GIDA
THE JOURNAL OF FOOD
E-ISSN 1309-6273, ISSN 1300-3070

Research/Araştırma
GIDA (2023) 48 (3) 499-508
doi: 10.15237/gida.GD23028

DETERMINATION OF ENDOSPORE STRUCTURE AND VOLATILE COMPOUNDS OF CLOSTRIDIAL SPECIES DURING SPORULATION

Pelin Ertürkmen[*1], Zübeyde Öner[2]

[1]Burdur Mehmet Akif Ersoy University, Burdur Food, Agriculture and Livestock Vocational School, Department of Food Processing, Burdur, Türkiye

[2]Suleyman Demirel University, Faculty of Engineering, Department of Food Engineering, Isparta, Türkiye

Received /Geliş: 04.02.2023; *Accepted* / Kabul: 27.03.2023; *Published online* / Online baskı: 03.04.2023

Ertürkmen, P., Öner, Z. (2023). Determination of endospore structure and volatile compounds of clostridial species during sporulation. GIDA (2023) 48 (3) 499-508 doi: 10.15237/ gida.GD23028

Ertürkmen, P., Öner, Z. (2023). Geç şişme etmeni clostridial türlerin sporulasyon süresince endospor yapısı ve uçucu bileşiklerinin belirlenmesi. GIDA (2023) 48 (3) 499-508 doi: 10.15237/ gida.GD23028

ABSTRACT

In this study, endospore structural and volatile compounds of clostridial species causing late blowing defect (LBD) in cheese were investigated during sporulation. The species were morphologically identified by Gram staining, catalase and oxidase tests, and anaerobic growth tests, and were confirmed by 16S rRNA gene sequence analysis. Strains with high gas production capacity were analyzed by Scanning Electron Microscopy (SEM) and gas chromatography-mass spectrometry (GC-MS) during sporulation. At the end of sporulation, alcohols such as butanol, pentanol, ethanol, hexanol, propanol, octanol, as well as sulfite, ester and acid group compounds were the prominent components. The cell wall residues and endospore structural components of *Clostridium sporogenes* strains with a total relative percentage area of >50% sulfite group compound were observed more intensively than *Clostridium butyricum* strains with high butanol production levels. The information obtained from this study will facilitate studies aimed at identifying endospore structural and volatile compounds of LBD clostridial species.

Keywords: *Clostridium* spp., GC-MS, SEM, late blowing, 16S rRNA

GEÇ ŞİŞME ETMENİ CLOSTRİDİAL TÜRLERİN SPORULASYON SÜRESİNCE ENDOSPOR YAPISAL VE UÇUCU BİLEŞİKLERİNİN BELİRLENMESİ

ÖZ

Bu çalışmada, çeşitli örneklerden izole edilen ve peynirde geç şişme kusuruna neden olan clostridial türlerin sporulasyon süresince endospor yapısal ve uçucu bileşikleri araştırılmıştır. Gram boyama, katalaz ve oksidaz testi, anaerobik gelişim testleri ile morfolojik olarak tanımlanan türler 16S rRNA gen sekans analizi ile doğrulanmıştır. Sporulasyon süresince, yüksek gaz üretim kapasitesine sahip suşlar Taramalı Elektron Mikroskobu (SEM) ve gaz kromatografisi-kütle spektrometresi (GC-MS) ile analiz edilmiştir. Sporulasyon sonunda bütanol, pentanol, ethanol, hexanol, proponol, oktanol gibi alkol bileşikleri; sülfit, ester ve asit grubu bileşikler öne çıkan bileşenlerdendir. Toplam bağıl yüzde alanı >%50 sülfit grubu bileşiğe sahip olan *Clostridium sporogenes* suşlarının hücre duvarı kalıntıları ve endospor yapısal bileşenleri; bütanol üretim düzeyi yüksek olan *Clostridium butyricum* suşlarına göre

[*] *Corresponding author* / Yazışmalardan sorumlu yazar:

✉: perturkmen@mehmetakif.edu.tr ✆: (+90) 248 213 2278

Pelin Ertürkmen; ORCID ID: 0000-0003-4321-7886
Zübeyde Öner; ORCID ID: 0000-0003-2557-0731

daha yoğun şekilde gözlemlenmiştir. Bu çalışmadan elde edilen bilgiler geç şişme etmeni clostridial türlerin endospor yapısal ve uçucu bileşiklerinin tespitine yönelik çalışmalara kolaylık sağlayacaktır.
Anahtar kelimeler: *Clostridium* spp., GC-MS, SEM, geç şişme, 16S rRNA

