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Identification of the bacteria and their metabolic activities associated with the microbial spoilage of custard cream desserts

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ABSTRACT

The famous French dessert "ile flottante" consists of a sweet egg white foam floating on a vanilla custard cream, which contains highly nutritive raw materials, including milk, sugar and egg. Spoilage issues are therefore a key concern for the manufacturers. This study explored the bacterial diversity of 64 spoiled custard cream desserts manufactured by 2 French companies. *B. cereus* group bacteria, coagulase negative *Staphylococcus*, *Enterococcus* and *Leuconostoc* spp. were isolated from spoiled products. Thirty-one bacterial isolates representative of the main spoilage species were tested for their spoilage abilities. Significant growth and pH decrease were observed regardless of species. While off-odours were detected with *B. cereus* group and staphylococci, yoghurt odours were detected with *Enterococcus* spp. and *Leuconostoc* spp. *B. cereus* group bacteria produced various esters and several compounds derived from amino acid and sugar metabolism. Most *Staphylococci* produced phenolic compounds. *Enterococcus* spp. and *Leuconostoc* spp. isolates produced high levels of compounds derived from sugar metabolism. Each type of spoilage bacteria was associated with a specific volatile profile and lactic acid was identified as a potential marker of spoilage of custard cream-based desserts. These findings provide valuable information for manufacturers to improve food spoilage detection and prevention of chilled desserts made with milk and egg.

1. Introduction

Spoilage is defined as any change in a food product that renders it unacceptable to the consumer from a sensory point of view (Sperber, 2009). Microbial growth and metabolic activities are the most important causes of food spoilage (Sperber, 2009). Microbial contaminants can use available substrates for growth and produce a range of metabolic by-products, such as organic acids and volatile compounds. These activities may result in changes in food appearance and texture, in pH modifications, and/or in the formation of undesirable odours or flavours. Spoilage issues are highly variable depending on the type of food, extent of processing and storage conditions (Nassos et al., 1988). Food spoilage mechanisms and chemical indicators are well-described for several foodstuffs such as fish and meat or derived products (Dainty, 1996; Nychas et al., 2008). Various volatile compounds have been proposed for early detection of the spoilage risk in various food products (Derevel et al., 1994) and for the characterization of bacteria involved in food spoilage issues (Ercolini et al., 2009; Joffraud et al., 2001). Some acids, such as lactic and succinic acids, have been associated with food spoilage and can serve as spoilage indicators for foods such as milk or egg products (Alamprese et al., 2004; Cousin, 1982; Dainty, 1996; Nassos et al., 1988; Stijve and Diserens, 1987).

The spoilage of custard cream has been poorly documented despite its extensive use in the formulation of various kinds of desserts particularly susceptible to spoilage. French custard cream, composed of milk, sucrose, egg products and minor components such as vanilla or starch is widely used in cream puffs, pastries, cakes or chilled dairy desserts. Custard cream is potentially a highly nutritive growth medium that can support the growth of bacteria and the production of metabolites that compromise shelf-life, even at chilled temperatures. Spoilage of commercial custard creams was reported to be associated with the presence of bacilli mainly belonging to the *B. cereus* group, staphylococci, lactic acid bacteria, and psychrotrophic Gram-negative rods (Arakawa et al., 2008).

Food quality control is usually achieved using traditional microbiological analyses that target specific bacterial groups that are

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detected using selective agars. However, this approach does not always provide sufficiently informative data due to ambiguity associated with specific microbiological methods and a lack of correlation with spoilage potential. More robust, sensitive approaches are thus required for a better control of spoilage phenomena. Moreover, it may be preferable to target metabolites associated with spoilage events rather than the microorganisms themselves.

The purpose of the present study was to fully characterize the bacterial spoilage association and relevant metabolites in spoiling custard cream to identify relevant spoilage markers. This work represents a first step in the development of efficient detection and prevention methods to support industry efforts to reduce spoilage events in chilled dessert manufacturing and distribution.

2. Material and methods

2.1. Origin of spoiled custard cream based desserts

A total of 64 spoiled custard cream-based desserts from two manufacturers referred to as A (n = 36) and B (n = 28) were analysed. The desserts were produced from May 2012 to August 2013, and were considered spoiled after "accelerated shelf-life tests" performed by incubation of samples at 30 °C for 24 h and 10 °C for 21 d. The criteria used by the manufacturers to qualify desserts as spoiled were based on pH modification and/or sensorial changes (mainly odours). When a spoilage event was detected, the dessert was kept refrigerated (< 4 °C) and was transported to our laboratory at refrigeration temperature (< 4 °C) for analysis within two days of reception.

2.2. Measurement of total aerobic microbial populations

Total mesophilic aerobic populations were measured by pour plate method. After removal of the egg white foam, custard was homogenized by vortexing. Ten grams of representative sample were then diluted in sterile glass bottle (Dutscher, Brumath, France) containing 90 mL of Tryptone Salt (0.1% Tryptone, AES Laboratoire, Combourg, France; 0.85% NaCl, Labogros, Buchs, France) (TS). Serial decimal dilutions prepared in TS, were applied to Brain Heart Infusion-Yeast Extract agar (BHI-YE) (3.7% BHI (Merck, Darmstadt, Germany); 0.1% yeast extract (Merck); 1.5% agar (Merck)), which were incubated under aerobic conditions for 24 h at 30 °C. After incubation, the colony-forming units (CFU) were counted and the results were expressed in log CFU/mL.

2.3. Assembly of a panel of representative spoilage bacteria

For each dessert, 8 to 10 colonies were randomly collected from the BHI-YE plates, resulting in a panel of 565 bacterial isolates. The isolates were maintained as frozen stocks at −80 °C in glycerol (25% w/w in water). All were identified by *16S rDNA* gene sequencing as previously described (Techer et al., 2015). *B. cereus* group isolates were identified at the phylogenetic group level by amplified *panC* gene sequence analysis, as defined by Guinebretiere et al. (2008, 2010). Polymerase chain reaction, purification, sequencing and affiliation to the phylogenetic groups using the resulting *panC* sequences were carried out as described in Techer et al. (2015).

2.4. Evaluation of in situ spoilage potential of bacterial isolates

A commercial UHT custard cream product was used for all experiments (Bridelice, Lactalis, Laval, France). According to the manufacturer, the cream contained 80% milk, 12% sucrose, 7% whole egg, 1% starch and unspecified quantities of vanilla aroma and stabilizer. The sterility of the custard cream was systematically verified by overnight incubation of 1 mL of custard and 14 mL of BHI-YE agar medium at 30 °C by pour plate method. For each experiment, a non-inoculated custard cream was used as a negative control.

Frozen isolates were thawed and twice propagated in BHI-YE at 30 °C for 24 h under aerobic conditions, without agitation. Each inoculum was diluted in TS in order to reach an initial population of 3 log CFU/mL in 5 mL sterile commercial custard cream in 15 mL Falcon tubes (Sarstedt, Nümbrecht, Germany). Initial concentrations were measured using a plate-counting micro-method (Baron et al., 2006) on BHI-YE agar, which was incubated for 24 h at 30 °C. The inoculated custard cream samples were placed in an incubator at 10 °C and held for 21 days under aerobic conditions, without agitation. The experiments were carried out at chilled temperature in order to get closer refrigeration conditions as specified in the French standard for shelf-life validation of perishable and refrigerated food (Afnor NF V 01–003, 2010) and to mimic the conditions of the ageing tests usually carried out in the chilled industry (Techer et al., 2015).

Microbiological, sensory, and biochemical analyses were performed at the end of the storage period. Total aerobic population was measured using the plate-counting micro-method described above.

2.5. Sensory analyses

Sensory analyses were performed by an untrained panel of 4 individuals who were tasked with the detection of changes in odour or appearance of custard cream using a free vocabulary. Main defects evaluated by judges were odours, which were classified into three groups (acceptable odours, off-odours and yogurt-type odours), and the presence (e.g. thickening, phase shift) or absence of visual defects expressed as (+) or (−).

2.6. pH measurements

pH was measured before and after custard cream inoculation and incubation (pH meter 315i, WTW, Weilheim, Germany). Results were expressed as ΔpH, corresponding to the difference between the pH of the custard cream before inoculation and after 21 d at 10 °C.

