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Challenging the problematic detection of clostridial isolates causing late-blowing defect with MALDI–TOF MS

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Abstract: The present study aimed to evaluate the *Clostridium* spp. counts in corn silage, raw milk and Kaşar cheese and to identify the clostridial isolates causing a late-blowing defect (LBD) potential using matrix-assisted laser desorption-ionization-time of flight mass spectrometry (MALDI-TOF MS). Lactate-fermenting clostridial spores (LFCS) were determined by the most probable number method (MPN) in 14 samples of corn silage, 12 samples of raw milk and 20 samples of Kaşar cheese. 181 isolates were obtained from MPN gas-positive tubes. Gram staining, catalase and oxidase activity, anaerobic development tests and Scanning Electron Microscopy (SEM) imaging showed that 95 isolates were typical clostridial bacteria. Sixty-six isolates could maintain viability during the passage and stock stages. A confirmatory identification technique was then performed using MALDI-TOF MS. The results revealed that (49 out of 66 isolates) of bacteria were correctly identified as 38 (77.55%) *Clostridium sporogenes*, 6 (12.24%) *Clostridium butyricum*, 3 (6.12%) *Clostridium beijerinckii*, 1 (2.04%) *Clostridium bifermentans* and 1 (2.04%) *Clostridium sartagoforme.* This study determined that clostridial isolates that cause LBD can be identified successfully and quickly by MALDI--TOF MS, a novel method for detecting anaerobic bacteria.

Keywords: butyric bacteria; cheese; *Clostridium* spp.; MALDI-TOF spectrometry; rapid identification

Clostridial spores can survive cheese-making processes and turn into vegetative cells that metabolise lactate during ripening. This includes the production of organic acids, butyric acid and CO_2-H_2 gas as the main fermentation products (Gómez-Torres et al. 2014). It is known that they are the primary agent of the problem known as a late-blowing defect (LBD), which is seen in the production of hard and semi-hard cheeses. In particular, the insolubility of H_2 leads to gas accumulation and eruptions during cheese ripening, forming large pores, cracks and crevices in the cheese, followed by blowing and the formation of unwanted odours (D'Incecco et al. 2018). Raw milk may contain more than one species of *Clostridium*, but not all clostridia affect the cheese quality (Zangerl 1993). Some authors stated that the spores belonging to species of *Clostridium* like *Clostridium tyrobutyricum*, *C. butyricum*, *C. sporogenes* and *C. beijerinckii*, which are the species most frequent-

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ly involved in the occurrence of LBD in cheese, can be found in milk (Arias et al. 2013).

Clostridial spores in milk mainly come from silage (Reindl et al. 2014). The silage plant may be contaminated with *Clostridium* spp. when harvested and dairy cows are fed contaminated silage, the spores pass from the gastrointestinal tract to the faeces, and manure can contaminate the teats during milking (Arnesson et al. 2011). It is believed that ensuring silage quality is important in preventing *Clostridium* spp. contaminating cheese, milk and cheese processing during fermentation (Vissers et al. 2007). LBD *Clostridium* can lead to considerable product loss, particularly in hard and semi-hard cheeses salted in brine, causing the formation of a rancid taste and resulting in large economic losses for businesses (Brändle et al. 2016; Silvetti et al. 2018).

LBD clostridia are very sensitive to oxygen, so their separation, cultivation and counting must be done in a short period without exposure to oxygen.

Alternatives to the conventional method used to detect clostridial isolates are PCR-based molecular techniques (Kaya et al. 2022) and proteomics-based methods like Matrix-assisted laser desorption–ionization-time of flight mass spectrometry (MALDI-TOF MS) (Burtscher et al. 2020; Podrzaj et al. 2020). The advantages and performance of MALDI-TOF MS over existing methods have been highlighted in several studies with MALDI-TOF MS have shown that the system is high-speed, requires very few samples, and applies to the identification of many microorganisms, including anaerobes (Chean et al. 2014; Hsu and Burnham 2014; Almogbel 2016; Schaumann et al. 2018).

To identify the LBD factor induced by clostridial species in cheese faster and more easily, innovative identification methods with high sensitivity and specificity have been required. The study aimed to identify clostridial isolates causing LBD obtained by phenotypic methods following the enumeration of lactate-fermentation clostridial spores (LFCS) populations in silage, milk and Kaşar cheese by employing MALDI-TOF MS.

