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In Vivo Efficacy of *Bacillus velezensis* Isolated from Korean Gochang Bokbunja Vinegar against Carbapenem-Resistant *Klebsiella pneumoniae* Infections

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Abstract

Outbreaks of carbapenem-resistant *Enterobacteriaceae* (CRE), especially *Klebsiella pneumoniae* (CRKP), are commonly reported as severe infections in hospitals and long-term care settings, and their occurrence is increasing globally. Conventional antibiotics used for treating CRE have become ineffective due to resistance development. Furthermore, their safety issues restrict their availability and use for CRE treatment. Therefore, developing new drugs different from existing drugs to combat this deadly menace is urgently needed. Probiotics can be a potential option in this context, as probiotics' efficacy against a variety of infectious illnesses has already been well established. Here, we report the effect of the *Bacillus velezensis* strain isolated from Gochang Bokbunja vinegar in Korea on CRE infection using two mouse models. Data showed that pretreatment with *B. velezensis* significantly reduced body weight loss and mortality of CRKP-infected mice in the preventive model. The oral administration of *B. velezensis* in a therapeutic model also decreased the mortality and illness severity in CRKP-infected mice. Moreover, a two-week oral acute toxicity assay in guinea pigs did not reveal any aberrant clinical signs. Our findings demonstrate the potential effectiveness of our candidate probiotic strain, *B. velezensis*, against CRKP, suggesting that it could be used as an antimicrobial agent for treating CRKP-related infections.

Keywords: *Bacillus velezensis*, *Klebsiella pneumoniae*, carbapenem-resistant *Enterobacteriaceae* (CRE), Gochang Bokbunja vinegar, probiotics

Introduction

Antimicrobial resistance has been considered one of the biggest threats to human health globally (Sulis et al. 2022). According to a recent report published in 2022, 4.95 million deaths were associated with antimicrobial resistance in 2019, including 1.27 million deaths directly attributable to antimicrobial resistance (Murray et al. 2022). To make matters worse, resistance to carbapenem, the preferred last resort drug for treating multidrug-resistant bacterial infections was first reported in *Enterobacteriaceae* strains in the early 1990s. These carbapenemase-producing isolates have spread worldwide, causing a global health crisis (Potter et al. 2016; Lutgring 2019). According to the Center for Disease Control and Prevention (CDC) report, in 2017, 13,100 carbapenem-resistant *Enterobacteriaceae* (CRE) infections were estimated among hospitalized patients, which resulted in 1,100 deaths in the USA (CDC 2019).

Meanwhile, *Klebsiella pneumoniae* is the species that causes the most cases of CRE infections (Logan and Weinstein 2017). It caused more than 55,700 deaths

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worldwide in 2019 (Murray et al. 2022). As such, with the significant increase in the use of carbapenems in clinical practice today, the evolution of carbapenem-resistant bacteria has become a significant concern (Sheu et al. 2019). Although standards for treating CRE infections are being developed in response to this human threat, problems due to resistance, treatment failure, and toxicity still exist (Tilahun et al. 2021). Therefore, new and effective anti-CRE medicines are desperately needed.

According to the Food and Agriculture Organization (FAO)/ World Health Organization (WHO), probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO 2001). As reported by many studies, probiotics have many functions, including providing nutrients, modifying biological activities, preventing pathogenic microorganisms, boosting immune responses, and enhancing growth (Oelschlaeger 2010). Some reports have shown the effects of probiotics against different diseases, such as necrotizing enterocolitis (Patel and Underwood 2018), acute infectious diarrhea (Allen et al. 2010), antibiotic-associated diarrhea (Kopacz and Phadtare 2022), metabolic disorders (Li et al. 2021a), and autoimmune diseases (De Luca and Shoenfeld 2019). Furthermore, probiotics could play an essential role in acute infectious diseases, namely: tuberculosis (Rahim et al. 2022), acquired immunodeficiency syndrome (AIDS) (Ceccarelli et al. 2019), *Pseudomonas aeruginosa* infection (Huang et al. 2020), and COVID-19 (coronavirus disease 2019) (Anwar et al. 2021). Probiotics have been extensively studied to treat various diseases, including infectious ones. Thus, the application of probiotics is considered valuable for treating CRE infections. This study aimed to determine the impact of a *Bacillus velezensis* strain isolated from Gochang Bokbunja vinegar in Korea on carbapenem-resistant *Klebsiella pneumoniae* (CRKP) infection.

Experimental

Materials and Methods

CRKP. CRKP was collected from the Pathogenic Resource Bank at Soonchunghyng University Hospital (Tajdozian et al. 2021). It was cultured in MacConkey broth (BD Difco, USA) and incubated at 37C for 18 h. Next, bacterial growth was determined using a spectrophotometer (DR 1900, HACH, USA), and optical density at 600 nm ($OD_{600\ nm}$) was adjusted to 1.0. After that, the bacterial number was enumerated through a colony-forming unit (CFU) assay. For further uses, stock culture in 60% glycerol was prepared and kept at –80°C.

