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Characterization of *Bacillus* Species from Market Foods in Beijing, China

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Abstract: Foodborne diseases have been witnessing a constant rising trend worldwide, mainly caused by pathogenic microorganisms, such as *Bacillus* spp., posing a direct threat to public health. The purpose of this study was to evaluate the biological risk of foodborne and probiotic *Bacillus* spp. in Beijing markets. A total of 55 *Bacillus* isolates, including 29 *B. cereus*, 9 *B. licheniformis* and 7 *B. subtilis*, mostly found in dairy products (32.7%), were recovered from 106 samples and identified by matrix-assisted laser desorption/ionization mass spectrometry and polymerase chain reaction methods. The susceptibility towards 16 antibiotics was determined using a broth microdilution method. *Bacillus* showed a high level of resistance to florfenicol (100%), lincomycin (100%), tiamulin (78.2%) and ampicillin (67.3%), while they were all susceptible or intermediate to vancomycin and rifampin. Additionally, we obtained the whole genome of 19 *Bacillus* strains using high-throughput sequencing, and the rates of resistance genes *van*, *fosB*, *erm* and *tet* were 57.9%, 57.9%, 21.1% and 26.3%, respectively. Moreover, 100%, 9.1%, 45.5% and 100% of these isolates carried virulence genes *nhe*, *hbl*, *cytK* and *entFM*, respectively. Lastly, 60% *Bacillus* strains were positive in hemolysis tests, and 3 *B. licheniformis* strains displayed an inhibitory activity on the growth of *S. aureus* ATCC 29213 using agar overlay technique. Our study outlines the characteristics of foodborne *Bacillus* spp. and provides information for the monitoring of food safety.

Keywords: emerging foodborne pathogens; *Bacillus*; probiotics; antimicrobial resistance

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1. Introduction

Foodborne diseases are now a widespread and growing problem to public health and the world economy [1–3]. It is estimated that about 600 million people are suffering from foodborne illnesses, such as malaise, diarrhea, etc., which even stimulates the possibility of cancer, leading to 420,000 deaths annually [4,5]. Food safety hazards are associated with the ingestion of poisonous toxins, chemicals, and mostly, bacteria, viruses, or parasites [6,7]. Notably, pathogens with the competence of producing toxins play a crucial role in foodborne illnesses [8].

Bacillus species are Gram-positive, spore-forming, rod-shaped, aerobic or facultative anaerobic bacteria, and they are ubiquitously distributed in soil, water, the environment as well as various food products [9,10]. By virtue of their multilayer-structured endospores, *Bacillus* spp. offer high tolerance towards acid, dehydration, γ-ray and ultraviolet radiation; they are stable during heat processing and low-temperature storage [11–14]. Several *Bacillus* strains have been screened for their potential probiotic functionalities in animal husbandry, bionematicides and antibiotic alternatives [15,16]. Additionally, they have also been verified to possess pathogen exclusion, anti-oxidant, immuno-modulatory and food fermentation abilities [17–20]. *Bacillus* and *Paenibacillus* spp. can yield potent antimicrobial lipopeptides, including polymyxins, octapeptins, polypeptins, iturins, surfactins, fengycins,

tridecaptins and kurstakins, which are usually secondary metabolites produced by non-ribosomal peptide synthetases (NRPSs) [21]. A mixture of D- and L-amino acids equips those lipopeptides with an enhanced ability to withstand proteolytic enzymes from target organisms, as well as human plasma proteases, and potentially enables treatment by oral administration and intravenous injection [21–23].