MATERIAL AND METHODS

Sample collection. Considering silage nutrition and seasonal effects, a total of 46 isolate materials, including corn silage (n = 14), raw milk (n = 12) and Kaşar cheese (n = 20) from farms after the third (December) and ninth (July) months in a silo, were collected in Türkiye. Late-blown cheese from the milk factory was sampled and analysed. Raw milk and silage samples were taken from the farms that supply milk to the milk factory where the late-blown cheese was produced. pH measurements were measured using a pH meter (SG23-FK2; Mettler Toledo, Switzerland).

Lactate fermenting clostridial spore (LFCS) counting. LFCS counts in raw milk, corn silage and Kaşar cheese were determined by the most probable number (MPN) method. Dilutions of raw milk samples were done with sterile 0.1% (w/v) peptone water in test tubes for each dilution. Ten grams of Kaşar cheese and corn silage samples were transferred to a stomacher bag and homogenised for 4 min in a laboratory stomacher blender (BagMixer®400; Interscience, France). Serial dilutions of the whole samples were inoculated into a series of three tubes containing 10 mL of Bryant Burkey Broth with Resazurine and Lactate (BBB; Merck, Darmstadt, Germany) and covered with sterile melted paraffin 2 cm thick (approximately 3 mL) to create an anaerobic condition. They were then heated to 80 °C for 10 min to kill vegetative cells. The MPN tubes were incubated at 37 °C for 7 days and tested daily for gas production. When evaluating the results, gas formation at the end of the incubation period was counted as positive points, while MPN numbers were expressed as log MPN·mL^{-1} or log MPN·g^{-1} (Garde et al. 2011).

Characterisation of clostridial isolates. For the isolation of clostridial strains, 0.1 mL of sample was taken from each BBB gas positive tube, then inoculated in Reinforced Clostridial Agar (RCA; Merck, Germany) using the riding and double-layer method. After this process, isolates were incubated for 72 h at 37 °C by an anaerobic kit (Anaerocult A; Merck, Germany) under anaerobic conditions. At the end of incubation, the typical colonies were selected. The colonies were re-inoculated into the BBB medium and incubated in anaerobic conditions for a further 7 days at 37 °C. Each bacterial isolate was replicated in two Petri dishes. Possible colonies were inoculated using the double-layer media method and left to incubate in an anaerobic jar using an anaerobic kit for up to 72 h at 37 °C. Isolates from each colony with a different morphology were randomly selected from each sample and again cultivated on RCA using the same conditions. Single colonies were applied from the typical colonies to the RCM media to ensure pure culture. (Garde et al. 2011). Then, only isolates forming Gram-positive, catalase and oxidase negative, and obligate or facultative anaerobic endospores were studied in detail.

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Endospore morphology. Clostridial isolates were incubated anaerobically in BBB at 37 °C for 3 days. Cells were washed twice in phosphate-buffered saline (PBS) solution and were centrifuged at 5000 rpm and 4 °C for 10 min each time. The suspension concentrations of spores varied by 10^7 to 10^9 spore·mL^{-1}. Scanning Electron Microscopy (SEM) (Quanta FEG 250; FEI, Eindhoven, The Netherlands) was used to study the shape of the bacteria and determine the morphology of spore structures. Observations were made using a gold-plating technique under low vacuum conditions.

The ability of clostridial isolates to produce gas. Clostridial isolates were inoculated into BBB for gas capacity testing. Inoculated tubes were sealed with sterile paraffin and incubated at 37 °C for 3 days. After incubation, the tubes were visually tested for gas production and measured paraffin plug displacement (cm).