Isolation and identification of a probiotic strain from naturally fermented vinegar. The candidate probiotic was obtained from naturally 14-days-fermented Gochang Bokbunja vinegar from Gochang County, North Jeolla Province, South Korea. Upon receiving the samples, they were streaked onto an MRS agar (BD Difco, USA) plate and incubated at 37°C for 24 h. After that, single colonies were picked and subcultured in MRS broth (BD Difco, USA). After incubating in a shaking incubator overnight at 37°C under aerobic conditions, bacterial growth was determined using the spectrophotometer. Stock in 60% glycerol was prepared and stored at –80°C for further use. This probiotic strain was sent to a company (Biofact Co, Korea) for 16S rRNA sequencing and species identification.

Biochemical tests. A biochemical test was conducted using an API 50CH kit (bioMerieux, France) to observe the carbohydrate fermentation pattern of the probiotic strain compared to other reference strains. The candidate probiotic strain was cultured on an MRS agar plate. A single colony was then picked and mixed with 1 ml suspension medium. After that, the suspension turbidity was determined using 2 McFarland, and 0.1 ml of this suspension was diluted in 10 ml of CHB medium (bioMerieux, France). Next, the strip was removed from the API kit and placed in a tray. The final prepared bacterial suspension was transferred into the strip's wells and incubated at 37°C for 48 h. Subsequently, color change in each well was observed, and an identification table was prepared as (+/–).

Quantitative RT-PCR assay. qRT-PCR is a well-established and robust approach for detecting and quantifying microorganisms (Kralik and Ricchi 2017). Thus, qRT-PCR was performed to identify the isolated strain. Genomic DNA was extracted from the isolated candidate probiotic strain using the QIAamp DNA Mini Kit (QIAGEN, Germany). Genomic DNAs were also extracted from *B. velezensis* KCTC 13417, *Bacillus amyloliquefaciens* KCTC 3002, *Bacillus subtilis* KCTC 3135, and *Bacillus lichenoformis* KCTC 1659. They were obtained from the Korean Collection for Type Cultures (KCTC) as reference organisms to help us to distinguish closely related species in the *Bacillus* genus. Extracted DNA was then diluted to run qRT-PCR using species-specific primers (Kwon et al. 2009; Huang et al. 2017a; Dunlap 2019; Bahuguna et al. 2020). A primer for the macrolactin gene was used to distinguish between *B. velezensis* and *B. amyloliquefaciens* (Fan et al. 2018; Li et al. 2021b). The used primers are listed in Table SI. We performed qRT-PCR in a final volume of 20 µl consisting of 10 µl of SYBER Green Supermix (BIO-RAD, USA), 4 µl nuclease-free water, 5 µl of genomic DNA, and 0.5 µl each of forward and reverse primers. Amplification used the following thermal cycling steps: an initial DNA denaturation step at 95°C for 3 min, followed

by 39 cycles of 10 s at 95°C for denaturation, 10 s at 55°C for annealing, and 30 s at 72°C for extension.

Cultivation of *B. velezensis* using a lab-scale fermenter based on food-grade media. We used food-grade medium (FGM) to cultivate our strain as an anti-CRE therapeutic agent that could be developed in the future. FGM was used for cultivation because this strain could grow well in this medium. In addition, FGM is safe for human consumption, and it is composed of glucose, yeast peptone, Tween 80, and magnesium sulfate based on an MRS medium (Table SII). The pH of the optimized FGM was 7.5. We adjusted its pH to 6.3 using 6 N HCl. Before growing in a fermenter system, the strain was cultivated in 30 ml FGM. After incubation at 37°C for 18 h, the OD and pH of the culture broth were checked with a spectrophotometer and a pH meter (Mettler-Toledo, Switzerland), respectively, every 2 h during the incubation period. After confirming the bacterial growth and the pH, the probiotic strain was grown in a lab-scale fermenter system (FMT-ST-S07, Fermentec, South Korea). The fermenter was first filled up with 2 l of FGM. The FGM-filled fermenter was then autoclaved at 121°C for 15 minutes. After that, when the medium reached the optimal temperature, 1.0% of probiotic culture was aseptically added into the fermenter system and fermented at 37°C for 10 h using a fermenter impeller. After incubation, the culture was recovered in a sterile container, centrifuged, washed, and resuspended with the culture supernatant at 1.5×10^9 CFU/200 µl for each mouse of the therapeutic model. FGM-cultured probiotics were used in the treatment model, but MRS-cultured probiotics were used in the prevention model and toxicity tests.

