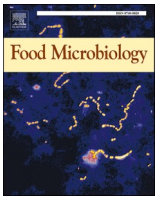
Food Microbiology 132 (2025) 104838

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Cold-tolerant *Clostridium* spp. related to meat spoilage in cattle farms in Austria

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ARTICLE INFO

Keywords:
C. estertheticum
Beef
Cow faeces
Hides
PCR

ABSTRACT

Many cold-tolerant *Clostridium* spp. are responsible for the spoilage of vacuum-packed meat. Cattle can ingest the bacteria via the soil and the environment when grazing or via the feed. Since cattle farming in Austria's Alpine regions is often practiced as pasture-based farming, the aim of this study was to investigate the prevalence of cold-tolerant clostridia in cattle in these regions, to identify the species detected, and to determine the growth temperature of the isolated clostridia. For this purpose, 260 faecal and 260 hide wipe samples were taken from 260 healthy adult cattle from 26 farms in the provinces of Salzburg and Tyrol. The samples were analysed using qPCR, sequencing, and cultural methods. Using qPCR, 22.3 % of the faecal samples were positive for *C. estertheticum*, 33.8 % for *C. tagluense*-like, 32.7 % for *C. bowmanii*, 11.2 % for *C. frigoriphilum*, and 14.2 % for *C. gasigenes*. The isolation rates of the species from the PCR-detected samples ranged from 4.7 % to 59.5 %. In addition, nine different *Clostridium* species were isolated by culture, with *C. subterminale*, *Lacrimispora algidixylanolytica* (syn. *C. algidixylanolytica*), *C. tagluense*, and *C. botulinum* being found most frequently. The prevalence of cold-tolerant clostridia in the investigated faeces was relatively high, while in the hide swab samples it was very low. The latter could be related to the lower contamination of the hide with dirt and faeces. The results of this study provide useful information for slaughterhouses, which should pay attention to cleanliness and hygiene during dehiding and evisceration to avoid further contamination of the meat.

1. Introduction

Meat spoilage caused by cold-tolerant clostridia, in particular the species *C. estertheticum*, can be partially recognised by the swelling of the packaging, known as blown pack spoilage (BPS, Fig. 1), which is caused by CO_2 and H_2 formation. These bacteria can produce enormous amounts of butanol, butyric acid, acetic acid, and butyl esters, which leads to the typical cheesy odour of the affected meat (Broda et al., 1996). *C. estertheticum* was first isolated in the United Kingdom in 1989 (Dainty et al., 1989), and since then the occurrence of cold-tolerant clostridia associated with this kind of spoilage has been reported worldwide, especially in countries with high meat production and/or consumption such as Brazil, Germany, Ireland, New Zealand, the United Kingdom, and the United States (Mang et al., 2021). Other cold-tolerant *Clostridium* spp. that have also been isolated from meat include *C. frigoriphilum*, *C. bowmanii,* and *C. tagluense*-like (Dorn-In et al., 2018), *C. algidicarnis* (Lawson et al., 1994; Mang et al., 2021), *C. gasigenes* (Broda et al., 2000b), and *C. frigidicarnis* (Broda et al., 1999).

As cold-tolerant clostridia are usually found in soils of temperate and subtropical climate zones, cattle can ingest the bacteria while grazing. This means that faeces and hides of slaughtered animals contaminated with soil and faeces are a potential source of contamination for beef. Nevertheless, there are only few studies on the presence of *C. estertheticum* and other cold-tolerant clostridia in these sample materials, e.g., in Ireland (Esteves et al., 2020; Moschonas et al., 2009), Switzerland (Wambui et al., 2021), and New Zealand (Esteves et al., 2021). The other studies focus on the detection of cold-tolerant clostridia in vacuum-packed meat. Cattle farming is of great importance in

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https://doi.org/10.1016/j.fm.2025.104838
Received 2 March 2025; Received in revised form 21 May 2025; Accepted 6 June 2025
Available online 6 June 2025

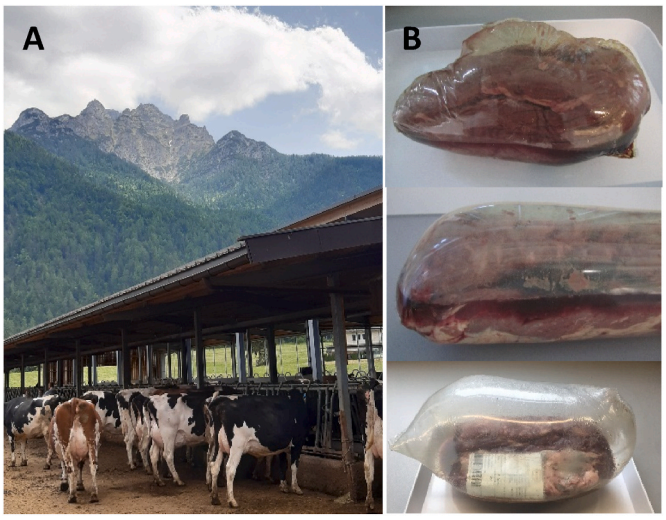

Fig. 1. **A**: Cattle (dairy) farm in the Alpine region in Austria and **B**: blown pack spoilage (BPS) of vacuum-packed beef caused by *C. estertheticum* (BPS).