MALDI-TOF MS analysis. The resulting pure cultures were stored in the BBB at 37 °C for 3 days. To prepare the samples and extract bacterial proteins, the ethanol-formic acid extraction procedure was used. A 1 mL of liquid culture was taken out and centrifuged at 14 000 g for 2 min to harvest the cells. The remaining precipitate was washed twice with sterile distilled water and air-dried for 10 min. The cells were lysed with 70% formic acid (the volume used was proportional to the size of the cell pellet), and an equal amount of acetonitrile was added. The mixture was homogenised entirely by using a pipette. The mixture was centrifuged for a second time at 14 000 g for 2 min, and 1 µL of supernatant was transferred to an MTP 384 Ground Steel Target (No. 8280784; Bruker, Germany). For each aliquot taken, a total of four sampling spots were analysed. After the sample spots were air-dried, they were coated with HCCA matrix solution [10 mg·mL^{-1} α-cyano-4-hydroxycinnamic acid (αCHCA), Bruker] and dried again before testing. Mass spectrometry analysis was conducted using MALDI-TOF MS (Autoflex Speed; Bruker Daltonics, Germany) and the MALDI Biotyper 3.1 software package. The results of the comparative evaluation of the received spectra with the reference spectra were expressed as the confidence score values.

Statistical analysis. Data analyses were accomplished using the Minitab (17. version) software program at a 95% confidence level. The Tukey test was used to group the samples according to the determined significance level. To determine the effect of the pH value of the isolate samples on the MPN values, a correlation test was performed. A statistical comparison of the proportional distribution of isolate's gas production between data values was used the Mann-Whitney U and Kruskal-Wallis H test method.

RESULTS AND DISCUSSION

The results of this study show that the value of the corn silage samples ranged from 0.84 to 1.32 log MPN·g^{-1} (r = 0.913, P < 0.05) and from 1.30 to 2.32 log MPN·g^{-1} (r = 0.797, P < 0.05); the value of Kaşar cheese samples ranged from 1.30 to 2.32 log MPN·g^{-1} (r = 0.853, P < 0.05) and from 1.30 to 3.04 log MPN·g^{-1} (r = 0.790, P < 0.05) in December and July, respectively (Table 1). The MPN values of raw milk samples taken in December ranged from 5.96 to 6.32 log MPN·mL^{-1} and from 5.87 to 6.66 log MPN·mL^{-1} in the samples taken in July. A strong positive correlation was observed between the pH and MPN values of the samples. The highest LFCS counts were determined in the corn silage and raw milk sample obtained in July. This increase in LFCS count may be due to the pH increase with high

Table 1. The pH and LFCS count of corn silage, raw milk and Kaşar cheese samples

Material	Dec		July	
	pH	log MPN	pH	log MPN
Corn silage	4.14 ± 0.02	1.32	5.38 ± 0.04	1.59
	3.93 ± 0.04	1.04	3.97 ± 0.02	1.30
	3.91 ± 0.01	1.04	5.10 ± 0.01	1.32
	3.86 ± 0.03	0.84	7.32 ± 0.03	2.32
	3.90 ± 0.01	1.04	6.10 ± 0.02	1.30
	3.89 ± 0.07	0.84	4.18 ± 0.04	1.30
Raw milk	6.67 ± 0.01	6.17	6.59 ± 0.01	6.32
	6.61 ± 0.02	5.96	6.78 ± 0.00	6.66
	6.77 ± 0.03	6.17	6.78 ± 0.00	6.17
	6.73 ± 0.02	6.17	6.69 ± 0.01	6.32
	6.78 ± 0.00	6.32	6.72 ± 0.01	5.96
	6.66 ± 0.01	6.17	6.65 ± 0.02	5.87
Kaşar cheese	5.72 ± 0.02	2.32	5.91 ± 0.02	2.32
	5.12 ± 0.02	1.32	5.30 ± 0.07	1.32
	5.31 ± 0.02	1.32	5.55 ± 0.15	1.96
	5.22 ± 0.02	1.30	5.14 ± 0.04	1.32
	5.31 ± 0.07	2.17	5.87 ± 0.03	3.04
	5.74 ± 0.05	2.32	5.72 ± 0.03	2.32
	5.84 ± 0.04	2.32	5.67 ± 0.02	1.30