Nevertheless, *Bacillus* is an opportunistic pathogen that may cause severe local or systemic infections, such as endophthalmitis and septicemia, when allowed access to mammalian tissues [16]. Few members of *Bacillus* spp., particularly *B. cereus* and *B. anthracis*, are infamous for producing emetic toxins (Cereulide) or enterotoxin. PA-LF (protective antigen- lethal factor) and PA-EF (edema factor) are *B. anthracis* generated toxins that induce the deadly disease anthrax in humans and animals [24,25]. Cereulide, produced by *B. cereus* and *B. weihenstephanensis*, is a major cause of foodborne intoxications through inhibiting the synthesis of RNA, causing expansion of mitochondria and formation of vacuoles in the protoplasm of target cells, thus bringing cell apoptosis and even fulminant liver failure [26,27]. Three enterotoxins of *B. cereus* that belong to the family of pore-forming toxins (PFTs), including non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl) and cytolysin K (CytK), are mainly responsible for diarrhea [28–30]. On the other hand, the presence of transferrable antimicrobial resistance genes (ARGs) will endow foods and probiotics containing *Bacillus* spp. as a reservoir for the transmission of antibiotic resistance [31,32]. Foodborne *Bacillus* spp. could serve as a vehicle to spread ARGs, while probiotic *Bacillus* spp. that are excreted through improperly treated animal waste would facilitate the horizontal gene transfer of mobile ARGs. Further, the emergence of antibiotic resistant strains, especially those resistant to multiple antibiotics, can cause routine treatments of *B. cereus* infection to fail [9,33,34]. Therefore, the mobile ARGs in *Bacillus* strains is another safety parameter that requires prompt attention.

The Centers for Disease Control and Prevention (CDC) website claimed that there were 619 confirmed *Bacillus*-related outbreaks from 1998 to 2015. *B. cereus* is also the second most frequently found causative agent of confirmed and suspected foodborne outbreaks (FBOs) in France after *Staphylococcus aureus* [35]. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks reported a total of 287 outbreaks caused by *B. cereus* toxins involving 3073 cases (about 8% hospitalization) in European Member States (MSs) in 2014, whereas 291 outbreaks involving 3131 cases (with 3% hospitalization) were reported by nine MSs in 2015 [36]. It is elucidated that rice, pasta, pastry and noodles are associated with emesis, whereas vegetables, meat products and milk products are connected with diarrhea, based on previous epidemiological data [12,37]. Dairy products such as infant formula in the Chinese market have caused a considerable number of *B. cereus*-induced FBOs [38]. Furthermore, the diarrheal-type disease has been reported more frequently in Northern Europe, such as in Finland and Norway, as well as the emetic-type disease in Japan and the UK, constituting a great challenge both in developed and developing societies [39,40]. Our study aims to investigate the potential virulence, molecular characteristics and antibiotic resistance profiles of *Bacillus* spp. isolated from market foods in Beijing, China, providing information about the prevalence and pathogenicity of *Bacillus* spp. to further ensure food safety.

2. Materials and Methods

2.1. Sample Collection

From September to December in 2020, we collected a total of 106 samples, including 31 Beijing specialty food, 29 dairy products, 15 rice products, 11 probiotics, 9 fermented food, 7 raw or cooked meat, 2 soybean milk and 2 snacks from different local markets and restaurants in Beijing, China. All samples were independently kept in sealed, sterile plastic bags, transported directly to the laboratory within 24 h and stored at 4 °C or −20 °C.

2.2. Bacterial Isolation and Identification

Liquid samples were serial-decimally diluted as needed, and solid samples were suspended in phosphate-buffered saline (PBS, pH = 7.2) as initial dilution before being plated onto the surface of Brilliance *Bacillus cereus* Agar (Oxoid) and incubated at 37 °C for 24 h. Blue/green colonies were considered as presumptive *B. cereus*. The colonies were then transferred into 1 mL brain heart infusion (BHI, Land Bridge Technology) broth and incubated at 37 °C for 24 h (200 rpm). The bacterial cultures were spread on BHI agar, incubated at 37 °C for another 24 h, and single colonies were chosen for further study.

The species-specific identification was performed by ① matrix-assisted laser desorption ionization-time of flight mass spectrometry (AXIMA Performance, Shimadzu, Japan). First, bacterial samples were grown on BHA agar plates, and a single colony was selected and smeared directly as a thin film on the steel sample plate; 0.8 µL of 70% formic acid and 1 µL of CCA matrix solution (prepared with 50% acetonitrile and 2.5% trifluoroacetic acid in pure water) were then dropped onto the smear successively. The loaded sample plate was left for several minutes at room temperature to dry before inserting it into the AXIMA for data acquisition. ② 16S rRNA sequencing: From cultures grown overnight in BHI at 37 °C, DNA of each isolate was extracted by centrifuging at 5000× *g* for 5 min and resuspending in 50 µL Tris-EDTA (TE, Amresco) buffer. The suspensions were boiled in water bath at 100 °C for 10 min. Then. the tubes were placed on ice immediately for 10 min. The procedure was repeated twice, and samples were centrifuged at 14,000× *g* for 5 min to obtain the genomic DNA of each isolate. All the extracted DNA was stored at –20 °C; 16S rRNA sequence analysis was used to further characterize the *Bacillus*-like strains, using primers 27F and 1492R [41]. PCR products were sent to Tsingke Biological Technology (Beijing, China) for sequencing. Genomic sequences were identified in NCBI nucleotides databases using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The strains with high similarity to 16S rRNA sequence of the reference strain (Evalue = 0 and Max identity $\geq$ 98%) were regarded as *Bacillus* spp. strains.

