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Sources of *Bacillus cereus* contamination in a pasteurized zucchini purée processing line, differentiated by two PCR-based methods

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Abstract

In previous work, raw materials used for processing pasteurized zucchini purée (zucchini, milk proteins and starch) and cultivation soil of zucchinis were found to be potential sources of stored product contamination with *Bacillus cereus*. 134 *B. cereus* strains originating from these sources and from the stored product were typed using coliphage M13 sequence-based polymerase chain reaction (M13-PCR) and DNA amplification fingerprinting. Combined patterns from the two methods were compared using Dice's coefficient and the unweighted pair group method with average cluster analysis. 16 combined profile groups and six unclustered strains were formed at 75% similarity level. Representative strains of each group were subsequently examined for growth at low temperature (4°C, 7°C and 10°C). Based on strain relatedness, five major groups and two minor groups among the nine groups containing zucchini strains were common to both zucchini and soil origins, indicating that the soil was the main initial source of contamination for zucchinis. Strains from zucchinis and soil were heterogeneous compared with strains from dehydrated ingredients (milk proteins and starch), and were mainly composed of psychrotrophic strains. Convergent results from molecular typing and growth at low temperature showed that psychrotrophic contaminants of the stored product originated from zucchinis, whereas non-psychrotrophic contaminants originated from milk proteins. The number of genetic groups decreased during the purée processing, suggesting selection of strains during zucchini washing operations, heat treatment and storage.
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Keywords: Pasteurized vegetable; Raw material; Molecular typing; M13-PCR; DAF; *Bacillus cereus*

1. Introduction

Bacillus cereus is a spore-forming bacterium found in many raw and processed foods [1–3]. Its endospore can survive mild heat treatment applied during food processing and some strains have the ability to grow at low temperatures during storage of food products. *B. cereus* has been incriminated in many cases of food poisoning [4]. Recently, vegetable purées contaminated with *B. cereus* caused a severe outbreak in France [5]. Hence it is important to monitor the potential sources of this pathogen in food processing plants to minimize product contamination.

Learning about the ecology of *B. cereus* can help identify potential sources of contamination and trace the spread of *B. cereus* in food plants. Traditional typing systems such as biotyping and serotyping provide little information for this purpose. Phage typing is more sensitive, but not all *B. cereus* isolates are typeable [6]. Furthermore, strong environmental pressure may lead to the emergence of similar phenotypes in distant lineages. Random amplified polymorphic DNA (RAPD) analysis and DNA amplification fingerprinting (DAF) are two similar genotypic DNA typing methods [7–9] that have been used successfully in epidemiological and ecological studies, notably for *B. cereus* [10–17]. Their advantages and disadvantages are well known. They are less laborious and time-consuming than other DNA-based techniques and can be employed for rapid identification of a large number of strains. They explore polymorphism at multiple sites of the whole genome. However, they may not be reproducible among different laboratories.

Previous studies [18,19] showed that numerous samples

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of cooked pasteurized and chilled vegetable purées, and particularly zucchini purée stored at 10°C, were contaminated with *B. cereus* (3.6–5.2 log CFU per g). In a recent investigation of the routes for *B. cereus* contamination of zucchini purée in a pasteurized vegetable purée processing plant [20], we showed that cultivation soil of zucchinis, zucchinis, and two ingredients (starch and milk proteins), carried *B. cereus* spores and were the most likely sources of contamination. This survey yielded a large number of *B. cereus* isolates. In the present study, genotyping was performed on these isolates with coliphage M13 sequence-based polymerase chain reaction (M13-PCR) and DAF, and the psychrotrophic property of selected types was studied. This study showed which of the four potential sources of contamination contributed to the final contamination of stored zucchini purée.

2. Materials and methods

2.1. Bacterial strains

196 strains belonging to the *B. cereus* group had been randomly isolated during the survey of a pasteurized purée processing plant in France [20], all derived from samples collected along the zucchini purée processing line and from the soil where the zucchinis were grown (Table 3). Among the strains isolated, some exhibited rhizoidal colonies or produced a yellow pigment on agar media. As strains isolated in the final product did not have these characteristics, they were not selected for the present study. Among the remaining strains, only strains derived from the possible sources of contamination and from the final stored product were examined. This allowed the characterization of 134 strains: 35 strains from soil, 28 from raw zucchinis, 17 from washed zucchinis, 14 from milk proteins, six from starch and 34 from the product stored at 10°C and 20–25°C (Table 1).

