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ORIGINAL ARTICLE

Genotypic identification of *Bacillus* sp. isolated from canned white asparagus (*Asparagus officinalis*) during the production/processing chain in northern Peru

Carmen Velezmoro · Elena Ramos · Carlos García · Doris Zúñiga

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Abstract *Bacillus* strains isolated from several stages of white asparagus production/processing before/after thermal treatment were characterized molecularly by BOX–PCR amplification and 16S ribosomal gene sequencing. Analysis of these sequences showed that the isolates belong to a variety of phylogenetic groups that are closely related to species of the genus *Bacillus*, such as *B. subtilis* subsp. *spizizenii* NRRL B-23049^T, *B. tequilensis* NRRL B-41771 T, *B. safensis* FO-036b^T, *B. licheniformis* ATCC 14580^T, *B. aryabhattai* B8W22^T, and *B. anthracis* ATCC 14578^T. Some *Bacillus* sp. strains were related to *B. endophyticus*, several isolates were associated with *B. tequilensis* and *B. subtilis* subsp. *inaquosorum*, and others were included in the same cluster of species as *B. subtilis* subsp. *subtilis*, *B. vallismortis*, *B. amyloliquefaciens* subsp. *plantarum*, and *B. methylotrophicus*. Strains closely related to *Paenibacillus peoriae* DSM 8320^T were also found.

Keywords *Bacillus* sp. · *Paenibacillus* sp. · Molecular characterization · *Asparagus officinalis*

Introduction

Cultivated asparagus (*Asparagus officinalis*) is a popular vegetable widely consumed worldwide. Peru has emerged has the leading exporter of asparagus, with a share of around 20% of the global market. Most of the major asparagus farms and associated processing industries are located in the northwest of the country, but asparagus is grown in almost all of the coastal regions (from Piura to Arequipa), totaling around 28 thousand hectares. In 2010, asparagus exports were worth US$426.4 million (CENTRUM Católica 2011). Because of the commercial value of Peruvian canned asparagus (US$105.5 million), most of which is exported to the USA and Europe, the industry focuses on the microbiological and texture aspects, such as quality parameters.

Food quality, particularly that of vegetables and fruits, can be adversely affected by microbiological spoilage. Such spoilage, although generally not harmful to human health, is undesirable commercially because it limits the shelf life or leads to quality complaints. More serious is the presence or growth of infectious or toxigenic microorganisms (foodborne pathogens), which represent the worst forms of quality deterioration due to the potential health risk to the consumer (ICMSF 1996). Contamination of industrial food processing plants and products with aerobe endospore-forming bacteria is a well-recognized and widespread problem (De Clerck et al. 2004). The persistence of these bacteria in the final product, due to their notorious longevity, wide nutritional versatility, and wide pH and temperature ranges for the growth and formation of endospores, which are much more resistant to heat, some chemicals, irradiation, and desiccation than vegetative forms, makes this group of bacteria an ever-present problem in various food processing industries (Brown 2000). Within the group of spore-forming bacteria, *Bacillus* sp. is commonly found on vegetal cultures. Some species of this genus are beneficial for plant growth, but others can cause human diseases or affect the quality of the product itself.

Despite the technological advances that have been made in recent years, food safety problems continue to exist, and thermal treatment remains the most applied technique to obtain pasteurization or sterilization of food products. However, thermal treatment may have a detrimental effect on the aroma,

C. Velezmoro (✉) · E. Ramos · C. García · D. Zúñiga
Laboratorio de Ecología Microbiana y Biotecnología
Marino Tabusso, Departamento de Ciencias,
Universidad Nacional Agraria La Molina,
Lima 12, Peru
e-mail: cevs@lamolina.edu.pe

flavor, and nutritive properties of the final product, as well as causing vitamin loss and protein denaturation (Winter 2003). Bearing in mind that canned asparagus is not subjected to very long heat treatments due to the degradation of texture, it is important that canned asparagus processors take into account the fact that bacteria can persist during the processing of food. Therefore, an optimal thermal treatment process needs to be designed which can destroy the microorganisms while preserving the fresh appearance and sensorial characteristics of the asparagus.

Molecular characterization based on repetitive element (rep)–PCR fingerprint (Versalovic et al. 1991; Martin et al. 1994; Versalovic et al. 1994) has proved to be a reliable technique for genomic analysis that enables identification of intraspecific diversity in bacteria. Repetitive regions present a higher variability than other genomic regions and can be used to analyze the genetic relationship between strains (Van Belkum et al. 1998; Kim et al. 2001). Lopez and Alippi (2007) used rep-PCR genomic fingerprinting for characterizing populations of *Bacillus* recovery from foods during the industrial process and to evaluate diversity. Cherif et al. (2003) evaluated the genetic relationship in the *Bacillus cereus* group also using this method. 16S rRNA gene sequence analysis is the most commonly used method for identifying bacteria or for constructing bacterial phylogenetic relationships (Woese, 1987; Vandamme et al. 1996; Joung and Cote 2002). The aim of the study reported here was to identify the diversity of *Bacillus* sp. bacteria present at different pre-thermal steps of the white canned asparagus process industrial chain through BOX-A1R-based repetitive extragenic palindromic–PCR (BOX–PCR) fingerprinting and 16S rRNA gene sequencing in order to achieve timely adjustments in the thermal process parameters and ensure a successful quality of the final product..

Materials and methods

Sampling procedure

Samples were taken in a two-phase study from one farm located in the northwest of Peru that produces asparagus for processing into canned white asparagus. The farmer and manufacturer have naturally adapted their growing conditions to satisfy the demanding protocols of Good Agricultural Practices and have the US-GAP, GLOBALGAP, and HACCP certifications.