2.3. Antimicrobial Susceptibility Tests

The minimum inhibitory concentrations (MICs) of isolated *Bacillus* spp. towards 16 kinds of antimicrobial agents (ampicillin, ceftriaxone, gentamicin, streptomycin, kanamycin, erythromycin, tetracycline, florfenicol, ciprofloxacin, vancomycin, rifampicin, linezolid, lincomycin, tiamulin, chloramphenicol, amoxicillin + clavulanate) were tested using a standard broth microdilution method (Clinical and Laboratory Standards Institutes [CLSI] Supplement M100). *Staphylococcus aureus* ATCC 29213 was used as the quality control strain.

2.4. Genome Sequencing and Bioinformatics Analysis

Bacterial genomes of 19 *Bacillus* isolates were extracted using a bacterial genome DNA extraction kit (Tiangen Biotech, Beijing, China) and sequenced using the Illumina HiSeq ×10 system (Annoroad, Beijing, China). The draft assemblies of the sequences were obtained with SPAdes 3.0, and antimicrobial resistance genes and virulence genes were screened using Center for Genomic Epidemiology (CGE).

2.5. Hemolysis Test and Detection of Bacterially Produced Inhibitory Compounds

(a). The *Bacillus* strains were cultured on sheep blood agar plate (5%) to identify the hemolytic properties after incubation at 37 °C for 24 h.

(b). A single colony of *Bacillus* was inoculated into 1 mL of BHI broth. Incubation took place over night with shaking (200 rpm) at 37 °C; 5 µL of bacterial suspension was pipetted on the BHA plate, and it was dried in sterile air and incubated at 37 °C for 24 h. The upper MHA agar layer was seeded with *E. coli* ATCC 25922/*S. aureus* ATCC 29213, and it was incubated at 37 °C for 24 h after solidification. The production of antimicrobial agents was indicated by the inhibition zone.

3. Results

3.1. Sample Collection and Bacterial Composition

A total of 55 *Bacillus* isolates were obtained from 41 samples (38.7%), including 29 *B. cereus* strains, 9 *B. licheniformis* strains, 7 *B. subtilis* strains, 5 *B. pumilus* strains, 2 *B. amyloliquefaciens* strains, 1 *B. taeanensis*, 1 *B. velezensis* and 1 *Paenibacillus cookii* strain (Figure 1, Figure 2). Moreover, 18 isolates were recovered from dairy products, and 11 isolates were from local specialty foods (Table 1). We found that the dominant species was *B. cereus* in dairy products, rice products and Beijing specialty foods, while the proportion varied slightly from different sources. The major bacteria in fermented foods and probiotics were *B. pumilus* and *B. licheniformis*, respectively. Only 1 *B. subtilis* strain was isolated from a ham. No *B. cereus* strain was found in probiotic products (Figure 3). Detailed information about the food source of each strain is listed in Table S1 (Supplementary Material). Taken together, it is clarified that *Bacillus* spp. was a probable contaminant in market foods, and the main threat could be ascribed to *B. cereus*.

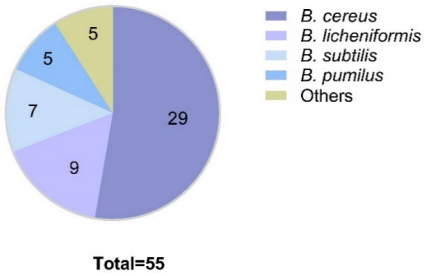

Figure 1. Proportion of different species of *Bacillus* isolates. *B. cereus*, *B. licheniformis*, *B. subtilis* and *B. pumilus* constitute 52.7%, 16.4%, 12.7% and 9.1% of the total number, respectively. Other species include *B. amyloliquefaciens* (3.6%), *B. taeanensis* (1.8%), *B. velezensis* (1.8%) and *Paenibacillus cookie* (1.8%).