MPN – most probable number method

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Clostridium spores in corn silage samples used for feeding. Some researchers investigating silage-induced milk contamination stated that silages with high spore content should not be used in the diet of dairy cows and that a significant proportion of clostridial spores in raw milk may be passed on from the silages used in animal feeding (Driehuis et al. 2016; Bava et al. 2017). Within the scope of this study, the number of spores found in late-blowing 14 (70%) Kaşar cheeses (> 0.36 log MPN·g^{-1}) in December was 20 to 210 MPN·g^{-1} and in July was 21 to 1 100 MPN·g^{-1}; the reason for the late-blowing problem was caused as a result of raw milk and corn silage containing high numbers of clostridial spores in July, and in Kaşar cheeses considered to be the result of its use in production. The highest value among the Kaşar cheese samples above the MPN limit was found in the Kaşar cheese sample, which had a pH of 5.87 with 3.04 log MPN·g^{-1}. The MPN values of the Kaşar cheese samples increased due to high pH. The authors stated that late-blowing defect could be significantly inhibited by reducing the pH below 5.5 and adding 400 IU·mL^{-1} nisin when the number of clostridial spores is below 300 spore·mL^{-1} in Kaşar cheese (Kaya et al. 2022).

One hundred and eighty-one spherical, with rough edges, slightly raised, granular, off-white, translucent or opaque and smooth colonies were characterised by phenotypic criteria. As a result of endospore staining, endospores of 48 isolates were detected in their central form, and the sporulation process identified competitive flora similar to *Bacillus* spp. It was determined that purification could not be achieved. Borreani et al. (2019) determined that *Clostridium* and *Bacillus* species were abundant in corn silage samples. The SEM showed that the cells were bacilli, subterminal spores and spores larger than the bacterial cell thickness. The length of the cells varied from 3 to 5 µm. Endospores were approximately 1 µm wide and 1.7 µm long (Figure 1). The shape and spacing of the isolates' measured size were determined to agree with the results of other research using gold-plating techniques and buffer solutions (Bassi et al. 2009). Twenty-seven isolates from corn silage, 37 isolates from raw milk, and 31 isolates isolated from Kaşar cheese were total possible 95 typical clostridial bacteria of anaerobic rod-shaped, Gram-positive, endospore-forming, moving, catalase and oxidase negative were selected for further research (Table 2). During isolation, 66 of 95 typical colonies, which could have been *Clostridium* spp., could survive the passage, stock and gas production stage. This suggests the necessity of working in an anaerobic cabinet, especially for anaerobic bacteria with a high sensitivity to oxygen.

The gas production values according to the source of the 66 isolates are shown in Figure 2. The Kruskal-Wallis H test results regarding the gas production capacity of the isolates and the source variable are shown