2.2. DNA isolation technique

DNA preparation was performed essentially as described previously [21] with minor modifications. The strains were plated onto J-agar plates [22] and grown overnight at 30°C. Cells (~10 μl) were suspended in 1 ml of sterile Milli-Q water, pelleted at 11 000×*g* for 15 min, and resuspended in 500 μl extraction buffer (1.7% sodium dodecyl sulfate, 200 mM Tris–HCl (pH 8), 20 mM EDTA, 200 mM NaCl). The suspension was incubated at 55°C for 1.5 h with 25 μl of proteinase K (10 μg μl^{-1}) and then at 65°C for 0.5 h with 25 μl of RNAase (10 μg μl^{-1}). DNA was extracted with one volume of phenol and subsequently with one volume of chloroform. The aqueous phase was precipitated with 2.5 volumes of cold 100% ethanol and centrifuged at 11 000×*g* for 20 min. The supernatant was discarded and the pellet washed once with 800 μl of cold 70% ethanol. After drying, the pellet was dissolved in 50 μl sterile Milli-Q water and stored at −20°C. DNA was quantified by absorbance at 260 nm on a MBA2000 spectrophotometer (Perkin-Elmer, Courtaboeuf, France).

2.3. Molecular typing

2.3.1. M13-PCR

The method used was adapted from Henderson et al. [16], using the sequence-specific primer PM13: 5′-GAGGGTGGCGGCTCT-3′. PCR mixture (25 μl) contained 100 ng of DNA template, 1.25 mM dNTPs mix (Eurogentec, Seraing, Belgium), 6 mM $MgCl_2$, 4.8 μM primer (Eurogentec), 10% (vol/vol) dimethyl sulfoxide, 1.5 U of Goldstar DNA polymerase (Eurogentec), and Goldstar buffer (Eurogentec). Thermal cycling was carried out in a PCR 9700 thermocycler (Perkin-Elmer) Parameters were 3 min at 94°C followed by 35×[1 min at 94°C, 1 min at 40°C, 8 min at 65°C] and 16 min at 65°C.

2.3.2. DAF

DAF was performed as described by Von Stetten et al. [11], with minor modifications. The applied primer was primer And2: 5′-CCGGCGGCG-3′ [13,14]. Amplification reactions (25 μl) contained 50 ng of DNA template, 200 μM dNTPs mix (Eurogentec), 4 mM $MgCl_2$, 4 μM primer (Eurogentec), 6 U of AmpliTac Stoffel fragment (Perkin-Elmer) and AmpliTac buffer. Parameters used with PCR 9700 thermocycler were 4 min at 95°C followed by 45× [1 s at 96°C, ramping −23.2°C min^{-1}, 1 s at 30°C, ramping+14.1°C min^{-1}].

2.3.3. Electrophoresis and data analysis

PCR products from M13-PCR and DAF (16 μl) were separated on 1.5% and 2% agarose gel, with the molecular mass DNA markers X and VII, respectively (Roche Diagnostic, Meylan, France). Gels were stained with ethidium bromide and digitized using a gel imager (Bioblock, Illkirch, France). Banding patterns were converted into 0–1 binary codes. Similarity coefficients for all pairwise combinations of M13-PCR/DAF profiles were determined by Dice's coefficient [23]. A dendrogram was constructed from the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA) [24]. Band detection, binary matrix, similarity coefficient and dendrogram were performed using the BioGene 99.04 software (Vilber Lourmat, Marne la Vallée, France).

The 134 strains were typed with both methods. 10 strains were tested in duplicate. Similar visual patterns were obtained between duplicates for the two typing methods.

