Efficacy of lyophilized Lactobacillus sakei as a potential candidate for the prevention of carbapenem-resistant Klebsiella infection

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Abstract

Background

Antimicrobial resistance has been considered one of the greatest threats to human health, according to the World Health Organization (WHO). Gram-negative bacteria, especially carbapenem-resistant Enterobacteriaceae (CRE), drive this alarming trend. Among CRE pathogens, carbapenem-resistant Klebsiella pneumonia (CRKP) has recently been reported as a highly infectious one responsible of a 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 mechanism of action from the existing drugs is urgently required to defeat this lethal menace. In these circumstances, probiotics intended for being a potential choice to be a therapeutic candidate and inhibit the pathogens. Thus, our research team has been focusing on probiotics for a long time to develop potential anti-CRKP drug agents.

Methods

After such efforts, we finally found a novel Lactobacillus sakei PMC104 derived from kimchi, a probiotic strain suitable for treating CRE infection. Next, as part of our expansion into therapeutic development, we did media optimization at food grade and then established a scale-up process to pilot scale. A lyophilizate was then obtained, which was subsequently used in a mouse model infected with CRKP.

Results

Data showed that treatment with L. sakei powder remarkably diminished the body weight loss, mortality, and illness severity in CRKP-infected mice which shows the preventive effect of our PMC 104 against CRKP infection.

Discussion

Our results exhibit the potential therapeutic effect of our candidate probiotic strain opposed to the CRKP, advocating that L. sakei can be congested as an antimicrobial candidate for treating CRKP infections. However, extensive studies such as toxicity tests and clinical trials are still needed to develop it as a new anti-CRE therapeutic agent.

Introduction

Antimicrobial resistance (AMR) caused by drugs used to treat infections can make drugs no longer practical for pathogens. It has emerged as a global public health threat of the 21st century (Hu et al., 2020). According to recently published data in 2022, 4.95 million deaths were associated with bacterial AMR 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). Infections due to CRE in healthcare settings are on the rise. They are assigned to the category of “urgent threats.” According to the Center for Disease Control and Prevention (CDC), 13,100 CRE infections were estimated among hospitalized patients, with 1,100 deaths in the USA in 2017 (Control and Prevention, 2019).

*Enterobacteriaceae* is a Gram-negative bacterium that has progressively developed the broadest spectrum of resistance to nearly all last-resort antibiotics (Atterby et al., 2019; Janda and Abbott, 2021). Among them, *K. 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), the resistance of *K. pneumoniae* to meropenem and imipenem has increased rapidly from 2.9% and 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 mechanisms of action to overcome the limitation of currently existing carbapenem drugs. Researchers are now trying to overcome this resistance problem by focusing on novel probiotics that can effectively fight against this deadly menace (Davies et al., 2013; Lawrence and Jeyakumar, 2013; Harikumar and Krishanan, 2022).

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 have enormous beneficial effects on the host by stimulating different types of immunomodulatory responses, lymphocyte proliferation, and the production of anti-inflammatory regulatory cytokines (Ashraf et al., 2014; Manuel et al., 2017). They also have medicinal properties. Thus, they are widely used as therapeutic candidates (Ishibashi and Yamazaki, 2001; Ukeyima et al., 2010; Ozyurt and Ötiles, 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 the 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., 2022b). Furthermore, they possess antibacterial effects against superbugs such as CRE, vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant *Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Thus, they could be used as an alternative treatment to nearly all pathogens (Sidjabat et al.; Manley et al., 2007; Mirnejad et al., 2013; McFarland, 2015; Kumar et al., 2016; Moghadam et al., 2018). Previous studies using in-vivo animal models have indicated that *Lactobacillus sakei* among probiotics can prevent pathogenic bacteria by reducing septic shock and body weight loss in mice and improve illness symptoms (Hong et al., 2014; Lim et al., 2016; Kwon et al., 2018; Ji et al., 2019; Jang and Min, 2020). Therefore, *L. sakei* has attracted particular attention. It is a good candidate for developing novel probiotic powder 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 pharma-biotic alternative among probiotic strains to treat infections caused by CRE, with terms of evaluating the scale-up study and novel food grade medium development.

**Materials and Methods**

**CRK clinical strain**

A CRK Clinical strains were collected from the Infectious Diseases Department of Soonchunhyang University. Upon receiving, they were cultured aerobically overnight at 37 °C in MacConkey broth (BD Difco, USA). Afterward, bacteria were cultivate to OD 1.0, 2×10⁹ CFU/mL at 600 and CRK colonies were isolated as described in previous paper (Tajdozian et al., 2021a).

