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Spoilage characteristics of sous-vide beef caused by *Clostridium estertheticum*

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ABSTRACT

The increasing popularity of sous-vide (SV) cooking necessitates research into the microbiological quality, sensory changes, and shelf life of SV products. Studies show that SV cooking significantly reduces the levels of meat microbiota and pathogens, positively affecting the shelf life and safety of SV products. However, the meat spoilage organism *Clostridium estertheticum* can survive SV cooking as it can produce heat-tolerant spores. Theses spores can germinate and multiply during storage at refrigerated temperatures, leading to spoilage of SV meat. Therefore, the aim of this study was to characterise the spoilage of SV beef caused by *C. estertheticum* compared to non-SV beef. In addition to the determination of spoilage characteristics, all beef samples were subjected to culture and qPCR analysis to determine the numbers of total bacteria, lactic acid bacteria, *Enterobacteriaceae*, yeasts, and *C. estertheticum*. Species identification of the colonies on the culture media was performed using MALDI-TOF MS. The tests were carried out at three different times (three repetitions). A total of 90 beef samples were analysed, of which 54 samples were artificially contaminated with three strains of *C. estertheticum* and vacuum-packed. Of these, 27 beef samples underwent SV cooking (55 °C, 70 min). After 28 days of storage at 4 °C, the SV beef samples exhibited significantly higher levels of gas and stronger spoilage odour compared to non-SV samples ($p < 0.05$). While drip loss and pH levels were also higher in SV beef, these were not considered specific spoilage characteristics caused by *C. estertheticum*. Microbiological and qPCR analyses revealed that all SV beef samples had very low bacterial and yeast counts but very high numbers of *C. estertheticum*, which strongly correlated with the sensory changes observed. We concluded that SV beef containing *C. estertheticum* has a shorter shelf life than contaminated non-SV beef. This is the first study to examine the spoilage of SV beef by *C. estertheticum*. The results may help raise awareness among meat producers and restaurants about the risk of meat losses due to spoilage caused by these bacteria.

1. Introduction

Vacuum packaging in combination with storage at chilled temperatures is an efficient method for extending the shelf life of fresh beef, as under these conditions, conventional aerobic spoilage bacteria such as *Pseudomonas* spp. either cannot grow or grow only very slowly (Dorn-In et al., 2023). Under anaerobic and cold storage conditions, however, meat can be spoiled by cold-tolerant facultative anaerobic microorganisms such as lactic acid bacteria (LAB) and *Enterobacteriaceae* (Dorn-In et al., 2023) and additionally by strictly anaerobic cold-tolerant *Clostridium* spp. (Bonke et al., 2016; Brightwell and Clemens, 2012; Dorn-In et al., 2018; Mang et al., 2021).

While there are numerous studies on the microbial spoilage of fresh and vacuum-packed meat, there are only few studies on spoilage of vacuum-packed heat-treated meat products such as sous-vide beef. Sous-vide (SV) is a technology in which food (vegetables or meat) is vacuum-packed and then heated in a sous-vide bath at a relatively low and constant temperature between 50 °C and 85 °C for a specific period of time (approximately 1 to 48 h) (Latoch et al., 2023). Temperature and duration depend on the type of food, weight and/or thickness of the meat (Latoch et al., 2023). The advantages of SV cooking are that it preserves moisture, natural flavours, and nutrients in the package. It also produces a uniform texture, maintains the desired colour, and prevents cross-contamination during storage (Baldwin, 2012; Douglas et al., 2023). This method has been used to produce ready meals since the 1960s and was used by restaurants in the 1970s. In the 1990s, food scientists became actively involved in SV processing, focusing particular on extending the shelf life of minimally processed foods (Baldwin, 2012). To this day, ready-made SV baths and SV devices are available on the market. This technology is increasingly used for the preparation of

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meat and vegetables in private households as well as in catering and restaurants.

In addition to preserving the nutritional value and creating specific sensory properties of meat, the SV process can extend the shelf life and improve safety. It can inactivate or stress common spoilage microorganisms such as LAB and *Enterobacteriaceae*, as well as pathogenic bacteria such as *Salmonella* spp., *Listeria monocytogenes*, and *E. coli*, by the heating process (Baldwin, 2012; Díaz et al., 2008; Latoch et al., 2023; Onyeaka et al., 2022; Patil et al., 2024). Mesophilic and spore-forming bacteria such as pathogenic *C. perfringens*, *C. botulinum*, or *Bacillus cereus* can survive this process (Baldwin, 2012; Latoch et al., 2023; Onyeaka et al., 2022), however, they generally do not grow under the storage conditions of SV meat, which are usually between 2 °C and 4 °C. Therefore, SV meat should be cooled rapidly after cooking to prevent the growth of these bacteria (Zavadlav et al., 2020).

Although the presence of spore-forming cold-tolerant and cold-loving clostridia in vacuum-packed fresh meat (beef, lamb, and game meat) has been widely reported, very little information is available on the extent to which these bacteria grow in SV meat and alter the properties of the meat. The occurrence of *C. estertheticum*, the best-known psychrophilic *Clostridium* species, is reported from all over the world, including Brazil, Canada, Germany, Ireland, New Zealand, Switzerland, the United Kingdom, and the United States (Mang et al., 2021). *C. estertheticum* can produce a large amount of CO_2 and H_2, along with significant amounts of butanol, butyric acid, acetic acid, and butyl esters (Broda et al., 1996). This leads to swelling of the pack, known as blown pack spoilage (BPS), and a cheesy odour (Boerema et al., 2007; Broda et al., 1996). Similar to other *Clostridium* species, they can produce heat-tolerant spores (Dorn-In et al., 2022; Moschonas et al., 2009), which can also play an important role in heat-treated meat and meat products stored under anaerobic conditions and at cool temperatures. The aim of this study is therefore to characterise the spoilage of SV meat (beef) caused by three strains of *C. estertheticum*. Spoilage characteristics investigated included gas formation, spoilage odour, loss of drip/meat juices, and pH changes. Samples were then analysed using culture methods and qPCR to determine the number of meat microbiota in SV and non-SV meat. Additionally, MALDI-TOF MS was performed to identify the species of other meat microbiota grown on agar plates.