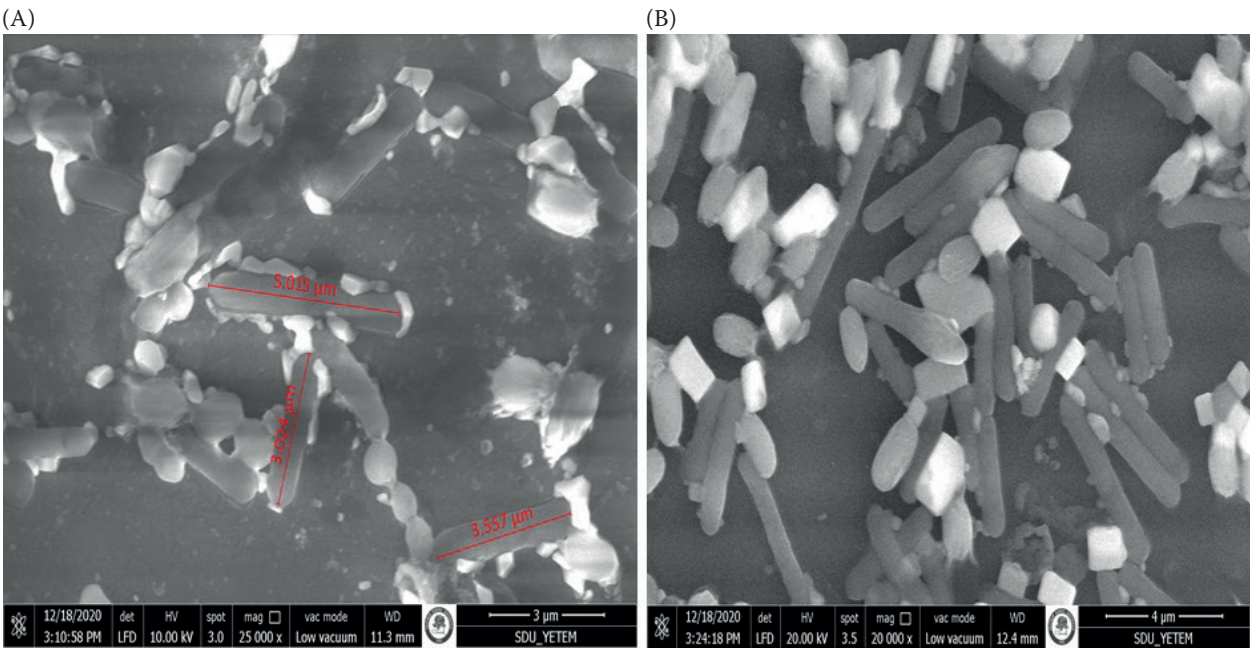

Figure 1. SEM pictures of *Clostridium* spp. under low vacuum with gold coating: (A) *C. sporogenes* PeCls68; (B) *C. butyricum* PeCls81

PeCls – strain code

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Table 2. Biochemical test results for the identification of the *Clostridium* spp.

Sample	Biochemical tests							
	isolates (*n*)	possible genus	microscopic morphology	endospore form	colony type	gram test	catalase test	oxidase test
Corn silage	59/13	other	B	–	R/M	–	–	–
	59/19	*Bacillus* spp.	B	S	M	+	+	–
	59/27	*Clostridium* spp.	B	T	R	+	–	–
Raw milk	76/17	other	B	–	R/M	–	–	–
	76/22	*Bacillus* spp.	B	S	M	+	+	–
	76/37	*Clostridium* spp.	B	T	R	+	–	–
Kaşar cheese	46/8	other	B	–	R/M	–	–	–
	46/7	*Bacillus* spp.	B	S	M	+	+	–
	46/31	*Clostridium* spp.	B	T	R	+	–	–

B – bacilli; + positive reaction; – negative reaction; T – spore form terminal; S – spore form central; M – mucoid colony; R – rough colony

in Table 3. The gas capacity of clostridial isolates obtained from raw milk samples was significantly higher than other isolates ($P < 0.05$). This result suggests that clostridial bacteria with high gas capacity obtained from raw milk may be a more causing factor in LBD. Silvetti et al. (2018) evaluated the effect of different stress conditions on LBD clostridial species' gas production and reduction potential.