**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 MRS agar (BD Difco, USA) plates containing vancomycin solution (Colombo et al., 2014) at 4 μg/mL (Sigma-Aldrich, USA) followed by incubation at 37 °C for 18 h using a microaerophilic chamber (Daeiltech, Korea). Next, single colonies were cultivated into MSR broth (Sigma-Aldrich, USA) and incubated under the same conditions. Subsequently, bacterial growth was measured, and bacteria were washed, centrifuged, and resuspended in 0.85% NaCl solution. The 16S rRNA sequencing analysis was then performed for probiotic culture at Biofact company, Korea.

**16S rRNA identification**

The 16S rRNA gene sequencing technology was conducted using sequencing protocol and the candidate probiotic was identified analyzed and analyzed at the BIOFACT Co, Korea sequencing company 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) 16S microbial database using NBLAST. The sequencing resulting hits were sorted first by e-value and then by bit score. The taxonomy of the hit with the highest scoring sequences was then reported.

**PCR identification of the candidate probiotic strain**

PCR amplification and qPCR analysis were conducted to confirm 16S 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 positive control), *Lactobacillus curvatus* KGMB 05016, and *Lactobacillus rhamnosus* KGMB 06348 (used as negative control) obtained from the Korean Gut Microbiome Bank (KGMB) as reference organisms. DNA quality and concentrations analyzed using0.8% agarose gel electrophoresis and Qubit-4 fluorometer.
(Thermo Fisher Scientific, UK), respectively. After that, PCR amplification was performed using species-specific primers (F: GATAAGCGTGAGGTCGATGGTT, R: GAGCTAATCCCCCATAATGAAACTAT) (Justé et al., 2008; Andani et al., 2012) with a Thermal Cycler Applied Biosystems platform (Thermo Fisher Scientific, USA). PCR reactions were carried out and quality of the PCR products were checked using agarose gel electrophoresis. The bands were visualized with a Molecular Imager ChemiDoc XRS + imaging system platform (Thermo Fisher Scientific, USA) after ethidium bromide staining. Quantitative analysis of agarose gel was obtained by an 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 2x Master mix; Qiagen, USA) with the same primers as described above. A triplicate reaction of each sample (Candidate stain, L. curvatus KGMB 05016, and L. rhamnosus KGMB 06348) and the PCR conditions optimized as described in (Seo et al., 2023).

**Biochemical tests**

The API 50CH Biochemical test was used to illustrate the biochemical profiles of our candidate probiotic. A single colony of our candidate strain culture was merged in a 1 mL suspension medium. Afterward, when turbidity caused by 2 McFarland reached its maximum, 1 mL of suspension was diluted in 10 mL of CHB medium (bioMerieux, France). Finally, the API kit strip (bioMerieux, France) wells were filled with the bacterial suspension and incubated at 37 °C for 48 h. Later, an identification table was assembled as (+/-) 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 cultivate 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 3M (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, the pH values of all FGMs except FGM 1 were adjusted to 6.4 using NaOH (Sigma Aldrich, USA). Initial pHs of FGM 2, FGM 3, FGM 4, FGM 5, FGM 6, and FGM 7 were 6.3, 6.2, 6.5, 6.1, 5.8, and 6.0, respectively. Next, all FGMs were sterilized at 121 °C for 15 min. The probiotic strain was then inoculated into the prepared FGMs and incubated at 37 °C for 18 h. Subsequently, the OD was checked with a spectrophotometer. Bacterial culture from the best grown FGM was stored (OD$_{600}$ = 1.0, 2 × 10$^8$ CFU/mL) at -80 °C using 20% glycerol.
Table 1
Ingredients of optimized food grade media (FGM)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRS media</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Yeast peptone standard type F</td>
<td></td>
</tr>
<tr>
<td>Proteose peptone No. 3</td>
<td></td>
</tr>
<tr>
<td>Beef extract</td>
<td></td>
</tr>
<tr>
<td>Yeast extract</td>
<td></td>
</tr>
<tr>
<td>Dextrose</td>
<td></td>
</tr>
<tr>
<td>Tween 80</td>
<td></td>
</tr>
<tr>
<td>Triammonium citrate</td>
<td></td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate 3M</td>
<td></td>
</tr>
<tr>
<td>magnesium sulfate</td>
<td></td>
</tr>
<tr>
<td>Manganese (II) sulfate</td>
<td></td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td></td>
</tr>
</tbody>
</table>

* mL

We also prepared edible FGM (EFGM) for pilot plant fermentation by optimizing the FGM so that our probiotic strain could grow the best. 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.

