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Biodiversity and identification of spore-forming bacteria in raw milk from Korean dairy farm environment using the culturomics approach

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ABSTRACT

Spores present in milk survive heat treatments and can persist during downstream processing. In Korea, the development of the dairy industry is also growing. However, studies related to the distribution and diversity of spore-forming bacteria for the quality control of raw milk and dairy products are insufficient. This study aimed to evaluate the contamination levels of spore-forming species in raw milk derived from five dairy farm environments in Korea. The isolated strains were also identified using 16S rRNA sequencing and characterized based on the environmental factors in farms, which affect raw milk production. Representative spore-forming bacteria communities in the samples include Bacillaceae, Lachnospiraceae, Paenibacillaceae, and Caryophanaceae at the family level, and *Bacillus*, *Clostridium,* and *Paenibacillus* were the most diverse and predominant at the genus level. A total of 1,102 isolates of 16 genera could be assigned to the genus *Bacillus* (67.3% and 742 isolates), *Clostridium* (12.3% and 135 isolates), *Paenibacillus* (9.3% and 102 isolates), and *Oceanobacillus* (5.9% and 65 isolates). Among 173 species, the prominent members were *Bacillus licheniformis, Bacillus kochii, Bacillus clausii,* and *Clostridium sporogenes*. Twenty-seven spore-forming species (*Bacillus licheniformis*, *Bacillus coagulans*, *Bacillus sonorensis*, *Bacillus sporothermodurans*, *Clostridium sporogenes*, *Clostridium tyrobutyricum*, and *Paenibacillus validus*) detected in raw milk were the same species found in the dairy farm environments. Our result clearly shows that specific provinces have distinct proportions of spore formers that may cause spoilage of raw milk and milk products, such as cheese and yogurt. The biodiversity of spore-forming bacteria in the dairy farms were greatly influenced by several factors such as dairy farm conditions and milking environments. Recommending proper management of hygienic and production practices, including strengthening laws and implementing Hazard Analysis and Critical Control Points (HACCP) principles, would be effective in eradicating contamination during the production of raw milk.

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Keywords: Spore-forming bacteria, Dairy farm, Raw milk, Culturomics, Biodiversity

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INTRODUCTION

In the dairy industry, the presence of spore-forming bacteria is a significant concern in dairy products. Spore-forming bacteria, such as *Bacillus* spp., *Paenibacillus* spp., and *Clostridium* spp., are commonly found in various dairy products and are ubiquitous in nature. They are also present in many raw materials and dry ingredients of processed foods. These organisms produce spores in response to environmental stresses, including nutrient limitation, osmotic pressure, and extreme temperature deviation [1, 2]. These spores are resistant to chemicals, pH changes, heat, and osmotic shock. When conditions become suitable for growth, spores can germinate into vegetative cells [3]. Bacterial contamination of raw milk can arise from various sources, including air, milking equipment, feed, soil, feces, and grass [4]. In addition, spore-forming bacteria can survive pasteurization conditions and grow in pasteurized fluid milk during refrigeration [5, 6], causing fluid milk spoilage and limiting further extension of its shelf life [7, 8]. *Bacillus* and related genera are found in raw and pasteurized milk, as well as in various environmental samples from dairy farms. This ubiquity suggests their involvement in the milk production chain from diverse sources.

Bacillus and closely related genera have been associated with the spoilage of raw and pasteurized milk, as well as other dairy products, including *Paenibacillus, Brevibacillus, Psychrobacillus, Viridibacillus, Anoxybacillus, Geobacillus*, and *Lysinibacillus*. In previous studies, these organisms are mostly caused by thermostable proteolytic and lipolytic enzymes or by recontamination of the sterilized milk during filling [9-11]. However, several *Bacillus* species that form highly heat-resistant spores capable of surviving industrial high-temperature and short-time and ultrahigh-temperature milk processing have been isolated [12, 13]. *Clostridium* and related species, such as *Clostridium butyricum*, *Clostridium sporogenes*, *Clostridium tyrobutyricum*, and *Clostridium beijerinckii*, are defined as Gram-positive, endospore-forming rods, with most species known to be obligate anaerobes with varying

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tolerance to oxygen. These species are associated with spoilage and the development of gas defects such as “late lowing” in cheeses. Incidences of butyric acid spoilage of cheese contributed by the presence of butyric acid bacteria spores in raw milk lead to considerable loss of product value and result in economic loss for the cheese industry [14]. *C. butyricum, C. tyrobutyricum*, and *C. sporogenes*, collectively known as 'butyric acid spores,' are commonly found in poor quality silage that has undergone aerobic deterioration. This deterioration results in insufficient acidification, thereby facilitating the germination and growth of *Clostridium* spores. [15, 16].

Culturomics refers to the strategy of directly culturing bacteria on a large scale to study the diversity and characteristics of microbial communities [17, 18]. With advances in bacterial culture technology and the importance of characterizing individual bacteria, analysis from a culturomics perspective has become crucial [19].

The objective of this study is to investigate the existence and source of the organisms associated within dairy farm (bedding material, manure, drinking water, feed, barn bottom, and soil) and milking parlor environments (rinse water, teat, used tower, dairy bottom, cooling chamber bottom, and tank surface). Therefore, we performed an in-depth study on the occurrence of spore-forming bacteria and their diversity associated within the raw milk production in the dairy farms of Korea.

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MATERIALS AND METHODS

Sample collection

Samples were collected at various sources (pen and milking parlor environments) from five dairy farms producing milk products in Korea. One farm is located in Chungnam (A) and two each in Jeonnam (B, C) and Jeonbuk (D, E). The pen environmental samples were collected in 6 points (bedding material, manure, drinking water, mixed feed, barn bottom, and soil) and the milking parlor environmental samples were collected from 7 points (rinse water, teat, used tower, dairy bottom, cooling chamber bottom, cooling tank surface, and raw milk). The information related to these samples is indicated in Table 1. All solid environmental samples (the bedding material, manure, soil, and mixed feed) were collected in sterilized packs and placed in a 25 g/mL stomacher bag added with 225 mL of 0.1% peptone water, which was homogenized for 2 min with a stomacher lab blender (FR/Bag Mixer; Interscience, St. Nom, France). Surface and bottom samples (barn bottom, teat, dairy bottom, cooling chamber bottom, and cooling tank surface) were collected by swabbing a 10 cm^2 area using Quick swab (3M, St Paul, MN, USA).

