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Isolation, characterization, and evaluation of *Bacillus thuringiensis* isolated from cow milk

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Abstract : Probiotics colonize the intestines and exert an antibacterial effect on pathogens. Therefore, probiotics could be used as a preventive agent against lethal infections. To isolate probiotic microorganisms, 116 bacterial strains were isolated from healthy cow's milk and were subjected to Gram-stain, morphology and biochemical analyses, Vitek analysis, and 16S rRNA analysis. One of the strains identified as *Bacillus* (*B.*) *thuringiensis* 87 was found to grow very well at pH 4.0~7.0 and to be resistant to high concentrations of bile salts (0.3~0.9% w/v). *B. thuringiensis* was susceptible to the antibiotics used in the treatment of bovine mastitis, yet it exhibited an antimicrobial effect against *Staphylococcus* (*S.*) *aureus* 305. Moreover, it protected mice from experimental lethal infections of *E. coli* O55, *Salmonella typhimurium* 01D, and *S. aureus* 305 through a significant induction of interferon-γ, even at four-week post-administration of *B. thuringiensis*. Although oral administration of *B. thuringiensis* 87 did not provide significant protection against these lethal challenges, these results suggest that *B. thuringiensis* 87 could be a feasible candidate as a probiotic strain.

Keywords : *Bacillus thuringiensis,* interferon-γ, probiotics

Introduction

Probiotics are live microorganisms that exhibit beneficial effects on a host when ingested in sufficient amounts [2]. Generally, probiotics improve symptoms caused by intestinal bacterial fermentation. They influence microflora congestion on the intestinal wall and produce various products that aid in the absorption and utilization of ingested nutrients [24]. They also generate substances that neutralize toxins produced by bacteria and inhibit pathogen growth and proliferation [9]. Probiotics include *Bacillus*, *Enterococcus, Streptococcus, Lactobacillus* species (*sp*), and yeast [7] and are commonly found in the fermentation process of dairy products, crude oil, and in the intestines of animals and humans. For example, *Lactobacillus sp* maintains an acidic pH in the intestines, which inhibits the growth of intestinal pathogens such as *E. coli, Clostridium sp*, or bacteria causing diarrhea, and supports a healthy intestinal microflora. It has been suggested that probiotics may be useful as therapeutics [1, 10, 17, 20, 23, 26].

Probiotics might be used to minimize the need to use antibiotics in feed. Antibiotics are commonly added into feed in the livestock industry to prevent diseases caused by pathogenic microorganisms, thus increasing the economic productivity of livestock. However, because of the emergence of antibiotic-resistant bacteria and antibiotic residuals that have recently emerged as a serious problem, the use of antibiotics tends to be regulated [6, 8, 22]. Therefore, the use of antibiotics is only encouraged for the treatment of diseases, with the use of probiotics being suggested for prevention or convalescence of diseases.

Probiotics in *Bacillus* (*B.*) species, including *B. subtilis, B. licheniformis*, and *B. coagulance,* are known to directly and indirectly prevent pathogenic damage through antagonistic action or secretion [12, 14]. In addition, probiotic microorganisms added to the feed can reach the intestine, where they can attach to the walls of epithelial cells, thus preventing other harmful pathogens from colonizing and improving the health of the animal. The aims of this study were to isolate the potential candidate strains from healthy cow's milk and to identify possible probiotic strains. Among the 116 bacterial strains isolated, *B. thuringiensis* 87 showed the best probiotic characteristics, as demonstrated by acid- and bile salt-resistances and a protective effect. These features imply for

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the first time that *B. thuringiensis* could be a feasible probiotic of livestock through the elicitation of nonspecific immunity for the prevention of animal diseases.

Materials and Methods

Bacterial strains, cultures, and animals

B. subtilis 1024 and *Staphylococcus* (*S.*) *aureus* 305 were purchased from American Type Culture Collection (ATCC1024 and ATCC305, respectively, USA). *Streptococcus* (*S.*) *pneumoniae* encapsulated type 2 strain D39 (NCTC7466) was cultured in brain heart infusion (BHI) broth or on BHI agar (Oxoid, UK) as described previously [18]. Commercially available *B. subtilis, B. coagulans,* and *B. mesentericus* strains were donated by Handong and Sungwon (Korea) for use as references. *E. coli* 055, *E. coli* 21, and *Salmonella* (*S.*) *typhimurium* 01D strains were isolated from pigs and cows in South Korea at the Animal, Plant and Fisheries Quarantine and Inspection Agency with intestinal diseases including diarrhea.

