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Involvement of *Clostridium gasigenes* and *C. algidicarnis* in 'blown pack' spoilage of Brazilian vacuum-packed beef

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ABSTRACT

The objectives of this study were to isolate psychrotrophic clostridia from Brazilian vacuum-packed beef cuts (spoiled or not) and to identify the isolates by using 16S rRNA gene sequencing. Anaerobic psychrotrophic microorganisms were also enumerated and samples were collected to verify the incidence of psychrotrophic clostridia in the abattoir environment. Vacuum-packed beef cuts (n = 8 grossly distended and n = 5 non-spoiled) and environmental samples were obtained from a beef packing plant located in the state of São Paulo, Brazil. Each sample was divided in three subsamples (exudate, beef surface and beef core) that were analyzed for vegetative forms, total spore-forming, and sulfide reducing spore-forming, both activated by alcohol and heat. Biochemical profiles of the isolates were obtained using API20A, with further identification using 16S rRNA gene sequencing. The growth temperature and the pH range were also assessed. Populations of psychrotrophic anaerobic vegetative microorganisms of up to 10^{10} CFU/(g, mL or 100 cm^2) were found in 'blown pack' samples, while in non-spoiled samples populations of 10^5 CFU/(g, CFU/mL or CFU/100cm^2) was found. Overall, a higher population of total spores and sulfide reducing spores activated by heat in spoiled samples was found. *Clostridium gasigenes* (n = 10) and *C. algidicarnis* (n = 2) were identified using 16S rRNA gene sequencing. Among the ten *C. gasigenes* isolates, six were from spoiled samples (C1, C2 and C9), two were isolated from non-spoiled samples (C4 and C5) and two were isolated from the hide and the abattoir corridor/beef cut conveyor belt. *C. algidicarnis* was recovered from spoiled beef packs (C2). Although some samples (C3, C7, C10 and C14) presented signs of 'blown pack' spoilage, *Clostridium* was not recovered. *C. algidicarnis* (n = 1) and *C. gasigenes* (n = 9) isolates have shown a psychrotrophic behavior, grew in the range 6.2–8.2. This is the first report on the isolation of psychrotrophic *Clostridium* (*C. gasigenes* and *C. algidicarnis*) in Brazil. This study shows that psychrotrophic *Clostridium* may pose a risk for the stability of vacuum-packed beef produced in tropical countries during shelf-life and highlights the need of adopting control measures to reduce their incidence in abattoir and the occurrence of 'blown pack' spoilage.

1. Introduction

Concerns on meat microbiology have been increasing through the years due to the increased commercialization and shipping of fresh or processed products to long distances (Nychas et al., 2008). Thus, several intervention strategies aiming at improving the microbiological quality of meat or meat products have been applied during processing or storage (Gill, 2009; Lee, 2010). Among these strategies, vacuum packaging is the most used technology by meat industries for preserving fresh chilled meat being well accepted by consumers (Skandamis and Nychas, 2002; Resurreccion, 2004). Vacuum packaging of meat under chilling storage greatly extends the product shelf-life (Seideman and Durland, 1982; Lee, 2010); however, vacuum-packed meat eventually spoils (Kalchayanand et al., 1989; Brightwell et al., 2007, 2009).

Vacuum-packed meat is particularly prone to the so-called 'blown-pack' spoilage that results in losses of large amounts of resources of meat industries (Adam et al., 2010). This spoilage is characterized by the production of large amounts of gas, offensive and putrid odors, presence of exudates, extensive proteolysis, changes in pH and color. These changes normally occur within 4–6 weeks of chilling storage (Kalchayanand et al., 1993; Broda et al., 1996a; Adam et al., 2010). Although *Enterobacteriaceae* was possibly associated with 'blown pack' spoilage (Brightwell et al., 2007), it is known that psychrotrophic clostridia such as *Clostridium algidicarnis*, *C. algidixylanolyticum*, *C. estertheticum*, *C. frigidicarnis* and *C. gasigenes* (Adam et al., 2010) can be the main causing-agents of this spoilage.

Studies in countries like the USA (Kalchayanand et al., 1989), the UK (Dainty et al., 1989; Lawson et al., 1994), New Zealand (Broda et al.,

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1996a,b), South Africa (Helps et al., 1999), Ireland (Byrne et al., 2009) and Canada (Yang et al., 2009b) have reported on incidence of 'blown pack' spoilage in vacuum-packed meat samples caused by psychrotrophic clostridia (Broda et al., 1996a,b).

Currently, Brazil top ranks World beef exportation and vacuum-packed beef accounts for approximately 75–80% of Brazilian beef exportations (ABIEC, 2010). Although the 'blown pack' spoilage was first described in the literature at the end of 80s (Kalchayanand et al., 1989) and considering that Brazilian meat industries have been increasingly facing this problem, to our knowledge there are no studies regarding the isolation and characterization of the viable microbiota that causes 'blown pack' spoilage in Brazil. Based on these considerations, the objectives of this study were to isolate and characterize the psychrotrophic clostridia in vacuum-packed beef (spoiled or not) produced in Brazil and to identify the isolates by means of 16S rRNA gene sequencing. The incidence of psychrotrophic clostridia in the abattoir environment was also investigated.