2.7. Biochemical analyses

2.7.1. Sugar and acid analyses

Lipids and proteins were removed from the samples by ultrafiltration at 4000 g for 45 min (Amicon Ultra-15, PLBC Ultracel-PL Membrane, 10 kDa, Millipore) before acid and sugar analysis. Lactic and succinic acids were quantified by high-performance liquid chromatography (HPLC DIONEX, Sunnyvale California, USA). Acids were separated on an Aminex *A*-6 ion-exchange column (Bio-Rad, Hercules, California) operated at 55 °C with 0.005 M sulfuric acid as the mobile phase (flow rate: 1.0 mL/min). Acids were detected by both UV at 210 nm (Dionex-UVD 1704) and refractometry (IOTA2, Dionex) and quantified according to standard calibration curves obtained with lactic acid (L2250, Sigma aldrich) and succinic acid (Merck 682, Merck) prepared in the range of 0–10 g/L in desionised water. Sucrose and lactose were quantified using commercial analytical kits (Megazyme K-MASUG and Megazyme K-LACGAR, respectively, Libios, Pontcharra-sur-Turdine, France) according to manufacturer's instructions.

2.7.2. Analysis of volatile organic compounds

Volatile organic compounds were extracted and analysed by head space-gas chromatography-mass spectrometry (GC-MS, Clarus 680 GC coupled to Clarus 600 T quadrupole MS, PerkinElmer, Courtaboeuf, France) as previously described (Pogačić et al., 2015). Briefly, samples (2.5 g of custard cream) were warmed for 15 min at 65 °C and volatiles were extracted at 207 kPa helium pressure, before being adsorbed on a Tenax™ trap at 35 °C. Trap load was performed twice for each sample. The trap was heated at 250 °C for 0.1 min and back flushed at 89 kPa, leading to desorption of the volatiles. Volatile metabolites were separated on an Elite-5MS capillary column (length, 60 m; inner diameter, 0.25 mm; PerkinElmer), with helium as the mobile phase. The

temperature of the oven was maintained at 35 °C for 5 min, and was then increased at a rate of 7 °C/min up to 140 °C and then at a rate of 13 °C/min up to 280 °C. The mass spectrometer was operated in the scan mode, within a mass range of m/z 25–300 at 5.5 scan/s. Ionization was done by electronic impact at 70 eV.

Data analysis was performed using XCMS software (Smith et al., 2006) implemented with the R-statistical language, as detailed in Pogačić et al. (2015). For the identification of volatile components, the NIST 2008 Mass Spectral library, and comparison with the spectra and retention indices of standards, were used.

Results were expressed as the relative abundance of each volatile compound in the spoiled custard cream, compared to the control sample (non-inoculated sterile custard cream), i.e. the ratio of the abundance of the volatile compound in the spoiled custard cream sample and of its abundance in the control sample.

2.7.3. Measurement of protein hydrolysis

To evaluate protein hydrolysis, the non-protein nitrogen fraction (NPN) of the custard cream was obtained after precipitation with 12% trichloroacetic acid (TCA) and filtration using Whatman filter N°40 (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The Total Nitrogen (TN) and NPN contents were determined using the Kjeldhal method, according to the IDF standard 20 B (IDF, 2001). The converting factors used for TN and NPN were 6.38 and 6.19, respectively (Karman and Van Boekel, 1986). Results were expressed as the ratio of the NPN/TN values x 100.

2.7.4. Measurement of lipid hydrolysis

Thin layer chromatography (TLC) followed by densitometry were used to monitor fat changes in the spoiled custard creams and to quantify different fat classes. Fat extraction and analysis were adapted from Bourlieu et al. (2012). Briefly, total lipids were extracted from around 0.5 g custard cream by the Folch method, using a chloroform:methanol mixture (2:1, v/v). A saline solution (NaCl 0.73% (p/v)) was added to the mixture to help the formation of a chloroform bottom phase containing fat. The total lipid extract dissolved in chloroform was spotted on silica gel plates (10 × 20 cm, 0.25 mm, Si G60, Merck) using Automatic TLC Sampler III (CAMAG, Muttenz, Switzerland). Plates were developed in hexane/diethyl ether/acetic acid (70:30:2 v/v/v) for 10–12 min, followed by 20 min of drying. The spots corresponding to the different classes of lipids were visualized by heating the plates for 20 min at 150 °C, after staining by immersion in a copper sulphate II/orthophosphoric acid solution. The nature of the spots was identified by comparison with different standards (Larodan, Solna, Sweden): triolein (0.163 mg/mL) for triacylglycerides (TAG), diolein (0.122 mg/mL) for diacylglycerides (DAG), monolein (0.065 mg/mL) for monoglycerides (MAG), oleic acid (0.122 mg/mL) for free fatty acids (FFA) and phosphatidylcholine (0.068 mg/mL) for phospholipids (PL). The intensity of the TLC bands was quantified by densitometry using the ImageQuant software (Image scanner III, GE Healthcare, Velizy-Villacoublay, France). For each identified band, results were expressed as a relative intensity (intensity of the band for the assay/intensity of the band for the negative control). Results were considered as significant above a threshold of 25%, calculated as the maximal coefficient of variation obtained from duplicate independent extractions and analyses.

Two positive controls were used, consisting in custard cream added with either a commercial lipase (reference 95608, Sigma-Aldrich, 0.1 mg/mL final concentration), or phospholipase (reference P6621, Sigma-Aldrich, 0.005 mg/mL final concentration) and incubated for 1.5 h at 30 °C. TLC was realized once for each sample, and the results were confirmed by two independent experiments for 5 randomly selected samples (coefficient of variation = 0.23).

2.8. Statistical analyses

All experiments (custard cream inoculation; microbiological, sensory and physicochemical analyses, extraction and quantification of chemical compounds) were performed in triplicate.

Statistical analyses were done using the R-software (R 2.13.0) to compare the spoiled custard cream samples to the control sample. The level of significance was at least 5%. Comparisons were performed with Kruskall Wallis test or one-way ANOVA followed by Tukey post hoc comparisons. Principal Component Analysis (PCA) was performed using the FactoMineR package of the R-software. Quantitative data used for PCA were sucrose and lactose concentrations, NPN/TN value, relative abundance of the different fat classes, abundances values of volatile compounds, lactic and succinic acids concentrations and ΔpH values. Sensory modifications (thickening, phase shift, odours) were considered as illustrative qualitative variables on the PC. Individuals are also identified according to their modality for the considered qualitative variables. For each modality of each qualitative variable, the individuals were considered as significantly different from the mean for student t-test P-values lower than 5%. The correlation between variables was determined from correlation matrix using the Pearson correlation coefficient.

3. Results

3.1. Selection of representative bacteria involved in custard cream spoilage

The total aerobic microbial populations of the 64 spoiled desserts varied from 4.5 to 10.5 log CFU/mL with an average value of 7.5 ± 1.3 log CFU/mL. The spoilage microorganisms mainly consisted of bacteria. Only 3 desserts were contaminated by yeasts in association with bacteria. As shown in Table 1, 81% of the spoiled desserts was contaminated with members of the *B. cereus* group. Coagulase negative *Staphylococcus*, *Enterococcus* spp. and *Leuconostoc* spp. were present in 25%, 19%, and 9% of the spoiled desserts, respectively. To a lower extent (less than 5%), 13 other genera were also detected, belonging to firmicutes, actinobacteria and proteobacteria (Table 1).

The main genera associated with spoilage, i.e. *Bacillus* spp., *Staphylococcus* spp. and *Enterococcus* spp., were isolated from desserts

Table 1
Type and distribution of bacteria isolated from 64 commercial custard cream-based desserts provided by companies A and B that were spoiled after incubation at 30 °C and/or 10 °C shelf-life testing temperatures.

Type of bacteria	Number of desserts (%)	Number of isolates	Shelf-life testing temperature	Company
B. cereus group	52 (81)	374	10 °C, 30 °C	A, B
Staphylococcus spp. (coagulase negative)	16 (25)	53	10 °C, 30 °C	A, B
Enterococcus spp.	12 (19)	49	10 °C, 30 °C	A, B
Leuconostoc spp.	6 (9)	37	10 °C	A
Bacillus spp. (other than the *B. cereus* group)	3 (5)	3	10 °C, 30 °C	A,B
Paenibacillus spp.	3 (5)	10	10 °C	A
Corynebacterium spp.	2 (3)	3	10 °C	A
Macrococcus spp.	2 (3)	3	10 °C	A
Aeromonas spp.	1 (2)	2	10 °C	A
Carnobacterium spp.	1 (2)	9	30 °C	A
Escherichia spp.	1 (2)	1	30 °C	B
Kocuria spp.	1 (2)	2	10 °C	A
Microbacterium spp.	1 (2)	2	10 °C	A
Micrococcus spp.	1 (2)	1	10 °C	B
Pseudoclavibacter spp.	1 (2)	7	30 °C	B
Raoultella terrigena spp.	1 (2)	4	10 °C	A
Rhodococcus spp.	1 (2)	1	10 °C	A
Stenotrophomonas spp.	1 (2)	4	30 °C	B