INTRODUCTION

Clostridium spp. are predominantly obligate anaerobic, Gram-positive, long rod-shaped bacteria that form endospores both in vivo and in vitro, with some being aerotolerant. They are catalase and peroxidase negative, most are mesophilic, except for *C. perfringens*, which is non-capsulated, motile with peritrichous flagella, and have a size of 4-6 µm-1 µm (Quinn et al., 2004; Keto-Timonen et al., 2006). *Clostridium* species are the only group of microorganisms that can undergo acetone-butanol-ethanol (ABE) fermentation, which they can do by various means during milk processing. Gas chromatography can be used to determine volatile carboxylic acids, providing information about ABE fermentation in case of doubt (Jakob, 2011). ABE fermentation consists of two phases, acidogenic and solventogenic. In the acidogenic phase, organic acids are produced, which are then converted to acetone and butanol in the solventogenic phase (Lee et al., 2008). In the solventogenic phase, the pH increases due to solvent formation, and the bacteria lose their ability to reproduce and form endospores (Qureshi and Ezeji, 2008). During cheese processing, endospores that survive thermal treatments of milk can transform into a vegetative form in cheese, fermenting sugars and producing acetic acid, butyric acid, carbon dioxide (CO_2) and hydrogen (H_2) gas (Matijasic et al., 2007; Gómez-Torres et al., 2014). In particular, the low solubility of H_2 gas leads to gas accumulation and explosions during cheese maturation, causing large pores, cracks, and fissures to form, followed by late blowing problems, and the production of bad odor and flavor (McSweeney, 2007; Lo´pez-Enr´ıquez et al., 2007; D'Incecco et al., 2018). The most important four bacterial species that cause late blowing in milk and cheese are *C. butyricum*, *C. sporogenes*, *C. beijerinckii* and *C. tyrobutyricum* (Julien et al., 2008; Garde et al., 2011).

Although various methods are used for the species-level identification of late blowing defect (LBD), errors can occur in the identification of isolates in some cases. Accurate identification is essential for determining which species are present in the sample under investigation, the reactions they cause, and for removing or monitoring the bacteria from the environment. PCR-based methods that identify the 16S rRNA region are commonly used molecular methods for the species-level identification of *Clostridium* spp. (Bassi et al., 2013; Chean et al., 2014; Turci et al., 2016).

The identification of clostridial species responsible for gas production and the determination of the structural compounds of endospores during sporulation are essential for preventing late blowing problems. For this purpose, scanning electron microscopy (SEM) and gas chromatography-mass spectrometry (GC-MS) were used together to analyze the endospore structural and volatile compounds of clostridial strains that were safely identified using PCR during sporulation.

MATERIALS AND METHODS

Clostridial strains and store conditions

17 clostridial strains isolated from Kashar cheese, milk and silage samples with phenotypic descriptions such as Gram-endospore staining, catalase–oxidase and mobility test by Ertürkmen and Öner (2023) which were used as material in this study. The strains were inoculated into the Bryant Burkey Broth with Resazurine and Lactate (BBB, Merck, Germany) and covered with 2 cm of sterile paraffin and incubated at 37 °C for 3 days by an anaerobic kit (Anaerocult A Merck 1.16275) under anaerobic conditions.

Identification of isolates by the 16S rRNA gene sequence

Genomic DNA extraction from pure liquid culture was performed by the GeneMATRIX Bacterial & Yeast Genomic DNA isolation kit (EURx Ltd., Polonia) following the manufacturer's instructions with an initial sample preparation protocol. Concentration and quality of DNA samples were measured by Nanodrop

2000 (Thermo Scientific, USA). 16S rRNA gene region was amplified with the universal primers 27F and 1492R (Lane 1991). For setting up PCR reactions were performed using a PCR Kyratec Thermal Cycler (Australia) and PCR Master Mix (5 x FIREPol® Master MixSolis BioDyne, Estonia). Each 35 µL of PCR reaction contained 3 µL of DNA template, 20 mM deoxynucleotide triphosphate, 10 mM primer, 25 mM $MgCl_2$, and 5 U of Taq DNA Polymerase (Solis Biodyne (Estonia) FIREPol® DNA Polymerase). The PCR program was 95 °C, 5 min; 35 cycles: 95 °C, 45 s; 57 °C, 45 s; 72 °C, 60 s; and a final extension at 72 °C for 5 min. A 10-µL aliquot of the PCR products from each reaction was electrophoresed in 1.5% agarose gel. Gel images were visualised using a Gel Viewing System (Vilber Lourmat, France). Used BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) (Macrogen Holland Laboratory) and ABI 3730XL DNA sequencer (Applied Biosystems, Foster City, California) to sequence the PCR products (forward and reverse primer). The generated sequences were compared with those present in the GenBank database, using BLAST sequence similarity searching [https:// blast.ncbi .nlm .nih .gov/ blast.cgi; ribosomal RNA sequences (bacteria)].

Scanning electron microscopy

SEM was used to observe the morphology of endospore structures during the sporulation process. These samples were incubated anaerobically in BBB at 37 °C for 3 and 7 days. Cells washed two times in phosphate buffered saline solution and were centrifuged at 4 °C for 10 min each time. Observations were made using the gold-plated technique under low vacuum conditions. Images of the samples were taken using QUANTA 250 FEG scanning electron microscope at the Energy Technologies Research Unit Center (ETRUC) of the Suleyman Demirel University.

