



Short Communication

Detection of *Clostridium algidicarnis* in beef in Thailand and comparison of their characteristics with strains from Central Europe

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Abstract

Cold-tolerant clostridia are important, albeit largely unexplored, spoilage microorganisms of vacuum-packed beef. Studies on these bacteria mainly take place in regions with a temperate climate, with very few conducted in the tropical countries. As beef consumption in Thailand is constantly increasing, this study aimed to investigate whether these bacteria exist in meat sold in the country. Vacuum-packed beef samples (n = 40) were collected from various supermarkets in two different provinces. Overall, 40.0% of samples tested positive for clostridia by PCR and 22.5% by culture. Of these, 14 samples were positive for *Clostridium algidicarnis*, while the other two samples were positive for *C. perfringens*. No correlation was found between positive beef samples and the level of gas production in meat packs. When compared with Austrian and German strains, all tested strains (n = 10) grew well in PYGS broth at 10°C and 25°C, grew slowly at 4°C, but showed no growth at 37°C. A low sporulation rate was observed during cultivation in pure media. Similarity in the MALDI-TOF-MS spectrum between the Thai and Austrian strains was observed. Although the detection rate is considered preliminary due to the small sample size, the results show that the cold-tolerant *C. algidicarnis* is present in vacuum-packed beef sold in Thailand.

Keywords: Cold-tolerant clostridia, Meat spoilage, Vacuum-packed beef

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INTRODUCTION

Psychrophilic and psychrotolerant clostridia, in particular *Clostridium estertheticum*, *C. frigorophilum*, *C. algidicarnis*, and *C. gasigenes*, have been widely recognized for their involvement in the spoilage of vacuum-packed fresh meat, especially beef, lamb, and game meat. The spoilage can sometimes be recognized by the swelling of the packaging, known as blown pack spoilage (BPS), which results from CO₂ and H₂ formation (Boerema et al., 2007; Adam et al., 2010). Due to the high butanol and butyric acid production of some *Clostridium* species, contaminated meat usually smells cheesy or like rancid butter (Lawson et al., 1994; Broda et al., 1996). The *Clostridium* species within this group are heterogeneous, with *C. estertheticum* being the most prominent and extensively studied species. Although consuming meat contaminated with *C. estertheticum* is unlikely to pose a direct health hazard, contamination substantially reduces the shelf life of vacuum-packed beef (BfR, 2010). The detection of cold-tolerant clostridia related to meat spoilage has been reported, especially in countries with high meat production and/or consumption located in temperate climate zones, such as Austria (Angerer et al., 2025; Dorn-In et al., 2025), Canada (Zhang et al., 2020), Germany (Bonke et al., 2016; Mang et al., 2021), Ireland (Bolton et al., 2015), New Zealand (Broda et al., 2009), Switzerland (Wambui et al., 2020), the United Kingdom (Dainty et al., 1989), and the United States (Kalchayanand et al., 1989). In tropical countries, with the exception of Brazil (Silva et al., 2011), there is limited data on the occurrence of cold-tolerant clostridia in meat, livestock, or along any stage of the production chain.

Beef production and consumption in Thailand are relatively low compared to countries in Europe, Oceania, or the Americas. Although demand is increasing, domestic cattle and beef production remains limited. To meet this growing requirement, Thailand imports up to 20-25% of its total domestic beef consumption (USDA, 2021; MLA, 2024). The primary beef suppliers for Thailand are Australia, New Zealand, Japan, and the United States. Imported beef (categorized as medium- and high-grade beef) is distributed to supermarkets, hotels, and restaurants, whereas domestically produced beef (generic beef) is usually sold in traditional or wet markets (USDA, 2021).

Within a globalized marketplace that facilitates international food trade, pathogens and food spoilage microorganisms can be transferred across borders. In this context, spoilage bacteria previously restricted to temperate regions with high beef consumption may also persist in tropical environments such as Thailand. Although there are no prior reports of cold-tolerant clostridia in Thailand, their presence in local retail beef cannot be ruled out. The first aim of this study was to determine the presence of psychrophilic and cold-tolerant clostridia in vacuum-packed beef retailed in supermarkets across two provinces in Thailand. The second aim was to compare the cold-tolerant clostridia isolated in this study with reference strains obtained from previous studies in Central Europe (Mang et al., 2021; Angerer et al., 2025), in terms of growth rate at various temperatures and spectral similarity, as determined by MALDI-TOF MS analysis.

MATERIALS AND METHODS

Beef samples and preparations

A total of 40 vacuum-packed beef samples were collected from seven supermarkets in the provinces of Phuket (n = 17) in southern Thailand and Chiang Mai (n = 23) in northern Thailand. Of these, 22 samples were derived from locally raised cattle, and 18 samples were imported beef. The samples were sourced from 16 different meat processing or packaging plants and collected between January and April 2024. The types and origins of the meat were documented, and the samples were stored at 4°C for up to 2-3 weeks beyond their expiry dates. This

extended storage period was employed to enrich clostridial cell populations, thereby increasing the probability of detection. The degree of gas formation (BPS scores 0-5) was then evaluated as described by Boerema et al. (2007). Then, the meat packages were opened, and the meat juice was collected in a sterile tube. Approximately 1 ml of the meat dripping was mixed with 9 ml of peptone yeast glucose starch (PYGS) broth (Lund et al., 1990) in a 15 ml glass tube. If no meat drip was present due to absorbent pads, the beef sample was rinsed with 20 ml of PYGS broth. Thereafter, 10 ml of this rinse suspension was transferred to a 15 ml glass tube. Because cold-tolerant clostridia grow relatively slowly, the incubation period for enrichment requires at least 3 to 4 weeks (Broda et al., 2009; Bonke et al., 2016). For this study, the tubes were anaerobically incubated at 4°C for 6 weeks.

Reference strains

Following the surveillance study, the growth rate, sporulation capacity, and MALDI-TOF MS spectral profile of the isolated species (*C. algidicarnis*) were determined. For comparative purposes, the type strain DSM 15099 and six other strains isolated from vacuum-packed beef in two previous studies conducted in Austria (Angerer et al., 2025) and Germany (Mang et al., 2021) were included. These archival strains were re-cultured from reference stock preserved at -80°C.