This study highlights the role of *C. estertheticum* in spoilage of SV beef compared to non-SV beef and provides additional insights into the complexity of the meat microbiota. Since meat production requires substantial resources, any spoilage-related loss contributes to economic losses and negatively impacts resource sustainability. Therefore, the results of this study should raise the awareness among meat producers and the food industry about preventing food waste caused by *C. estertheticum* and support consumer protection efforts.

2. Material and methods

2.1. Samples

2.1.1. Clostridium estertheticum

Three strains of *C. estertheticum* were used to inoculate beef samples, namely strain DSM 8809 (C1), one strain isolated from vacuum-packed beef (C2, Dorn-In et al., 2022) and another strain from bovine faeces (C3, in this study, unpublished). The latter two isolates were identified by specific qPCR (see Section 2.4) and sequencing of the 16S rRNA gene. All *C. estertheticum* strains showed haemolytic activity on Columbia blood agar (CBA, Columbia agar with 5 % sheep blood, BioMérieux™). Each strain was subcultured on CBA and anaerobically incubated at 10 °C for 3 weeks. The grown colonies on CBA were subcultured in peptone-yeast-glucose-starch broth (PYGS, Lund et al., 1990) and incubated anaerobically at 10 °C for 8, 10, and 16 weeks for the first, second, and third biological replication, respectively. The long incubation period was intended to stimulate sporulation of *C. estertheticum* so that they could survive heat treatment during SV cooking of beef samples.

To prepare an inoculum of each *C. estertheticum* strain, 1.5 ml of each PYGS enrichment was filled into two 2 ml tubes, centrifuged at 15,000 ×*g* for 2 min, the supernatant discarded, and the tube was filled up with 1.5 ml of 0.90 % NaCl, then vortexed for 10 s or until the pellet had completely dissolved. The bacterial suspensions from both tubes were pooled, and 100 µl were used to inoculate each beef sample.

Then, 200 µl of each inoculum were subjected to DNA extraction in duplicate, followed by qPCR with standards to quantify *C. estertheticum* (cfu/ml) in the inoculum. To determine the exact number of culturable *C. estertheticum* (C1, C2, and C3), each inoculum was serially diluted to 10^{-5} with 0.90 % NaCl (1:10) in a 1.5 ml tube. In addition, to simulate the SV cooking conditions, 0.5 ml of each inoculum was filled in a 1.5 ml tube, treated at 55 °C for 70 min in a Thermomixer (Eppendorf), and serially diluted to 10^{-5}. The CBA plate was divided into 6 sectors, and then 50 µl of each dilution were dropped in duplicate onto each section of the CBA. The CBA plates were incubated anaerobically at 10 °C for 18 (±3 days), and the clostridial colonies were counted.

2.1.2. Beef samples

The tests were carried out at three different times with three different beef samples (three biological replications/batches). These beef samples (approx. 10 kg each) were purchased from three different butchers in Vienna, Austria. They were cut into pieces; each piece (subsample) was about 2.0 to 2.5 cm thick and weighed between 240 and 260 g. There was a total of 10 sample groups (groups 1–10, Table 1), with each group containing n = 3 subsamples per batch (n = 9 for three batches). A total of n = 30 subsamples of beef were therefore required for one batch. Accordingly, a total of n = 90 beef subsamples were used for all three batches (Table 1). Fresh beef (sample group 1, n = 9) was analysed for the number of original microbiota, group 2 (n = 9) for the effect of sous vide temperature on the reduction of meat microbiota, and groups 3–10 (n = 72) for the spoilage characteristics of the beef and the number of spoilage meat microbiota.

Beef sample groups 3–5 and groups 7–10 were contaminated with 100 µl of *C. estertheticum* inoculum (see Table 1). Beef sample groups 2–10 were vacuum packed using a vacuum packing machine (model: Max, Boss GmbH) with an automatic program (high vacuum level). The vacuum-packed beef sample groups 2–6 were placed in the SV bath (model: SVGVA16, GGM Gastro International, GmbH) at a temperature of 55 °C for 70 min. This followed the recommendation that a 2.5 cm thick beef to be used as a medium-rare steak should be cooked at this

Table 1
Sample groups (1−10) for all three biological replicates with a total of n = 90 beef subsamples.

Group	Sample*	n	Sous-vide cooking	Storage temperature	Investigating day
1	Fresh beef	9	–	–	1
2	Beef	9	✓	–	1
3	Beef & C1	9	✓	4 °C	28
4	Beef & C2	9	✓	4 °C	28
5	Beef & C3	9	✓	4 °C	28
6	Beef	9	✓	4 °C	28
7	Beef & C1	9	–	4 °C	28
8	Beef & C2	9	–	4 °C	28
9	Beef & C3	9	–	4 °C	28
10	Beef	9	–	4 °C	28

* Fresh beef samples (group 1) are non-vacuumed. Beef contaminated with *C. estertheticum*: C1 = DSM 8809; C2 = isolated from vacuumed packed beef; C3 = isolated from cow faeces.

temperature (Grillfuerst, 2024). All samples were then stored at 4 °C for 28 days.

2.2. Spoilage determination of beef

After 28 days of storage at 4 °C, all stored meat samples (groups 3–10, n = 72) were analysed for their spoilage characteristics, i.e., the blown pack spoilage (BPS) score, spoilage odour, percentage of drip loss, and pH value.

Firstly, the BPS value was determined as described by Boerema et al. (2007) (see Fig. 1). After opening the meat package, the meat drip was taken using a 10 ml pipette, filled into a 50 ml Falcon tube, and weighed. The weight of the drip was related to the weight of the meat (w/w), giving the results of the drip loss as a percentage (Stella et al., 2019). The surface of the beef was then cut into small pieces with sterile scissors and tweezers, weighed to 10 g, placed in a stomacher bag, and stored at 4 °C for microbiological investigation, which was performed immediately after the sensory testing was completed. The next step was to evaluate the spoilage odour after opening the package for at least 10 min using 5 scores as described previously (Dorn-In et al., 2023). As the last step of the sensory determination, the pH value was determined using the Testo 230 device (Testo). The determination of BPS score and spoilage odour was carried out by two persons (Mang et al., 2021).

2.3. Microbiological examination

All beef samples (groups 1–10, n = 90) were subjected to microbiological examination. For each sample, 10 g of beef in a stomacher bag were homogenised with 90 ml of peptone water in a Bag Mixer (Interscience) for 30 s. Then 10 ml of meat homogenate (dilution 10^{-1}) were filled into a Falcon tube and serially diluted (1:10 ml) to 10^{-3} for fresh meat (group 1) and for SV meat (groups 2–6), and to 10^{-5} for non-SV meat (groups 7–10). Subsequently, 100 µl of the last three dilutions were spread in duplicate to the respective agar.