2.4. Growth at low temperatures

A total of 43 strains were tested for growth at low

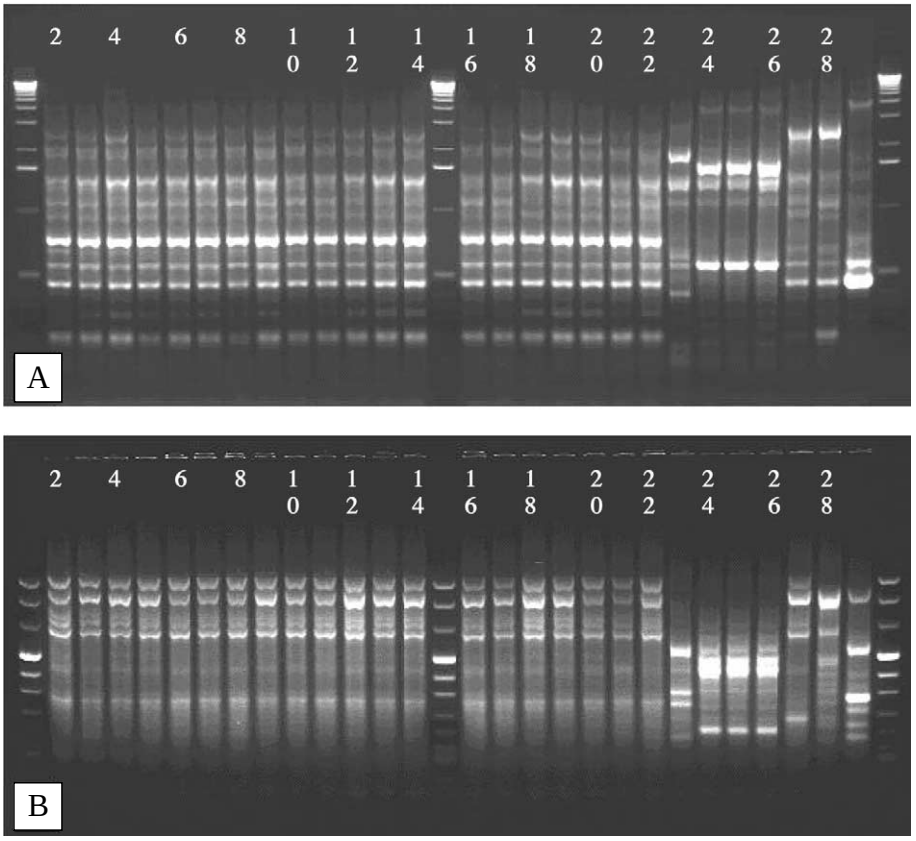

Fig. 1. Example of M13-PCR patterns (A) and DAF patterns (B) generated for *B. cereus* strains. Lanes 2–11: strains 20, 21, 24, 28, 29, 30, 31, 32, 33, 34 from purée stored at 20–25°C. Lanes 12–26: strains PC, PD, PE, PH, PI, PJ, PK, PL, PM, PN, PA, PB, PF, PG from milk proteins. Lane 27: strain A3 from starch. Lane 28: strains 23 and 22 from purées stored at 20–25°C. Lanes 1, 15, 30: DNA molecular mass marker X (Roche).

temperature. Strains were randomly selected in each M13/DAF group, depending on the total number and the heterogeneity of strains in each group. The strains tested in each group are given in Table 2.

The strains were grown in a water bath stabilized at 4 ± 0.2°C, 7 ± 0.1°C, 10 ± 0.1°C and 30°C, in 20-ml tubes containing 5 ml of J-Broth [22] supplemented with 0.1% of agar and hermetically sealed. Incubation at 30°C served to control the physiological state of the strains. *Bacillus weihenstephanensis* WSCB 10204T and *Bacillus alvei* CIP 66.18 were used, respectively, as positive and negative controls at low temperatures. Cultures were examined for visible growth after 3, 7, 14 and 21 days at low temperatures for low temperature tests and after 2 and 5 days for controls at 30°C.

3. Results

3.1. Patterns

Fig. 1 shows an example of fingerprints for *B. cereus* strains generated using M13-PCR and DAF. Fingerprints were obtained for all strains. The number of DNA bands detected varied from five to 12 bands ranging between 100 and 4000 bp for M13-PCR patterns and from four to nine bands ranging between 150 and 1200 bp for DAF patterns. The total number of distinct DNA-amplified fragments corresponding to the different sizes taken into account for conversion into binary matrices was 59 and 42 fragments for M13-PCR and DAF, respectively. Data from the two typing systems were combined, giving more discriminative and informative results. The similarity coefficient (Dice's coefficient) among duplicates was $\geq 80\%$ for the combined M13-PCR and DAF method, representing the resolution achieved by automated band detection.