Investigation of the effect of *B. velezensis* in a CRKP-infected mouse model. In this study, preventive and therapeutic models were applied to evaluate the effectiveness of probiotics in a CRKP-infected mouse model, and 9-week-old female BALB/c mice (Dooyeol Biotech, Korea) were used. The prevention model was used to see preventive effects by administering probiotics before infection. In the treatment model, probiotics were administered the day after inducing infection to see the therapeutic effect. At the time of treatment with probiotics in both models, the probiotic-treatment group received *B. velezensis*. However, the infection and control groups received distilled water through oral gavage for making the same treatment condition. In both models, gastric neutralization was induced by oral administration of sodium bicarbonate ($NaHCO_3$) to increase animal severity through the increased intestinal reach of live pathogens (Czuprynski and Faith 2002). In addition, cyclophosphamide was intraperitoneally injected 3 days before infection to induce neutropenia (Pan et al. 2015). 5-Fluorouracil (5-FU) was also administered intraperitoneally to induce additional immunosuppression (VanderVeen et al. 2020). Disease severity score, body weight, and survival of mice were checked in both models during the experimental period.

We examined the preventive effect of our candidate probiotic strain, *B. velezensis*, in CRKP-infected BALB/c mice. The probiotic strain was administered at 1.5×10^9 CFU/mouse in 200 µl daily as a single dose by oral gavage for three days before infection. Cyclophosphamide (Sigma Aldrich, USA) at 450 mg/kg (200 µl) was also injected intraperitoneally three days before infection. Three days after neutropenia, infection was induced twice per week through oral administration at a dose level of 6.7×10^9 CFU/200 µl/mouse on day 0 and 9×10^9 CFU/200 µl/mouse on days 2, 10, and 12. $NaHCO_3$ (0.2 M, 200 µl) (Sigma Aldrich, USA) was administered along with the infection. 5-FU (50 mg/kg, 200 µl) (Sigma Aldrich, USA) was administered intraperitoneally on days 13 and 15 after inducing infection.

We investigated the therapeutic potential of our candidate probiotic strain, *B. velezensis*, in CRKP-infected BALB/c mice. In the therapeutic mouse model, 200 µl of cyclophosphamide (450 mg/kg) was injected intraperitoneally three days before infection. Infection was then induced by oral administration of 200 µl CRKP at 9×10^9 CFU/mouse on days 0, 2, and 6 with pretreatment of $NaHCO_3$ (0.2 M, 200 µl). Mice were treated with *B. velezensis* at 1.5×10^9 CFU/200 µl/mouse twice the next day after the infection treatment. They were intraperitoneally injected on days 10 and 14 with one dose of 5-FU (50 mg/kg, 200 µl).

Evaluation of repeated oral acute toxicity of probiotics in guinea pigs. A 2-week repeated oral acute toxicity evaluation of probiotics in guinea pigs was performed according to a previous report (Lee et al. 2021). Adult male guinea pigs (weight range 1,000 to 1,280 g) were used and administered orally, and sterile water was used as the vehicle. In the case of the treatment group, probiotics (2×10^8 CFU/ml/200 µl) were administered once a day, and clinical symptoms, mortality, and weight changes were observed over the entire period.

Review of animal experiment ethics. All animal experiments were conducted in a biosafety level 2 facility (LML 20–591) of Soonchunhyang University, according to the Ministry of Food and Drug Safety (Registration, MFDS, No. 657). This study's animal experiments protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Soonchunhyang University.

Results

Identification based on 16S rRNA gene sequencing. The 16S rRNA gene sequencing analysis was done for the isolated strain (Table I). The result was compared to sequences stored in the National Center for

Table I
Identification of isolated bacterial strain based on 16S rRNA gene sequence analysis.

NCBI references	Organisms	Length	Score	Identities	Gaps
NR_075005.2	*Bacillus velezensis* strain FZB42	1,550	2,728 bits (1,477)	1,492/1,499 (99%)	2/1,499 (0%)
NR_116240.1	*Bacillus velezensis* strain CBMB205	1,445	2,615 bits (1,416)	1,427/1,432 (99%)	2/1,432 (0%)
NR_112685.1	*Bacillus amyloliquefaciens* strain NBRC 15535	1,475	2,699 bits (1,461)	1,470/1,475 (99%)	0/1,475 (0%)
NR_041455.1	*Bacillus amyloliquefaciens* strain NBRC 15535	1,472	2,697 bits (1,460)	1,468/1,472 (99%)	0/1,472 (0%)
NR_102783.2	*Bacillus subtilis* subsp. *subtilis* strain 168	1,550	2712 bits (1,468)	1,489/1,499 (99%)	2/1,499 (0%)
NR_118996.1	*Bacillus licheniformis* strain DSM 13	1,545	2,579 bits (1,396)	1,466/1,500 (98%)	4/1,500 (0%)
NR_116023.1	*Bacillus licheniformis* strain BCRC 11702	1,468	2,545 bits (1,378)	1,439/1,469 (98%)	2/1,469 (0%)

Biotechnology Information (NCBI) databases. The 16S rRNA gene sequencing of the probiotic strain shared 99% sequence identities with 16S rRNA gene sequences of *B. velezensis* strain FZB42, *B. velezensis* strain CBMB205, *B. amyloliquefaciens* strain NBRC 15535, and *B. subtilis* subsp. *subtilis* strain 168. It also shared 98% sequence identities with *B. licheniformis* strain DSM 13 and *B. licheniformis* strain BCRC 11702. These findings suggest that the isolated strain belongs to the *Bacillus* genus.