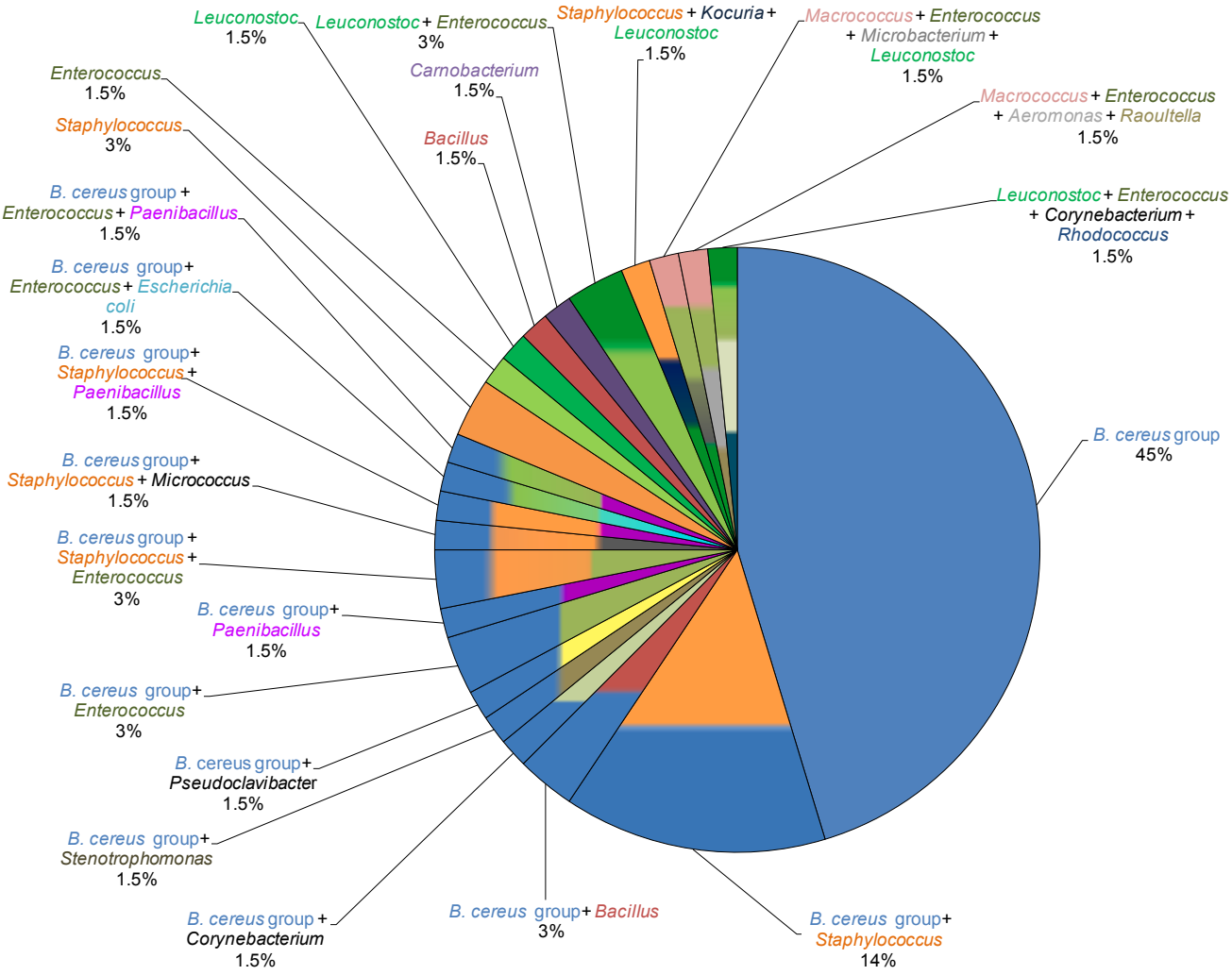


Fig. 1. Bacterial associations identified in the spoiled custard cream-based desserts provided by companies A and B after incubation at 30 °C and 10 °C shelf-life testing temperatures (n = 64).

provided by the 2 companies involved in the study (A and B) after incubation at both shelf-life testing temperatures (10 °C and 30 °C) (Table 1).

More than half of the desserts contained only one bacterial group (Fig. 1). *B. cereus* group isolates were either present as the sole bacterial type (45% of samples) or in association with 1 or 2 other types of bacteria (35% of samples). Only 9% of the desserts contained *Staphylococcus* spp., *Enterococcus* spp., *Leuconostoc* spp., or non *B. cereus* group bacilli as the sole bacterial type. Less than 15% of spoiled desserts contained 3 or 4 bacterial types (Fig. 1).

A subset of 31 isolates, belonging to the 4 main bacterial types identified in the spoiled desserts, was selected and further characterized to determine their impact on the sensorial, physicochemical, and biochemical properties of sterile custard cream (Table 2). The isolates from each genus were selected, on the basis of their occurrence in the spoiled desserts and their origin (i.e. company A or B, shelf-life testing temperature). This bacterial collection included 14 *B. cereus* group isolates, 7 *Staphylococcus* spp., 7 *Enterococcus* spp., and 3 *Leuconostoc* spp. Bacteria were singly inoculated.

3.2. Microbiological, physicochemical and sensory changes due to in vitro custard cream bacterial spoilage

Bacterial populations ranged from 7 to 9.3 log CFU/mL after incubation at 10 °C for 21 d (Table 2). Before inoculation, the average pH of the commercial custard cream was 6.95 ± 0.1. Except for one *Staphylococcus* isolate, a decrease in pH was systematically observed after 21 d incubation. The ΔpH values ranged between 0.3 and 2 units (Table 2). The average final pH values of the spoiled custard creams were 6.26 ± 0.2, 6.67 ± 0.13, 5.43 ± 0.10, and 4.95 ± 0.10 after 21 d incubation with *B. cereus* group bacteria, *Staphylococcus* spp., *Enterococcus* spp., and *Leuconostoc* spp., respectively.

Most isolates (28/31) led to changes in the custard cream texture, either by thickening (87%) and/or by phase shift (13%). All the samples displayed odour changes (Table 2). Off-odours were detected in samples inoculated with *B. cereus* group bacteria and *Staphylococcus* spp., and yoghurt-type odours for both lactic acid bacteria (Table 2).

3.3. Biochemical changes due to in vitro custard cream bacterial spoilage

3.3.1. Evolution of the main custard cream substrates during bacterial spoilage

3.3.1.1. Sugar consumption. The sucrose and lactose concentrations of 120 and 27 g/L, respectively, were measured in the control custard creams (Fig. 2). These values were consistent with those provided by the manufacturer. The consumption of sucrose and lactose was highly genus and isolate – dependent. Only leuconostocs consumed significant amounts of sucrose, whereas lactose was consumed by most of the *B. cereus* group isolates and, to a smaller extent, by enterococci and leuconostocs.

Table 2
Microbiological, physicochemical and sensory changes of the custard creams inoculated by *B. cereus* group bacteria, coagulase negative *Staphylococcus, Enterococcus* spp., or *Leuconostoc* spp. and incubated for 21 d at 10 °C. Each value is the mean ± standard deviations of 3 independent experiments. Presence (+) and absence (−) of the defect.