The ability of clostridial isolates to produce volatile compounds

Clostridial isolates were inoculated into BBB for gas capacity testing. Inoculated tubes were sealed with sterile paraffin and incubated at 37 °C for 7 days. Strains were analyzed for volatile compounds by GC-MS (Model; QP 2010 ultra-series, Shimadzu, Tokyo, Japan). Volatile compounds were extracted by automated solid-phase microextraction (SPME, Supelco, Germany). A 5 mL amount of liquid culture was placed in an amber headspace vial and sealed with a PTFE coated silicone septum (Supelco, Bellefonte, PA) that was placed on an autosampler tray and subjected to solid-phase microextraction. The equilibration and extraction phases were carried out at 45 °C for 15 minutes. Carboxen-Polydimethylsiloxane (CAR/ PDMS) coated fiber (Supelco, Bellefonte, PA) was used for headspace extraction. Chromatographic separation was carried out in a column (Restek Rx-5 Sil MS 30 m × 0.25 mm, 0.25 µm) with a helium flow of 1 mL/min. Relative abundances of compounds were expressed as percentages of their peak areas over the peak area of the internal standard cyclohexanone.

RESULTS AND DISCUSSION

Identification results of *Clostridium* spp.

The identification of 17 *Clostridium* spp. strains at the species level and the similarity rates ranging from 99-100% are presented in Table 1. After PCR reaction, agarose gel electrophoresis was performed on the DNA samples, and it was observed that the *Clostridium* strains formed a single pattern of approximately 1500 bp in size with reliable bands (Figure 1).

The results showed that sixteen bacterial isolates were found to be the genus *Clostridium* supported an identification of *C. sporogenes* using BLAST analysis and identical (100%) match with a *C. sporogenes* (*n*:11) MF062498.1 and MT356160.1 with 100% and 99.56% of sequence homology and with other *C. sporogenes* (*n*:1) LC153544.1 with 99.71% of homology identify 16S rRNA sequences in the GenBanks database, correctly. CL81, CL99 and CL105 were identified as *C. butyricum* strain MT510294.1 with 99.17% and 100% of homology respectively and one isolate did not result in a species level match either. In the study conducted by Cremonesi et al. (2012), the authors identified *C. beijerinckii*, *C. butyricum*, *C. sporogenes* and *C. tyrobutyricum* from milk, hard cheese, and silage using a multiplex PCR test, and

determined that the method had good specificity. The potential of clostridial species to cause cheese spoilage can vary among different strains. In our study, based on the 16S rRNA sequence analysis of clostridial strains isolated from silage, raw milk, and Kashar cheese, *C. sporogenes* was determined to be the most common species among all of our isolates. The findings of this study are consistent with the literature data indicating a high *C. sporogenes* prevalence, according to some researchers (Garde et al., 2011; Bakhtiary et al., 2018; Oliveira et al., 2018).

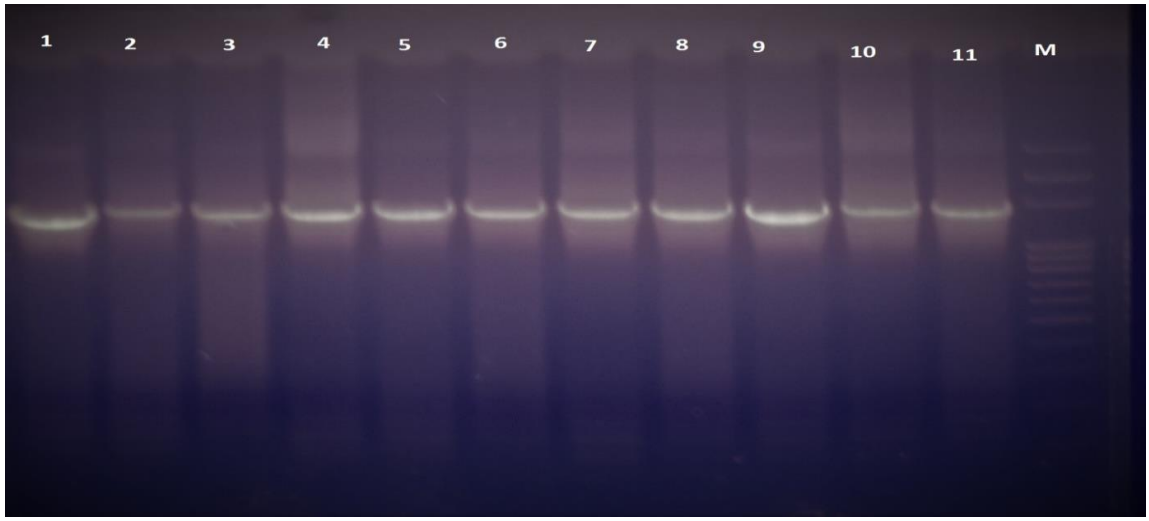

Figure 1. Shows the agarose gel image of PCR products of *Clostridium* spp. strains using 27F and 1492R universal primers, along with the Marker DNA.