Carbon utilization assay of the isolated probiotic strain by API kit. To identify and characterize phenotypes of the probiotic strain, biochemical characterization was performed using an API 50CH Biochemical Kit. Utilization patterns of carbon sources of the isolated probiotic strain were compared with *B. velezensis* KCTC 13417, *B. amyloliquefaciens* KCTC 3002, and *B. lichenoformis* KCTC 1659, obtained from KCTC. Results are shown in Table II. The candidate strain showed carbon source utilization patterns more similar to *B. velezensis* than to *B. licheniformis* and *B. amyloliquefaciens* but showed some differences with the KCTC *B. velezensis*.

Identification of isolated probiotic strain by qRT-PCR assay. The qRT-PCR assay was conducted to identify the candidate probiotic strain using species-specific primer sets (Table III). We analyzed the resulting threshold cycle (C_t value). We found that the *B. velezenesis*-specific primer was specific for both *B. velezensis* KCTC 13417 and our candidate probiotic strain, but it could not detect other closely related species. We used a *B. amyloliquefaciens*-specific primer and observed amplification result for *B. amyloliquefaciens* KCTC 3002, *B. velezensis* KCTC 13417, and the candidate probiotic strain. We found that this primer could not work specifically. Therefore, to distinguish between *B. amyloliquefaciens* and *B. velezensis*, we ran the PCR using a macrolactin-specific primer. Macrolactin is a gene cluster detected only in *B. velezensis* (Fan et al. 2017). After running the PCR using a macrolactin-specific primer, we noticed the amplification cycle of *B. velezensis* KCTC 13417 and our candidate probiotic strain but not *B. amyloliquefaciens* KCTC 3002. This result strongly proves that our candidate probiotic strain is *B. velezensis*. Species-specific primers for *B. lichenoformis* and *B. subtilis* showed the amplification for *B. lichenoformis* KCTC 1659 and *B. subtilis* KCTC 3135, respectively, while they did not show the amplification for other strains. These results confirm that our isolated probiotic strain is *B. velezensis*.

Cultivation using lab-scale fermenter and FGM. The probiotic strain was cultivated in FGM. Bacterial growth and pH were checked every 2 h (Fig. 1). Fig. 1a shows the growth curve of our candidate probiotic strain. Its OD reached 0.8 after 10 h of incubation. The pH of the broth culture was also checked. We noticed a reduction of pH after 2 h of incubation. It continued to

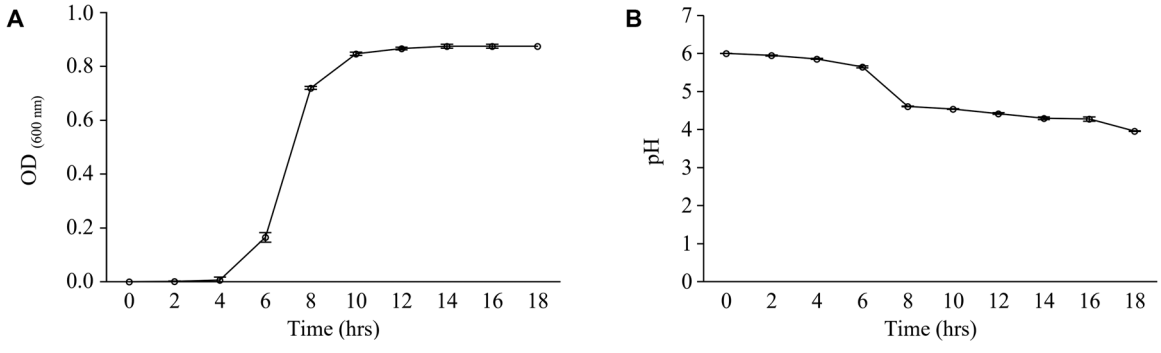

Fig. 1. Bacterial growth and pH measurement in food grade medium with time. The probiotic strain was inoculated and cultivated. Its A) growth and B) pH were checked every 2 h.

Table II
The ability of the candidate probiotic strain to dehydrate carbon sources.