Austria, and Alpine pasture and grazing are widespread in this context. The aim of this study is therefore to provide an initial overview of the prevalence of *C. estertheticum* and other cold-tolerant clostridia in cattle faeces and hides in Austria. The results of this study should help to assess the risk of contamination of meat with *C. estertheticum* and other cold-tolerant clostridia. Strategies for the delivery of cattle for slaughter and hygiene measures during slaughter can be evaluated in order to minimise the risk of contamination.

2. Material and methods

2.1. Samples and preparations

2.1.1. Clostridial reference strains

The following reference *Clostridium* species were used: *C. estertheticum* subsp. *estertheticum* (DSM 8809), *C. frigoriphilum* (DSM 17811), *C. algidicarnis* (DSM 15099), *C. gasigenes* (DSM 12272), *C. bowmanii*, and *C. tagluense*-like (all previously isolated from meat, confirmed by 16S rRNA gene sequencing). These clostridial strains were subcultured in peptone-yeast-glucose-starch (PYGS) broth (Lund et al., 1990) and incubated at 10 °C for four weeks. The pure cultures of *C. estertheticum*, *C. frigoriphilum*, *C. bowmanii*, *C. algidicarnis*, *C. gasigenes*, and *C. tagluense*-like were subjected to DNA extraction, and the DNA extracts were used as PCR positive controls. The species *C. estertheticum*, *C. algidicarnis,* and *C. gasigenes* were artificially contaminated in faecal suspensions and then subjected to the test the efficiency of the DNA extraction methods.

2.1.2. Animal samples and preparation

A total of 520 animal samples, including 260 faecal and 260 hide wipe samples, were taken from 260 healthy cows from 26 dairy farms in the Federal States Salzburg and Tyrol, Austria. The number of cattle per sampled farm varied between 12 and 140. To avoid stress from catching and restraining of the animals, sampling was carried out during feeding, whereby the animals were restrained in their usual feeding fences. Ten adult animals were sampled per farm. A faecal sample and a hide wipe sample were taken from the same animal. The faecal samples were taken directly from the rectum or from the floor if the cows had shed fresh faeces and the animal could be clearly identified. The veterinary gloves containing the faecal samples were knotted to prevent faecal leakage and then placed in a polystyrene box with cool packs for transport.

To obtain the hide wipe samples, one square metre of an area on the hindquarters of the cow was wiped several times vertically and horizontally with a moistened sterile sponge (S/Budget sponge cloth, 18 × 10 × 0.3 cm, autoclaved at 125 °C for 15 min). The sponge cloth was then packed in a stomacher bag and placed in the polystyrene box with cool packs for transport. During transport, the temperature in the polystyrene box was approximately 10 (±2) °C. The samples were stored at 4 °C and prepared for enrichment within 24 h after sampling.

To prepare PYGS enrichment, 50 ml of PYGS broth was added to each of the hide wipe samples in the stomacher bags, while 10 g of faeces were weighed into a stomacher bag and filled with 90 ml of PYGS broth. The samples were then homogenised in a laboratory stomacher (Bag-Mixer, Interscience) for 30 s. The sample suspension (14 ml) was transferred to a sterile glass tube and incubated anaerobically (with AnaeroGen™, Thermo Scientific™/Oxoid™) at 4 °C for 4–5 weeks.

2.2. Molecular biological examinations

2.2.1. DNA extraction

Faecal samples usually contain various PCR inhibitors such as phytic acid that prevent PCR amplification (Thornton and Passen, 2004). Therefore, the efficiency of the DNA extraction method for faecal samples plays an important role in the success of qPCR. To test the efficiency of the DNA extraction method, 0.9 ml of bovine faecal sample in the PYGS broth (concentration: 1 g per 10 ml) was artificially contaminated with 100 µl of different clostridial species, namely *C. estertheticum*, *C. algidicarnis*, and *C. gasigenes*. The contaminated and native faecal suspensions were subjected to the three DNA extraction methods, each in duplicate.

DNA extraction with the High Pure PCR Template Preparation Kit (Roche) and the QIAamp Fast DNA Stool Mini Kit (Qiagen) was performed according to the manufacturer's instructions. The method using the combination of InhibitEX Buffer (Qiagen) and High Pure Template Preparation Kit (Roche) was performed as follows: 200 µl of the sample suspension or PYGS enrichment was transferred to a 1.5 ml tube. Then, 1 ml of InhibitEX buffer was added and vortexed for 15 s. The suspension was then heated to 95 °C for 5 min in a thermomixer (Eppendorf) and vortexed for 15 s. To separate the solid components of the sample, the mixture was centrifuged at 20,000×*g* for 1 min. Finally, 200 µl of the supernatant was transferred to a new 1.5 ml tube, and DNA extraction was continued using the High Pure PCR Template Preparation Kit (Roche) as described by the manufacturer.