In phase I, cultured soil and white asparagus samples were selectively taken from diverse field plots in high traffic areas and other areas where contamination can occur. Sampling was performed on three occasions during a period of 6 months, with the crops in different growth stages (vegetative state, hoeing, and harvesting). Each composite soil sample comprised 15–20 cores collected in a grid pattern, as previously described by the NRCS-USDA (Natural Resources Conservation Service–U.S. Department of Agriculture 2002). The soil samples were kept aseptically in Ziploc bags, transported briefly in insulated coolers, and maintained at 4°C until ready for transport to the university laboratories for analysis, arriving at the lab after no more than 6 h after collection. Asparagus samples at harvest time were also collected aseptically in Ziploc bags (up to approx. 800 g) and subsequently treated in the same way as the soil samples.

In phase II, asparagus products were sampled three times at the processing plant, during a period of 6 months. Samples were taken along the process industrial chain upon arrival at the receiving dock, after washing with water–air sparging, after hydro-cooling, after cutting and peeling, and 1 and 3 h after filling and sealing the cans (pre-thermal processing). The samples were collected in the same way as the asparagus in phase I, cooled at 4°C, and transported to the lab within 6 h after sampling.

Isolation of spore-forming strains

The first step in the isolation of spore-forming bacteria strains was to eliminate vegetative cells by heat treatment of the samples. Serial decimal dilutions were made and inoculated onto plates tryptone glucose extract agar (TGE) plates. The plates were then incubated at 35° and 55°C under aerobic and anaerobic conditions for isolation of mesophilic and thermophilic microorganisms (APHA 1998). Colonies of strains were chosen that represented all of the colony types that could be distinguished visually, and these were purified by reculturing on the same medium. Isolates were identified as *Bacillus* sp. or *Paenibacillus* sp. by morphological analysis. These strains were Gram-positive, catalase-positive, spore-forming microorganisms. Pure cultures were stored in Casoy broth supplemented with glycerol (2:1, v/v) at −80°C.

Molecular characterization

DNA extraction

Total genomic DNA of strains of spore-forming bacteria were extracted from overnight liquid culture in TGE broth grown at 35°C using the AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Scientific, Union City, CA). DNA quality was verified and quantified by electrophoresis in 1% agarose gels by comparison to a 1- kb lambda DNA ladder (Fermentas, Burlington, ON, Canada) after staining with 0.5 μg/ml ethidium bromide.

BOX–PCR amplification

PCR reactions and amplification were performed by using primer BOX A1R (5′-CTACGGCAAGG CGACGCTGACG-3′) as described by Versalovic et al. (1991). DNA was amplified in 25-µl volumes of master mix containing 1–8 µl DNA, 1.25 mM dNTPs, 7.5 mM $MgCl_2$, 0.80 pmol/ml primer BOX A1R, 0.08 U/µl Taq DNA polymerase (Fermentas), 10% DMSO, and 1× buffer KCl. The cycling conditions were: 95°C for 3 min, 25 cycles of 93°C for 45 s (denaturation), 53°C for 1 min (annealing), and 65°C for 8 min (extension), followed by a final extension at 65°C for 16 min. Genomic fingerprints were generated and DNA fragments separated in a 1.5% agarose gel, photographed after ethidium bromide staining, and the bands visually recorded. GeneRuler 1-kb DNA Ladder Plus (Fermentas) was used as the molecular marker.

Different BOX–PCR patterns were assigned to strains having at least one different band and representative strains were chosen for 16S rRNA analysis.

16S rRNA gene amplification and sequencing and phylogenetic relationship

The 16S rRNA gene was amplified by PCR using fD1 (5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCT CAG-3′) and rD1 (5′-CCCGGGATCCAAGCT TAAGGAGGTGATCCAGCC-3′) primers as described by Versalovic et al. (1991). DNA was amplified in 25-µl volumes of PCR reaction mixtures containing 5 µl DNA, 0.5 mM DNTPs, 1.5 mM $MgCl_2$, 0.5 pmol/ml of each primer, 0.5 U/µL Taq DNA polymerase (Fermentas), and 1×buffer KCl. DNA was amplified under the following cycling conditions: an initial denaturation at 93°C for 2 min, 30 cycles of 93°C for 45 s (denaturation), 62°C for 45 s (annealing), and 72°C for 2 min (extension), with a final extension at 72°C for 5 min. The PCR product was cleaned using the AxyPrep PCR Cleanup Kit (Axygen Scientific) according to the manufacturer's instructions and subsequently commercially sequenced by Macro Gen Inc. (Seoul, Korea).

The sequences of type and reference strains of related species registered in the National Center for Biotechnology Information data bank (http://www.ncbi.nlm.nih.gov) were identified by BLASTN searches (Altschul et al., 1990) and EzTaxon (Chun et al., 2007). Phylogenetic analysis was performed by the neighbor joining (NJ) method (Saitou and Nei, 1987), and the bootstrap test was calculated for 1,000 subsets with genetic distances computed with the Kimura's two-parameter model using Mega4 software (Tamura et al. 2007).

Biochemical characterization

The API 50 CHB kit (bioMérieux, Marcy l'Étoile, France) was used to identify the representatives of each BOX–PCR profile group. Strains were subcultured onto TGE agar plates and incubated for 18–24 h at 30°C. The API systems were inoculated in accordance with the manufacturer's instructions and incubated at 30°C for 24 and 48 h. Each identification system was read following the user guide and the identity of the microorganism provided for the API web database system. The results were compared with identification by 16S rRNA sequencing.

Results and discussion

The aim of preserving asparagus is to slow down/reduce microbial growth during the shelf life of the canned product and reduce the health risk from eating it. The objective of canning is to destroy harmful microbes present in food; however, with improper handling, lack of quality control in the canning process, or a temperature that is too low to destroy the microbes, contamination and/or spoilage may result. Some of the *Bacillus* sp. can be facultative anaerobic bacteria that develop during the shelf life of the canned food product. The diversity of the bacteria found in the asparagus production chain is shown below.