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Quantitative microbiological analyses

Ten milliliters of each environmental sample were transferred into a sterile tube and heat-treated at 85°C for 10 min to kill vegetative cells and to select for spore-forming bacteria. Then, the samples were serially diluted in 0.1% peptone water and cultured at 37°C for 5 days under aerobic and anaerobic conditions on brain heart infusion agar (BHI agar, Difco, USA). The total number of spore-forming bacteria from the surface and bottom samples was determined in colony-forming unit (CFU)/cm^2, and the other samples (solid and liquid) as CFU/g or CFU/mL.

Isolation of spore-forming bacteria from dairy farm environments

Bacterial colonies present on the BHI agar of all heat-treated samples were visually examined, and 5–10 colonies with different morphologies were isolated and streaked for purity on the BHI agar, and incubated at 37ºC for 48h under aerobic and anaerobic conditions. Purified isolates were frozen at −80°C in 15% glycerol for further processing.

Identification of isolates by 16S rRNA sequencing

The genomic DNA was extracted from the isolates using the Powerfood Microbial DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene was amplified using universal primers, 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′- GGT TAC CTT GTT ACG ACT TC-3′), and the purified PCR products were sequenced using ABI 3730xl Genetic Analyzer (Applied Biosystems, Forster, CA, USA). The sequences were aligned in the Gene bank database using the BLASTN program at the National Centre for Biotechnology information, and the percent homology score was obtained to identify the organism at the genus and species levels.

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RESULTS

Culturomics approach for spore-forming bacteria

Because dairy farms are complex environments with various microbial ecosystems, especially those contaminated with spoilage and spore-forming bacteria, dairy hygiene management has an impact on raw milk at the stage of milk production. The aim was to count heat-resistant spore-forming bacteria under aerobic and anaerobic conditions from dairy environmental factors to evaluate the hygienic qualities. The average heat-resistant spore-forming bacteria counts for various control points (pen environment and milking parlor environment) in the selected five dairy farms are depicted in Table 2. Overall, the counts of soil, bedding material, manure, and feed in the pen environment were higher than those of the barn bottom, drinking water. The counts in the bedding material and manure of environmental samples from the five dairy farms ranged from 5.7×10^4 to 9.1×10^6 CFU/g and 2.8×10^4 to 1.7×10^6 CFU/g under aerobic culture conditions, respectively. Similarly, heat-treated bedding material and manure samples had the highest counts for spore-forming bacteria initially and throughout the refrigerated storage, starting at 4.25–6.64 log CFU/g on day 1 and reaching 5.43–8.27 log CFU/g on day 21 [5]. The impact of the farm environment (silage, feed, animal manures, bedding, soil, etc.) are associated with poor hygienic practices and affect the quality of raw milk [20-22]. In the milking parlor environment, the counts in the rinse water, teat, used tower, and cooling tank surface of farm E were found to be undetectable under aerobic culture conditions. In contrast, the rinse water counts of farm A, B, and C were higher than the detectable levels. The dairy bottom, where the cattle milking takes place, from farms A–D showed higher counts at a range of 1.3×10^2–1.1×10^4 CFU/cm^2, in contrast to farm E, which had <2 CFU/cm^2. The surface of the raw milk cooling tank from four out of five dairy farms showed a range of <2 CFU–9.3×10^1 CFU/cm^2, except for farm E. The heat-treated raw milk

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from farms A–E were detected to have higher counts (8.7×10^1–2.4×10^2 CFU/mL) under aerobic culture conditions. In a previous report, after heating at 80°C for 10 min, the mesophilic and thermophilic bacterial spore counts of raw and pasteurized camel's milk were both 2 log - CFU/mL [23]. Aerobic bacterial spores in dairy cow's milk are within 2.3 log CFU/mL [24], and this concentration is comparable to the concentration reported by [25], which was 2.1 log CFU/mL.

All the heat-treated environmental samples showed that spore formers survived the pasteurization process and grew under aerobic and anaerobic culture conditions. The dairy farm environment is a typical source of contamination for spore-forming bacteria in raw milk. This study provides a more accurate quantitative portrait of the microflora of spore formers and depicts that the composition of the dairy and milking environments varies from one farm to another. However, confirmation on these findings will only come with the analysis of the DNA sequences of the spore formers isolated from raw milk and environmental samples.

Richness of spore-forming bacteria isolates

This part of the study specifies an insight into the biodiversity of spore-forming bacteria from various sources of the dairy farm environment that contaminate raw milk. To succeed in this goal, we employed phenotypic and genotypic tactics involving the sequencing of taxonomically related target genes to identify spore-forming isolates of the *Bacillus* and *Clostridium* groups as precisely as possible. In our study, 1,102 spore-forming bacteria colonies were obtained from the samples collected from the five dairy farms, which are divided into the pen environment (n = 637) and milking parlor environment (n = 465). The selection of colonies was based on differences in the shape and color under aerobic and anaerobic culture conditions. A total of 1,102 isolates could be assigned to family Bacillaceae (842 isolates [76.4%] and 10 genera), Lachnospiraceae (135 isolates [12.3%] and 1 genus), Paenibacillaceae (110 isolates

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[10.0%] and 3 genera), and Caryophanaceae (15 isolates [1.4%] and 2 genera). The most diverse and abundant isolated family was Bacillaceae, with 842 isolates found in all five dairy farms (A, 80 isolates [59.7%]; B, 65 isolates [73.0%]; C, 219 isolates [80.5%]; D, 254 isolates [80.9%]; and E, 224 isolates [76.5%]) (Table 3, Figure 1a).

Of the total 16 genera isolated, *Bacillus, Paenibacillus,* and *Clostridium* were found to be frequent with a greater number of isolates (Table 3, Figure 1b). *Bacillus*, *Paenibacillus, Solibacillus,* and *Clostridium* were the only genera detected in all five dairy farms, whereas *Lysinibacillus*, *Virgibacillus*, and *Oceanobacillus* were prevalent in four dairy farms. Characteristically, the number of *Oceanobacillus* isolates from farms D and E was higher than that of other farms. *Aneurinibacillus* and *Rummeliibacillus* were present only on farm C. *Fictibacillus*, *Gracilibacillus,* and *Pseudogracilibacillus* were found to be only frequent with a lower number of isolates from dairy farms D and E.

