, the obtained sequences were compared with those in the NCBI (Table S2). Results showed that our isolated strain shared 99% sequence similarities with 16 S rRNA gene sequences of *L. sakei* strains, including DSM 20,017, NBRC 15,893, and CCUG31331. Additionally, its sequences were similar to those of other strains of the *Lactobacillus* genus, with similarities ranging from 96 to 99%. Additionally, NBALST similarity scores showed 186 hits for *L. sakei* among unique sequences submitted to NCBI (Table S3).

PCR identification of isolated probiotic

PCR assay was performed to identify the isolated probiotic strain using a species-specific primer set (Fig. S1). DNA was extracted, quantified, and amplified. The PCR product was then checked by agarose gel electrophoresis. The resulting data showed clear bands for both the isolated probiotic and positive control strains (*L. sakei* KGMB 05070). However, non-*L. sakei* strains (*L. curvatus* KGMB 05016 and *L. rhamnosus* KGMB 06348) showed no visible bands (Fig. S1 A). Furthermore, a qRT-PCR assay was performed to quantify the isolated probiotic strain. The presence of the isolated strain was detected by a low threshold cycle (Ct) value (21.56 ± 1.19), whereas the two non-*L. sakei* strains presented high threshold values (~40) (Fig. S1 B). Moreover, the *curvatus* species-specific primer could only detect the *L. curvatus* KGMB 05016 (Fig. S1 C). These results confirmed that the isolated probiotic strain was *L. sakei*.

Wholegenome analysis results of the selected strain

Whole genome result showed that the PMC 104 strain had a genome size of 2,015,612 bp with an average GC content of 41.1% (Fig. 1). According to functional categorization system Clusters of Orthologous Groups (COG), 1,960 coding sequences (CDSs) were predicted. Among these CDSs, 1,780 proteins were assigned to families of COG. Biological functions could be identified for 1,249 proteins, while 531 CDSs were homologous to conserved proteins whose functions were unknown in other organisms. The other 180 hypothetical proteins did not match any known proteins in the database. Furthermore, 66 tRNA and 21 rRNA genes were predicted. PMC 104 sequences were later submitted to NCBI. Bio Project ID: PRJNA945229.

OrthoANI genomic similarity

OrthoANI analysis was performed to obtain similarity values between strains with substantial similarities in 16 S rRNA analyses using whole genome sequencing data of PMC 104 (Fig. 2). ANI values between PMC 104 and all publicly available *L. sakei* genomes, including *Lacticaseibacillus* subsp. strain ELA14391, DSM 20,017, subsp. strain TMW, AMBR8, and DS4 were 99.11%, 98.84%, 98.86%, 97.83%, and 92.53%, respectively, significantly above the cutoff value of 95% for species delineation. Likewise, ANI values of our strain with other *Lacticaseibacillus* species, such as *L. rhamnosus* AMBR4, *L. casi* 12 A, and *L. fermentum* PMC 101, were 67.11%, 66.57%, and 65.55%, respectively, which were all below 80%. These results strongly suggest that the newly discovered strain PMC 104 is *L. sakei*.

Different genomic characteristics between *L. Sakei* species strains

Different genomic characteristics between PMC 104 and other strains of *L. sakei*, such as DSM20017, TMW, AMBR8, DS4, and ELA214391, were investigated. Results demonstrate that our isolated strain is newly discovered (Table 2). The PMC 104 strain differed from other *L. sakei* strains in genomic size, G+C content, and CDS. Thus, PMC 104 is a newly discovered strain of *L. sakei*.

Biochemical characterization of *L. Sakei* PMC 104

A biochemical characterization was performed to investigate the phenotypic properties of our probiotic strain (Table 3). According to previous studies, an API kit can identify *Lactobacillus* strains phenotypically and biochemically following complete fermentation based on color changes from purple to yellow in the strip capsule (Casaburi et al. 2016). The test enables a strain to be classified and specified by its ability to utilize 49 different carbohydrates. Results of the API kit showed that the isolated strain could ferment four carbohydrates (galactose, fructose, mannose, and N-acetylglucosamine), similar to the carbon utilization pattern of other *L. sakei* strains (Bajpai et al. 2016).

Cultivation of *L. Sakei* based on food-grade medium

The probiotic strain was cultured in seven types of FGMs. All the cultures started at the same OD600 (0.043 ± 0.01) at time 0 h and incubated to reach endpoint growth. Bacterial growth was then checked after 18 h (defined as the endpoint growth of *L. sakei* in different media) using a spectrophotometer (Fig. 3). Data showed that our probiotic strain grew the best in medium 2 (OD_{600}