* M: Marker, Strains 1-11 are labeled, 1:CL79, 2:CL81, 3:CL97, 4:CL99, 5:CL101, 6:CL105, 7:CL135, 8:CL136, 9:CL148, 10:CL171, 11:CL177, size of the amplikon; 1500 bp

Table 1. Isolates of *Clostridium* spp. by sequence 16S rRNA gene sequencing

Isolate	Source	Species similarity*	Genbank Accession No	Total Number of Bases	Similarity score	Nucleotide identity (%)
CL5	Cheese	*Clostridium sporogenes*	LC153544.1	1382	2531	99.71
CL12	Cheese	*Clostridium sporogenes*	MF062498.1	1362	2516	100
CL41	Cheese	*Clostridium sporogenes*	MF062498.1	1379	2547	100
CL79	Cheese	*Clostridium sporogenes*	MT356160.1	1372	2534	100
CL81	Cheese	*Clostridium butyricum*	MT510294.1	1090	1960	99.17
CL54	Milk	*Clostridium sporogenes*	MF062498.1	1378	2545	100
CL64	Milk	*Clostridium sporogenes*	MF062498.1	1378	2545	100
CL68	Milk	*Clostridium sporogenes*	MF062498.1	1264	2335	100
CL97	Milk	*Clostridium sporogenes*	MT356160.1	1356	2505	100
CL99	Milk	*Clostridium butyricum*	MT510294.1	1090	1960	99.17
CL101	Milk	*Clostridium sporogenes*	MT356160.1	1382	2553	100
CL105	Milk	*Clostridium butyricum*	MT510294.1	1138	2084	100
CL135	Silage	*Clostridium sporogenes*	MT356160.1	1375	2540	100
CL136	Silage	*Clostridium sporogenes*	MT356160.1	1364	2468	99.56
CL148	Silage	*Clostridium sporogenes*	MT356160.1	1379	2519	99.57
CL171	Silage	*Sequence failed*	-	-	-	-
CL177	Silage	*Clostridium sporogenes*	MT356160.1	1375	2516	99.85

*The species were identified by means of 16S universal primers when sequence similarity was ≥98% in the GenBank database, using BLASTn sequence similarity searching (https://blast.ncbi.nlm .nih.gov/Blast.cgi).

Fermentation product of *Clostridium* spp.
In addition to accurate identification of *Clostridium* spp., it is also important to detect fermentation products during sporulation. After sporulation, Gas production and displacement of the paraffin plugs were observed in the tested tubes of the strains. The volatile compounds highlighted by GC-MS were sulfite, ester, alcohol, and acid group compounds (Figure 2).

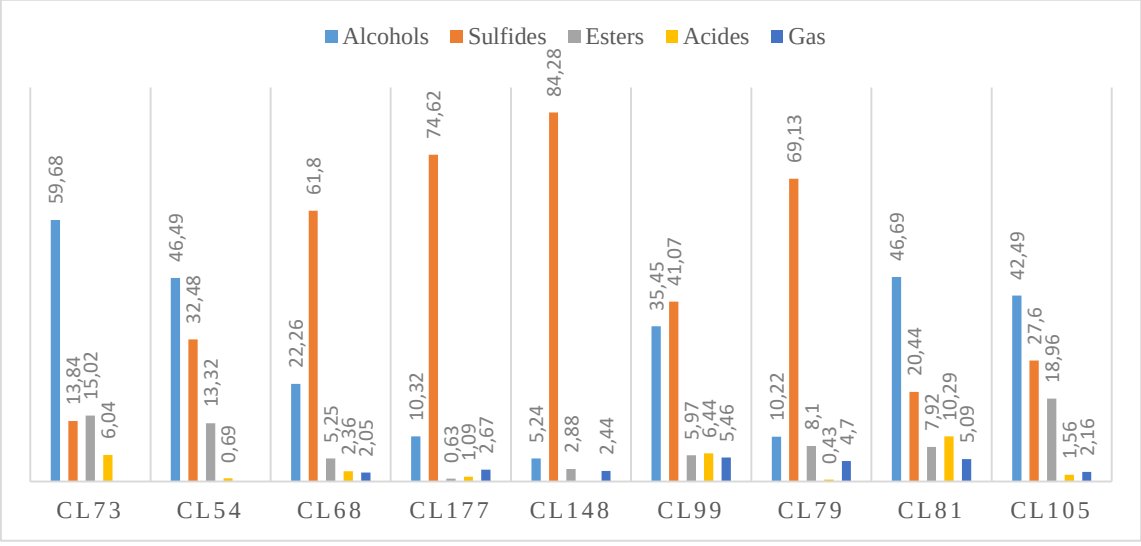

Figure 2. The prominent components of clostridial strains causing late-blowing in cheese analyzed by GC-MS

In a study characterizing volatile metabolites in *Clostridium* species using GC-MS-SPME, it was found that the molecules contained both saturated and aromatic sulfur-containing heterocyclic compounds, sulfides, esters, carboxylic acids and alcohols (Rees et al., 2016). The researchers indicated that the butyric acid, acetic acid, CO_2 and H_2 gases produced by clostridial strains were responsible for late blowing in semi-hard and hard cheeses, leading to unpleasant odors and flavors and causing significant economic losses for businesses (Doyle et al., 2015; Brändle et al., 2016; Silvetti et al., 2018). Disulfide, trisulfide, and tetrasulfide compounds were the most common among the sulfite group compounds and had the highest proportion in this study. Butyrate, acetate, butanoate, valerate, and heptanoate esters were among the ester group compounds. *C. sporogenes* was found to produce butyrate using lactate, pyruvate, and mostly free amino acids (Le Bourhis et al., 2007; Rainey et al., 2009).