The most diverse and abundant isolated genus was *Bacillus* (67.3%) with 742 isolates and 73 species found to be overly represented in all the five dairy environments (farms A, B, C, D, and E). More than 200 isolated genera of *Bacillus* were detected in farm D (226 isolates) and C (212 isolates), >150 from farm E (178 isolates), and <70 from farm A (64 isolates) and B (62 isolates). Aerobic spore-forming bacteria associated with the dairy environment predominantly belong to the genus *Bacillus*, and affects dairy contamination produced using raw milk [26]. The second most isolated genera (12.3%) were *Clostridium* (135 isolates, 34 species), followed by (9.3%) *Paenibacillus* (102 isolates, 33 species). *Clostridium* is an anaerobic spore-former that is problematic for the dairy industry, and the genus constitutes most of the groups. In this study, *Clostridium* was detected from all environmental factors of the five dairy farms, except from the milking parlor environment of farm B (Table 3, Figure 1b). A previous study reported that *Clostridium* spp. were first detected in milk and dairy products during the early 20th century [27]. *Paenibacillus* spp. are another group of aerobic

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bacilli associated primarily with the spoilage of milk and milk products [5, 28]. Previously, this genus has been found to comprise over 95% of the bacterial population present in milk after prolonged refrigeration and is strongly linked to the spoilage of milk stored for more than 10 days [28, 29].

Diversity of spore-forming bacteria species

The abundance of spore-forming bacteria in the dairy environment and raw milk analyzed at the species level resulted in the identification of 173 species. Of the identified species, *Solibacillus*, *Brevibacillus*, *Rummeliibacillus*, *Pseudogracilibacillus*, *Ornithinibacillus*, *Psychrobacillus*, *Terribacillus,* and *Aneurinibacillus* were isolated to only one species. Among 73 species and 742 isolates of *Bacillus* identified in this study, *B. licheniformis* (32.3%) was especially more abundant in four dairy farms, except for farm B (Figure 2a, Table S1). Specifically, when analyzed on the basis of environmental factors, farms C and D had the most abundant and evenly distributed contaminants for both raw milk production from the pen and milking parlor environments (Table 4, Table S5). Likewise, several studies reported that *B. licheniformis* were thermotolerant spore-forming organisms and along with *B. amyloliquefaciens* and *B. pumilus* may play a role in food poisoning associated with dairy food processing and dairy foods [30-32]. The second, third, and fourth abundant species, *B. kochii* (5.8%), *B. clausii* (5.5%), and *B. cereus* (4.3%), respectively, were isolated from four dairy farms (except farm B) (Table 4, Table S5). Previously, *B. kochii* has been reported to be detected in two farms from raw milk collected from four dairy farms in New Zealand during the summer and winter seasons [33], and the investigation was performed by comparing the aerobic spore-forming flora in milk from organic and conventional dairy farms, isolating *B. clausii,* a mesophilic spore-forming bacteria, from both of those conditions [4].

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Next to the most abundant *Bacillus* species, among 34 species and 135 isolates of *Clostridium* identified in this study, *C. sporogenes* (25.9%), *C. tyrobutyricum* (8.1%), and *C. amygdalinum* (8.1%) dominated in the dairy farms (Figure 2b, Table S2). *C. sporogenes* was detected on four dairy farms (except farm B), but was particularly more abundant in farm D (12 out of 36 *Clostridium* genus isolates), which was detected in the pen (manure, feed, and barn bottom) and milking parlor environments (teat and dairy bottom), and even in raw milk. *C. tyrobutyricum* and *C. amygdalinum* were only isolated from farms A and D, and farm A, respectively, but *C. tyrobutyricum* was even identified in raw milk from farm D alone (Table 4, Table S5). *C. sporogenes* is an anaerobic Gram-positive straight rod commonly found as a spoilage organism in canned foods and dairy products [14, 34]. *C. sporogenes, C. tyrobutyricum,* and *C. butyricum* are the most important anaerobic bacteria involved in the spoiling of cheese.

Our study showed that spore-forming contaminants belonging to the family Paenibacillaceae were the third-largest flora. Among them, *Paenibacillus* spp. are the most common aerobic psychrotrophic thermophilic species associated primarily with the spoilage of milk (stored in an excess of 10 days) and milk products [5, 28, 29]. This genus has previously been found to comprise over 95% of the bacterial population present in milk after prolonged refrigeration [28, 29]. Correspondingly, in our study, we identified 33 species and 102 isolates of *Paenibacillus*, with *Paenibacillus timonensis* (16.7%) and *Paenibacillus jilunlii* (7.8%) as the most abundant species dominating the dairy farms (Figure 2c, Table S3). In particular, *P. timonensis* was isolated from four dairy farms (except farm D); however, 14 out of 17 isolates as contaminants from the milking parlor environment (only rinse water) in farm E (Table S4 and S5).

The majority of the species of *Bacillus*, *Clostridium*, and *Paenibacillus* encountered in various factors of the dairy environment are also present wherever cows are raised or milked,

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and wherever raw milk is collected and stored. With respect to the other minor spore-former species (Figure 2d, Table S4), few species are isolated only once in a single or two farms. Species from *Aneurinibacillus* (1 species), *Brevibacillus* (4 species), *Fictibacillus* (2 species), *Gracilibacillus* (2 species), *Ornithinibacillus* (1 species), *Pseudogracilibacillus* (1 species), *Psychrobacillus* (1 species), *Psychrobacillus* (1 species), *Rummeliibacillus* (1 species), and *Terribacillus* (1 species) were the representative species. Six species of *Oceanobacillus* were found to be more dominant in farm E (32 out of 65 isolates) (Table S4). Eight species of *Lysinibacillus* was absent in farms A–E (except farm B) (Table S4).

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DISCUSSION

The identification of a comprehensive dairy farm environment and raw milk collection (n = 1,102) of spoilage-associated spore-forming isolates revealed a very large taxonomic diversity covering as many as 173 species from 16 different genera with conditions of different stages of raw milk production. These results corroborate well with the findings of previous studies on dairy production environments. Ninety-five percent of the total isolates were assigned either to *Bacillus*, *Clostridium*, *Paenibacillus*, and *Oceanobacillus*, which is in line with previous reports listing these spore-forming genera as the prominent ones in the dairy sector.