No.	Type of test	Candidate strain		*Bacillus velezensis* KCTC 13417		*Bacillus amyloliquefaciens* KCTC 3002		*Bacillus licheniformis* KCTC 1659	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
0	Control	–	–	–	–	–	–	–	–
1	Glycerol	+	+	+	+	+	–	+	+
2	Erythritol	–	–	–	–	–	–	–	–
3	D-arabinose	–	–	–	–	–	–	–	–
4	L-arabinose	–	+	+	+	+	+	–	+
5	D-ribose	+	+	+	+	+	+	+	+
6	D-xylose	–	+	+	+	+	+	–	–
7	L-xylose	–	–	–	–	–	–	–	–
8	D-adonite	–	–	–	–	–	–	–	–
9	Methyl-βD-xylopyranoside	–	–	–	–	–	–	–	–
10	D-galactose	+	+	+	+	–	–	–	–
11	D-glucose	+	+	+	+	+	–	+	–
12	D-fructose	+	+	+	+	+	–	+	–
13	D-mannose	+	+	+	+	+	–	+	–
14	L-sorbose	–	–	–	+	–	–	–	–
15	L-rhamnose	+	+	+	+	–	–	–	–
16	Dulcitol	–	–	–	–	–	–	–	–
17	Inocitol	+	+	+	+	+	+	–	–
18	D-mannitol	+	+	+	+	+	–	+	–
19	D-sorbito	+	+	+	+	+	–	+	+
20	Methyl-αD-mannopyranoside	–	–	–	–	–	–	–	–
21	Methyl-αD-glucopyranoside	–	+	+	+	+	+	+	+
22	N-acetylglucosamine	+	+	–	–	+	–	+	–
23	Amygdaline	+	+	+	+	–	+	+	–
24	Arbutine	+	+	+	+	+	–	+	–
25	Esculine	+	+	+	+	+	+	+	+
26	Salicin	+	+	+	–	+	+	+	–
27	D-Cellibiose	+	+	+	+	+	+	+	+
28	D-Maltose	+	+	+	+	+	+	+	+
29	D-Lactose	+	+	–	–	+	+	+	+
30	D-Melibiose	–	+	–	+	+	+	–	+
31	D-Sacharose	+	+	+	–	+	+	+	–
32	D-Trehalose	+	+	+	+	+	+	–	–
33	Inulin	–	–	–	+	–	–	–	–
34	D-Melezitose	+	–	–	–	–	–	–	–
35	D-Raffinose	+	+	+	+	+	+	+	+
36	Amidon	+	+	+	–	+	–	+	–
37	Glycogen	–	+	+	–	+	–	+	–
38	Xylitol	–	–	–	–	–	–	–	–
39	Gentibiose	+	–	–	–	–	–	+	–
40	D-Turanose	–	–	+	+	–	–	+	+
41	D-Lyxose	–	–	–	–	–	–	–	–
42	D-Tagatose	+	+	+	–	–	–	–	–
42	D-Fucose	–	–	–	–	–	–	–	–
44	L-Fucose	–	–	–	–	–	–	–	–
45	D-arabitol	–	–	–	–	–	–	–	–
46	L-arabitol	–	–	–	–	–	–	–	–
47	Potassium gluconate	+	–	–	–	–	–	–	–
48	Potassium 2 ketogluconate	–	–	–	–	–	–	–	–
49	Potassium 5 ketogluconate	–	–	–	–	–	–	–	–

(+) – positive reaction (yellow) (No. 25: black); (–) – negative reaction (red)

Table III
Identification of the isolated bacterial strain by qRT-PCR.

Species-specific primer for-	Oligo name	Amplification results				
		Candidate probiotic strain	*B. velezensis* KCTC 13417	*B. amyloliquefaciens* KCTC 3002	*B. subtilis* KCTC 3135	*B. licheniformis* KCTC 1659
Bacillus velezensis	Bvel	+	+	–	–	–
Bacillus subtilis	YtcP	–	–	–	+	–
Bacillus amyloliquefaciens	spBamyphes	+	+	+	–	–
Bacillus licheniformis	Blich	–	–	–	–	+
Macrolactin	Mln	+	+	–	–	–

(+) – amplification, (–) – no amplification

reduce slowly until 18 h (Fig. 1b). We cultured our strain in the lab-scale fermenter system based on these results.