DNA extraction

The remaining meat drip or rinse suspension was stored at -20°C until DNA extraction, which was performed simultaneously with DNA extraction from the drip enrichments in PYGS broth. To extract genomic DNA, 10 µl of lysozyme (final concentration 1 mg/ml) was added to 200 µl of each drip sample and drip enrichment sample. The mixture was then incubated at 37°C for 30 min. The samples were then subjected to DNA extraction using the High Pure PCR Template Preparation Kit (Roche).

The DNA extraction of the colonies on agar plates was performed by transferring a single colony to 100 µl of sterile water in a 1.5 ml reaction tube. Then 5 µl of 1 mg/ml lysozyme was added and incubated at 37°C for 30 min. After that, 10 µl of 600 mAU/ml proteinase K was added, incubated at 70°C for 10 min, followed by heating the colony suspension at 99°C for 15 min. The sample was then centrifuged for 1 min at 18,000 × g, and the supernatant was used for PCR.

Real-time PCR and sequencing analysis

The extracted DNA samples were analyzed using four real-time PCR assays with probes (PCR 1, 2, 3, and 4; Table 1). The first real-time PCR (PCR 1) for universal bacteria was used to ensure that no PCR inhibitors were present in the DNA-extracted samples. The remaining three PCR assays (PCRs 2, 3, and 4) were used to detect the following *Clostridium* species: *C. estertheticum*, *C. frigoriphilum*, *C. algidicarnis*, *C. bowmanii*, *C. tagluense*-like, *C. gasigenes*, and *C. putrefaciens*. Each 20 µl real-time PCR reaction contained 0.25 µM of each primer, 0.1 µM of each probe, 10 µl of SensiFast™ Probe No-Rox Master Mix (Bioline™, Meridian™), 2 µl of the DNA sample and RNA-free water to fill up to the final volume of 20 µl. PCR amplification was performed using the CFX Opus™ 96 Real-Time PCR System (Bio-Rad™). The real-time PCR protocol started with initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 5 sec, then annealing and elongation at 62°C for 20 sec for PCR 1 (for universal bacteria) and at 60°C for 30 sec for PCR 2, 3, and 4 (for clostridia). As positive controls for all four real-time PCRs, the DNA extracts of reference strains of *C. estertheticum* (DSM 14864), *C. frigoriphilum* (DSM 17811), *C. algidicarnis* (DSM 15099), *C. putrefaciens* (DSM 1291), *C. gasigenes* (DSM 12272), *C. bowmanii*, and *C. tagluense*-like were used. For the no-template control reaction, RNA-free water was used instead of the DNA sample to ensure the purification of the prepared PCR master mix solution.

Table 1 List of primers and probes and the specificity of the four real-time PCR methods used in this study

PCR*	Primer/probes	Sequence and fluorescens for probes (direction 5'-3')	Specificity
1	Uni335-F	CADACTCCTACGGGAGGC	Universal bacteria
	Uni923-R	CTTGTGCGGGCCCCCGT	
	Uni799	Cy5.5-AACAGGATTAGATACCCTGGTAGTC-BBQ-650	
2	CI94-F	CGGCGGACGGGTGAGTAAC	<i>Clostridium</i> spp.
	CI642-R	CCTCTCCTGCACTCTAGA	
	CI555	FAM-CCTTTACRCCCAGTAAATCCGGAC-BHQ-1	<i>Clostridium</i> spp.
	Cest	Hex-CAAAGGAATTTTCGGAATTTCACTTTGAG-BHQ-1	<i>C. estertheticum</i>
	Cfgrpl	Cy3.5-CAAAGGAATAGTCTTCGGATTATTTCACT-BHQ-2	<i>C. frigidophilum</i>
	Cpal	Cy5-ACCCCATACATAGCATTATCGCATG-BHQ-2	<i>C. algidicarnis</i> & <i>C. putrefaciens</i>
3	Ctag-like	Cy5.5-CAAAGGATTTTCTTCGGAATTTCCAC	<i>C. tagluense</i> -like
	CI94-F	CGGCGGACGGGTGAGTAAC	<i>Clostridium</i> spp.
	CI642-R	CCTCTCCTGCACTCTAGA	
	CI555	FAM-CCTTTACRCCCAGTAAATCCGGAC-BHQ-1	<i>Clostridium</i> spp.
	Cest	Hex-CAAAGGAATTTTCGGAATTTCACTTTGAG-BHQ-1	<i>C. estertheticum</i>
	Cfgrpl	Cy3.5-CAAAGGAATAGTCTTCGGATTATTTCACT-BHQ-2	<i>C. frigidophilum</i>
	Cbow	Cy5-CAAAGGATTCTCTTCGGAGATTCCAC-BHQ-2	<i>C. bowmanii</i>
	Cgas	Cy5.5-TCGGGCTCAACCCGAGAAGTGC-BBQ-650	<i>C. gasigenes</i>
	Cpal1423- F	CTAACGCGTAAGCGAGGCAG	<i>C. algidicarnis</i> & <i>C. putrefaciens</i>
	Cpal-ITS-R	CTTTGCTTTTAAACCATATGCAG	
	Cal-ITS	FAM-TTGTAGCTYGTCTATAACTAGTAC-BHQ-1	<i>C. algidicarnis</i>
	Cput-ITS	HEX-TTATAGCTAGTCTATAACTCTTAATAC-BHQ-1	<i>C. putrefaciens</i>

*Real-time PCR 1, 2, 3 (Dorn-In et al., 2025), and 4 (Mang et al., 2021).