For the spoilage-associated isolates, in the case of dairy farms A and B, the level of contamination with spore-forming bacteria isolated from dairy environments where cows are raised and milked did not lead to raw milk production in stages unlike other dairy farms (C, D, and E (Table 4). *B. licheniformis* were the most common spore-forming bacteria isolated from the farm environment and raw milk collected in farms C and D (Table 4). Previous studies [35-37] have highlighted the prevalence of *B. licheniformis* among *Bacillus* species in raw milk and throughout the dairy processing chain. Although not recognized as a significant human pathogen, this species has the potential to spoil milk and dairy products, thereby affecting the organoleptic and functional characteristics [38]. Additionally, *B. licheniformis*, being psychrotolerant spore-forming bacteria, can thrive at refrigeration temperatures, posing a threat to the quality of dairy products [39]; however, some of these isolates were able to grow at a higher temperature of 55°C [40, 41]. This study shows the distribution of the lesser-known *B. clausii* isolated from various dairy farm environments (manure, mixed feed, barn bottom, soil, dairy bottom, and cooling chamber bottom) and raw milk in dairy farm E (Table S5). Detection of *B. clausii* in raw milk provides extra support for feed as an important source of

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contamination [4], even though this species is isolated from feed concentrate samples, but not from raw milk [42]. In the present study, *B. coagulans* is a spore-forming Gram-positive *Bacillus* and was only detected in the milking parlor environment (rinse water and dairy bottom) and raw milk in farm C (Table 4, Table S1, and Table S5). Interestingly, this bacterium was known to exhibit lactic acid-producing and spore-forming capabilities similar to of *Lactobacillus* species, employing spore formation as a survival strategy within the host's intestines and functioning as probiotics [43].

In this study, *Clostridium* species are abundant in mixed feed, barn bottom, soil, teat, and dairy bottom, showing that these are common sources of raw milk contamination. Spores from the pen environment can be transferred via feces and soil contamination of the udder, eventually contaminating milk during milking. In particular, *C. tyrobutyricum*, *C. butyricum*, and *C. beijerinckii* isolated from various dairy farm environments and raw milk samples in this study have been found to be associated with butyric acid fermentation and have potential to cause late blowing defects in different cheese types, including Gouda, Emmental, and Grana Pardano [44, 45]. *C. tyrobutyricum*, considered as the principle causative agent of late blowing in cheeses, was detected in the raw milk samples investigated in the present study (dairy farm D). A total of two isolates of *C. tyrobutyricum* from farm D were identified, one from the barn bottom and the other from raw milk (Table 4, Table S2, and Table S5). However, *C. butyricum* and *C. beijerinckii*, associated with butyric acid fermentation and also late blowing in cheeses, were not detected in any of the raw milk samples in all five farms, but were isolated from the different dairy farm environments. A total of 33 species of *Paenibacillus* were identified in this study, of which one species (*Paenibacillus lactis*) in farm D and six species (*P. amylolyticus*, *P. barengoltzii*, *P. borealis*, *P. jilunlii*, *P. pasadenensis, P. xylanexedens*) in farm E were all detected from raw milk (Table S5). In addition, *Paenibacillus* can contaminate the entire chain from the dairy farm environment to raw milk production stage, and two isolates of *P. validus*

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with one each detected on the surface of cooling tank and one each in the raw milk of farm C were confirmed (Table 4). In a previous study [46], *C. tyrobutyricum*, *C. beijerinckii*, and *Paenibacillus* spp. were detected with relatively small differences in their incidences in the different sample types (cow feces, silage, and cooling tank milk), with proportions of 67%, 58%, and 60% for *C. tyrobutyricum*, 44%, 59%, and 61% for *C. beijerinckii*, and 69%, 47%, and 36% for *Paenibacillus* spp., respectively. These three species indicate the occurrence of anaerobic conditions, despite the close contact with oxygen [47, 48].

Other spore-forming bacteria species in this study, *Oceanobacillus aidingensis* and *Oceanobacillus polygoni,* were only detected in the entire chain from the dairy farm environment to the raw milk production stage from both farms D and E (Table S4 and Table S5). *Oceanobacillus sojae* was even identified in raw milk on farm C (Table S5). *Virgibacillus proomii* is a facultative anaerobe and mesophilic spore-former, with two isolates detected in this study, one from the bottom of the cooling chamber and the other from raw milk in farm D (Table 4 and Table S5).

To the best of our knowledge, this is the first study that investigated the diversity and sources of the spore-forming bacteria in a milk chain of five dairy farms in the Republic of Korea. Based on the results of the diversity of spore-forming bacteria, the microbial distribution needs serious attention to prevent hampering the quality of raw milk and dairy products by properly managing hygienic and production practices.

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CONCLUSIONS

This study provides new evidence of the presence of spore-forming bacteria, including *Bacillus*, *Clostridium*, *Paenibacillus* species, and others, which pose risks of food poisoning and public health issues. *Bacillus* and *Clostridium* spores are particularly relevant to the dairy industry due to their role in spoilage and as human pathogens. Our findings highlight the need to reduce spore-former levels in dairy products and identify contamination sources in raw milk. Developing hygienic practices and aseptic preservation techniques is crucial for milk handling. Overall, this information will be helpful for dairy farms to develop innovative production processes and comprehensive strategies to eliminate spoilage bacteria and eradicate its contamination in milk processing industries.

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Competing interests

No potential conflict of interest relevant to this article was reported.

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Not applicable.

Availability of data and material

Upon reasonable request, the datasets of this study are available from the corresponding author

Authors’ contributions

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ACCEPTED

Ethics approval and consent to participate

This study does not require IRB/IACUC approval because there are no human and animal participants.