All *Bacillus sp* strains, *S. aureus* ATCC305, *S. pneumoniae* D39, and *S. typhimurium* 01D were grown in BHI broth or on BHI agar, and *E. coli* 055 was grown in Luria-Bertani (LB) broth or on LB agar. All inoculated broth or agar plates were incubated at 37°C, and colonies were counted after 18 h incubation. CD1 mice were purchased from Samtako (Korea).

Isolation and identification of microorganisms from healthy cow's milk

In our search for an effective probiotic, microbial strains were collected from fresh raw milk from 35 dairy farms at 22 geographically different locations from March to August 2001 in South Korea. To avoid contamination, the first part of the milk was not collected. Microorganisms were isolated from milk only when the somatic cell count was less than 30,000 according to an automatic somatic cell counter (Fossomatic series 4000; Foss Electric, Denmark). Raw milk was kept on ice after sampling and was directly processed for determination of total colony. For total colony count, 1 mL of the sample suspension was diluted decimally and was pour plated in duplicate directly on milk plate count agar (Oxoid). All plates were incubated at 30°C for 72 h prior to count colonies. For isolation of potential probiotics, and after cooling on ice, 1 mL was immediately spread plated in duplicate onto petri dishes containing BHI broth supplemented with filter-sterilized vitamin B_{12} (1 mg liter^{-1}; Sigma, USA), pH 6.8 and bacteriological agar no. 1 (15 g liter^{-1}; Oxoid). All visibly different colonies were picked off (n = 116). Pure cultures of these isolates were stored at −80°C. After the samples were selected, they were further incubated on a blood agar plate at 37°C for 16 h. Initially, 116 strains comprising *Bacillus, Enterococcus,* and *Lactobacillus* were isolated from fresh milk and were further cultured on BHI agar, and their morphologies and cultural and physiological characteristics including blood agglutination, Gram staining, were carried out according to Bergey's manual. Also antibiotic resistance was determined by disc diffusion method as suggested by guidelines of the Clinical and Laboratory Standard Institute [5]. The bacterial strains were also screened using the GPI card of the Vitek system (BioMerieux, USA).

Identification of *Bacillus* strains

To further identify the *Bacillus* strains, the carbohydrate utilization pattern and nucleotide sequence of 16S ribosomal RNA were determined via PCR methods using conserved sequences. The conserved 16S rRNA sequences in *Bacillus* strains used for amplification were 16S-27F (5′AGR GTT TGA TCM TGG CTC AG 3′) and 16S-1492R (5′ GGY TAC CTT GTT ACG ACT T 3′) [19]. To further identify the *Bacillus* species, conserved 16S rRNA sequences were used to amplify the rDNA using an ABI thermal cycler; forward primers 16S-F23 (5'GGC GGC GTG CCT AAT ACA TGC AAG TCG3') and 16S-F310 (5'CGG CCC AGA CTC CTA CGG GAG GCA GCA3') and reverse primer 16S-R770 (5'GCG TGG ACT ACC AGG GTA TCT AAT CC3') [19]. Amplified PCR products were subsequently separated using gel electrophoresis in a 1.2% agarose gel, were cut from the agarose gel and used for nucleotide sequence determination. Nucleotide sequences were determined using the above primers and a BigDye terminator cycle sequencing kit (Applied Biosystems, USA) in an automatic DNA sequencer (Applied Biosystems Model 310) according to the manufacturer's instructions. The nucleotide sequence was deposited into GeneBank (Accession no. HQ845263). Using the BLASTN tool, the nucleotide sequences were compared against those in the National Center for Biotechnology Information GenBank. The identified strain was deposited into the Korean Agricultural Culture Collection (KACC 91098).

Screening for pH- and bile-salt-tolerant strains

BHI broth adjusted to pH 1~7 with HCl (Sigma) was used to culture all of the *Bacillus* strains, which were then incubated at 37°C for 18 h followed by measurement of absorbance at 620 nm. Bile salt tolerance was determined in BHI broth after adding bile salt (Difco, USA) to a final concentration of 0.3%, 0.6%, or 0.9%. Then, the *Bacillus* strains were cultured at 37°C for 16 h, and the absorbance at 620 nm was measured to assess growth.

Antimicrobial resistance testing

Antimicrobial resistance was determined by disc diffusion method according to the guidelines of the Clinical and Laboratory Standard Institute [5]. Antibiotic discs (BD, USA) were placed onto a lawn of freshly plated bacteria on the Muller-Hinton agar containing 3% (v/v) sheep blood, and antimicrobial resistance was determined by measuring the diameter of the inhibition zone after incubation of the plate at 37°C for 16 h. The antibiotics in each disc included ampicillin (10 μg), amikacin (30 μg), cephalothin (30 μg), cloxacillin (1 μg), erythromycin (15 μg), enrofloxacin (30 μg), gentamy-

cin (10 μg), kanamycin (30 μg), lincomycin (2 μg), neomycin (30 μg), oxacillin (1 μg), penicillin G (10 U), streptomycin (10 μg), tetracycline (30 μg), or vancomycin (30 μg).