The following agars and incubation conditions were applied: Plate Count Agar (PCA; Merck) for total aerobic/anaerobic plate count (PC, aerobic/anaerobic, 30 °C for 72 h), Violet Red Bile Dextrose Agar (VRBD, Merck) for *Enterobacteriaceae* (anaerobic, 30 °C for 48 h). DeMan Rogosa Sharpe Agar (MRS; Merck) was used for lactic acid bacteria (LAB, anaerobic, 25 °C for 72 h), while yeasts were cultured on Wort Agar (Merck, aerobic, 25 °C for 72 h). The AnaeroGen™ (Thermo Fisher Scientific, Oxoid) was used to create anaerobic conditions in the bags or jars with PCA (for anaerobic plate count), VRBD, and MRS agar plates.

2.4. Molecular biological examination

The number of *C. estertheticum* in all samples was determined by qPCR. The High Pure Template Preparation Kit (Roche) was used to extract DNA from beef homogenate (dilution 10^{-1}). DNA extraction and qPCR for *Clostridium* spp. were performed as previously described by Dorn-In et al. (2018, 2022, 2023). The qPCR for clostridia included primer pair Cl93-F and Cl642-R and five TaqMan probes, namely for *Clostridium* spp. (Cl555-FAM), *C. estertheticum* (Cest-Hex), *C. frigoriphilum* (Cfgrpl-Cy3.5), *C. bowmanii* (Cbow-Cy3.5), and *C. tagluense*-like (Ctag-like-Cy5.5). Standard dilutions of *C. estertheticum* (DSM 8809) from 10^{-6} to 10^{-1} cfu/g were used to quantify *C. estertheticum* in the inocula and beef samples (Dorn-In et al., 2023). The probes for *C. frigoriphilum*, *C. bowmanii*, and *C. tagluense*-like were included in the qPCR to check whether the samples were naturally contaminated with these clostridial species.

2.5. MALDI-TOF MS

Matrix Assisted Laser Desorption Ionisation — Time of Flight Mass Spectrometry (MALDI-TOF MS) was used to identify the species of colonies on PCA (aerobic and anaerobic), MRS, VRBD, and Wort agar.

The *Enterobacteriaceae* colonies on VRBD agar were first subcultured on CBA and then incubated aerobically at 30 °C for 24 h before being

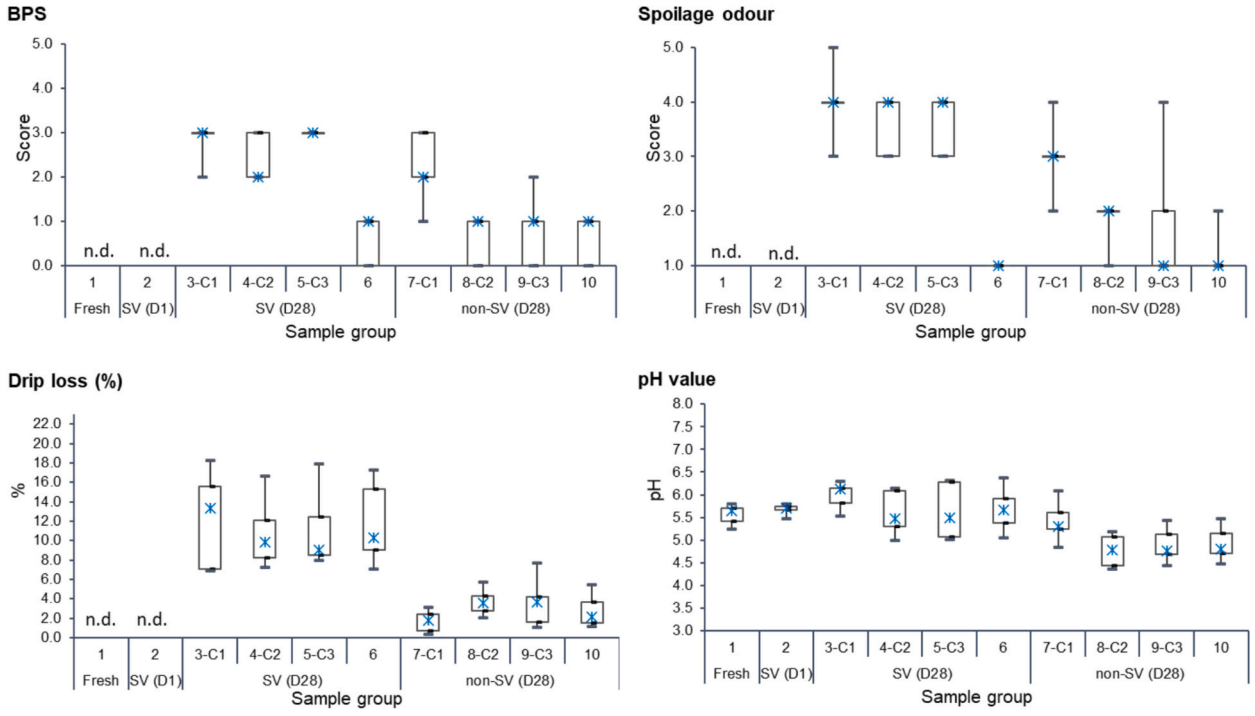

Fig. 1. Spoilage characteristics of beef samples (groups 1–10, see Fig. 3). BPS score: 0: no gas production, 1: small bubbles in drip, package intact, 2: loss of vacuum through gas production, 3: blown, puffy packs, 4: fully distended, without tightly stretching the pack, 5: overblown, tightly stretched packs/packs leaking. Spoilage odour score: 1: fresh, almost odourless; 2: slight deviation, still acceptable; 3: distinct deviation; 4: spoilage odour (unpleasant and repellent); 5: distinct spoilage odour. n.d. = not done.

analysed by MALDI-TOF MS. The colonies on the other agar plates (PCA, MRS, and Wort agar) were directly subjected to MALDI-TOF MS. Up to five colonies with different morphologies were taken per sample and culture medium. Colonies were subjected to protein extraction using the extended transfer method as described in the instruction manual (Bruker Daltonik GmbH, Germany). Samples were spotted in duplicate on a polished steel plate. The generated protein spectra were compared with the MALDI-TOF biotyper database (Bruker Daltonik GmbH, Germany).