Evaluation of the preventive effect of *B. velezensis* in CRKP-infected mice. The preventive effect of our candidate strain was evaluated (Fig. 2). Throughout the test phase, the body weights of untreated mice decreased significantly more compared to the mice treated with *B. velezensis* (Fig. 2a). Body weight reduction rate was 8.4% for the treated group on days 18 and 20. In contrast, it was 35.8% for the untreated group. Illness severity scores of the probiotic-treated group and infection group were observed from day 0 to day 21 (Fig. 2b). The group not treated with probiotics increased steadily throughout the experiment and then increased more rapidly at 2 to 3 weeks but to a lesser extent in the probiotic-treated group. The survival rates of mice in both groups are shown in (Fig. 2c). The untreated group showed a survival rate of 60% on day 20, and finally, all mice died on day 21, whereas 80% of mice survived in the treated group until the end of the experiment. Fig. 2d shows the images of mice in different groups. After finishing the experiment, we found that all mice in the infection group without probiotic treatment died (left image), whereas mice in the probiotic-treatment group were healthy (right image). These results showed a preventive effect of our candidate probiotic strain against CRKP infection in a mouse model.

Evaluation of the therapeutic effect of *B. velezensis* on CRKP-infected mice. Therapeutic effects of *B. velezensis* on lethal CRKP-infected mice were evaluated (Fig. 3). On days 13 and 18, the untreated group lost 29.5% of their body weight. In contrast, the treated

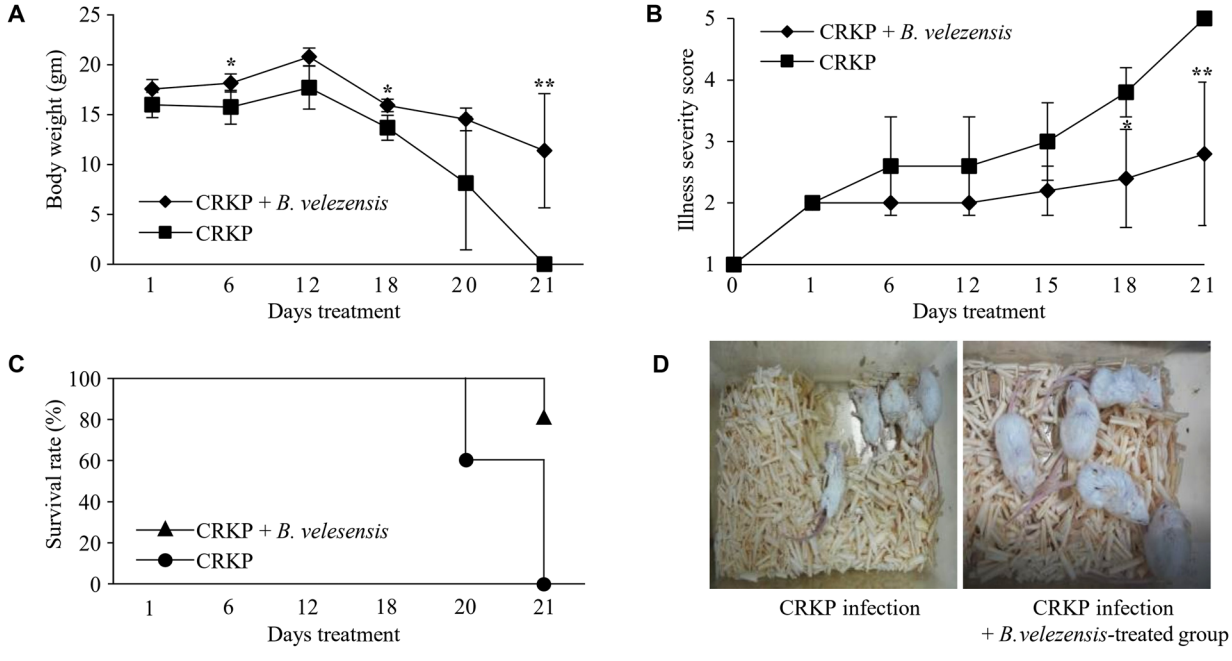

Fig. 2. Effect of *B. velezensis* on a clinical isolate of CRKP in the preventive infection murine model.
A) Body weight and B) illness severity were observed during the experimental period. Illness severity score was evaluated as (1 – healthy, 2 – minimally ill, 3 – moderately ill, 4 – severely ill, 5 – dead). C) Survival rate was observed during the entire experimental period. D) CRKP treated group (left image), CRKP + *B. velezensis* treated group (right image).
Statistical significance with the control was analyzed using unpaired Student's *t*-test (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