The DNA extraction method for the clostridial or bacterial colonies recovered from agar plates was performed using the direct (heating) extraction method as described in previous studies (Mang et al., 2021)

2.2.2. Quantitative PCR (qPCR)

The DNA extracts were tested using three qPCR methods (qPCR 1, 2, and 3, see Table 1). qPCRs 1 and 2 were used to check for the presence of clostridia and to identify the species. Samples that gave an inconclusive or negative result in qPCRs 1 and 2 (for clostridia) were then subjected to qPCR 3 (for universal bacteria) to confirm the success of the DNA extraction.

Primer pairs and probes were carried over from previous studies (Table 1), except for the probe for the species *C. gasigenes*, which was newly developed in this study. The validation of the specificity of the probe for the detection of *C. gasigenes* (qPCR 2, Cgas) was performed as described in a previous study (Dorn-In et al., 2018). Table S1 (supplementary data) shows the specificity of this probe, which was tested by qPCR 2 with 51 bacterial species/strains. The target gene of all three qPCR methods is the 16S rRNA gene. The amplicon size is approximately 566 bp for qPCR 1 and 2 (for clostridia) and 604 bp for qPCR 3 (for universal bacteria).

The master mix for qPCR contained the following components: 0.25 µM per primer, 0.1 µM per probe, 10 µl SensiFast™ Probe No-Rox sample (Bioline™, Meridian™), 2 µl of the respective DNA sample, and filled up with RNA-free water to the total volume of 20 µl per reaction. The qPCR protocol in the CFX Opus Real-Time PCR Systems (Bio-

Table 1
Primer and probes for three qPCR methods.

qPCR	Specificity	Primer/ probes	Fluorescence (5′-3′)		Sequence (direction 5′-3′)	Reference[a]
1	*Clostridium* spp.	Cl94-F	–	–	CGGCGGACGGGTGAGTAAC	1, 2
		Cl642-R	–	–	CCTCTCCTGCACTCTAGA	2
	Clostridium spp.	Cl555	FAM	BHQ-1	CCTTTACRCCCAGTAAATCCGGAC	3
	C. estertheticum	Cest	Hex	BHQ-1	CAAAGGAATTTTTCGGAATTTCACTTTGAG	2
	C. frigoriphilum	Cfgrpl	Cy3.5	BHQ-2	CAAAGGAATAGTCTTCGGATTATTTCACT	2
	C. putrefaciens & *C. algidicarnis*	Cpal	Cy5	BHQ-2	ACCCCATAACATAGCATTATCGCATG	4
	C. tagluense-like	Ctag-like	Cy5.5	BBQ-650	CAAAGGATTTTTCTTCGGAAAATTCCAC	2
2	*Clostridium* spp.	Cl94-F	–	–	CGGCGGACGGGTGAGTAAC	1, 2
		Cl642-R	–	–	CCTCTCCTGCACTCTAGA	2
	Clostridium spp.	Cl555	FAM	BHQ-1	CCTTTACRCCCAGTAAATCCGGAC	3
	C. estertheticum	Cest	Hex	BHQ-1	CAAAGGAATTTTTCGGAATTTCACTTTGAG	2
	C. frigoriphilum	Cfgrpl	Cy3.5	BHQ-2	CAAAGGAATAGTCTTCGGATTATTTCACT	2
	C. bowmanii	Cbow	Cy5	BHQ-2	CAAAGGATTCTCTTCGGAGATTCCAC	2
	C. gasigenes	Cgas	Cy5.5	BBQ-650	TCGGGCTCAACCCGAGAACTGC	This study
3	Universal bacteria	Uni335-F	–	–	CADACTCCTACGGGAGGC	5
		Uni923-R	–	–	CTTGTGCGGGCCCCCGT	6
	Universal bacteria	Uni799	Cy5.5	BBQ-650	AACAGGATTAGATACCCTGGTAGTC	6

[a] Reference: 1: Brightwell and Clemens (2012); 2: Dorn-In et al. (2018); 3: Dorn-In et al. (2022); 4: Mang et al. (2021); 5: Dorn-In et al. (2015); 6: Dorn-In et al. (2023).

Rad™) device started with initial denaturation at 95 °C for 5 min. For each of the following 40 cycles, denaturation at 95 °C for 5 s was followed by annealing and elongation at 60 °C for 30 s for qPCR 1 and 2 (for clostridia) and at 62 °C for 20 s for qPCR 3 (for universal bacteria).

Positive and negative controls were included to demonstrate the validity of the qPCR. For each PCR run, DNA extracts of six *Clostridium* species were used as positive controls: *C. estertheticum* subsp. *estertheticum*, *C. frigoriphilum*, *C. bowmanii, C. tagluense*-like, *C. algidicarnis,* and *C. gasigenes*. The constant Ct-values of the positive controls were defined in relation to the cut-off of the relative fluorescence unit (RFU). If one of the amplified samples yielded an RFU value that was below the cut-off value, the corresponding sample was evaluated as negative.