2.6. Statistical analysis

The values of spoilage parameters of SV and non-SV samples artificially contaminated with *C. estertheticum* strains (groups 3–5 and groups 7–9, respectively) were statistically analysed using the Microsoft Excel *t*-test. Differences were considered statistically significant if the *p*-value was <0.05.

In addition, the Pearson's correlation coefficient (*r*, Microsoft Excel, 2020) was used to determine the correlation between numbers of *C. estertheticum* and spoilage characteristics of vacuum-packed beef samples stored for 28 days. The strength of the correlation was interpreted as follows: $r = 0$–0.19 is considered as very weak, 0.20–0.39 as weak, 0.40–0.59 as moderate, 0.60–0.79 as strong, and 0.8–1.0 as very strong (Evans, 1996). Subsequently, the *p*-value was calculated based on a two-tailed *t*-test to determine whether the correlation was statistically significant ($p < 0.05$, Dorn-In et al., 2023).

3. Results and discussion

3.1. Spoilage determination of beef

Stored beef samples (groups 3–10, n = 72) were analysed for their spoilage characteristics (see Fig. 1), while sample groups 1 (n = 9) and group 2 (n = 9) were only subjected to microbiological investigation. In general, the storage life or shelf life of vacuum-packed fresh beef is at least 10 to 12 weeks, when stored below 4 °C @@@@(CSIRO, 2003). The storage life may be shorter if the initial bacterial contamination of the beef is high. For this reason, the storage life of SV beef (sample group 6) must be longer than that of fresh beef. However, the aim of the present study was not to investigate the shelf life of SV beef, but to reveal the role of *C. estertheticum* as a spoilage agent in SV beef. Sample groups 6–10 were used for comparison with the results of sample groups 3–5. In the first replicate, the BPS values of the beef packs were recorded every week. After about 3 to 4 weeks, sample groups 3–5 (contaminated and SV) showed significantly higher gas production than samples from groups 7 to 9 (contaminated, non-SV), indicating that *C. estertheticum* accelerates the spoilage of SV beef. Therefore, the storage time for all replicates was designed at 4 weeks (28 days).

The stored meat with a BPS score of 1 (small bubbles in the drip, packaging intact) is not considered spoiled if the spoilage odour (score ≥ 2) was not present. Carbon dioxide can be released from the meat muscle and nitrogen from the fat tissue, resulting in small gas bubbles after the meat has been vacuum-packed for hours and increasing during storage (CSIRO, 2001). After 28 days of storage, all SV control samples (group 6, n = 9) were considered fresh as no signs of spoilage were detected. In the non-SV control samples (group 10, n = 9), one sample from the third replicate showed spoilage odour with score 2 (slight deviation).

The values of spoilage parameters of SV and non-SV samples contaminated with *C. estertheticum* (groups 3–5 and groups 7–9, respectively) were compared and statistically analysed to determine whether SV cooking accelerates the growth of *C. estertheticum* and thus the spoilage of vacuum-packed beef due to reduced competitive microbiota. Overall, SV samples contaminated with *C. estertheticum* (groups 3–5) showed statistically significantly higher BPS and spoilage odour scores than non-SV samples (groups 7–9, $p < 0.05$).

The gas produced by *C. estertheticum* is a combination of hydrogen and carbon dioxide. Hydrogen can combine with sulphur, which is present in some amino acids, to form hydrogen sulphide, resulting in an unpleasant sulphurous or foul odour (Broda et al., 1996; Broda et al., 2000; Dainty et al., 1989). In addition, various volatile compounds, especially butanol, butyl ester, and butyric acid, produced by *C. estertheticum* contribute to the spoilage odour of meat, which is regularly described as cheesy or dairy off-odour (Dainty et al., 1989; Kalchayanand et al., 1989; Broda et al., 1996). On the contrary, the spoilage odour caused by LAB is usually described as sour and acidic due to the high production of lactic acid (Casaburi et al., 2015; EFSA, 2016; Jääskeläinen et al., 2012). In the present study, these spoilage odours in almost all SV beef samples contaminated with *C. estertheticum* (groups 3–5) were on average at score 4 (unpleasant and repellent). Lower scores of 1 to 2 were determined for almost all non-SV beef samples that were contaminated with the same clostridial strain (groups 7–9, Fig. 1).

When looking at the individual samples, it was found that sample groups 8 and 9 (non-SV and contaminated with *C. estertheticum* strains isolated from beef (C2) and from cow faeces (C3), respectively) had similar BPS, and spoilage odour scores as control samples (group 6: SV and group 10: non-SV). This indicates that these *C. estertheticum* strains (C2 & C3) may not grow very well in vacuum-packed beef samples, as the conditions are not optimal with respect to some influence factors like numbers of meat microbiota and pH values (see Sections 3.2 and 3.4 for further discussion).

In the present study, the term drip loss, which is normally used for vacuum-packed beef samples, also refers to the cooking loss of SV samples. The cooking loss, or meat drip, is a combination of water and soluble substances such as minerals (Przybylski et al., 2021; Aaslyng et al., 2003). In general, the term cooking loss is used for cooked meat and refers to the weight loss after cooking the meat, either with traditional cooking methods or with SV cooking (Jeong et al., 2020). During the cooking process, the muscle fibres shrink and condense, resulting in a deformation of the muscle fibres and a reduction in the physical space in which free water is retained in the meat, thereby increasing the cooking loss (Shin et al., 2023). For the reasons described, the percentage drip loss is statistically significantly higher for all SV samples than for non-SV samples ($p < 0.05$). Accordingly, percentage drip loss is not appropriate parameters for distinguishing whether SV meat is contaminated with *C. estertheticum*.