Isolation of spore-forming strains

One hundred and thirty-five strains were isolated during phase I of this study, of which 110 were isolated from the soil samples and 25 from the shoots collected during the harvest. Seventy strains were isolated from samples of asparagus at different stages of the production chain (phase II).

Molecular characterization

BOX–PCR amplification

The fingerprints of 194 isolates were generated by BOX–PCR amplification (Figs. 1, 2). Since microbial isolates that showed the same BOX–PCR pattern belong to the same species, profiles of each strain were grouped by their similar bands in the fingerprints. According to the molecular results, there were 13 fingerprint patterns from the 135 strains tested in phase I; these were denoted strains A to H, respectively, and are shown in Figs. 1 and 2. In phase II, 59 strains were selected from 70 spore-forming bacteria isolated at the processing plant; there were 11 different fingerprint patterns. The BOX–PCR amplification profiles were the same as those found in phase I.

Identification	Representative isolate	Isolation source
B. subtilis subsp. *spizizenii* [Pattern A1]	2M2CE	10
	2M3BE19	8
	2M3BE14	1
	M3BE36	1
	2M1BE4	9
	2M1BE3	9
	M3BE32	1
	2M2BE1	3
	2M2BE4	5
	2M2BE10	8
	2M2BE14	5
	2M2BE16	8
	2M2BE22	1
	2M2BE25	1
	2M2BE26	1
	2M2BE28	1
	2M2BE30	9
	2M2BE31	3
	2M2BE33	3
	2M2BE40	1
	2M2BE41	8
	2M2BE42	3
	3MBE131	8
	3MBE120	7
	3MBE238	4
B. subtilis subsp. *spizizenii* [Pattern A2]	M3BE11	1
	M3BE30	5
	M2BE53	1
	M2BE59	1
	M3BE10	1
	M3BE31	1
	M2BE50	1
	M1BE4	1
	M1BE11	1
	M1BE15	1
	M1BE16	1
	M1BE20	1
	M2BE17	1
	M1BE22	1
	M1BE30	1
	M1BE31	1
	M1BE32	1
	M2BE8	1
	M1BE33	2
	M2BE10	1
	M2BE11	1
	M2BE26	2
	M2BE27	1
	M2BE30	1
	M2BE32	1
	M2BE34	1
	M2BE36	1
	M2BE37	1
	M2BE40	1
B. subtilis subsp. *spizizenii* [Pattern A3]	M3BE18	2
	M3BE19	2
	M3BE23	2
	M3BE4	2
	3MBE105	6
B. tequilensis [Pattern A4]	M3BE27	1
	M3BE1	1
	M2BE52	1
	M2BE60	1
	M2BE61	1
	M2BE33	1
	M2BE35	1
	M2BE47	1
	M3BE22	1
	M3BE26	1
	M3BE35	1
	M3BE38	1
	M3BE25	1

Identification	Representative isolate	Isolation source
B. tequilensis [Pattern A4]	2M2BE19	8
	2M2BE20	5
	2M2BE21	1
	2M2BE34	3
	2M2BE36	1
	2M2BE37	1
	2M2BE39	4
Bacillus sp. associated with *B. tequilensis* and *B. subtilis* subsp. *inaquosorum* [Pattern A5]	M1BE8	1
	M1BE18	1
	M2BE21	1
	M1BE24	2
	M1BE25	1
	M1BE27	1
	M2BE4	1
	M2BE5	2
	M2BE28	1
	M3BE33	1
	M3BE34	1
	2M2BE29	9
	2M2BE38	1
	3MBE122	7
	3MBE124	7
	3MBE111	4
Bacillus sp. associated to *B. subtilis* subsp. *subtilis*, *B. vallismortis, B. amyloliquefaciens* subsp. *plantarum* and *B. methylotrophicus* [Pattern B]	M2BE41	2
	M2BE49	1
	M2BE48	1
	M1BE21	2
	M1BE28	2
	M2BE15	1
	M1BE29	2
	2M2BE35	9
B. safensis [Pattern C]	2M3BE12	9
	2M1BE2	9
	M3BE12	1
	M3BE6	1
	M3BE7	1
	M3BE8	2
	M3BE13	1
	M3BE28	1
	M3BE3	1
	M3BE40	1
	M3BE41	1
	M1BE9	2
	M1BE12	1
	M1BE2	1
	M2BE16	2
	M1BE23	1
	M1BE5	1
	M1BE7	1
	M1BE14	1
	M2BE25	2
	M3BE29	2
	2M2BE2	3
	2M2BE3	1
	2M2BE5	9
	2M2BE6	4
	2M2BE9	3
	2M2BE12	3
	2M2BE13	8
	2M2BE17	8
	2M2BE43	3
	2M2BE44	9
	M3BE39	1
	3MBE119	7
	3MBE112	4
	3MBE217	6
	3MBE219	6
	3MBE213	5
	3MBE203	4
	3MBE204	4
	3MBE176	3

◀ **Fig. 1** BOX-A1R–based repetitive extragenic palindromic–PCR (BOX–PCR) patterns (*A–C*) of representative microbial strains isolated from the asparagus production/processing chain, their phylogenetic relationship based on 16S rRNA sequencing, and isolation sources (*1* harvest soil, *2* fresh asparagus at harvest, *3* upon arrival at receiving dock, *4* after washing with water–air sparging, *5* after hydro-cooling, *6* without peeling and cutting, *7* after cutting and peeling, *8*, *9* canned asparagus waiting for thermal processing with a delay of 1 h (*8*) or 3 h (*9*), *10* spoiled can of a failed sterilization)

16S rRNA gene amplification and sequencing and phylogenetic relationship

Strains from every fingerprint pattern group were selected for further 16S rRNA gene sequencing. Nearly complete sequences of 16S rRNA genes were obtained and compared with those held in Genbank, which enabled determination of the relationship amongst species after construction of a phylogenetic tree (Fig. 3).