	Isolate (*B. cereus* group phylogenetic assignation)	Bacterial population (log CFU/mL)	ΔpH	Thickening	Phase shift	Odour
B. cereus group (n = 14)	A-C1 (II)	7.7 ± 0.2	0.81 ± 0.02	+	–	Off-odours
	AM-C1 (IV)	7.0 ± 0.3	0.74 ± 0.05	+	–	Off-odours
	BK-C9 (VI)	7.6 ± 0.3	0.84 ± 0.08	+	–	Off-odours
	BL-C9 (VI)	7.7 ± 0.3	0.82 ± 0.08	+	–	Off-odours
	BM-C3 (VI)	7.6 ± 0.4	0.81 ± 0.02	+	–	Off-odours
	BN-C2 (VI)	7.8 ± 0.2	0.83 ± 0.07	+	–	Off-odours
	BO-C4 (VI)	7.5 ± 0.4	0.77 ± 0.04	+	–	Off-odours
	BP-C2 (II)	7.6 ± 0.1	0.81 ± 0.01	+	–	Off-odours
	BR-C10 (II)	8.1 ± 0.8	0.90 ± 0.02	+	–	Off-odours
	K–C3 (II)	7.8 ± 0.2	0.36 ± 0.04	–	–	Off-odours
	L-C6 (II)	8.2 ± 0.3	0.32 ± 0.11	–	–	Off-odours
	L-C9 (II)	7.7 ± 0.2	0.31 ± 0.04	–	–	Off-odours
	N–C3 (IV)	7.4 ± 0.5	0.60 ± 0.03	+	–	Off-odours
	O–C3 (IV)	7.3 ± 0.2	0.77 ± 0.10	+	–	Off-odours
Staphylococcus spp. (n = 7)	AO-C3	9.1 ± 0.1	0.26 ± 0.06	+	–	Off-odours
	AO-C8	8.9 ± 0.2	0.29 ± 0.04	+	–	Off-odours
	AP1-C2	8.5 ± 0.5	0.25 ± 0.08	+	–	Off-odours
	AV2-C1	8.5 ± 0.2	0.33 ± 0.10	+	–	Off-odours
	AY-C4	9.0 ± 0.3	0.33 ± 0.10	+	–	Off-odours
	AY-C5	8.8 ± 0.5	0.38 ± 0.09	+	–	Off-odours
	S–C4	7.9 ± 0.1	0.14 ± 0.24	–	–	Off-odours
Enterococcus spp. (n = 7)	AK-C8	8.6 ± 0.1	1.60 ± 0.04	+	–	Yoghurt
	AL-C3	8.4 ± 0.1	1.69 ± 0.05	+	–	Yoghurt
	BP-C8	8.7 ± 0.4	1.37 ± 0.13	+	–	Yoghurt
	G-C6	8.8 ± 0.2	1.51 ± 0.10	+	–	Yoghurt
	P–C9	9.0 ± 0.1	1.39 ± 0.07	+	–	Yoghurt
	S–C5	8.8 ± 0.1	1.53 ± 0.05	+	–	Yoghurt
	S–C7	8.9 ± 0.2	1.45 ± 0.05	+	–	Yoghurt
Leuconostoc spp. (n = 3)	AJ-C2	9.3 ± 0.2	1.96 ± 0.10	+	+	Yoghurt
	AL-C10	9.1 ± 0.5	1.97 ± 0.14	+	+	Yoghurt
	G-C10	9.3 ± 0.1	1.89 ± 0.06	+	–	Yoghurt

3.3.1.2. Protein hydrolysis. Out of the 31 isolates studied, only those belonging to the *B. cereus* group produced significant amounts of NPN, with NPN/TN values ranging between 10 and 20%. In contrast, amounts measured for the three other bacterial groups tested were of the same order of magnitude as the control, *i.e.* around 5% (Fig. 3).

3.3.1.3. Lipid hydrolysis. When a commercial lipase was added to the custard cream, the concentration of TAG significantly decreased whereas that of DAG and MAG increased concomitantly. The addition of a commercial phospholipase C led to a significant decrease in PL concentration (*i.e.* phospholipolysis) and increase in DAG concentration (Fig. 4A).

In the presence of *B. cereus* group bacteria, the TLC profiles of custard cream were close to those obtained with phospholipase C. For two isolates, BP-C2 and BR-C10, the decrease in PL and the increase in DAG were less pronounced (Fig. 4B). In contrast, the *Staphylococcus* spp., *Enterococcus* spp., and *Leuconostoc* spp. isolates did not display any detectable lipolytic activity (data not shown).

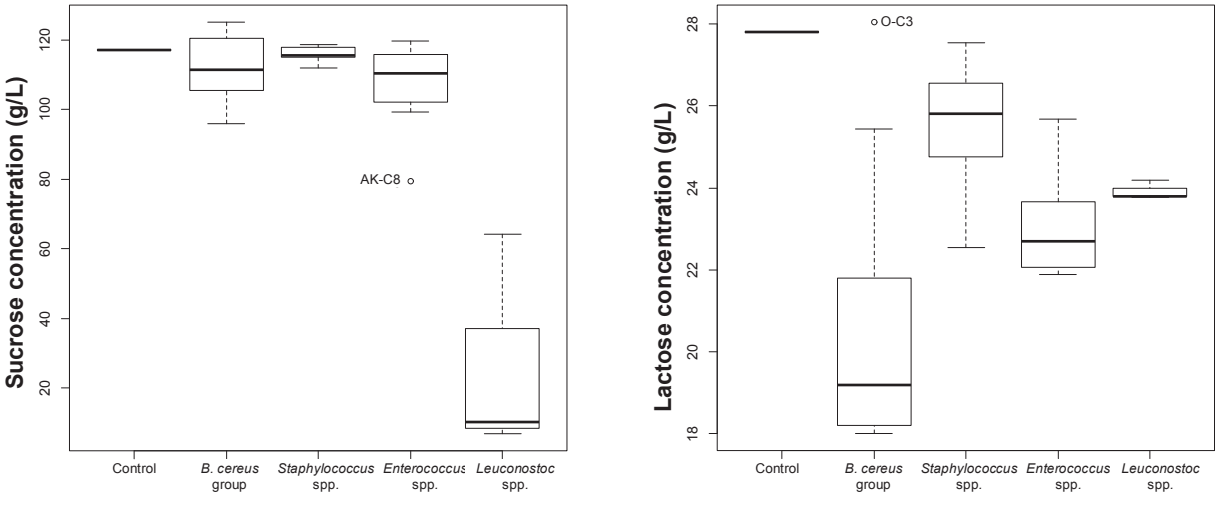


Fig. 2. Boxplots relative to the sucrose and lactose residual concentrations in the custard creams contaminated with *B. cereus* group, *Staphylococcus* spp., *Enterococcus* spp. or *Leuconostoc* spp. isolates, and incubated for 21 d at 10 °C.

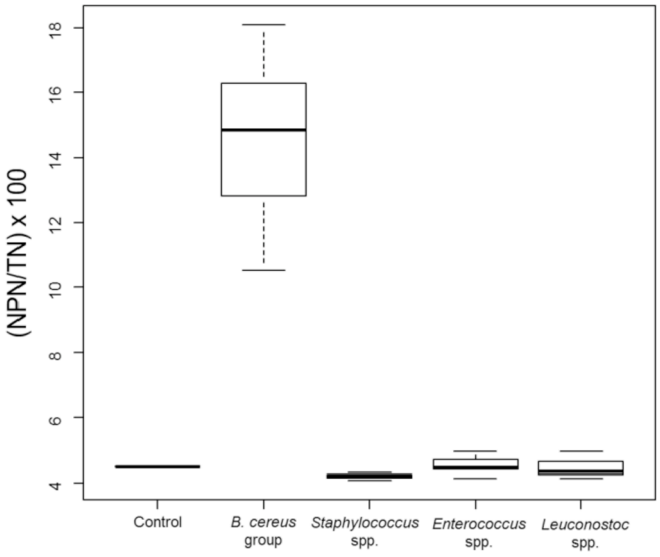


Fig. 3. Boxplots relative to proteolysis, expressed as the ratio of the non-protein nitrogen fraction and total nitrogen (NPN/TN*100)) in the custard creams contaminated with *B. cereus* group, *Staphylococcus* sp., *Enterococcus* sp., or *Leuconostoc* sp. isolates, and incubated for 21 d at 10 °C.

3.3.2. Production of metabolites during bacterial spoilage

3.3.2.1. Lactic and succinic acid production. Lactic acid was produced by the 31 isolates, with concentrations ranging from 0.79 to 1.08 and from 0.11 to 0.56 mg/mL for the *B. cereus* group and *Staphylococcus* spp., respectively. The highest concentrations were produced by *Enterococcus* spp. and *Leuconostoc* spp., and ranged from 2.5 to 4.4 mg/mL and 1.9–2.9 mg/mL, respectively. Succinic acid was exclusively produced by isolates from the *B. cereus* group, with concentrations ranging from 0.02 to 0.08 mg/mL (Fig. 5).

3.3.2.2. Volatile compound profile. Analysis of the volatile fraction of the spoiled custard creams revealed significant production of 33 volatile compounds (Table 3). Their relative abundance in contaminated custard cream *versus* in controls was genus and isolate – dependent. For example, most isolates (27/33, 82%) were able to produce 2-hydroxypropanone, with higher amounts produced by *Staphylococcus* spp. (relative abundances ranging from 25 to 68) compared to the *B. cereus* group and *Enterococcus* spp. (relative abundances ranging from 12 to 32 and from 7 to 21, respectively, Table 3). In the samples spoiled by the *B. cereus* group, the main compounds detected were 2-hydroxypropanone, 1-hexanol, 1-propanol, 1-butanol, 2-methylpropanol, 3- and 2-methylbutanoic acids, ethyl propanoate, ethyl butanoate, ethyl hexanoate, and ethyl 3-hydroxybutanoate (Table 3). Concerning *Staphylococcus* spp., 2-hydroxypropanone only was produced by all the isolates (Table 3). Except for S–C4, all *Staphylococcus* isolates produced diacetyl, 2,3-pentanedione, 3- and 2-methylbutanoic acids, ethyl propanoate, phenol and 2-methoxyphenol (Table 3). Lactic acid bacteria produced hexanol, diacetyl, acetoin, hexanoic acid, acetic acid and 2-methylpropanol (Table 3).