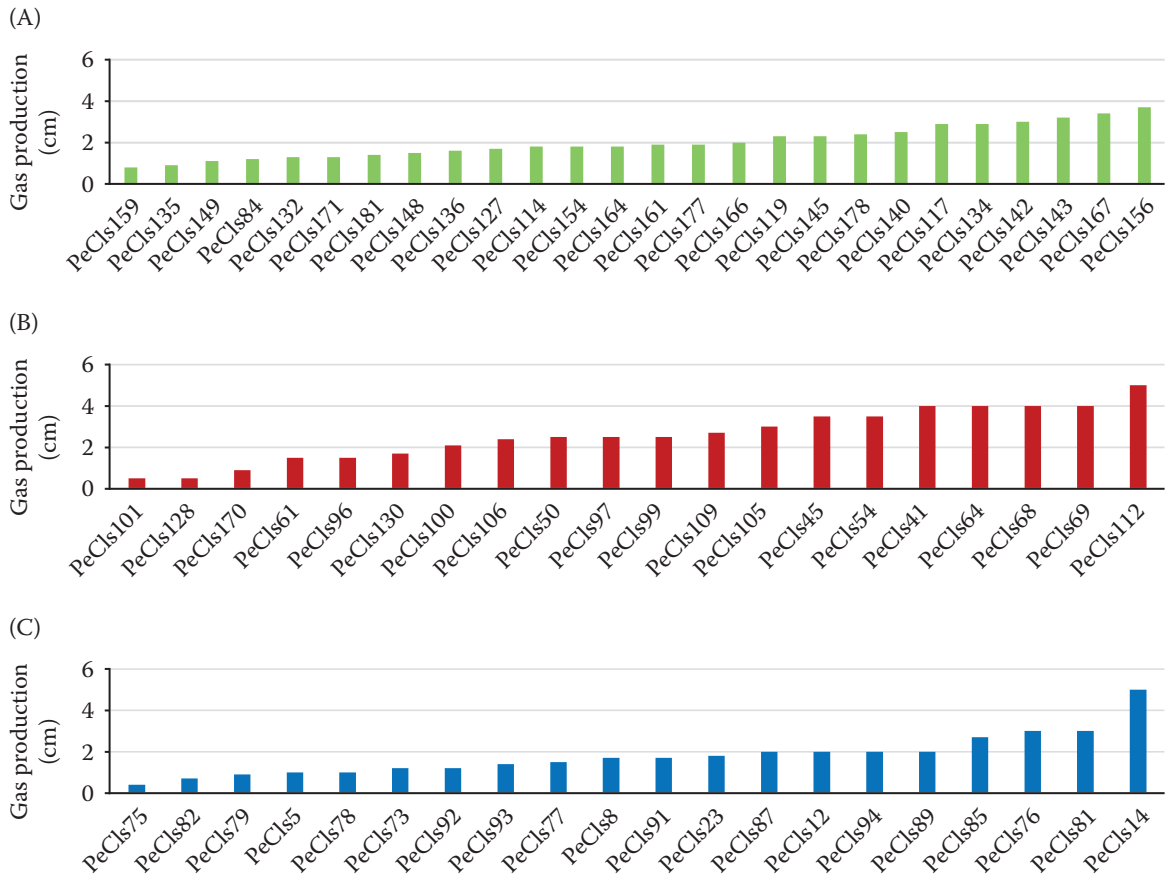

Figure 2. The values of the gas capacity (cm) of the clostridial strains isolated from (A) corn silage (n = 26), (B) raw milk (n = 20), and (C) Kaşar cheese (n = 20)

PeCls – strain code

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Table 3. Kruskal-Wallis H test results for gas production capacities by isolating the source of clostridial isolates

Isolation source	*n*	Mean	d*f*	χ^2	*P*
Corn silage	26	32.3			
Raw milk	20	41.5	2	5.908	< 0.05
Kaşar cheese	20	27.0			

The identification of clostridial isolates using MALDI-TOF MS is presented in Table 4. Mass spectra are given in Figure 3. A total of 49 out of 66 isolates among these specimens were identified as 38 (77.55%) *C. sporogenes*, 6 (12.24%) *C. butyricum*, 3 (6.12%) *C. beijerinckii*, 1 (2.04%) *C. bifermentans* and 1 (2.04%) *C. sartagoforme*. The results showed that 14 bacterial isolates were found to be < 1.70 score level as genus *Clostridium*. Three isolates did not result in a clostridial genus and species level match either. Of the isolates identified as *C. sporogenes*, 44.73% (17/38) had a score of ≥ 2.0 at a species level, as shown in Table 4, and 55.2% (21 out of 38) had a score from 1.7 to 2.0 at the genus level. For *C. butyricum*, 16.6% (1 out of 6) of the isolates were identified to the species level with a score ≥ 2.0 and 83.3% (5 out of 6) of the isolates to the genus level with a score from 1.7 to 2.0. *C. beijerinckii* (3), *C. bifermentans* (1) and *C. sartagoforme* (1) isolates were identified at the genus level with a score from 1.7 to 2.0. To evaluate score values in MALDI-TOF MS identification, modified cut-off criteria for anaerobic species were used regarding Hsu and Burnham (2014) and Chean et al. (2014). Moreover, prolonged disintegration, vortexing, or bead use will be applied during the ethanol-formic acid extrac-

Table 4. Identification of clostridial isolates caused LBD using MALDI-TOF MS at a log score ≥ 2.000 (species level) and ≥ 1.700 (genus level)

Species	MALDI-TOF MS at log (score)	
	≥ 2.000	≥ 1.700
Clostridium sporogenes	17	21
Clostridium butyricum	1	5
Clostridium beijerinckii	0	3
Clostridium bifermentans	0	1
Clostridium sartagoforme	0	1
Total	18	31