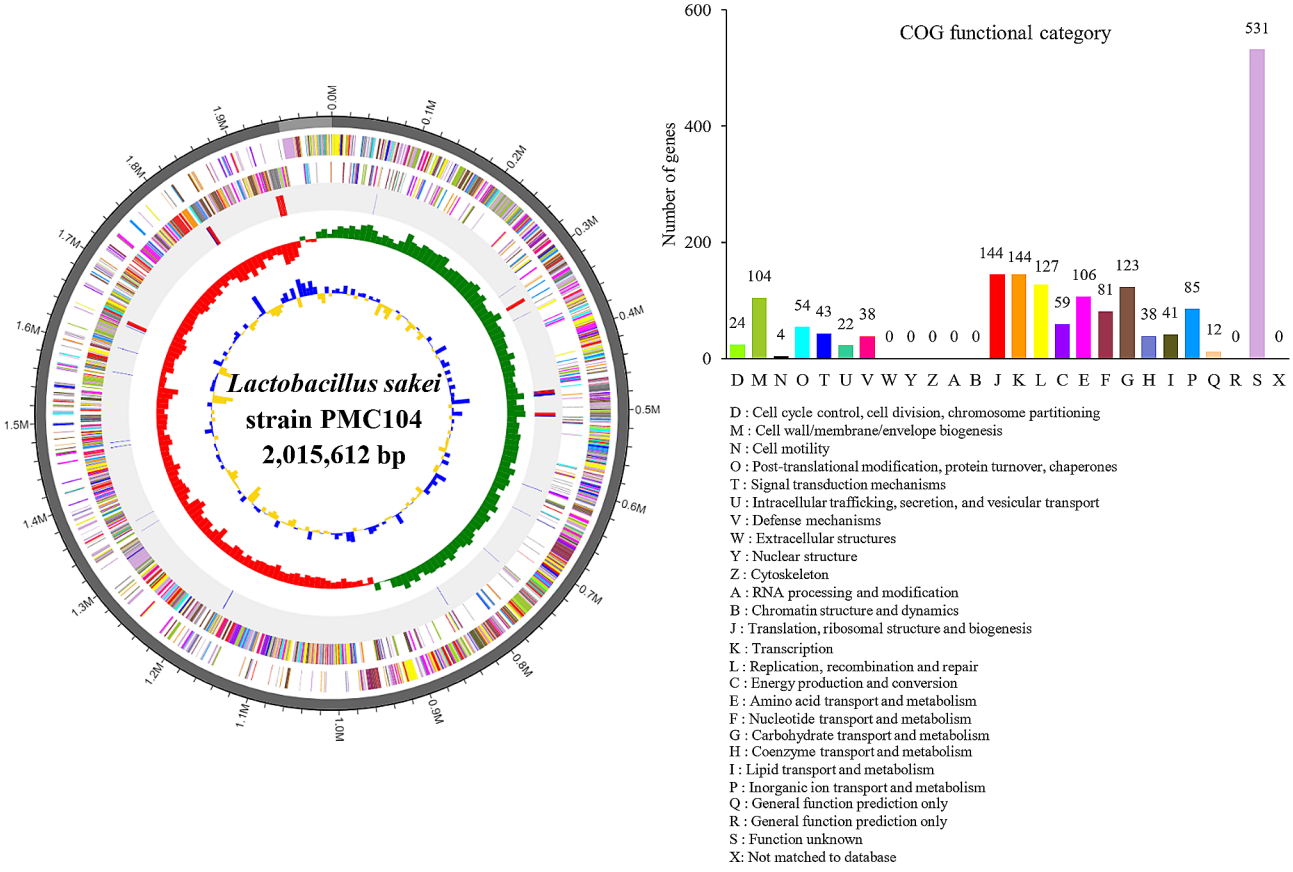

Fig. 1 High-throughput genome sequencing results of *L. sakei* strain PMC 104. Circular map of *L. sakei* PMC 104 genome. Antisense and sense strands (colored according to COG categories) and RNA genes (red, tRNA; blue, rRNA) are shown from the outer periphery to the center. Inner circles show GC skew, with yellow and blue indicating positive and negative values, respectively. GC content is indicated in red and green. This genome map was visualized using CLC genomics. Relative abundance of cluster of orthologous groups (COG) functional categories of genes is shown

=1.3). In contrast, it had the lowest growth in medium 1 (OD_{600}=0.38) and medium 4 (OD_{600}=0.39). Based on this result, medium 2 was selected for culturing our probiotic strain using a lab-scale fermenter. Later, we prepared EFGM by optimizing ingredients used in medium 2.

Pilot fermentation, powder production, and packaging

The proposed industrial process scale-up steps from laboratory to packaging are shown in Fig. 4 and Fig. S7. Figure 4A depicts a schematic diagram of our study. Residual glucose concentration and bacterial growth rate were checked simultaneously during the scale-up fermentation process (Fig. 4B and C). Data showed that at 0 to 8 h of fermentation, bacteria had no significant growth. Thus, residual glucose was not utilized before 8 h of fermentation. However, after 8 h of incubation, significant growth and utilization of glucose were observed, which continued until 16 h. We also observed that bacterial growth became stable after 18 h of fermentation. After fermentation, bacterial cells were harvested and freeze-dried, and 3.0 kg of probiotic powder was obtained. The powder contained a bacterial load of 3×10^8 CFU/g. Next, 2.5 kg of freeze-dried powder out of the 3.0 kg was mixed with some food additives and then packed using polyethylene material, with each pack containing 4 g of powder.

Cytotoxicity of *L. Sakei*

Cytotoxicity of *L. sakei* extract at various concentrations was evaluated using macrophage RAW 264.7 cells (Fig. 5) (Kim et al. 2020, 2023b; Xu et al. 2022). Data showed that *L. sakei* extract had no significant cytotoxicity at a concentration up to 20×10^7 CFU/mL, in which 100% corresponds to untreated cells (control) group (Fig. 5A). Cell viability was also checked using methylene blue staining. Cells were then observed under a light microscope (Fig. 5B). No significant changes in the morphology of RAW 264.7 cells were observed after treatment with *L. sakei* extract at 2.5×10^6 to 20×10^7 CFU/mL.

Acute oral dose toxicity of *L. Sakei*

The acute oral toxicity of *L. sakei* was evaluated in both guinea pig and mouse models to investigate the potential side effects of the *L. sakei* strain and powder. No

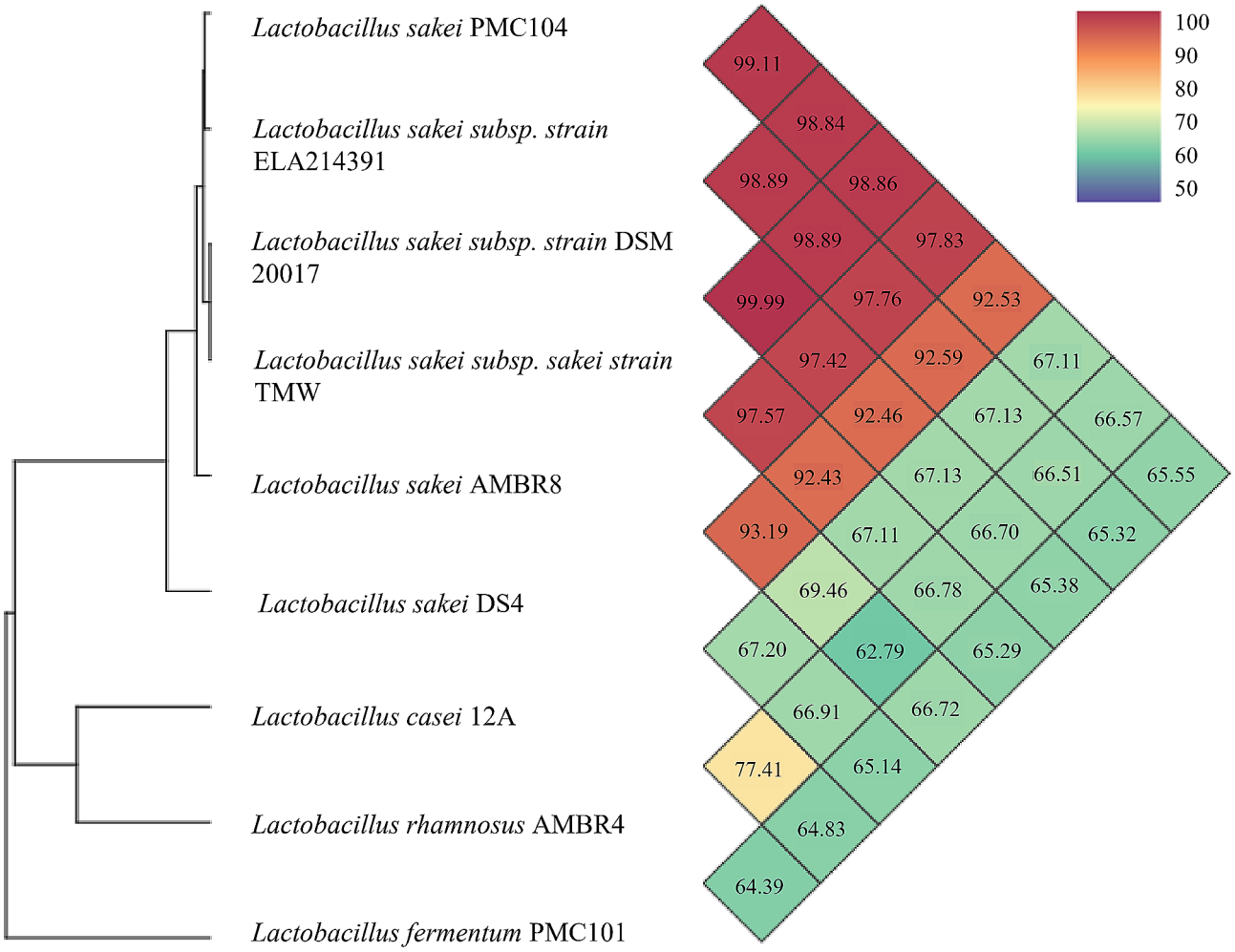

Fig. 2 OrthoANI results calculated from available genomes of *Lacticaseibacillus* species. OrthoANI value of *L. sakei* PMC 104 and *L. sakei* subsp. strain ELA214391 was 99.11%, higher than 96.0%, the standard for determining the same species

Table 2 Comparison of chromosomal characteristics of *L. sakei* strains

Strain	PMC 104	DSM 20,017	TMW	AMBR8	DS4	ELA214391
Sources	Korean kimchi	Moto starter of sake	Raw sausage	Human nasopharynx	Korean kimchi	Foregut
Genome size (bp)	2,015,612	1,910,247	1,942,056	1,997,834	2,103,571	2,069,589
G + C content (%)	41.1	45.0	57.0	73.0	89.0	42.0
Predicted CDS	1,960	1,885	1,891	2,012	1,992	2,046
Number of rRNA genes	21	3	18	7	21	20
Number of tRNA genes	66	44	66	58	65	66

clinical symptoms or changes in body weight were found between the group treated with the candidate probiotic strain and those treated with sterile water. All guinea pigs and mice survived during the experiment, and no adverse or lethality effects were observed in any experimental group (Fig. 6A and B, and Tables S4, S5).

