Incidence and Differentiation of Cold-tolerant *Clostridium* Species in Vacuum-Packed Beef Produced and Retailed in Germany

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Meinen Eltern

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List of Abbreviations

B.	*Bacillus*
bp	base pair
C.	*Clostridium*
CBA	Columbia Blood Agar
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig
ITS	Internal Transcribed Spacer
LAB	Lactic acid producing bacteria
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry
mm	millimetre
P.	*Pseudomonas*
PCR	Polymerase Chain Reaction
PYGS	Peptone Yeast Glucose Starch
qPCR	quantitative Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal ribonucleic acid
sp., spp.	species (singular/plural)
subsp.	subspecies

1 Introduction

Cold-tolerant clostridia are known as causative agents for spoilage of vacuum-packed meat. The first case of so-called blown pack spoilage occurred in 1989 in the UK where meat was found to be spoiled by *Clostridium* (*C.*) *estertheticum* (Dainty et al., 1989). Since then, meat spoiled by cold-tolerant clostridia has been reported worldwide and different species such as *C. gasigenes*, *C. frigoriphilum* and *C. tagluense* have been discovered (Broda et al., 2000a; Pecheritsyna et al., 2007; Suetin et al., 2009). Whereas *C. estertheticum* and *C. gasigenes* produce high amounts of gas and therefore lead to swelling of the package (blown pack spoilage), other species such as *C. frigoriphilum*, *C. bowmanii* and *C. frigidicarnis* produce only little or no gas (Broda et al., 1999; Dorn-In et al., 2018; Moschonas et al., 2010). In this study, the term cold-tolerant (psychrotolerant) clostridia refers to species which are able to grow at temperatures lower than 5 °C, such as psychrophilic (cold-loving) and psychrotrophic (cold-growing) *Clostridium* spp. (Adam et al., 2010).

Since culturing of cold-tolerant clostridia is time-intensive, molecular biological methods, such as Polymerase Chain Reaction (PCR), are often used as an alternative to detect cold-tolerant clostridia in samples. Specific PCRs for the detection of *C. estertheticum* and *C. estertheticum*-like, *C. bowmanii*, *C. frigoriphilum* and *C. tagluense*-like have already been developed (Brightwell and Clemens, 2012; Broda et al., 2003a; Dorn-In et al., 2018). However, there may be other cold-tolerant *Clostridium* species, which may be present in samples but stay undetected, since no specific PCR has yet been developed to detect them directly in meat samples.

C. putrefaciens and *C. algidicarnis* are both psychrotrophic species and were first described in 1911 and 1994, respectively (Lawson et al., 1994; McBryde, 1911). They were often associated with deep tissue spoilage (bone taint) and ham souring but have recently also been found in vacuum-packed meat (Boyer, 1926; Broda et al., 1996a; Hernández-Macedo et al., 2012). Both species show a high similarity of their 16S rRNA genes and can therefore not be differentiated by sequencing (Broda et al., 2000b). To this point, all methods to differentiate between the two require pure isolates and are thus rather time consuming.

To this day, no health issues have been associated with cold-tolerant clostridia but the financial losses in the meat industry caused by this type of spoilage are severe, which makes

an early detection of a possible contamination within abattoirs and cutting plants an important issue. Although incidences of cold-tolerant clostridia were reported worldwide, only a few investigations on this topic were conducted in Germany. Most studies concentrate on the prevalence of cold-tolerant clostridia in meat from retail or in abattoirs in Germany (Böhm, 2013; Bonke et al., 2016; Eckardt, 2015; Ziegler, 2009).

The aim of this study was to obtain the prevalence of cold-tolerant clostridia in beef processed and retailed in Germany, as well as in processing plants and abattoirs of the investigated beef samples by tracing back the information provided on the package. For this purpose, 60 vacuum-packed beef samples were purchased in supermarkets and butchers in Greater Munich and investigated for the appearance of said clostridia using culturing methods, multiplex qPCR and sequencing as partly described by Dorn-In et al., (2018). For a definite species identification of cold-tolerant clostridia found in some samples, a further aim of this study was to develop a Restriction Fragment Length Polymorphism (RFLP) and, additionally, a multiplex quantitative Polymerase Chain Reaction (qPCR) as practical methods to differentiate between *C. putrefaciens* and *C. algidicarnis*.

2 Literature

2.1 Microbial Spoilage of Meat

Meat spoilage is a natural process leading to sensory deviations, such as changes in colour, odour, flavour and texture of the meat. Globally, around 20% of the produced meat products are lost or wasted at some point in the food supply chain, mainly due to commencing spoilage (FAO, 2011). Apart from the massive food waste, it also accounts for immense economic losses in the meat industry.

Meat spoilage can have several causes unrelated to microbiota, such as physical damage, chemical reactions and pest infestation. However, microbial growth represents the main cause (Gram et al., 2002).

2.1.1 Spoilage of Meat under Aerobic Conditions

Bacteria belonging to the order Pseudomonadales are the main spoilage microorganisms in meat stored under aerobic conditions. Representative spoilage genera in this order are *Pseudomonas* (Family Pseudomonadaceae), as well as *Moraxella* and *Acinetobacter* (Family Moraxellaceae).

Pseudomonas (*P.*) species (spp.), predominantly *P. fragi*, *P. lundensis*, *P. fluorescens* and *P. putida*, are the main cause for microbial meat spoilage under aerobic and chilled conditions (Delaquis et al., 1992; Koutsoumanis et al., 2006; Sundheim et al., 1998). *Pseudomonas* spp. produce high amounts of methyl acetate and ethyl acetate, utilising glucose and amino acids, and their growth comes along with foul, cabbage-like odours and slime production (Stanborough et al., 2018).

The incidence of meat spoilage caused by *Moraxella* spp. and *Acinetobacter* spp. is generally significantly lower than by *Pseudomonas* spp. (Farber and Idziak, 1984; Ingram and Dainty, 1971; McMeekin, 1975). *Moraxella* spp. proportionally increases when the meat is salted, while *Pseudomonas* spp. decreases due to osmotic stress (Juven and Gertshovki, 1976). *Acinetobacter* spp. can act as a major spoilage cause, when meat has a high ultimate pH, since the initial numbers of *Acinetobacter* spp. are proportionally higher under these circumstances (Gill and Newton, 1978).

In rarer cases, mainly when the surface becomes dry, moulds and yeasts, such as *Candida* spp., *Cryptococcus* spp. and *Sporotrichum* spp., can play a role in aerobic meat deterioration (Abunyewa et al., 2000; Nielsen et al., 2008).

2.1.2 Spoilage of Meat under Anaerobic Conditions

Spoilage of meat under anaerobic conditions can be caused by obligate anaerobic microorganisms (e.g. *Clostridium* spp.), facultative anaerobic (e.g. *Enterobacteriacaeae* and *Brochothrix thermosphacta*) or aerotolerant microorganisms (e.g. lactic acid producing bacteria).

When maintaining hygienic slaughtering conditions and a constant low storing temperature, lactic acid producing bacteria (LAB) are dominating in an anaerobic atmosphere, as found in vacuum-packed meat (Borch et al., 1996; Yost and Nattress, 2002). Commonly found species are *Lactobacillus* spp., *Leuconostoc* spp. and *Carnobacterium* spp. (Hernández-Macedo et al., 2011). Generally, their spoilage potential is rather low. If spoilage occurs, it is often caused by LAB strains producing butyric acid (Jones, 2004). Short chain fatty acids, such as lactic acid, acetic acid and butyric acid account for the typical acidic and cheesy odour of meat spoiled by lactic acid producing bacteria (Gill, 1983; Jääskeläinen et al., 2012; Jones, 2004).

In case of temperature abuse, *Enterobacteriaceae*, such as *Serratia proteamaculans*, *Hafnia alvei, Proteus* spp. and *Klebsiella* spp., can be a relevant cause of vacuum-packed meat spoilage (Gamage et al., 1997; Gill and Newton, 1978; Hanna et al., 1979). The spoilage is characterised by offensive odours and greening of the meat (Brightwell et al., 2007).

B. thermosphacta plays an important role in meat deterioration (Hernández-Macedo et al., 2011). It can grow under aerobic as well as anaerobic conditions and preferably metabolises glucose (Pin et al., 2002). With residual oxygen being present in vacuum-packs, its main products are acetoin and diacetyl, causing an offensive, sweet and cheesy odour. Under strict anaerobic conditions, spoilage caused by *B. thermosphacta* is less severe since lactic acid and ethanol are the main metabolites resulting from its utilisation of glucose. (Gribble and Brightwell, 2013; Pin et al., 2002).

So-called blown pack spoilage (see **Fig. 1**) of vacuum-packed and constantly chilled meat has been related to psychrotolerant *Clostridium* spp. since 1989 (Broda et al., 1999; Dainty et al., 1989; Silva et al., 2011). The spoilage is characterised by an immense gas production which

leads to gas bubbles in the drip and eventually to a distension of the vacuum film (Boerema et al., 2007). The cheesy odour is caused by butanol, butyl esters and butyric acid being volatile compounds of gas produced by psychrotolerant clostridia (Broda et al., 1996b). Since Dainty et al. (1989) first reported on meat spoiled by *C. estertheticum* in the United Kingdom, cases of blown pack spoilage have occurred all over the world (Bolton et al., 2015; Bonke et al., 2016; Broda et al., 1996b; Wambui et al., 2020; Zhang et al., 2020).

While blown pack spoilage was long believed to be solely caused by *C. estertheticum* and *C. gasigenes*, more recent studies have shown that *Enterobacteriaceae* and LAB are also able to produce gas and cause this kind of spoilage. In the United States, commercially obtained, gas-swollen ground beef chubs were tested for microbial spoilage and revealed to be contaminated with *Hafnia alvei* (Kang et al., 2002). In New Zealand, cases of blown pack spoilage were also found to be unrelated to psychrotolerant clostridia. Further investigations revealed a contamination with species of *Enterobacter*, *Serratia*, *Hafnia* and *Rahnella* (Brightwell et al., 2007). Similar findings were reported by Hernández-Macedo et al. (2012) in meat from Brazil. Chaves et al. (2012) tested different species of *Enterobactericeae* and lactic acid bacteria recovered from meat and abattoir samples for their blown pack spoilage potential. Various species, such as *Hafnia alvei*, *Klebsiella pneumoniae*, *Leuconostoc mesenteroides* and *Lactobacillus brevis*, showed commencing gas production after 14 days and a moderate to hard distension of the packaging after 21 days of storage at 4 °C.

Studies showed that other cold-tolerant *Clostridium* species than *C. estertheticum* and *C. gasigenes*, such as *C. bowmanii*, *C. frigoriphilum* and *C. frigidicarnis*, were able to produce only small amounts of gas (Broda et al., 1999; Dorn-In et al., 2018; Moschonas et al., 2010). Thus, the spoilage appearance of vacuum-packed meat caused by these *Clostridium* species was rather similar to spoilage caused by *Enterobacteriaceae* and LAB. In some cases, the co-contamination of meat with different bacterial groups has to be considered. The routine culturing method may not detect cold-tolerant clostridia, since they grow much slower and require lower growth temperatures than *Enterobacteriaceae* and LAB. Additionally, specific media for culturing cold-tolerant clostridia are not yet available, thus the current quantification method is solely based on qPCR (Bonke et al., 2016; Brightwell and Clemens, 2012; Dorn-In et al., 2018). Therefore, developing specific primers and probes to detect and to quantify a wide range of cold-tolerant *Clostridium* species in meat samples is required.

Unlike *Enterobacteriaceae*, *B. thermosphacta* and LAB, cold-tolerant clostridia are uncommon in vacuum-packed meat. Therefore, when present in high numbers in meat, they may be considered as a main spoilage microorganism in that product.