ACCEPTED

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Table 1. Sample list of dairy farm environmental factors

	Samples
Pen environment	Bedding material (P1), Manure (P2), Drinking water (P3), Mixed feed (P4), Barn bottom (P5), Soil (P6)
Milking parlor environment	Rinse water (M1), Teat (M2), Used tower (M3), Dairy bottom (M4), Cooling chamber bottom (M5), Cooling tank surface (M6), Raw milk (M7)

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Table 2. Enumeration of spore-forming bacteria from various sources in the dairy farm environment and raw milk in Korea

Isolation source		Total microbial counts (CFU/mL or CFU/g or CFU/cm^2)									
		A		B		C		D		E	
		aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
Pen environmental factors	Bedding material (P1)	5.7×10^{4}	1.5×10^{4}	7.2×10^{6}	5.8×10^{5}	9.1×10^{6}	1.5×10^{5}	2.0×10^{6}	7.9×10^{5}	1.3×10^{6}	7.0×10^{5}
	Manure (P2)	2.8×10^{4}	7.7×10^{4}	1.7×10^{6}	7.8×10^{4}	1.1×10^{6}	1.1×10^{4}	3.1×10^{5}	3.0×10^{4}	1.6×10^{5}	1.0×10^{5}
	Drinking water (P3)	2.3×10^{1}	6.7×10^{1}	7.0×10^{3}	3.3×10^{0}	6.5×10^{3}	2.2×10^{1}	5.6×10^{3}	2.6×10^{3}	1.3×10^{1}	ND
	Mixed feed (P4)	2.6×10^{4}	2.4×10^{4}	2.8×10^{5}	6.1×10^{5}	1.1×10^{6}	9.4×10^{4}	1.1×10^{6}	1.4×10^{4}	1.0×10^{5}	1.3×10^{5}
	Barn bottom (P5)	3.0×10^{2}	3.5×10^{2}	6.3×10^{1}	1.1×10^{2}	1.2×10^{3}	1.5×10^{2}	9.5×10^{2}	6.4×10^{1}	6.3×10^{2}	2.4×10^{2}
	Soil (P6)	2.1×10^{6}	1.4×10^{6}	2.1×10^{6}	7.1×10^{4}	2.1×10^{5}	1.7×10^{6}	6.1×10^{6}	1.3×10^{6}	5.7×10^{5}	3.0×10^{3}
Milking parlor environmental factors	Rinse water (M1)	1.9×10^{3}	3.3×10^{2}	3.7×10^{2}	3.3×10^{1}	1.7×10^{2}	6.7×10^{1}	3.3×10^{1}	ND	ND	6.6×10^{1}
	Teat (M2)	4.7×10^{1}	2.7×10^{1}	5.7×10^{0}	6.7×10^{0}	3.4×10^{1}	3.0×10^{1}	1.3×10^{2}	4.1×10^{1}	ND	3.1×10^{1}
	Used tower (M3)	5.0×10^{0}	<1 CFU	3.0×10^{0}	3.3×10^{0}	6.8×10^{2}	1.3×10^{2}	2.3×10^{1}	3.2×10^{0}	ND	<2 CFU
	Dairy bottom (M4)	1.1×10^{4}	3.0×10^{3}	3.0×10^{2}	1.3×10^{2}	5.2×10^{2}	7.1×10^{1}	1.3×10^{2}	1.2×10^{2}	<2 CFU	3.9×10^{1}
	Cooling chamber bottom (M5)	3.3×10^{1}	1.7×10^{1}	8.7×10^{0}	7.7×10^{0}	9.2×10^{2}	4.5×10^{1}	4.7×10^{2}	5.3×10^{1}	6.2×10^{4}	2.0×10^{1}
	Cooling tank surface (M6)	<2 CFU	<2 CFU	9.3×10^{1}	2.8×10^{1}	<2 CFU	3.3×10^{1}	2.0×10^{1}	<2 CFU	ND	1.7×10^{1}
	Raw milk (M7)	1.0×10^{2}	2.0×10^{2}	1.0×10^{2}	2.0×10^{2}	2.0×10^{2}	1.3×10^{2}	8.7×10^{1}	3.7×10^{1}	2.4×10^{2}	3.3×10^{0}

ND, not detectable

Values are the means of three replicates of each sample.

A to E indicate the location of each farm. A; Chungnam, B and C; Jeonnam, D and E; Jeonbuk.

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Table 3. Taxonomic classification of spore-forming bacteria isolated from the dairy farm environmental factors in Korea

Farm	Family	Genus	Pen environmental factors		Milking parlor environmental factors	
			No of species	No of isolates	No of species	No of isolates
A	Bacillaceae	*Bacillus*	13	38	12	26
		Lysinibacillus	3	3	-	-
		Oceanobacillus	3	7	1	5
		Virgibacillus	-		1	1
	Caryophanaceae	*Solibacillus*	1	1	-	-
	Lachnospiraceae	*Clostridium*	7	27	4	10
	Paenibacillaceae	*Brevibacillus*	1	1	-	-
		Paenibacillus	9	12	1	3
		Total (8 genera)	**35**	**89**	**19**	**45**
B	Bacillaceae	*Bacillus*	16	45	8	17
		Psychrobacillus	1	1	-	-
		Terribacillus	1	1	-	-
		Virgibacillus	1	1	-	-
	Caryophanaceae	*Solibacillus*	1	3	-	-
	Lachnospiraceae	*Clostridium*	5	10	-	-
	Paenibacillaceae	*Paenibacillus*	5	10	1	1
		Total (7 genera)	**30**	**71**	**9**	**18**
C	Bacillaceae	*Bacillus*	19	97	18	115
		Lysinibacillus	2	3	-	-
		Oceanobacillus	1	2	2	2
	Caryophanaceae	*Rummeliibacillus*	1	1	1	4
		Solibacillus	1	2	-	-
	Lachnospiraceae	*Clostridium*	6	17	1	3
	Paenibacillaceae	*Aneurinibacillus*	1	1	-	-

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Farm	Family	Genus	Pen environmental factors		Milking parlor environmental factors	
			No of species	No of isolates	No of species	No of isolates
		Brevibacillus	2	3	2	3
		Paenibacillus	6	7	7	12
		Total (9 genera)	**39**	**133**	**31**	**139**
D	Bacillaceae	*Bacillus*	35	146	19	80
		Fictibacillus	1	1	1	1
		Lysinibacillus	3	4	2	3
		Oceanobacillus	3	10	3	7
		Virgibacillus	-	-	1	2
	Caryophanaceae	*Solibacillus*	1	1	-	-
	Lachnospiraceae	*Clostridium*	12	24	8	12
	Paenibacillaceae	*Paenibacillus*	11	20	2	3
		Total (8 genera)	**66**	**206**	**36**	**108**
E	Bacillaceae	*Bacillus*	25	89	18	89
		Gracilibacillus	1	1	1	1
		Lysinibacillus	1	1	1	1
		Oceanobacillus	5	13	4	19
		Ornithinibacillus	1	1	1	1
		Pseudogracilibacillus	-	-	1	3
		Virgibacillus	1	2	2	3
	Caryophanaceae	*Solibacillus*	1	2	1	1
	Lachnospiraceae	*Clostridium*	10	23	7	9
	Paenibacillaceae	*Paenibacillus*	4	6	7	28
		Total (10 genera)	**49**	**138**	**43**	**155**

A to E indicate the location of each farm. A; Chungnam, B and C; Jeonnam, D and E; Jeonbuk.

