DETECTION AND CHARACTERIZATION OF MICROORGANISM IN BLOWN PACK DETERIORATION

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***Abstract*— Brazil is a major producer of beef in the world and, despite the importance that the meat industry represents to the country there are few studies about the microorganisms involved in the problem of distension in packs of vacuum chilled meat, also known such as deterioration of "blown pack". This deterioration is attributed to psichrophilic *Clostridium* and other species such as *Enterobacteriaceae* and lactic acid bacteria. The culture isolation for detection is difficult in anaerobic species like *Clostridium*, because require equipment that enable the growth in anaerobic condition. Furthermore, using culture conventional methods may not be possible to detect species. These limitations can be overcome with the use of molecular techniques based on PCR, such as T-RFLP used in this work. The present study evaluated ten samples of vacuum packed meat with Blown Pack deterioration. The T-RFLP analysis showed contamination mainly *Enterobacteriaceae* (*H. alvei* and *S. liquefaciens*), were also found contamination by *Clostridium putrefaciens; C. algidicarnis* and *L. sakei*. Were also performed gas and volatile composition analysis, the results of these tests showed that CO_2 is the main gas in the samples, and organic acids, sulfur compounds, aldehydes and alcohol were found in the volatile analysis.**

***Index Terms*—blown pack, T-RFLP, vacuum packaging, gas and volatile composition**

I. INTRODUCTION

Early deterioration of vacuum packed chilled meat, with normal pH, without temperature abuse or packaging failures have been reported and associated with the bacteria psichrophilic of *Clostridium* genus (Lawson et al. 1994) and some *Enterobacteriaceae* (Brightwell et al., 2007).The presence of these organisms on the meat is not detected by routine microbiological tests, and the consequences of their growth are evident in few weeks. Thus, molecular methods can be an alternative to conventional microbiological test for meat spoilage microorganisms detection (Broda *et al*. 2002). PCR based molecular techniques such as TRFLP (*Terminal Restriction Fragment Length Polymorphism)*, is used to evaluate various environmental samples such as soil (Burke et al., 2010). In foods this method was used to study bacterial communities in dairy products like cheese and yogurt (Sanchéz et al., 2006; Redemaker et al., 2006). In this work, T-RFLP was used for detction of main spoilage microorganism in vacuum chilled meat and was performed gas and volatile composition too.

II. MATERIALS AND METHODS

A. Bacterial strains and growth conditions

The bacterial reference strains used in this study included five clostridia, *C. algidicarnis* (DSM 15099); *C. estherteticum* (DSM *8809*); *C. frigidicarnis* (DSM 12271); *C. gasigenes* (DSM *12272*); *C. putrefaciens* (DSM *1291*). Two Lactobacilli: Lactobacillus sakei ATCC *15521*) and Leuconostoc mesenteroides (ATCC 8293), three Enterobacteriaceae: Hafnia alvei ATCC 11604); Serratia liquefaciens (ATCC *35551);* Serratia *marcienscens ATCC 14756)*. The clostridia were propagated according to German Collection of Microorganisms and Cell Cultures (DSMZ) catalogue (http://www.dsmz.de/microorganisms/main.php?menu_id=2). The lactobacilli were routinely propagated in MRS broth (Mann, Rogosa & Sharpe, DIFCO) at 30^{o}C for 48 hours and enterobacteriaceae were propagated in Nutrient-broth at 26^{0}C for 48 hours.

B. Meat Sample and DNA extraction

In this study were used ten vacuum chilled meat samplings. For DNA extraction were collected swab and exudated from each samples. The swabs were washed in 1mL saline solution 0,08 % NaCl and collected 1mL of exudated, both

was performed using High Pure PCR template Preparation Kit (Roche Diagnostics, Mannheim Germany) according to the protocol described by the manufacture.

C. T-RFLP analysis

T-RFLP analysis was performed using the conserved bacterial primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTAGGACTT 3'). Primer 27 F was 5' end-labeled with 6-carboxyfluorescein (6-FAM). Each 50 mL PCR mixture contained 1 µl DNA, 0.1 mM each primer, 200 mM of deoxynucleoside triphosphate, 1X PCR-buffer, 1mM $MgCl_2$, 400 µg/ml BSA and 1.25U Taq- Polymerase (Invitrogen). PCR cycling consisted of initial denaturation at 94 ^{0}C for 3 min, followed by 35 cycles of 94 ^{0}C for 30 s, 59 ^{0}C for 45 s and 1 min at 72 ^{0}C, and a final extension at 72 ^{0}C for 15 min. The amplified product was purified with the GFX^{TM} PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) according to the manufacturer's recommendation. Subsequently 5 µL of the fluorescently labeled PCR product was digested for 3hours at 37 ^{0}C with 5U of the Hae III, Hha I, MseI and RsaI restrictions enzymes (Invitrogen, Breda, The Netherlands) in 15 µL reaction and inactivated at 65 ^{0}C for 10 min.