2.2.3. Sequencing

The DNA of bacterial colonies identified as *Clostridium* spp. by qPCR was subjected to sequencing analysis. For this purpose, the PCR amplicons from qPCRs 1 or 2 were purified using the E.Z.N.A.® Cycle Pure Kit (Omega) according to the manufacturer's instructions and subsequently sequenced at Microsynth Austria GmbH (Vienna, Austria). The forward primer (Cl94-F) was used for sequencing. The sequences obtained were compared with those of the reference sequences in the database of the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to identify or confirm the species of the isolated clostridia.

2.3. Cultural examination

PYGS enrichments from faeces and hide wipe samples were incubated at 4 °C for 4–5 weeks for the first isolation phase and 14–16 weeks for the second isolation phase.

All PYGS enrichments (n = 520) were subjected to the culture method in the first isolation phase. For this purpose, 0.5 ml of PYGS enrichment was transferred to a 1.5 ml reaction tube and heated in a ThermoMixer™ (Eppendorf™) at 80 °C for 5 min to kill the vegetative cells of the accompanying microorganisms (Dorn In et al., 2022). Subsequently, 50 µl of the heat-treated PYGS were dropped onto a blood agar plate (CBA, Columbia blood agar + 5 % sheep blood, BioMérieux™) and incubated anaerobically at 10 °C for 3–4 weeks. Colonies grown on CBA with different morphologies were subcultured onto two CBA plates, one of which was incubated aerobically and the other anaerobically at 10 °C for 3 weeks. The colonies that grew only under anaerobic conditions were subjected to DNA extraction using a direct (heating) method. The DNA extracts were subjected to qPCR 1 and qPCR 2 and, in some cases, qPCR 3 if qPCR 1 gave negative results. If the tested colonies were positive for clostridia, their PCR products were subjected to sequencing. If qPCR was negative for universal bacteria, the bacterial suspension was again subjected to DNA extraction using the High Pure Template Preparation Kit (Roche) and retested with qPCR 1, 2, and if necessary, with qPCR 3.

The clostridial isolates from the first isolation phase were subjected to the test of growth temperature. For this purpose, the colonies of the same isolate were subcultured onto five CBA plates using an inoculation loop. The CBA plates were then incubated anaerobically for 3 weeks at 4 °C, 10 °C, 22 °C, 30 °C, and 37 °C, then growth was categorised as good (++) if the colonies grew closely together, as weak (+) if only small single colonies grew, or as having no growth (−).

In the second isolation phase, only the PYGS enrichment samples that were qPCR-positive for *C. estertheticum* and *C. frigoriphilum* were subjected to the culture method. Heat treatment and subcultivation on CBA were performed as in the first isolation phase. In addition, PYGS enrichments (without heat treatment) were streaked on CBA using a 10-µl inoculation loop, anaerobically incubated at 10 °C for 3 weeks. Colonies grown on CBA with different morphologies were then suspended and pooled in 0.5 ml of sterile water in a 1.5 ml tube using an inoculation loop. These pooled colonies were subjected to DNA extraction and then qPCR 1. The pooled colony samples that yielded positive results for *C. estertheticum* or *C. frigoriphilum* in qPCR were subcultured onto new CBA plates using a 10 µl inoculum loop and incubated anaerobically at 10 °C for 3 weeks. The DNA of the single colonies with different morphologies was then extracted using the direct (heating) DNA extraction method. The extracted DNA was subjected to qPCR 1 to check whether it was *C. estertheticum* or *C. frigoriphilum,* and qPCR 3 if all probes of qPCR 1 gave negative results.

3. Results

3.1. Molecular biological examination

Supplementary data Table S2 shows the Ct-values of the probe Cl555 of the three extraction methods tested for pure isolates of *C. estertheticum*, *C. gasigenes,* and *C. algidicarnis*, as well as the Ct-values of a native faecal sample and three faecal samples contaminated with the aforementioned *Clostridium* species. Overall, a combination of the InhibitEX Buffer and the High Pure Template Preparation Kit (Roche) showed the lowest Ct-values for all contaminated faecal samples and was therefore used to extract clostridial DNA from all faecal and wipe

samples.

The number and percentage of farms and animals that tested positive by qPCR for *Clostridium* spp. and the species *C. estertheticum*, *C. frigoriphilum*, *C. bowmanii*, *C. tagluense*-like, *C. algidicarnis,* and *C. gasigenes* are shown in Table 2 for faecal samples and in Table 3 for hide wipe samples. The Ct-values of the probes for the respective clostridia species were categorised as strongly positive (Ct-values <30), moderately positive (Ct-values between 30 and 36), and weakly positive (Ct-values >36). All samples that showed no Ct-values (RFU below the cut-off value) were assessed as negative.