Antagonism test

To determine the *in vitro* antagonistic effects of the probiotic strains against *S. aureus,* both probiotic strain and *S. aureus* ATCC305 strain were cultured overnight, and the next day they were inoculated into a fresh medium until they reach mid-log phase. Subsequently, probiotic strain was added into culture of *S. aureus* ATCC305 to a final volume of 1% (1/100 dilution). Then the mixed culture was incubated at 37°C for 1, 3, 6, or 24 h, and viable cell numbers of *S. aureus* were determined after plating on Baird-Parker agar containing 5% egg yolk.

***In vivo* challenge experiments**

To determine the presence of a protective effect against virulent pathogens in the probiotic strains *in vivo*, CD1 mice (four-week-old males) were injected intraperitoneally (*i.p*) with 100 μL of *B. thuringiensis* 87 (approximately 1×10^8 CFU suspended in PBS). This experiment was also conducted to identify the potential risk of toxigenicity and hypersensitivity of strain.

Three days post-administration, each mouse was challenged *i.p.* with 100 μL of a lethal dose of virulent pathogen (2.5×10^9 CFU of *E. coli* 055, 2×10^8 CFU of *S. aureus* 305, or 7.3×10^6 CFU of *S. typhimurium* 01D) suspended in sterile PBS. In the case of the *S. aureus* challenge, one week post-administration of *B. thuringiensis* 87, 100 μL of *S. aureus* ATCC305 (2×10^8 CFU/mL in PBS) was injected *i.p.* To differentiate *S. aureus* from *Bacillus, S. aureus* was selected from Baird-Parker agar containing 5% egg yolk. For each strain, at least two experiments were performed. Survival of the mice was checked every day.

Determination of interferon-γ

The interferon (IFN)-γ level in serum was determined using an ELISA kit (BD) according to manufacturer's instructions as follows. One hundred μL of serum was added into microtiter plate well, and primary antibody diluted to 2,000 pg/mL was added. After incubation of the mixture at room temperature for 2 h, the titer plate was washed 5 times with PBS. Subsequently, secondary antibody diluted 250 fold was added and incubated for 30 min at room temperature. After washing 5 times, detector solution was added and incubated 1 h further. The titer plate was washed 7 times and substrate (3,3',5,5'-tetramethyl benzidine: TMB) was added and incubated at room temperature for 30 min in dark place. To stop the reaction, stop solution was added, and OD at 450 nm was determined.

Statistics

For statistical analysis, the OD differences among groups were calculated using the one-way ANOVA Newman-Keuls test. Statistical differences between the group medians were analyzed using the Mann-Whitney U test (two-tailed unpaired). ELISA data were expressed as the average of triplicate wells ± SEM. Statistically significant differences were defined as $p < 0.05$.

Results

Identification of *B. thuringiensis* from bacterial strains isolated from fresh cow milk

In total, 116 bacterial strains were isolated from fresh raw milk, which was chosen only when it contained less than 30,000 somatic cells. Gram staining and morphological observation showed the isolated strains were either Gram-positive bacilli or cocci. The strains were also identified using the Vitek system (bioMrieux, France), which indicated the presence of *Bacillus, Enterococcus,* and *Lactobacillus* species (data not presented).

To further identify the strain, sequencing of 16S rRNA nucleotide was performed. Results showed 99% homology with the *B. thuringiensis* species (Table 1), and the strain was named *B. thuringiensis* 87.

pH and bile salt resistance of *B. thuringiensis* 87

First, pH sensitivity was studied over a range of pH from 1 to 7 and *B. thuringiensis* 87 as well as other *Bacillus.* Results showed that *B. thuringiensis* 87, as well as other *Bacillus* strains such as *B. subtilis, B. coagulans,* and *B. mesentericus,* grew at pH 5 or above but did not grow in conditions less than pH 4 (Fig. 1A).