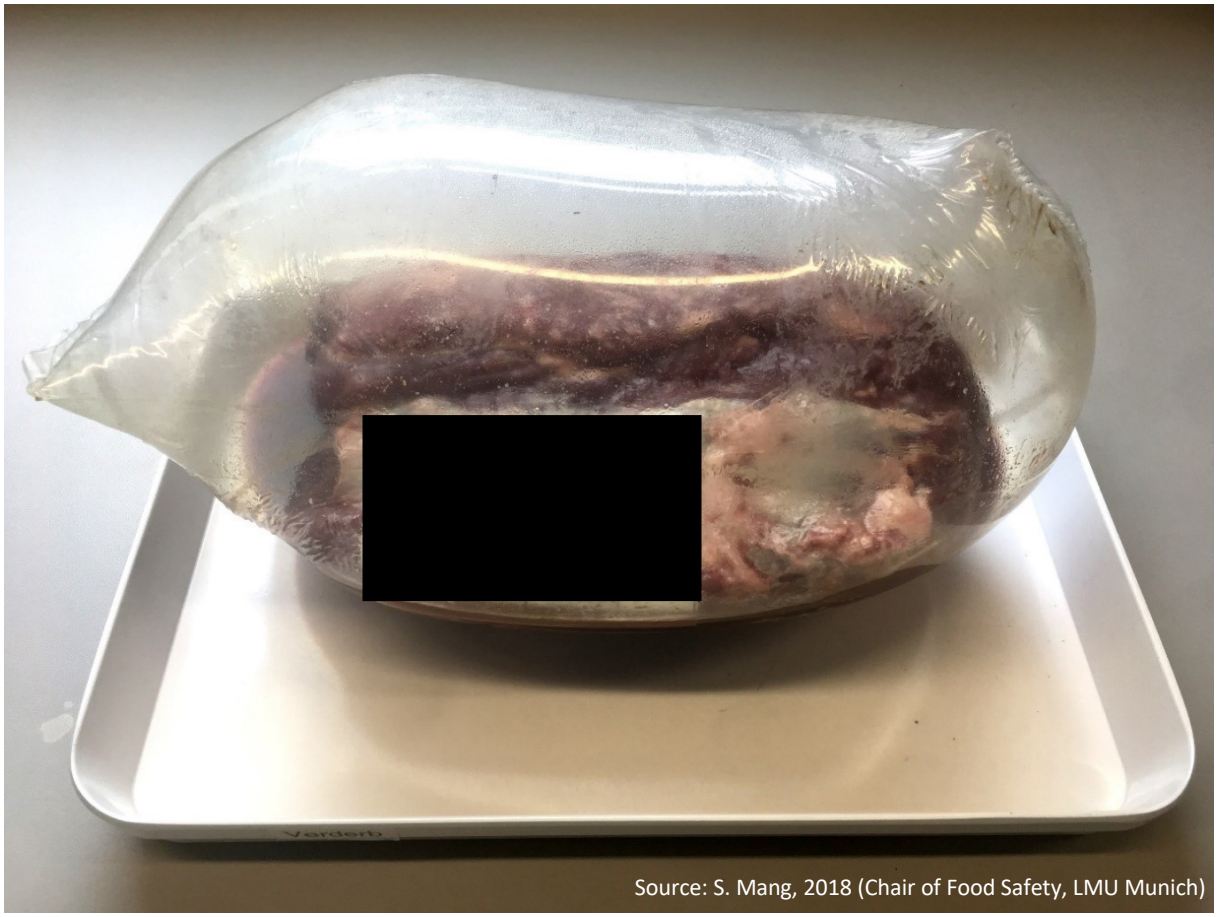

Figure 1: Vacuum-packed beef sample with severe distention of the vacuum foil due to gas production of *C. estertheticum* (label blackened).

2.2 Psychrotolerant Clostridia

2.2.1 Appearance and Characteristics

Like all *Clostridium* species, psychrotolerant (cold-tolerant) clostridia are spore-forming, gram-positive, rod-shaped anaerobes. Psychrophilic (cold-loving) and psychrotrophic (cold-growing) clostridia are characteristically able to grow at very low temperatures. The definitions of the terms psychrophilic, psychrotrophic and psychrotolerant as used in this study followed

descriptions from previous studies (see Table 1).

Table 1: Temperature range for growth of psychrophilic, psychrotrophic and psychrotolerant microorganisms (Olson and Nottingham, 1980; Adam et al., 2010)

	minimum range	**optimum range**	**maximum range**
psychrophilic	-5 - +5 °C	12 – 15 °C	15 – 20 °C
psychrotrophic	-5 - +5 °C	25 – 30 °C	30 – 35 °C
psychrotolerant*		capable of growing at < 5 °C	

*covers psychrophilic, psychrotrophic and mesophilic strains with low minimum growth temperatures (Broda et al., 1997).

All psychrotolerant *Clostridium* species produce gas, however, in various amounts. While *C. estertheticum* and *C. gasigenes* are known to produce large volumes of gas, other species show significantly less gas production (Broda et al., 1999; Moschonas et al., 2010). The main compounds of the produced gas are carbon dioxide and hydrogen. Butyric and volatile sulphur compounds, as well as ammonia and diamines, lead to the putrid odour of meat spoiled that way (Broda et al., 2000a). The appearance and selected characteristics of the most relevant species of psychrotolerant clostridia are summarised in **Table 2**.

Table 2: Appearance and characteristics of selected cold-tolerant *Clostridium* species

species	morphology of colonies	temperature optimum	pH range	ß - haemolysis
C. estertheticum subsp. *estertheticum* [1,2,3]	1 - 2 mm, round with often coarsely granulated margins, smooth, slightly raised, cream-white to greyish, semi-transparent to opaque	6 - 8 °C	5.5 - 7.8	- [1] / + [2,3]
C. estertheticum subsp. *laramiense* [1,4]	small, greyish white, smooth, convex	15 °C	4.5 - 7.5	+
C. gasigenes [5]	0.7 - 3.0 mm, white to grey, circular, convex and shiny	20 - 22 °C	5.4 - 8.9	+
C. putrefaciens [6,7]	cottony, small, filamentous	20 -25 °C	6.0 - 9.0	n. d.
C. algidicarnis [8]	2 - 3 mm, raised, convex, creamy grey	25 - 30 °C	n. d.	+
C. tagluense [9]	round, cream-coloured, convex, 1 - 2 mm	15 °C	6.0 - 8.0	n. d.
C. frigoris [1]	1 - 2 mm, round with often coarsely granulated margins, smooth, slightly raised, cream-white to greyish, semi-transparent to opaque	5 - 7 °C	5.5 -7.5	-
C. lacusfryxellense [1]		8 - 12 °C	6.0 - 7.3	-

[1] Spring et al. (2003) [2] Helps et al. (1999) [3] Yang et al. (2010) [4] Kalchayanand et al. (1993) [5] Broda et al. (2000a) [6] McBryde (1911) [7] Sturges and Drake (1927) [8] Lawson et al. (1994) [9] Suetin et al. (2009)
n. d. = not determined

2.2.2 Sources of Contamination of Meat

Knowing about the relevant sources of cold-tolerant clostridia is a major requirement for an effective prevention of contamination.

While species like *C. estertheticum* subsp. *estertheticum*, *C. gasigenes*, *C. frigidicarnis C. putrefaciens* and *C. algidicarnis* were first found in spoiled meat samples, a significant number of psychrophilic species, such as *C. tagluense*, *C. frigoriphilum*, *C. algoriphilum*, *C. frigoris*, *C. lacusfryxellense*, *C. bowmanii* and *C. psychrophilum*, were first isolated from

extreme, non-animal related environments like permafrost or Antarctic mat samples (Broda et al., 1999; Broda et al., 2000a; Collins et al., 1992; Lawson et al., 1994; McBryde, 1911; Shcherbakova et al., 2005; Spring et al., 2003; Suetin et al., 2009). However, strictly psychrophilic clostridia are not restricted to permanently cold territories. Several of the above-mentioned species were later found in animal-related samples and spoiled meat from areas with a moderate climate like Germany, Ireland, New Zealand and the United Kingdom (Broda et al., 2009; Cavill et al., 2011; Dorn-In et al., 2018; Moschonas et al., 2010).

There have been various studies investigating prime sources of cold-tolerant clostridia and the point of contamination of the carcass. Broda et al. (2002) took swabs of the hide, faeces and tonsils of 100 slaughter animals and 33 environmental samples at various points of a venison processing chain in New Zealand and tested them for the occurrence of cold-tolerant clostridia. In 6% of hide samples and in 5% of faecal samples they found *C. gasigenes*. This species could not be isolated from tonsil swabs or environmental samples. Other species that could not be fully identified but showed high similarities to *C. estertheticum* were also mainly found in faecal samples, fewer in hide or environmental samples. Tonsil swabs were continuously negative for cold-tolerant clostridia.

The studies of Boerema et al. (2003), also conducted in New Zealand, revealed similar results: 39 samples were taken from the abattoir and its environment and tested for *C. estertheticum* and *C. gasigenes*. Both species were mainly found in hide, faeces and soil samples, as well as in samples taken from the stockyard pen and significantly less in samples taken from the slaughter floor or the boning room. Both authors consequently concluded that the source of contamination with cold-tolerant clostridia is most likely spores on animal hide contaminated with faeces which are then transferred to the carcass during the dressing process.

This conclusion was supported by Moschonas et al. (2009), after testing 1680 samples of animals and the surroundings of four different Irish slaughterhouses for the occurrence of *C. estertheticum* and *C. gasigenes*. Samples positive for one or both of the two species predominantly originated from hide or faeces samples and from predressing areas, while positive samples from postfleece removal areas were rare.

Broda et al. (2009) included two additional species – *C. putrefaciens* and *C. algidicarnis* – in their study and made an important finding. After taking and testing 357 samples, they found that, in accordance with former studies, *C. estertheticum* and *C. gasigenes* were mainly found in samples of fleece, faeces and soil. Neither of the two species could be detected in postfleece

removal areas. *C. putrefaciens*/*C. algidicarnis* (no further differentiation was made), however, could rarely be detected in faecal or farm samples but mainly in swabs taken from surfaces of the boning room, suggesting that contamination happens only after the carcass breakdown. These results support other authors in their assumption that *C. putrefaciens* and *C. algidicarnis* may proliferate in deep tissues and the lymphatic system and thus could be present in living animals (Boyer, 1926; Broda et al., 1996a; Haines and Scott, 1940; Mundt and Kitchen, 1951).

Dorn-In et al. (2018) obtained 110 isolates of cold-tolerant clostridia from 108 samples of different types of meat, as well as from skin and faeces samples of wild boar. While *C. estertheticum* and *C. frigoriphilum* were isolated mainly from faeces samples, *C. frigidicarnis*, *C. bowmanii* and *C. tagluense*-like were predominantly isolated from skin of wild boars.

2.2.3 Methods of Detection and Differentiation

2.2.3.1 Culturing

Since psychrophilic and psychrotrophic clostridia grow at low temperatures the culturing process is slow and not always successful (Boerema et al., 2003; Broda et al., 1998; Dainty et al., 1989). To this day, there is no selective culture medium which makes isolation difficult. Commonly used are Columbia Blood Agar (CBA) as a solid and Peptone Yeast Glucose Starch (PYGS) as a liquid culture medium (Broda et al, 1998). PYGS broth is used in order to enrich the number of cold-tolerant clostridia, if present in samples. Suspicious colonies growing on CBA have to be further confirmed by either biochemical methods, molecular biological methods or Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), as described below. Culturing methods used for cold-tolerant clostridia are therefore only for qualitative purposes.

2.2.3.2 Molecular Biological Methods

There are several molecular biological methods to detect cold-tolerant clostridia. The first PCR to detect *C. estertheticum* was developed by Helps et al. (1999). The used primers bind to the 16S rRNA gene of said species and produce amplicons with a size of 641 bp. Broda et al. (2003a) established a method to detect *C. estertheticum* and *C. gasigenes* using

two primer sets (16SDBF/16SDBR to detect *C. gasigenes* and 16SEF/16SER to detect *C. estertheticum* and *C. estertheticum*-like) binding to the 16S rRNA gene and one primer set (EISRF/EISRR to detect *C. estertheticum*) binding to the Internal Transcribed Spacer (ITS) region. However, since then, new species of cold-tolerant clostridia have been discovered and several studies have shown that primer pair 16SEF/16SER is also able to bind to the 16S rRNA gene of species like *C. bowmanii* and *C. lacusfryxellense* (Brightwell and Clemens, 2012; Cavill et al., 2011).

Boerema et al. (2002) designed a primer set to detect 16S rRNA gene fragments of *C. algidicarnis* and *C. putrefaciens* in meat drip. Since both species show a high rate of similarity in the target region, a differentiation was not possible.

Brightwell and Clemens (2012) developed a real time PCR assay for the detection of *C. estertheticum* and *C. estertheticum*-like, such as *C. bowmanii* and *C. lacusfryxellense*, in a variety of different matrices. While the detection of said species in meat, hide, blood/drip and environmental samples was successful (limit of detection: 3 spores per ml), the detection in faeces and soil proved to be more difficult and enrichment prior to the DNA extraction is recommended.

Dorn-In et al. (2018) developed the first multiplex qPCR to identify four different cold-tolerant *Clostridium* species, namely *C. estertheticum*, *C. frigoriphilum*, *C. bowmanii* and *C. tagluense*-like and thereby provided a practical method for a rapid identification of said species.

qPCR based methods, as described above, are used to detect and at the same time quantify target microorganisms directly in samples such as meat drip, faeces or environmental swabs. However, the provided PCR methods cannot detect all species of cold-tolerant clostridia and many of them are not able to differentiate between species. Therefore, further molecular biological methods have to be applied for exact species identification.

Broda et al. (2000b) used Restriction Fragment Length Polymorphism (RFLP) analysis of the 16S rRNA gene to differentiate six different species of cold-tolerant clostridia, namely *C. estertheticum*, *C. algidicarnis*, *C. putrefaciens*, *C. fimetarium*, *C. vincentii* and *C. botulinum* (type B and E). Out of all eight restriction enzymes tested, a combination of four endonucleases (AluI, HaeIII, TaqI, CfoI) allowed a distinct differentiation of all species involved.