The relatedness of the isolates identified in phase I to seven species of the family *Bacillaceae* and one species of family *Paenibacillaceae*, all members of class Bacilli and phylum *Firmicutes*, was established. The spore-forming bacteria of genus *Bacillus* found in this study cause important problems in the food processing industry due to the resistance of their endospores, which may be associated with psychrophilic or acidophilic properties of the vegetative cells (Andersson et al., 1995). Additionally, some strains showed BOX–PCR fingerprint patterns (A5 and B) with high a similarity to more than one species, suggesting that these strains should be sequenced for other housekeeping genes to determine their identity. The use of protein-encoding genes as phylogenetic markers is now a common approach for constructing bacterial phylogenetic relationships (Yamamoto and Harayama 1998; Ko et al. 2004; Chelo et al. 2007).

A wide diversity of spore-forming microorganisms were present in the asparagus rhizospheric soil based on the identification of 110 bacterial strains isolated from samples collected during the harvest. Based on our results, the strains belonged to seven *Bacillus* and one *Paenibacillus* species (Table 1), with 30.91% being closely related to *B. subtilis* subsp. *spizizenii* NRRL B-23049^T (patterns A1–A3) with >99.93% similarity. *B. tequilensis* NRRL B-41771^T (pattern A4) and *B. safensis* FO-036b^T (pattern C) were identified with 100% similarity in 14.55% of isolates

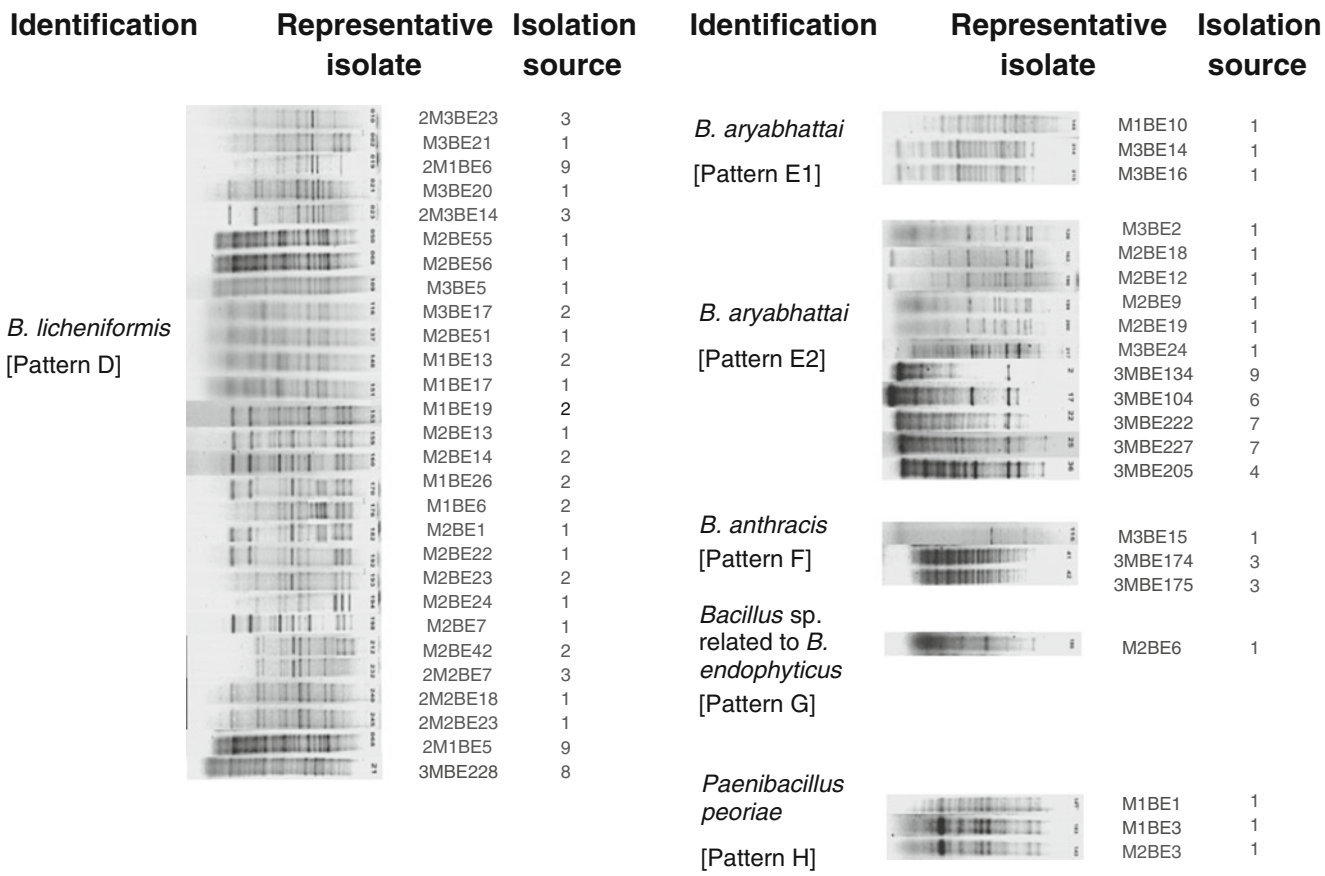
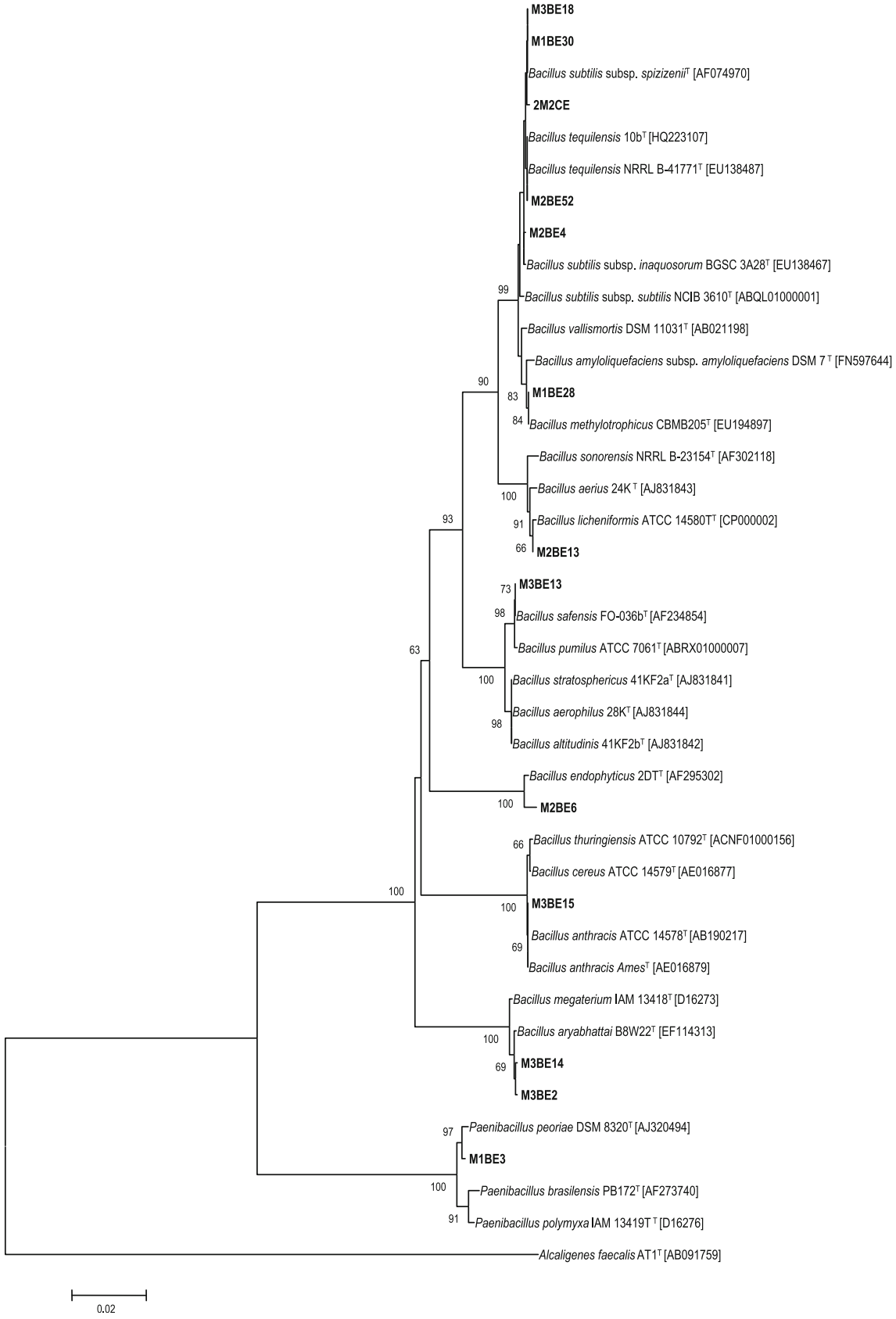