3.4. Global overview of custard cream bacterial spoilage

A Principal component analysis (PCA) was performed to provide a global overview of the effect of bacterial spoilage on custard cream attributes, including sucrose and lactose concentrations, NPN/TN value, relative abundance of the different fat classes, abundance of volatile compounds, lactic and succinic acids concentration, ΔpH and sensory modifications (thickening, phase shift, odours).

The first two principal components (PC) accounted for 54% of the total variance of the data set. PCA highlighted several groups based on the main chemical changes that occurred in the spoiled custard creams. The first PC distinguished *B. cereus* group isolates, positively associated with PC1, from the control and the other genera studied, i.e.

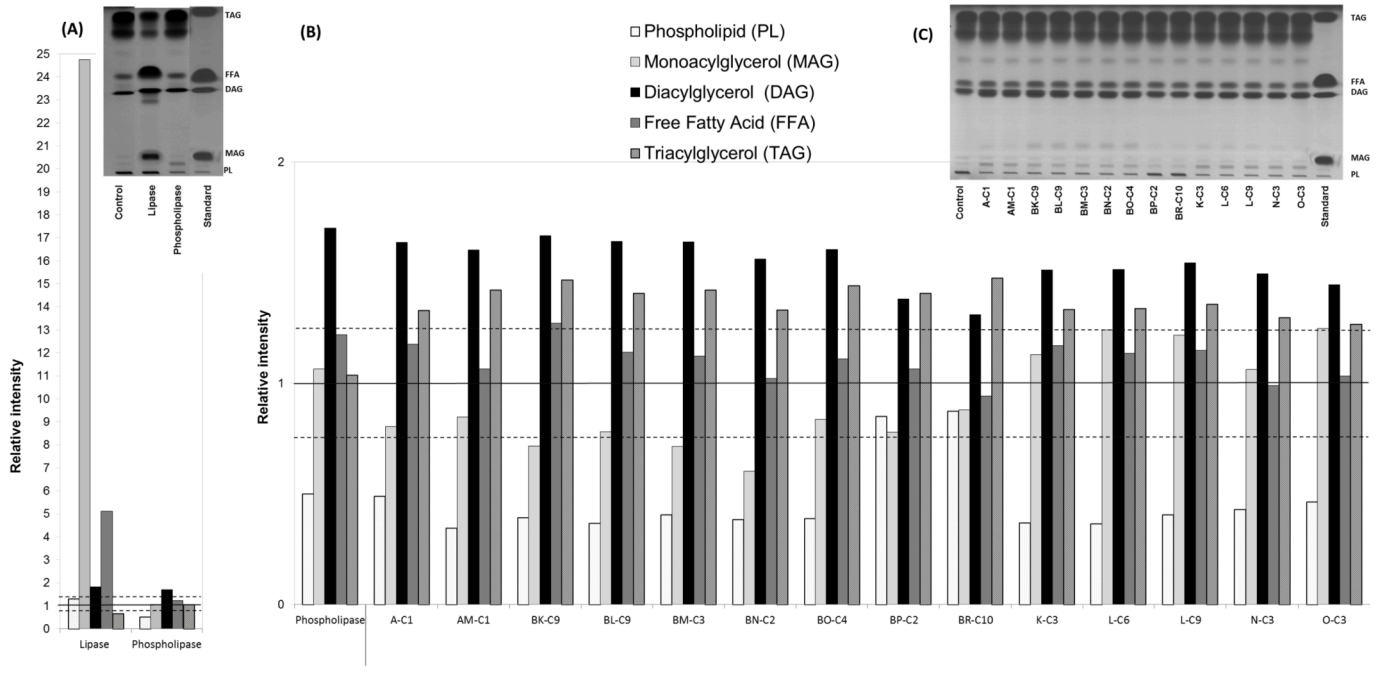


Fig. 4. Fat hydrolysis in the custard creams incubated with (A) commercial enzymes (lipase and phospholipase) for 1.5 h à 30 °C or (B) contaminated with *B. cereus* group isolates and incubated for 21 d at 10 °C. Lipid classes were separated by thin layer chromatography, identified by comparison with standards and quantified by densitometry. (C): results expressed as relative intensities compared to the controls, calculated as the ratio of the intensity of the spots in contaminated custard creams and that of the control. Repeated experiments showed a coefficient of variation smaller than 25%, considered as threshold to consider the results as different (indicated by the dashed lines).

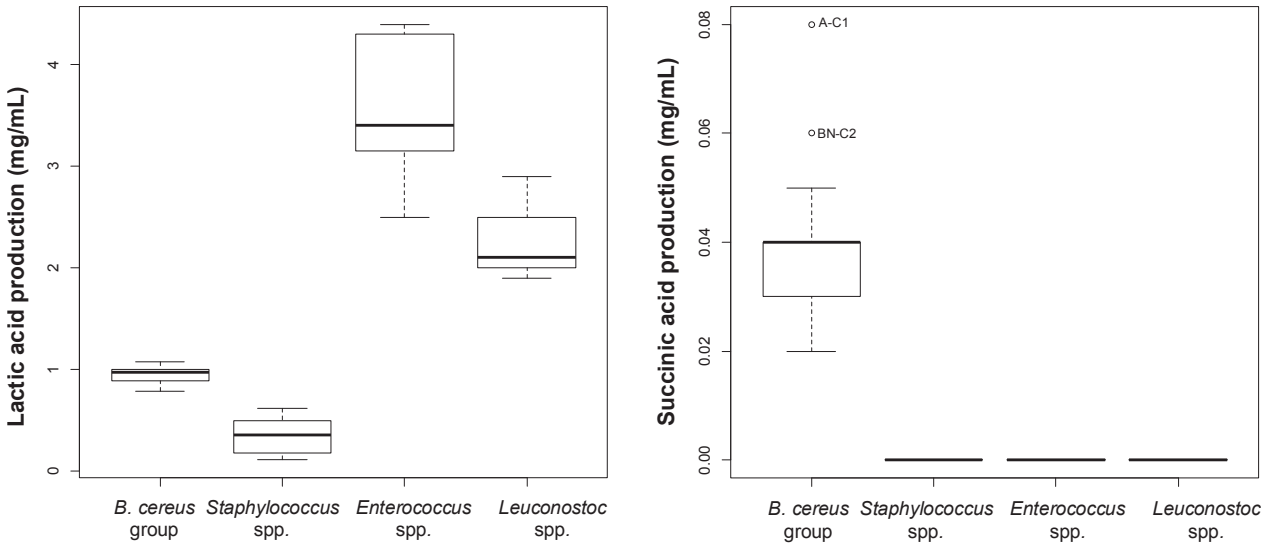


Fig. 5. Boxplots relative to the production of lactic and succinic acids in custard creams contaminated with *B. cereus* group, *Staphylococcus* spp., *Enterococcus* spp., or *Leuconostoc* spp. isolates and incubated for 21 d at 10 °C.

Staphylococcus spp., *Enterococcus* spp. and *Leuconostoc* spp., negatively associated with PC1. *B. cereus* group was associated with a high proteolysis, phospholipolysis, and the production of a range of compounds including esters, compounds derived from sugar metabolism such as succinic acid, and compounds derived from amino acid catabolism, such as 2- and 3-methylbutanoic acid, 2-methylpropanol and sulfur-containing compounds (dimethyl disulfide, dimethyl trisulfide and mercaptoacetone). In the *B. cereus* group, a subgroup of 8 isolates (A-C1, BK-C9, BL-C9, BM-C3, BN-C2, BO-C4, BP-C2 and BR-10) was particularly associated with the production of branched-chain volatile compounds, while a second subgroup of 6 isolates (L-C6, L-C9, K–C3, N–C3, O–C3 and AM-C1) was characterized by their inability to use sucrose.

The second PC distinguished *Staphylococcus* spp. from lactic acid bacteria. All but one of the *Staphylococcus* spp. isolates were associated with the production of phenolic compounds, whereas the remaining *Staphylococcus* isolate was associated with high concentrations of branched-chain aldehydes (Fig. 4). *Enterococcus* spp. and *Leuconostoc* spp. isolates were mainly associated with (i) high levels of compounds derived from sugar metabolism, such as diacetyl, acetoin, 2, 3-pentanedione, acetic and lactic acids, (ii) high pH decrease and (iii) sensory defects such as occurrence of yoghurt-odours and custard cream phase shift (Fig. 6).