3.2. Genetic relationship between strains

To determine the relatedness between strains, a dendrogram based on combined M13-PCR and DAF fingerprint data was constructed using Dice's similarity coefficients and the UPGMA clustering methods (Fig. 2). A broad genotypic variety was observed among *B. cereus* strains. Taking into account our typing system, the threshold of similarity used as a working definition of a clone should be the 80% level, as stated above. According to the criteria of Tenevor at al. [25], we established three categories of genotypic relatedness. The first category consisted of strains showing very similar band patterns (number and size of bands) by visual comparison on electrophoresis gels; after

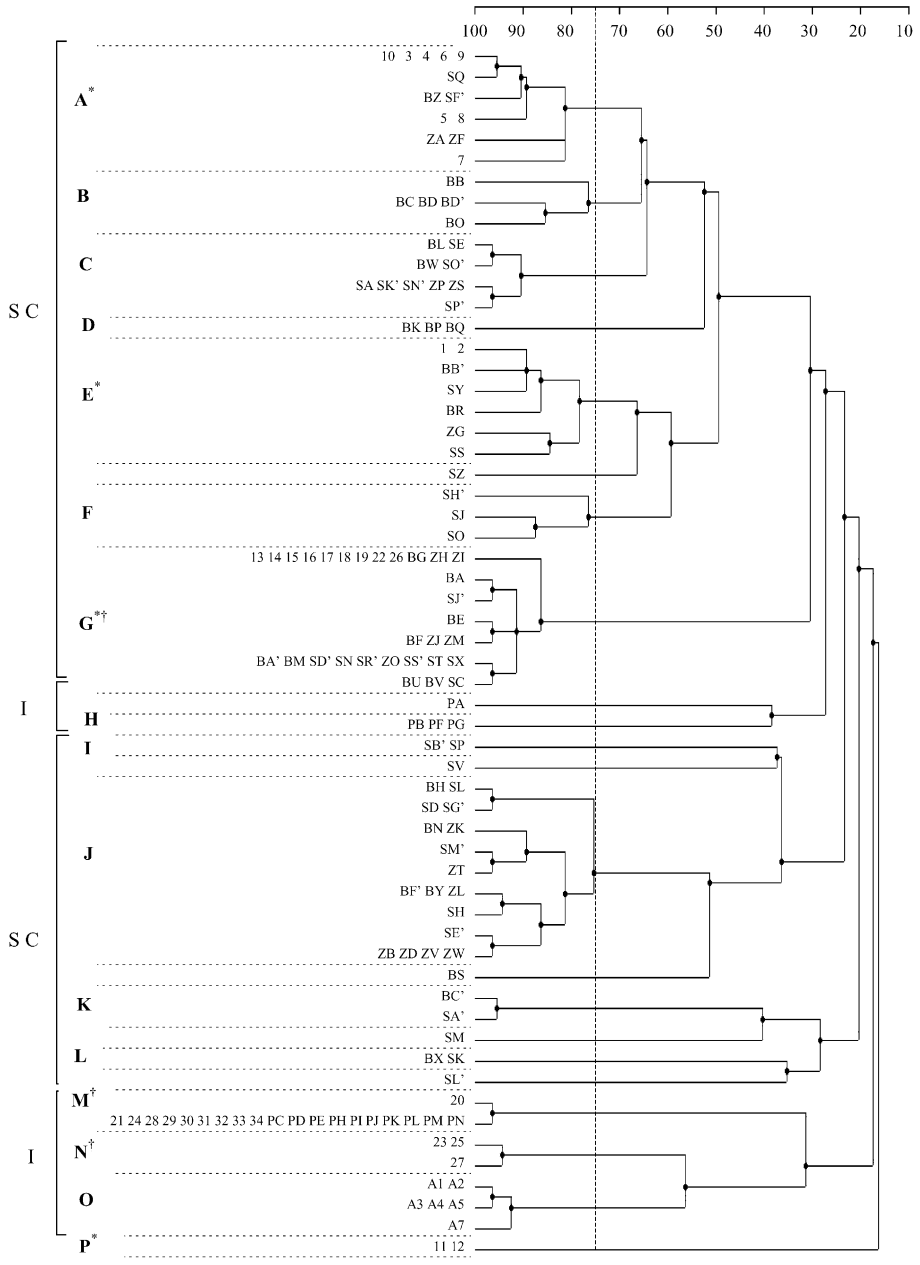

Fig. 2. Dendrogram showing relationships between *B. cereus* strains, based on the combined data from M13-PCR and DAF. The percentage similarities (represented by the horizontal scale) were calculated using Dice's coefficient and clustering by UPGMA. The letters in boldface represent the different groups formed at 75% similarity level. *, groups containing strains isolated from purées stored at 10°C. †, groups containing strains isolated from purées stored at 20–25°C. SC, strains originating from soil and zucchinis. I, strains originating from dehydrated ingredients (milk proteins and starch).