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Table 4. Diversity of spore-forming bacteria isolated in the entire chain from the dairy environment to the raw milk production stage.

Farm	Species	No of isolates	Isolation source												
			Pen environmental factors						Milking parlor environmental factors						Raw milk
			P1	P2	P3	P4	P5	P6	M1	M2	M3	M4	M5	M6	
C	*Bacillus clausii*	12		1	1		2				3	2	2		1
	Bacillus coagulans	3							1			1			1
	Bacillus licheniformis	115	16	5	5	10	3	1	9	11	8	8	7	2	30
	Bacillus paralicheniformis	2										1			1
	Bacillus sporothermodurans	2							1						1
	Oceanobacillus sojae	3	2												1
	Paenibacillus validus	2												1	1
	Total (7 species)	**139**													
D	*Bacillus aryabhattai*	2		1											1
	Bacillus cereus	9		2		1		5							1
	Bacillus gibsonii	3		2											1
	Bacillus kochii	8			2		2	1		1					2
	Bacillus licheniformis	79	26		18		3	3	5	2		4	2	2	14
	Bacillus sonorensis	18	2		1		1		1			3		1	9
	Bacillus subtilis	11	5				2		1			1			2
	Bacillus tequilensis	10	5	1	1		1								2
	Bacillus thermoamylovorans	3	2												1
	Clostridium punense	2											1		1
	Clostridium sporogenes	12		2		2	4			1		2			1

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	Clostridium tyrobutyricum	2					1								1
	Oceanobacillus aidingensis	7	1	2		2									2
	Oceanobacillus polygoni	5				1			3						1
	Virgibacillus proomii	2											1		1
	Total (15 species)	**173**													
E	*Bacillus aerius*	15				3		2		2			3	1	4
	Bacillus circulans	3										1		1	1
	Bacillus clausii	27		6		2	7	1				3	2		6
	Bacillus kochii	26	9		1			1		7		1	6		1
	Bacillus pumilus	19	2			2						7	4	1	3
	Bacillus rhizosphaerae	6		1			1	2				1			1
	Bacillus safensis	9	1	1		1				3			1	1	1
	Bacillus tequilensis	3											1		2
	Oceanobacillus aidingensis	8			1	1		1							5
	Oceanobacillus caeni	2						1							1
	Oceanobacillus polygoni	16	4					2		3		2	1		4
	Total (11 species)	**134**													

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P1; bedding material, P2; manure, P3; drinking water, P4; mixed feed, P5; barn bottom, P6; soil, M1; rinse water, M2; teat, M3; used tower, M4; dairy bottom, M5; cooling chamber bottom, M6; cooling tank surface
C to E indicate the location of each farm. C; Jeonnam, D and E; Jeonbuk.