Evaluation of long-term treatment of CRKP-infected mice with *L. Sakei*

We investigated the long-term preventive effect of our probiotic strain (Fig. 7). Figure 7A depicts a schematic diagram of our study. During the experimental period, survival rates in the untreated and treated groups were 45% and 80%, respectively (Fig. 7B). We also observed body weight changes. Mice in the untreated group showed severe weight loss compared to those in the *L. sakei* treated group ($p<0.001$) (Fig. 7C). Mice in the *L. sakei* treated group also showed lower ($p<0.05$) scores of illness severity than those in the untreated group (Fig. 7D). The CFU of infection in fecal samples of mice were obtained at day 1, 4, 7 and 8 (Fig. 7E).

Table 3 Biochemical proprieties of the candidate strain based on carbohydrate fermentation using API 50 CH kit

Substrates	Results	Substrates	Results
Control	-	D-Fucose	-
Glycerol	-	L-Fucose	-
Erythritol	-	D-arabitol	-
D-arabinose	-	L-arabitol	-
L-arabinose	-	Potassium gluconate	-
Ribose	-	Potassium 2 ketogluconate	-
D-Xylose	-	Potassium 5 ketogluconate	-
L-Xylose	-	D-Tagatose	-
Adonitol	-	Methyl-αD-mannopyranoside	-
Methyl-βD-xylopyranoside	-	Methyl-αD-glucopyranoside	-
D -galactose	+	N-acetylglucosamine	+
D-glucose	-	Amygdaline	-
D-fructose	+	Arbutin	-
D-mannose	+	Esculin	-
L-sorbose	-	Salicin	-
L-rhamnose	-	D-Cellobiose	-
Dulcitol	-	D-Maltose	-
Inositol	-	D-Lactose	-
D-mannitol	-	D-Melibiose	-
D-sorbitol	-	D-Saccharose	-
D-Melezitose	-	D-Trehalose	-
D-Raffinose	-	Inulin	-
Starch	-	D-Turanose	-
Xylitol	-	D-Lyxose	-
Gentibiose	-		

Efficacy of *L. Sakei* in preventing CRKP infection in a mouse model

The treatment effect of *L. sakei* powder on CRKP infection was evaluated (Fig. 6). Figure 8A depicts a schematic diagram of our study. During this study, we observed the survival rate of mice and found that mice in the untreated group died 4 days after the first infection. On the other hand, all mice in the treatment group survived not only until the end of the study but also for a long time during post-study observation without any exceptions (Fig. 8B). We also observed body weights of mice and found that mice without probiotic treatment showed significant weight losses ($p<0.001$) and died on day 4 while *L. sakei*-treated mice were almost stable throughout the observation period (Fig. 8C). Illness severity scores of both groups were also observed. We noticed a significant difference between the two groups (Fig. 8D). Mice in the infected group suffered from diarrhea and developed weakness, leading to higher illness scores. In contrast, those in the probiotic powder-treated group showed low illness scores ($p<0.001$).

Effects of *L. sakei* treatment on pH levels and SCFA concentration

To determine the effects of *L. sakei* on pH and SCFA levels in mouse serum, mice in the treatment group received a therapeutic dose of probiotics for 10 days during the experiment. Results of pH analysis demonstrated notable differences in fecal samples' pH levels between the group treated with *L. sakei* and the control group. *L. sakei* administration decreased pH levels in mouse serum samples (Fig. 9A), although pH values (8.0 ± 0.2) were comparable between the two groups before the initial treatment with *L. sakei*.

Moreover, the abundance of SCFAs in mouse serum, as well as acetic acid, propionic acid, butyric acid, and valeric acid levels in fecal samples, collected from both control and probiotic-treated mice, was analyzed at 10 days after the initial *L. sakei* treatment. Results revealed that the probiotic-treated group showed significantly higher levels of SCFAs in serum samples than the control group (Fig. 9B and C). The initial SCFA concentration (4.5 ± 0.5) was evaluated before the treatment of *L. sakei.*

Time-kill assay with CFCS of *L. Sakei* strain on CRKP pathogens

A time-kill assay was executed to determine the bactericidal activity of *L. sakei* against the CRKP pathogen. Results revealed that the survivability of CRKP in the presence of *L. sakei* CFCS was decreased, illustrating a powerful kill effect of *L. sakei* CFCS against indicator pathogens. The CFCS of *L. sakei* in both MRS and EFGM completely prevented the growth of CRKP after 4 h and 6 h of incubation, respectively. In contrast, the viability of the pathogen in the control tube was gradually enhanced according to the passage of time. These results support the presence of antibacterial substances such as organic acid and bacteriocin in the CFCS of the *L. sakei* strain (Fig. 10).

Discussion

Most studies have shown that CRE strains are among the main urgent threats due to the emergence of resistance to carbapenem (Caliskan-Aydogan and Alocilja 2023). Over the last few decades, increasing mortality due to CRE infections has been progressively reported (Centers for Disease and Prevention 2013; Li et al. 2024; Zhang et al. 2024).

Recently, probiotics have been a significant focus of research due to their solid antibacterial abilities to increase defense against antibiotic-resistant pathogens and prevent infections that can produce variable morbidity and mortality (Manley et al. 2007; Karska-Wysocki et al. 2010; Nagasaki et al. 2010; Asahara et al. 2016; Olaimat et al. 2020; Reikvam et al. 2020; Lopez-Santamarina et al. 2021). Probiotics, known as immunomodulators, regulate