Table 1. Number of samples and bacterial isolates of different sources.

Sample Source	No. of Samples	No. of Isolates
Beijing specialty food	31	11
Dairy products	29	18
Rice products	15	8
Probiotics	11	9
Fermented food	9	8
Raw or cooked meat	7	1
Soybean milk	2	0
Snacks	2	0
Total	106	55

Dairy products include pasteurized milk, yogurt and cheese; rice products include foods made from flour, rice, noodles and cakes; fermented foods include pickles, kimchi and preserved beancurd.

3.2. Antimicrobial Susceptibility of Bacillus Isolates

We subjected all the 55 isolates to antimicrobial susceptibility tests. Generally, *Bacillus* isolates showed complete resistance to florfenicol and lincomycin. The resistance rates towards ampicillin, amoxicillin + clavulanate and ceftriaxone were 67.3%, 54.5% and 70.9%, respectively (Figure 4). A large proportion of isolates exhibited resistance to streptomycin and tiamulin with levels of 56.4% and 78.2%. None of the isolates showed tolerance to either rifampin or vancomycin. It is noteworthy that all 29 *B. cereus* strains were resistant to ampicillin and ceftriaxone, while other *Bacillus* spp. were mostly sensitive to these two antibiotics (Table 2). *B. pumilus* isolates were susceptible to 12 antibiotics except for ceftriaxone, florfenicol, lincomycin and tiamulin. Compared with other *Bacillus* species, *B. cereus* showed more severe resistance to antibacterial agents. In terms of different food sources, *Bacillus* isolated from dairy products and rice products are highly resistant to penicillin–ampicillin and amoxicillin + clavulanate, for which isolates from vegetables,

meat and probiotics displayed more sensitivity. *Bacillus* from probiotics expressed very low resistance to ampicillin, streptomycin, kanamycin, erythromycin, florfenicol, linezolid and lincomycin. Collectively, the presence of resistant strains revealed that antimicrobial resistance may have widely disseminated through *Bacillus* spp. in the food chain.

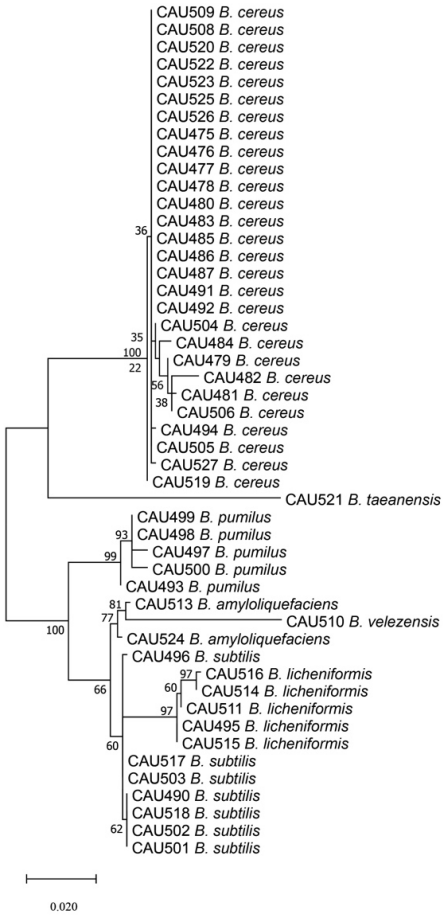

Figure 2. Phylogenetic tree of *Bacillus* isolates based on 16S rRNA sequences. The tree was constructed using maximum likelihood method, and genetic distances were generated using Kimura 2-parameter model. The numbers at the branches are bootstrap confidence percentages from 1000 bootstrapped trees.

