STUDY OF THE MICROBIOTA CAUSING "BLOWN PACK" SPOILAGE OF VACUUM-PACKED BEEF

ESTUDO DA MICROBIOTA ENVOLVIDA NA DETERIORAÇÃO "BLOWN PACK" DE CORTES CÁRNEOS EMBALADOS A VÁCUO

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SUMMARY

"Blown pack" spoilage is characterized by abundant production of gas and off-odor leading to swelling of the packing of vacuum-packed chilled meat cuts. The aim of this study was to determine possible microorganisms involved in this spoilage by quantifying and characterizing the population of *Enterobacteriaceae*, quantifying lactic-acid bacteria, as well as researching *Clostridium estertheticum* and *Clostridium gasigenes* by PCR in meat proper for consumption and meat presenting "blown pack" spoilage. Fifty-four vacuum-packed meat cuts were analyzed, of which 27 were spoiled and 27 non-spoiled. Average enterobacteria population was $1.7x10^6$ CFU/mL and $5.5x10^3$ CFU/mL for spoiled and non-spoiled samples, respectively. Average lactic-acid bacteria population was $5.5x10^8$ and $1.0x10^5$ CFU/mL for spoiled and non-spoiled samples, respectively. The most prevalent enterobacteria species was *Hafnia alvei*. Meat showing "blown pack" spoilage had higher frequency of positive samples for *Clostridium estertheticum*. No statistically significant difference was observed in the population of *Clostridium gasigenes* between "blown pack" spoilage and non-spoiled meat. It was concluded that the most efficient method to control "blown pack" spoilage is to prevent meat contamination by fecal material.

KEY-WORDS: "Blown pack" spoilage. Enteric bacteria. Psychrophilic clostridia. Vacuum-packed meat.

RESUMO

A deterioração "blown pack" é caracterizada por abundante produção de gás e odor desagradável, induzindo à completa distensão da embalagem durante o processo de estocagem sob refrigeração de cortes cárneos embalados a vácuo. Assim, o objetivo deste trabalho foi o de determinar os possíveis microrganismos envolvidos nessa deterioração através da quantificação e caracterização das populações de *Enterobacteriaceae* e quantificação de bactérias ácido-lácticas, além da pesquisa de *Clostridium estertheticum* e *Clostridium gasigenes*, através da PCR, em carnes próprias para o consumo e em carnes que apresentaram a deterioração "blown pack". Foram analisadas 54 peças de carne embaladas a vácuo, sendo 27 com deterioração e 27 sem deterioração. As populações médias de enterobactérias foram de $1,7x10^6$ UFC/mL para amostras deterioradas e de $5,5x10^3$ UFC/mL nas não deterioradas, de bactérias ácido-lácticas foram, respectivamente, de $5,5x10^8$ e $1,0x10^5$ UFC/mL. Dentre as enterobactérias, a espécie de maior prevalência foi *Hafnia alvei*. A maior frequência de amostras positivas para o *Clostridium estertheticum* foram aquelas apresentando a deterioração "blown pack". Não houve diferença estatística significativa para a presença do *Clostridium gasigenes* entre amostras com deterioração "blown pack" e carnes não deterioradas. Concluiu-se que a principal forma de controle desta deterioração é a prevenção da contaminação da carne por material fecal.

PALAVRAS-CHAVE: Carne embalada vácuo. Clostrídios psicrofílicos. Deterioração "blown pack". Enterobactérias.

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INTRODUCTION

"Blown pack" spoilage of vacuum packed chilled meat is characterized by abundant gas production that leads to swelling of the package during storage. After opening the package, a foul odor is exhaled with or without apparent sulfur staining. The gases present in the package are carbon dioxide and hydrogen, in addition to several butyric types resulting from the fermentation metabolism (JONES & WOODS, 1986).

Since 1989, when the relationship between "blown pack" spoilage of vacuum packaging and psychrophilic clostridia was first established (DAINTY et al., 1989), research has been conducted to determine the source of contamination processes in order to reduce deterioration caused by members of the genus *Clostridium*, which are considered to be the major causative agents of this type of spoilage (MOSCHONAS et al., 2009; YANG et al., 2009).