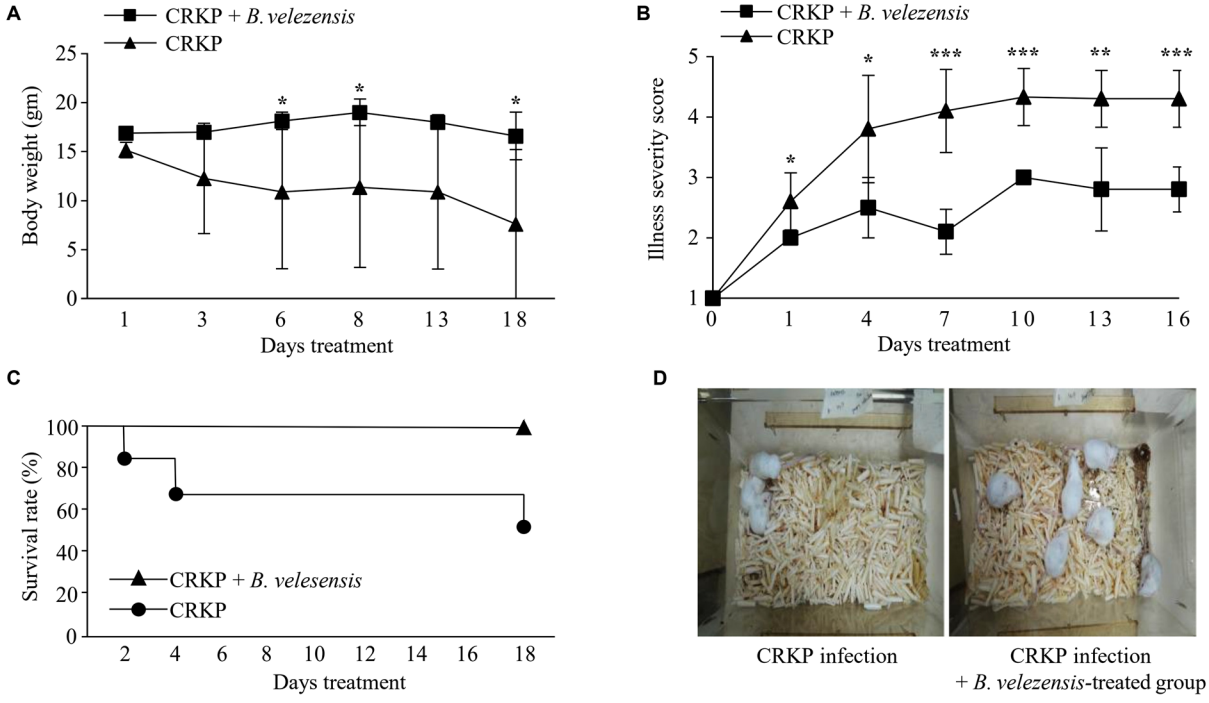

Fig. 3. Effect of *B. velezensis* on a clinical isolate of CRKP in a therapeutic infection murine model.

A) Body weight, B) severity of illness, and C) survival rate were observed during the experimental period. Illness severity score was evaluated (1 – healthy, 2 – minimally ill, 3 – moderately ill, 4 – severely ill, 5 – dead). D) CRKP treated group (left image), and CRKP + *B. velezensis* treated group (right image). Statistical significance with the control was analyzed using unpaired Student's *t*-test (*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

group showed insignificant body-weight reduction (Fig. 3a). The severity of illness was measured for both the probiotic-treated group and the infection group from day 0 to day 20 (Fig. 3b). We observed a variation in sickness scores between the groups. The survival rate of mice was determined for both groups (Fig. 3c). The untreated group's survival rate had decreased to 50.2%, whereas all mice remained alive in the treated group. Fig. 3d shows images of mice after CRKP infection. The infection group developed sickness and eventually died (left image), while mice with probiotic treatment were healthy (right image).

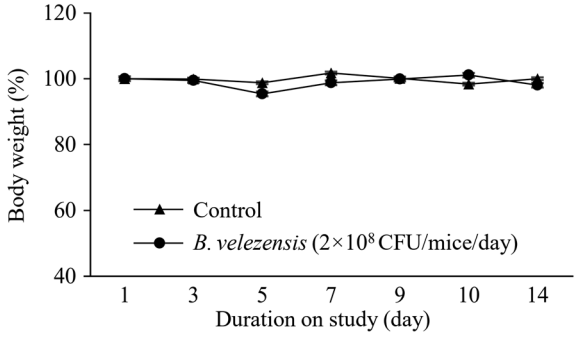

Fig. 4. Two-week repeated oral dose toxicity test of *B. velezensis* using guinea pigs. The body weights of guinea pigs were measured on days 1, 3, 5, 7, 9, 10, and 14. The candidate probiotic was orally administered at 2×10^8 CFU/mice/day at 200 µl per animal once a day, every day, for 2 weeks.

Acute oral dose toxicity of *B. velezensis*. Acute oral toxicity of *B. velezensis* was evaluated using guinea pigs through oral administration for two weeks. No significant variations in body weight were observed between the groups (Fig. 4). Additionally, unusual clinical symptoms or death in guinea pigs treated with *B. velezensis* were not observed (Table SIII).

Discussion

CRKP strains are causing a significant public health concern across the globe because of their capacity to spread quickly in the hospital setting with a high mortality rate and their extensive antimicrobial resistance characteristics (Brink 2019). Currently, infections with CRE have a limited number of therapeutic options, which have been utilized rarely due to concerns about their effectiveness and toxicity (Morrill et al. 2015). Therefore, new anti-CRE drug agents that differ from existing drug regimens are desperately needed. Under such circumstances, probiotics can be a potential option as their effectiveness against different infectious diseases has already been well established.