While almost all faecal samples were strongly and moderately positive for *Clostridium* spp., only a few hide wipe samples were found positive in these categories. As almost all DNA extracts from hide wipe samples were only weakly positive for *Clostridium* spp., they were tested with a qPCR for universal bacteria to check whether DNA extraction was successful. A total of 97.0 % ($n = 252$) of the hide wipe samples showed Ct-values below 30 PCR cycles (strongly positive), and 3.0 % ($n = 7$) had Ct-values between 30 and 36 PCR cycles. This confirms that the DNA extraction of the hide wipe samples worked well, as the DNA of the universal bacteria was detected in large quantities, but these wipe samples contained little clostridial DNA.

3.2. Cultural examination

The PYGS enrichments ($n = 520$) were incubated for 4–5 weeks when they were subjected to the first isolation phase: A total of 690 colonies were isolated and subcultured, of which 223 (32.3 %) grew exclusively under anaerobic conditions. Of these, $n = 152$ (42.2 %) isolates proved positive in the clostridia qPCR and were confirmed by sequencing of the 16S rRNA gene. Table 4 shows the prevalence of the identified clostridia and summary of their growth temperatures. The detailed growth temperature and haemolysis activities of the individual isolates are listed in the supplementary data, Table S3. The haemolysis activities of some strains vary depending on the incubation temperature and growth rate. The most frequently isolated species are *C. subterminale*, *C. gasigenes*, *Lacrimispora algidixylanolytica, C. tagluense*, *C. tagluense*-like, and *C. botulinum, La. algidixylanolytica* was previously named as *Clostridium algidixylanolyticum* by Broda et al. (2000a) and was reclassified by Haas and Blanchard (2020). In qPCR-positive samples, the isolation rate of *C. gasigenes* is relatively high compared to the isolation rates of *C. tagluense*-like and *C. bowmanii* (see Table 6). The species *C. algidicarnis* was weakly detected in the enrichment of two hide wipe samples from one farm by qPCR and could be isolated. In contrast, *C. estertheticum* and *C. frigoriphilum* could not be isolated from any sample of the first isolation attempt, although they showed stronger signals than *C. algidicarnis*. There is no correlation between the species isolated from faeces and from hide wipe samples from the same animal. This means that the same *Clostridium* species was isolated from either faecal or hide wipe samples, but never from both samples from the same animal.

C. estertheticum is the best-known *Clostridium* species to cause BPS in vacuum-packed meat. Therefore, it is important that this and its closely related species, *C. frigoriphilum,* are isolated as pure cultures to ensure that the PCR results were correct. For this reason, all PYGS enrichments of faecal and hide wipe samples reacted positively to these two species were subjected to the second isolation phase (see Table 5). At this point, the PYGS enrichments had already been incubated at 4 °C for 14–16 weeks. Of the untreated PYGS enrichments, all CBA plates were overgrown by other accompanying microbiota, and only pooled colonies collected from three CBA plates contained DNA from *C. estertheticum*. Attempts to isolate individual colonies of *C. estertheticum* from these pooled colony samples were unsuccessful (see Table 5). In contrast, isolation rates from PYGS enrichments subjected to heat treatment gave better results, i.e., *C. estertheticum* could be isolated from 35.6 % ($n = 21/59$) and *C. frigoriphilum* from 17.6 % ($n = 6/34$) PCR-positive samples (see Tables 5 and 6). It should be noted that the high isolation rate of both species originates from strongly PCR-positive PYGS enrichments (see Table 5).

4. Discussion

The habitat of the cold-tolerant clostridia is permafrost and soil in temperate and subtropical climate zones (Spring et al., 2003; Suetin et al., 2009). Cattle can ingest these bacteria when grazing on pastures or through contaminated feed. The conditions in the animals' intestines are not optimal for the growth of the psychrophilic, but possibly some cold-tolerant *Clostridium* species. The spores are excreted with the faeces and can germinate and multiply if the conditions in the stable or on the pasture are favourable. The animals can then become re-contaminated on their hides which can later lead to contamination of the carcasses and meat in the abattoir.

Faecal and hide wipe samples were first enriched in a PYGS broth in order to increase the number of spores and thus the chance of successful detection and isolation (Brightwell and Clemens, 2012; Broda et al., 1998b; Lund et al., 1990; Moschonas et al., 2009). Using qPCR, *C. estertheticum* was detected in 22.3 %, *C. frigoriphilum* in 11.2 %, *C. bowmanii* in 32.7 %, *C. tagluense*-like in 33.8 %, and *C. gasigenes* in 14.2 % of the 260 faecal samples (see Table 2). Only very few hide wipe samples were PCR-positive for these clostridial species (see Table 3).