A discrimination and identification of cold-tolerant clostridia using internal transcribed spacer polymorphism analysis was attempted by Broda et al. (2003b). However, due to the presence

of interstrain and a lack of interspecies ITS polymorphism, the method proved to be unsuitable for the purpose of identification.

Genetic sequence analysis of the 16S rRNA gene (about 1500 bp) presents another method to identify cold-tolerant *Clostridium* species. Using universal primer sets, the sequence is amplified in a PCR run and, after purification and sequencing, can be compared to 16S rRNA gene sequences of other cold-tolerant clostridia, which are provided in public databases (e.g. NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Even though sequencing is generally a very reliable method for identification, it cannot be applied to all cold-tolerant *Clostridium* species. The 16S rRNA gene sequences of some cold-tolerant *Clostridium* species, such as *C. putrefaciens* and *C. algidicarnis*, are highly homogenous and can therefore not be differentiated that way (Broda et al., 2000b).

Since the application of RFLP analysis and sequencing of the 16S rRNA gene require pure isolates, culturing has to be performed first. Therefore, a variability of materials is needed and the working process is time intensive until the contaminating species is finally identified.

2.2.3.3 MALDI-TOF MS

Another way of differentiating cold-tolerant *Clostridium* species is by using MALDI-TOF MS. Similar to RFLP analysis and sequencing of the 16S rRNA gene, pure isolates are required as starting material. The wide range of standard protein spectra of each cold-tolerant *Clostridium* species has to be established and included in the databank. There are still many unknown cold-tolerant *Clostridium* species and variable protein structures within the same species may lead to insufficient results of the identification, as reported by Dorn-In et al. (2018).

2.3 *C. putrefaciens* & *C. algidicarnis*

2.3.1 Reported Cases Worldwide

C. putrefaciens was first described in 1911 by McBryde in a bacteriological study. Back then, statistics showed that 1.675.000 pounds of ham produced annually in the United States were seized because of souring. Based on that, McBryde concluded a total annual loss of approximately a quarter of a million dollars in the meat packaging industry. He defined a sour ham as ham that has a scent that deviates from the normal, often described as sour or – in severe cases – putrefactive. As initial cause, McBryde determined the growth of an anaerobic

bacterial species that he assigned to the genus *Bacillus* (*B.*) and named it *B. putrefaciens*. According to McBryde, the bacillus and its spores are common in dust and dirt and might contaminate the ham by being present on thermometers, pumping needles and billing hooks which are used during the curing process. By examining ham taken from the killing floor only 45 minutes after slaughtering, Boyer (1926) later concluded that *B. putrefaciens* must already be present at the moment of slaughter and could possibly be found in the blood and tissue of living animals. A year later Sturges and Drake (1927) published a complete description of the species and rightly assigned it to the genus *Clostridium*. In the following years *C. putrefaciens* was found to be the cause of bone taint in cured ham in various studies (Ingram, 1952; Mundt and Kitchen, 1951; Ross, 1965).

C. algidicarnis was first described by Lawson et al. (1994) who isolated the species from spoiled vacuum-packed, cooked pork. Since then, it has been associated with bone taint in temperature-abused beef and deep tissue spoilage of vacuum-packed lamb (Boerema et al., 2002; Broda et al., 1996a; De Lacy et al., 1998).

In recent years, both species were found in vacuum-packed beef in the UK, New Zealand and Brazil (Broda et al., 2009; Cavill et al., 2011; Hernández-Macedo et al., 2012; Silva et al., 2011). So far, there are no reported findings of *C. putrefaciens* or *C. algidicarnis* within the mainland of Europe.

2.3.2 Methods to Differentiate Between Both Species

C. putrefaciens and *C. algidicarnis* are very closely related. Their 16S rRNA genes show a similarity of 99 – 100% (Broda et al., 2000b; Stackebrandt and Swiderski, 1999), which excludes sequencing of said genes as a possible method for differentiation. In most studies that involved the investigation of cold-tolerant *Clostridium* species, no differentiation between the two was made (Boerema et al., 2002; Broda et al., 2009; Cavill et al., 2011). Broda et al. (2000b) used RFLP analysis to differentiate between various species of psychrophilic and psychrotrophic clostridia. *C. putrefaciens* and *C. algidicarnis* could be differentiated by using the restriction endonuclease CfoI/HhaI for digestion of the former amplified 16S rRNA. The obtained restriction patterns are clearly distinguishable. However, the method requires pure isolates of both species, similar to MALDI-TOF MS.

To this day, there is no method available that can be used to distinguish between both species directly from sample material such as meat juice or swab samples.

3 Publication

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High incidence of cold-tolerant *Clostridium frigoriphilum* and *C. algidicarnis* in vacuum-packed beef on retail sale in Germany

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ABSTRACT

Sixty vacuum-packed beef samples retailed in Germany were investigated for the occurrence of cold-tolerant *Clostridium* spp. After a storage period at 4 °C for eight weeks, meat juice from all samples was processed for culturing, DNA extraction and SYBR green qPCR for *Clostridium* species. After that, a previously developed multiplex qPCR, sequence analysis of the 16S rRNA gene, and MALDI-TOF MS were applied in order to identify *Clostridium* spp. found in samples. Subsequently, 23 samples were found positive for *C. frigoriphilum* ($n = 19$), *C. estertheticum* ($n = 2$), *C. tagluense* (n = 1) and *C. lacusfryxellense/C. frigoris* (n = 1). By using a new multiplex qPCR and a new RFLP method developed in this study, a further 15 meat juice samples were revealed to be contaminated with *C. algidicarnis*. With some samples being co-contaminated with two different species, 53% ($n = 32$) of all investigated vacuum-packed beef samples were found to be positive for cold-tolerant clostridia. This is the first report of detection and identification of *C. algidicarnis* in meat samples in Germany and Central Europe.

1. Introduction

Cold-tolerant clostridia are known to be causative agents of meat spoilage under anaerobic conditions. So-called Blown Pack Spoilage (BPS) of vacuum-packed meat is often caused by *Clostridium estertheticum* (Brightwell and Clemens, 2012; Broda et al., 2009; Byrne et al., 2009; Cavill et al., 2011). There are no studies indicating a health risk associated with cold-tolerant clostridia, but BPS could be responsible for severe economic losses within the meat industry. Since the first reported case of meat spoiled by *C. estertheticum* imported to the UK (Dainty et al., 1989), meat spoilage caused by various cold-tolerant *Clostridium* spp. have been reported from countries all over the world, such as Brazil (Silva et al., 2011), Canada (Zhang et al., 2020), Germany (Bonke et al., 2016; Dorn-In et al., 2018), Ireland (Bolton et al., 2015), New Zealand (Broda et al., 1996a), Switzerland (Wambui et al., 2020) and the United States (Kalchayanand et al., 1989). Besides *C. estertheticum*, other cold-tolerant clostridia isolated from vacuum-packed meat, such as *C. algidicarnis* (Lawson et al., 1994), *C. bowmanii* (Moschonas et al., 2010), *C. frigidicarnis* (Broda et al., 1999), *C. frigoriphilum* (Dorn-In et al., 2018) and *C. gasigenes* (Broda et al., 2000a), have been shown to spoil meat either with or without signs of BPS. Meat marketed in Europe has been found to be contaminated with different species of cold-tolerant bacteria as described above (Bonke et al., 2016; Byrne et al., 2009; Cavill et al., 2011). However, most previous investigations did not report whether imported meat from other countries had been packed in the country of export or if they were cut and packed in the country of import. In order to determine the prevalence of cold-tolerant clostridia in beef produced and marketed solely in Germany, a total of 60 vacuum-packed beef samples from German retail outlets were investigated in this study. Besides *C. frigoriphilum*, a significant number of beef samples were thus revealed to be contaminated with *C. putrefaciens* or *C. algidicarnis*. Both species could not be differentiated by sequencing of the 16S rRNA genes, since they are very closely related (99–100% similarity, Broda et al., 2000b). Previous methods for differentiation require pure isolates. Therefore, besides the investigation of the incidence of cold-tolerant clostridia in vacuum-packed beef produced in Germany, a further aim of this study was to develop an RFLP method and, additionally, a multiplex qPCR, which allow a rapid differentiation of *C. putrefaciens* and *C. algidicarnis* directly from meat juice samples.

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2. Material and methods

2.1. Samples

2.1.1. Beef

A total of 60 vacuum-packed chilled beef samples were included in this study. They were purchased from retail shops within Greater Munich (Bavaria, Germany) during April 2019. All samples were cuts and weighed between 180 g and 1.4 kg. Twenty-six samples were already vacuum-packed at the time of purchase, while 19 samples were bought at the meat counter and therefore unpacked. The remaining 15 samples were packed under protective atmosphere. Samples in modified atmosphere packaging were unwrapped and, as well as the unpacked samples, vacuum-packed immediately after purchase. Samples were chosen on the condition that at least one production step, from birth to packing, had taken place in Germany. Fifty beef samples were completely processed within Germany. Four samples were partly processed in Austria, three samples in Ireland, two samples in the Netherlands and one sample in Italy. Fig. S1 shows the Federal Republic of Germany and where the abattoirs and cutting plants are located in which the beef samples in this study were originally processed (as indicated on the package) and is provided as supplementary data. Table 1 shows a summary of sample numbers and origins of their processing steps.

2.1.2. Reference bacteria

All bacterial species used for validation of the specificity and sensitivity of the developed RFLP method and multiplex qPCR are listed in Table S1 (see supplementary data). Besides *Clostridium* species ($n = 28$), representatives of facultative anaerobic and aerotolerant microorganisms such as bacterial species in the family *Enterobacteriaceae* ($n = 10$) and in the order Lactobacillales (lactic acid producing bacteria, $n = 10$) were included in this study.

2.2. Storage of meat and spoilage appearance

All vacuum-packed beef samples were stored for eight weeks at 4 °C. By that time, all samples had passed their shelf life by six to eight weeks. They were evaluated weekly for their level of gas production and loss of meat juice. In order to grade their ongoing pack distension, a Blown Pack Spoilage Scoring System (BPS score) from 0 to 5 was applied (Boerema et al., 2007). After eight weeks, the integrity of the vacuum packaging, the BPS score, the loss of meat juice, as well as the odour, colour and texture of all samples were evaluated. The sensory analysis was carried out by two people. In terms of the odour of the meat juices, samples were put in one of three categories: no or very slight change, repellent & sour smell and severe acidic & cheesy odour. The colour and texture of the meat was compared to fresh, vacuum-packed meat and changes were noted.

2.3. Culture of cold-tolerant clostridia

After eight weeks of incubation, meat juice from each sample was collected using a sterile syringe (5 ml, B. Braun Melsungen AG, Germany) and needle (30G, Becton Dickinson GmbH, United States). The resulting perforation was sealed with multiple strips of transparent adhesive tape and the packs were further stored at 4 °C until cultivation and identification was completed. Meat juice was streaked on two Columbia Blood Agar Plates (CBA, Oxoid) which were incubated anaerobically for at least three weeks at 4 °C and 10 °C, respectively. Additionally, two 9 ml volumes of peptone yeast glucose starch broth (PYGS, Lund et al., 1990) were inoculated with 1 ml of meat juice and likewise anaerobically incubated at 4 °C and 10 °C for at least three weeks. All macroscopically different looking colonies grown on CBA were picked using inoculating loops (1 µl, VWR International, United States), subcultured on a new CBA, and anaerobically incubated at 4 °C or 10 °C until colonies were visible. Then, the subcultured colonies were processed for species identification using MALDI-TOF MS (see Section 2.5.4). To confirm the results obtained, all colonies that were identified as *Clostridium* spp. with MALDI-TOF MS, were further processed for DNA extraction (direct extraction, as described in Section 2.4) and PCR amplification using the SYBR green qPCR method described in Section 2.5.1.

The species *C. estertheticum* subsp. *estertheticum* (DSM 8809) and *C. algidicarnis* (DSM 15099) were used to establish standard dilutions for qPCR in order to quantify cold-tolerant clostridia in meat juice samples. They, too, were subcultured on CBA and anaerobically incubated at 10 °C for 10 days. After that, the colonies were suspended in sodium chloride solution (0.9%) and the cell counts were performed using a microscope and a Thoma cell counting chamber. Suspensions of both species were serially diluted from 10^8 to 10^1 cells/ml in a meat juice sample, which had previously been found negative for *Clostridium* species. The serial dilutions were further processed for DNA extraction.

Table 1
Meat samples from abattoirs and cutting plants found positive for cold-tolerant *Clostridium* spp. and their countries/states of origin.