The organic acids formed in the acidogenic phase of the ABE fermentation prevent the inhibition caused by organic acids from reaching critical levels by turning into acetone and butanols in the solventogenic phase (Jones and Woods, 1986). In our study, alcohols such as butanol, pentanol, ethanol, hexanol, propanol, and octanol, and acids such as butanoic and acetic acid were commonly detected in all *Clostridium* strains (Figure 3).

Butanol was detected in *C. butyricum* strains as the highest in CL81 (43.38%) and CL105 (38.11%). In a study, cultures containing *C. tyrobutyricum* and *C. beijerinckii* were found to produce 0.5 mM - 0.7 mM 1-butanol at low concentrations (Driehus et al., 2016). It is stated that butanol has a toxic effect on *Clostridium* spp. and stops metabolism at concentrations above 20 g/L (Qureshi and Blaschek, 1999). In *C. butyricum* CL81 strain, the production of butyric and acetic acids was determined as 2.12% and 8.18%, respectively. The acetic acid production of *C. sporogenes* CL68 and CL73 strains was found to be 6.04% and 2.36%, respectively. *C. sporogenes* CL54 and CL177

strains were also found to produce low levels of pentanoic and butanoic acids in addition to acetic acid. Driehuis et al. (2016) stated that *C. tyrobutyricum* and *C. beijerinckii* strains completely consumed lactic acid and produced acetic and butyric acids using glucose.

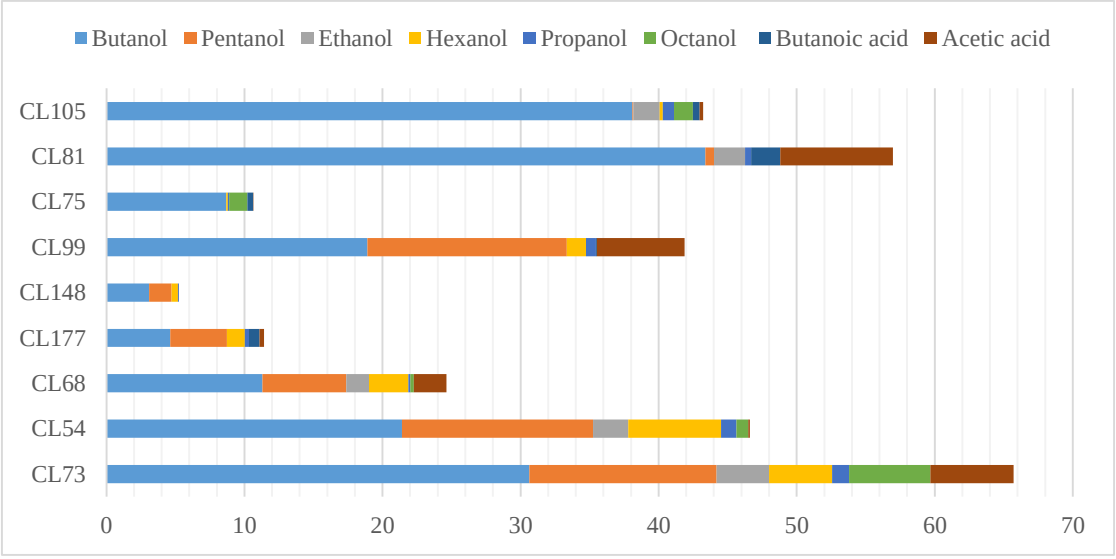

Figure 3. Alcohol and acid group compounds detected in clostridial strains

Endospore morphology of clostridial strains

Chemical changes such as the release of calcium dipicolinate from structural components of endospores and determination of endospore location can be achieved by SEM imaging (Cabrera-Martinez et al., 2003). In this study, SEM images taken at the beginning of sporulation showed that the cells were rod-shaped and belonged to the genus *Bacillus*, forming terminal and subterminal endospores. The length of the cells varied between 3-5 µm (Figure 4). The use of PBS buffer before SEM imaging, observation under a low vacuum and a gold plating technique provided a sharp border appearance in the clostridial cells. The size and range of the isolated clostridial bacteria were found to be consistent with the results of other researchers who used gold coating and buffer solutions (Bassi et al., 2009).