4. Discussion

The control of spoilage bacteria is as a key challenge for reducing economic losses and food waste, which are among the most significant factors that impact food availability worldwide (FAO, 2013). The objective of this study was to identify the main bacteria involved in the spoilage of custard cream-based desserts and to investigate their spoilage potential.

This study showed that the main custard cream spoilage bacteria were Gram-positive including the *B. cereus* group, coagulase negative staphylococci, and lactic acid bacteria.

In a previous study on spoiled custard cream, the same species were also identified amongst Gram-positive bacteria, but the dominant species was the psychrotrophic Gram-negative *Achromobacter denitrificans,* which was detected in 6 of 7 analyzed samples (Arakawa et al., 2008). These bacteria are also known as major food spoilers in other food products, such as milk, fish, meat and derived products (Casaburi et al., 2015; Remenant et al., 2015; Ternstrom et al., 1993).

The microbiological quality of custard cream-based desserts was recently presented in the studies of Puangburee et al. (2016), Amin (2018), and Park et al. (2017). The first two publications were in agreement with our study, highlighting high prevalence of *B. cereus* group bacteria after storage of the creams (45 and 32% of positive samples in the studies of Puangburee et al. (2016) and Amin (2018), respectively). Acceptable levels of bacterial concentrations were highlighted, according to the corresponding public health guidelines. In Thai pandan custard-filled products, the bacterial concentration was no more than 2 log CFU/mL after 2 days at 30–32 °C (Puangburee et al., 2016). Lower levels, in the range of less than 1 to 2 log CFU/mL were reported by Amin (2018) after custard-based desserts analysis collected from different dairy and Pastry shops in Egypt. In these studies, the relatively low levels of *B. cereus* group bacteria may explain the fact that they did not focus on spoilage issues, contrary to what we argue in the present study. It is important to underline that the types of creams differ from one study to another, and consequently the type of contaminants and their behavior. It is particularly difficult to analyze our results in light of the study of Park et al. (2017) who demonstrated the good microbial quality of custard tarts thanks to the baking process, which not only kills pathogens but also creates a surface too dry to support microbial growth. These conditions differ quite considerably from the processing conditions of the French custard cream dessert.

In the present study, bacterial diversity was relatively low in spoiled custard cream-based desserts, since only 1 to 3 genera were identified in the majority of products tested. Similar results have been reported in spoiled foodstuffs such as mozzarella cheese, salads, and boiled eggs (Pothakos et al., 2014). Conversely, a high bacterial diversity, with more than 8 bacterial genera in the same batch, was reported in spoiled foods such as milk, meat, and derived compounds (Ercolini et al., 2009; Mace et al., 2013). Bacterial diversity is highly dependent on specific factors, such as food composition, process, and storage conditions. The low bacterial diversity observed in custard cream could be ascribed to the process of production, which comprises a thermal treatment which decreases the initial bacterial load, and which generates a lower risk of microbial contamination compared to the production of other foodstuffs such as those derived from meat or fish.

The presence of a complex spoilage flora complicates the clarification of the role of each group in a spoilage event. In the present study, the spoilage potential of the three groups was thus further evaluated by characterizing several isolates of each groups for their ability to induce chemical and sensorial changes in custard cream at low temperature.

Table 3
Production of volatile compounds in the custard creams inoculated with isolates of the *B. cereus* group, *Staphylococcus* spp., *Enterococcus* spp., and *Leuconostoc* spp., and incubated for 21 d at 10 °C. Results are expressed as the relative abundance of each volatile compound in the spoiled custard cream, calculated as the ratio of the abundance of the volatile compound in the spoiled custard cream and that of the control (sterile custard cream). Values in bold were considered as significantly different from the control ($p < 0.05$).

	Groupe B. cereus									
	A-C1	AM-C1	BK-C9	BL-C9	BM-C3	BN-C2	BO-C4	BP-C2	BR-C10	K–C3
Sugar catabolism										
2-Hydroxypropanone	**18**	**15**	**21**	**27**	**24**	**21**	**20**	**32**	**24**	**27**
1-Hexanol	**20**	**18**	**40**	**40**	**38**	**42**	**43**	**56**	**50**	**18**
2,3-Butanedione (diacetyl)	**4**	1	**2**	**3**	**3**	**2**	**3**	2	**2**	2
3-Hydroxy-2-butanone (acetoin)	**11**	2	**5**	**5**	**5**	**4**	**5**	**4**	**4**	**5**
Hexanoic acid	**3**	2	**4**	**5**	**5**	**3**	**3**	**4**	**5**	**3**
1-Propanol	**44**	**16**	**14**	**15**	**14**	**15**	**16**	**49**	**46**	**18**
2,3-Pentanedione	3	1	**5**	**5**	**4**	**4**	**5**	**5**	3	2
1-Butanol	**42**	**41**	**69**	**71**	**69**	**72**	**80**	**126**	**133**	**54**
2-Butanone	**2**	1	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**2**
Butanoic acid	3	**5**	3	**5**	**5**	3	4	3	5	5
Acetone	2	1	**2**	**3**	**2**	**2**	**2**	1	1	2
Acetic acid	1	2	1	1	1	1	1	1	1	1
Amino acid catabolism										
Benzaldehyde	0	0	0	0	0	0	0	0	0	0
3-Methylbutanoic acid	**61**	**18**	**77**	**107**	**75**	**65**	**52**	**60**	**101**	**27**
2-Methylbutanoic acid	**22**	**4**	**74**	**146**	**91**	**68**	**67**	**31**	**179**	**16**
2-Methylpropanal	**11**	**4**	**5**	**6**	**5**	**5**	**5**	**13**	**12**	**4**
1-Mercapto-2-propanone	**13**	2	**473**	**519**	**479**	**515**	**504**	**6**	**7**	**9**
3-Methylbutanal	2	1	**2**	**3**	2	**3**	**3**	**2**	**3**	2
Dimethyl disulfide	2	1	**12**	**17**	**17**	**13**	**13**	3	2	2
Dimethyl trisulfide	1	1	**8**	**9**	**9**	**7**	**6**	2	2	1
2-Methylbutanal	1	1	**4**	**5**	**3**	**5**	**3**	2	2	2
2-Methylbutanol	1	1	1	2	1	2	1	1	2	2
Esters										
Ethyl propanoate	**11**	**8**	**7**	**7**	**6**	**8**	**8**	**25**	**25**	**7**
Ethyl butanoate	**9**	**4**	**9**	**10**	**9**	**10**	**9**	**12**	**10**	**10**
Ethyl hexanoate	**6**	**5**	**8**	**8**	**8**	**9**	**8**	**8**	**7**	**7**
Ethyl 3-hydroxybutanoate	**139**	**53**	**280**	**299**	**297**	**279**	**300**	**179**	**179**	**158**
Ethyl decanoate	**4**	**2**	**3**	**3**	**3**	**3**	**3**	**4**	**3**	**3**
Propyl acetate	1	2	**2**	**2**	**2**	**2**	**2**	**2**	**3**	**2**
Fatty acid catabolism										
2-Heptanol	1	**5**	1	1	1	1	1	1	1	1
2-Undecanone	1	1	**2**	**2**	**2**	**2**	**2**	1	1	1
a										
Phenol	1	1	1	2	2	1	1	1	2	2
2-methoxyphenol (guaiacol)	1	1	1	1	1	1	1	1	4	1

	Groupe B. cereus				Staphylococcus spp.						
	L-C6	L-C9	N–C3	O–C3	AO-C3	AO-C8	AP1-C2	AV2-C1	AY-C4	AY-C5	S–C4
Sugar catabolism											
2-Hydroxypropanone	**27**	**30**	**12**	7	**68**	**38**	**41**	**45**	**44**	**42**	**25**
1-Hexanol	**17**	**16**	**16**	**18**	1	2	1	1	1	1	1
2,3-Butanedione (diacetyl)	1	2	1	1	**3**	**3**	**4**	**4**	**4**	**4**	2
3-Hydroxy-2-butanone (acetoin)	**5**	**6**	1	1	2	2	2	2	2	3	3
Hexanoic acid	2	2	2	2	2	**3**	2	2	1	2	1
1-Propanol	**18**	**19**	**14**	**15**	1	1	1	1	1	1	1

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Table 3 (*continued*)