Country born & raised	Beef samples		Abattoirs			Cutting plants		
	n investigated	*n* positive	Location[a]	*n* investigated	*n* positive	Location[a]	*n* investigated	*n* positive
Germany (DE)	44	24	BV	13	8	BV	16	9
			BW	3	1	NW	6	4
			NI	2	2	BW	2	2
			NW	2	1	ST	1	1
			MV	1	1	MV	1	1
			HE	1	0	SH	1	0
	6	4	Unknown	–	–	Unknown	–	–
Austria (AT)	3	2	AT	1	1	AT & NW	1	1
			AT	1	1	AT & NW	1	1
			AT	1	0	AT & ST	1	0
	1	0	Unknown	–	–	Unknown	–	–
Ireland (IR)	1	0	IR	1	0	IR & NW	1	0
	2	1	Unknown	–	–	Unknown	–	–
Netherlands (NL)	1	1	NL	1	1	NW	1	1
	1	0	Unknown	–	–	Unknown	–	–
Italy (IT)	1	0	IT	1	0	IT & NW	1	0
Total	60	32	–	28	16	–	33	20

[a] Federal States of Germany (DE): BV = Bavaria, BW = Baden-Württemberg, HE = Hessen, MV = Mecklenburg-Vorpommern, NI = Lower Saxony, NW = North Rhine-Westphalia, SH = Schleswig-Holstein, ST = Saxony-Anhalt (see Fig. S1, supplementary data).

2.4. DNA extraction

DNA extraction of meat juice and of PYGS enrichment was carried out using the High Pure PCR Template Preparation Kit (Roche Life Science). For better lysis of Gram-positive bacteria, 200 µl of the sample suspension was mixed with 10 µl of lysozyme (20 mg/ml in 10 mM Tris HCl, pH 8.0: final concentration 1 mg/ml) and incubated at 37 °C for 30 min. Further steps, including treatment with proteinase K and mixing with binding buffers, were conducted as recommended in the instruction manual of the kit.

For colonies subcultured on CBA and identified as *Clostridium* spp. with MALDI-TOF MS, DNA extraction was performed using a direct extraction method developed in this study. Colonies were mixed into 100 µl of molecular biological water. Lysozyme (5 µl, final concentration 1 mg/ml) was mixed thoroughly into the solution and the samples were then incubated for 30 min at 37 °C. After incubation, 10 µl of proteinase K (final concentration 600 mAU/ml) was added, the samples were mixed and incubated for 10 min at 70 °C. Then the mixture was centrifuged and incubated once more at 99 °C for 15 min. The sample was then centrifuged for 1 min at 20.200 ×*g* and 80 µl of supernatant was transferred to a new tube and subsequently used for PCR.

2.5. Identification of Clostridium spp. in meat samples

2.5.1. Specific qPCR for genus Clostridium spp.

A specific SYBR green qPCR with primer pair TM-F (Brightwell and Clemens, 2012) and Cl642-R (Dorn-In et al., 2018) was applied as a screening method to determine whether meat juices and inoculated PYGS bouillons were positive for *Clostridium* spp. A 20 µl reaction mixture contained 0.2 µM of each primer, 10 µl of SensiFAST™ SYBR No-ROX Kit (Bioline), and 2 µl of DNA, with the remainder of the mixture being comprised of water. All PCR runs were carried out in a BioRad CFX96 Touch™ thermocycler (BioRad, United States). The following protocol was used: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 5 s and 72 °C for 15 s. A melt curve analysis was carried out. Samples with Ct values lower than 36 cycles and melting temperatures between 87 °C and 88 °C were considered positive for *Clostridium* spp. and were further identified to species level using a multiplex qPCR as described in Section 2.5.2. Samples where both meat juice and inoculated PYGS broth showed negative results in SYBR qPCR were classified as negative for cold-tolerant clostridia.

2.5.2. Multiplex qPCR for cold-tolerant clostridia

Samples positive for *Clostridium* species by SYBR green qPCR, as described in Section 2.5.1, were processed for a multiplex qPCR as previously described (Dorn-In et al., 2018). This multiplex qPCR comprises five probes. The first probe detects eight different cold-tolerant *Clostridium* spp. (*C. estertheticum*, *C. frigoris*, *C. frigoriphilum*, *C. lacusfryxellense*, *C. bowmanii*, *C. psychrophilum*, *C. tagluense* and *C. tagluense*-like). The other four probes are used to identify the species *C. estertheticum*, *C. frigoriphilum*, *C. bowmanii* and *C. tagluense*-like, respectively. A standard curve was established using *C. estertheticum* subsp. *estertheticum* (DSM 8809) in order to quantify cold-tolerant clostridia in meat juice. Samples that could not be identified with this multiplex qPCR were processed for PCR product purification and subsequent sequencing of the 16S rRNA gene (see Section 2.5.3).

2.5.3. Sequencing of the 16S rRNA gene

The PCR amplicons of the 16S rRNA gene (about 566 bp) obtained from SYBR green qPCR (Section 2.5.1) were purified with the QIAquick PCR purification kit (Qiagen, Netherlands) according to the manual instructions. The purified amplicons were submitted for sequencing (Eurofins, Germany). The sequences obtained were aligned with the sequences of the reference strains provided by the Basic Local Alignment Search Tool (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5.4. MALDI-TOF MS

Protein from subcultured colonies was extracted using the formic acid extraction method as described in the instruction manual (Bruker Daltonik GmbH, Germany). Extracted samples were then spotted on a polished steel plate in triplicate. Reference protein-spectra of 244 strains of 87 *Clostridium* spp. are provided in the databank of Bruker Daltonik GmbH (Germany). Additionally, protein-spectra of 24 cold-tolerant clostridia (see Table S1, supplementary data) were previously established at the Chair of Food Safety (in-house; LMU Munich, Germany) and included in the database.

2.6. Method development for differentiation of C. putrefaciens and C. algidicarnis

2.6.1. Restriction Fragment Length Polymorphism (RFLP)

RFLP of the 16S rRNA gene (about 1315 bp) described by Broda et al. (2000b) was first used to differentiate between the isolates of *C. putrefaciens* and *C. algidicarnis*. However, in order to identify both species directly in meat juice samples without culturing of isolates, another RFLP method was developed in this study.

For this purpose, the sequence of probe Cpal168-Cy5 was used as a forward primer (Cpal168-F, without Cy5 and BHQ2 dye). Its combination with reverse primer Cpal-ITS-R (1529 bp, sequences see Table 2) is specific exclusively for *C. putrefaciens* and *C. algidicarnis*. Thus, it could be used directly with meat juice samples to detect both species. The design of new primers was based on the sequences of the 16S rRNA gene and the internal transcribed spacer (ITS) of both species provided in GenBank (NCBI, https://www.ncbi.nlm.nih.gov/nucleotide/) and resulted from sequencing (see Section 2.5.3). This primer pair was validated for its specificity and sensitivity using bacterial cultures and meat juice of all beef samples (see Section 2.1 and Table S1, supplementary data). The PCR mix and PCR protocol were performed as described in Section 2.5.1, except that elongation at 72 °C was extended to 20 s. The PCR amplicons were then purified using the QIAquick PCR purification kit (Qiagen, Netherlands) and subsequently digested with the restriction endonuclease HhaI/CfoI (GCGC, New England Biolabs, United States). Restriction digests contained 10 units of enzyme, 1× of the provided buffer, 1 µg of DNA and were filled up with water to a total volume of 50 µl. The tube was incubated for 60 min at 37 °C in a thermomixer (Eppendorf, Germany). To stop the reaction, the tube was

Table 2
List of primers and probes used in the developed multiplex qPCR.

Primers and probes		Specificity	Sequence (direction 5′-3′)	Reference
Set 1	TM-F	–	CGG CGG ACG GGT GAG TAA C	Brightwell and Clemens (2012)
	Cl642-R	–	CCT CTC CTG CAC TCT AGA	Dorn-In et al. (2018)
	Cpal168-Cy5[a]	*C. putrefaciens* & *C. algidicarnis*	CY5-ACC CCA TAA CAT AGC ATT ATC GCA TG-BHQ2	This study
Set 2	Cpal1423-F	–	CTA ACG CGT AAG CGA GGC AG	This study
	Cpal-ITS-R	–	CTT TGC TTT TAA ACC ATA TGC AG	This study
	Cput-ITS-Hex	*C. putrefaciens*	HEX-TTA TAG CTA GTC TAT AAC TCT TAA TAC-BHQ1	This study
	Cal-ITS-Fam[b]	*C. algidicarnis*[b]	FAM-TTG TAG CTY GTC TAT AAC TAG TAC-BHQ1	This study

[a] Sequence of Cpal168-Cy5 was also used as a forward primer and combined with the reverse primer Cpal-ITS-R for RFLP analysis (see Section 2.6.1).

[b] Apart from *C. algidicarnis*, the probe is also able to slightly bind to the DNA of other cold-tolerant clostridia such as *C. tagluense*, *C. frigoriphilum*, but not *C. putrefaciens*.

heated for 20 min at 65 °C. Digested products were separated by gel electrophoresis using 2.0% Certified™ Molecular Biology Agarose (BioRad, United States) running at 120 V for 60 min in 0.5% TBE buffer. The HyperLadder™ 50 bp (Bioline, United Kingdom) was used as a fragment size marker. The gel was stained with ethidium bromide and the restriction pattern of digested DNA was visualized by ultraviolet transillumination.

2.6.2. Multiplex qPCR for C. putrefaciens and C. algidicarnis

Since the multiplex qPCR described in Section 2.5.2 could not detect *C. putrefaciens* and *C. algidicarnis*, an additional multiplex qPCR with four primers and three probes (see Table 2) was developed in this study in order to detect and to differentiate between both species. Primer pair TM-F & Cl642-R (566 bp, as used in Section 2.5.1) was combined with probe Cpal168-Cy5, while primer pair Cpal-1423F & Cpal-ITS-R (360 bp) was combined with probes Cput-ITS-Hex and Cal-ITS-Fam which were specific for *C. putrefaciens* and *C. algidicarnis*, respectively. The design of new primers and probes and the validation for their specificity and sensitivity were performed as described for primers used for RFLP (see Section 2.6.1). Each PCR reaction contained a 20 µl mixture that consisted of 0.25 µM of each primer, 0.1 µM of each probe, 10 µl of SensiFAST™ Probe No-ROX Kit (Bioline, United Kingdom), 2 µl of DNA and was filled up with water. After initial denaturation for 5 min at 95 °C, the target sequences were amplified in 40 cycles. Each cycle started with denaturation for 10 s at 95 °C, followed by annealing and elongation for 30 s at 60 °C. Furthermore, the reference strain *C. algidicarnis* (DSM 15099) was used to establish a standard dilution for multiplex qPCR in order to quantify *C. putrefaciens* and *C. algidicarnis* in meat juice samples (see Sections 2.1.1 and 2.2).

3. Results

3.1. Detection and isolation of cold-tolerant clostridia from meat samples

After storage at 4 °C for eight weeks, meat juice from all 60 vacuum-packed beef samples was processed for culturing, DNA extraction and PCR amplification to identify cold-tolerant *Clostridium* spp. Table 3 summarises the results of all samples and methods included in this study. A total of 31 meat juice samples were found positive using SYBR green qPCR for *Clostridium* spp. (see Section 2.5.1). All positive samples were further tested with multiplex qPCR (see Section 2.5.2) and the species *C. frigoriphilum* and *C. estertheticum* were detected in 19 and two samples, respectively. Two samples were positive using the probe used to detect cold-tolerant clostridia but negative using all species-specific probes. The other eight samples, tested with multiplex qPCR, were negative. After that, PCR products of all 10 samples contaminated with unidentified *Clostridium* species were processed for sequencing (see Section 2.5.3). Seven of eight samples negative by multiplex qPCR were revealed to be contaminated with *C. putrefaciens/C. algidicarnis*. The remaining sample was false positive and was identified as a non-*Clostridium* sp. (*Acidaminococcus* sp.). One sample positive in multiplex qPCR was identified as *C. tagluense* and one as *C. lacusfryxellense/C. frigoris*, respectively.

The other 29 meat juice samples were negative for clostridia using the SYBR green qPCR, so their PYGS enrichments were processed using the same method (see Table 3). In two of these 29 samples clostridia were detected. The sequencing results showed that both samples were contaminated with *C. putrefaciens/C. algidicarnis*. Collectively, 32 (53%) vacuum-packed beef samples were identified as positive for cold-tolerant clostridia, whereas the other 28 (47%) samples were finally classified as negative.

A total of eight strains of *Clostridium* spp. were isolated from eight meat samples positive for *C. putrefaciens/C. algidicarnis*. Colonies growing on Columbia Blood Agar had a creamy, greyish colour, no haemolysis, were slightly raised and about 2 mm in diameter. All of them were identified as *C. algidicarnis* using MALDI-TOF MS and the RFLP method described by Broda et al. (2000b). However, the sequences of their 16S rRNA genes showed that all were 99–100% identical to the sequences of *C. putrefaciens* (accession no. NR 024995.1) and *C. algidicarnis* (accession no. NR 119083.1) provided in GenBank (NCBI). These isolates were later used to validate the specificity and sensitivity of the new RFLP and a multiplex qPCR method (see Section 3.2).