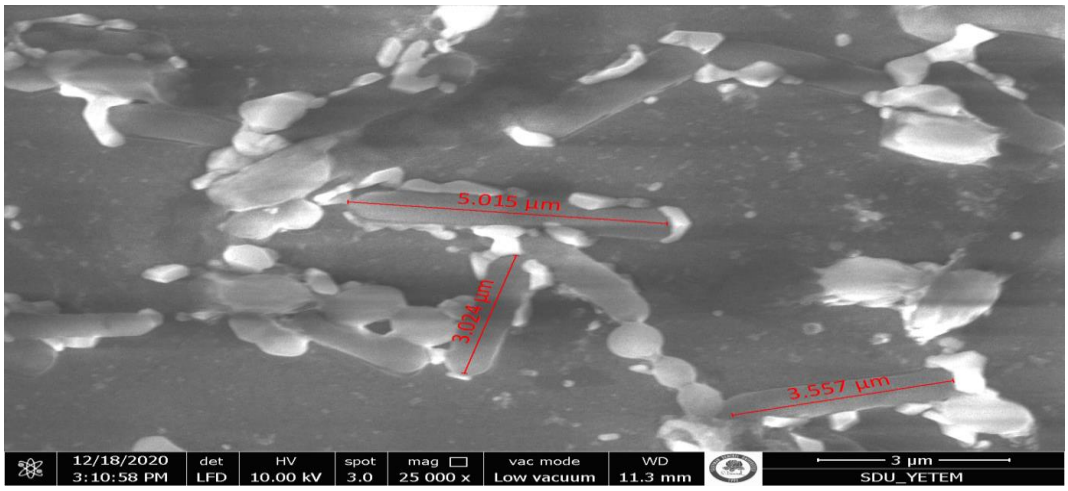

Figure 4. SEM image of *C. sporogenes* CL68 strain at the beginning of sporulation

During sporulation, SEM imaging and GC-MS analysis revealed that the cell wall remnants and endospore structural components of *Clostridium sporogenes* CL177 and CL68 strains, which have a total relative percentage area greater than >50% for the sulfide group compound, were more intensively present compared to *Clostridium butyricum* CL81 strain with high butanol production (Figure 5).

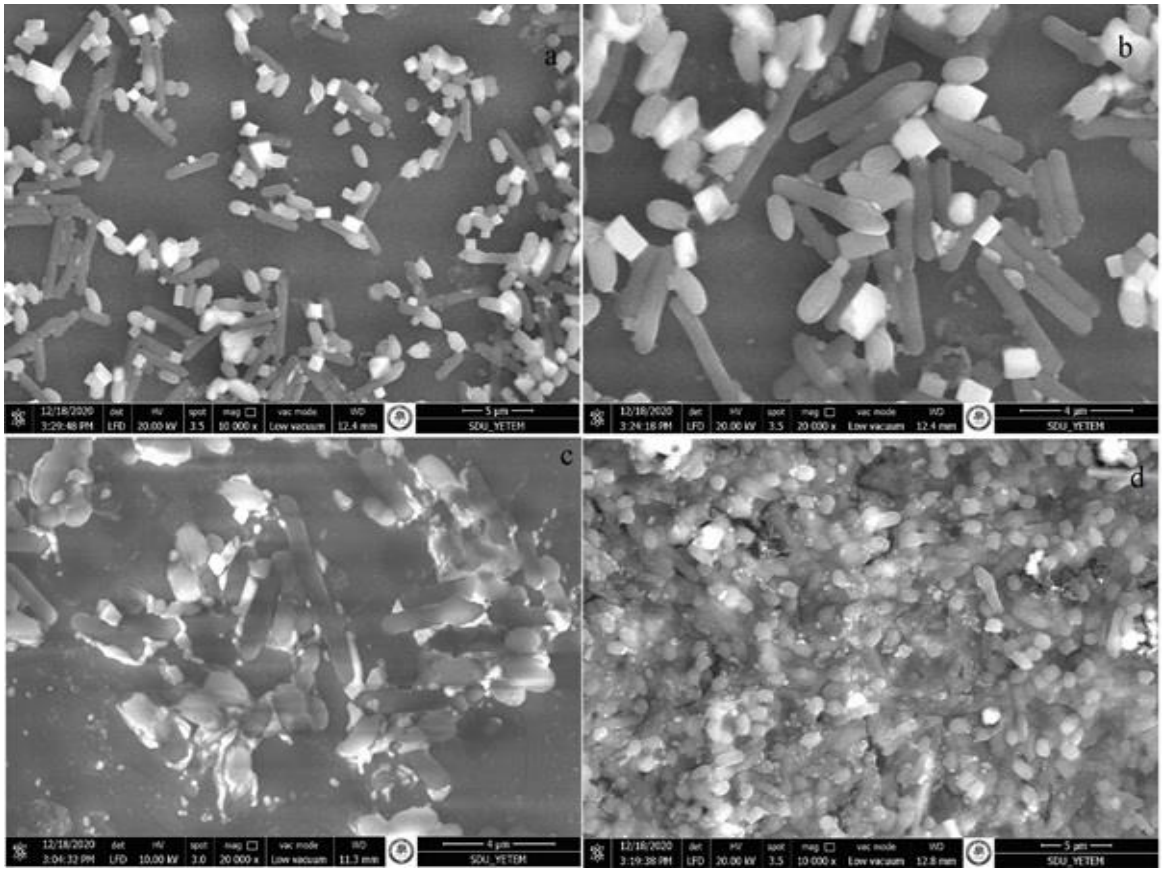

Figure 5. SEM images of *C. sporogenes* and *C. butyricum* strains during sporulation
*a: *C. butyricum* CL81 strain at the beginning of sporulation; b: *C. butyricum* CL81 strain at the end of sporulation; c: *C. sporogenes* CL68 strain at the beginning of sporulation; d: *C. sporogenes* CL68 strain at the end of sporulation

During the final stage of sporulation, SEM imaging revealed that sporulation had progressed and spores had changed in size to approximately 1 μm in width and 1.7 μm in length. Some cells had started to form nuclei and exosporium (Figure 5). In a study on SEM imaging of *C. sporogenes*, it was found that the exosporium surrounds all the spores and has a rough, oval appearance with a typical length of 3 mm. It was also observed that exosporium played a role in adhering to cells after sporulation. Some studies on SEM imaging support this finding (Faille et al., 2007; Lequette et al., 2011).