Although studies by DAINTY et al. (1989) and BRODA et al.(2000) pointed at the psychrophilic species *Clostridium estertheticum* and *Clostridium gasigenes* as the swelling causing agents of meat cuts kept chilled at temperatures between -1.5 and 2°C, other studies carried out in an attempt to establish the prevalence of these clostridia in chilled meat have suggested the possibility of *Enterobacteriaceae* as also determinant agent in the "blown pack" spoilage, since they were found in all samples that present this type of spoilage, regardless the presence of cold-tolerant clostridia (BRODA, 1997; BRIGHTWELL et al., 2007).

The primary sources of carcass contamination in the meat industry are microorganisms of either clostridia or *Enterobacteriaceae* groups present in dust, water and animal feces that attach to the animal skin (BOEREMA et al., 2003) . Based on the foregoing considerations and the fact that "blown pack" spoilage has led to significant economic losses, this study focus on determining presence and number of bacteria of the *Enterobacteriaceae* family and the lactic acid producing bacteria, as well as the frequency of *Clostridium estertheticum* and *Clostridium gasigenes* in meat suitable for consumption and spoiled meat. This information should assist in the adoption of measures to help prevent this problem.

MATERIAL AND METHODS

Sampling

Samples used in the trial were 54 pieces of vacuum packed beef, produced under sanitary control by the Federal Inspection Service. From these samples, 27 presented "blown pack" spoilage and were provided by the industry, while the 27 non-spoiled meat samples were obtained from retail shops, chosen randomly, provided that they were in perfect condition, presenting typical physical and sensory characteristics. The samples came from several industries from the states of Mato Grosso, Mato Grosso do Sul, São Paulo, Minas Gerais, Goiás, Pará and Paraná. The beef cuts used were sirloin steak, fillet mignon, rump, rib steak, flank, top side, shoulder and tail.

Samples presenting "blown pack" spoilage were visibly swollen due to accumulation of gases, displayed physical and sensory changes, often a greenish color and pungent odor, high proteolytic activity and excessive sweating, which made the product unacceptable for consumption. The non-spoiled samples presented uniform aspect, no dark or light spots, no slime on the surface, the color ranged from dark red to pinkish red, with marble and shiny appearance, firm, compact, elastic and slightly moist, mild and pleasant odor, characteristic of meat fit for consumption.

Identification and counts of the *Enterobacteriaceae* and lactic acid bacteria (BAL)

These determinations were performed according to methods described in ICMSF (2000) in the exudate collected from inside the package. Enterobacteria was quantified using violet red bile agar (VRBA) with glucose and dilution up to 10^{-7} of the exudates (APHA, 2001), while for *Lactobacillus,* Man, Rogosa & Sharpe (MRS) double layer was used to count lactic acid bacteria.

In order to characterize the enterobacteria, ten typical colonies were isolated and subjected to two steps purification in VRBA. After 24 hours incubation at 37°C, the pure colonies were transferred to slanted nutrient agar and incubated the same way. Smears were then stained by Gram and oxidase test confirmed pure cultures (MacFADIN, 1976). Cultures shaped as Gram-negative bacilli, non sporulated and oxidase negative were considered as belonging to the *Enterobacteriaceae* family. Subsequently, biochemical tests identified the genera, according to "Bergey`s Manual of Systematic Bacteriology" (HOLT et al., 1994).

Clostridium estertheticum and *Clostridium gasigenes*

Using a Pasteur pipette, 2 mL of exudate from each sample was transferred aseptically to a test tube containing 20 mL of brain heart infusion (BHI, Difco) in duplicate. The BHI tubes were placed in anaerobic jars (Gas Pack™, BD), incubated for 10 days in the fridge at 10ºC. After this period, the samples were processed for subsequent DNA extraction. The genomic DNA extraction was performed according to modified methodology proposed by Van Soolingem et al. (1991).