Fresh beef normally has a pH value of 5.5 to 5.8 (Broda et al., 1997; Węglarz, 2010). The pH value may increase after SV cooking as the heat exposure contributes to a reduction of available carboxyl groups in the cooked meat (Berdigaliuly et al., 2022; Gómez et al., 2019). However, Bhat et al. (2020) concluded that SV cooking has only a small effect on the pH of beef, while some other studies show that SV cooking increases the pH of beef (Berdigaliuly et al., 2022) or other meats such as chicken breast (Hasani et al., 2021). The results of the present study showed that the pH of SV beef (group 2, pH 5.7 ± 0.1) was slightly higher than that of fresh beef (group 1, pH 5.6 ± 0.2), suggesting that SV cooking can increase the pH of meat, but only with minimal effects, similar to those observed by Bhat et al. (2020). After the samples were stored at 4 °C for 28 days, the pH values of the non-SV samples (groups 7–10, pH 5.0 ± 0.4) decreased statistically significantly compared to the SV samples (groups 3–6, pH 5.7 ± 0.4, $p < 0.05$). This supposed to be a consequence of the growth of LAB in the non-SV beef samples (see Fig. 4).

3.2. Microbiological and molecular examination

C. estertheticum strains (C1, C2, and C3) in inocula were quantified by qPCR and culture methods. Fig. 2 shows that the number of all three *Clostridium* strains detected by qPCR and culture was similar. In almost all samples, the number of clostridia in fresh culture is slightly higher than in samples treated at 55 °C for 70 min, but not statistically significant ($p < 0.05$). This indicates that the heat treatment conditions did not significantly affect the viability of all tested *C. estertheticum* strains that were enriched in the PYGS broth and incubated at 10 °C for a long

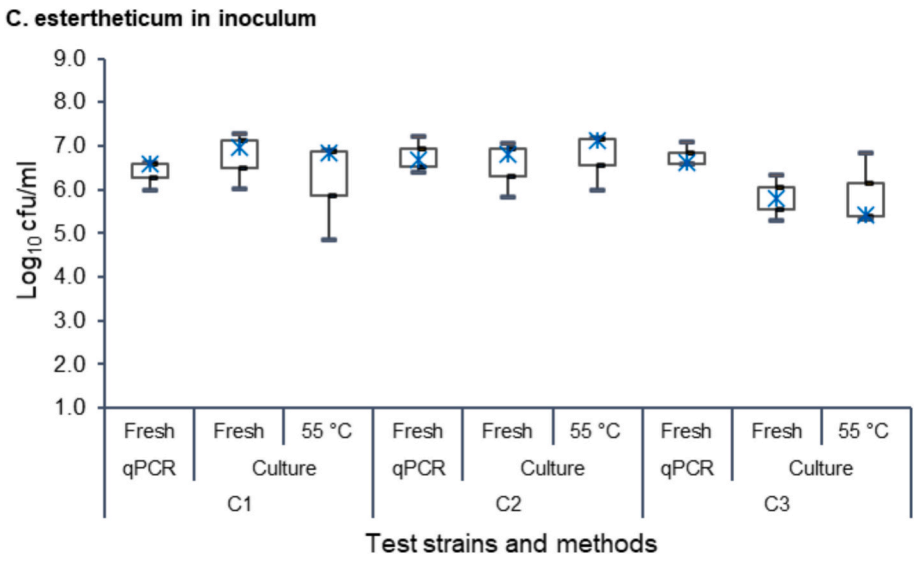

Fig. 2. *C. estertheticum* (strains C1: DSM 8809; C2: isolated from beef; C3: isolated from bovine faeces) in inocula, quantified by qPCR (for fresh culture) and culture method (for fresh culture and culture treated at 55 °C for 70 min).

period of time (8, 10, and 16 weeks). This prolonged incubation allows them to produce heat-resistant spores and thus survive the conditions of SV meat production. From these results, the number of viable cells of strains C1, C2, and C3 artificially contaminated in beef was on average 2.4, 2.6, and 2.0 $\log_{10}$ cfu/g beef, respectively.

In general, enumeration of *C. estertheticum* in fresh meat and non-SV samples (groups 1, and 7–10) using the culture method is very difficult due to the overgrowth of the other meat microbiota, especially LAB. Although the culture method can probably be used to quantify *C. estertheticum* in SV beef samples (groups 3–5), the qPCR method was chosen for this purpose as it is suitable for all sample groups (fresh beef, SV, and non-SV beef). As the same analysis method is used, the results of all sample groups can be reliably compared.

Fig. 3 shows the initial contamination of the fresh meat samples (group 1) from all three biological replicates. Fresh beef from the first replication contained the lowest number of microbial contaminations, while that from the third replication has the highest contamination rate. Clostridia were not detected in any of the samples of fresh beef (<2.0 $\log_{10}$ cfu/g).

Fig. 4 shows the number of microorganisms analysed in all samples (groups 1–10, n = 90). In some samples of group 2 (directly after SV cooking), only aerobic plate counts were detected. After a 28-day storage of SV beef samples (groups 3–6), total aerobic/anaerobic bacterial counts were detected in 50 % (n = 18/36) of the samples. They were found from the detection limit (2.0 $\log_{10}$) up to about 3.5 $\log_{10}$ cfu/g in SV beef @@@samples of the 2nd and 3rd replication but could not be detected from SV samples of the 1st replication. In all these stored SV samples (groups 3–6), LAB, *Enterobacteriaceae*, and yeasts were not detected (<2.0 $\log_{10}$ cfu/g). In this context, the applied SV cooking conditions may deactivate the majority of vegetative and non-spore forming meat microbiota. The high survival rate was observed in meat that initially contained a high number of meat microbiota, as observed