	Groupe B. cereus				Staphylococcus spp.						
	L-C6	L-C9	N–C3	O–C3	AO-C3	AO-C8	AP1-C2	AV2-C1	AY-C4	AY-C5	S–C4
2,3-Pentanedione	2	3	1	1	**10**	**9**	**10**	**10**	**11**	**11**	1
1-Butanol	**53**	**52**	**33**	**40**	0	3	0	0	0	0	**12**
2-Butanone	**2**	**2**	1	1	1	1	1	1	1	1	1
Butanoic acid	3	2	3	4	3	**7**	**4**	4	1	2	0
Acetone	2	2	1	1	**2**	**2**	**2**	**2**	**2**	**2**	1
Acetic acid	1	1	1	1	1	1	1	1	1	1	1
Amino acid catabolism											
Benzaldehyde	0	0	0	0	0	0	0	0	0	0	1
3-Methylbutanoic acid	**16**	**19**	**14**	**11**	**17**	**23**	**15**	**11**	**6**	**7**	2
2-Methylbutanoic acid	**5**	**9**	**4**	**3**	**28**	**36**	**13**	**8**	**4**	**4**	2
2-Methylpropanal	**4**	**4**	**3**	**4**	0	0	0	0	0	0	**54**
1-Mercapto-2-propanone	**9**	**10**	2	2	2	2	2	1	2	1	2
3-Methylbutanal	2	2	1	1	1	1	1	1	1	1	**365**
Dimethyl disulfide	2	2	0	0	0	0	0	0	0	0	1
Dimethyl trisulfide	1	1	0	2	0	0	0	0	0	0	1
2-Methylbutanal	2	2	0	0	0	1	1	0	1	1	**113**
2-Methylbutanol	1	1	2	1	1	4	1	1	1	1	**4**
Esters											
Ethyl propanoate	**7**	**8**	**7**	**8**	**3**	**3**	**3**	**3**	**2**	**3**	1
Ethyl butanoate	**11**	**11**	**4**	**4**	1	1	1	1	1	1	1
Ethyl hexanoate	**8**	**8**	**4**	**5**	1	1	1	1	1	1	1
Ethyl 3-hydroxybutanoate	**152**	**156**	**49**	**48**	1	4	4	3	5	4	1
Ethyl decanoate	**3**	**3**	**2**	**2**	1	1	1	1	1	1	1
Propyl acetate	**2**	**3**	1	2	1	2	1	1	1	1	1
Fatty acid catabolism											
2-Heptanol	1	1	**4**	**5**	1	1	1	1	1	1	1
2-Undecanone	1	1	1	1	1	1	1	1	1	1	1
a											
Phenol	2	2	1	1	**28**	**28**	**8**	**7**	**9**	**8**	1
2-methoxyphenol (guaiacol)	1	2	1	1	**44**	**47**	**8**	**6**	**8**	**6**	1

	Enterococcus spp.							Leuconostoc spp.		
	AK-C8	AL-C3	BP-C8	G-C6	P–C9	S–C5	S–C7	AJ-C2	AL-C10	G-C10
Sugar catabolism										
2-Hydroxypropanone	**11**	**9**	**11**	**21**	1	**7**	**7**	**6**	1	1
1-Hexanol	**4**	**4**	**4**	**8**	**3**	**14**	**16**	**5**	**4**	**5**
2,3-Butanedione (diacetyl)	**22**	**29**	**26**	**13**	**43**	**21**	**23**	**14**	**13**	**10**
3-Hydroxy-2-butanone (acetoin)	**50**	**46**	**52**	**26**	**39**	**30**	**39**	**5**	**6**	**1**
Hexanoic acid	**5**	**5**	**3**	**4**	**3**	**4**	**4**	**5**	**5**	**5**
1-Propanol	**4**	**5**	**4**	**2**	1	**2**	**2**	1	1	1
2,3-Pentanedione	**14**	**52**	**19**	1	1	**54**	**60**	**15**	**12**	1
1-Butanol	1	1	1	1	1	1	1	1	1	0
2-Butanone	1	1	1	**2**	1	**4**	**4**	1	1	1
Butanoic acid	**5**	**5**	4	**5**	4	**5**	4	**4**	**4**	**5**
Acetone	1	1	1	1	1	2	2	1	1	1
Acetic acid	**4**	**4**	**2**	**6**	**5**	**6**	**6**	**35**	**26**	**52**
Amino acid catabolism										
Benzaldehyde	0	0	1	**2**	1	**2**	**2**	0	0	0
3-Methylbutanoic acid	3	3	4	**4**	2	2	3	**6**	**3**	**4**
2-Methylbutanoic acid	2	2	3	2	3	2	2	**4**	3	3
2-Methylpropanal	1	2	1	2	0	**3**	**4**	0	0	0

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Table 3 (*continued*)

	Enterococcus spp.						Leuconostoc spp.				
	AK-C8	AL-C3	BP-C8	G-C6	P-C9	S-C5	S-C7	AJ-C2	AL-C10	G-C10
1-Mercapto-2-propanone	**3**	2	2	**6**	**4**	**7**	**6**	2	2	1
3-Methylbutanal	2	2	**2**	**2**	**3**	**2**	**2**	1	1	1
Dimethyl disulfide	2	1	1	**6**	1	**16**	**12**	1	1	1
Dimethyl trisulfide	1	1	0	4	0	**8**	**6**	1	0	1
2-Methylbutanal	1	1	1	1	1	1	1	0	0	0
2-Methylbutanol	1	1	1	1	1	1	1	1	1	1
Esters										
Ethyl propanoate	**3**	2	**2**	**6**	**2**	2	**2**	1	1	1
Ethyl butanoate	1	1	1	1	1	**2**	**2**	1	1	1
Ethyl hexanoate	2	2	2	2	2	2	2	1	2	**2**
Ethyl 3-hydroxybutanoate	1	1	3	1	1	0	2	3	1	1
Ethyl decanoate	1	1	1	1	1	1	1	1	1	1
Propyl acetate	1	1	1	1	1	1	1	**2**	**2**	1
Fatty acid catabolism										
2-Heptanol	2	**3**	2	1	1	1	**2**	1	1	**3**
2-Undecanone	1	1	1	1	1	1	1	1	1	1
a										
Phenol	1	1	1	1	1	1	2	1	1	1
2-methoxyphenol (guaiacol)	1	1	1	1	1	1	1	1	1	1

The custard creams spoiled by *B. cereus* group isolates were characterized by bacterial populations > 7 log CFU/mL, small (< 0.9) pH decreases and the development of off-odours (Table 2). These defects are correlated with the ability of the *B. cereus* group isolates to use sugars and proteins as substrates, and to produce a range of volatile and non-volatile metabolites. Unexpectedly, most *B. cereus* group isolates (93%) used lactose as carbon source. Previous findings on strains of milk or dairy origin suggested low occurrence (less than 20%) of lactose positive *B. cereus sensu lato* (De Jonghe et al., 2010; Ivy et al., 2012; Te Giffel et al., 1997). This difference may be ascribed to the fact that conditions such as temperature can impact the β-galactosidase activity in the *B. cereus* group, as suggested by Ivy et al. (2012). These authors indicated that a *Bacillus weihenstephensis* strain (belonging to the *B. cereus* group) isolated from milk exhibited β-galactosidase activity, specifically at low temperature, as previously shown for *Planoccocus* spp. (Hu et al., 2007). In the present study, lactose consumption was tested *in situ* in the food itself and at 10 °C rather than at 30–37 °C in an agar-based medium. Lactic acid is one of the main fermentation products of *B. cereus* group bacteria grown under anaerobic conditions (Wang and Wang, 2002). The production of lactic acid has been associated with a pH decrease and the formation of acidulated flavours and odours by Montel et al. (1998). The volatile compound profile in custard cream spoiled by *B. cereus* group was globally characterized by the presence of high amounts of primary alcohols, esters, and products from the catabolism of sulfur and branched-chain amino acids. These results are the first report, to our knowledge, concerning volatile compounds produced by *B. cereus* group isolates despite their frequent involvement in food spoilage issues. Some of these volatiles are odorant molecules possibly responsible for the off-flavours detected in spoiled custard creams. On the basis of their relative intensity, 1-propanol, 1-butanol and 1-hexanol can be regarded as the main spoilage molecules produced from sugar by *B. cereus* group isolates. To our knowledge, these alcohols, associated with fermented, fruity, alcoholic and/or green flavor descriptors (Curioni and Bosset, 2002), have never been highlighted in custard cream-based dessert or any dairy products contaminated with *B. cereus* group cells. Rather, they are frequently highlighted in spoiled meat and fish products (Hernandez-Macedo et al., 2012; Jaaskelainen et al., 2013; Jaffres et al., 2011). *B. cereus* group isolates also hydrolysed custard cream proteins and produced a range of odorant compounds likely derived from amino acid catabolism (Fig. 3, Table 3). Branched-chain acids derive from leucine, isoleucine, and valine degradation (Yvon and Rijnen, 2001) and are associated with fruity or cheesy odours (Curioni and Bosset, 2002; thegoodscentscompany.com). Sulfur volatile compounds (1-mercapto-2-propanone, dimethyl disulfide and dimethyl trisulfide) derive from sulfur amino acid catabolism and are associated with sulfurous, cabbage and/or onion-like odours (Curioni and Bosset, 2002). A range of ethyl esters were also produced in custard cream by *B. cereus* group bacteria. They result from enzymatic esterification of carboxylic acids with ethanol and are mainly associated with fruity odours (Collins et al., 2003). Most esters were produced by all the *B. cereus* group isolates, suggesting that the ester-synthetizing activity is a common characteristic of this group. Moreover, most *B. cereus* isolates exhibited a phopholipolytic activity towards custard cream phospholipids (Fig. 3), in agreement with previous reports (Mossel et al., 1967). In milk, the phospholipolytic activity of *B. cereus* group bacteria leads to fat globule destabilization, known as “bitty cream” defect (Meer et al., 1991), but such a visual defect is difficult to assess in custard cream.