To confirm that weakly PCR-positive samples contained clostridial DNA, the amplified 16S rRNA gene fragments (566 bp) from PCR 2 or PCR 3 were purified using the EZNA™ Cycle Pure Kit (Omega Bio-Tek™) and sequenced by Microsynth™ AG (Vienna, Austria). Species identification was performed by comparing the obtained sequences with reference sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Cultural examination

The PCR-positive PYGS enrichment samples were forwarded for cultivation. From each enrichment, 500 µl was transferred into each of two 2.0 ml reaction tubes, which were subsequently treated with heat or ethanol to inactivate accompanying non-spore-forming microbiota and selectively allow clostridial spores to survive (Moschonas et al., 2009). The first tube was treated with heat (80°C, 5 min) in a ThermoMixer™ (Eppendorf™). Subsequently, 500 µl of the heat-treated tubes were spread on a blood agar plate (CBA, Columbia blood agar + 5% sheep blood, BioMérieux™). The second tube was treated with 50% ethanol, for which 500 µl enrichment was mixed with 500 µl of 99.9% ethanol. Then these tubes were incubated at room temperature for 15 min and centrifuged at 16,000 x g for 2 min; the supernatant was discarded, and the pellet was suspended in 500 µl of 0.9% NaCl. Then 500 µl of each ethanol-treated sample was spread on CBA plates. All CBA plates were incubated anaerobically at 10°C for 3 to 4 weeks. Isolated colonies were collected and subjected to DNA extraction, real-time PCR, and MALDI-TOF MS for species identification.

Growth rate determination

To prepare the inoculum, ten individual colonies of *C. algidicarnis* strains (one DSM strain, three Austrian strains, three German strains, and three Thai strains) were subcultured in 10 ml of PYGS broth. The tubes were then incubated at 10°C for eight weeks to ensure the *C. algidicarnis* strains reached the plateau growth phase. The PYGS enrichment was then serially diluted in PYGS to a dilution of 10⁻⁶,

and 50 μ l of each dilution was dropped onto the CBA plate in duplicate. The plates were then incubated anaerobically at 10°C for 14 days, after which the colonies were counted.

Based on the colony counts, the bacterial suspensions were adjusted to a target concentration of approximately 100 cfu/ml in PYGS broth. Then, 1 ml of each cell suspension was inoculated into four glass tubes containing 9 ml of PYGS broth. These tubes were then anaerobically incubated for 42 days at temperatures of 37°C, 25°C, 10°C, and 4°C, respectively. PYGS enrichment was examined on days 0, 3, 7, 14, 21, 28, 35, and 42 to determine the growth rate and the degree of sporulation of all isolates. To determine sporulation, the PYGS enrichment was treated with 50% ethanol, as described in the cultural examination section. The PYGS enrichment (both untreated and treated with ethanol) was diluted 1:10 in new PYGS broth to 10^{-6} ; then, 50 μ l of each dilution was dropped onto a CBA plate and incubated anaerobically at 10°C for two weeks. The colonies were then counted. This experiment was performed in two independent biological replicates.

MALDI-TOF MS

To identify species, colonies from CBA plates were collected for protein extraction using an extended extraction method involving 70% formic acid and acetonitrile, as described by the manufacturer (Bruker Daltonik GmbH, Germany).

For spectral and similarity analysis, all ten *C. algidicarnis* strains previously evaluated for growth rate and sporulation were subcultured onto CBA plates and incubated anaerobically for 3 days at an optimum growth temperature of 25°C. A single colony (approximately 1 mm in diameter) was selected from each plate for protein extraction using the extended extraction method. For each strain, the protein extract was applied to six spots on the target plate and measured three times, yielding 18 replicates per strain. MSP-Compass-Explorer software was used to analyze the mass spectra and generate a dendrogram illustrating the spectral similarities among the strains originating from the three countries (Austria, Germany, and Thailand).

RESULTS

Detection and isolation of *Clostridium* spp.

All DNA extracts from meat drip and enrichment samples tested with universal PCR for bacteria (PCR 1) achieved Ct-values below 20 PCR cycles, indicating that the DNA extracts did not contain any PCR inhibitors. The detailed information of each vacuum-packed beef sample and the result of the test, i.e., the BPS score, the Ct-values obtained by real-time PCR for clostridia before sample enrichment, and the isolation results, was demonstrated in Table 2, with summary results provided in Table 4. The samples with Ct-values < 30 PCR cycles were evaluated as strongly positive, Ct-values between 31 and 35 as moderately positive, and Ct-values > 35 as weakly positive. The results of the fourth PCR method (Table 1) show that 14 of 16 beef drip samples, prior to enrichment, were PCR positive for *C. algidicarnis*. Of these, 6 samples showed a strong positive result. Two samples domestically produced (no. 35 and 38; Table 2) were weakly positive for the genus *Clostridium* but negative for the species *C. algidicarnis*. All positive samples originated from the same six meat processing or packaging plants. The real-time PCR probes for *C. estertheticum*, *C. frigorophilum*, *C. bowmanii*, *C. tagluense*-like, *C. gasigenes*, and *C. putrefaciens* showed negative results for all meat samples.

Table 2 Meat information and results of BPS scores and Ct-values of a real-time PCR probe for the genus *Clostridium* and culturally isolated species