**Inoculum preparation for a scale-up process**

For a scale-up process, we prepared *L. sakei* inoculum from lab scale to industrial scale step by step. 1 mL of stored probiotic culture broth was inoculated into 10 mL of EFGM and incubated at 37 °C 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 °C 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 °C with only agitation at 25 rpm (no sparging of air or N₂ gas) and the pH was maintained at 6.0 using 28% (w/v) NH₄OH. During fermentation, the culture broth was collected aseptically 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 5% (w/v), 1.5 kg of reconstituted skim milk (RSM). Next, to produce probiotic powder from harvested cells, freeze-drying was performed at -60 °C for 72 h under a pressure of 6.7 × 10⁻² mbar using a 50 L freeze-dryer (PVTFD 50R, Korea). 2.5 kg out of 3.0 kg produced powder was packed for further experiments. Before packaging, some food additive ingredients including D-glucose (5 kg), dextron (1 kg), and cornmeal (1 kg) were added into the powder and blended by 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 in further experiments.

Experimental design for in vivo efficacy test

Animal experiments using specific pathogen-free (SPF) BALB/c female mice (9 weeks old) purchased from Doo Yeol Biotech company, Korea were performed and approved as described previously (Tajdozian et al., 2021b) (approval number: SCH23-0004). All mice were randomly divided into two groups (infection and treatment groups; n = 5 per group) for both models. Briefly, two preventive mice experiments were performed to estimate the efficacy of our candidate probiotic against CRE infection. In the first model, we investigated the long-term preventive effect by administering probiotic culture before infection. In the second model, we treated mice with probiotic powder instead of culture. In both models, the group treated with probiotic received L. sakei culture or L. sakei powder during treatment with probiotic. In contrast, the infection group only received sterile saline through oral gavage similarly under the same conditions. Sodium bicarbonate (NaHCO₃) was administered before CRE infection to both models to increase the severity of infection by neutralizing stomach acidity (Czuprynski and Faith, 2002). Additionally, the neutropenia was induced three days prior to the infection at 450 mg/kg intraperitoneally by 200 µl of
cyclophosphamide injection (Sigma Aldrich, USA) (Pan et al., 2015). The survival rate, body weight, and illness score of mice in both models were obtained during the experimental.

**Long-term treatment efficacy of L. sakei in CRE-infected mice model**

We estimated the long-term preventive effect of our probiotic strain *L. sakei* in CRE-infected BALB/c mice. The probiotic culture was prepared using EFGM and adjusted to an approximate concentration of $3 \times 10^9$ CFU/mouse (200 µL/mouse) using NaCl solution. The probiotic culture was administered twice a day by oral gavage from 3 days before infection to day 29. Three days after neutropenia, 200 µL of CRE was administered to mice orally at $4 \times 10^{10}$ CFU/mouse and $6 \times 10^9$ CFU/mouse on days 0 and 1, respectively. NaHCO$_3$ (0.2 M, 200 µl) (Sigma Aldrich, USA) was administered before infection.

**Preventive effect of L. sakei on CRE-infected mice model**

We investigated the preventive potential of *L. sakei* powder in CRE-infected BALB/c mice. The probiotic powder was first prepared using sterile water and administered to mice at $1 \times 10^8$ CFU/mouse (200 µL per mouse) orally as a single dose from day −3 to day 7. At the same time, $3 \times 10^8$ CFU/mL of probiotic powder was provided through drinking water. In this model, infection was induced orally of CRE at $4 \times 10^{10}$ CFU/mouse (200 µL) on day 0 and $6 \times 10^9$ CFU/mouse on days 1 and 3 with pretreatment of NaHCO$_3$ (0.2 M, 200µL).

**The study of acute toxicity of candidate probiotics**

To examine the oral toxicity of *L. sakei*, a two weeks toxicity assay using adult male guinea pigs was performed and approved, as previously described (Lee et al., 2021), (IACUC) approved number was SCH-22-0111. Animals were separated into two groups (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 $1 \times 10^8$ CFU/animal (200 µL per animal) orally for 14 days whereas the control group received just saline. During the study the body weight and mortality measured and the clinical symptoms obtained.

**Whole Genome Sequencing**

In order to indicate a better identification for our candidate probiotic, Whole-genome sequencing test was done at the WGS service company (Chunlab Inc. Seoul, Korea). Briefly, Genomic DNA (gDNA) of *L. sakei* strain was extracted using the QIAaamp DNA Mini kit (Qiagen, Germany). Then the PacBio sequencing results were obtained from the HGAP2 protocol using PacBio SMRT Analysis 2.3.0 and, Circlator 1.4.0 (Sanger Institute, UK) was performed resulting in circulated contigs from PacBio sequencing.