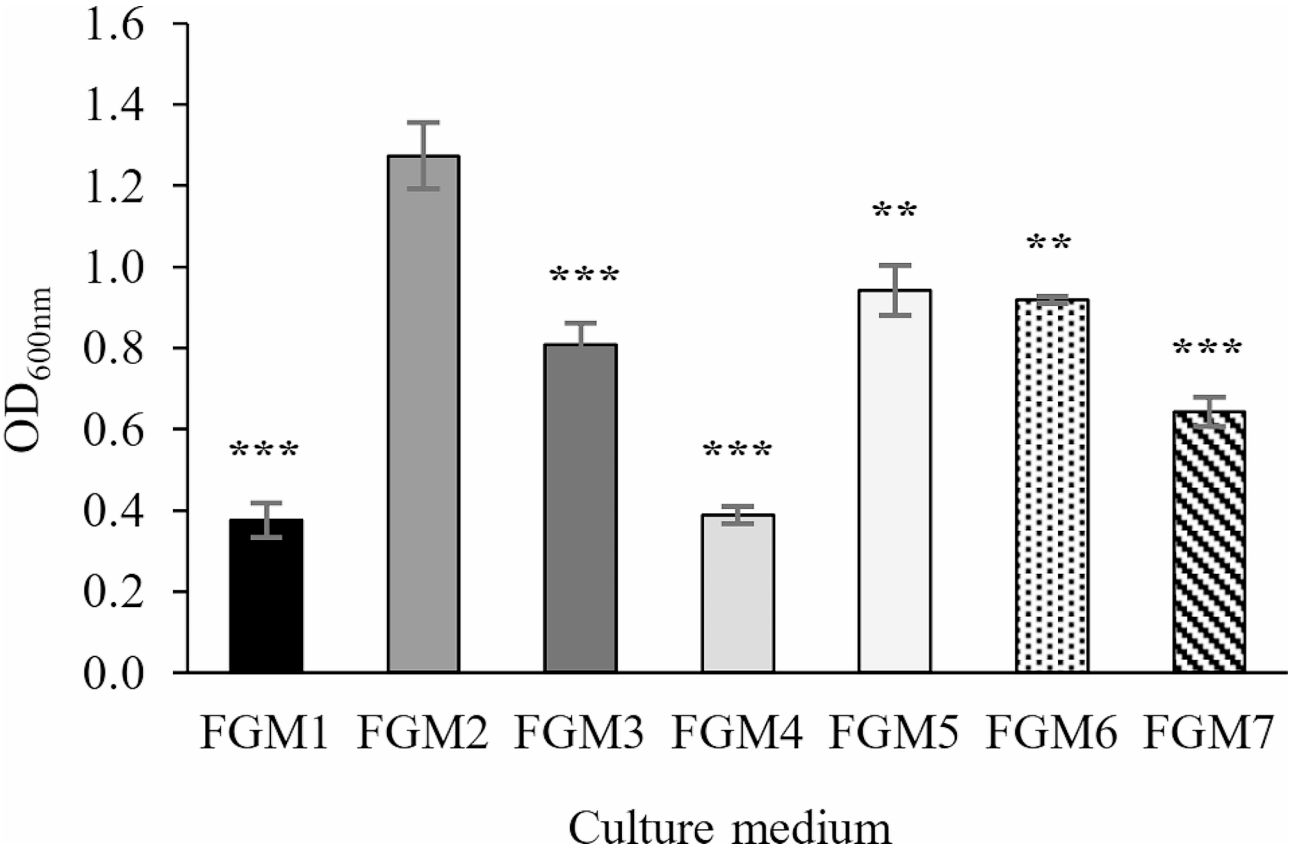

Fig. 3 Culture of *L. sakei* using a lab scale fermenter based on FGMs. *L. sakei* strain was inoculated into seven different types of FGMs and incubated at 37 ℃ for 18 h to reach its endpoint growth in different media. The bacterial culture growth rates were checked using a spectrophotometer. All data are presented as mean ± SD. They were log-transformed for each experiment. Significant differences are indicated by asterisks (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) using unpaired Student's t-test. A *p*-value of less than 0.05 was considered statistically significant

innate and adaptive immune responses through mechanisms such as vitamin and fatty acid production, bacteriocin release, and cell adhesion, thereby contributing to pathogen exclusion and microbiota balance in different body sites during pathogenic infections (Coudeyras et al. 2008; Ishikawa et al. 2010; Plaza-Diaz et al. 2019; Raheem et al. 2021; Yan et al. 2021). Therefore, we aimed to develop a new probiotic candidate to investigate its potential as an antibiotic alternative against CRE infection and evaluate its effect as a novel treatment.

Throughout our examinations of the potential effects of probiotic strains (isolated from traditional Korean kimchi) against the CRE pathogen, one strain demonstrated excellent anti-infective activity against the CRE pathogen. It was expected to inhibit deadly infection caused by CRE. The isolated strain was confirmed to be *L. sakei* by 16srRNA sequencing. Its detailed genetic characteristics and DNA sequences were obtained through whole sequencing methods. A real-time PCR validation process was performed to support and ensure the accuracy of NGS data. Moreover, this specific strain was evaluated as a new strain of *L. sakei* as it showed a different carbon use pattern from an existing standard *L. sakei* strain (Ghorbanian et al. 2022).

According to previous studies, the *L. sakei* strain has a significant and remarkable effect against many bacterial pathogens, including *Enterococcus faecium*, *E. coli*, *K. pneumoniae*, *Listeria ivanovii subsp. ivanovii*, *Listeria monocytogenes*, *Pseudomonas spp.*, *Staphylococcus aureus*, *Streptococcus caprinus*, and *Streptococcus spp* (Todorov et al. 2012; Ciandrini et al. 2017). *L. sakei*, as one of the main lactic acid bacterial (LAB) strains, can protect the host against infections with potential preventive effects, as its bacteriocin can control multi-drug resistant pathogens and bacterial infections (Diep et al. 2000; Vaughan et al. 2001; Mandal et al. 2016; Benítez-Chao et al. 2021; Riesute et al. 2021). Moreover, previous reports have indicated that *L. sakei* can enhance IgA production and improve the host's immune systems to regulate the intestinal environment (Miyoshi et al. 2021). Therefore, we decided to use this particular strain for our further experiments.

According to FDA (U.S. Food and Drug Administration) regulation, probiotics may be classified under the Food, Drug, and Cosmetic Act (Code 2013), depending