The results of the faecal samples from this study are consistent with the results of Moschonas et al. (2009) and Wambui et al. (2021), which were conducted in Ireland and Switzerland, respectively. In slaughterhouses in Ireland, Moschonas et al. (2009) found that 17.9 % and 25.4 % of cattle faecal samples were PCR-positive for *C. estertheticum* and *C. gasigenes,* respectively, while in Switzerland a *C. estertheticum*-like group was detected in 39 % of faecal samples by RT-PCR (Wambui et al., 2021). In faecal samples from cattle farms in Ireland, 8.5 % and 45 % of samples were qPCR-positive for *C. estertheticum* and *C. gasigenes,* respectively, while in New Zealand the corresponding species were detected in 4 % and 18 % of samples (Esteves et al., 2020, 2021).

There is little information available on the contamination of cold-tolerant clostridia on bovine hide. A single relevant study was conducted by Moschonas et al. (2009), who found that 13.3 % and 18.8 % of animal hides were PCR-positive for *C. estertheticum* and *C. gasigenes*, respectively. This result differs significantly, as only 0.8 % of the hide wipe samples analysed in the present study were positive for *C. estertheticum*, while *C. gasigenes* was not detected in a single hide wipe

Table 2
Number and percentage of farm and faecal samples tested positive by qPCR.

Species	Positive farm		Positive faecal samples							
			strong		moderate		weak		Total	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Clostridium spp.	26	100.0	191	73.5	64	24.6	3	1.2	258	99.2
C. estertheticum	23	88.5	15	5.8	28	10.8	15	5.8	58	22.3
C. frigoriphilum	10	38.5	14	5.4	9	3.5	5	1.9	29	11.2
C. bowmanii	17	65.4	26	10	46	17.7	13	5.0	85	32.7
C. tagluense-like	21	80.8	19	7.3	52	20	17	6.5	88	33.8
C. gasigenes	5	19.2	17	6.5	11	4.2	9	3.5	37	14.2
C. algidicarnis	0	0	0	0	0	0	0	0	0	0

Table 3
Number and percentage of farm and hide wipe samples tested positive by qPCR.

Species	Positive farm		Positive hide wipe samples							
			strong		moderate		weak		Total	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
Clostridium spp.	26	100.0	3	1.2	36	13.8	187	71.9	226	86.9
C. estertheticum	1	3.8	0	0	1	0.4	0	0	1	0.4
C. frigoriphilum	2	7.7	0	0	2	0.8	4	1.6	6	2.3
C. bowmanii	1	3.8	0	0	0	0	3	1.2	3	1.2
C. tagluense-like	0	0	0	0	0	0	0	0	0	0
C. gasigenes	0	0	0	0	0	0	0	0	0	0
C. algidicarnis	1	3.8	0	0	0	0	2	0.8	2	1.6

Table 4
Isolated species from the first isolation phase and their growth temperatures (*n* farm = 26; *n* faecal samples = 260; *n* hide wipe samples = 260; *n* total animals = 260).

Species identified by sequencing	Pos. farm		Isolated from Faeces		Hide		Total		Growth temperature [a]				
	n	%	*n*	%	*n*	%	*n*	%	4 °C	10 °C	22 °C	30 °C	37 °C
La. algidixylanolytica	6	23.1	2	0.8	18	6.9	20	7.7	+/−	++/+	++	++	++/ ±
C. botulinum	4	15.4	4	1.5	7	2.7	11	4.2	+/−	++	++	++	++
*C. bowmanii***	3	11.5	3	1.2	1	0.4	3	1.2	+/−	++	++	++	++
C. butyricum	1	3.8	1	0.4	0	0	1	0.4	–	++	++	+	+
C. frigidicarnis	3	11.5	1	0.4	2	0.8	3	1.2	+	++	++	++	++
*C. gasigenes***	7	26.9	22	8.5	0	0	22	8.5	++	++	++/+	++/ ±	++/ ±
C. algidicarnis	1	3.8	0	0	2	0.8	2	0.8	+	++	++	+	+
C. sartagoforme	4	15.4	3	1.2	1	0.4	4	1.5	+/−	+	++	++	++
C. subterminale	14	53.8	11	4.2	15	5.8	26	10.0	+/−	++	++	++/ ±	++/ ±
C. sulfidigenes	1	3.8	1	0.4	0	0	1	0.4	+	++	++	++	++
C. tagluense	5	19.2	19	7.3	0	0	19	7.3	+/−	++	++	++	++
C. tagluense-like**	8	30.8	18	6.9	0	0	18	6.9	++/ ±	++	++	++	++
C. vincentii	1	3.8	1	0.4	0	0	1	0.4	–	++	++	+	–
Clostridium spp.	12	46.2	19	7.3	2	0.8	21	8.1	++/ ±	++	++	++/ ±	++/ ±

[a] Growth grading: good (++), weak (+), or no growth (−); *** were also identified by qPCR 1 or 2 (see Table 1).