To assess bile salt tolerance, the growth rates of a variety of *Bacillus* strains were determined at different concentrations of bile salt in BHI broth. The results showed that *B. thuringiensis* 87 and several other *Bacillus* strains survived at bile salt proportions of 0.3% to 0.9%. At a 0.9% bile salt concentration, the absorbances of *B. thuringiensis* 87 and *B. coagulans* were similar, whereas those of commercially available probiotics *B. subtilis* (A), *B. subtilis* (B) and *B. mesentericus* were lower. *B. subtilis* had a low survival rate throughout the various concentrations of bile salt. These data suggest that *B. thuringiensis* 87 is resistant to a high bile salt

Table 1. Identification of *Bacillus thuriengiensis* 87 via DNA sequence comparison in GenBank

	Genebank Accession No.	Identity	Total length	Homology region
Bacillus thuringiensis str. Al Hakam	CP000485.1	1508/1512 (99%)	5257091	258211-259722
Bacillus thuringiensis serovar konkukian str	AE017355.1	1505/1510 (99%)	5237682	9320-10829

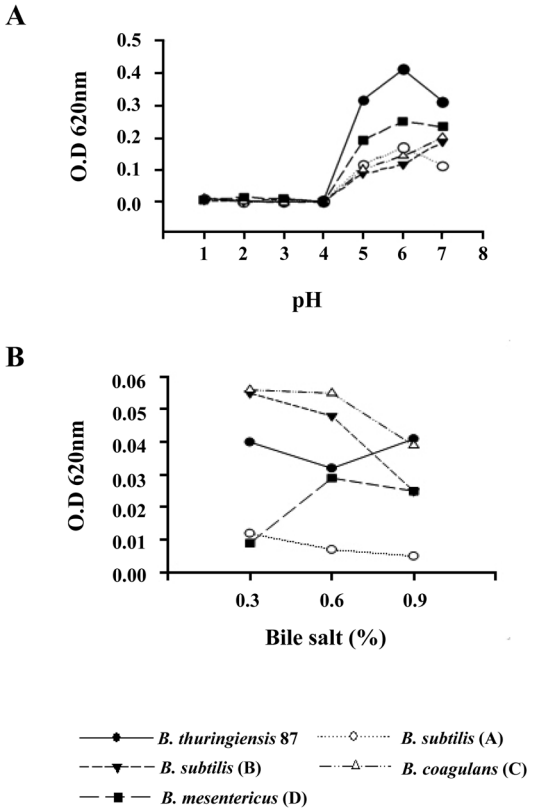

Fig. 1. Effects of pH (A) and bile salts (B) on the growths of various *Bacillus* species at 37°C. To check pH and bile-salt resistance, the growth of *Bacillus* species in BHI broth was determined by OD at 620 nm after adjusting the culture media to the specific pH or salt concentration.

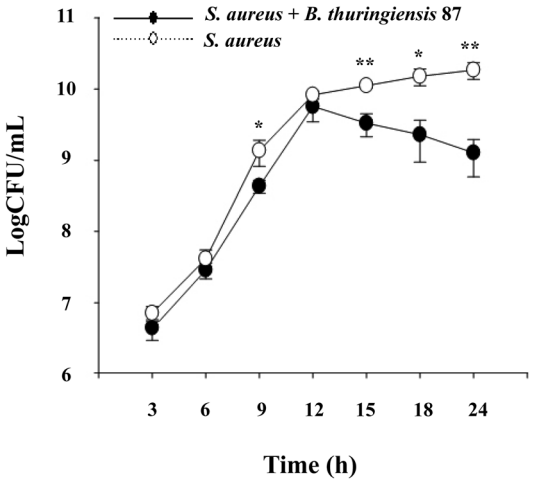

Fig. 2. Antimicrobial activity of *Bacillus* (*B.*) *thuringiensis* 87 against pathogenic *Staphylococcus* (*S.*) *aureus* 305. To determine their antimicrobial activities, *B. thuringiensis* 87 was inoculated into *S. aureus* 305 cultures to a final volume of 1% (1/100 dilution). Then the mixed culture was incubated, and a viable cell number of *S. aureus* was selected by plating on Baird-Parker agar containing 5% egg yolk.

concentration (Fig. 1B).

In vitro antimicrobial activity of *B. thuringiensis* 87

Results showed that the growth of the pathogenic strain was significantly decreased after 6 and 24 h of co-culture compared to that of the control group, indicating that *B. thuringiensis* 87 had a detectable antibiotic effect (Fig. 2). Also antibiotic effect of *B. thuringiensis* 87 was the same or better than those of the other commercially available strains (data not shown). This result indicates that *B. thuringiensis* 87 has desirable antibacterial effects against the pathogenic *S. aureus* strain.