3.2. Differentiation between C. putrefaciens and C. algidicarnis

MALDI-TOF MS and the RFLP method by Broda et al. (2000b) could be applied to differentiate the isolates of *C. putrefaciens* and *C. algidicarnis*, but required pure cultures for identification. By 16S rRNA gene analysis, a differentiation of the two species was not possible. In order to detect and to identify both species in meat juice samples without culturing of isolates, a RFLP method and a multiplex qPCR were developed in this study. All eight isolates of *C. algidicarnis*, the control bacteria listed in Table S1, 60 meat juice samples and two positive PYGS enrichments were used for the validation of the specificity of both methods (see Table 2). Primer pair Cpal168-F & Cpal-ITS-R was specific exclusively for *C. putrefaciens* and *C. algidicarnis* and was applied for the RFLP method. Using this method, seven meat juices and two PYGS enrichments were finally identified as *C. algidicarnis*. Additionally, a co-contamination with two *Clostridium* spp. was found in six meat samples (see Table 3). Thus, a total of 13 meat juice samples and two PYGS enrichments were found positive for *C. algidicarnis*. Fig. 1 shows an example of the RFLP pattern obtained from reference strains

Table 3
Number of samples positive for cold-tolerant clostridia by each laboratory method.

Sample type	Laboratory methods and number of positive (pos.)/negative (neg.) samples				
	SYBR PCR	Multiplex qPCR 1[a]	Sequencing of 16S rRNA gene	RFLP[b]	Multiplex qPCR 2[b]
Meat juice ($n = 60$)	31 (pos.)	19 (*C. frigoriphilum*)	nd	5[c] (*C. algidicarnis*)	5[c] (*C. algidicarnis*)
		2 (*C. estertheticum*)	nd	1[c] (*C. algidicarnis*)	1[c] (*C. algidicarnis*)
		2 (unidentified)	1 (*C. tagluense*)	neg.	neg.
			1 (*C. lacusfryxellense/C. frigoris*)	neg.	neg.
		8 (neg.)	7 (*C. putrefaciens/C. algidicarnis*)	7 (*C. algidicarnis*)	7 (*C. algidicarnis*)
			1 (*Acidaminococcus* sp.)	neg.	neg.
	29 (neg.)	neg.	nd	neg.	neg.
PYGS enrichment ($n = 29$)	2 (pos.)	neg.	2 (*C. putrefaciens/C. algidicarnis*)	2 (*C. algidicarnis*)	2 (*C. algidicarnis*)
	27 (neg.)	neg.	nd	neg.	neg.
Isolate (n = 8)	8 (pos.)	neg.	8 (*C. putrefaciens/C. algidicarnis*)	8 (*C. algidicarnis*)	8 (*C. algidicarnis*)

nd = not determined, pos. = positive, neg. = negative.

[a] Multiplex qPCR according to Dorn-In et al. (2018) to detect some cold-tolerant clostridia and the species *C. estertheticum*, *C. frigoriphilum*, *C. bowmanii* and *C. tagluense*-like.

[b] RFLP and multiplex qPCR developed in this study were to detect and to differentiate *C. putrefaciens* and *C. algidicarnis* (see Section 2.6).

[c] Number of samples with co-contamination (five samples with *C. frigoriphilum* & *C. algidicarnis* and one sample with *C. estertheticum* & *C. algidicarnis*).

C. putrefaciens (2 DNA fragments, 835 and 617 bp) and *C. algidicarnis* (2 DNA fragments, 835 and 332–359 bp), four isolates of *C. algidicarnis* and their original meat juice samples. The other four isolates and all meat juice/PYGS enrichments positive for *C. algidicarnis* also show similar RFLP patterns to the reference strain of *C. algidicarnis* (DSM 15099).

A multiplex qPCR to detect and differentiate *C. putrefaciens*/*C. algidicarnis* was also developed in this study (Section 2.6.2), validating with the same samples and controls as for the RFLP method. Primer pair TM-F & Cl642-R in combination with probe Cpal168-Cy5 was specific solely for *C. putrefaciens* and *C. algidicarnis*. Primer pair Cpal1423-F & Cpal-ITS-R was combined with two probes. The first probe, Cput-ITS-Hex, was specific exclusively for *C. putrefaciens*. The second probe, Cal-ITS-Fam, was designed for *C. algidicarnis*. It was not specific for *C. putrefaciens* but was able to bind slightly to the ITS sequences of other cold-tolerant clostridia such as *C. bowmanii*, *C. frigoriphilum*, *C. frigoris*, *C. lacusfryxellense* and *C. tagluense*. Therefore, results of probe Cal-ITS-Fam had to be interpreted only as long as probe Cpal168-Cy5 showed a positive signal.

Table 3 summarises the results of all samples tested for cold-tolerant clostridia and all methods used. Analogous to the results obtained by RFLP, 13 meat juice samples, two PYGS enrichments and all of the eight pure isolates were found to be positive for *C. algidicarnis* by this multiplex qPCR.

3.3. Relation between the number of cold-tolerant clostridia and spoilage appearance

Serial dilutions from 10^8 to 10^1 cells/ml (meat juice) of reference strains *C. estertheticum* subsp. *estertheticum* (DSM 8809) and *C. algidicarnis* (DSM 15099) were used to quantify cold-tolerant clostridia in all positive meat juice samples. The Ct value of 10^8 cells/ml meat juice was 19.3 (± 0.2) cycles for *C. estertheticum* subsp. *estertheticum* and 21.8 (± 0.3) cycles for *C. algidicarnis*, respectively. However, the limit of quantification of both species was 10^2 cells/ml and the limit of detection was 10^1 cells/ml meat juice.

Fig. S2 shows the relation between the number of cold-tolerant clostridia ($\log_{10}$ cells/ml meat juice) and the given blown pack spoilage score (BPS score) of all meat samples and is provided as supplementary data. Two samples positive for *C. estertheticum* (7.0–8.0 $\log_{10}$ cfu/ml) showed a high gas production with BPS scores of 4 and 5, respectively. Samples positive only for *C. frigoriphilum* with 7.0–8.0 $\log_{10}$ ($n = 10$) had no correlation to their gas production, since their BPS scores ranged from 2 to 5. All samples positive only for *C. algidicarnis* (containing 5.0–8.8 $\log_{10}$) showed no or only little gas production with BPS scores from 0 to 3. The co-contamination of *C. algidicarnis* and *C. frigoriphilum* ($n = 5$) showed no synergy effect, since the BPS score of those samples ranged from 0 to 4, similar to the BPS scores obtained from samples contaminated only with *C. frigoriphilum*. Two other samples positive for *C. tagluense* and *C. lacusfryxellense*/*C. frigoris* showed BPS scores of 2 and 4, respectively. Even though 12 of the samples that were negative for cold-tolerant clostridia had a BPS score of 0, the remaining 16 samples all showed signs of gas production and reached BPS scores of up to 3. Of 41 samples that showed gas production, 26 samples reached their final BPS score within the first five weeks of storage. Only 15 samples showed ongoing gas production in the following three weeks.

Table 4 shows further spoilage appearances of all 60 meat samples. The investigation was performed qualitatively by observation of two investigators. The loss of meat juice was classified as none/very little or a large amount. The visible signs of spoilage of the meat itself were changes of colour towards green and changes in consistency like softening of the meat. In terms of the odour of the meat juices, it occurred in three categories: no or very slight change, repellent & sour smell and severe acidic & cheesy odour.

Twenty-seven out of 32 positive samples and 25 out of 28 negative samples, showed an extensive amount of meat juice loss after eight weeks of storage time. Only few positive and negative samples ($n = 3$ and $n = 2$, respectively) showed changes in colour or consistency of the meat. Most negative meat juices ($n = 26/28$) had no change in odour. Among 32 positive samples, 12 meat juices had a conspicuously acidic and cheesy smell, seven samples showed a repellent and sour smell, whereas the other 13 meat juices had no change in odour.

3.4. Distribution of cold-tolerant clostridia in abattoirs and cutting plants in Germany

According to the information on the package of each beef sample, it could be traced back to the country/location of each production step, from the country where the calves were raised to the states of Germany where the cattle were slaughtered and where their meat was processed for cutting and packaging. Table 1 (see Section 2.1.1) shows the number of meat samples positive for cold-tolerant clostridia and their production origins. All 60 samples were processed in at least 28 different abattoirs and 33 different cutting plants. Positive samples were processed in 16 different abattoirs (13 in Germany, two in Austria and one in the Netherlands) and 20 different cutting plants. For 10 samples, the exact abattoir and cutting plant were unknown.

Different species such as *C. frigoriphilum* and *C. algidicarnis* were found in meat samples processed in abattoirs and cutting plants all over Germany. There were three abattoirs and four cutting plants where at least two cold-tolerant *Clostridium* spp. were found in their meat samples.

4. Discussion

A total of 60 vacuum-packed beef samples, purchased in retail in Greater Munich, were investigated in this study in order to determine the prevalence of cold-tolerant clostridia in beef and their corresponding production plants in Germany. At the day of purchase, all samples were within their shelf-life span (three days to two weeks before use-by date)

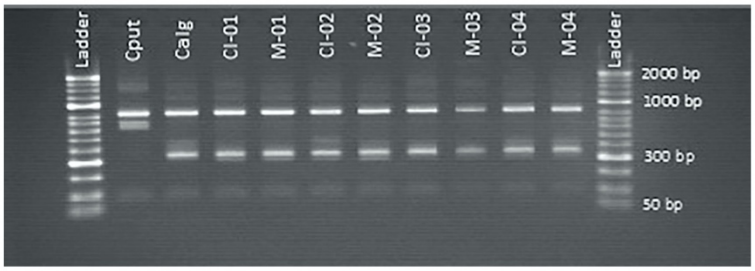

Fig. 1. RFLP patterns using primer pair Cpal168-F & Cpal-ITS-R and endonuclease HhaI/CfoI (see Section 2.6.1). Shown are patterns from *C. putrefaciens* (Cput, DSM 1291), *C. algidicarnis* (Calg, DSM 15099), four isolates of *C. algidicarnis* (Cl-01 – Cl-04) and their original meat juice samples (M-01 – M-04).

Table 4
Possible signs of spoilage observed in the packs and their relationship to gas production during storage at 4 °C for 8 weeks.

Samples positive for	Total number of samples	Number of samples (BPS scores[a])						
		Loss of meat juice		Change of colour & consistency		Change of odour		
		None/little	High amount	No	Yes	None/slightly	Repellent & sour	Acidic & cheesy
C. algidicarnis	7	–	7 (0–3)	7 (0–3)	–	2 (0; 2)	1 (3)	4 (0–3)
C. algidicarnis (PYGS enrichment)	2	–	2 (2; 3)	2 (2; 3)	–	1 (3)	–	1 (2)
C. estertheticum	1	–	1 (4)	–	1 (4)	–	–	1 (4)
C. estertheticum & *C. algidicarnis*	1	–	1 (5)	1 (5)	–	–	–	1 (5)
C. frigoriphilum (>6.0 $\log_{10}$)	10	2 (2; 4)	8 (3–5)	10 (2–5)	–	4 (2–4)	4 (3–4)	2 (4; 5)
C. frigoriphilum (<3.0 $\log_{10}$)	4	1 (0)	3 (0–2)	3 (0–2)	1 (0)	4 (0–2)	–	–
C. frigoriphilum & *C. algidicarnis*	5	1 (0)	4 (2–4)	4 (2–4)	1 (0)	2 (2; 3)	1 (0)	2 (3; 4)
C. lacusfryxellense/*C. frigoris*	1	1 (4)	–	1 (4)	–	–	–	1 (4)
C. tagluense	1	–	1 (2)	1 (2)	–	–	1 (2)	–
Negative samples	28	3 (0–1)	25 (0–3)	26	2 (0; 2)	26 (0–3)	2 (2; 3)	–
Total	60	8	52	55	5	39	9	12

[a] Blown Pack Spoilage (BPS) scores (Boerema et al., 2007): score 0, no gas production; score 1, small bubbles in drip, package intact; score 2, loss of vacuum through gas production of present bacteria; score 3, blown, puffy packs; score 4, fully distended, without tightly stretching the pack; score 5, overblown, tightly stretched packs/packs leaking.

and did not show any signs of spoilage, thus may have contained only a low level of contaminating cold-tolerant clostridia, if present at all. Therefore, to enhance the possibility of detecting the target bacterium, all beef samples were stored (incubated) at 4 °C for eight weeks before they were processed for investigation.