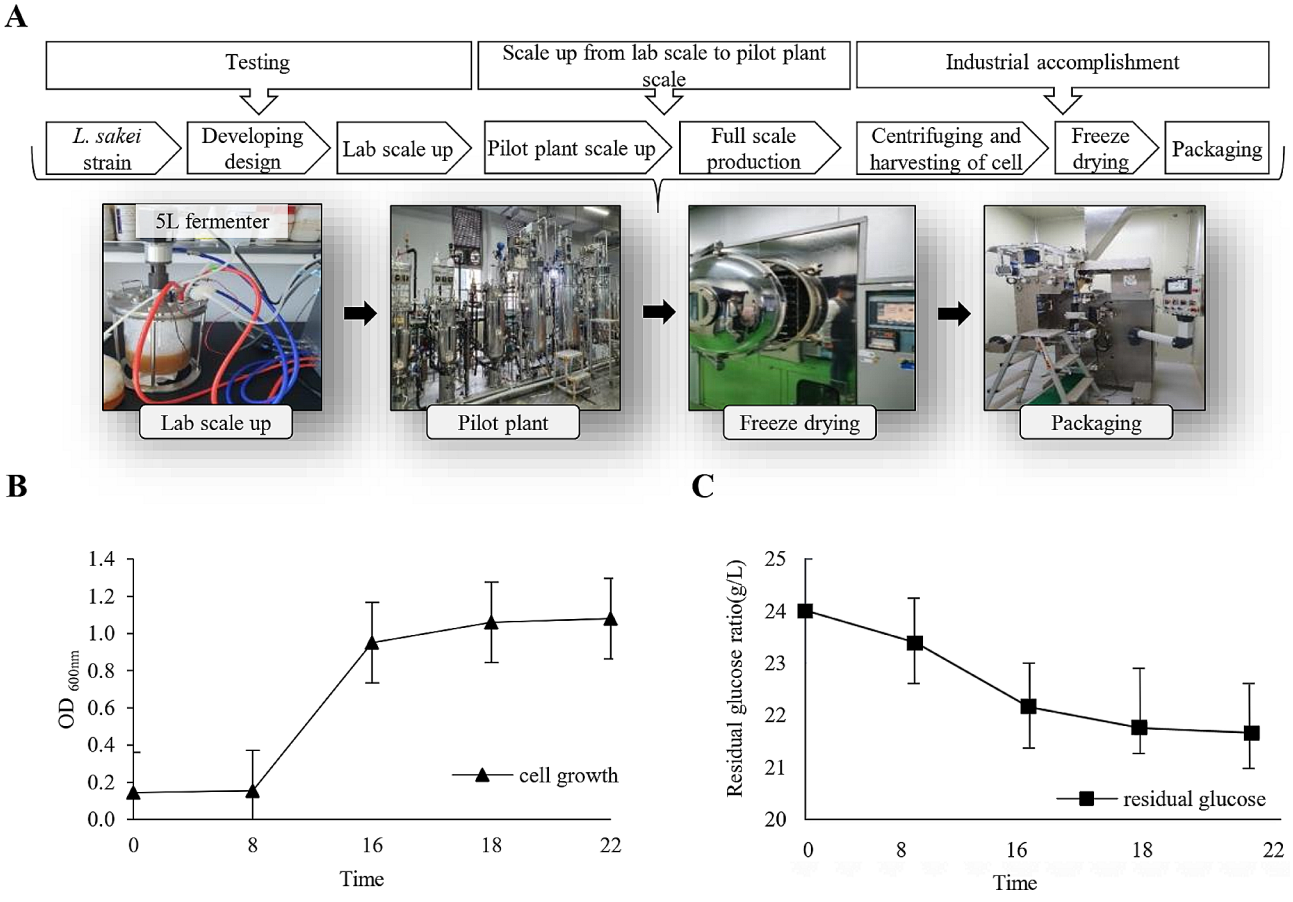

Fig. 4 Pilot scale-up fermentation of *L. sakei*.**A** A schematic diagram showing the whole process from lab scale-up steps to packaging. *L. sakei* culture was transferred from lab scale-up culture (5 L) to pilot scale-up fermentation (200 L). After the fermentation process, a freeze-dried powder was obtained using a freeze-drying process and packed using a packaging method. **B** Cell growth rate was checked at 0, 8, 16, 18, and 22 h of fermentation. **C** Residual glucose concentration was also measured during fermentation at 0, 8, 16, 18, and 22 h. All data are presented as mean ± SD

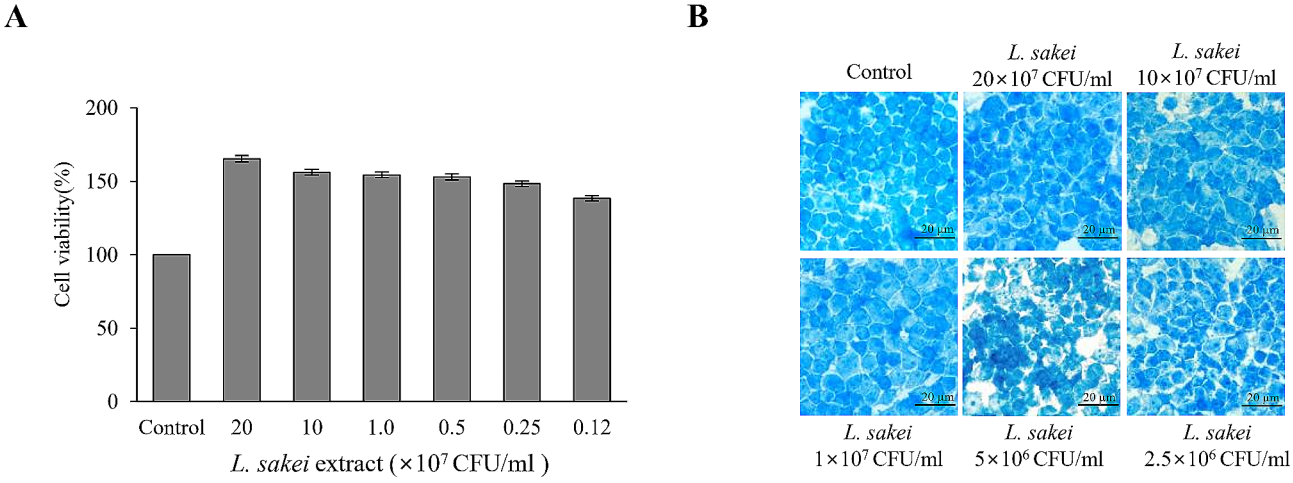

Fig. 5 Cytotoxicity of *L. sakei* extract to RAW 264.7 cells. **A** The cytotoxicity of *L. sakei* extract at various concentrations in a macrophage cell line were evaluated by a WST cell viability assay. Cell viability in untreated group (control) was considered to be 100%. **B** Cell viability was checked by methylene blue staining. Scale bar = 20 μm. Experiments were performed in triplicate. Values are expressed as mean values and standard deviations. All data are presented as mean ± SD

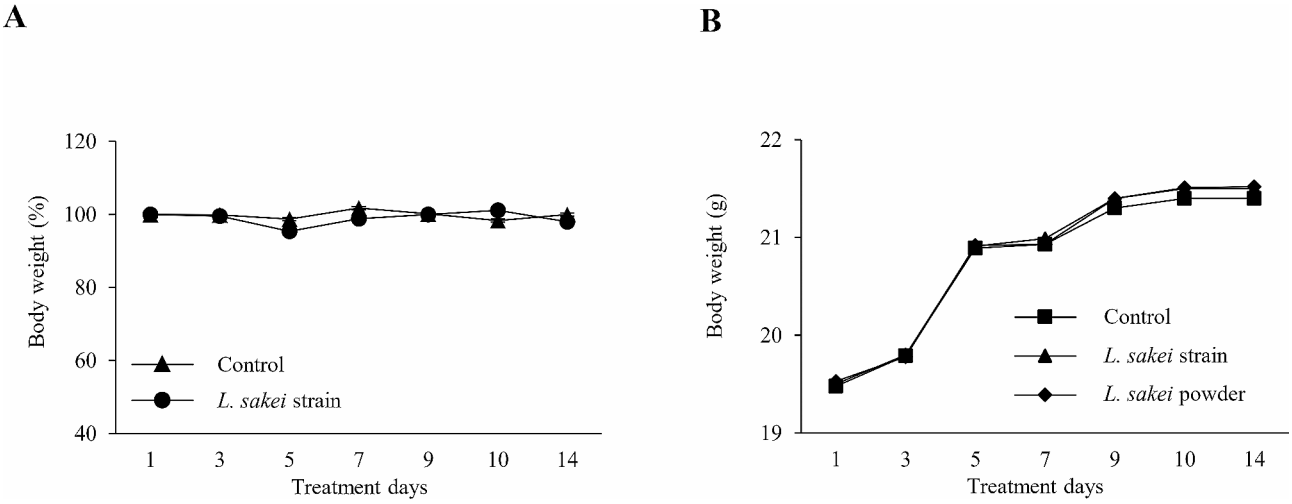

Fig. 6 Two-week repeated oral dose toxicity test of *L. sakei*. **A** Guinea pigs in the treatment groups were administered *L. sakei* orally at 2×10^8 CFU/animal (200 µL per animal) for 14 days, whereas the control group was treated with sterile water. During the experimental period, body weights of all guinea pigs were measured on days 1, 3, 5, 7, 9, 10, and 14. **B** Mice in treatment groups received *L. sakei* strain and its powder at a concentration of 3×10^9 CFU/mL through drinking water daily and the single dose administration 3×10^9 CFU/animal (200 µL per animal) once a day using NaCl solution from day 1 to day 14, respectively. In contrast, the control group was treated with sterile water. During the experimental period, the body weights of all mice were measured every 2 days from day 1 to day 10. All data are presented as mean ± SD

on the intended use of the product (Food 1938; Degnan 2008). Production of probiotic powder reported as an antibiotic alternative has increased dramatically due to their potential benefits for human health (Suvarna and Boby 2005; da Cruz et al. 2007; Cao et al. 2019; Obianwuna et al. 2023).