CONCLUSION

This study investigated LBD clostridial strains isolated from different sources through 16S rRNA sequence analysis, and the strains were identified as *C. sporogenes* and *C. butyricum* at the species level. The GC-MS analysis of volatile compounds produced by clostridial strains during fermentation revealed that these strains produce a range of products such as acids, alcohols, sulfites, esters that can cause significant product loss in hard and semi-hard cheeses. During SEM imaging, the use of PBS buffer, low vacuum observation, and gold coating technique provided

a sharp boundary appearance in clostridial cells, allowing successful observation of endospore structural compounds. In future studies, SEM can be used as a sensitive tool to gain more information about chemical changes in LBD clostridial strains, such as internal spore structure and calcium dipicolinate release, with additional techniques for evaluation.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

AUTHORS' CONTRIBUTIONS

Pelin Ertürkmen: Conceptualization, methodology, investigation, formal analysis, writing - original draft. Zübeyde Öner: Project administration, supervision, conceptualization, methodology, writing-review and editing. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This study was part of a doctoral dissertation (Pelin Ertürkmen, FDK-2019-6943) supported by the Scientific Research Projects Unit of Suleyman Demirel University, Isparta, Türkiye.

REFERENCES

Bakhtiary, F., Sayevand, H.R., Remely, M., Hippe, B., Indra, A., Hosseini, H., Haslberger, AG. (2018). Identification of *Clostridium* spp. derived from a sheep and cattle slaughterhouse by Matrix-Assisted Laser Desorption and Ionizationtime of Flight Mass Spectrometry (MALDI-TOF MS) and 16S rDNA sequencing. *International Journal of Food Science Technology*, 55(8):3232-3240.

Bassi, D., Cappa, F., Cocconcelli, P.S. (2009). A combination of SEM technique and X-ray microanalysis to study spore germination process of *Clostridium tyrobutyricum*. *Research in Microbiology*, 160:322-329.

Bassi, D., Fontana, C., Zucchelli, S., Gazzola, S., Cocconcelli, P.S. (2013). Taqman Real Time-Quantitative PCR targeting the phosphotransacetylase gene for *Clostridium tyrobutyricum* quantification in animal feed, faeces, milk and cheese. *International Dairy Journal*, 33:75-82.

Brändle, J., Domig, K.J., Kneifel, W. (2016). Relevance and analysis of butyric acid producing Clostridia in milk and cheese. *Food Control*, 67:96–113.

Brunt, J., Cross, L.K., Peck, M.W. (2015). Apertures in the *Clostridium sporogenes* spore coat and exosporium align to facilitate emergence of the vegetative cell. *Food Microbiology*, 51:45-50.

Cabrera-Martinez, R.M., Tovar-Rojo, F., Vepachedu, V.R., Setlow, P. (2003). Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. *Journal of Bacteriology*, 185(8):2457-2464.

Chean, R., Kotsanas, D., Francis, M.J. (2014). Comparing the identification of *Clostridium* spp. by two matrix-Assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry platforms to 16S rRNA PCR sequencing as a reference standard: A detailed analysis of age of culture and sample preparation. *Anaerobe*, 30(12):85–89.

Cremonesi, P., Vanoni, L., Silvetti, T., Morandi, S., Brasca, M. (2012). Identification of *Clostridium beijerinckii, C. butyricum, C. sporogenes, C. tyrobutyricum* isolated from silage, raw milk and hard cheese by a Multiplex PCR assay. *Journal of Dairy Research*, 79:318–323.

D'Incecco, P., Pellegrino, L., Hogenboom, J.A., Cocconcelli, P.S., Bassi, D. (2018). The late blowing defect of hard cheeses: behaviour of cells and spores of *Clostridium tyrobutyricum* throughout the cheese manufacturing and ripening. *LWT*, 87:134-141.

Doyle, C.J., Gleesonc, D., Jordan, K., Beresford, T.P., Ross, R.P., Fitzgerald, G.F. (2015). Anaerobic sporeformers and their significance with respect to milk and dairy products. *International Journal of Food Microbiology*, 197:77–87.

Driehuis, F., Hoolwerf, J., and Rademaker, J.L.W. (2016). Concurrence of spores of *Clostridium tyrobutyricum, Clostridium beijerinckii* and *Paenibacillus polymyxa* in silage, dairy cow faeces and raw milk. *International Dairy Journal*, 63:70–77.

Ertürkmen P., Öner Z. (2023). Challenging the problematic detection of clostridial isolates

causing late-blowing defect with MALDI–TOF MS. *Czech Journal Food Science*, 41(1):36-43.

Faille, C., Tauveron, G., Le Gentil-Lelievre, C., Slomianny, C. (2007). Occurrence of *Bacillus cereus* spores with a damaged exosporium: Consequences on the spore adhesion on surfaces of food processing lines. *Journal of Food Protection*, 70, 2346-2353.