Antibiotic resistance of *B. thuringiensis* 87

Since antibiotic-susceptible probiotics are preferred, we determined the antibiotic resistance of *B. thuringiensis* 87 to several antibiotics. Since the guidelines for antibiotic resistance are only set for human clinical isolates and guidelines for antibiotic resistance for probiotic organisms are not available, we could not define true antibiotic resistance for *B. thuringiensis* 87. However, the results showed that *B. thuringiensis* 87 had the same resistance pattern as did the other commercially available reference *Bacillus* strains used in this study. These results indicate that *B. thuringiensis* 87 was susceptible to antibiotics such as ampicillin, amikacin, cephalothin, cloxacillin, erythromycin, enrofloxacin, gentamycin, kanamycin, lincomycin, neomycin, oxacillin, penicillin G, streptomycin, tetracycline, and vancomycin (Supplementary Table 1).

Virulence attenuation of *B. thuringiensis* 87

Invasion of pathogenic bacteria from the intestine into the blood can lead to high morbidity, septic shock, and death. Since *B. thuringiensis* 87 has a similar antimicrobial effect to those of other commercially available *Bacillus* species, the virulence of *B. thuringiensis* 87 was examined using the septicemia model. *B. thuringiensis* 87 (7×10^7 CFU) or *S. pneumoniae* D39 (1×10^4 CFU) was inoculated intraperitoneally (*i.p*) into mice, and survival time was determined. The results showed that *B. thuringiensis* 87, along with PBS as the negative control, caused no mice mortalities within seven days (Fig. 3A), demonstrating that the virulence of *B. thuringenisis* 87 was absent or significantly attenuated and suggesting that *B. thuringenisis* 87 might be a highly feasible candidate as a probiotic.

Protection from lethal infections by *B. thuringiensis* 87

To check the probiotic effect of the strain in question, groups of ten or 11 mice were inoculated *i.p* with 7×10^7 CFU of *B. thuringenisis* 87. Three days post-infection, mice were challenged *i.p* with either 2.5×10^9 CFU of pathogenic *E. coli* 055 or 7.3×10^6 CFU of pathogenic *S. typhimurium* 01D. Ninety percent of mice were killed within six days when challenged with *E. coli* 055 and within two days when challenged with *S. typhimurium* 01D. In contrast, mice that received *B. thuringenisis* 87 prior to challenge with *E. coli*

Supplementary Table 1. Antibiotic resistances of *Bacillus* (*B.*) *thuringiensis* 87 and other *Bacillus* species

Strain	Antibiotic resistance, growth inhibition diameter (mm)													
	AN	N	S	K	TE	P	E	ENR	VA	GM	OX	AM	CF	CX
B. thuringiensis 87	21	21	24	24	28	18	30	28	22	22	–	19	21	–
B. subtilis[a]	27	25	20	28	22	13	12	ND[*]	25	32	11	16	25	15
B. subtilis[b]	30	23	20	11	25	33	30	ND	28	30	24	32	42	15
B. coagulans[c]	26	21	18	24	19	30	25	ND	20	26	23	30	40	22
B. mesentericus[d]	22	23	20	30	19	27	27	ND	20	26	17	25	40	17

AM: ampicillin (10 μg), AN: amikacin (30 μg), CF: cephalothin (30 μg), CX: cloxacillin (1 μg), E: erythromycin (15 μg), ENR: enrofloxacin (30 μg), GM: gentamycin (10 μg), K: kanamycin (30 μg), L: lincomycin (2 μg), N: neomycin (30 μg), OX: oxacillin (1 μg), P: penicillin G (10 U), S: streptomycin (10 μg), TE: tetracycline (30 μg), VA: vancomycin (30 μg). [a,b,c,d]Commercially available products. [*]ND: Not determined.