Table 5
Number and percentage of qPCR positive PYGS enrichment and culturable samples for *C. estertheticum* and *C. frigoriphilum*.

qPCR positive-PYGS	*n*	n (%) Culturable PYGS			
		Untreated		Heated (80 °C, 5 min)	
		Pooled[a]	Single**	Pooled[a]	Single**
Strongly positiv					
C. estertheticum	15	1 (6.7 %)	–	15 (100 %)	14 (93.3 %)
C. frigoriphilum	14	–	–	4 (28.6 %)	3 (21.4 %)
Moderately positiv					
C. estertheticum	29	2 (6.9 %)	–	5 (17.2 %)	3 (10.3 %)
C. frigoriphilum	11	–	–	2 (18.2 %)	3 (27.3 %)
Weakly positiv					
C. estertheticum	15	–	–	4 (26.7 %)	4 (26.7 %)
C. frigoriphilum	9	–	–	1 (11.1 %)	–
Total (both species)	93	3 (3.2 %)	–	31 (33.3 %)	25 (26.8 %)

[a] PCR positive in pooled colonies; ** Single (pure) colony could be isolated and identified as *C. estertheticum* or *C. frigoriphilum* by qPCR 1.

Table 6
Number and percentage of positive animals (either faecal or hide wipe samples) tested by qPCR and culture methods and species isolated by culture from qPCR-positive samples (total animals, *n* = 260).

Species	Positive animal				
	qPCR		Culture		%
	n	%	*n*	%	Culturable
C. estertheticum[a]	59	22.7	21	8.1	35.6
C. frigoriphilum[a]	34	13.1	6	2.3	17.6
C. bowmanii	85	32.7	4	1.5	4.7
C. tagluense-like	88	33.8	18	6.9	20.5
C. gasigenes	37	14.2	22	8.5	59.5
C. algidicarnis	2	0.8	2	0.8	100.0

[a] Can only be culturally isolated in the second isolation phase after the incubation period has been extended to 14–16 weeks.

sample. This could be due to differences in the sampling method and the degree of contamination of the hide with dirt or faeces. The study by Moschonas et al. (2009) took place in the abattoir, and pieces of the animals' hide were taken for examination, whereas in the present study the samples were obtained by wiping the hide of cattle on the farm where the animals normally live. The contamination of animal hides with faeces or soil was not explicitly scaled in this study or recorded in the study of Moschonas et al. (2009). However, on each farm visit it was found that the animals were kept dry in the barn and no significant amounts of faeces or other dirt particles were found on the skin of the cattle during sampling.

To isolate cold-tolerant clostridia, a minimum of eight weeks is required, or longer if the first cultivation attempt was unsuccessful. Faecal samples contain a wide variety of microorganisms, including cold-tolerant facultatively anaerobic bacteria, which can grow excessively on the non-selective CBA plates, as shown by the results of the cultivation of the untreated PYGS enrichment in this study (see Table 5). However, CBA is rich in nutrients and is, in principle, suitable as a culture medium for bacteria that are difficult to cultivate. It has already been used in many studies for the isolation of cold-tolerant clostridia (e.g., ; Broda et al., 2009; Moschonas et al., 2009). Two methods are usually used to reduce the number of non-spore-forming accompanying microorganisms in the sample suspensions before they are spread on CBA plates, i.e., by heating at 80 °C for 10 min or by treatment with ethanol 50 % v/v (Broda et al., 1998b; Moschonas et al., 2009; Wambui et al., 2021).

The first isolation test was performed after the PYGS broths (*n* = 520) had been enriched for 4–5 weeks. The clostridial isolates were subjected to the test of the growth temperature. Almost all isolates can grow very well at temperatures between 10 °C and 30 °C, while only isolates of *C. gasigenes* (*n* = 22) can grow very well at 4 °C–30 °C (see Table 4). It is possible that many of the isolated clostridia are unable to germinate at 4 °C. The spores simply survive this temperature and germinate after activation by heat and grow at an incubation temperature of 10 °C. This

assumption is consistent with the results that *La. algidixylanolytica*, *C. subterminale, C. botulinum,* and *C. algidicarnis* were isolated from 18, 15, 7, and 2 hide wipe enrichments, respectively, although the PCR results of these samples are only weakly positive for *Clostridium* spp. (Ct-values >36). In addition, the pure isolates of these three species do not grow or grow poorly at 4 °C. Among the isolated clostridia, the species *C. botulinum*, including psychrotrophic strains, are considered pathogenic as they can produce toxins (Broda et al., 1998a; Dahlsten et al., 2015). *C. botulinum* was isolated from 4.2 % of the animals examined in this study. All strains showed haemolytic activity and can grow well at temperatures between 10 °C and 37 °C; no other characteristics of these strains were analysed in the present study. However, it should be noted that the toxin production potential of these *C. botulinum* strains remains to be fully assessed in a follow-up study to determine whether they may pose a risk to animal or human health. This can be done using qPCR specific for botulinum toxin types A-F (Hill et al., 2010; Lindström et al., 2001; Prévot et al., 2007) and toxicity testing using cell culture-based methods (Hong et al., 2016; Rust et al., 2017).