Garde, S., Arias, R., Gaya, P., Nuñez, M. (2011). Occurrence of *Clostridium* spp. in ovine milk and Manchego cheese with late blowing defect: Identification and characterization of isolates. *International Dairy Journal*, 21:272–278.

Gómez-Torres, N., Ávila, M., Gaya, P., Garde, S. (2014). Prevention of late blowing defect by reuterin produced in cheese by a *Lactobacillus reuteri* adjunct. *Food Microbiology*, 42:82–88.

Jakob, E. (2011). Analytik Rund Um Die ButtersäAuregäArung. In Alp Forum (Vol. 85, Pp.1-23.

Jones, D.T., Woods, D.R. (1986). Acetone-butanol fermentation revisited. *Microbiological Reviews*, 50:484-524.

Julien, M.C., Dion, P., Lafrenie`re, C., Antoun, H., Drouin, P. (2008). Sources of clostridia in raw milk on farms. *Applied and Environmental Microbiology*, 74:6348–6357.

Keto-Timonen, R., Heikinheimo, A., Eerola, E., Korkeala, H. (2006). Identification of *Clostridium* species and DNA fingerprinting of *Clostridium perfringens* by amplified fragment length polymorphism analysis. *Journal of Clinical Microbiology*, 44:4057–65.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, Editors. Nucleic Acid Techniques in Bacterial Systematics. New York: Wiley. Pp. 115–175.

Le Bourhis, A.G., Dore, J., Carlier, J.P., Chamba, J.F., Popoff, M.R., Tholozan, J.L. (2007). Contribution of *C. beijerinckii* and *C. sporogenes* in association with *C. tyrobutyricum* to the butyric fermentation in emmental type cheese. *International Journal of Food Microbiology*, 113:154–63.

Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S. (2008). Fermentative butanol production by clostridia. *Biotechnology and Bioengineering*, 101(2):209-228.

Lequette, Y., Garenaux, E., Tauveron, G., Dumez, S., Perchat, S., Slomianny, C., Lereclus, D., Guerardel, Y., Faille, C. (2011). Role played by exosporium glycoproteins in the surface properties of *Bacillus cereus* spores and in their adhesion to stainless steel. *Applied and Environmental Microbiology*, 77:4905-4911.

Lo´pez-Enr´ıquez, L., Rodr´ıguez-La´zaro, D., Herna´ndez, M. (2007). Quantitative detection of *Clostridium tyrobutyricum* in milk by Real-time PCR. *Applied and Environmental Microbiology*, 73(37), 47–51.

Matijasic, B.B., Rajsp, M.K., Perko, B., Rogelj, I. (2007). Inhibition of *Clostridium tyrobutyricum* in cheese by *Lactobacillus gasseri*. *International Dairy Journal*, 17:157–166.

Mc Sweeney, P.L.H. (2007). What is late gas blowing and how may this defect be avoided. In Mcsweeeny (Ed.) Cheese Problems Solved Boca Raton, USA:CRC Press, 198–199.

Oliveira, R.B.A., Lopes, L.S., Baptista, R.C., Chincha, A.A.I.A., Portela, J.B., Nascimento, J.S. (2018). Occurrence, populations, diversity, and growth potential of spore-forming bacteria in "Requeijão Cremoso". *LWT-Food Science and Technology*, 89, 24–31.

Quinn, P.J., Markey, B.K., Carter, M.E., Donnelly, W.J., Leonard, F.C. (2002). *Veterinary Microbiology and Microbial Disease*. Oxford, UK: Blackwell Science.

Qureshi, N., Blaschek, H.P. (1999). Production of Acetone-Butanol-Ethanol (ABE) by a hyper-butanol producing mutant strain of *Clostridium beijerinckii* BA101 and recovery by pervaporation. *Biotechnology Progress*, 15:594–602.

Qureshi, N., Ezeji, T.C. (2008). Butanol, "A Superior Biofuel" production from agricultural residues (Renewable Biomass): Recent Progress in technology. *Biofuels, Bioproducts and Biorefinery,* 2:319-330.

Rainey, F. A., Hollen, B. J., Small, A. (2009). Genus I. *Clostridium prazmowski*, 1880, 23al. In P. Vos, G. Garrity, D. Jones NR. Krieg, W. Ludwig, F. A. Rainey (Eds.), Bergey's Manual of Systematic Bacteriology. The Firmicutes New York: Springer, 3:736-827.

Rees, C. A., Shen, A., Hill, J.E. (2016). Characterization of the *Clostridium difficile* volatile metabolome using comprehensive two-dimensional gas chromatography time-of-flight Mass Spectrometry. *Journal* of *Chromatography*, 1039:8-16.

Silvetti, T., Morandi, S., Brasca, M. (2018). Growth factors affecting gas production and reduction potential of vegetative cell and spore inocula of dairy-related *Clostridium* species. *LWT*, 92:32-39.

Turchi, B., Pero, S., Torracca, B., Fratini, F., Mancini, S., Galiero, A. (2016). Occurrence of *Clostridium* spp. in Ewe's milk: Enumeration and identification of isolates. *Dairy Science and Technology*, 96(5):693-701.