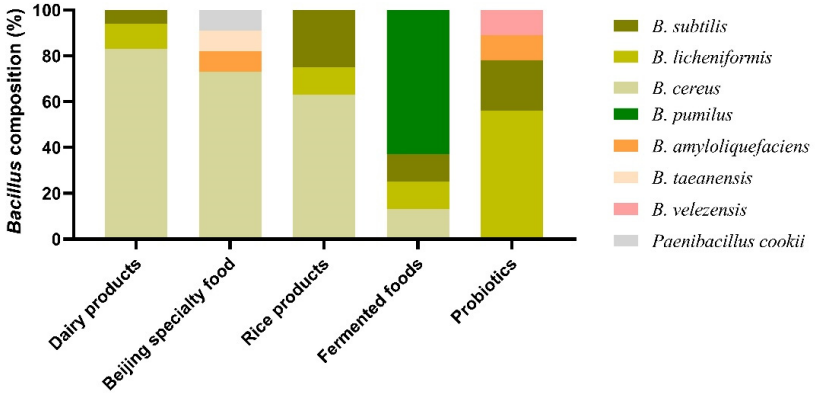

Figure 3. Compositions of *Bacillus* species in the samples from different sources. *B. cereus* is the dominant species in the isolates recovered from dairy products, Beijing specialty foods and rice products. *B. pumilus* and *B. licheniformis* are the most prevalent groups of fermented foods and probiotics-derived *Bacillus* strains, respectively.

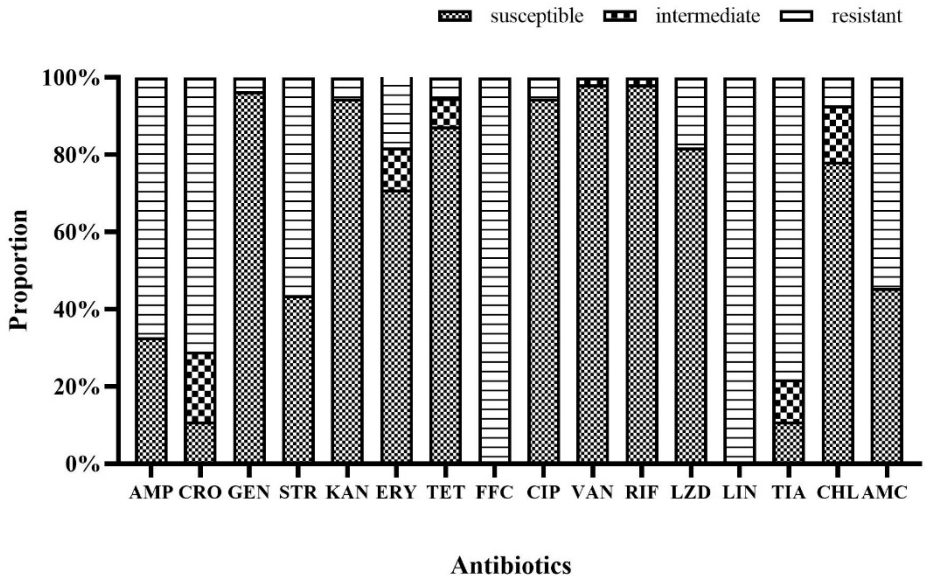

Figure 4. The proportions of susceptible, intermediate and resistant strains among 55 isolated *Bacillus* strains to 16 antibiotics. AMC: ampicillin, CRO: ceftriaxone, GEN: gentamicin, STR: streptomycin, KAN: kanamycin, ERY: erythromycin, TET: tetracycline, FFC: florfenicol, CIP: ciprofloxacin, VAN: vancomycin, RIF: rifampicin, LZD: linezolid, LIN: lincomycin, TIA: tiamulin, CHL: chloramphenicol, AMC: amoxicillin + clavulanate.

Table 2. Proportion of *Bacillus* strains of different species resistant to antibiotics.

Antibiotics	Fraction of Resistant Isolates			
	B. cereus	***B. licheniformis***	***B. subtilis***	***B. pumilus***
AMP	29/29	3/9	1/7	0
CRO	29/29	3/9	1/7	5/5
GEN	2/29	0	0	0
STR	15/29	7/9	7/7	0
KAN	1/29	1/9	0	0
ERY	2/29	7/9	0	0
TET	2/29	0	0	0
FFC	29/29	9/9	7/7	5/5
CIP	2/29	0	0	0
VAN	0	0	0	0
RIP	0	0	0	0
LZD	6/29	1/9	3/7	0
LIN	29/29	9/9	7/7	5/5
TIA	23/29	9/9	4/7	4/5
CHL	1/29	2/9	0	0
AMC	29/29	0	0	0