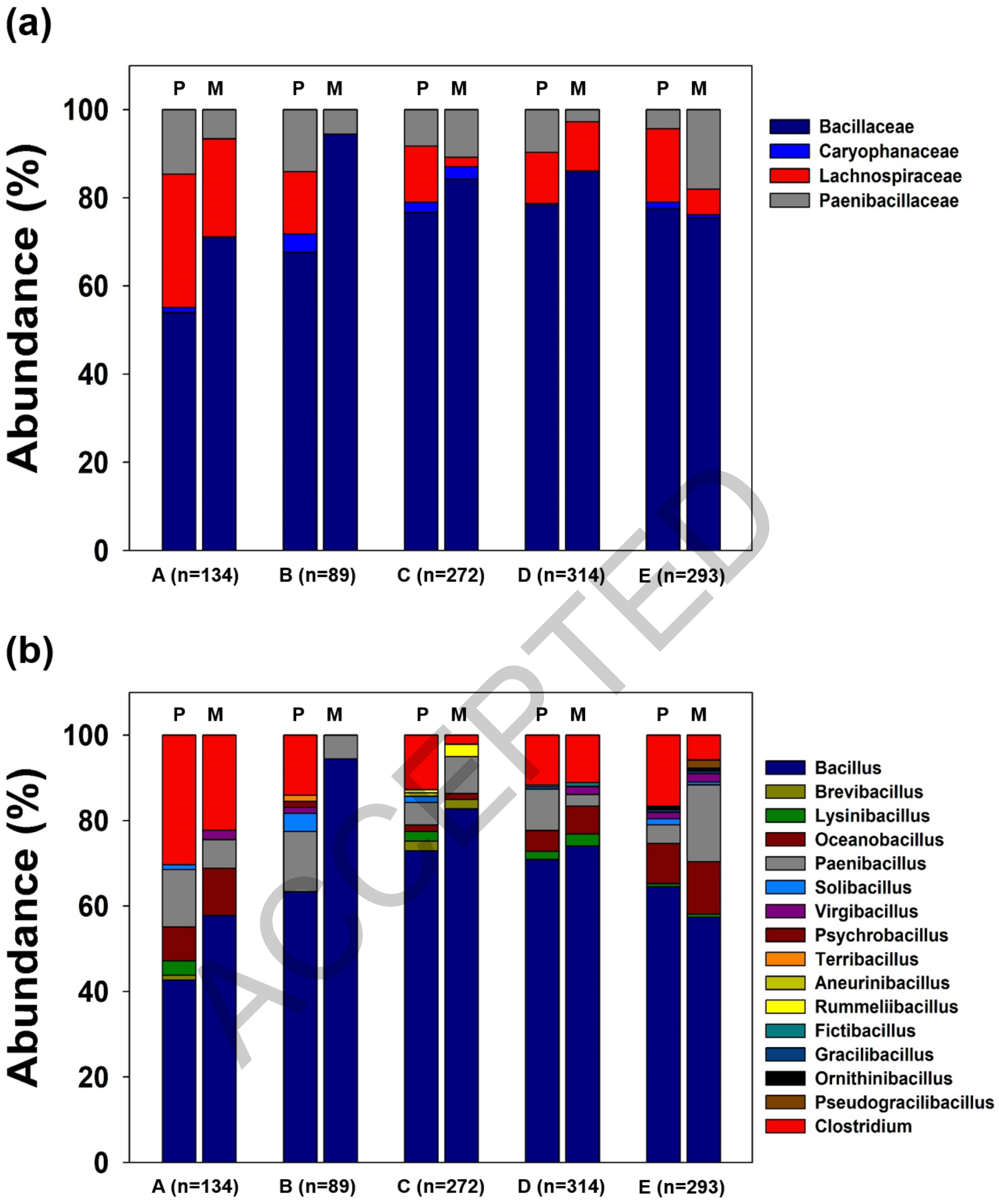

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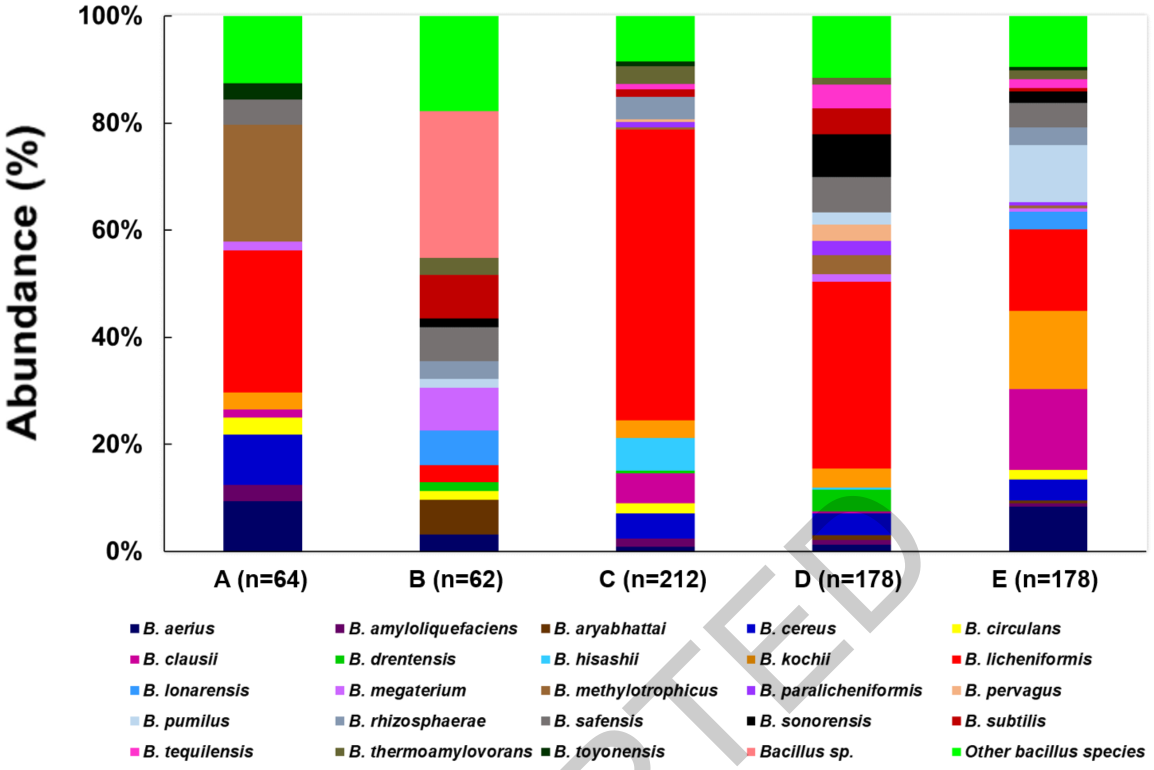
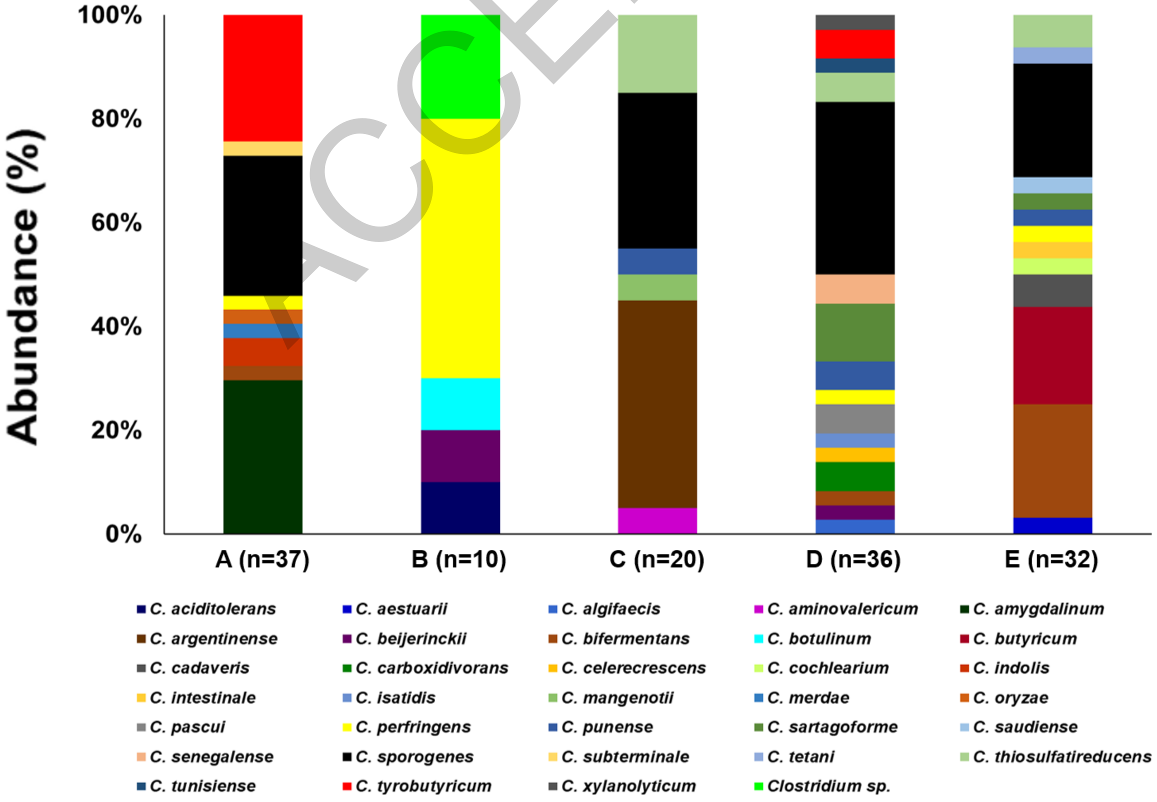
Figure 1. Relative distribution of spore-forming bacteria at (a) family and (b) genera levels across different dairy farm environments and raw milk in Korea. Pen environmental factors (P), Milking parlor environmental factors (M). A to E indicate the location of each farm. A; Chungnam, B and C; Jeonnam, D and E; Jeonbuk.