Meat no.	Product type	Source of meat	Weight (g)	Absorbent pad ¹	Purchased from	BPS score ²	Ct-values ³		Cultural results of enrichment ⁴
							Meat drip	Enrichment	
1	Knuckle steak dry aged	Thailand	200	Yes	Retail 1, Phuket	1	-	-	n.d.
2	Rib Eye steak	Australia	160	Yes	Retail 1, Phuket	2	-	-	n.d.
3	Striploin steak	New Zealand	180	Yes	Retail 1, Phuket	1	-	-	n.d.
4	Striploin steak	Australia	238	No	Retail 1, Phuket	2	26.54	38.16 ⁵	negative
5	Beef rib exe	Thailand	200	Yes	Retail 2, Phuket	1	26.36	24.29	<i>C. algidicarnis</i> (Th 1)
6	Rib eye	Thailand	180	Yes	Retail 2, Phuket	1	31.24 ⁵	-	n.d.
7	Lean meat	Thailand	250	Yes	Retail 2, Phuket	1	-	-	n.d.
8	Blades steak	Australia	350	No	Retail 2, Phuket	2	-	-	n.d.
9	Beef round	Thailand	250	Yes	Retail 3, Phuket	0	-	-	n.d.
10	Beef diced	Thailand	200	Yes	Retail 3, Phuket	0	-	-	n.d.
11	Sliced beef shank	Thailand	150	Yes	Retail 3, Phuket	1	26.01	31.11	<i>C. algidicarnis</i> (Th 2)
12	Striploin steak	Australia	160	No	Retail 3, Phuket	2	-	-	n.d.
13	Beef diced	Australia	200	Yes	Retail 4, Phuket	2	-	-	n.d.
14	Chuck roll steak	Australia	200	Yes	Retail 4, Phuket	1	33.88 ⁵	-	n.d.
15	Beef diced	Thailand	250	Yes	Retail 4, Phuket	2	-	-	n.d.
16	Brahman beef chuck roll steak	Thailand	250	Yes	Retail 4, Phuket	1	27.69	34.45	<i>C. algidicarnis</i> (Th 3)
17	Brahman beef diced	Thailand	300	Yes	Retail 4, Phuket	1	30.51	24.43	<i>C. algidicarnis</i> (Th 4)
18	Rib eye steak	Australia	150	No	Retail 5, Chiang Mai	2	27.67	22.18	<i>C. algidicarnis</i> (Th 5)
19	Striploin steak	Australia	150	No	Retail 5, Chiang Mai	2	-	-	n.d.
20	Meltique beef loin fillet	Australia	150	No	Retail 5, Chiang Mai	2	-	-	n.d.
21	Tenderloin	Thailand	180	Yes	Retail 6, Chiang Mai	1	36.25 ⁵	39.63	<i>C. algidicarnis</i> (Th 6)
22	Diced beef meat	Thailand	180	Yes	Retail 6, Chiang Mai	1	27.76	37.67 ⁵	negative
23	Chuck eye sliced	Thailand	120	Yes	Retail 6, Chiang Mai	1	-	-	n.d.
24	Sirloin tip	Thailand	250	Yes	Retail 6, Chiang Mai	2	35.68	26.05	<i>C. algidicarnis</i> (Th 7)
25	AU Angus beef rib eye steak	Australia	262	Yes	Retail 7, Chiang Mai	1	-	-	n.d.
26	AU beef top round steak	Australia	312	Yes	Retail 7, Chiang Mai	1	-	-	n.d.
27	Thai angus beef picanha	Australia	244	Yes	Retail 7, Chiang Mai	1	-	-	n.d.
28	AU beef rib eye steak	Australia	194	Yes	Retail 7, Chiang Mai	1	-	-	n.d.
29	T-bone steak	Thailand	260	No	Retail 8, Chiang Mai	2	30.61	27.09	<i>C. algidicarnis</i> (Th 8)
30	Shank slices	Thailand	300	No	Retail 5, Chiang Mai	2	-	-	n.d.
31	Tenderlion TH-Charolais	Thailand	150	No	Retail 5, Chiang Mai	2	-	-	n.d.
32	Top blade	Thailand	200	No	Retail 5, Chiang Mai	2	-	-	n.d.
33	US beef rib eye steak	United States	230	No	Retail 7, Chiang Mai	2	-	-	n.d.
34	US beef striploin steak	United States	298	No	Retail 7, Chiang Mai	2	-	-	n.d.
35	Rib eye steak	Thailand	280	No	Retail 8, Chiang Mai	2	37.63 ⁵	-	n.d.

36	Top round steak	Thailand	280	No	Retail 5, Chiang Mai	2	-	-	n.d.
37	Brisket	Thailand	200	No	Retail 5, Chiang Mai	2	36.68	22.65	<i>C. algidicarnis</i> (Th 9)
38	Tenderloin	Thailand	150	No	Retail 5, Chiang Mai	2	36.49 ⁵	-	n.d.
39	AUS. grass fed rib eye	Australia	200	No	Retail 5, Chiang Mai	2	-	-	n.d.
40	Slide wagyu brisket	Japan	100	No	Retail 5, Chiang Mai	1	33.20 ⁵	-	n.d.

¹ Presence of absorbent pad (yes or no)

² BPS score as described by Boerema et al. (2007): score 0: no gas production; score 1: small bubbles in the drip, packaging intact; score 2: loss of vacuum due to small gas production.

³ Ct-values of probe CI555 of PCR 1 (Table 1) specific for the genus *Clostridium*, tested with meat drip and enrichment samples, - = no amplification

⁴ Cultural results of enrichment: n.d. = not done (as the enrichment samples were negative for clostridia in the real-time PCR)

⁵ PCR products of these samples (n = 8) were subjected to sequencing of the 16S rRNA gene (supplementary data, Table S2)

Table 4 Results of the real-time PCR for *Clostridium* spp. for the meat drip and meat drip enrichment samples

Beef origin	n of samples in total	Meat drip				Meat drip enrichment			
		strong	moderate	weak	total	strong	moderate	weak	total
Australia	14	2	1	-	3	1*	-	1	2
Japan	1	-	1	-	1	-	-	-	-
New Zealand	1	-	-	-	-	-	-	-	-
United States	2	-	-	-	-	-	-	-	-
Thailand	22	4	3	5	12	6*	1*	1*, 1	9
Total	40	6	5	5	16	7	1	4	11

* Enrichment samples from which *C. algidicarnis* could be isolated

Regarding different packaging types, 40.9% (n = 9/22) of beef samples from packs with absorbent pads and 38.9% (n = 7/18) of beef samples from packs without absorbent pads tested positive for clostridia by real-time PCR. In the present study, beef samples that tested positive for *C. algidicarnis* by PCR showed gas production similar to that of PCR-negative beef samples, as BPS scores ranged from score 1 (small bubbles in the drip, packaging intact) to score 2 (loss of vacuum due to slight gas production). Although spoilage odors were not formally graded in this study, all vacuum-packed beef samples exhibited off-odors indicative of spoilage upon opening.

After 6 weeks of enrichment, 11 samples were positive for *C. algidicarnis*, while five samples turned out to be negative (Table 2, Table 5). PCR products of eight samples with weakly PCR-positive results (6 from drip samples and 2 from enrichment samples) were sequenced to confirm that the DNA originated from *Clostridium* spp. and was not non-specific amplification (Table 2, Table 4). Sequences from six of those samples (no. 4, 6, 14, 21, 22, and 40; Table 5) showed the highest identity (99.8%) to the 16S rRNA gene of *C. algidicarnis* (NCBI accession number, e.g., NR_041746.1) and *C. putrefaciens* (NCBI accession number, e.g., NR_119282.1). The sequences of drip samples no. 35 and 38, which were the only two samples positive for clostridia but negative for *C. algidicarnis*, were identified as *C. perfringens* by sequencing. Both sequences showed 99.8% similarity to the 16S rRNA genes of numerous *C. perfringens* strains provided in GenBank (NCBI accession number, e.g., NR_113204).