**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 ($1.5 \times 10^5$ cells/mL) into a 96-well plate
(SPL life science, Korea) and incubated at 37 °C overnight with 5% CO₂. After that, RAW cells submitted to the *L. sakei* extract diluted to various concentrations using fresh media, and incubated again at 37 °C for 24 h. Following incubation, 10 µl of WST solution was added to each well. After incubation for 30 min, absorbance was measured and cell viability was checked by a Victor Nivo Multiplate reader (Perkin Elmer, USA) at 450 nm and methylene blue staining, respectively. Cells were seeded onto 2-well cell culture slides (SPL life sciences, Korea), and submitted to our *L. sakei* strain extract. Finally, cells were counted by a hemocytometer (Marienfeld) using an optical microscope (AX10, Carl Zeiss, Germany).

**Results**

**16S rRNA-based identification of isolated strain**

Among colonies isolated from fermented products, a kimchi-derived strain was an effective candidate against CRE infection. After 16S rRNA gene sequencing was conducted for this strain (Table 2), sequences were compared with sequences deposited in the database of NCBI. Results showed that our isolated strain indicates 99% sequence similarities with 16S rRNA gene sequences of *L. sakei* strains, including DSM 20017, NBRC 15893, and CCUG31331. Additionally, its sequences were similar to those of other strains of the *Lactobacillus* genus, with similarities ranging from 96–99%. Additionally, NBALST similarity scores showed 186 hits for *L. sakei* among unique sequences submitted to NCBI (Table S2). These findings identify the isolated strain as a *Lactobacillus* genus strain.
Table 2
Identification of the isolated bacterial strain based on 16S rRNA gene sequence analysis

<table>
<thead>
<tr>
<th>NCBI reference</th>
<th>Organism</th>
<th>Length</th>
<th>Score</th>
<th>Identities</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR_042443.1</td>
<td><em>Lactobacillus sakei subsp. sakei</em> strain DSM 20017</td>
<td>1561</td>
<td>2778</td>
<td>1508/1510</td>
<td>0/1510 (0%)</td>
</tr>
<tr>
<td>NR_113821.1</td>
<td><em>Lactobacillus sakei</em> strain NBRC 15893</td>
<td>1499</td>
<td>2763</td>
<td>1498/1499</td>
<td>0/1499 (0%)</td>
</tr>
<tr>
<td>NR_104208.1</td>
<td><em>Lactobacillus sakei subsp. casei</em> strain CCUG</td>
<td>1414</td>
<td>2571</td>
<td>1407/1414</td>
<td>4/1414 (0%)</td>
</tr>
<tr>
<td>NR_115172.1</td>
<td><em>Lactobacillus sakei subsp. sakei</em> strain DSM 20017</td>
<td>1406</td>
<td>2590</td>
<td>1405/1406</td>
<td>1/1406 (0%)</td>
</tr>
<tr>
<td>NR_042438.1</td>
<td><em>Lactobacillus graminis</em> strain G90</td>
<td>1548</td>
<td>2712</td>
<td>1497/1511</td>
<td>2/1511 (0%)</td>
</tr>
<tr>
<td>NR_114916.1</td>
<td><em>Lactobacillus graminis</em> strain LMG 9825</td>
<td>1466</td>
<td>2593</td>
<td>1447/1467</td>
<td>6/1467 (0%)</td>
</tr>
<tr>
<td>NR_113334.1</td>
<td><em>Lactobacillus curvatus</em> strain NBRC 15884</td>
<td>1491</td>
<td>2673</td>
<td>1480/1495</td>
<td>6/1495 (0%)</td>
</tr>
<tr>
<td>NR_114915.1</td>
<td><em>Lactobacillus curvatus</em> strain LMG 9198</td>
<td>1401</td>
<td>2505</td>
<td>1388/1403</td>
<td>3/1403 (0%)</td>
</tr>
<tr>
<td>NR_042437.1</td>
<td><em>Lactobacillus curvatus</em> strain DSM 20019</td>
<td>1559</td>
<td>2708</td>
<td>1497/1511</td>
<td>5/1511 (0%)</td>
</tr>
<tr>
<td>NR_112753.1</td>
<td><em>Lactobacillus fuchuensis</em> strain B5M10</td>
<td>1495</td>
<td>2564</td>
<td>1463/1499</td>
<td>6/1499 (0%)</td>
</tr>
<tr>
<td>NR_104976.1</td>
<td><em>Lactobacillus fuchuensis</em> strain LMG 21669</td>
<td>1524</td>
<td>2571</td>
<td>1469/1506</td>
<td>6/1506 (0%)</td>
</tr>
<tr>
<td>NR_112157.1</td>
<td><em>Lactobacillus fuchuensis</em> strain JCM 11249</td>
<td>1329</td>
<td>2193</td>
<td>1293/1342</td>
<td>15/1342 (1%)</td>
</tr>
</tbody>
</table>