Fig. 2 BOX-PCR patterns (*D–H*) of representative microbial strains isolated from the asparagus production/processing chain, their phylogenetic relationship based on 16S rRNA sequencing, and isolation sources (*1* harvest soil, *2* fresh asparagus at harvest, *3* upon arrival at receiving dock, *4* after washing with water–air sparging, *5* after hydro-cooling, *6* without peeling and cutting, *7* after cutting and peeling, *8*, *9* canned asparagus waiting for thermal processing with a delay of 1 h (*8*) or 3 h (*9*)


M3BE18
M1BE30
Bacillus subtilis subsp. spizizenii[T] [AF074970]
2M2CE
Bacillus tequilensis 10b[T] [HQ223107]
Bacillus tequilensis NRRL B-41771[T] [EU138487]
M2BE52
M2BE4
Bacillus subtilis subsp. inaquosorum BGSC 3A28[T] [EU138467]
Bacillus subtilis subsp. subtilis NCIB 3610[T] [ABQL01000001]
Bacillus vallismortis DSM 11031[T] [AB021198]
Bacillus amyloliquefaciens subsp. amyloliquefaciens DSM 7[T] [FN597644]
M1BE28
Bacillus methylotrophicus CBMB205[T] [EU194897]
Bacillus sonorensis NRRL B-23154[T] [AF302118]
Bacillus aerius 24K[T] [AJ831843]
Bacillus licheniformis ATCC 14580T[T] [CP000002]
M2BE13
M3BE13
Bacillus safensis FO-036b[T] [AF234854]
Bacillus pumilus ATCC 7061[T] [ABRX01000007]
Bacillus stratosphericus 41KF2a[T] [AJ831841]
Bacillus aerophilus 28K[T] [AJ831844]
Bacillus altitudinis 41KF2b[T] [AJ831842]
Bacillus endophyticus 2DT[T] [AF295302]
M2BE6
Bacillus thuringiensis ATCC 10792[T] [ACNF01000156]
Bacillus cereus ATCC 14579[T] [AE016877]
M3BE15
Bacillus anthracis ATCC 14578[T] [AB190217]
Bacillus anthracis Ames[T] [AE016879]
Bacillus megaterium IAM 13418[T] [D16273]
Bacillus aryabhattai B8W22[T] [EF114313]
M3BE14
M3BE2
Paenibacillus peoriae DSM 8320[T] [AJ320494]
M1BE3
Paenibacillus brasilensis PB172[T] [AF273740]
Paenibacillus polymyxa IAM 13419T[T] [D16276]
Alcaligenes faecalis AT1[T] [AB091759]
99
90
83
84
100
93
91
66
73
98
63
100
98
100
66
100
100
69
100
69
97
100
91
0.02