Hence, we used the *L. sakei* strain to develop an antibiotic alternative by constructing industrial processes from upscaling in a pilot plant to packaging, as Large-scale production is essential for assessing the commercial viability of *L. sakei* as a potential therapeutic candidate (Behera et al. 2020). To ensure reliable manufacturing processes, *L. sakei* was cultured in a modified EFGM medium (edible) to enhance the FGM from laboratory grade to a safe and edible medium grade, potentially suitable for human consumption (Fenster et al. 2019). Consequently, *L. sakei* culture was scaled up to a 200 L pilot scale. *L. sakei* powder of 3.0 kg was obtained after freeze-drying and packaging processes. Accordingly, the *L. sakei* powder was packed with polyethylene material at a ratio of 1:3 in order to ensure the highest level of bacteria preservation, thereby enabling the execution of its preventive and therapeutic effects (Vaudant 2008; Fonseca et al. 2015; do Morais et al. 2016; Quintana et al. 2017).

In this study's in vivo experiment, compared to long-term oral treatment with *L. sakei* culture, the *L. sakei* powder showed a more significant effect in reducing illness severity with an inhibitory effect on CRKP strain because all infected mice survived in the long term during this study. Other studies have also reported that using probiotic powder instead of bacteria culture is preferred due to the higher viability of the product during storage and ease of use(Naksing et al. 2019; Oktavia et al. 2020). A probiotic powder of several Gram-negative and Gram-positive pathogens can also prevent infections and reduce antibiotic-resistant infections in in vivo experiments (Kechagia et al. 2013; Ghoneum and Abdulmalek 2021).

Comprehensive examinations were subsequently carried out to confirm the safety of probiotic powder for consumption and its potential impact on human health. A two-week acute oral toxicity study was first performed using a guinea pig mouse model due to its biological similarities to humans (Padilla-Carlin et al. 2008). Further, a cytotoxicity study using RAW 264.7 cell line was conducted to determine the effect of powder on the viability of macrophages (Du et al. 2022). In vivo and in vitro studies demonstrated that *L. sakei* exhibited no toxicity during experiments. However, further studies are necessary to investigate the biological mechanisms involved in the antimicrobial effect of *L. sakei* powder and any potential development of resistance, considering the probiotic's ability to serve as a reservoir for antibiotic resistance (Gueimonde et al. 2013). Our findings show the preliminary result in such research. Additional extensive studies, including the determination of optimal dosing, and relevant validation through clinical trials at the pharmaceutical grade or level are also needed to support the main aspects of this research and develop a new drug candidate with high efficacy against CRKP infections.

Despite the experimental results, there are some limitations in this study. First, in our study CRKP infection was significantly reduced in preventive animal models indicating the promising effect of our probiotic candidate. However, extensive animal models such as therapeutic

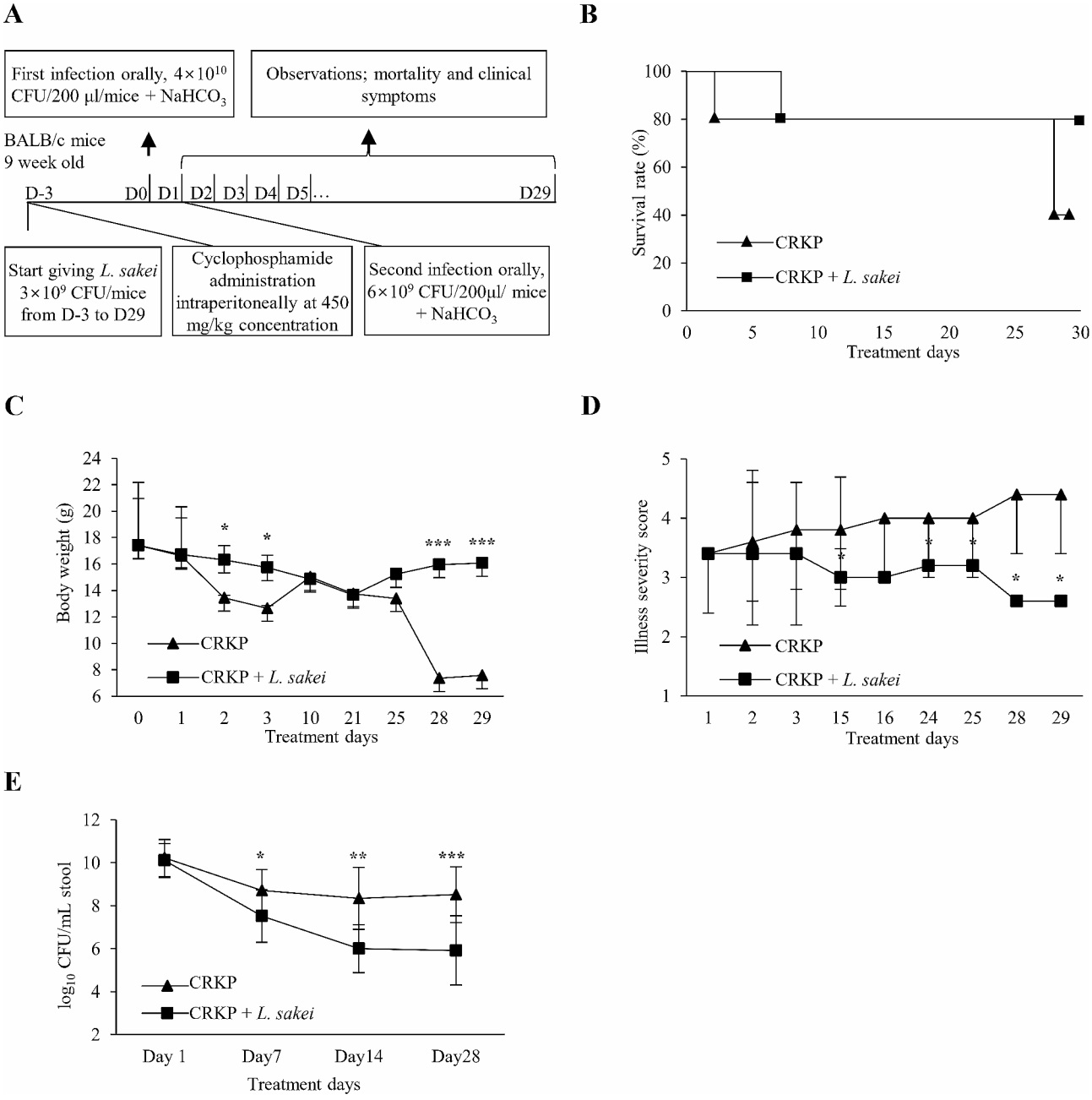

Fig. 7 Prophylactic effect of *L. sakei* strain in a CRKP-infected mice model. **A** Candidate probiotic strain *L. sakei* was administered 3 days before infection to observe its preventive effect during 29 days of the experimental period. **B** The survival rate of mice was observed for 30 days post-infection. **C** Body weight was measured for 29 days. **D** Illness severity scores (score parameters: 1, healthy; 2, minimally ill; 3, moderately ill; 4, severely ill; 5, dead) of all mice were evaluated during 29 days. **E** The burden of CRKP infection in the stools was significantly reduced, especially on day 8. Significant differences are indicated by asterisks (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) using unpaired Student's t-test. A p-value of less than 0.05 was considered statistically significant. All data are presented as mean ± SD

and evaluation with CRKP infection drug as a control is needed. Second, although toxicity assay was performed in this study, more in-depth toxicity assay such as GLP toxicity should be performed. Finally, to truly understand immune regulatory effect of *L. sakei*, extensive experiments including analysis of histopathology and probiotic impact on mice organs are important to perform. Although this study had these limitations, our findings and can be used as primary data for clinical level.