PCR identification of isolated probiotic

The PCR assay was performed to identify the isolated probiotic strain using a species-specific primer set (Fig. 1). To do that, 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. 1A). The fold change of the signal that appeared in the imaging system was analyzed. The signal was increased 0.9-fold for the isolated probiotic strain and 1.0-fold for the positive control strain, whereas it was 0.12 or 0.05-fold for the two non-*L. sakei* strains (*L. curvatus* KGMB 05016 and *L. rhamnosus* KGMB 06348) (Fig. 1B).

Furthermore, 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. 1C). These results confirmed that our isolated probiotic strain was *L. sakei*.

**Wholegenome analysis results of the selected strain**

The 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 percent (Fig. 2). According to the 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 t-RNA and 21 r-RNA genes were anticipated. PMC 104 sequences were later submitted to NCBI (National Center for Biotechnology Information).

**OrthoANI genomic similarity**

The OrthoANI analysis was performed to obtain similarity value between strains with substantial similarities in 16S rRNA analyses using the whole genome sequencing data of PMC 104 (Fig. 3). The ANI values between PMC 104, and all publicly available *L. sakei* genomes, including *Lacticaseibacillus* subsp. strain DSM 20017, subsp. strain TMW, and AMBR8 were 98.84, 98.86, and 97.83 percent, respectively, significantly above the cutoff value of 95% for species delineation. Likewise, the ANI values of our strain with other *Lacticaseibacillus* species, including *rhamnosus* AMBR4, *casi* 12A, and *fermentum* PMC 101, ranged from 66.57, 67.11, and 65.55 percent, respectively which were all below 80%. These results strongly suggest that the newly discovered strain PMC 104 is *L. sakei*.

**The genomic characteristics difference between *L. sakei* species strains**

The differences of genomic characteristics between PMC 104 and other strains of *L. sakei*, such as DSM20017, TMW, AMBR8, DS4, and ELA214391 was investigated to shows that our isolated strain is newly discovered (Table 3). The PMC 104 strain differed from other *L. sakei* strains in genomic size, G + C content, and CDS. The finding showed that PMC 104 is a newly discovered strain of *L. sakei*. 
Table 3
Comparison of chromosomal properties of *L. sakei* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>DSM 20017</th>
<th>TMW</th>
<th>AMBR8</th>
<th>DS4</th>
<th>ELA214391</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sources</td>
<td>Moto starter of sake</td>
<td>Raw sausage</td>
<td>Human nasopharynx</td>
<td>Korean kimchi</td>
<td>Foregut</td>
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<tr>
<td>Genome size (bp)</td>
<td>1,910,247</td>
<td>1,942,056</td>
<td>1,997,834</td>
<td>2,103,571</td>
<td>2,069,589</td>
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<tr>
<td>G + C content (%)</td>
<td>45.0</td>
<td>57.0</td>
<td>73.0</td>
<td>89.0</td>
<td>42.0</td>
</tr>
<tr>
<td>Predicted CDS</td>
<td>1,885</td>
<td>1,891</td>
<td>2,012</td>
<td>1,992</td>
<td>2,046</td>
</tr>
<tr>
<td>Number of rRNA genes</td>
<td>3</td>
<td>18</td>
<td>7</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Number of tRNA genes</td>
<td>44</td>
<td>66</td>
<td>58</td>
<td>65</td>
<td>66</td>
</tr>
</tbody>
</table>

Biochemical characterization of PMC 104

The biochemical characterization was performed to investigate the phenotypic properties of our probiotic strain (Table 4). According to previous studies, an API kit can be used to 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 the strain to be classified as specified by its ability to utilize 49 different carbohydrates. The result of 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).
Table 4
Biochemical properties of the candidate strain based on carbohydrate fermentation using API 50 CH kit

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Results</th>
<th>Substrates</th>
<th>Results</th>
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<tbody>
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<td>Control</td>
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<td>D-Fucose</td>
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<tr>
<td>Glycerol</td>
<td>-</td>
<td>L-Fucose</td>
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<tr>
<td>Erythritol</td>
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<td>D-arabitol</td>
<td>-</td>
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<tr>
<td>D-arabinose</td>
<td>-</td>
<td>L-arabitol</td>
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<tr>
<td>L-arabinose</td>
<td>-</td>
<td>Potassium gluconate</td>
<td>-</td>
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<tr>
<td>Ribose</td>
<td>-</td>
<td>Potassium 2 ketogluconate</td>
<td>-</td>
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<tr>
<td>D-Xylose</td>
<td>-</td>
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<td>D-Tagatose</td>
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<td>Adonitol</td>
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<td>Methyl-αD-mannopyranoside</td>
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<tr>
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<td>Methyl-αD-glucopyranoside</td>
<td>-</td>
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<tr>
<td>D-galactose</td>
<td>+</td>
<td>N-acetylglucosamine</td>
<td>+</td>
</tr>
<tr>
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<td>Amygdaline</td>
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<tr>
<td>Gentibiose</td>
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</table>