3.3. The Antimicrobial Resistance and Virulence Genes of Bacillus Isolates

According to the results of MIC tests, 19 *Bacillus* isolates were subjected to next-generation sequencing, and the whole genomic sequences were screened for ARGs and virulence genes. It is interpreted that 57.9%, 26.3% and 21.5% of *Bacillus* strains carried fosfomycin resistance gene *fosB*, tetracycline resistance gene *tet* and erythromycin resistance gene *erm*, respectively (Table 3). Vancomycin resistance gene *van* was present in 11 (100%) *B. cereus* isolates. In addition, 2 *B. subtilis* strains and 1 *B. pumilus* strain were found to harbor kanamycin resistance gene *aadK*, and 1 *B. pumilus* isolated from fermented vegetable and 1 *B. cereus* from dairy product carried chloramphenicol resistance gene *cat*. There was a poor consistency between the phenotypical and genetic antimicrobial resistance traits of tested strains. Interestingly, *B. cereus* was the only species carrying virulence genes, with

the rates of 100%, 9.1%, 45.5% and 100% for *nhe*, *hbl*, *cytK* and *entFM*, respectively. Other virulence factor related genes include *PLC*, *hlyIII*, *clo*, *InhA2*, and so on. Therefore, *Bacillus* spp. derived from foods are likely to develop resistance to drugs and produce toxins that cause relevant disease.

Table 3. The antibiotic resistance genes and virulence genes of *Bacillus* isolates.

Strains	Species	Sources	ARGs	VGs
CAU475	*B. cereus*	Dairy products	*fosB*, *van*	*clo*, *entFM*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU476	*B. cereus*	Dairy products	*fosB*, *tetA*, *van*	*clo*, *cytK*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU479	*B. cereus*	Dairy products	*fosB*, *van*	*clo*, *cytK*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU480	*B. cereus*	Dairy products	*aac*, *cat*, *fosB*, *van*	*clo*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU481	*B. cereus*	Dairy products	*fosB*, *van*	*clo*, *cytK*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU482	*B. cereus*	Dairy products	*fosB*, *van*	*clo*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU484	*B. cereus*	Dairy products	*fosB*, *van*	*cesH*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU486	*B. cereus*	Dairy products	*fosB*, *van*	*cesH*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU504	*B. cereus*	Rice products	*fosB*, *van*	*clo*, *entFM*, *entS*, *hblA*, *hblC*, *hblD*, *hlbB*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU505	*B. cereus*	Rice products	*fosB*, *van*	*clo*, *cytK*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU506	*B. cereus*	Rice products	*fosB*, *tetL*, *van*	*clo*, *cytK*, *entFM*, *entS*, *hlyIII*, *inhA2*, *nheA*, *nheB*, *nheC*, *nprA*, *PLC*, *sph*
CAU495	*B. licheniformis*	Fermented vegetable	*ermD*	–
CAU511	*B. licheniformis*	Probiotics	*ermD*	–
CAU514	*B. licheniformis*	Probiotics	*ermD*	–
CAU516	*B. licheniformis*	Probiotics	*ermD*	–
CAU498	*B. pumilus*	Fermented vegetable	*aadK*, *mphK*, *tetL*	–
CAU500	*B. pumilus*	Fermented vegetable	*cat*	–
CAU501	*B. subtilis*	Ham	*aadK*, *mphK*, *tetL*	–
CAU502	*B. subtilis*	Rice products	*aadK*, *mphK*, *tetL*	–

ARGs = antimicrobial resistance genes; VGs = virulence genes.

3.4. The Hemolytic Ability and Antibacterial Effect of Bacillus Isolates

Cytotoxicity is an important factor involved in pathogenesis of bacterium. We found that 33 out of the 55 (60%) *Bacillus* strains caused hemolysis. Among the hemolytic isolates, 75.8% were *B. cereus*, which was recognized as a dangerous pathogen correlated with food poisoning. All 5 *B. pumilus* strains were hemolytic, while *B. licheniformis* were completely non-hemolytic. Of seven *B. subtilis* strains, two resulted in hemolysis, suggesting that a large number of *Bacillus* spp. have the capability of rupturing red blood cells.

We observed that 3 *B. licheniformis* strains (CAU495, CAU511, CAU514) exerted an inhibitory effect on the growth of *S. aureus* ATCC 29213, and none of the bacteria inhibited *E. coli* ATCC 25922 (Figure 5). *B. licheniformis* CAU495, CAU511 and CAU514 were obtained from fermented vegetables, pet probiotics and livestock probiotics, respectively, with diameters of the zone presented to be 2, 1.1 and 1.9 cm. Thus, foodborne *Bacillus* spp. showed the potential of developing into a probiotic strain.