Several human studies have recently reported the effectiveness of probiotics for various gut-related diseases, such as necrotizing enterocolitis and antibiotic-associated diarrhea (Cremonini et al. 2002; AlFaleh and

Anabrees 2014). Besides, probiotics can help maintain intestinal barrier integrity (Hemert et al. 2013). Moreover, probiotic administration can protect and enhance the gut microbiota of mice by improving the number of beneficial bacteria while decreasing the number of pathogenic bacteria (Li et al. 2019). With this background, our research team is interested in using probiotics to counter the threat to human health caused by CRE infections.

Our research team isolated various probiotic strains, such as *Bacillus*, during a vinegar microbiome study, and the *Bacillus* genus was applied to the CRE infection treatment study. Vinegar has long been considered fermented food for its many health benefits, including its antioxidant activity, ability to improve hypercholesterolemia, prevent metabolic syndromes, and regulate the gut immune system (Urtasun et al. 2020; Sui et al. 2021;). However, *in vivo* effects of probiotics isolated from vinegar on CRKP infection have not been reported yet. Here we report the efficacy of a probiotic strain isolated from Gochang Bokbunja vinegar against CRKP infections.

The strain discovered in the vinegar was confirmed as *B. velezensis* through 16S rRNA gene sequencing analysis and additional species-specific PCR. It was judged to be a new strain as it showed a different carbon use pattern from the existing standard *B. velezensis* strain. As a result of additional efficacy evaluation *in vivo*, this candidate probiotic strain's prophylactic and therapeutic efficacies were confirmed, similar to a recent study (Tajdozian et al. 2021) showing the infection prevention and therapeutic effect of probiotics in a mouse model. Such effects are believed to be related to their diverse ability to adhere to epithelial surfaces and induce immunological responses (Wagner et al. 1997). Moreover, it has recently been reported that *Bacillus* treatment might have a solid immunostimulatory effect on the host to defend against infections (Plaza-Diaz et al. 2014; Mazaya et al. 2015; Suva et al. 2016). According to the results of related studies on *B. velezensis* as a candidate strain in this study, *B. velezensis* naturally produces various antibiotic compounds such as bacteriocins, short-chain fatty acids, and organic acids that can protect the gastrointestinal tract from diseases (Lee et al. 2019). There have also been claims that the administration of *B. velezensis* in animals can increase intestinal *Lactobacillus* and *Ruminococcus* and decrease *Acinetobacter* and that the effectiveness of *B. velezensis* on CRE might be related to this phenomenon (Li et al. 2019).

Probiotics are classified as Generally Regarded as Safe (GRAS) by the United States Food and Drug Administration (FDA) (Martín and Langella 2019). Probiotics have been used for a long time, and their safety has been established. These findings allow their usage as food or supplements from a scientific perspective (Martín and Langella 2019). Although probiotics are commonly considered safe (Williams 2010), some strains can cause bacteremia and septicemia (Kulkarni 2019). In consideration of these safety issues, a repeated two-week oral acute toxicity test was performed using guinea pigs. No abnormal clinical symptoms, weight loss, or animal death were observed during the experiment period.

Concerning safety, this study used probiotic strains cultured in FGM. FGM is one medium allowed to come into direct contact with foods, and it does not cause any hazards or change the taste and flavor, making it suitable for human consumption (Sawatari et al. 2006). Besides, FGM is less expensive and easier for bulk production; therefore, many *in vivo* studies rely on FGM (Huang et al. 2016; 2017b).

Many studies have found that the gastrointestinal tract's stability and beneficial bacterial population enhance the host's immune system and antioxidant capacity and prevent the spread of pathogenic bacteria in the gut (Khalid et al. 2021; Li et al. 2019). Moreover, according to a report, the probiotic strain *B. velezensis* can also generate secondary metabolites such as polyketides, lipopeptides, and peptides that have antibacterial properties (Ye et al. 2018). However, here, the in-depth mechanism of action based on the metabolite mentioned above and pathology studies to support the effectiveness of this probiotic strain remains a limitation.

Conclusions

In summary, in this study, a new probiotic strain, the *B. velezensis* strain, was isolated from Korean Gochang Bokbunja fermented vinegar. Its efficacy against CRKP infections was confirmed *in vivo*. Results of this study suggest the potential for developing *B. velezensis* as a live biotherapeutic agent for CRKP-associated infections. To develop CRKP therapeutics, extensive and in-depth research is required, including extensive animal efficacy investigations, GLP toxicity studies, clinical trials, and research regarding synergistic effects with existing drugs.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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