◀ **Fig. 3** Neighbor-joining tree showing the taxonomic location of a representative from each BOX–PCR group based on 16S rRNA sequences. Strains (*in bold*) were isolated from the white asparagus production/processing chain grown in northern Peru. Reference strains are also indicated. The significance of each branch is indicated by a bootstrap value calculated for 1,000 subsets. *Scale bar*: 2% of sequence divergence [2 nucleotides (nt) substitution per 100 nt]

each; meanwhile, 12.73% of strains had 99.93% similarity with respect to *B. licheniformis* CP000002^T (pattern D). Then, 11.8% of isolates were associated with *Bacillus sp.* included in the same cluster of species as *B. tequilensis* and *B. subtilis* subsp. *inaquosorum* (pattern A5) (Fig. 3). Strains closely related to *B. aryabhattai* B8W22^T (patterns E1–E2((>99.87% similarity), *B. anthracis* ATCC 14578^T (pattern F) (100% similarity), and strains included in the same cluster of species *B. subtilis* subsp. *subtilis*, *B. vallismortis*, *B. amyloliquefaciens* subsp. *plantarum* and *B. methylotrophicus* (pattern B) as well as *Bacillus* sp. strains related with 99.44% similarity to *B. endophyticus* 2DT^T (pattern G) were present in fewer than 8.18% of the tested isolates. The remaining 2.73% of strains were closely related to *P. peoriae* DSM 8320^T (pattern H) with 99.72 % similarity. Based on their results from rhizosphere studies, Garbeva et al. (2008) reported the isolation of 11 and seven different *Bacillus* species from maize and barley, respectively, based on the development of a 16S rRNA clone library. On the other hand, *Paenibacillus peoriae* (previously *Bacillus peoriae*) was first isolated from soil (Montefusco et al. 1993) and has been associated with the production of antimicrobial substances, a capability for molecular nitrogen fixation, and the production of chitinases and proteases. However, other *Paenibacillus* species have been related to contamination of food products, including pasteurized pureed vegetables (Berge et al. 2002). The genetic diversity of this family has also been studied by rep-PCR (Loncaric et al. 2009) and found to be a reliable tool for establishing phylogenetic relationships.

Fresh asparagus at harvest, which were initially contaminated through the soil in which they grew, had the same *Bacillus* species than the soil samples. Of the 25 isolates, 32% were closely related to *B. licheniformis* ATCC 14580^T with 99.93% similarity; 24% to *B. subtilis* subsp. *spizizenii* NRRL B-23049^T with >99.93% similarity; 20% to *B. safensis* FO-036b^T with 100% similarity. Moreover, 16% of strains were associated as *Bacillus* sp. included in the same cluster of species as *B. subtilis* subsp. *subtilis*, *B. vallismortis*, *B. amyloliquefaciens* subsp. *plantarum*, and *B. methylotrophicus* (pattern B). Additionally, 8% were associated with *Bacillus* sp. included in

Table 1 Molecular characteristics and phylogenetic classification of the bacteria strains from the asparagus processing/production chain isolated in this study

16S rRNA gene sequenced strains	BOX–PCR pattern	Closest related type strains based on 16S rRNA gene	Accession numbers of the closest type strains	Similarity (%)
2M2CE	A1	*Bacillus subtilis* subsp. *spizizenii* NRRL B-23049^T	AF074970	99.93
M1BE30, M1BE15, M3BE11, M2BE30	A2	*B. subtilis* subsp. *spizizenii* NRRL B-23049^T	AF074970	100.00
M3BE18	A3	*B. subtilis* subsp. *spizizenii* NRRL B-23049^T	AF074970	100.00
M2BE52, M2BE35, M2BE47, M2BE61, M3BE27	A4	*B. tequilensis* NRRL B-41771^T	EU138487	100.00
M2BE4, M1BE8, M2BE28	A5	*B. tequilensis* 10b^T	HQ223107	99.93
		B. subtilis subsp. *inaquosorum* BGSC 3A28^T	EU138467	99.91
M1BE28, M2BE48, M1BE21	B	*B. subtilis* subsp. *subtilis* NCIB 3610^T	ABQL01000001	99.80
		B. vallismortis DSM 11031^T	AB021198	99.73
		B. amyloliquefaciens subsp. *plantarum* FZB42^T	CP000560	99.73
		B. methylotrophicus CBMB205^T	EU194897	99.72
M3BE13, M1BE12, M2BE42, 2M3BE12	C	*B. safensis* FO-036b^T	AF234854	100.00
M2BE13, M3BE5, 2M3BE23	D	*B. licheniformis* ATCC 14580^T	CP000002	99.93
M3BE14, M1BE10	E1	*B. aryabhattai* B8W22^T	EF114313	99.87
M3BE2, M2BE18	E2	*B. aryabhattai* B8W22^T	EF114313	99.93
M3BE15	F	*B. anthracis* ATCC 14578^T	AB190217	100.00
M2BE6	G	*B. endophyticus* 2DT^T	AF295302	99.44
M1BE3, M2BE3	H	*Paenibacillus peoriae* DSM 8320^T	AJ320494	99.72

BOX–PCR, BOX-A1R–based repetitive extragenic palindromic-PCR

the same cluster of *B. tequilensis* and *B. subtilis* subsp. *inaquosorum* (pattern A5).