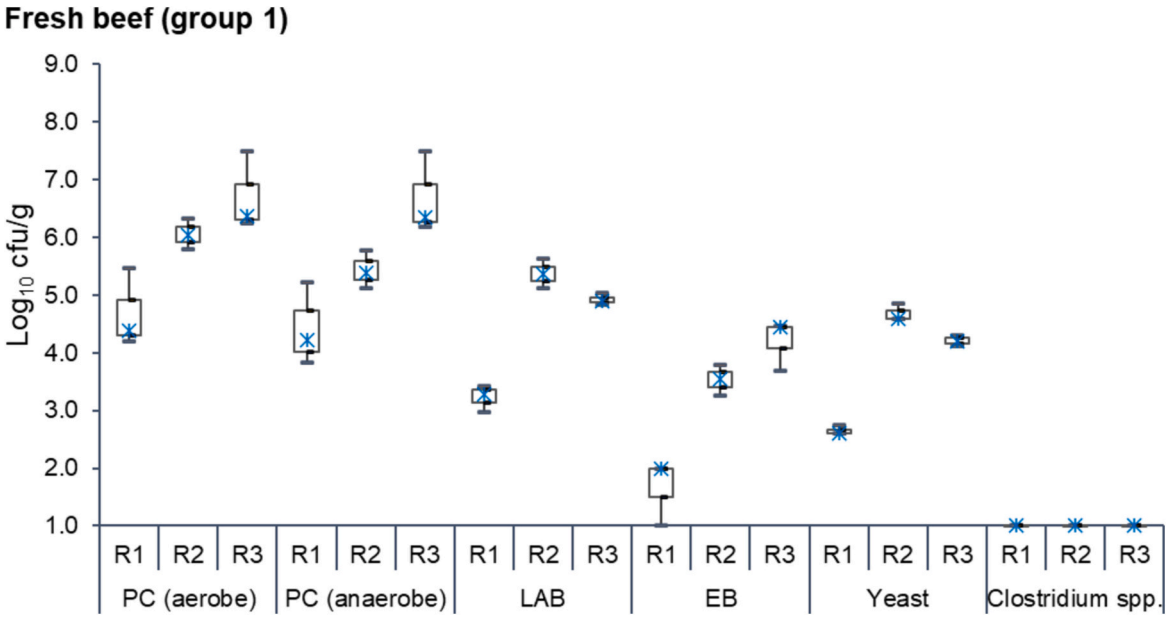

Fig. 3. Number of microorganisms ($\log_{10}$ cfu/g) in fresh beef (group 1) from all three biological replicates (R1 to R3), determined by the culture method (for PC, LAB, *Enterobacteriaceae*: EB, and yeast) and qPCR (for *Clostridium* spp.).

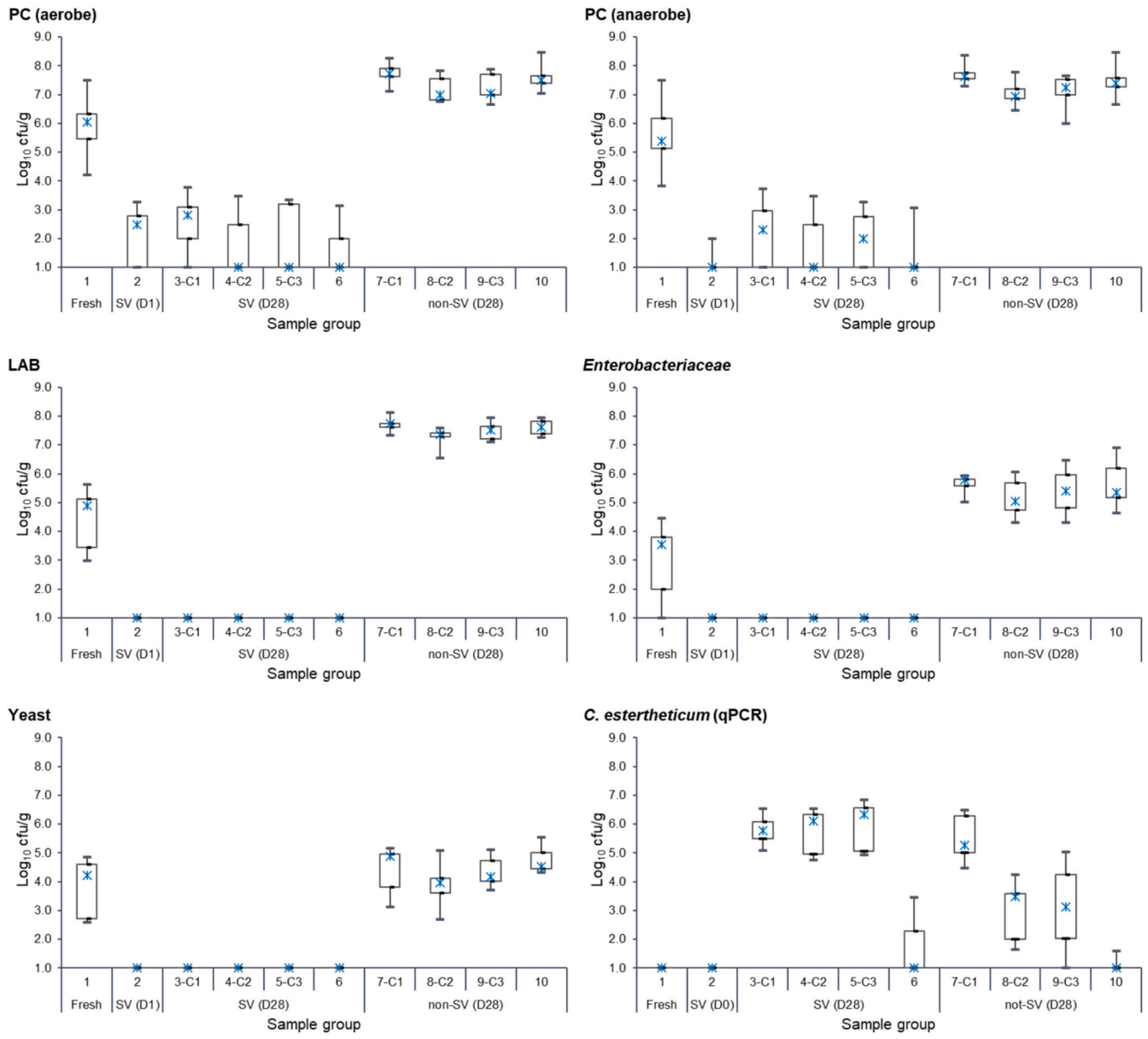

Fig. 4. Number of microorganisms (log_{10} cfu/g) in each sample (groups 1–10, see Table 1) determined using culture method (PC, LAB, *Enterobacteriaceae*, and yeast) and qPCR (for *C. estertheticum*). The limit of detection for all target microorganisms was 2.0 log_{10} cfu/g.

in fresh beef (group 1) from the 2nd and 3rd replicates (see Fig. 3).