Table 5 Sequencing results of the 16S rRNA gene of samples with weakly PCR-positive results

Meat sample No.	Sequence
6 (meat drip, Ct-value = 31.24)	GGGGATAGCCTTCCGAAGGAAGATTAATACCCATAACATAGCATTATCGCATGATAAAGTTATTAAGGAGT AATCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAA TATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAGCTCTGT CTTCGGGGACGATAATGACGGTACCCGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA GGTGGCAAGCGTTGTCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATACTTAAGTGGGATGTGAAATACCC GGGCTCAACCCGGGTGCTGCATTCCAACTGGGTATCTAGAG
14 (meat drip, Ct-value = 33.88)	GGGGATAGCCTTCCGAAGGAAGATTAATACCCATAACATAGCATTATCGCATGATAAAGTTATTAAGGAGT AATCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAA TATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAGCTCTGT CTTCGGGGACGATAATGACGGTACCCGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA GGTGGCAAGCGTTGTCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATACTTAAGTGGGATGTGAAATACCC GGGCTCAACCCGGGTGCTGCATTCCAACTGGGTATCTAGTGAGGGGAGAGGATTGTCATTGGATACTGGAAGTC TAGAGTGACAGGAGAGGAATTAACCCGATGCTCCACCGCTGGTG
21 (meat drip, Ct-value = 36.25)	TAGCCTTCCGAAGGAAGATTAATACCCATAACATAGCATTATCGCATGATAAAGTTATTAAGGAGTAATCC GCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAGCCGAC CTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG CACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAGCTCTGTCTTCG GGGACGATAATGACGGTACCCGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG CAAGCGTTGTCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATACTTAAGTGGGATGTGAAATACCCGGGCT CAACCCGGGTGCTGCATTCCAACTGGGTATCTAGAG
35 (meat drip, Ct-value = 37.63)	GGATAGCCTTCCGAAGGAAGATTAATACCGCATAATGTTGAAAGATGGCATCATCATTCAACCAAAGGAGCAA TCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAGCCG ACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT TGCACAATGGGGAACCCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAGCTCTGTCTT TGGGGAAGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG TGGCGAGCGTTATCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATGATTAAGTGGGATGTGAAATACCCGG GCTCAACTTGGGTGCTGCATTCCAACTGGTTATCTAGAG
38 (meat drip, Ct-value = 36.49)	TAGCCTTCCGAAGGAAGATTAATACCGCATAATGTTGAAAGATGGCATCATCATTCAACCAAAGGAGCAATCC GCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAGCCGAC TGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG ACAATGGGGAACCCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAGCTCTGTCTTTGG GGAAGATAATGACGGTACCCAAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG CGAGCGTTATCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATGATTAAGTGGGATGTGAAATACCCGGGCT CAACTTGGGTGCTGCATTCCAACTGGTTATCTAGAG
40 (meat drip, Ct-value = 33.20)	TAGCCTTCCGAAGGAAGATTAATACCCATAACATAGCATTATCGCATGATAAAGTTATTAAGGAGTAATCC GCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAGCCGAC CTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG CACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAGCTCTGTCTTCG GGGACGATAATGACGGTACCCGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGG CAAGCGTTGTCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATACTTAAGTGGGATGTGAAATACCCGGGCT CAACCCGGGTGCTGCATTCCAACTGGGTATCTAGAG
4 (PYGS-drip, Ct-value = 38.16)	GGGGATAGCCTTCCGAAGGAAGATTAATACCCATAACATAGCATTATCGCATGATAAAGTTATTAAGGAGT AATCCGCTATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAA TATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAGCTCTGT CTTCGGGGACGATAATGACGGTACCCGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA GGTGGCAAGCGTTGTCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATACTTAAGTGGGATGTGAAATACCC GGGCTCAACCCGGGTGCTGCATTCCAACTGGGTATCTAGACGATTTCTCCTGACTTGTGAAGGGTCTTCGCAT TGATTCAATTAACACATGCTCCACCGCTTGTGGGGTCCCCGTCATTCCTTTGAGTTTCAGCCTTGCGGCCG TACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTTCAGCACTGAGGGGCGGAAACCCCAAC
22 (PYGS-drip, Ct-value = 37.67)	GCCTTCCGAAGGAAGATTAATACCCATAACATAGCATTATCGCATGATAAAGTTATTAAGGAGTAATCCG TATGAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAGGCCACGATGCGTAGCCGACCT GAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA CAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAGCTCTGTCTTCGGG ACGATAATGACGGTACCCGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA AGCGTTGTCCGGATTACTGGGCGTAAAGGGAGCGTAGGCGGATACTTAAGTGGGATGTGAAATACCCGGGCTCA ACCCGGGTGCTGCATTCCAACTGGGTATCTAGAG

According to the PCR results, the detection rate of clostridia in the analyzed beef samples was 40.0% ($n = 16/40$). As *C. perfringens* is considered a mesophilic clostridial species, the detection rate of cold-tolerant clostridia in beef sold in supermarkets in Thailand was therefore 35.0% ($n = 14/40$). Overall, 45.5% of domestically produced beef and 22.2% of imported beef were found to be contaminated with cold-tolerant clostridia.

All drip enrichments ($n = 11$) that were positive for clostridia by PCR were subjected to cultivation. To increase the probability of isolating pure isolates of *C. algidicarnis*, two methods were used to culture clostridial spores, namely treatment with heat (80°C for 5 min) and with 50% ethanol. *C. algidicarnis* could be isolated from four enrichment samples both by heat treatment and by treatment with 50% ethanol. From two and three enrichments, respectively, *C. algidicarnis* could only be cultivated by heat treatment or by treatment with ethanol. Overall, clostridia could be isolated from 81.8% ($n = 9/11$) of PCR-positive enrichments (Table 2). All clostridial colonies grown on CBA were identified as *C. algidicarnis* by the fourth PCR (Table 1) and MALDI-TOF MS. Two drip enrichment samples (beef samples no. 4 and 22; Table 2) with weakly PCR-positive results were not cultivatable.