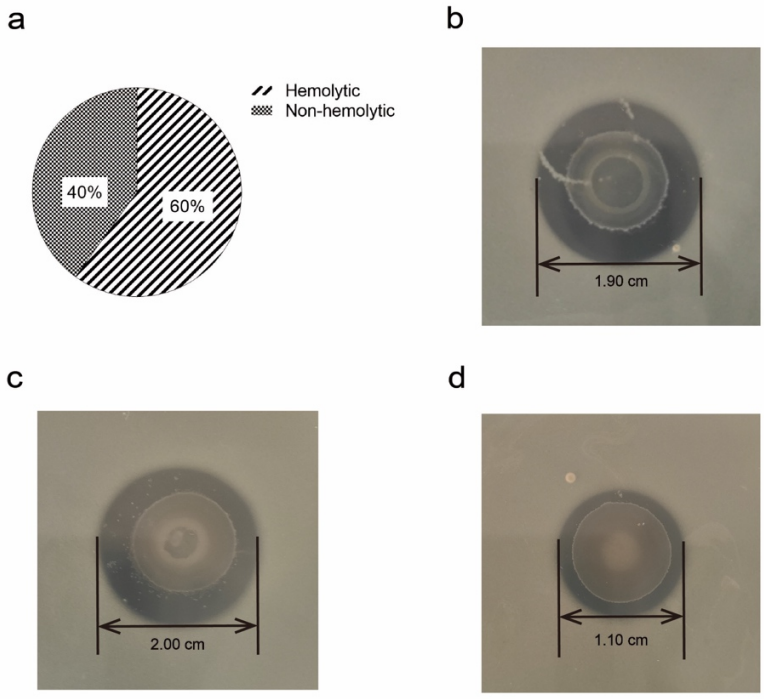

Figure 5. Results of the hemolysis test and agar overlay technique. (**a**) proportion of hemolytic and non-hemolytic *Bacillus* strains; 60% of *Bacillus* strains were hemolytic on sheep blood. (**b**–**d**) are the inhibition zones of *B. licheniformis* CAU514, CAU495 and CAU511, respectively, with *S. aureus* ATCC 29213 added in the soft-agar overlay.

4. Discussion

Our study confirmed the presence of *Bacillus* spp. in several kinds of foods. The highest prevalence was in dairy and rice products, illustrated by the antimicrobial resistance of 55 *Bacillus* isolates mainly towards florfenicol, lincomycin and florfenicol and lincomycin. Meanwhile, the virulence genes were common in *B. cereus*, and 3 out of 55 strains displayed the capability of attacking other organisms. In spite of the rapid advancement in the fields of food science and technology, and a growing concern raised by various international groups on food safety, prevalence of foodborne illness still remains a substantial cause of morbidity and preventable mortality [42]. *Bacillus* is designated as a group of soil inhabitants that also can be isolated from varied sources including vegetables and food. It represents the most heterogeneous group considering their phenotypic and genotypic characters. Some distinct species such as *B. cereus* have also been recognized as opportunistic pathogens or toxin producers in human or animal hosts. In this study, we collected 95 food samples from local markets in Beijing and 11 probiotic products purchased from other areas. A total of 55 *Bacillus* strains were obtained from 41 samples, mostly composed of *B. cereus* (29/55). Among different categories of food products, dairy products were the most important niche, followed by specialty foods, probiotics and rice products. However, Beijing specialty foods are theoretically a sub-type of rice products, which implies that both rice products and dairy products were eligible residences for *Bacillus* spp. In Japan, *B. cereus* was found in 66 out of 101 (65.3%) domestically pasteurized milk samples, which is moderately higher than the rate (51.7%) of our study [43]. In addition, a previous study found that nearly half of the 65 isolated *Bacillus* spp. strains from 34 commercial probiotic products harbored multiple antimicrobial resistance genes, coupled with mobile genetic elements, and were capable of producing hazardous toxins, while no *B. cereus* was recovered from the 11 probiotics we collected, indicating that those samples were free from the contamination of *B. cereus* and its toxins [44].