The primer pair used for *Clostridium estertheticum* detection was outlined by Helps et al. (1999) and it is composed of *forward* primers RFP (5´TGA TCG CAT GAT CTT AAC ATC AAA G-3´) and *reverse* RRP (5´TCG ACC CCC GAC ACC TAG TAT T-3´) found in positions 173-197 and 813-792, respectively, subunit 16S of RNAr of *C. estertheticum* (access number GenBank® S46734). This pair amplifies fragment 641 base pairs (bp) of *Clostridium estertheticum* DNA.

To detect *Clostridium gasigenes*, the primers *forward* 16SDBF (5´GAG AGG AGT TCT TCG GAA CGA-3´) and *reverse* 16SDBR (5´AAG CSA CTT

CCC CAA TTA C-3′) were used (BRODA et al., 2003). These primers are found in regions 61-81 and 995-997, respectively, in the reference sequence of the microorganism (GenBank®, access number AFO92548 and AF143692), amplifying the fragments 935pb.

The following components were used in each reaction of PCR 50 µL:

- Primers RFP/RRP: amplicon 641 pb, DNA (100 ng), primers (0.3 mM), dNTP (0.2 mM), $MgCl_2$ (1.5 mM), Taq DNA polymerase (2 U), buffer 10X (tris-HCl pH 8.3) and KCl (500 mM);

- Primers 16SDBF/16SDBR: amplicon 935 pb, DNA (100 ng), primers (0.5 mM), dNTP (0.2 mM), $MgCl_2$ (1,. mM), Taq DNA polymerase (2.5 U) and buffer 10X (tris-HCl pH 8.3) and KCl (500 mM).

The amplification reaction was performed using a thermocycler Eppendorf® for a total of 40 cycles for RFP/RRP and 30 cycles for 16SDBF/16SDBR.

The conditions of the RFP/RRP amplification were as follows: initial denaturation at 95°C/5 minutes, denaturation at 94°C/1 minute, annealing at 60°C/1 minute, extension at 72°C/1 minute and final extension at 72°C/10 minutes. While amplification conditions of 16SDBF/16SDBR were: initial denaturation at 93°C/3 minutes, denaturation at 92°C/1 minute, annealing at 55°C/1 minute, extension at 72°C/1 minute and final extension at 72°C/3 minutes.

After amplification, PCR products underwent electrophoresis on agarose gel at 1%, with initial alignment 115V for 15 minutes and run at 100V for 40 minutes. A standard molecular weight marker 100 pb DNA Ladder (Invitrogen®) was used. The gels were stained in ethidium bromide solution (0.6 g/mL), visualized on an ultraviolet transilluminator and photo-documented in a digital system (BIO RAD®).

Statistical analysis

The variables, number of enterobacteria and lactic acid bacteria were analyzed by GLM (General Liner Model), SAS, using a mathematical model that includes the effects of treatments (spoiled and non-spoiled samples) and the interaction between them. The comparison of the means was performed by Tukey test at 5%. On the other hand, the effect of the treatments on the presence and absence of *C. estertheticum* and *C. gasigenes* was analyzed by Chi-square test.

RESULTS AND DISCUSSION

Counts of enterobacteria

The counts of enterobacteria population was divided into three intervals for spoiled and non-spoiled samples that can be seen in Table 1.

Populations of enterobacteria in the spoiled and non-spoiled samples were significantly different ($p<0.05$). The mean $1.7x10^6$ UFC/mL for spoiled samples was significantly higher than $5.5x10^3$ UFC/mL found for non-spoiled samples. From the 27 spoiled samples, 21 (77.77%) had enterobacteria population greater than 10^6 UFC/mL, while from the 27 non-spoiled samples 18 (66.66%) had populations of less than 10^5 UFC/mL. These results are in agreement with the ones reported by Borch et al. (1996), Riddell & Korkeala (1997) and Yost et al. (2002) while studying the microbiology of non-spoiled vacuum packed meat.

Characterization of microorganisms of the *Enterobacteriaceae* family

The results from biochemical tests carried out in 270 colonies isolated from spoiled samples showed high incidence of *Hafnia alvei* (18.5%), followed by *Proteus vulgaris* (16.7%), *Klebsiella* spp. (8.5%), *Enterobacter aerogenes* (8.5%), *Serratia liquefaciens* (8.1%) and *Edwardsiella ictaluri* (7.7%). Results that corroborate the ones reported by Hanna et al. (1979) and Boerema et al. (2002), who identified three species of the family *Enterobacteriaceae* - *Serratia liquefaciens, Enterobacter aerogenes* and *Hafnia alvei* -, as probable spoiling agents of vacuum packed meat showing "blown pack" spoilage.