Growth curve and sporulation of *C. algidicarnis*

The growth and sporulation of ten strains were tested in PYGS broths that were incubated at different temperatures. The DSM 15099 strain and the German and Austrian strains (isolated from other studies as mentioned in section Reference strains) were used for comparative purposes alongside the strain isolated in this study. None of the tested strains were able to grow at 37°C and were unculturable. While the growth curves of all strains were similar at 25°C and 10°C, those at 4°C differed individually (Figure 1). The optimal growth temperature for all strains was 25°C, as they entered the exponential growth phase by day 3 and reached the stationary phase (approximately 7.5-9.1 log₁₀ cfu/ml) by day 7. At 10°C, the stationary phase was reached by day 21 (9.3-10.3 log₁₀ cfu/ml), achieving higher cell counts than at 25°C. Incubation at 4°C resulted in substantially delayed growth; only six strains reached concentrations of 4.2-6.6 log₁₀ cfu/ml by day 42. The remaining four strains (A1, G3, T1, and T3, Fig. 1) survived but could not grow, maintaining cell densities between 10 and 100 cfu/ml. Although all *C. algidicarnis* strains grew well and underwent a stationary phase lasting five weeks at 25°C and three weeks at 10°C, only three strains (A3, G2, and T3) incubated at 25°C formed spores, and even then, only in small numbers (Figure 2).

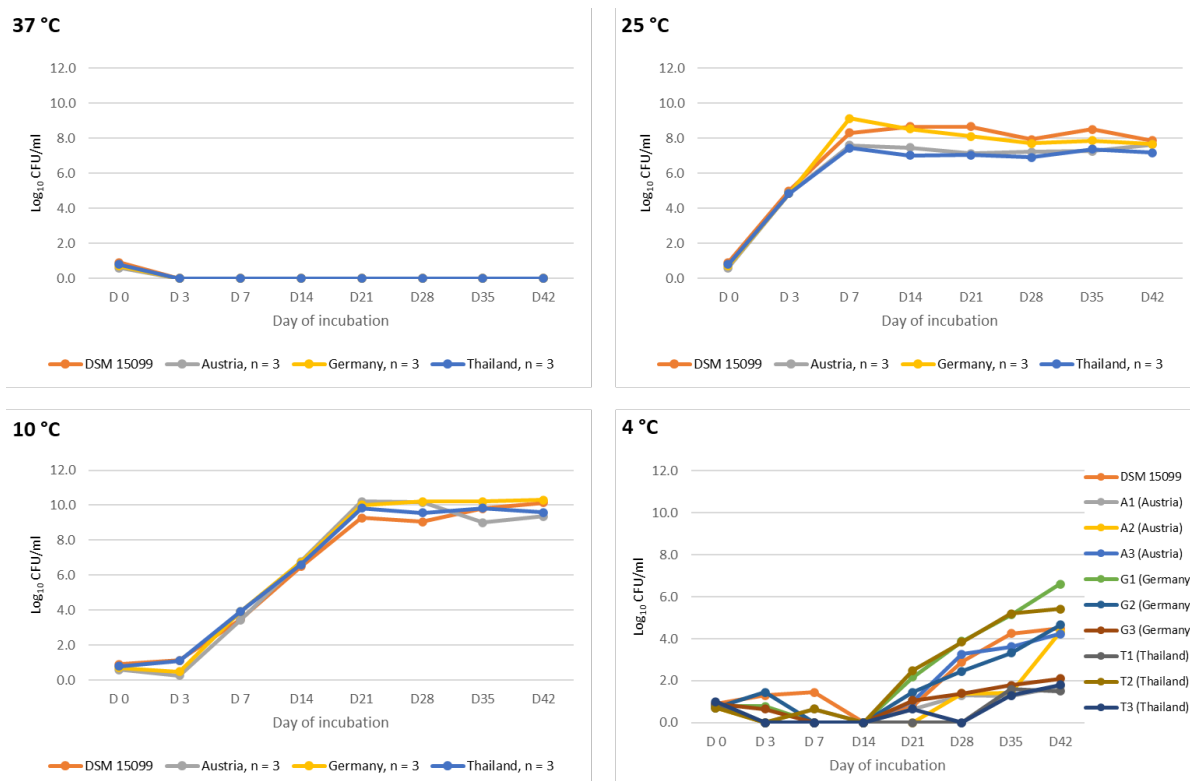


Figure 1 Growth curve of *C. algidicarnis* (DSM 15099) and the other 9 strains isolated from vacuum-packed beef in Austria (n = 3), Germany (n = 3), and this study in Thailand (n = 3). The strains were anaerobically incubated in PYGS broth at four different temperatures (37°C, 25°C, 10°C, and 4°C) for 42 days. At 4°C, the growth curves of the individual strains differed significantly from one another, so they are shown separately.

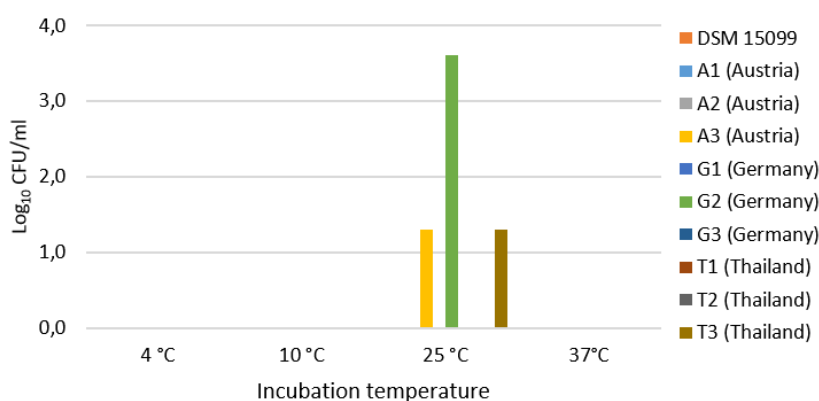


Figure 2 Sporulation of *C. algidicarnis* strains after incubation at the corresponding temperature for 42 days. Only strains A3, G2 and T3 produced spores (detected by treating with 50% ethanol prior to cultivation), albeit at a very low level, when incubated at 25°C.