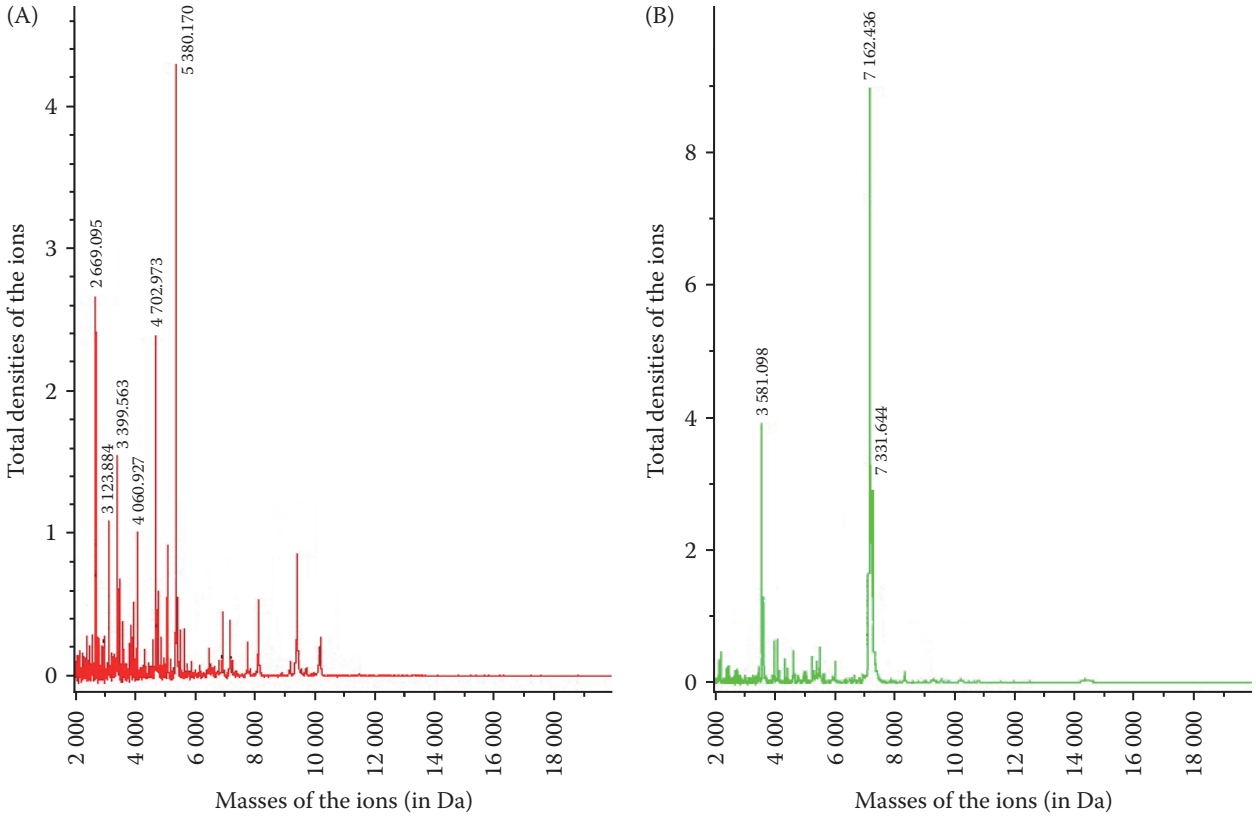

Figure 3. MALDI-TOF MS mass spectra of (A) *Clostridium sporogenes* and (B) *Clostridium butyricum*

Da – Dalton, distinctive peak graph of the strains is inspected; the mass: charge ratio is represented by the *m*/*z* number, which corresponds to the molecular weight of the protein with a single positive charge

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tion procedure to improve the extraction efficiency and spectra quality, which may be an effective strategy.

CONCLUSION

The study contributed to increasing the knowledge of LFCS level in the farm environment in corn silage, raw milk and Kaşar cheese. The use of SEM imaging ignited ideas on endospore morphology. MALDI-TOF MS was used to identify clostridial isolates causing LBD, which caused shortened identification and oxygen exposure time. The MALDI-TOF MS results showed that *C. sporogenes* was the most common species among all the isolates and shared the same habitats and milk contamination pathway during cheese ripening. However, to what extent each strain contributes to cheese quality or LBD remains unclear. To develop the MALDI-TOF MS database, it is necessary to repeat such studies and increase the useability of LBD clostridial species.

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