automatic detection of bands, a zero-to-three-band difference was observed on combined profiles. On the dendrogram, the strains of this category had a similarity coefficient of $\geq 80\%$. The second category consisted of related strains that showed a difference of four to six bands on combined profiles, after automatic band detection. The strains of this category had similarity coefficients of between 75 and 79% on the dendrogram. Strains with similarity coefficients lower than 75% were considered different and consisted of those that demonstrated more than seven-band differences in combined patterns.

Based on a similarity threshold of 75%, the 134 *B. cereus* strains were distributed into 16 combined profile groups, and six strains remained unclustered (Fig. 2, Table 2). The strains and the clustered strains preferentially grouped according to their origin; groups A–G and I–L contained most of the strains isolated from soil and zucchinis, whereas groups M–O contained most of the strains isolated from milk proteins and starch (Fig. 2). A higher heterogeneity was observed for strains isolated from soil and zucchinis, resulting in the formation of 11 groups and five unclustered strains at 75% similarity level. Strains from zucchinis were often closely related to strains originating from cultivation soil of zucchinis (Fig. 2), five ma-

Table 1
Description of *B. cereus* strains used in this study

Strain designation	Origin
SA, SC, SD, SE, SH, SJ, SK, SL, SM, SN, SO, SP, SQ, SR, SS, ST, SV, SX, SY, SZ, SA′, SB′, SD′, SE′, SF′, SG′, SH′, SJ′, SK′, SL′, SM′, SN′, SO′, SP′, SR′	Soil where the zucchinis used for processing were grown
BA, BB, BC, BD, BE, BF, BG, BH, BK, BL, BM, BN, BO, BP, BQ, BR, BS, BU, BV, BW, BX, BY, BZ, BA′, BB′, BC′, BD′, BF′	Raw zucchinis
ZA, ZB, ZD, ZF, ZG, ZH, ZI, ZJ, ZK, ZL, ZM, ZO, ZP, ZS, ZT, ZV, ZW	Washed zucchinis
PA, PB, PC, PD, PE, PF, PG, PH, PI, PJ, PK, PL, PM, PN	Milk proteins
A1, A2, A3, A4, A5, A6	Starch
1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	Zucchini purée stored 21 days at 10°C
20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34	Zucchini purée stored 5 days at 20–25°C

jor groups (A, C, E, G, J) and two minor groups (K, L) being common to both sources (Table 2).

Six groups were found in the final stored purées and their occurrence depended on the storage conditions (Table 2). Groups A, E, G and P were found in purées stored at 10°C and groups G, M and N in purées stored at 20–25°C. Groups A and E, related at 49% similarity level, and group G dominated in purées stored at 10°C. All were recovered in zucchinis and cultivation soil of zucchinis. Group M, dominant in purées stored at 20–25°C, was recovered in milk proteins. In each of these groups (A, E, G and M) strains showed clonal relatedness with 80–100% similarity levels. Group N, minor in purées stored at 20–25°C, was not recovered but was related at a 56% similarity level to group O found in starch. Finally, group P, minor in purée stored at 10°C, was not recovered and was not related to any known sources of contamination.

3.3. Growth at low temperature

Among the strains representing the different groups, no strain grew at 4°C. All representative strains in a given group showed similar growth characteristics at 7°C and 10°C. Representative strains of groups A–F and unclustered strain SZ had the most rapid growth at 7°C and 10°C (Table 2). All these groups and strain SZ were related, forming a separate group at 49% similarity level (Fig. 2). All originated from soil and zucchinis. In contrast, representative strains of groups originating from starch (group O), milk proteins (groups M and H, unclustered strain PA) and purée stored at 20–25°C (group N) had the lowest growth at 10°C; the first bacterial growth was noted after 21 days or was not observed. The remaining strains, representing groups G, I–L and unclustered strains BS, SM and SL′, had intermediate growth properties at low temperature and originated from soil and zucchinis.