On the contrary, the non-SV-cooked beef samples (groups 7–10), which were also stored for 28 days, showed a high number of aerobic and anaerobic mesophilic bacteria (>6.5 log_{10} cfu/g), LAB (>7.0 log_{10} cfu/g), and *Enterobacteriaceae* (>5.0 log_{10} cfu/g, Fig. 4). Yeasts were found at similar levels (approximately 3.0–5.0 log_{10} cfu/g) as in fresh meat, indicating that they do not grow very well under the incubation conditions. LAB usually grow slowly at refrigeration temperatures and reach a concentration of 7.0 to 8.0 log_{10} cfu/g after about 6 weeks of storage (CSIRO, 2003). They will stay at this level for the rest of the storage life of the product but do not produce signs of spoilage until several weeks after the maximum population of bacteria is reached (CSIRO, 2003). Therefore, only a weak to moderate correlation was found between spoilage parameters such as odour changes and LAB numbers (Dorn-In et al., 2023). Generally, meat considered as spoiled when there are changes in sensory properties. However, regarding food safety and consumer protection aspects described in the regulation of the European Union, total plate count and *Enterobacteriaceae* in fresh beef at retail should not exceed 6.5, and 5.0 log_{10} cfu/g, respectively (European Commission Regulation (EC) No 2073/2005, n.d.). In addition, the European Food Safety Authority recommends that LAB on meat should not exceed 7.0 log_{10} cfu/cm^2 (EFSA, 2016). Therefore, some of beef samples without sensory spoilage evidence (e.g., groups 10) may be considered unfit for human consumption due to high bacterial contamination as described above and in Article 14 (Food safety requirements) of the European Commission Regulation (EC) No 178/2002 (n.d.).

Although *C. estertheticum* was not detected in any sample of fresh beef (group 1) from all three replicates, it was detected in sample group 6 (SV beef) and group 10 (non-SV beef) in the first replicate. This means that these samples were naturally or accidentally contaminated with *C. estertheticum* but were not detected in fresh meat as the contamination rate was below the detection limit of qPCR (2.0 log_{10} cfu/g). However, the contamination rate is rather low, and the typical spoilage caused by *C. estertheticum* was not observed in these samples as they had a BPS and spoilage odour score of 1.

The comparison between SV samples (groups 3–5) and non-SV samples (groups 7–9) showed that the number of *C. estertheticum* was higher in SV samples than in non-SV samples. The largest differences were particularly observed between samples of group 4 and group 8 (contaminated with strain C2 isolated from beef) and between samples of group 5 and group 9 (contaminated with strain C3 isolated from bovine faeces). This result indicates that the conditions of the SV beef sample in this study (pH values of about 5.7 ± 0.4 and low number of meat microbiota) favour the growth of all *C. estertheticum* strains. As observed, *C. estertheticum* strain C1 was highly adaptable compared to strains C2 and C3, as it grew well in all non-SV beef samples (group 7) and caused spoilage close to the level of SV beef samples contaminated with this clostridial strain. Overall, the present study showed that the growth of *C. estertheticum* could be inhibited by the meat microbiota, especially LAB. On the other hand, the growth of other meat microbiota (aerobic/anaerobic plate count, LAB, *Enterobacteriaceae* and yeast) was not influenced by any *C. estertheticum* strain, as the number of these bacteria in sample groups 7–9 (contaminated and non-SV) was at a similar level as in the control sample group 10 (uncontaminated and non-SV).

As mentioned in the Introduction section, SV cooking is of interest to extend shelf life and ensure safety of meat, as the combination of temperatures (50–85 °C) and time (1–48 h) can kill almost all spoilage microorganisms and pathogens (Baldwin, 2012; Díaz et al., 2008; Latoch et al., 2023; Onyeaka et al., 2022; Patil et al., 2024). This assumption is correct, as shown by sample group 6 (uncontaminated and SV cooking), which showed no signs of spoilage and whose meat microbiota count remained low at least until the study date (day 28 of storage). When contaminated with *C. estertheticum*, the storage life of the SV beef samples decreased significantly in terms of signs of spoilage, although the number of other meat microbiota was very low. This point is important because *C. estertheticum* or other cold-tolerant clostridia are not

Table 2
Species of meat microbiota identified using MALDI-TOF MS.

No.	Species	Samples and number of colonies												Total
		Replicate 1				Replicate 2				Replicate 3				
		1	2	3–6	7–10	1	2	3–6	7–10	1	2	3–6	7–10	
1	*Acidovorax temperans*	1												1
2	*Acinetobacter albensis*	1												1
3	*Aeromonas eucrenophila*									5				5
4	*Aeromonas hydrophila*									1				1
5	*Aeromonas* sp.									1				1
6	*Aeromonas veronii*									5				5
7	*Brochothrix thermosphacta*					5			4	3				12
8	*Buttiauxella gaviniae*	5			3	2								10
9	*Candida zeylanoides*	1			1	3			3					8
10	*Carnobacterium divergens*	17			23	3		13	15	9		4	14	98
11	*Carnobacterium maltaromaticum*				3			4	6	3			2	18
12	*Cutaneotrichosporon curvatum*	3				1								4
13	*Hafnia alvei*	1			11	2			3	1			10	28
14	*Janthinobacterium lividum*	1												1
15	*Kocuria rhizophila*	2					4	11			1	5	5	28
16	*Kocuria salsicia*	1									3			4
17	*Latilactobacillus curvatus*					2								2
18	*Latilactobacillus sakei*	9			11	6			35	5			18	84
19	*Lactococcus piscium*				8	2			7					17
20	*Latilactobacillus fuchuensis*				4	2			2	2			3	13
21	*Leuconostoc carnosum*					2			6	8			12	28
22	*Leuconostoc gelidum*	1			11	3			4				7	23
23	*Leuconostoc mesenteroides*	5							1					6
24	*Macrococcus caseolyticus*						2	1						3
25	*Microbacterium phyllosphaerae*	1												1
26	*Micrococcus luteus*							1	2					3
27	*Moraxella osloensis*					2	2				1			5
28	*Paracoccus yeei*				2									2
29	*Pseudomonas antarctica*					1								1
30	*Pseudomonas brenneri*									1				1
31	*Pseudomonas cedrina*								1					1
32	*Pseudomonas extremorientalis*					2			1					3
33	*Pseudomonas fragi*				7	1		1	14	1			11	35
34	*Pseudomonas kilonensis*								1					1
35	*Pseudomonas libanensis*								1					1
36	*Pseudomonas lundensis*				3	2		5	3				2	15
37	*Pseudomonas oleovorans*	1												1
38	*Pseudomonas taetrolens*				1	1		4	7				2	15
39	*Rahnella aquatilis*				1					5			2	8
40	*Rahnella inusitata*	2			6	2				4			11	25
41	*Serratia grimesii*					2			2					4
42	*Serratia liquefaciens*				2	5			3	7			2	19
43	*Serratia proteamaculans*				3	2			4	2			12	23
44	*Staphylococcus capitis*				1									1
45	*Staphylococcus epidermidis*				2				1				1	4
46	*Staphylococcus hominis*							3						3
47	*Staphylococcus warneri*				1			2						3
48	*Yarrowia deformans*					1								1
49	*Yersinia enterocolitica*				3				1				1	5
50	*Yersinia ruckeri*				3				4				19	26
51	Not identifiable	14			18	2	2	2	23	2	2	2	25	91

routinely investigated in almost all laboratories and affected samples may only be subjected to microbiological examination of the usual meat microbiota. Specialised procedures are required to culture clostridia and the isolation process takes time (Dorn-In, 2018). Therefore, qPCR may be a better option for screening and then culture can be performed for the PCR-positive samples if required (Mang et al., 2021).