In phase II, the taxonomic position of spore-forming bacteria isolated from the asparagus production/processing chain was also elucidated by 16S rRNA sequence analysis. Both *B. safensis* FO-036b^T (100% similarity) and *B. subtilis* subsp. *spizizenii* NRRL B-23049^T (>99.93 similarity) were the most frequent closely related type strains associated to isolates from this phase (27.14% each). *Paenibacillus* species and *Bacillus* sp. strains related to *B. endophyticus* 2DT^T with BOX–PCR pattern G, both present in phase I samples, were absent in phase II samples (Fig. 4). In addition, *B. anthracis* only was present in small quantities until upon arrival at the receiving dock in the processing plant.

An acidic (pH 5.3) standard liquid (water, salt, organic acids, and probably additives) was added the canned white asparagus tested in our study, and it is therefore necessary to develop a thermal treatment to destroy surviving microorganisms. Microbial assays and molecular characterization of the isolated strains from the canned asparagus before the thermal process with a delay of 1 and 3 h showed that >80.00% of spore-forming microorganisms found at those critical points in the processing chain (stages 8 and 9 of the production chain in Fig. 4) were closely related to *B. subtilis* subsp. *spizizenii* NRRL B-23049^T with >99.93% similarity (38.10% of isolates), *B. safensis* FO-036b^T with 100% similarity (28.57% of strains), and *B. licheniformis* ATCC 14580^T with 99.93% similarity (14.29%); all of these strains are members of the *B. subtilis* group. The washing, peeling and bleaching procedures and and the low pH of the canned asparagus are sufficiently efficient measures to destroy these microbes (Fig. 4). The all other phylogenetically related species found in soil samples were also present in the canned asparagus but in considerably smaller numbers; the exceptions were strains related to *B.*

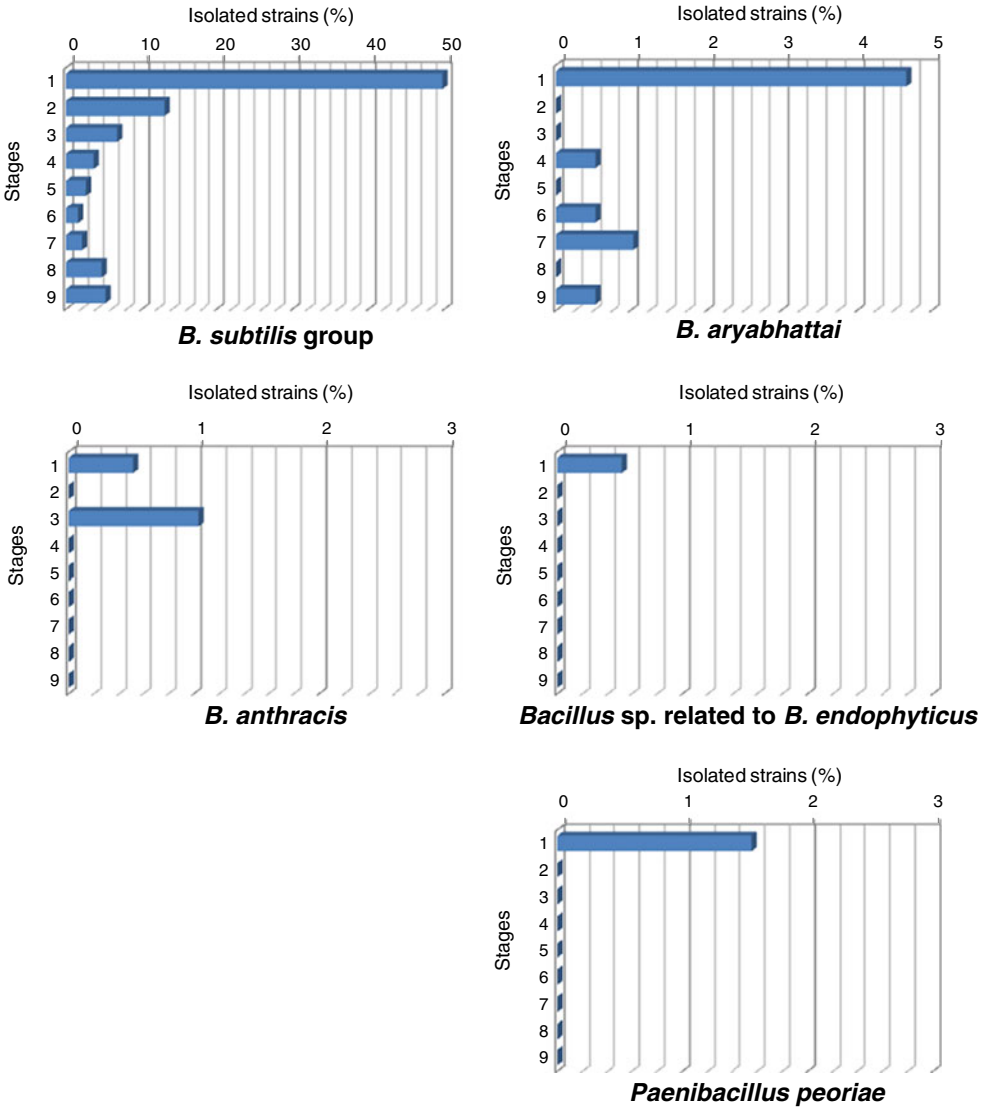


Fig. 4 Diversity of spore-forming bacteria along the production/processing chain of canned white asparagus according to stages of the production: *1* harvest soil, *2* fresh asparagus at harvest, *3* upon arrival at receiving dock, *4* after washing with water–air sparging, *5* after hydro-cooling, *6* without peeling and cutting, *7* after cutting and peeling, *8*, *9* canned asparagus waiting for thermal processing with a delay of 1 h (*8*) or 3 h (*9*). Percentages are given based on the total number of spore-forming bacteria molecularly characterized in this work

anthracis ATCC 14578^T, *Bacillus* sp. related to *B. endophyticus*, and *P. peoriae* DSM 8320^T, which were absent (Fig. 4).