(a)


Abundance (%)
100%
80%
60%
40%
20%
0%
A (n=64)
B (n=62)
C (n=212)
D (n=178)
E (n=178)
B. aerius
B. amyloliquefaciens
B. aryabhattai
B. cereus
B. circulans
B. clausii
B. drentensis
B. hisashii
B. kochii
B. licheniformis
B. lonarensis
B. megaterium
B. methylotrophicus
B. paralicheniformis
B. pervagus
B. pumilus
B. rhizosphaerae
B. safensis
B. sonorensis
B. subtilis
B. tequilensis
B. thermoamylovorans
B. toyonensis
Bacillus sp.
Other bacillus species

(b)


Abundance (%)
100%
80%
60%
40%
20%
0%
A (n=37)
B (n=10)
C (n=20)
D (n=36)
E (n=32)
C. aciditolerans
C. aestuarii
C. algifaecis
C. aminovalericum
C. amygdalinum
C. argentinense
C. beijerinckii
C. bifermentans
C. botulinum
C. butyricum
C. cadaveris
C. carboxidivorans
C. celerecrescens
C. cochlearium
C. indolis
C. intestinale
C. isatidis
C. mangenotii
C. merdae
C. oryzae
C. pascui
C. perfringens
C. punense
C. sartagoforme
C. saudiense
C. senegalense
C. sporogenes
C. subterminale
C. tetani
C. thiosulfatireducens
C. tunisiense
C. tyrobutyricum
C. xylanolyticum
Clostridium sp.

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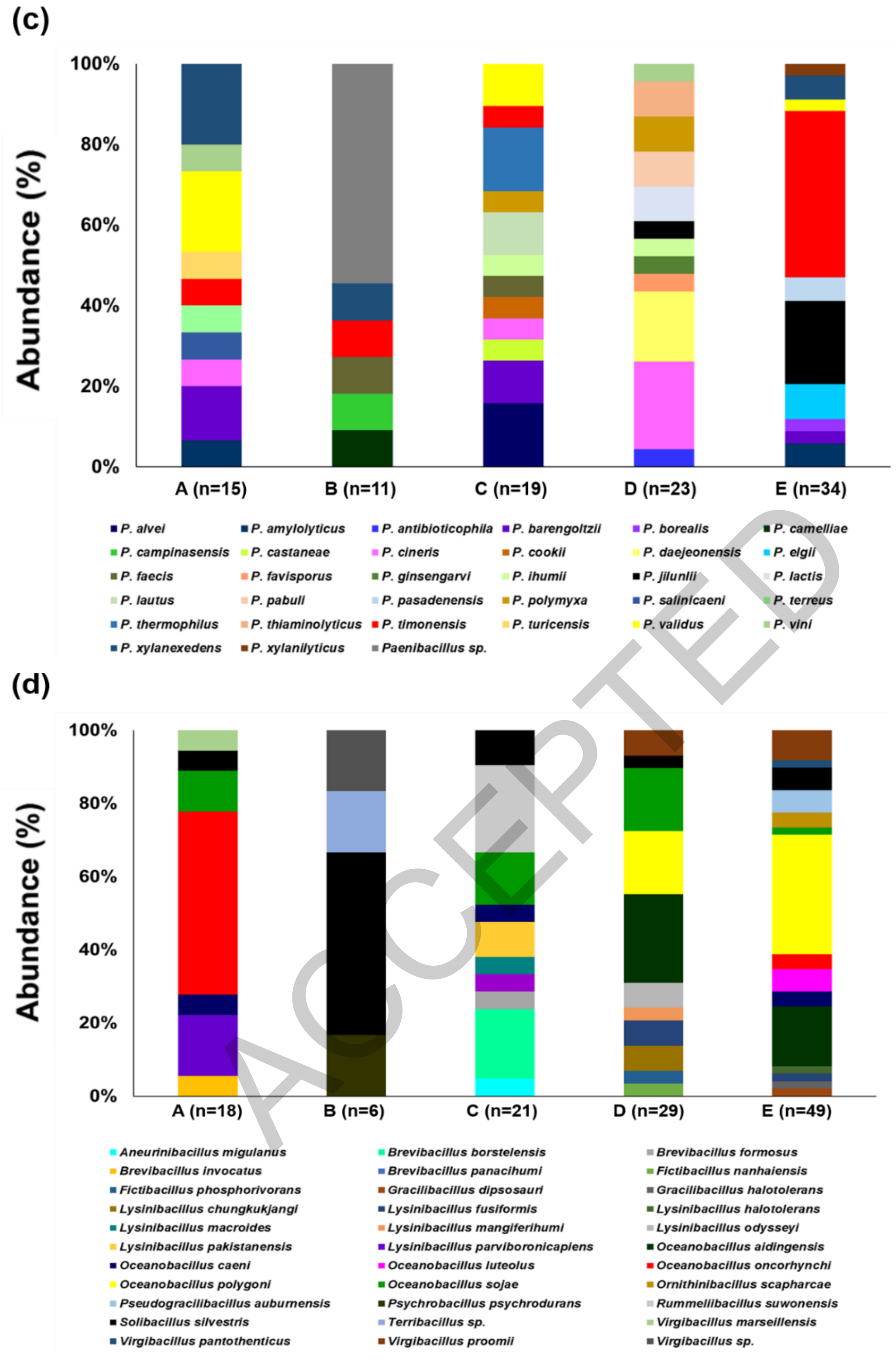

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Figure 2. Relative distribution of spore-forming bacteria at species levels across different dairy farm environments and raw milk in Korea. (a) *Bacillus*, (b) *Clostridium*, (c) *Paenibacillus* species, (d) other spore-forming bacteria species. A to E indicate the location of each farm. A; Chungnam, B and C; Jeonnam, D and E; Jeonbuk.