**Cultivation of** *L. sakei* **based on the food grade medium**
The probiotic strain was cultured in seven types of FGMs, and bacterial growth after 18 h was checked with a spectrophotometer (Fig. 4). The data showed that our probiotic strain grew best in medium 2 ($\text{OD}_{600} = 1.3$), whereas it had the lowest growth in medium 1 ($\text{OD}_{600} = 0.38$) and medium 4 ($\text{OD}_{600} = 0.39$), (Fig. 4). 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. 5 and Fig. S1. Figure 5A depicts a schematic diagram of our study. Residual glucose concentration and bacterial growth rate were checked at the same time during the scale-up fermentation process (Fig. 5B, C). Data showed that at 0 to 8 h of fermentation, there was no significant growth of bacteria. Thus, there was no utilization of residual glucose before 8 h. 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 produced. The powder contained a bacterial load of $3 \times 10^8$ CFU/g. Next, 2.5 kg of freeze-dried powder out of 3.0 kg was mixed with some food additives and then packed using polyethylene material, in which each pack contained 4 g of powder.

**Evaluation of long-term treatment of CRE infected mice with L. sakei**

We investigated the long-term preventive effect of our probiotic strain (Fig. 6). Figure 6A depicts a schematic diagram of our study. During the experimental period the survival rate of the untreated group and treated group were 45% and 80%, respectively (Fig. 6B). We also observed the body weights changes of mice showed severe weight loss in the untreated group compared to the $L. \text{sakei}$ treated group ($p < 0.001$) (Fig. 6C). Mice in the treated group also showed lower scores of illness severity than those in the untreated group ($p < 0.05$), (Fig. 6D).

**Efficacy of L. sakei in preventing CRK infection in a mouse model**

The treatment effect of $L. \text{sakei}$ powder on CRK infection was evaluated (Fig. 7). Figure 7A depicts a schematic diagram of our study. During this study, we observed the survival rate of mice and found that mice belongs to untreated group died after 4 days of first infection. On the other hand, mice in the treatment group survived during the experimental period (Fig. 7B). We also observed the body weights of mice and found that mice without probiotic treatment indicated significant weight loss ($p < 0.001$) and died on day 4 while the treated mice were almost stable throughout the study period (Fig. 7C). Illness severity scores of the both group were also observed and we noticed a significant difference between the
groups (Fig. 7D). Mice in the infected group suffered from diarrhea and developed weakness leading to higher illness scores, whereas probiotic powder-treated group showed low illness scores ($p < 0.001$).

**Cytotoxicity of L. sakei**

The cytotoxicity of *L. sakei* extract at various concentrations was evaluated using macrophage RAW 264.7 cells (Fig. 8). Data showed that *L. sakei* extract had no significant cytotoxicity at a concentration up to $20 \times 10^7$ CFU/mL (Fig. 8A). Cell viability was also checked using methylene blue staining. Cells were then observed under a light microscope (Fig. 8B). No significant changes in the morphology of RAW 264.7 cells were observed after treatment with *L. sakei* extract at $2.5 \times 10^6$ to $20 \times 10^7$ CFU/mL.

**Acute oral dose toxicity of L. sakei**

Acute oral toxicity of *L. sakei* was performed through orally administered to guinea pigs at $2 \times 10^8$ CFU/mL. No clinical symptoms or body weight changes were found between the group treated with candidate probiotic strain and the group treated with sterile water and all guinea pigs survived until the end of the experiment (Fig. 9) (Table S3).

**Discussion**

Most studies have shown that CRE strains are among the main urgent threats due to the emergence of resistance to carbapenem. Over the last few decades, increasing mortality due to CRE infection has been progressively reported (Centers for Disease and Prevention, 2013).