Table 1 – Distribution of enterobacteria and lactic acid bacteria in non spoiled samples and samples presenting "blown pack" spoilage UFC/mL of exudates.

	Sample					
	Enterobacteria			**Lactic acid bacteria**		
Population (UFC/mL)	**Spoiled**	**Non spoiled**	**Total**	**Spoiled**	**Non spoiled**	**Total**
	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)
$< 10^5$	6(22.22)	18(66.66)	24(44.45)	0(0.00)	13(48.15)	13(24.08)
10^5 - 10^7	12(44.45)	5(18.52)	17(31.48)	6(22.23)	6(22.23)	12(22.22)
$> 10^7$	9(33.33)	4(14.82)	13(24.07)	21(77.77)	8(29.62)	29(53.70)
Total	27(100)	27(100)	54(100)	27(100)	27(100)	54(100)

As for the 270 colonies isolated from non-spoiled samples, the following microorganisms occurred: *Hafnia alvei* (38.9%), followed by *Edwardsiella ictaluri* (11.5%), *Serratia liquefaciens* (10.3%) and *Enterobacter* spp. (8.6%).

Both spoiled and non-spoiled samples showed prevalence of *Hafnia alvei*, which was identified in 155 (28.70%) of the 540 studied cultures. According to Lindberg et al., (1998) and Ridell & Korkealla (1997), 50% of the isolates from chilled meat belong to the species *Hafnia alvei.* These data justify the wide distribution found in the studied samples.

Brenner (1992) indicated the genera *Citrobacter, Enterobacter, Hafnia, Klebsiella, Proteus, Providencia, Serratia, Escherichia* and *Yersinia* as spoilers of meat products; however, in this study these genera were isolated in both spoiled and non-spoiled samples. These results suggest that the determining factor in the spoiling process is the number these microorganisms are found and the conditions that favor their development, since spoiled samples had enterobacteria population much larger than non-spoiled.

The results are troublesome because even though fewer in number, some potentially pathogenic bacteria of public health interest such as, *Salmonella* sp., *Shigella* spp., *Yersinia* spp., *Klebsiella* spp. were isolated. According to Holt et al. (1994), *Enterobacteriaceae* are responsible for 50% of nosocomial infections most commonly caused by *Escherichia coli*, *Klebsiella*, *Enterobacter, Proteus*, *Providencia* and *Serratia*, which were also isolated in the studied samples.

According to Smith et al. (1993) and Dainty et al. (1986), the main species isolated from vacuum packed chilled meat responsible for the potential production of biogenic amines are *Hafnia alvei* and *Serratia liquefaciens*, which in this study were found more often in non-spoiled samples. Therefore, they could be ingested without the consumer realizing any sensory changes in the meat, and cause subsequent health problems.

Counts of lactic acid bacteria

The population count of LAB (lactic acid bacteria) was divided into three intervals for spoiled and non-spoiled samples that can be seen in Table 1.

Lactic acid bacteria was highly prevalent in samples with "blown pack" spoilage. The populations of lactic acid bacteria were significantly higher ($p<0.05$) in spoiled samples compared to non-spoiled, means of $5.5x10^8$ UFC/mL and $1.0x10^5$ UFC/mL, respectively. From the 27 spoiled samples analyzed, 21 (77.77%) displayed populations higher than 10^7 UFC/mL, while only 8 (29,62%) out of 27 non-spoiled samples had populations greater than 10^7 UFC/mL as shown in Table 1.

According to Borch et al. (1996), spoilage with formation of gas within the packaging of chilled meat and odor change as well, only happen when spoiling agents are present at levels ranging from 10^8 UFC to 10^9 UFC/g. These authors also conclude that the interaction between lactic acid bacteria (dominant microbiota) and enterobacteria intensifies the degree of deterioration of the product.