3.3. MALDI-TOF and species identification

Table 2 shows the microorganisms identified and the number of colonies in the three replicates. A total of 699 colonies were subjected to species identification using MALDI-TOF MS. Of these, 608 colonies were identified, and 91 were not identifiable. 289 (41.3 %) colonies belonged to LAB, while 138 (19.7 %) colonies were classified as *Enterobacteriaceae*. The remainder (37.1 %) belonged to other bacterial groups such as *Pseudomonas* spp., *Brochothrix thermosphacta*, *Kocuria* spp., and *Staphylococcus* spp. Most of the yeasts could not be identified, only 13 (1.9 %) colonies were identifiable. The most abundant species were *Carnobacterium divergens* (14.0 %), followed by *Latilactobacillus sakei* (12.0 %) and *Pseudomonas fragi* (5.0 %). The species *Hafnia alvei*, *Kocuria rhizophila*, *Leuconostoc carnosum*, *Leuconostoc gelidum*, *Rahnella inusitata*, *Serratia proteamaculans*, and *Yersinia ruckeri* were found in between 3.0 % and 4.0 % of the colonies subjected to MALDI-TOF MS.

The detected bacterial species, especially those belonging to the LAB and *Enterobacteriaceae*, have also been found in vacuum-packed meat in other studies (Dorn-In et al., 2023; Hernández-Macedo et al., 2011). In general, *Pseudomonas* spp. play an important role as spoilage organisms in meat stored under aerobic conditions (EFSA, 2016). In vacuum-packed meat, *Pseudomonas* spp. can utilise the residual oxygen in the packaging and grow during the first days of storage. In the present study, they were detected using PC plates. *Pseudomonas fragi* was the predominant species, as it has a short storage phase and therefore grows very rapidly even at refrigeration temperatures (Hernández-Macedo et al., 2011).

3.4. Correlation between number of C. estertheticum and spoilage parameter

In general, and as previously described, the other spoilage bacteria can also produce gas, resulting in a low BPS score (Mang et al., 2021). However, as shown in Fig. 1, all non-SV meat samples had a lower BPS score than the SV samples. This indicates that in the SV samples, where the number of other meat microbiota is very low, all *C. estertheticum* strains can grow very well, leading to a significant increase in gas production and the typical spoilage odour. In line with this, strong correlations were found between the number of clostridia and the BPS value ($r = 0.75$), and the number of clostridia and the odour ($r = 0.87$). These correlations were found to be statistically significant ($p = 5.1E{-}14$ and $p = 2.9E{-}23$, respectively). Although the correlations between the number of clostridia and drip loss ($r = 0.27$) and pH ($r = 0.27$) were very weak, they were found to be statistically significant ($p = 0.02$ for both correlations). However, this result should be interpreted with caution. As mentioned in Section 3.1, the drip loss of meat was a result of the SV process.

There are several factors that can promote the growth of *C. estertheticum* in SV beef. For example, high drip loss can contribute to the rapid growth of clostridia, as the meat juice is a source of water-soluble minerals that are released from the meat and are available to the clostridia. In addition, there are only a small number of other meat microbiota in SV meat; thus, there was little or no competition with other microorganisms for the uptake of nutrients. Moreover, the growth of other meat microbiota, especially LAB, leads to an increase of substances such as lactic acid that lower the pH of the meat, resulting in growth inhibition of *C. estertheticum* in non-SV meat. Almost all non-SV meat samples containing very low numbers of *C. estertheticum* had pH values of 5.0 ± 0.4, while the SV meat samples containing high numbers of *C. estertheticum* had pH values of 5.7 ± 0.4. Since *C. estertheticum* can grow at a pH of 5.5 to 7.5, with an optimal range between 5.8 and 6.8 (Wambui, 2019), the pH values of SV beef provide another optimal condition for the growth of these bacteria. In addition, SV treatment at moderately high temperature over a long period of time (55 °C, 70 min) can lead to activation of the spores for germination.

These results indicate that a high number of *C. estertheticum* leads to increased gas production (BPS value) and to the typical spoilage odour, regardless of whether it is SV or non-SV beef. On the other hand, the ability of LAB to produce lactic acid, which lowers the pH sufficiently to inhibit the growth of *C. estertheticum*, may extend the shelf life of vacuum-packed meat caused by at least some strains of *C. estertheticum*. As shown in this study, the field strains isolated from vacuum-packed beef (strain C2) and from bovine faeces (strain C3) did not grow very well when the competing meat microbiota was present in large numbers.

4. Conclusion

In general, the SV process can extend the shelf life of beef because the common spoilage bacteria are killed, inactivated or stressed so that they cannot grow very well. However, the results of this study show that in vacuum-packed beef samples contaminated with *C. estertheticum* and subjected to SV cooking, the clostridial spores survive the SV process and can grow very well in SV beef because of the lack of competing microorganisms (especially LAB). In addition, the high volume of meat drip produced during SV processing can serve as a nutrient source for these bacteria. The growth of *C. estertheticum* leads to remarkable gas production and a spoilage odour after 28 days of storage at 4 °C. Therefore, the shelf life of SV beef containing *C. estertheticum* is generally shorter than that of non-SV meat containing *C. estertheticum*.

CRediT authorship contribution statement

Samart Dorn-In: Writing – original draft, Visualization, Project administration, Methodology, Funding acquisition, Conceptualization. **Riem El-Seniti:** Writing – original draft, Investigation, Data curation. **Karin Schwaiger:** Writing – review & editing, Supervision, Resources.

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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.

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Data availability

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

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