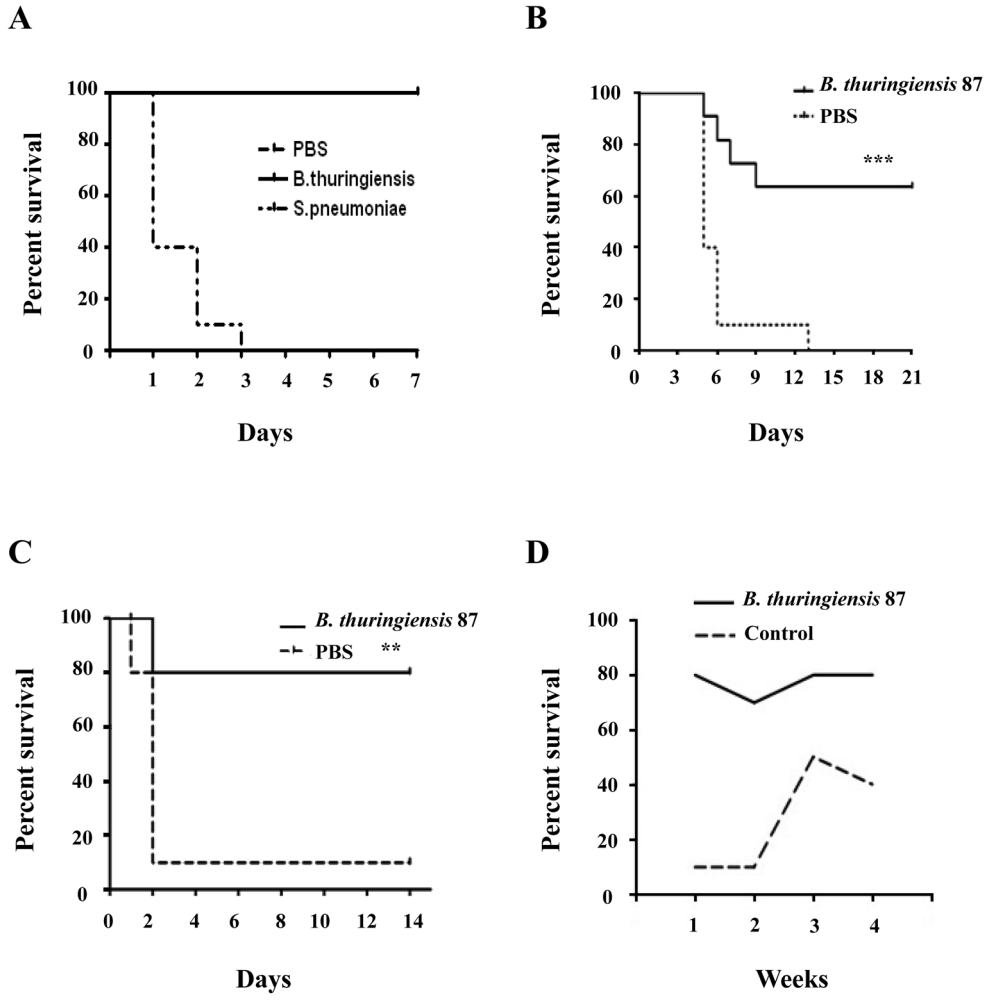

Fig. 3. Probiotic effects of *B. thuringiensis* 87 against pathogens. (A) Virulence attenuation of *B. thuringiensis* 87 *in vivo* after intraperitoneal inoculation. A group of 10 mice was inoculated with 7×10^7 CFU of *B. thuringiensis* 87, 1×10^4 CFU of *S. pneumoniae* D39, or PBS as a control. (B, C) Protection from *E. coli* 055 and *S. typhimurium* 01D challenges. Groups of ten or 11 mice were intraperitoneally inoculated with 7×10^7 CFU of *B. thuringenisis* 87, three days prior to an intraperitoneal inoculation with either 2.5×10^9 CFU of *E. coli* 055 (B) or 7.3×10^6 CFU of *S. typhimurium* 01D (C). (D) Long-term protection by *B. thuringenisis* 87 against *S. aureus* 305 challenge. Forty mice were intraperitoneally immunized with *B. thuringenisis* 87 (1.1×10^8 CFU) and then divided into 4 groups, and subsequently challenged by *S. aureus* 305 (2×10^8 CFU) 1, 2, 3, and 4 weeks post-inoculation. The survival was recorded every day and was analyzed using the Prism survival test ($p = 0.0016$, $^{**}p < 0.01$, $^{***}p < 0.001$).