From the 27 non-spoiled samples, 13 (48.15%) presented lactic acid bacteria population lower than 10^5 UFC/mL, while none of the spoiled samples had LAB lower than 10^5 UFC/mL. Hanna et al. (1979) have shown that *Lactobacillus* spp. heterofermentative are capable of producing gas within the vacuum package when stored at temperatures ranging from 1 to 3°C during three weeks. Thus, the presence of *Lactobacillus* in spoiled meat in large numbers may have contributed significantly to "blown pack" spoilage.

Despite the fact that *Lactobacillus* produce bacteriocins and other compounds that inhibit the growth of pathogens (AYMERICH & HUGAS, 1998), its population should he controlled by hygienic and sanitary measures to prevent them from contributing to the deterioration of the product, thus reducing shelf life. Considering that many of these microorganisms are in the digestive tract of ruminants (HOVE et al., 1999), direct or indirect contamination of the carcass with fecal material should be avoided by preventive measures.

It should be noted that in non-spoiled samples, 14.82% (4/27) presented population of enterobacteria larger than 10^7 UFC/mL and 29.62% (8/27) of the samples had populations of lactic acid bacteria larger than 10^7 UFC/mL, rising questions about the true role of these groups of microorganisms in "blown pack"spoilage. There are several variables that lead to "blown pack" spoilage, not only microbial population size. Another factor to be considered is the permeability of the film used in the packaging that could allow selective passage of some gases in different packaging. Any gas exchange that takes place through the film may cause significant change in the gaseous atmosphere inside the package and favor the development of specific spoilage microorganisms or even the fact that some packages can retain some gas molecules causing the typical swelling in certain samples and not others.

Tsigarida & Nychas (2001) showed that the film used in packaging influences the development of different microbial groups, as well as type and concentrations of the metabolites produced. Other factors such as pH of different muscle groups, initial bacterial contamination and temperature variations to which the product is submitted during the process must also be considered. Each of these variables reflects in different rates of deterioration (EGAN & SHAY, 1982) or different rates of development of the bacteria responsible for spoilage (TSIGARIDA et al., 2000).

***Clostridium estertheticum* and *Clostridium gasigenes* detection**

Figure 1 shows the amplification of the DNA of *Clostridium estertheticum* and *C. gasigenes* found in the samples of chilled vacuum packed meat, represented by amplicons of 641 pb and 935 pb, respectively.

The results in Table 2 show that from the total of 54 samples, 68.52% (37) were positive and 31.48% (17)

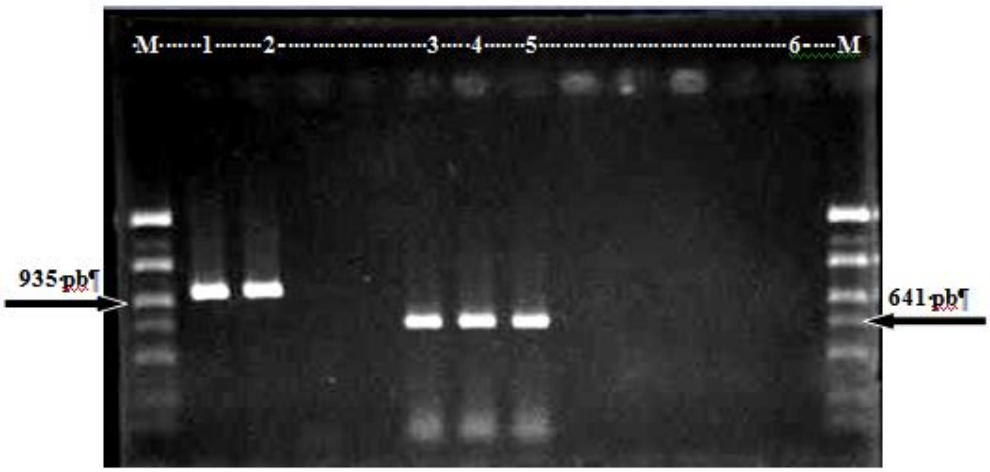

Figura 1 - Produtos de PCR obtidos de *C. estertheticum* e *C. gasigenes*. M: marcador molecular (DNA ladder 100 pb); 1: Controle positivo para *C. gasigenes* (DSM12272); 2: Amostra positiva para o *C. gasigenes*; 3: Controle positivo *C. estertheticum* (DSM 8809); 4, 5: Amostras positivas para *C. estertheticum*; 6: Controle negativo.