2. Material and methods

2.1. Collection of spoiled ('blown pack') and non-spoiled vacuum-packed beef samples

Eight vacuum-packed beef cuts showing grossly distended (spoiled) and five non-spoiled vacuum-packed beef samples were collected in a slaughter and beef packing factory located in the state of São Paulo, Brazil. The spoiled beef cuts (C1, C2, C3, C7, C9, C10, C11 and C14) analyzed were Strip loin (*Longissimus dorsi*), ox-rump (*Trapezius Thoracis*), and rump cover (*Biceps femoris*) (Table 1). All packages of non-spoiled beef samples (strip loin; codes C4, C5, C6, C12 and C13) did not show any signs of distension. At the time of collection, the packages were stored between 1/4 and 12 months at −10 °C.

2.1.1. Characterization of samples of vacuum-packed beef

All packages of spoiled and non-spoiled samples were sanitized with 70% ethanol solution under aseptic conditions before opening. Then, they were characterized regarding their appearance (distension or not) (Brightwell et al., 2007), the presence of gas (H_2 and CO_2) (FDA, 1998), odor, color, texture of beef surface and core, presence of exudate, pH of exudate and color of exudate and fat.

2.1.2. Preparation of subsamples (exudate, beef surface and beef core) of vacuum-packed beef

Each vacuum-packed beef sample was divided into subsamples (exudate, beef surface and beef core) that were analyzed separately summing 104 samples. Subsamples were prepared as described by Sveum et al. (1992) and Broda et al. (1996a,b) in tubes containing 9 mL of pre-reduced Reinforced *Clostridium* Medium (RCM) (Oxoid, Basingstoke, UK).

2.2. Enumeration of psychrotrophic anaerobic vegetative and spore-forming microorganisms in beef

Portions (1 mL) of inoculated tubes containing subsamples (Section 2.1.2) homogenized with pre-reduced RCM broth were used for enumeration of psychrotrophic anaerobic vegetative microorganisms. Pre-exhausted dilution fluid was used for the preparation of serial dilutions. The enumeration of psychrotrophic anaerobic vegetative microorganisms was performed by surface-plating portions of 0.1 mL of the dilutions onto pre-reduced (0.1 mL of catalase [Sigma, Germany] sterile solution at 2000 IU) modified Reinforced *Clostridium* agar (RCA) supplemented with 5% defibrinated blood sheep, 0.5% glucose and 1.5% agar.

The enumeration of psychrotrophic anaerobic spore-forming microorganisms was carried out after activating spores possibly present in the sub-samples by ethanol and heat (Broda et al., 1998a,b). Activation of spores by ethanol was performed by adding 4 mL of each sub-sample to 4 mL of ethanol (96%) with further incubation at 15 °C/60 min followed by neutralization in 8 mL of pre-reduced RCM broth (Johnston et al., 1964; Koransky et al., 1978). Heat activation was performed by adding 1 mL of sub-sample to 9 mL of dilution fluid with further heating at 80 °C/10 min followed by quick cooling (Broda et al., 1998a,b). Serial dilutions were prepared using dilution fluid and portions of 0.1 mL were surface-plated onto Perfringens Agar Base

Table 1
Characterization of spoiled vacuum-packaged beef cuts samples analyzed in this study[a].

Beef cuts	Spoiled vacuum-packaged beef cuts							
	Strip loin			Ox-rump		Strip loin		Rump cover
Sample codes	C1	C2	C3	C7	C9	C10	C11	C14
Time of storage at −10 °C until analysis (months)	5	6	6	1	4	4	4	4
Package Distension[b]	++++	++++	++++	+++++	+++	+++++	+++	++++
Gas incorporated to beef and drip[c]	++	++	++	++	+	++	++	++
Presence of H_2 (hydrogen)[d]	−	+	−	+	−	+	+	+
Presence of CO_2 (carbon dioxide)[d]	+	−	+	+	−	+	+	+
Putrid odor	−	Intense	−	slight	−	Slight	Slight	−
Butyric odor	Intense	−	Slight	−	Slight	−	−	Slight
Color of beef surface	Greenish	Meat greening at the extremities	Pale red	greenish	Pale red	greenish	greenish	greenish
Color of beef core	Greenish	Bright red	Bright red	Reddish-green	Bright red	Reddish-green	Reddish-green	Pale red
Texture of beef surface	Soft	Soft	Integral	Soft	Integral	Soft	Soft	Soft
Texture of beef core	Soft	Soft	Integral	Soft	Integral	Soft	Soft	Soft
Presence of exudate	++	++	++	+	+	+	+	+
pH of exudate[e]	6.01	7.17	5.50	6.5	6.2	7.0	6.9	6.9
Color of exudate	Dark brown	Dark brown	Maroonish-green	Reddish-green	Dark brown	Reddish-green	Reddish-green	Reddish-green
Color of fat	Greenish	Greenish	Light yellow	Yellowish	Yellowish	Greenish	Greenish	Greenish

[a] Expiration date is 1 year when cuts are stored at −18 °C. Package material is Ethylene vinyl acetate (EVA). Ox-rump and rump-cover cuts were within 1–2 kg. Strip loin was between 4 and 6 kg.
[b] Where: (+++) = 'blown'; puffy packs; (++++) = fully distended packs; (+++++) = overblown packs.
[c] (−) No gas bubbles in the drip; (+) = small numbers of gas bubbles in the drip; (++) = loss of vacuum.
[d] (−) Absence of gas; (+) = presence of gas.
[e] Exudate was classified according to the amount of drip found (+ = small) or (++ = large).