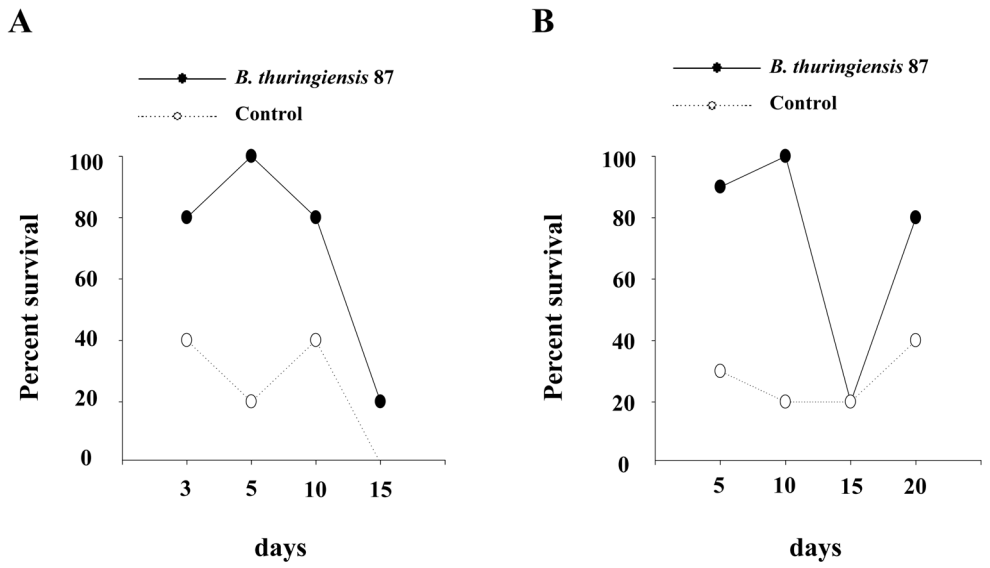

Fig. 4. Protection from lethal challenge with *E. coli* 21 or *S. aureus* 305 by heat-killed *B. thuringiensis* 87. 1.1×10^8 CFU of the heat-killed *B. thuringiensis* 87 was inoculated prior to lethal challenge. For lethal challenge, either *S. aureus* 305 (2×10^8, panel A) or *E. coli* 21 (2.5×10^9 CFU, panel B) was inoculated *i.p*. Each group consisted of five mice.

055 showed a 64% survival rate 21 days post-challenge ($p < 0.001$; Fig. 3B). Consistently, mice given *B. thuringenisis* prior to challenge with *S. typhimurium* 01D showed an 80% survival rate 14 days post-challenge ($p < 0.01$; Fig. 3C). These results demonstrate that *B. thuringenisis* 87 could serve as a probiotic to protect animals from invasive infection by pathogenic bacteria.

To further corroborate the probiotic effect of *B. thuringenisis* 87 against Gram-positive pathogens, *S. aureus* 305 was used as the challenge organism. Before determination of the probiotic effect of *B. thuringenisis* 87, the virulence of the *S. aureus* 305 strain was determined after *i.p* inoculation. When 8×10^8 CFU or 4×10^8 CFU of *S. aureus* 305 was inoculated into 5 mice *i.p*, all five mice were dead within a day, whereas *i.p* inoculation of 2×10^8 CFU of *S. aureus* 305 resulted in the death of four mice within a day (data not shown). Therefore, to determine the probiotic effect of *B. thuringenisis* 87, we inoculated *B. thuringenisis* 87 *i.p*, and then, 1-, 2-, 3-, and 4-weeks post-inoculation, mice were challenged *i.p* with 2×10^8 CFU of *S. aureus* 305. One- to four-weeks post-administration, the mice that received *B. thuringenisis* 87 showed significantly increased survival rates compared to those of the control group. The average survival rate of the *B. thuringenisis* 87 pre-administered group was 77.5%, whereas that of the control group was 27.5% (Fig. 3D). These data confirm that *B. thuringenisis* 87 inoculation could be used for probiotic protection against invasive virulent pathogens in animals.

Although inoculation of sonicated lysate of 7×10^7 CFU *B. thuringenisis* 87 did not provide protection from *S. aureus* 305 three days post-inoculation (data not shown), inoculation of heat-killed *B. thuringenisis* 87 provided protection from *S. aureus* 305 three to ten days post-inoculation (Fig. 4A). Consistently, heat-killed *B. thuringenisis* 87 provided protection from *E. coli* 21 five to ten days post-inoculation (Fig. 4B). These results demonstrate that live or dead *B. thuringenisis* 87, but not the lysates, can be used to prevent invasive pathogen infection.

To corroborate the probiotic effect of the dead *B. thuringenisis* 87, it was *i.p*. inoculated twice over a one-week interval and was challenged one week after the last inoculation with either *S. aureus* 305 or *E. coli* 21. When mice were inoculated twice with heat-killed *B. thuringiensis* 87 followed by *S. aureus* 305 challenge, we observed a 100% survival rate, whereas the control without *B. thuringiensis* 87 inoculation showed no survival (data not shown). Consistent with this, two inoculations of heat-killed *B. thuringiensis* 87 followed by *E. coli* 21 challenge provided 80% survival, whereas the control group showed only 10% survival (data not shown). These results indicate that inoculation with heat-killed *B. thuringenisis* 87 could be useful for protection against invasive pathogen infection.