In conclusion, our study aimed to investigate the efficacy of *L. sakei* as a therapeutic candidate for CRKP infection through preliminary investigations. The results of this study indicate that the *L. sakei* strain can potentially be used as a therapeutic candidate to treat

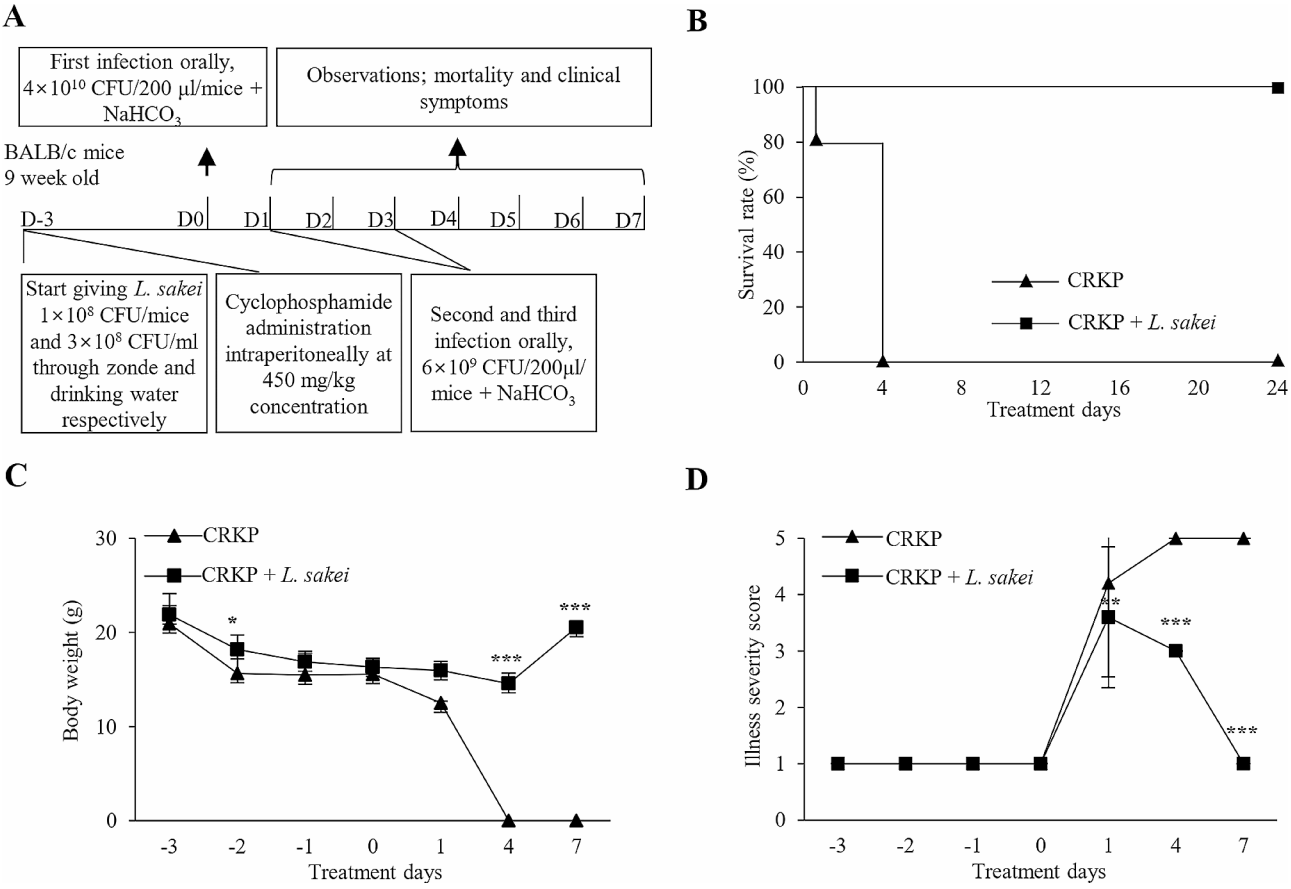

Fig. 8 Preventive effect of *L. sakei* powder in a CRKP-infected mice model. **A** *L. sakei* powder was administered 3 days before infection and observed for 24 days to investigate the preventive effect of our candidate strain. **B** Mice were monitored for one week for mortality. **C** Body weight was checked at 7 days. **D** Illness severity scores (score parameters: 1, healthy; 2, minimally ill; 3, moderately ill; 4, severely ill; 5, dead) of all mice were measured during probiotic treatment for up to 7 days. Statistical difference in the number of microorganisms from different treatment groups was determined. Significant differences are indicated by asterisks (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) using unpaired Student's t-test. A p-value of less than 0.05 was considered statistically significant. All data are presented as mean ± SD

infections caused by various multi-resistant pathogens, such as CRKP in the future as a novel antibiotic alternative.