Recently, probiotics have been among the main topics of research studies due to their strong pathogen-killing abilities to eliminate antibiotic-resistant superbugs, including multidrug-resistant *Acinetobacter baumannii*, vancomycin-resistant *Enterococcus* (VRE), multidrug-resistant *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), and viral pathogens such as SARS-CoV-2 and HIV infections (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). Probiotics have been known for their bacterial mechanisms, such as controlling microbiota compositions at different body sites, killing enteric pathogens directly in the gastrointestinal tract, and enhancing host immunity (Coudeyras et al., 2008; Ishikawa et al., 2010; Yan et al., 2021). However, the effectiveness of probiotics as CRKP alternative drugs against CRE infections has yet to be thoroughly investigated. Therefore, we aimed to develop a new probiotic compound against CRE infection and examine its effect as a novel alternative treatment.
Results of our examination on the effect of probiotic strains against CRE pathogen revealed that the *L. sakei* strain (isolated from traditional Korean kimchi) had an excellent anti-infective activity to inhibit this deadly infection. The isolated strain was confirmed by 16srRNA sequencing and PCR methods. It was judged to be a new strain of *L. sakei*. It showed a different carbon use pattern from the existing standard *L. sakei* strain (Ghorbanian *et al*.; Rahim *et al*., 2022a). According to previous studies, the *L. sakei* strain has an incredible impact against many bacterial pathogens, including *Enterococcus faecium*, *E. coli*, *K. pneumoniae*, *Listeria ivanovii subsp. ivanovii*, *Listeira 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 according to its bacteriocin production which has been frequently reported to be able to 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).

According to FDA (U.S. Food and Drug Administration) regulation, probiotics may be drugs under the Food, Drug, and Cosmetic Act (Code, 2013), depending on the intended use of the product (Food, 1938; 1978; 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).

Hence, we used the *L. sakei* strain to develop a probiotic powder as an antibiotic alternative by constructing industrial processes from upscaling in the pilot plant to packaging. To administer reliable manufacturing processes, large-scale industrial supplements of candidate probiotics demand a safe and low-cost alternative medium with a high density for novel probiotic strains as alternatives to antibiotics (Fenster *et al*., 2019). Food grade medium has been shown to be safe in terms of food manufacture without causing any changes in industrial scale, making it potentially suitable for human consumption (Sawatari *et al*., 2006). Therefore, before scaling up from laboratory to pilot plant, *L. sakei* was grown in modified EFGM medium (edible) in order to improve the FGM from laboratory grade to edible medium grade. To start an industrial application, developing a pilot plant scale is the primary step between laboratory scale-up and new commercial production (Levin, 2001). Therefore, *L. sakei* culture was transferred to a 200 L pilot scale, and 3 kg of freeze-dried powder was obtained due to *L. sakei* pilot scale-up fermentation. Freeze-drying and packaging processes were performed to produce *L. sakei* powder, with its efficacy preserved while avoiding damage or death of lactic acid bacteria during freeze-drying and packaging processes. Freeze-drying and packaging are popular and necessary processes in developing industrial products to improve the shelf-life stability of the final product for a long time as the viability of probiotic bacteria should be maintained to the highest level in order to be able to execute its preventive and therapeutic effects (Vaudant, 2008; Fonseca *et al*., 2015; do Vale Morais *et al*., 2016; Quintana *et al*., 2017). Accordingly, the *L. sakei* powder was packed with polyethylene material at a ratio of 1:3 to preserve its treatment efficacy. From an industrial point of view, using probiotic powder instead of bacteria culture is preferred due to the higher viability of the product during storage and the ease of use.
(NAKSING et al., 2019; Oktavia et al., 2020). In addition, our data showed that *L. sakei* powder had higher efficacy in an in vivo model than *L. sakei* culture.

In the *in vivo* experiment of this study, unlike the long-term oral treatment with *L. sakei* culture, the *L. sakei* powder showed a more significant effect in reducing the illness severity with an inhibitory effect on CRK strain because all infected mice survived until the end of this study. Other studies have also reported that the probiotic powder can prevent infections by several Gram-negative and Gram-positive pathogens and reduce antibiotic-resistant infections in *in vivo* experiments (Kechagia et al., 2013; Ghoneum and Abdulmalek, 2021).

Moreover, as probiotic powder must be safe for consumption, a two-week acute oral toxicity study using a mouse model and a cytotoxicity study using macrophage cell lines were performed using *L. sakei* powder. Both *in vivo* and *in vitro* studies showed that *L. sakei* had no toxicity during treatment.

In conclusion, the results of this study indicate that *L. sakei* powder can potentially be used as a therapeutic candidate to treat infections caused by multi-resistant pathogens such as CRK in the future as a novel antibiotic alternative. However, further studies such as toxicity tests and clinical trials at the pharmaceutical grade level are needed to develop it as a new drug candidate with high efficacy against CRK infections.