MALDI-TOF spectrum

As shown in Figure 3A, the Thai strains exhibited high spectral similarity to one another and clustered closely with two Austrian strains. One German strain and one Austrian strain were highly similar to each other as well as to the type strain DSM 15099. Overall, the results suggest that most of the tested strains displayed similar mass spectra, with the exception of two German strains (G1 and G3), which exhibited distinct spectral profiles. Figure 3B displays representative mass spectra illustrating spectral variations among strains DSM 15099, A1 (Austria), G1 (Germany), and T1 (Thailand). According to the dendrogram, strain G1 diverged from the remaining strains, although its overall spectrum appeared visually similar to those of the other strains.

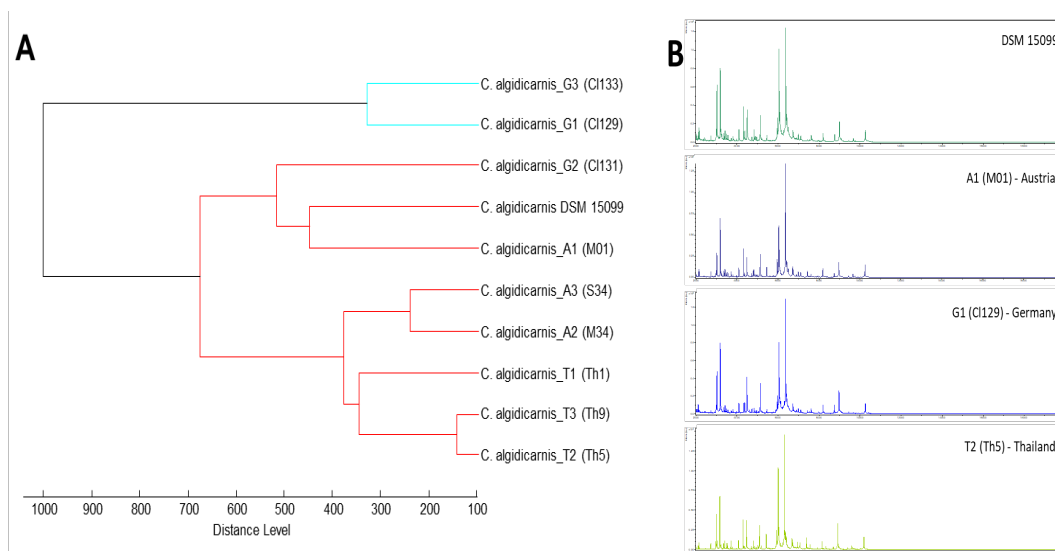


Figure 3 (A) Similarity of MALDI-TOF MS spectra of *C. algidicarnis* strains from Austria (A1-A3), Germany (G1-G3) and Thailand (T1-T3), (B) Representative mass spectra of the four test trains. The internal numbers of the strains used in the research unit are shown in brackets.

DISCUSSION

A total of 40 vacuum-packed beef samples were collected from two different provinces located in the north and south of Thailand. Overall, 40.0% and 35.0% of tested vacuum-packed beef samples were found to be contaminated with clostridia and, in particular, cold-tolerant clostridia, respectively. No significant difference in detection rates was observed between beef samples packed with and without absorbent pads (40.9% and 38.9%, respectively), even when considering that sample preparation differed between these two packaging groups. When analyzing the origin of the samples, a higher positive rate (45.5%) was found in locally produced beef samples than in imported beef samples (22.2%). The overall detection rate is relatively high and is comparable to findings from other studies conducted in Europe. For example, 31.0% of vacuum-packed beef produced in Austria (Angerer et al., 2025) and 34-53% of samples in Germany (Bonke et al., 2016; Mang et al., 2021) were positive for cold-tolerant clostridia. It should be noted that the total number of meat samples tested in this study was smaller than in those conducted in Austria (n = 150) and Germany (n = 96 by Bonke et al., 2016; n = 60 by Mang et al., 2021). Moreover, in the present study, beef samples were collected from only two provinces, meaning the detection rate should be considered preliminary.

C. algidicarnis was the only cold-tolerant *Clostridium* species detected by PCR and isolated in this study. The two methods used to prepare the samples prior to isolation, namely, heat treatment at 80°C for 5 min and treatment with 50% ethanol, show no significant difference in the isolation rate of *C. algidicarnis*. Altogether, these bacteria could be isolated from 9 out of 11 positive drip enrichments. Two drip enrichment samples (no. 4 and 22) that yielded weakly PCR-positive results may have contained only dead or inactive *C. algidicarnis* cells. Consequently, no growth was observed during enrichment, and the cells could not be recovered by cultivation. Interestingly, the Ct-values of these two meat drip samples prior to enrichment were classified as strongly positive. The significant difference in Ct-values between the meat drip and enrichment samples (10-12 PCR cycles) of these two samples suggests that the bacteria died during sample preparation or incubation in PYGS broth rather than entering a viable but non-culturable state. If the latter were the case, the difference in Ct-values between the meat drip and the enrichment sample would be expected to be small. Clostridia produce spores to survive under harsh conditions, including in the presence of oxygen. It can be hypothesized that these strains may grow well in vacuum-packed beef samples while producing fewer spores. During sample preparation, vegetative cells are usually exposed to air, resulting in cell death, followed by DNA degradation during the six-week incubation period. The few surviving cells may have been unable to compete with the meat microbiota, which grows faster in PYGS broth.

In the present study, beef samples testing positive for *C. algidicarnis* showed only minimal gas production, similar to the negative beef samples. These results are consistent with those of other studies, which reported that *C. algidicarnis*, in contrast to *C. estertheticum*, produces very little gas (Lawson et al., 1994; Mang et al., 2021; Angerer et al., 2025). Unlike the BPS score, spoilage odors were not graded in this study. However, all vacuum-packed beef samples were classified as spoiled based on their odor deviations due to storage beyond their shelf life. Indeed, the role of these strains in the spoilage of vacuum-packed beef should be investigated in depth. This could include investigating their ability to produce spoilage indicators such as butanol and butyric acid, their growth in the meat matrix, and their effects on the shelf life of contaminated vacuum-packed beef. To clarify these aspects, challenge tests should be carried out in future studies.