3.4. B. cereus contamination along the zucchini purée processing line

Table 3 shows the distribution of the various genetic groups along the zucchini purée processing line. Most groups found in soil were recovered in zucchinis. After washing, the number of groups present in zucchinis decreased. New groups of *B. cereus* appeared with the addition of ingredients (milk proteins and starch). After pasteurization and storage of purées, groups C and J from washed zucchinis and groups O and H from starch and milk proteins were not recovered in the final stored products. Groups A, E and G survived two heat treatments (steam cooking and pasteurization).

4. Discussion

Combination of M13-PCR and DAF techniques provided discriminative and informative results for tracing the spread of *B. cereus* contamination in a food plant. These techniques, derived from RAPD, have previously been used with success for *B. cereus* and *B. anthracis* typing [11,16]. They benefit from the rapidity and low cost of RAPD. The size of primer PM13 (15 bp) for M13-PCR and the particular amplification cycles for DAF [9] make those techniques more reproducible than the classical RAPD technique. In our work, some adaptations were made to the initial protocols to improve reproducibility and typeability (modification of reaction medium, increase of hybridization temperature, modification of amplification cycles and selection of primer). These methods can be easily standardized at the laboratory level using the same batch of PCR reagents, the same equipment and the inclusion of control strains in each PCR experiment. The choice of a typing method depends on the objective of the work, the timeliness of information needed, and the financial and technical resources available. Even though less rapid or more expensive techniques based on restriction analysis (ribotyping, AFLP and PFGE) are recommended for studies implying the setting up of a common data base between laboratories (where equipment and reagents often differ from each other), RAPD-derived techniques seem to be well appropriated for intra-laboratory studies, providing a rapid, easy, discriminative and low-cost identification of a large number of strains.

Strains isolated from zucchinis (45 strains) seem to be more diverse than strains isolated from the dehydrated

Table 2
Distribution of *B. cereus* strains according to molecular group and origin, and growth at low temperature for representative strains

Group[a]	Number of strains	Strain origin						Number of strains per group	Representative strains	Growth at[b]	
		Soil	Zucchini	Milk proteins	Starch	Purées stored at 10°C	Purées stored at 20–25°C			7°C	10°C
A	13	2	3			8		13	6, 8, SQ, BZ, ZA	++	+++
E	7	2	3			2		7	1, BB′, BR, ZG	++	+++
G	30	8	13			7	2	30	15, BE, BG, BU, BV, ZO, ZM	−	++
M	20			10			10	20	32, PC, PJ	−	+
N	3						3	3	25	−	+
P	2					2		2	11	−	++
B	5		5					5	BB, BC	++	+++
C	10	6	4					10	SA, BL, ZP, ZS	++	++
D	3		3					3	BK	++	+++
F	3	3						3	SO	++	+++
I	2	2						2	SB′	−	++
J	17	6	11					17	BN, BY, ZB, ZT	−	++
K	2	1	1					2	BC′	+	++
L	2	1	1					2	SK	−	++
H	3			3				3	PF	−	−
O	6				6			6	A3	−	+
SZ	1	1						1	SZ	++	+++
SV	1	1						1	nt	nt	nt
BS	1		1					1	BS	−	++
SM	1	1						1	SM	−	+
SL′	1	1						1	SL′	−	++
PA	1			1				1	PA	−	−
Total number of groups	22	13	10	3	1	4	3	22	21		
Total number of strains	134	35	45	14	6	19	15	134	43		

[a]Z, SV, BS, SM, SL′, PA, unclustered strains included independently in the calculation of group number.
[b]+++, growth after 7-day incubation; ++, growth after 14-day incubation; +, growth after 21-day incubation; −, no growth observed; nt, not tested.