The methods primarily used for detection and identification of *Clostridium* species in meat juice samples were based on the multiplex qPCR described by Dorn-In et al. (2018) and the sequence analysis of the 16S rRNA gene. The efficiency of the multiplex qPCR in this study was similar to the efficiency of the multiplex qPCR developed by Dorn-In et al. (2018), since the difference in Ct values of 10^8 cells/ml of both multiplex qPCRs was only 0.3 PCR cycles. For species identification it was not vital to include the probe detecting eight different cold-tolerant *Clostridium* spp. in the multiplex qPCR, since samples that were positive using the SYBR green qPCR and could not be clearly identified using the multiplex qPCR were further processed for sequencing. Therefore, this probe could have been omitted since no further information was gained by its results. Additionally, MALDI-TOF MS was applied to pure cultures of isolates. A total of 32 (53%) meat samples tested in this study were positive for cold-tolerant clostridia. Almost all clostridia present in meat juices could be identified down to species level and were revealed to be *C. frigoriphilum* ($n = 19$), *C. estertheticum* ($n = 2$) and *C. tagluense* (n = 1). However, a significant number of samples ($n = 8$ and n = 2 for meat juice and PYGS enrichment, respectively) were found to be positive for *C. putrefaciens*/*C. algidicarnis* by sequencing of the 16S rRNA gene. Further identification was not possible without culturing of isolates.

Since the culturing of cold-tolerant clostridia is labour-intensive, two independent methods (multiplex qPCR and RFLP) were additionally developed in this study in order to differentiate between *C. putrefaciens* and *C. algidicarnis* directly from meat juice samples. As probe Cal-ITS-Fam, used to detect *C. algidicarnis* in the newly developed multiplex qPCR, can slightly bind to some other cold-tolerant *Clostridium* spp. (but not *C. putrefaciens*), the results of this probe should only be interpreted as long as probe Cpal168-Cy5 shows a positive signal. Both newly developed methods (multiplex qPCR and RFLP) showed identical results and could be applied independently from each other to detect and to differentiate between *C. putrefaciens* and *C. algidicarnis* directly from meat juice. However, the developed multiplex qPCR is more practical, since the method is less labour-intensive than the RFLP method. By using the two multiplex qPCR methods (as described by Dorn-In et al. (2018) and the one developed in this study) and additional confirmation by the developed RFLP method, it was revealed that five samples contaminated with *C. frigoriphilum* and one sample with *C. estertheticum* were also contaminated with *C. algidicarnis*. Thus, a total of 25% ($n = 15/60$) of all meat samples were identified to be contaminated with *C. algidicarnis*. None of the investigated beef samples were found to be positive for *C. putrefaciens*.

The first isolate of *C. algidicarnis* (DSM 15099) was originally found in vacuum-packed cooked pork in the United Kingdom (Lawson et al., 1994). The species *C. putrefaciens* (DSM 1291) was first isolated from sour ham and was mistakenly identified as a *Bacillus* species by McBryde (1911). It was later reclassified as *C. putrefaciens* by Sturges and Drake (1927). Both species have been found as a cause of deep tissue spoilage (bone taint) in lamb legs, pork and dry cured ham (Boyer, 1926; Broda et al., 1996b). In multiple studies examining lamb legs, *C. algidicarnis* could be detected in swabs of the joint, but not in external swabs or meat juice, suggesting that this species may proliferate in deep tissues and may already be present in the joint at slaughter (Boerema et al., 2002; Broda et al., 1996b). Broda et al. (2009) detected *C. algidicarnis* mainly in swabs taken from the boning room and significantly less in faeces and farm samples, which may indicate that the environment of abattoirs is being contaminated after dissection of the carcass and that external areas of the slaughter animal, such as fleece and faeces, may not play such a relevant role as a source of contamination for this species. *C. algidicarnis* was also found in beef samples with signs of BPS (Cavill et al., 2011; Hernández-Macedo et al., 2012; Silva et al., 2011). In Germany and in Central Europe, this study is the first report to detect and identify the species *C. algidicarnis* in vacuum-packed beef.

C. estertheticum has often been found to be the most common causative agent of Blown Pack Spoilage (BPS) in vacuum-packed beef (Adam et al., 2010). However, among the *Clostridium* spp. found in this study, only 6% ($n = 2/32$) thereof were *C. estertheticum*, whereas the species *C. frigoriphilum* and *C. algidicarnis* were more frequently found in this study, namely in 59% ($n = 19/32$) and 47% ($n = 15/32$) of all positive samples, respectively. However, as mentioned above, the samples of this study were "vacuum-packed, chilled beef", stored for eight weeks. Therefore, the results of this study represent the incidence of cold-tolerant clostridia in vacuum-packed, chilled and fresh beef marketed in retail, but not in samples with signs of BPS.

In this study, all investigated samples were different cuts of non-marinated beef. Therefore, we cannot make a statement on the prevalence of the detected species in other types of meat or in farm and slaughterhouse environments. However, our results are similar to the results obtained by Brightwell and Horváth (2018), where the majority of *C. algidcarnis*-like isolates were obtained from bovine and ovine sources, while other *Clostridium* spp. (e. g. *C. gasigenes* and *C. tagluense*-like) were mainly found in venison carcasses and their processing environments or in lamb. Focussing on the nature of spoilage associated with different cold-tolerant *Clostridium* spp. could be of interest in future studies.

Among cold-tolerant clostridia, *C. estertheticum* and *C. gasigenes* are the species that primarily lead to BPS by producing an extensive amount of gas (Collins et al., 1992; Broda et al., 2000a). Similar to the results of other research, the two samples positive for *C. estertheticum* in this study consistently showed a high gas production. Although the number of *C. algidicarnis* found in beef samples was moderate to very high, their BPS scores only ranged from 0 to 3, which is similar to the samples negative for *Clostridium* spp. There was no clear correlation between the level of gas production and the amount of *C. frigoriphilum*, which was also previously reported by Dorn-In et al. (2018). Some other *Clostridium* spp., such as *C. bowmanii* (Moschonas et al., 2010) and *C. frigidicarnis* (Broda et al., 1999), can also cause spoilage of meat, with, however, only little or no signs of BPS.

Sixteen of 28 samples negative for cold-tolerant clostridia showed signs of gas production and reached BPS scores of up to 3. These findings support the conclusion that other cold-tolerant anaerobes such as *Enterobacteriaceae* and lactic acid producing bacteria are able to cause a moderate blown pack spoilage (Brightwell et al., 2007; Hernández-Macedo et al., 2012). Regarding the loss of meat juice, it was observed that all beef samples contaminated with *C. estertheticum* and *C. algidicarnis* lost a high amount of meat juice. However, the amounts of drip loss could not be related to the presence of cold-tolerant clostridia, since 89% of negative samples and 84% of positive samples lost an extensive amount of meat juice during the time of storage. These results support the conclusion that loss of meat juice is increased by the use of vacuum packaging and might not be related to a certain group of bacteria (Schluter et al., 1994; Doherty et al., 1996; Payne et al., 1998). The changes in colour and consistency of the meat were also not related to the presence of cold-tolerant clostridia. In terms of odour, a significant difference between samples negative and samples positive for cold-tolerant clostridia was observed in this study. Whereas 93% of negative samples had no change in odour, 59% of positive samples smelled noticeably off. The smell, regularly described as cheesy, is often associated with cold-tolerant clostridia and stems from butanol, butyl esters and butyric acid being volatile compounds of gas produced by these bacteria (Broda et al., 1996a; Dainty et al., 1989), while the acidic and sour smell is mostly caused by lactic acid producing bacteria (Jääskeläinen et al., 2012; Casaburi et al., 2015).

The 60 vacuum-packed beef samples examined in this study were processed in at least 28 different abattoirs and 33 cutting plants all over Germany with a majority being processed in the Federal State of Bavaria (see Fig. S1, supplementary data). By including this many different production plants located all over the country, the obtained results can be considered a good representation of the prevalence of cold-tolerant clostridia in vacuum-packed beef produced and processed within Germany. In this study, 16 abattoirs (57%) and 20 cutting plants (61%) were classified as positive, since at least one beef sample thereof was positive for at least one cold-tolerant *Clostridium* spp. It has to be considered that all investigated beef samples were not sampled in the abattoir or cutting plant but were purchased in retail. The results regarding abattoirs and cutting plants positive for cold-tolerant clostridia were obtained by trace back using the information on the package. Therefore, it cannot be definitively concluded if positive samples were contaminated while being processed in the abattoir, the cutting plant, or potentially even in both locations.

5. Conclusion

A total of 32 (53%) from 60 investigated vacuum-packed beef samples purchased in retail were identified to be positive for various cold-tolerant *Clostridium* spp., such as *C. frigoriphilum*, *C. algidicarnis*, *C. estertheticum* and *C. tagluense*. Positive beef samples were originally processed in 16 different abattoirs and 20 different cutting plants. The results obtained in this study prove the high incidence of these bacteria in vacuum-packed beef and in their processing plants in Germany. This is the first report of the detection and identification of *C. algidicarnis* in beef samples in Germany and Central Europe. Additionally, the multiplex qPCR as well as the RFLP method developed in this study allow a precise differentiation between the very closely related species *C. putrefaciens* and *C. algidicarnis* and can be directly applied with DNA extracts of meat juice samples.

In conclusion, to obtain a fast result of contamination with cold-tolerant clostridia, we recommend the DNA extraction directly from meat juice of suspicious vacuum-packed meat. After that, the SYBR green qPCR for *Clostridium* spp. should first be performed. In case of positive results, the multiplex qPCR by Dorn-In et al. (2018) and the multiplex qPCR developed in this study should be carried out respectively. If clear species identification cannot be achieved that way, sequencing of the 16S rRNA gene from SYBR qPCR can be further applied. However, if meat samples are contaminated with at least two cold-tolerant *Clostridium* spp., sequencing may be difficult due to mixed sequences of the 16S rRNA gene. Therefore, the development of species specific PCRs for other cold-tolerant *Clostridium* spp. are required.

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Declaration of competing interest

None.

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Table S1: List of bacterial species used for validation of the specificity and sensitivity of the developed RFLP method and the developed multiplex qPCR for *C. putrefaciens* and *C. algidicarnis*

No.	Species	Source[a]
Psychrophilic and psychrotolerant clostridia		
1	*Clostridium acidisoli*	DSM 12555
2	*Clostridium akagii*	DSM 12554
3	*Clostridium algidicarnis*	DSM 15099
4	*Clostridium algidixylanolyticum*	DSM 12273
5	*Clostridium algoriphilum*	DSM 16153
6	*Clostridium bowmanii*	DSM 14206
7	*Clostridium estertheticum* subsp. *estertheticum*	DSM 8809
8	*Clostridium estertheticum* subsp. *laramiense*	DSM 14864
9	*Clostridium fimetarium*	DSM 9179
10	*Clostridium frigidicarnis*	DSM 12271
11	*Clostridium* sp. (*frigoriphilum*)	DSM 17811
12	*Clostridium frigoris*	DSM 14204
13	*Clostridium gasigenes*	DSM 12272
14	*Clostridium homopropionicum*	DSM 5847
15	*Clostridium hydroxybenzoicum*	DSM 7310
16	*Clostridium jejuense*	DSM 15929
17	*Clostridium lacusfryxellense*	DSM 14205
18	*Clostridium psychrophilum*	DSM 14207
19	*Clostridium puniceum*	DSM 2619
20	*Clostridium putrefaciens*	DSM 1291
21	*Clostridium schirmacherense*	DSM 17394
22	*Clostridium tagluense*	DSM 17763
23	*Clostridium uliginosum*	DSM 12992
24	*Clostridium vincentii*	DSM 10228
Mesophilic clostridia		
25	*Clostridium butyricum*	DSM 10702
26	*Clostridium pascui*	DSM 10364
27	*Clostridium perfringens*	DSM 756
28	*Clostridium sporogenes*	DSM 1664
Enterobacteriaceae		
29	*Citrobacter freundii*	LMU
30	*Escherichia coli*	LMU
31	*Hafnia alvei*	LMU
32	*Klebsiella pneumoniae*	LMU
33	*Morganella morganii*	LMU
34	*Pectobacterium carotovorum*	LMU
35	*Proteus mirabilis*	LMU
36	*Salmonella enteritidis*	LMU
37	*Serratia proteamaculans*	LMU
38	*Yersinia intermedia*	LMU

Lactobacillales		
39	*Carnobacterium maltaromaticum*	LMU
40	*Enterococcus faecalis*	LMU
41	*Lactobacillus agilis*	LMU
42	*Lactobacillus fuchuensis*	LMU
43	*Lactococcus lactis*	LMU
44	*Lactococcus vividescens*	LMU
45	*Leuconostoc citreum*	LMU
46	*Leuconostoc lactis*	LMU
47	*Streptococcus agalactiae*	LMU
48	*Streptococcus infantis*	LMU

[a] Species with DSM numbers were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The other species were isolated from meat samples and were identified by MALDI-TOF MS in-house, at the Chair of Food Safety, Ludwig-Maximilians-University (LMU) of Munich, Germany.