Table 2 – Distribution of *Clostridium estertheticum* and *Clostridium gasigenes* in non spoiled and "blown pack" spoiled meat samples.

	Samples					
	Clostridium estertheticum			*Clostridium gasigenes*		
	Spoiled	**Non spoiled**	**Total**	**Spoiled**	**Non spoiled**	**Total**
	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)
Presence	23(85.18%)	14(51.85)	37(68.52)	8(29.62)	2(7.40)	10(18.52)
Absence	4(14.82)	13(48.15)	17(31.48)	19(70.38)	25(92.60)	44(81.48)
Total	27(100)	27(100)	54(100)	27(100)	27(100)	54(100)

were negative for *C. estertheticum*. These data confirm the wide distribution of *C. estertheticum* in chilled vacuum packed meat.

The spoiled samples were significantly more positive compared to non-spoiled ($p<0.01$). Of the 27 samples with "blown pack" spoilage, 23 (85.18%) were positive for *C. estertheticum,* while non-spoiled samples 14 (51.85%) were positive, as shown in Table 2. This result agrees with the one reported by Rauecker (2007), who detected the microorganism in 85.18% of the spoiled samples. However, in the same study, while analyzing non-spoiled samples, *Clostridium estertheticum* was found in only 9.52% of the samples.

Although non-spoiled samples presented a significantly lower positive number for *C. estertheticum,* the presence of this microorganism in

51.85% of the samples may be an indicative of the wide distribution of this microorganism and that their presence alone is not enough to cause spoilage. There must be other factors that influence the onset and evolution of the deterioration process including permeability of the packaging as previously discussed. Temperature change during storage, use of sanitizers when washing carcasses and interaction between different microbial populations present in contaminated meat should also be considered.

The prevalence of *Clostridium gasigenes* in all samples was low. The results in Table 2 show the ratio of total positives (10/54 – 18.52%) and negatives (44/54 – 81.48%) for *C. gasigenes*. The number of positive samples for spoiled and non-spoiled meat was low, only 8/27 (29.62%) and 2/27 (7.40%), respectively, with no significant difference between them. Rauecker (2007) also found *C. gasigenes* in only 18.52% (5/27) of meat samples with "blown pack" spoilage.

The results presented in this paper are consistent with earlier studies by Dainty et al. (1989) and Brod et al. (2000) who also identified *C. estertheticum* and *C. gasigenes* in chilled vacuum packed beef.

Helps et al. (1999) found these clostridia in soil, gut and feces of animals. Broda et al. (2009) isolated clostridia that causes "blown pack" spoilage on the floor of walk-in cold rooms, in slaughter rooms before the skin is removed and deboning rooms. Rauecker (2007) isolated these microorganisms in several places within the meatpacking industry, on the conveyor rollers, drains, deboning rooms, walk-in cold rooms and others, thus justifying the wide distribution of clostridia in meat samples. These data show that the problem can be directly related to indirect and/or direct fecal contamination and lack of hygiene during processing.

CONCLUSIONS

The microorganisms of the family of the *Enterobacteriaceae* and lactic acid bacteria were widely distributed in all meat samples, but appeared in greater numbers in samples that presented "blown pack" spoilage. Among the *Enterobacteriaceae* family, the predominant genera were *Hafnia*, *Proteus, Klebsiella*, *Enterobacter*, *Serratia* and *Edwardsiella*, with marked presence of the species *Hafnia alvei. C. estertheticum* was more prevalent in spoiled samples compared to non spoiled. However, no significant difference was observed for *C. gasigenes* occurrence in non spoiled samples and meat that presented "blown pack" spoilage. It was concluded that the best way to prevent "blown pack" spoilage is by avoiding meat contamination by fecal material.

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