C. estertheticum and *C. frigoriphilum* could not be isolated from any sample of the first isolation phase. Similarly, Moschonas et al. (2009) showed that *C. estertheticum* can be isolated to a very small extent with a 10-min treatment at 80 °C. However, in a previous experiment (Dorn-In et al., 2022), the survival rate was higher after heating the PYGS enrichment of contaminated meat juice. In the present study, *C. estertheticum* and *C. frigoriphilum* could only be isolated from the PCR-positive samples after a long incubation period of 14–16 weeks from the second isolation attempt. The isolation rate of *C. estertheticum* from all PCR-positive samples was 35.6 %, while the isolation rate from strongly PCR-positive samples was 93.3 %, which is very high. In contrast, the isolation rate from samples with moderately and weakly positive PCR results was very low.

The detection of cold-tolerant clostridia in faecal and environmental samples is difficult due to their slow growth and the lack of selective culture media. Therefore, in addition to cultivation, molecular biological methods such as PCR have often been used in parallel. The positive rate of cold-tolerant clostridia determined by the culture method is very low compared to that determined by qPCR, as shown in this and other studies (Esteves et al., 2020; Moschonas et al., 2009; Wambui et al., 2021). In this context, the PCR results should be interpreted with caution, as PCR-positive samples could be due to inactivated or non-culturable forms of clostridia, especially the enrichments that produce Ct-values >36. Enrichment steps are required for faecal and environmental samples, and a strongly positive PCR result (Ct-values <30) is to be expected if clostridia multiply in the enrichment. On the other hand, the cultivation conditions for these bacteria may not be optimal, as the vegetative cells died during handling of the sample and therefore cannot be cultivated. Furthermore, different clostridial strains can behave differently in terms of sporulation and even their robustness to environmental changes. The incubation period of 4 weeks at 4 °C is generally sufficient to detect the growth of cold-tolerant clostridia by qPCR. However, it is not certain that they can produce sufficient numbers of spores at this stage to survive a 5-min treatment at 80 °C and can be detected in a small volume (50 µl) of PYGS dropped onto the CBA. If the enrichment contains only a small number of spores, increasing the volume of heat-treated PYGS spread onto the CBA can increase the isolation rate. However, due to the large number of samples and replicates analysed, this was not performed for reasons of space and time constraints.

5. Conclusion

A total of 260 faecal and 260 hide wipe samples were taken from 260 cattle from 26 farms in Austria. Molecular biological and cultivation methods were applied to all samples. The results show that cold-tolerant clostridia are widespread in Austrian cattle farms. The prevalence of cold-tolerant clostridia associated with meat spoilage, such as *C. estertheticum*, *C. frigoriphilum* and *C. gasigenes*, in faecal samples can be classified as high. However, they could only be detected in a few skin wipe samples, as the animals were housed in a good housing systems and the animals' skin was kept dry and clean. In general, dairy cows in pasture-based housing systems, such as those in this study, are kept under good hygienic conditions, especially when compared to other housing systems such as free-range or confined housing systems, where animals may be more intensively exposed to soil or faecal contamination. The results of this study clearly demonstrate that cattle faeces represent a habitat of cold-tolerant clostridia. Thus, hides contaminated with faeces can also be a significant source of contamination for meat. As the dehiding of animals is one of the critical steps in the cattle slaughter process, it is important that cattle farms pay attention to the cleanliness of the cattle delivered for slaughter. At the same time, slaughterhouses must also ensure strict hygiene during dehiding in order to minimise contamination of the carcasses and meat. Beef production is associated with high consumption of resources such as agricultural land, feed and water, and significantly contributes to environmental problems. In addition, to reduce microbial spoilage and ensure food safety, effective hygiene measures and close co-operation among all stakeholders are essential to prevent microbial contamination and minimise avoidable meat losses.

CRediT authorship contribution statement

Samart Dorn-In: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Vanessa Zand:** Writing – review & editing, Investigation, Data curation. **Joachim Angerer:** Writing – review & editing, Investigation. **Kahraman Özbek:** Investigation. **Cassandra Eibl:** Data curation. **Karin Schwaiger:** Writing – review & editing, Resources.

Funding

This work was financially supported by the "BIOS Science Austria" (www.bios-science.at).

Declaration of competing 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.

Acknowledgments

We would like to thank Andrea Pauker, Dimitri Sofka, Michaela Mach, and Morten Sorig for their support and help with the laboratory work at the Unit of Food Hygiene and Technology (Centre for Food Science and Veterinary Public Health, University of Veterinary Medicine Vienna). Great thanks to Prof. Dr. Johannes Lorenz Khol from the Research and Teaching Extension for Alpine Ruminant Medicine (Clinical Centre for Ruminant and Camelid Medicine, Clinical Department for Farm Animals and Food System Science, University of Veterinary Medicine Vienna) for supporting part of the samples for investigation and required information.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2025.104838.

Data availability

No data was used for the research described in the article.

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