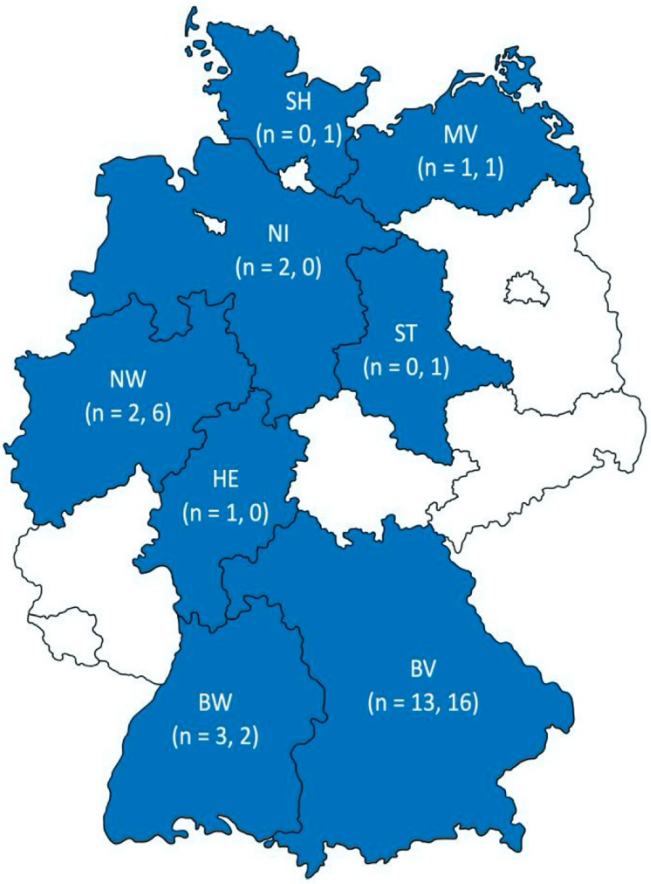

Fig. S1: The Federal Republic of Germany and the number (n) of abattoirs and cutting plants, respectively, where meat samples were originally produced. BV = Bavaria, BW = Baden-Württemberg, HE = Hessen, MV = Mecklenburg-Vorpommern, NI = Lower Saxony, NW = North Rhine-Westphalia, SH = Schleswig-Holstein, ST = Saxony-Anhalt

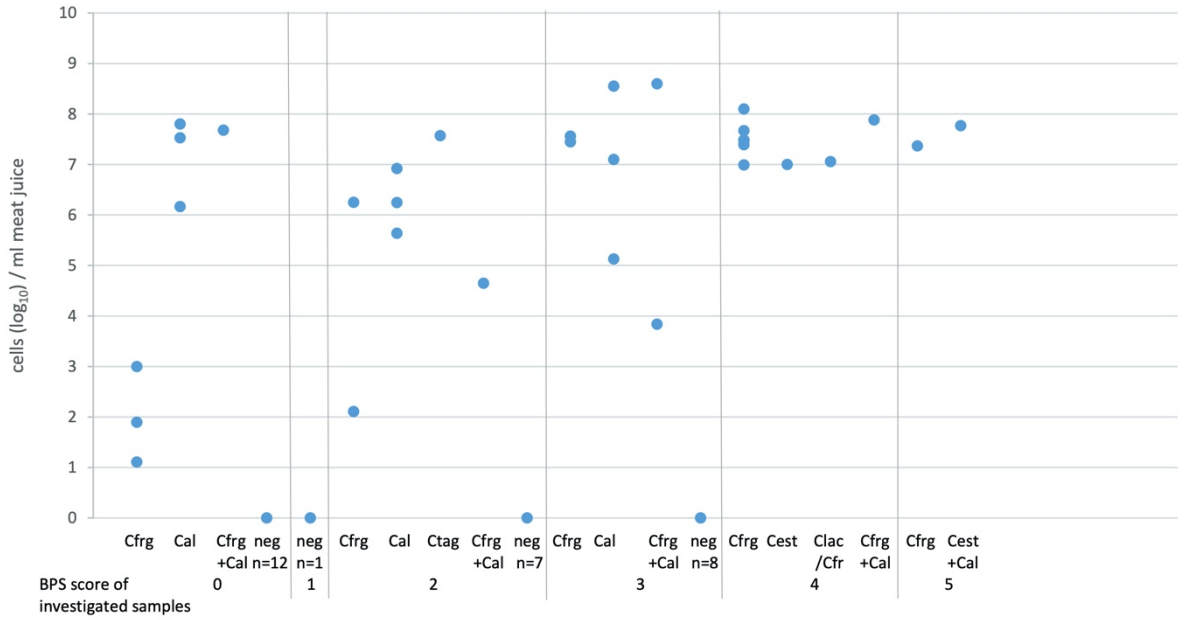

Fig. S2: Distribution of samples according to their Blown Pack Spoilage (BPS) scores in relation to the amount of psychrophilic clostridia found in the meat juice. Abbreviations: Cfrg = *C. frigoriphilum*, Cal = *C. algidicarnis*, Ctag = *C. tagluense*, Clac = *C. lacusfryxellense*, Cfr = *C. frigoris*, Cest = *C. estertheticum*, neg = negative.
BPS scores: score 0, no gas production; score 1, small bubbles in drip, package intact; score 2, loss of vacuum through gas production of present bacteria; score 3, blown, puffy packs; score 4, fully distended, without tightly stretching the pack; score 5, overblown, tightly stretched packs/packs leaking

4 Discussion

4.1 Beef Samples from German Retail

In this study, 60 vacuum-packed beef samples purchased in German retail were tested for the occurrence of cold-tolerant clostridia in their meat juice. At the day of purchase, all samples were within their shelf-life span and did not show any signs of spoilage. All beef samples were stored at 4 °C for a further eight weeks after purchase in order to increase the chance of detecting a possible contamination with cold-tolerant clostridia. After storage, the meat was investigated for its spoilage appearance (BPS, odour, loss of meat juice, colour and consistency). Meat juice was taken and investigated for the presence of cold-tolerant clostridia.

Until this day, there is very little information about the incidence of cold-tolerant clostridia in German processing plants and in meat sold in Germany. The few studies that were conducted regarding this issue concentrated on *C. estertheticum* and *C. estertheticum*-like organisms, further species identification was not carried out (Böhm, 2013; Bonke et al., 2016, Eckardt, 2015; Ziegler, 2009). The study by Dorn-In et al. (2018) was the first study that identified other cold-tolerant clostridia than *C. estertheticum*, isolated from meat juice, skin or faeces samples from Germany, down to species level.

To obtain more information about the prevalence and the different species of cold-tolerant clostridia in processing plants and meat from Germany, samples investigated in this study were chosen on the condition that at least one of the production steps had taken place in Germany. For 50 samples, all steps from birth to cutting were conducted in Germany.

4.2 Development of Detection Methods

As mentioned in literature Section 2.2.1, clostridia that are able to grow at cold temperatures, can be psychrophilic or psychrotrophic *Clostridium* species. In this work, the term “cold-tolerant clostridia” includes all of these species.

The detection method of cold-tolerant clostridia in this work was primarily based on the qPCR developed by Dorn-In et al. (2018). After DNA extraction of meat drip, samples were tested using a SYBR green qPCR detecting the 16S rRNA gene of almost all *Clostridium* spp.. Samples that tested positive were further tested using the multiplex qPCR with probes being specific

for *C. estertheticum*, *C. frigoriphilum*, *C. bowmanii* and *C. tagluense*-like (Dorn-In et al., 2018). PCR products of samples that could not be identified this way were purified and sent off for sequencing of the 16S rRNA gene. For meat drip samples that tested negative in the SYBR green qPCR, a DNA extraction of their PYGS enrichment was carried out and the detection process was repeated as described above. Only once the PYGS enrichment tested negative, samples were finally classed as negative.

However, following this process allowed a definite species identification of only 22 out of 32 positive samples. Sequencing results showed that nine samples were positive for *C. algidicarnis*/*C. putrefaciens* and one sample was positive for *C. frigoris*/*C. lacusfryxellense*. To this day, further differentiation of those species directly from meat juice using molecular biological methods was not possible, since their 16S rRNA genes are highly similar (Broda et al., 2002; Broda et al., 2000b). Exact differentiation of those species using biochemical and morphological analysis of isolates can be difficult and further method development is needed to simplify identification. This is a conclusion that led to the second objective of the study: To develop a practical method to differentiate between *C. putrefaciens* and *C. algidicarnis* using molecular biological methods, namely RFLP and multiplex qPCR. For this purpose, samples contaminated with *C. algidicarnis*/*C. putrefaciens* were submitted to culturing in order to obtain pure isolates for the validation of the developed methods. As a result, eight pure cultures of *C. algidicarnis* were obtained and species identification was confirmed using MALDI-TOF MS.

As mentioned above, the 16S rRNA genes of both species are very similar, thus the newly developed primers and probes are a combination of the 16S rRNA gene and the Internal Transcribes Spacer (ITS). The ITS region is located between the 16S rRNA and the 23S rRNA region and is known as a non-conserved sequence. Therefore, it is often used for species and intra-species differentiation of bacteria (Barry et al., 1991; Scheinert et al., 1996). Both newly developed differentiation methods (RFLP and multiplex qPCR) show a sensitivity and specificity of 100%, tested with 48 pure cultures of different bacterial species including reference strains of *C. algidicarnis* (DSM 15099) and *C. putrefaciens* (DSM 1291), eight filed isolates of *C. algidicarnis* that were previously identified using MALDI-TOF MS and all 60 vacuum-packed beef samples. They allow a clear identification and differentiation of both species and can be independently applied. Comparing the two, the multiplex qPCR represents the more efficient method, since it is certainly less time-intensive than the RFLP method.

Additionally, the multiplex qPCR can be used for quantification of target species in the meat juice samples, as performed in this study. However, the RFLP method can serve as a practical alternative if available equipment does not allow for implementation of the multiplex qPCR.

By using the multiplex qPCR to differentiate between *C. algidicarnis* and *C. putrefaciens*, an interpretation concept has to follow, since it was not possible to design a probe detecting solely *C. algidicarnis*. Probe Cal-ITS-Fam does not detect *C. putrefaciens*, but despite *C. algidicarnis* it also detects some other cold-tolerant *Clostridium* species, such as *C. frigoriphilum* and *C. tagluense*, however, with significantly less sensitivity. This problem was solved by including probe Cpal168-Cy5 combined with primer pair TM-F & Cl642-R into the method. This probe is exclusively specific for *C. algidicarnis* and *C. putrefaciens*. As long as results of probe Cal-ITS-Fam are only evaluated when probe Cpal168-Cy5 shows a positive signal, an exact identification of *C. algidicarnis* is ensured.

During the process of validation of the RFLP method and multiplex qPCR, it was revealed that five beef samples positive for *C. frigoriphilum* and one beef sample positive for *C. estertheticum* were additionally positive for *C. algidicarnis*. By following the process that was used for detection, this co-contamination was initially missed, since the samples were determined as positive for *C. frigoriphilum* and *C. estertheticum* and were not further tested.

Although the multiplex qPCR described by Dorn-In et al. (2018) and the one developed in this study are a useful and rapid method for the identification of cold-tolerant clostridia, the application of the SYBR PCR, which is specific for almost all clostridia, may be even more effective as a screening method. If the aim is to investigate vacuum-packed beef stored at refrigerated temperatures for a contamination with any *Clostridium* spp., the SYBR PCR provides useful results and is a good starting point for further investigations, if required. Sequencing of the 16S rRNA gene can be a practical method for species identification. Yet a co-contamination of several species, as found in this study, could lead to incorrect or unclear results. The close relation between certain cold-tolerant *Clostridium* spp. is another issue that can result in unspecific species identification when using sequencing methods. Species-specific multiplex qPCRs are a rapid and reliable way to identify the cold-tolerant *Clostridium* spp. down to species level. However, more probes specific for further species such as *C. frigoris*, *C. lacusfryxellense* and *C. tagluense* detected in this study need to be developed.

4.3 Detected Species

By using the sequence analysis of the 16S rRNA gene, the multiplex qPCR described by Dorn-In et al. (2018) and the multiplex qPCR and RFLP method developed in this study, 32 beef samples were found to be positive for the following cold-tolerant *Clostridium* species: *C. frigoriphilum* (n = 14), *C. algidicarnis* (n = 9), *C. estertheticum* (n = 1), *C. tagluense* (n = 1), *C. lacusfryxellense*/*C. frigoris* (n = 1), *C. frigoriphilum* & *C. algidicarnis* (n = 5), *C. estertheticum* & *C. algidicarnis* (n = 1).