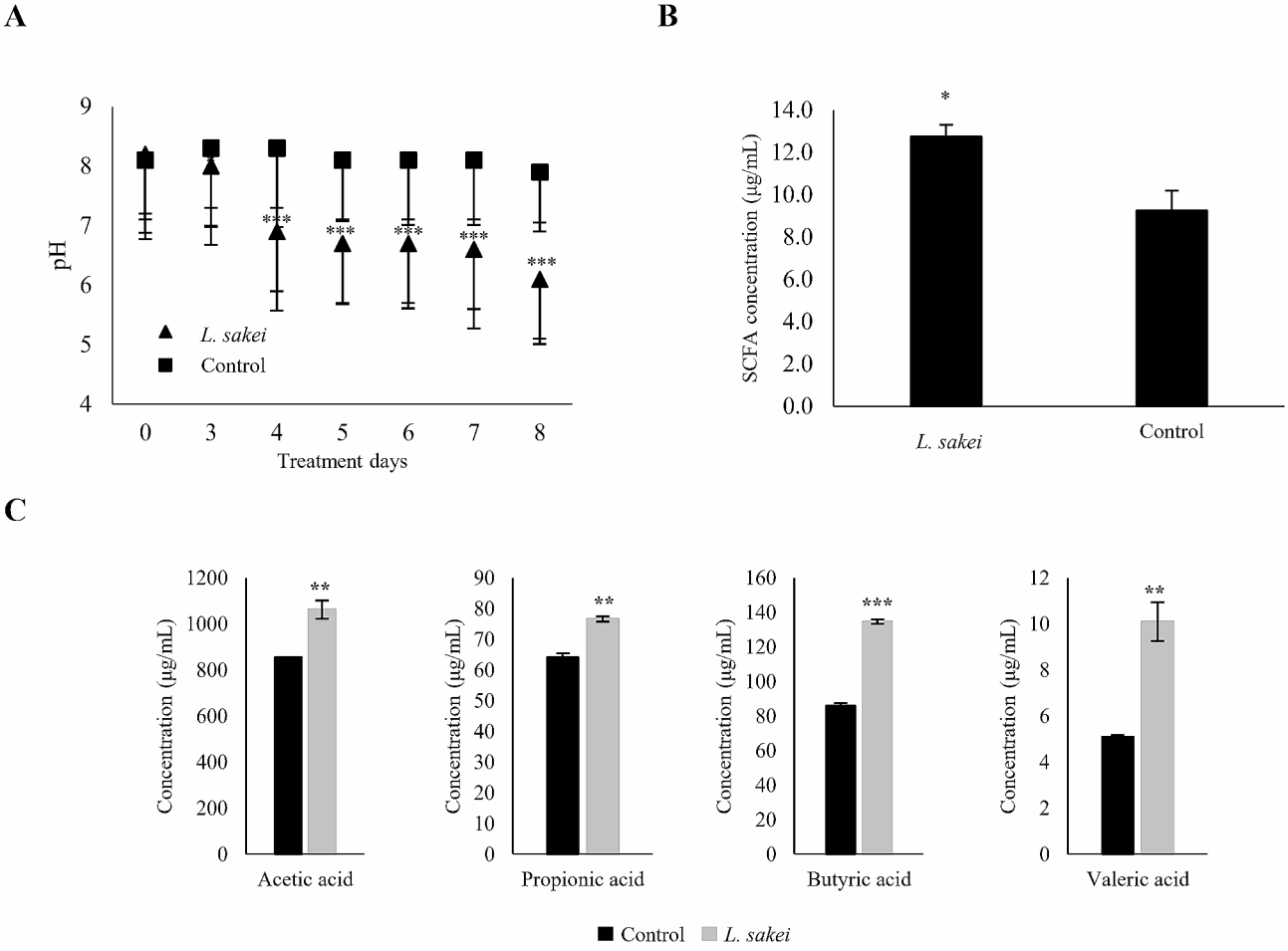

Fig. 9 Effect of *L. sakei* treatment on pH levels and SCFA concentration. **A** The effect of the *L. sakei* strain on fecal pH in mice was determined after receiving *L. sakei* treatment for 10 days. **B** The effect of *L. sakei* on the concentration of short-chain fatty acids (SCFAs) was also determined in both *L. sakei* treatment and control mice using a mouse short-chain fatty acids (SCFAs) ELISA kit. **C** The concentration of different short-chain fatty acids in the feces of mice was measured after 10 days. The statistical difference in the number of microorganisms from different treatment groups was determined. Significant differences are indicated by asterisks using unpaired Student's t-test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). A p-value of less than 0.05 was considered statistically significant. All data are presented as mean ± SD

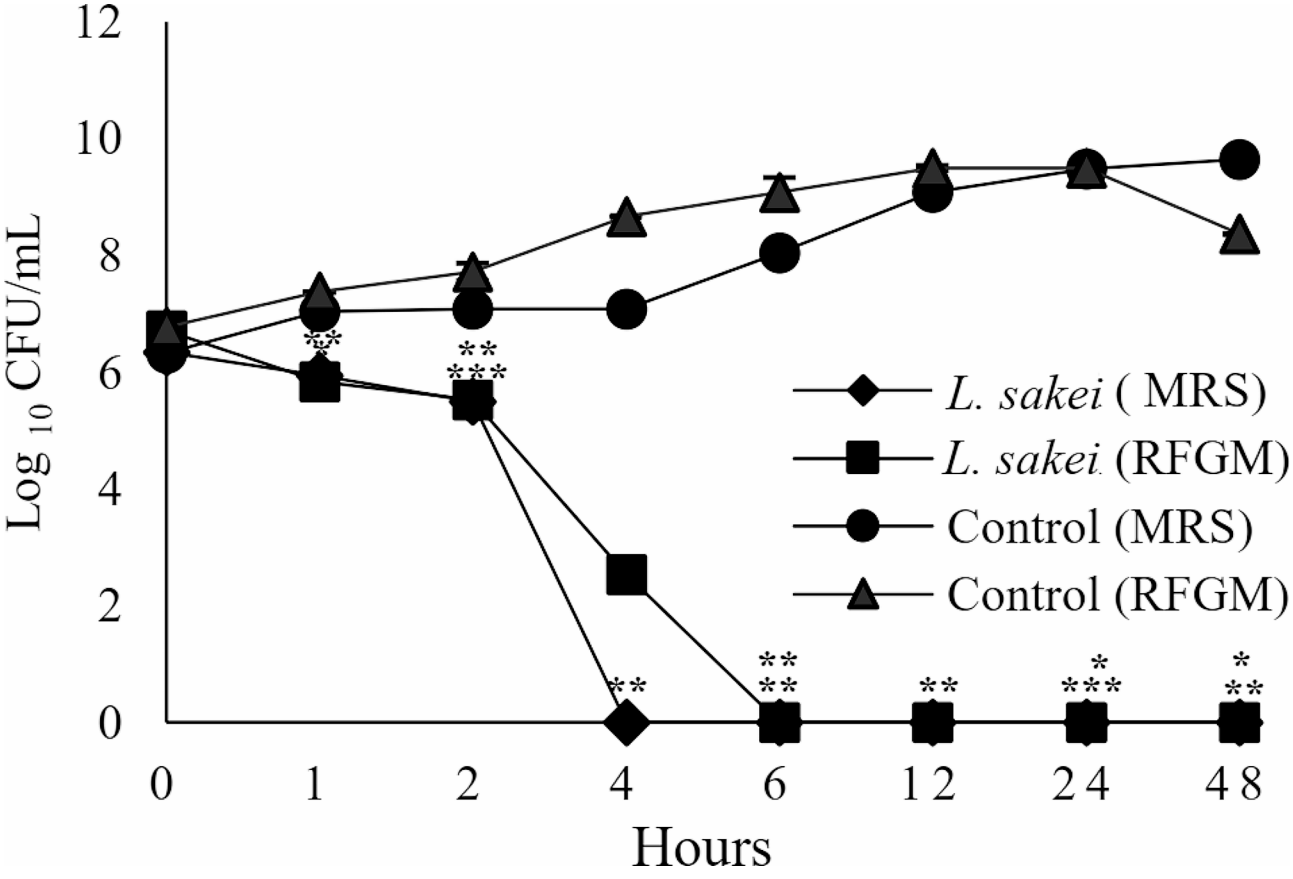

Fig. 10 Time-kill assay conducted with *L. sakei* strain against CRKP pathogen at different time intervals. To evaluate the antimicrobial effect of *L. sakei*, CFU of CRKP was counted on MacConkey agar after exposure to the cell-free-culture supernatant (CFCS) of *L. sakei* strain. Values are presented as standard deviation (SD) of kill assay data conducted in triplicate. Significant differences are indicated by asterisks using unpaired Student's t-test (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). A p-value of less than 0.05 was considered statistically significant

Abbreviations

CRE	Carbapenem-resistant Enterobacteriaceae
CRKP	Carbapenem-resistant Klebsiella pneumoniae
L. sakei	Lactobacillus sakei
L. curvatus	Lactobacillus curvatus
L. rhamnosus	Lactobacillus rhamnosus
AMR	Antimicrobial resistance
FGM	Food grade media
EFGM	Edible FGM
MRSA	Methicillin-resistant Staphylococcus aureus
VRE	Vancomycin-resistant Enterococcus
MRS	Rogosa and Sharpe
CFCS	Cell-free-culture supernatant
SCFA	Short-chain fatty acid
WGS	Whole-genome sequencing
gDNA	Genomic DNA
IACUC	Institutional Animal Care and Use Committee
NCBI	National Center for Biotechnology Information
KGMB	Korean Gut Microbiome Bank
COG	Clusters of Orthologous Groups
CDS	Coding sequences
LAB	Lactic acid bacterial
MOTIE	Ministry of Trade Industry, and Energy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-024-01773-8.

Supplementary Material 1

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Author contributions

Conceptualization: HYS; Methodology: HT, YL, FG, FS, YJ; Former analysis: HT, SK; Resources: SKK, JJ, BO, CK; Writing—review, and editing: HT, MR, HS; Visualization: HS, SK; Supervision: HYS; Project administration: HT, HS, SL.

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Data availability

The datasets used or analyzed for this manuscript are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All animal experiments using specific pathogen-free (SPF) BALB/c female mice were performed after they were approved by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University (approval number: SCH23-0004). The two-week toxicity assay using adult male guinea pigs was performed after it was approved by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University (IACUC approval number: SCH-22-0111).

Consent for publication
All authors have read and approved the final manuscript. The authors declare that the article is original, has not already been published in a journal, and is not currently under consideration by another journal.

Competing interests
The authors have no conflicts of interest relevant to this study to disclose.

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