During the course of this study it was possible to obtained a spoiled can of white asparagus due to a failed thermal treatment. This can was tested for microbial growth according to BAM–FDA guidelines (U.S. Federal Drug Administration 2001), and one strain was isolated. A 99.93% similarity between this strain (2M2CE) and the type strain of *B. subtilis* subsp. *spizizenii* NRRL B-23049^T was established (Fig. 3). Previous studies by Kotzekidou (1996) revealed the presence of *B. subtilis* as the most frequent microorganism in spoiled canned foods, often in the presence of *B. megaterium*, *B. brevis*, and *B. pumilus*. These microorganisms are also common in other kind of foods, with *B. subtilis*, *B. pumilus*, and *B. licheniformis*, all members of the *B. subtilis* group, generally constituting the predominant mesophilic spore-forming species in raw milk (Scheldeman et al. 2005).

After *B. cereus*, *B. subtilis*, is the most common microbial species associated with food contamination, often meat, pastries, and rice dishes with meat or seafood. It has been reported that when present in numbers of more than 10^5 cfu/g, this species can cause diseases (Lund 1990; Granum and Baird-Parker, 2000), with patients' symptoms generally consisting of vomiting after a short incubation period (Lund 1990; Jenson and Moir 2003). *B. subtilis* spores are notoriously difficult to denature, and they can survive moist heat (100°C at atmospheric pressure) with a D value (decimal reduction time—the time required to lower viability by a factor of 10) of 20–30 min. Moreover, spores survive approximately 1000-fold longer in dry heat than in moist heat (Fox and Eder 1969; Nicholson et al. 2000). Because of their high thermal resistance, *Bacillus* species have used as indicators in thermal process designs and have served as experimental models for exploring the molecular mechanisms underlying the incredible longevity of spores and their resistance to environmental insults (Nicholson et al. 2000).

Biochemical characterization

Using the biochemical assay API 50 CHB, we explored the phylogenetic relationship of the isolated strains (Table 2). Seventeen strains with diverse BOX–PCR patterns were tested, and although there were no general similarities between the identities based on the molecular assays and those based on biochemical methods, the characterization was understandable. Biochemical analysis confirmed the phylogenetic relationship of strains with BOX–PCR pattern D, and *B. licheniformis* was identified with >99.8% ID. Similarly, both methods revealed the identity of *B. anthracis* for strains with BOX-PCR pattern F.

Molecular techniques such as 16S rRNA sequencing analysis require a large and very up-to-date genetic sequence database of prokaryotic strains, such as GenBank® or EzTaxon, against which the sequence of an unknown strain can be compared. However, the API system

Table 2 *Bacillus* sp. identification by molecular and biochemical methods

Code	Molecular characterization		Biochemical characterization	
	BOX–PCR pattern	Best match	Taxa	% ID
2M1BE6	D	*B. licheniformis*	*B. licheniformis*	99.9
2M2BE4	A1	*B. subtilis* subsp.*spizizenii*	*B. subtilis / amyloliquefaciens*	99.5
3MBE131	A1	*B. subtilis* subsp. *spizizenii*	*B. subtilis / amyloliquefaciens*	97.3
2M2BE41	A1	*B. subtilis* subsp. *spizizenii*	*B. subtilis / amyloliquefaciens*	89.5
2M2BE29	A5	*Bacillus* sp.	*B. licheniformis*	53.2
			B. subtilis / amyloliquefaciens	34.6
3MBE122	A5	*Bacillus* sp.	*B. subtilis / amyloliquefaciens*	88.1
3MBE104	E2	*Bacillus aryabhattai*	*B. megaterium*	99.9
2M2BE5	C	*Bacillus safensis*	*B. pumilus*	99.9
2M2BE6	C	*Bacillus safensis*	*B. pumilus*	99.9
3MBE176	C	*Bacillus safensis*	*B. pumilus*	99.9
3MBE204	C	*Bacillus safensis*	*B. pumilus*	99.9
3MBE213	C	*Bacillus safensis*	*B. pumilus*	99.9
3MBE217	C	*Bacillus safensis*	*B. pumilus*	99.9
3MBE228	D	*B. licheniformis*	*B. licheniformis*	99.8
3MBE222	E2	*Bacillus aryabhattai*	*B. megaterium*	97.9
3MBE134	E2	*Bacillus aryabhattai*	*B. megaterium*	99.9
3MBE174	F	*Bacillus anthracis*	*B. anthracis*	79.6

has a limited database that contains no more than 29 species and strains; *B. subtilis* subsp. *spizizenii* (BOX–PCR pattern A1), *B. safensis* (BOX–PCR pattern C), or *B. aryabhattai* (pattern E2) are not included in its database. Thereby, when each of these strains was tested by API, they were associated to *B. subtilis/amyloliquefaciens*, *B. pumilus*, and *B. megaterium*. Although there was no correspondence between species, the strains were clustered together and belong to the same group. Thus, *B. subtilis* subsp. *spizizenii* and *B. subtilis*/*amyloliquefaciens* are close members of the *B. subtilis* group, *B. safensis* and *B. pumilus* show a high similarity and also belong to the *B. subtilis* group, while *B. aryabhattai* and *B. megaterium* are in the same *B. megaterium* group (Table 2).

Additionally, none of the methods could identify strains with BOX–PCR pattern A5 and other methods may be required.

Although a diversity of spore-forming microorganisms were found in the cultured soil where the asparagus grew, most decreased in numbers during the production process; the remaining microorganisms were members of the *B. subtilis* group (as *B. subtilis* subsp. *spizizenii*, *B. tequilensis*, *B. safensis*, and *B. licheniformis*) and *B. megaterium* group (as *B. aryabhattai)*. Based on these results, we suggest that the resistance of the spores of these microorganisms as well as the resistance of spore-forming pathogenic bacteria should be considered in the design of thermal treatments to guarantee the quality and sterility of the end product.

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