**Declarations**

**Ethics approval**

All animal experiments using specific pathogen-free (SPF) BALB/c female mice, were performed and approved as described by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University (approval number: SCH23-0004). The two weeks’ toxicity assay using adult male guinea pigs was performed and approved by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University, (IACUC) approved number was SCH-22-0111

**Consent for publication**

All authors of the manuscript have read and approved the final manuscript. That the article is original, has not already been published in a journal, and is not currently under consideration by another journal.

**Availability of data and materials**

The datasets used or analyzed during the preparation of the review manuscript are available from the corresponding author at reasonable request.

**Authors’ contributions**

Conceptualization: HYS; Methodology: HT, YL, AH, 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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Conflict of interest

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

References

1. GB v, Lackner *Cal. App. 3d*. Cal: Court of Appeal, 1st Appellate Dist., 3rd Div., Vol. 80, p. 64
15. Code U (2013) Title. Chapter III• Articles L413-1 et seq., R413-1 et seq. • Articles L362-2, L362-3, R362-1 and A362-2• Article L310-12


29. Ghoneum M, Abdulmalek S (2021) KDP, a lactobacilli product from kimchi, enhances mucosal immunity by increasing secretory IgA in mice and exhibits antimicrobial activity. Nutrients 13:3936


40. Jang S-E, Min S-W (2020) Lactobacillus sakei S1 Improves Colitis Induced by 2, 4, 6-Trinitrobenzene Sulfonic Acid by the Inhibition of NF-κB Signaling in Mice


54. Manuel PM, Elena B, Carolina MG, Gabriela PJJON, Metabolism I (2017) Oral probiotics supplementation can stimulate the immune system in a stress process. 8:29–40


77. Sidjabat HE, Cottrell K, Bordin A, Cervin ARreviving conventional microbiology approaches for probiotic discovery and development


**Figures**
Figure 1

**Identification of the isolated candidate strain.** Representative results of PCR experiment for identifying *L. sakei* strain using *L. sakei* species-specific primers, non-*L. sakei* strains (*L. curvatus* KGMB 05016 and *L. rhamnosus* KGMB 06348) as negative controls, and *L. sakei* KGMB05070 as a reference and positive control. (A) Agarose gel image data showed clear bands for the isolated probiotic and positive control strains, unlike the two non-*L. sakei* strains. (B) The imaging system showed a fold change of the signal, indicating that the signal was increased 0.9-fold for the isolated probiotic strain and 1.0-fold for the positive control strain. For the two non-*L. sakei* strains, it remained at 0.12 and 0.05, respectively. (C) Amplification cycles of *L. curvatus* KGMB 05016 and *L. rhamnosus* KGMB 06348 and candidate strain obtained from qRT-PCR analysis which detected the presence of the isolated strain with a low threshold cycle (Ct) value (21.56 ± 1.19). The *p*-value is shown (***, *p* < 0.001; **, *p* < 0.01; *, *p* < 0.05).
Figure 2

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. a. 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.
Figure 3

OrthoANI results calculated from available genomes of *Lacticaseibacillus* species. The OrthoANI value of *L. sakei* PMC 104 and *L. sakei* subsp. strain DSM 20017 was 99.84, higher than 96.0%, the standard for determining the same species.
Figure 4

Cultivation 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 °C for 18 h. During the incubation period, their growth rate was checked using a spectrophotometer.
Figure 5

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 has been transferred from lab scale-up cultivation (5L) to pilot scale-up fermentation (200L). After the fermentation process, the freeze-dried powder was obtained using a freeze-drying process and packed using a packaging method. (B) The 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.
Figure 6

Prophylactic effect of *L. sakei* strain in a CRK-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. The *p*-value is shown (***, *p* < 0.001; **, *p* < 0.01; *, *p* < 0.05).
Figure 7

Preventive effect of *L. sakei* powder in a CRK-infected mice model. (A) *L. sakei* powder was administered 3 days before infection and observed for 7 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. The *p*-value is shown (***, *p* < 0.001; **, *p* < 0.01; *, *p* < 0.05).
Figure 8

Cytotoxicity of *L. sakei* extract to RAW 264.7 cells. (A) The cytotoxicities of *L. sakei* extract at various concentrations in a macrophage cell line were evaluated by WST cell viability assay. (B) The viability of cells was checked by methylene blue staining. Experiments were performed in triplicate. Values are expressed as mean values and standard deviations.
Figure 9

Two-week repeated oral dose toxicity test of *L. sakei* with guinea pigs. Treatment groups were administered the probiotic strain, while the control group was treated with sterile water. During the experimental period, the body weights of all guinea pigs were measured on days 1, 3, 5, 7, 9, 10, and 14.

Supplementary Files

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