Induction of IFN-γ by administration of *B. thuringiensis* 87

To further investigate the underlying mechanism of the protective effect of *B. thuringenisis* 87, the strain was inoculated *i.p*. and the viability of *B. thuringenisis* 87 and IFN-γ levels in blood were determined two and four weeks post-administration. Although no viable cells were detected at these times (data not shown), IFN-γ was increased 7.1-fold significantly after 2 weeks post-administration of *B. thuringenisis* 87 (Fig. 5). Moreover, three to 20 days post-inoculation with heat-killed *B. thuringenisis* 87, the level of IFN-γ

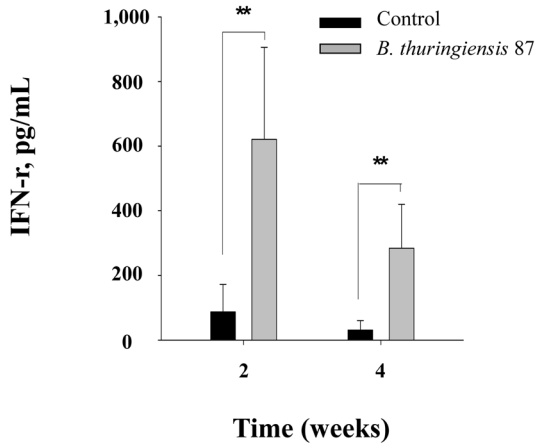

Fig. 5. Induction of interferon (IFN)-γ by *B. thuringiensis* 87 injection. 1.1×10^8 CFU of *B. thuringiensis* 87 was inoculated *i.p*. Two and four weeks post-administration of *B. thuringiensis* 87, a blood sample was taken and the IFN-γ level was determined using ELISA. Three mice per group were used.

was maintained at a higher level than that of the control group (data not shown), indicating that administration of *B. thuringenisis* 87 could activate the immune system and protect the host from invading pathogens.

Discussion

Some *Bacillus* species posses probiotic characteristics such as resistance to strong acids from stomach and bile acids; tolerance to heat, acids, and humidity; and formation of polypeptide antibiotics like bacitracin [12]. B. thuringiensis, the spore-forming bacterium, is well known as a bioinsecticide that controls plant diseases. The strain produces several plasmid-encoded Cry proteins in large quantities during sporulation and which are packaged into intracellular crystal inclusions. Inclusions ingested by insect larvae are solubilized and converted to active toxins in the midgut. Subsequently, toxins are inserted into the membrane, resulting in the formation of cation-selective channels and lethality due to osmotic lysis [3, 4, 25]. Moreover, in the mammalian intestinal tract, *B. thuringiensis* has been shown to germinate and transfer DNA [28]. However, the role of *B. thuringiensis* as a probiotic has not received much attention. This is the first study to isolate and characterize *B. thuringiensis* 87 from fresh cow milk and to provide evidence that it is a probiotic strain.

In this study we demonstrate that the *B. thuringiensis* 87 isolated has acid- and bile-resistance, broad carbohydrate utilization, and growth inhibitory effects against harmful bacteria. Moreover, we demonstrate the non-toxicity of *B. thuringiensis* 87 *in vivo* and its protective effect against lethal pathogenic infections including *S. aureus* 305, *E. coli* 21 and *S. typhimurium* 01D. Although heat-killed *B. thuringiensis* 87 could protect mice from lethal challenges, live *B. thuringiensis* 87 has stronger protective effects. Since *B. thuringiensis* 87 can protect against lethal septicemia even four weeks post-inoculation, it is highly feasible that it could be an excellent probiotic for the prevention of lethal diseases due to invasive pathogens in animals. Upon infection with pathogens, specific pathogen-associated molecular patterns are recognized by phagocyte receptors, resulting in the triggering of the type-1 early cytokine (IFN-γ, interleukin 12, interleukin 23, interleukin 17) signaling pathway. Activation of this pathway initiates microbicidal mechanisms in phagocytes [27].

The protective effects of *B. thuringenisis* 87 can be explained by 1) the antimicrobial and/or microcidal activity of *B. thuringenisis* 87, 2) activation of the immune system to result in pathogen removal, and 3) colonization in the host. The antimicrobial activity of *B. thuringenisis* 87 was detected. Moreover, *i.p*. injections of *B. thuringiensis* 87 provided protection against lethal challenges, although oral administration of *B. thuringiensis* 87 did not provide protection against lethal challenge (data not shown). Since administration of *B. thuringiensis* 87 significantly induced IFN-γ, a key cytokine that induces mouse and human macrophages to kill pathogens through both oxidative burst and nonoxidative mechanisms [13, 15, 16, 21], and mice deficient for IFN-γ-receptor are highly susceptible to attenuated Salmonella Typhimurium [11], our results suggest that colonization is not the major mechanism through which *B. thuringiensis* provides protection against lethal challenge. Rather, other mechanisms such as antimicrobial activity and immune system activation might play major roles in mediating the protective effects of the probiotic *B. thuringiensis* 87.

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