We found that 55 *Bacillus* isolates were all resistant to florfenicol, lincomycin and had significant resistance to tiamulin, ampicillin, amoxicillin + clavulanate and ceftriaxone.

Since *B. cereus* are able to produce β-lactamase, they are intrinsically resistant to β-lactams. It is also reported that ABC (ATP binding cassette) efflux transporters of *B. subtilis* can generate tolerance to lincosamide, which is consistent with our results [45]. Concerning the resistance genes, we disclosed that *B. cereus*, *B. subtilis* and *B. licheniformis* mainly carried fosfomycin resistance gene *fosB*, tetracycline resistance gene *tet* and erythromycin resistance gene *erm*, separately. *B. cereus* isolates, obtained from vegetables in South Korea, were susceptible to imipenem, vancomycin, gentamicin, erythromycin, ciprofloxacin and chloramphenicol, and unlike the tendency of our results, 40.5% from romaine lettuce were resistant to rifampin and 6% of isolates from garlic chives exhibited resistance to tetracycline [46]. Moreover, 147 *B. cereus* sensu lato strains isolated from German market food showed resistance against the β-lactam antibiotics such as penicillin G and cefotaxim (100%), as well as amoxicillin/clavulanic acid combination and ampicillin (99.3%), while most strains were susceptible to ciprofloxacin (99.3%), chloramphenicol (98.6%), imipenem (93.9%), erythromycin (91.8%), gentamicin (88.4%) and tetracycline (76.2%), which are higher than our results to a mild extent [47]. Our findings suggest that *Bacillus* is a group of potential foodborne pathogens harboring mobile ARGs and might undermine the therapeutic effect of antibiotics.

B. cereus produces a wide array of virulence factors, including pore-forming toxins, cereulide, hemolysins, enterotoxins, proteases and phospholipases [48]. We discovered here that virulence genes were undetectable in *Bacillus* spp., except *B. cereus.* The detection rates of *nhe*, *hbl* and *cytK* were 100%, 9.1% and 45.5%, respectively, and *ces* was 0. Another study focusing on *B. cereus* in Chinese markets, distinguished by the sample volume (860) and sites (39 cities), revealed that 35% of ready-to-eat (RTE) food was contaminated with *B. cereus*, with 39%, 83%, 68% and 7% of the isolated strains harboring the enterotoxin-encoding gene clusters *hblACD*, *nheABC*, *cytK* and emetic toxin-encoding gene *cesB*, respectively. The majority of the isolates were resistant to most β-lactam antibiotics and rifamycin [49], which is largely consistent with our findings. From 2013 to 2015, Kui Zhu et al. isolated 18 *B. cereus group* strains from 15 probiotics and discovered that all strains produced the enterotoxin Nhe, 15 strains additionally produced Hbl, and nearly half of them harbored the antimicrobial resistance gene *tet(45)* [50]. In Egypt, 6.9 and 8.5% of *B. cereus* were recovered from milk powder and Ras-cheese, respectively, and *nhe* gene was detected and dominated in all isolates (100%) from both products [51]. A South Korean survey involving 496 samples of food from environmental and clinical origin, found that 92.3% and 59.5% of *B. cereus* strains carried *nhe* and *hbl*, respectively [52]. Those studies have presented that almost all *B. cereus* harbor the *nhe* gene, including the emetic *B. cereus*, which explains that vomiting symptoms are often accompanied with diarrhea. Regarding the hemolytic activity, most *B. cereus* could destroy blood cells, denoting their virulence towards target cells to some extent. Apart from the biological perils that have been discussed, we found encouraging evidence of 3 *B. licheniformis* strains that showed antimicrobial activities towards Gram-positive bacteria, indicating the potential of being probiotic candidates or antibiotic alternatives. Thus, among isolated *Bacillus* strains, *B. cereus* constitutes a principal part in generating antimicrobial resistance and virulence. Other species of *Bacillus* spp., on the other hand, have different phenotypical features and could even release antibacterial components.

5. Conclusions

Our findings connote that *Bacillus* spp. are distributed in a variety of food products with a potential of secreting virulent substances and disseminating antimicrobial resistance. They are also possible resources for new antibiotic substitutes. Therefore, corresponding guidelines regarding the sterilization, pasteurization and monitoring of toxins in the whole food chain, especially on-shelf foods, should be developed to further promote public health.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9050866/s1, Table S1: Food sources of each *Bacillus* strain.

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