Table 3
Locations of *B. cereus* and genotypic groups in the purée processing plant

	Process flow chart	Type of sample collected[a]	Detection of *B. cereus*[a]	Genotypic groups found at various locations	
				groups[b]	*unclustered strains*
Fields		Cultivation soil of zucchinis	+	**A, E, G**, C,J, I, F, K, L,	*SL', SM, SV, SZ*
Processing	Washing of vegetables	Unwashed zucchinis	+	**A, E, G**, C, J, K, L, B, D	*BS*
	↓	Washed zucchinis	+	**A, E, G,** C, J	
	Steam cooking of vegetables	Cooked zucchinis	+		
	↓				
	Grinding of vegetables	Equipment surfaces	-		
	↓				
	Addition of ingredients and mixing	Equipment surfaces	-		
	↓	Milk proteins	+	**M,** H	*PA*
		Starch	+	O	
		UHT cream	-		
		salt	-		
	Packaging	Equipment surfaces	-		
	↓				
	Pasteurization in final package				
Storage		Purée stored 21 days at 4°C	-		
		Purée stored 21 days at 10°C	+	**A, E, G,** P	
		Purée stored 5 days at 20-25°C	+	**M, G,** N	

[a]From a previous work [20]. [b]Genotypic groups from stored purée with identified source are in boldface.

ingredients milk proteins and starch (20 strains). The number of strains compared between each origin was significantly different. However, the fact that 80% of the ingredient strains (16 strains out of 20) were clustered in only two groups at 90% similarity level (Fig. 2) argues for a lower diversity in dehydrated ingredients. Another argument for this is that the selective pressure due to the processing of dehydrated ingredients (heat treatments) and the low water activity in those products are not favorable conditions for diversity. In contrast, soil and vegetables are favorable environments for metabolic activities and transfer of genetic material between strains.

Strains from soil/zucchinis and strains from milk proteins/starch formed two separate clusters (Fig. 2), one at the top of the dendrogram and the other at the end. As an exception, four milk protein strains were grouped with soil/zucchini strains. Soil is also considered as a source of *B. cereus* contamination for milk [26–28]. These four milk protein strains might have initially originated from soil. Interestingly, the ability of representative strains to grow at low temperature decreased with their position from the higher to the lower part of the dendrogram. This underlines the differences between strains of the two main different origins (soil/zucchinis and dehydrated ingredients) and may reflect a particular adaptation of strains to a particular habitat or niche. Te Giffel [3] showed the absence of psychrotrophic *B. cereus* strains in milk powder and ascribed this finding to the process generally used to make powder. Zucchinis contributed to the transfer of psychrotrophic *B. cereus* strains to the final product, whereas milk proteins and starch contributed to the transfer of non-psychrotrophic strains. This may explain the distribution of groups in stored purées according to storage temperature, illustrated by the recovery of groups A and E in purées stored at 10°C, M and N in purées stored at 20–25°C and of the intermediate group G at the two storage temperatures.

The diversity of strains observed in zucchinis increases the chances of strains persisting throughout the processing line and contaminating the final product. Washing of zuc-

chinis, heat treatments and storage at low temperature contribute to a selection of zucchini strains, as only groups A, E and G were recovered in stored purée. Groups J, B and C were good candidates for the contamination of purées, as they were among dominant groups in zucchinis. Washing of zucchinis, heat treatments and/or storage conditions probably did not permit them to survive.

In this study, molecular typing enabled us to trace the dissemination of *B. cereus* from the potential sources of contamination to the finished stored product. In contrast with the case of pasteurized milk, where *B. cereus* contamination was often due to the installation of particular genotypes on dairy plant equipment [10,29,30], contamination of zucchini purée was directly associated with raw material used for processing, such as vegetable and dehydrated ingredients. The soil was the initial source of contamination for zucchinis. In the purée processing plant, the type of equipment, easily accessible for washing solutions and efficient cleaning procedures using *B. cereus* sporicide, probably do not allow the installation of particular genotypes on equipment surfaces [20]. Among sources of contamination, milk proteins represent a low risk for the stored product as it contains non-psychrotrophic strains and zucchini purée is refrigerated during its commercialization. *B. cereus* contamination carried by zucchinis calls for particular attention, as it is composed to a large extent of psychrotrophic strains. Optimization of washing procedures and heat treatments applied to zucchinis would help decrease contamination.

This work emphasizes the role of soil as a reservoir for *B. cereus* contamination of foods. Spores from soil, highly diverse, persisted throughout the process and adapted to storage conditions of the processed food (i.e. represent the *B. cereus* strains that would be ingested by consumers). In contrast, texturing agents such as milk proteins and starch did not contribute to the contamination of the product when properly stored at cold temperatures. This illustrates the need for typing methods able to discriminate the respective contributions of the various contamination sources.

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