Regarding the detection of *C. perfringens* DNA in meat drips, these samples tested negative for clostridia after incubation at 4°C for 6 weeks in PYGS broth. An attempt to isolate *C. perfringens* from these meat samples could not be performed, as they had already been disposed of after the meat drips had been taken for enrichment purposes. The disappearance of *C. perfringens* DNA may be explained by either the presence of dead cells only or the inability of viable cells to proliferate at the low incubation temperature of 4°C.

All tested strains obtained from this study, as well as those from Germany and from Austria and the reference DSM 15099 strain, did not grow at 37°C. Instead, a temperature of 25°C was identified as their optimum growth temperature. This is consistent with findings reported by Lawson et al. (1994). There was a discrepancy between the growth curve and the sporulation rate. Although all strains grew well at 25°C and 10°C, they produced few or no spores. On day 42 of incubation, sporulation was observed in only three strains (A3, G2, and T3) incubated at 25°C. At 4°C, the tested strains grew very slowly. This may have been due to the low initial inoculation number combined with the low incubation temperature, which created unfavorable conditions for multiplication. However, the growth and sporulation rates of the Thai strains in pure culture contradicted the culture results obtained from meat drip enrichment incubated at 4°C. In the latter case, the tested strains produced a large quantity of spores that survived treatment with heat or ethanol and could therefore be isolated.

The growth and sporulation of *C. algidicarnis* can be influenced by various factors, including the incubation temperature and in vitro conditions (i.e., pure media), as well as the real meat matrix and the interaction with the meat microbiota

present in the enrichment medium. Growing in pure PYGS enrichment without co-occurring microorganisms appeared to be associated with little or no spore formation, despite the cultures reaching the stationary phase within three weeks at 10°C and one week at 25°C. Previous studies (Mang et al., 2021; Angerer et al., 2025) have also reported a low isolation rate of *C. algidicarnis* from strongly PCR-positive enrichment of meat drip, which could be a result of a low sporulation rate. As reported by Angerer et al. (2025), increasing the incubation time to 16 weeks led to an increase in the number of spores that survived heat or ethanol treatment and could subsequently be isolated. This could be because good growth conditions (such as a nutrient-rich medium) lead to low sporulation requirements. Similar observations were found for *C. estertheticum*, as it grew at similar rates in meat juice and PYGS broth at temperatures between -2°C and 17°C. However, at 20°C, growth was only observed in meat juice, not in PYGS broth (Yang et al., 2009). These characteristics lead to difficulties in predicting the isolation rate of cold-tolerant *Clostridium* species. Although *C. algidicarnis* cannot be easily isolated and does not appear to reliably produce spores under laboratory conditions, it was nevertheless found to be the main *Clostridium* species detected in vacuum-packed beef sold in Austria (Angerer et al., 2025) as well as in Germany (Mang et al., 2021). In addition, it was previously detected in Brazil (Silva et al., 2011). To increase the success of culturing these cold-tolerant clostridia, further studies are required to investigate conditions that enhance sporulation, such as the use of specialized media or different incubation conditions.

MALDI-TOF MS is widely used for the species identification of microorganisms. Beyond this predominant application, attempts have been made to use MALDI-TOF MS to differentiate between bacterial strains within the same species, particularly between toxin- and non-toxin-producing strains like *Clostridioides difficile* or *C. botulinum* (Bano et al., 2017; Abdrabou et al., 2023). However, difficulties remain with this application, and further tools for clustering the spectra may help to distinguish subgroups of *C. botulinum* (Abdrabou et al., 2023). In this study, in addition to using MALDI-TOF MS for species identification, it was also used to analyze the similarity of the protein spectra among *C. algidicarnis* strains isolated from two different regions: Central Europe (Austria and Germany) and Southeast Asia (Thailand). The spectra of the three Thai strains were highly similar, and they were more closely related to the Austrian strains than to the German strains. However, it should be noted that only three strains were tested from each country; thus, any interpretation of their similarity should be treated with caution. For future studies, other tools such as whole genome sequencing should be used to compare the genotypes of strains obtained from different regions.

CONCLUSIONS

Of the 40 investigated vacuum-packed beef samples sold in Thailand, 35.0% and 22.5% were found to be contaminated with *C. algidicarnis* using real-time PCR and culture methods, respectively. Although this detection rate is relatively high, it is preliminary data due to the small sample size. Consistent with the fact that the beef samples were investigated after their use-by dates, all were classified as spoiled based on sensory detection of off-odors. However, because *C. algidicarnis* produces only a small amount of gas, the level of gas production cannot be used as an indicator of spoilage for this clostridial species. As an ancillary food safety screen, *C. perfringens*, a potential toxin producer, was detected by PCR and sequencing in two meat samples.

Characterization in terms of growth rate and sporulation revealed that *C. algidicarnis* can grow well in PYGS broth when incubated at 25°C or 10°C; however, the rate of spore production is low. This may be due to their favorable growth conditions in the absence of co-existing meat microbiota, resulting in no environmental stress and consequently minimal spore formation. MALDI-TOF MS spectral analysis did not reveal any significant differences between strains from the

two regions (Southeast Asia and Central Europe). Given its impact on food waste and the economy, limiting meat spoilage is an important objective across the supply chain. It remains to be determined whether this bacterium poses a significant spoilage problem for the Thai meat industry; this should be assessed in collaboration with beef producers. Further work is needed to identify the sources of contamination. To achieve this, close collaboration with Thai cattle slaughterhouses and meat processing plants will be required.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Phakamas Ngamsanga: Data curation, Investigation.

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Julian Bleicher: Data curation, Investigation, Writing - review & editing.

Karin Schwaiger: Resources, Writing - review & editing.

Samart Dorn-In: Conceptualization, Funding acquisition, Project administration, Methodology, Data curation, Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing.

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