C. estertheticum and *C. gasigenes* are generally believed to be the most important cold-tolerant *Clostridium* species leading to meat spoilage. In former studies, they were the main species isolated from meat with signs of blown pack spoilage or from environmental samples from the abattoir area (Bolton et al., 2015; Byrne et al., 2009; Moschonas et al., 2009). In most studies, however, the molecular methods used for detection were specific for those two species, which might be an explanation as to why those species are overrepresented. Additionally, the primer pair developed by Broda et al. (2003a) for the detection of *C. estertheticum* was later found to also detect so-called *C. estertheticum*-like species, such as *C. bowmanii* and *C. frigoriphilum*, so samples might have been wrongly concluded as positive for *C. estertheticum*.

The results of the presented study show that other species than *C. estertheticum* and *C. gasigenes* can be of high significance regarding the spoilage of vacuum-packed beef. *C. frigoriphilum* has rarely been detected in former studies but was found in 19 of 32 positive samples in this study. These results are in accordance with the results obtained by Dorn-In et al. (2018), where *C. frigoriphilum* was detected in 8 of 20 vacuum-packed beef samples with signs of blown pack spoilage, collected in Germany. Concluding from the results of these two studies, it appears that the prevalence of *C. frigoriphilum* in Germany is significantly higher than the prevalence of *C. estertheticum*.

C. algidicarnis was found to be the cause of bone taint, as well as spoilage of vacuum-packed meat. Cases have been reported from several countries such as Brazil, the UK and New Zealand (Boerema et al., 2002; Cavill et al., 2011; De Lacy et al., 1998; Silva et al., 2011). The presented study is the first report of *C. algidicarnis* in Germany and the mainland of Europe. Although Dorn-In et al. (2018) applied similar methods (SYBR qPCR and sequencing), none of their investigated samples were positive for *C. algidicarnis*. However, many vacuum-packed meat samples investigated in the study of Dorn-In et al. (2018) were suspicious samples

obtained from meat processing plants, which occasionally had problems with blown pack spoilage, while the samples in this study were fresh meat processed in various processing plants. In 2018, when the study of Dorn-In et al. was conducted, a multiplex qPCR for the detection of *C. algidicarnis* was not yet available so the incidence of this species was not inclusively investigated. Additionally, unlike *C. estertheticum*, *C. algidicarnis* does not produce much gas (see Section 3, Table 4 of the Publication and Section 4.4). Thus, the spoilage of meat caused by this bacterium may be suspected to be caused by other spoilage microorganisms such as lactic acid producing bacteria and/or *Enterobacteriaceae*.

4.4 Cold-tolerant Clostridia and Signs of Spoilage

As mentioned in the publication (see Section 3), loss of meat drip or change of consistency cannot be used as a reliable indicator of meat being spoiled by cold-tolerant clostridia. However, a change of odour to acidic and cheesy may imply a contamination with these bacteria.

The typical spoilage appearance, so-called Blown Pack Spoilage, results from the immense gas production that is observed in samples contaminated with *C. estertheticum*. The detected amounts of *C. algidicarnis* in beef samples stored for 8 weeks were quite high (quantified by qPCR) and, therefore, may very well be the cause of spoilage. However, the production of gas was rather similar to samples negative for cold-tolerant clostridia. Since the spoilage appearance caused by this species does not differ from the spoilage appearance caused by other bacteria, the samples may be falsely considered as not contaminated with clostridia and the investigation may be targeted at lactic acid producing bacteria or *Enterobacteriaceae*. This could lead to the application of unspecific hygienic and disinfection measures for the processing plants or meat industries. A common cleaning process applied for other spoilage microorganisms such as *Pseudomonas*, *Enterobacteriaceae* and lactic acid producing bacteria may not be effective against endospores of clostridia, since they are strongly resistant against a wide range of disinfecting agents and high temperatures (Böhm, 2013; Broda, 2007).

Similar to the results obtained by Dorn-In et al. (2018), high amounts of *C. frigoriphilum* found in beef samples in this study could not be correlated to the level of gas production. This may be partly due to other spoilage microorganisms present in meat samples, such as LAB and/or *Enterobacteriaceae*. Furthermore, meat or muscle type, as well as other components in meat

such as fat, may influence the level of gas production. Since this parameter was not included in the analysis, it may be an interesting matter for further studies.

4.5 Distribution of Abattoirs and Cutting Plants where Positive Samples were Processed

To obtain representative results, the chosen samples were processed in a large number of abattoirs and cutting plants, namely 28 and 33, respectively. From those, 22 abattoirs and 27 cutting plants were located in Germany. The origins of the samples were traced back using the given information on the packaging. Since all samples were purchased in supermarkets and butchers in Greater Munich, the majority of abattoirs and cutting plants (13/22 and 16/27, respectively) were located in the state of Bavaria. Positive samples were processed in 16 abattoirs and 20 cutting plants all over Germany, as well as in Austria, the Netherlands and Ireland. Evaluating the obtained results, there is no noticeable difference of the prevalence of cold-tolerant clostridia in the different federal states of Germany. Regarding the distribution of different species, *C. frigoriphilum* and *C. algidicarnis* – the two main species detected in positive samples – were found in samples processed throughout Germany. This goes to show, that in a significant amount of meat processing plants a contamination with cold-tolerant clostridia seems to be an issue and that this problem is not a local occurrence. These results are in accordance with the results obtained by Eckardt (2015), where in all three investigated German meat-processing plants spores of various cold-tolerant *Clostridium* species were found. However, it has to be mentioned that from the available information about the origin, it cannot be concluded if contamination of the sample happened in the abattoir, the cutting plant or potentially even in both locations. Nevertheless, the results of this study show that more than half of the abattoirs and cutting plants where investigated samples were processed are potentially contaminated with cold-tolerant clostridia. Further investigations to see whether cold-tolerant clostridia can be found in abattoirs and processing plants and whether they can cause a contamination between batches, could therefore be of interest. The information obtained could be useful for meat processing plants in order to develop effective hygienic concepts, disinfection and cleaning methods against endospores of cold-tolerant clostridia and, thus, reduce the possibility of contamination.

5 Summary

Psychrotolerant (cold-tolerant) *Clostridium* spp. are known to play a crucial part in the spoilage of vacuum-packed, chilled beef, leading to immense economic losses within the meat industry. Cases have been reported from countries all over the world. However, until now there were only a few reports about the incidence of these bacteria in vacuum-packed beef from German retail and about the origins of the meat. Therefore, the presented study was targeted at investigating the prevalence of cold-tolerant clostridia in German abattoirs and cutting plants.

A total of 60 beef samples were purchased in supermarkets and at butchers in Greater Munich. To enhance the possibility of detecting a potential contamination, samples were stored at 4 °C for eight weeks after purchase. Subsequently, samples were tested for the occurrence of cold-tolerant clostridia using culturing methods, SYBR green/multiplex qPCR (Dorn-In et al., 2018), MALDI-TOF MS and sequencing. It was not possible to differentiate *C. algidicarnis* from *C. putrefaciens* using sequencing analysis since their 16S rRNA genes are highly similar. Therefore, a RFLP method and a multiplex qPCR to differentiate the two species were additionally developed in this study. Both methods show a high specificity and sensitivity and, therefore, allow for a precise differentiation between *C. algidicarnis* and *C. putrefaciens*. As a result of the investigations, 32 out of 60 samples proved to be positive for the following species: C. *frigoriphilum* (n = 14), *C. algidicarnis* (n = 9), *C. estertheticum* (n = 1), *C. tagluense* (n = 1), *C. lacusfryxellense*/*C. frigoris* (n = 1), *C. frigoriphilum* & *C. algidicarnis* (n = 5), *C. estertheticum* & *C. algidicarnis* (n = 1). This study is the first report of the occurrence of *C. algidicarnis* in Germany and the whole of mainland Europe.

The typical spoilage appearance associated with cold-tolerant clostridia, so-called blown pack spoilage, was observed in samples contaminated with *C. estertheticum*, whereas high amounts of *C. frigoriphilum* could not be related to immense gas production and consequent swelling of the package. Likewise, *C. algidicarnis* produced only small amounts of gas, although present in high amounts in meat samples. Further spoilage appearances, such as loss of meat drip and changes in colour or consistency of the meat, could neither be used as a reliable indicator for spoilage by cold-tolerant clostridia, while a change of odour towards repellent, acidic and cheesy may indicate a contamination with these bacteria.

According to the information on the package, the 32 samples that were contaminated with cold-tolerant clostridia were processed in 16 different abattoirs and 20 different cutting plants, mainly located in Germany. Considering the results of this study, the prevalence of cold-tolerant clostridia in vacuum-packed beef processed in Germany is very high. Since the contamination of meat is likely to happen during the process of slaughtering or cutting, it can, therefore, be concluded that a substantial number of abattoirs and cutting plants in Germany are contaminated with vegetative cells or spores of these bacteria.

6 Zusammenfassung

Psychrotolerante *Clostridium* spp. sind dafür bekannt, eine entscheidende Rolle im Verderbsprozess von vakuumverpacktem, gekühltem Rindfleisch zu spielen, was immense wirtschaftliche Verluste in der Fleischindustrie zur Folge hat. Fälle von auf diese Weise verdorbenem Fleisch wurden bereits aus der ganzen Welt gemeldet. Bisher wurde jedoch nur wenig über das Vorkommen dieser Bakterien in vakuumverpacktem Rindfleisch aus dem deutschen Einzelhandel und über die Herkunft des Fleisches berichtet. Ziel der vorliegenden Studie war es daher, die Prävalenz kältetoleranter Clostridien in deutschen Schlachthöfen und Zerlegebetrieben zu untersuchen.

Zu diesem Zweck wurden 60 Rindfleischproben aus Supermärkten und Metzgereien im Großraum München erworben. Um die Chancen auf einen Nachweis einer möglichen Kontamination zu erhöhen, wurden die Proben im Anschluss für weitere 8 Wochen bei 4 °C gelagert. Anschließend wurden die Proben unter Verwendung von Kultivierung, SYBR green/multiplex-qPCR (Dorn-In et al., 2018), MALDI-TOF MS und Sequenzierung auf das Vorkommen kältetoleranter Clostridien untersucht. Die Spezies *C. algidicarnis* und *C. putrefaciens* konnten mittels Sequenzierung nicht eindeutig voneinander unterschieden werden, da die 16S rRNA Sequenzen beider Spezies sehr ähnlich sind. Deshalb wurden in dieser Studie eine RFLP Methode sowie eine multiplex qPCR entwickelt, um beiden Spezies nachweisen und unterscheiden zu können. Beide Methoden zeigen eine hohe Sensitivität und Spezifität und eignen sich daher für eine eindeutige Differenzierung von *C. algidicarnis* und *C. putrefaciens*. Insgesamt 32 der 60 untersuchten Rindfleischproben waren positiv für folgende Spezies: *C. frigoriphilum* (n = 14), *C. algidicarnis* (n = 9), *C. estertheticum* (n = 1), *C. tagluense* (n = 1), *C. lacusfryxellense*/*C. frigoris* (n = 1), *C. frigoriphilum* & *C. algidicarnis* (n = 5), *C. estertheticum* & *C. algidicarnis* (n = 1). Die vorliegende Arbeit dokumentiert erstmalig den Nachweis von *C. algidicarnis* in Deutschland und dem gesamten europäischen Festland.

Das typische, als 'Blown Pack Spoilage' beschriebene Verderbsbild kältetoleranter Clostridien, konnte in mit *C. estertheticum* kontaminierten Proben beobachtet werden. Hohe Gehalte an *C. frigoriphilum* konnten jedoch nicht mit einer starken Gasproduktion und einem damit verbundenen Aufblähen der Verpackung in Zusammenhang gebracht werden. Auch *C. algidicarnis* produzierte lediglich geringe Mengen Gas, obwohl diese Spezies in einigen

Proben in hohen Gehalten nachgewiesen werden konnte. Weitere Verderbsanzeichen, wie der Verlust von Fleischtropfsaft und Veränderungen in Farbe oder Konsistenz des Fleisches, konnten nicht als verlässliche sensorische Indikatoren für den Verderb durch kältetolerante Clostridien identifiziert werden. Veränderungen des Geruchs in Richtung abstoßend, sauer und käsig könnten jedoch auf eine Kontamination mit diesen Bakterien hinweisen.

Die 32 Proben, in denen kältetolerante Clostridien nachgewiesen werden konnten, wurden den Informationen auf der Verpackung zufolge in 16 verschiedenen Schlachthöfen und 20 verschiedenen Zerlegebetrieben verarbeitet, von denen die meisten ihren Standort in Deutschland haben. In Anbetracht der Ergebnisse der vorliegenden Studie kann davon ausgegangen werden, dass die Prävalenz von kältetoleranten Clostridien in vakuumverpacktem Fleisch aus deutschen fleischverarbeitenden Betrieben sehr hoch ist. Da eine Kontamination des Fleisches mit kältetoleranten Clostridien in der Regel im Laufe der Verarbeitungskette stattfindet, lässt sich darauf schließen, dass die Inzidenz von Sporen und/oder vegetativen Zellen kältetoleranter Clostridien in deutschen Verarbeitungsbetrieben beträchtlich hoch ist.

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