Custard cream spoilage by *Staphylococcus* was characterized by very high (> 8 log CFU/mL) bacterial load, a slight pH decrease (< 0.4), the development of off-odours, lactose decrease, the production of phenolic compounds and branched-chain acids (Table 3). All the metabolites identified in this study had previously been reported in meat fermented by coagulase-negative *Staphylococcus* (Montel et al., 1998; Olesen et al., 2004; Sondergaard and Stahnke, 2002; Stahnke, 1999), except for 2-methoxyphenol. The latter, also referred to guaiacol, is associated to

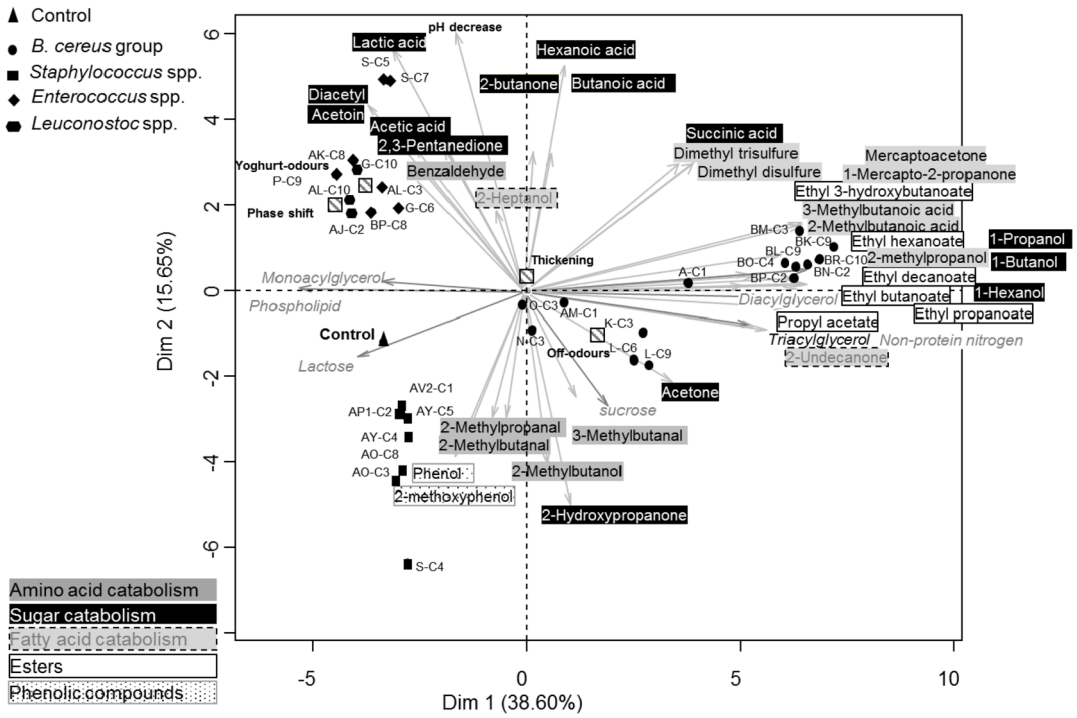


Fig. 6. Biplot of principal component analysis (PCA) of sensory and chemical custard cream changes after contamination by *B. cereus* group, *Staphylococcus* spp., *Enterococcus* spp., or *Leuconostoc* spp. isolates. The compounds resulting from bacterial spoilage are classified according to their chemical nature or their putative metabolic origin.

medicinal, woody, smoky odours and responsible for odour defects in a variety of spoiled foods and beverages. Guaiacol production likely results from the bioconversion of vanillin, an ingredient of the commercial custard creams used in this work, which has been described in various bacteria including bacilli and staphylococci (Ito et al., 2016). Similarly, *Alicyclobacillus* spp. produces guaiacol from vanillin (Bahceci et al., 2005) and vanillic acid (Niwa and Kuriyama, 2003).

Lactic acid bacteria reached very high populations (> 8.4 log CFU/mL) and their growth was accompanied by a sharp pH decrease (> 1.4) and the development of a marked yoghurt-like odour (Table 2). The change in odour likely results from the production of diacetyl and acetoin, compounds that are associated with buttery, creamy odours (Casaburi et al., 2015; thegoodscentscompany.com). *Enterococcus* spp. and *Leuconostoc* spp. are able to produce diacetyl and acetoin from citrate fermentation (Quintans et al., 2008). Citrate is present in milk, the main ingredient of custard cream, at a concentration of approximately 1.5 g/L (McSweeney and Sousa, 2000). Citrate metabolism varies from one species to another and, within a species, from one strain to another (Bourel et al., 2001; Devoyod and Poullain, 1988). In our study, amongst all isolates studied, two *Enterococcus* spp. isolates (S–C5 and S–C7) and one *Leuconostoc* spp. isolate (G-C10) consumed all the available citrate (data not shown), suggesting that acetoin and diacetyl production could partly come from citrate metabolism.

This study provides a global overview of custard cream spoilage events and highlights some specific features of the three groups of bacteria responsible. Amongst the biochemical changes documented in this study, lactic acid production was the sole common feature shared by all the isolates tested, even if produced at greater amounts by *Enterococcus* spp. and *Leuconostoc* spp. isolates. Therefore, lactic acid is a relevant candidate marker of custard cream spoilage, as previously reported in other foods such as pork, beef, lamb meats and meat products (Borch et al., 1996; Dainty, 1996). The validation of this marker should include further work to evaluate if the metabolite fulfill all the requirements for spoilage assessment. According to Jay (1986), a metabolite that can be used for spoilage assessment has to be (i) absent or at least present at only low levels in the market food, (ii) increasing during its storage, and (iii) be produced by the dominant flora and in good correlation with the sensory score.

Supported by a PCA analysis performed on data from the metabolism *in situ* of a large collection of spoilage bacteria, our study suggests that the metabolome allows assigning custard cream spoilage to specific bacteria groups (Fig. 6). The detection of high quantities of 1-butanol or esters, such as ethyl 3-hydroxybutanoate, could predict spoilage by *B. cereus* group bacteria. The presence of phenolic compounds could indicate staphylococci-related spoilage, whereas the detection of high quantities of diacetyl/acetoin could indicate spoilage due to *Enterococcus* spp. or *Leuconostoc* spp. and perhaps other lactic acid bacteria. Testing more isolates would strengthen the relevance of these potential spoilage markers.

The detection of spoilage markers in combination with sensory analysis would offer an attractive quality control approach for food dessert manufacturers, who need objective criteria (i) for setting the expiry date of their products and (ii) to anticipate potential claims from customers through accelerated shelf-life testing. Some specific spoilage markers have been described for other foodstuffs, such as fish and meat. For example, the spoilage of cold-smoked salmon by *Carnobacterium piscicola* has been associated with the production of diacetyl, 2,3-pentanedione, and a butter odour (Joffraud et al., 2001). In spoiled meat, the presence of *Carnobacterium maltaromaticum*, *Pseudomonas fragi* and *Serratia proteamaculans* has been correlated to the production of 2-ethyl-1-hexanol, decanal, and ethyl decanoate, respectively (Ercolini et al., 2009). Due to the complex nature and evolution of microbial volatile spoilage compounds, as well as the wide range of processing, packaging and storage conditions of the same food worldwide, it is difficult to imagine that a common single marker would be associated with the spoilage of a specific type of food. The alternative consisting in multiple instead of single compound analysis could enhance the efficiency of spoilage detection and further facilitate the design of new analytical tools for early spoilage detection.

In conclusion, the present study contributes substantially to enhance knowledge about the mechanisms involved in the spoilage of chilled custard cream-based desserts by different bacteria. By highlighting

relevant and specific spoilage markers, it represents the first step towards the development of prediction tools and the implementation of effective prevention of spoilage.

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