The precise lengths of the T-RFLP fragments from the amplified rDNA products were determined by multicapillary electrophoresis with a model 3100 Genetic Analyzer automated sequencer (Applied Biosystems). After electrophoresis the produced sequencing files (.ab1 format) were readjusted in Genescan files (.fsa format). The lengths of the FAM fluorescently labeled fragments were analyzed by comparison of each sample pecks with strain type peck using Peack Scanner v 1.0.

III. RESULTS AND DISCUSSION

The T-RFLP analysis of ten samples studied showed the presence of *Clostridium*, *Enterobactereaceae* and Lactic Acid Bacteria (LAB). To detect these microorganisms in the samples were used five restriction enzymes (*HaeIII, HhaI, MseI, MspI and RsaI*). When the samples to showed the same length of T-RF (Terminal Restriction Fragment - peak) with strains patterns in at least three enzymes, they were considered contaminated. Program Peak Scanner v1.0 analysis allowed the following results: pyschrotolereant *Enterobacteriaceae* species were detected by T-RFLP, *H. alvei* in 1,2,3,4 and 7 samples; *S. liquefaciens* in 1,7,8 and 10 samples. Samples 1 and 7 had peaks (T-RF) in the same position of patterns *S. liquefaciens* e *H. alvei* (Fig. 1, example showing only one of the restriction enzymes). *Serratia liquefaciens* was observed in 8 and 10 samples too (Tabela 1). These two species were most frequent isolated from meat samples and were reported like possible involvement in blown pack spoilage too (Brightwell et al., 2007) because they can moderate or major produce gas in anaerobic condition. The presence of this genus is often used as an indicator of the inappropriate processing or post-processing contaminati. The volatile compounds analysis detected the presence of alcohols, ketones, aldehydes, acids and sulfur compounds in samples contaminated with Enterobacter (Table 2). Was not detected the presence of *S. marcienscens* in the study.

In the sample five was observed contamination by *L. sakei* in using three restriction enzymes (Tabela 1). This microorganism is a food-borne bacterium naturally found in meat and fish products and can restrict the growth of unwanted organisms like *L. mocytogenes*, *C. jejuni* and *C. estherteticum* through competition and/or generation of antagonistic molecules such as organic acids, bacteriocins and hypothiocyanate (Jones et al., 2009). *L. sakei* was reported associated whit meat spoilage in vacuum-packaged meat products (Aznar and Chenoll, 2006). This microorganism is facultative heterofermentive, being able to produce CO_2 via the phosphogluconate pathway from gluconate and pentoses (not present in the meat products analyzed) (Chenoll et al., 2007). It supports the main vacuum packed spoilage in the samples will be another microorganism. The heterofermentative *Lactic Acid Bacteria* (LAB) metabolize glucose and produce lactic acid, isobutanoic acid, isopentoic acid and acetic acid, among others. These, acidify the meat and can also cause changes in flavor and odor. The volatile compounds analysis detected the presence of alcohol and ketone (Table 2).

In this study was detected too the presence of *C. algidicarnis* and *C. putrefaciens* in samples 8,9 and 10. In the 8 and 10 samples the T-RF (peck) showed also *S.liquefaciens* contamination using the restriction enzymes *HaeIII*, *HhaI* e *RsaI* (Table 1). Psychrophilic *Clostridium* spp., particularly, *C. estertheticum* and *C. gasigenes*, have been established as causative agents of blown pack spoilage (Broda et al., 2002). In T-RFLP analysis were found *C. algidicarnis* and *C. putrefaciens* contamination, the first microorganism is not known to cause "blown pack" spoilage, it has been previously isolated from spoiled vaccum packaged pork (Kalchayanand et al., 1989), vaccum-packed chilled lamb and recently from lamb-processing plants (Broda et al., 2009). *C. putrefaciens* is a blown pack associated microorganism, it is strict anaerobe and markedly proteolytic, has hydrogen sulphide slight production and synthesized acid and gas in glucose fermentation. Generally the clostridia blown pack associated are sacrolitic, able to ferment fructose, glucose, sucrose, xylose and inulin, producing large quantities of butyric acid, 1-butanol, acetic acid, lactic acid, formic acid and ethanol (Spring et al., 2003). During the process of "blown pack" deterioration, the gas present inside the package consists mainly of carbon dioxide, nitrogen and hydrogen. These compounds resulting from the metabolism Butyric fermentation, as well as decarboxylation of amino acids that produce sulfur compounds as final products volatile ammonia and diamines characterizing the odor of "blown pack" meat deterioration (Broda et al., 2000). The volatile compounds analysis of the *Clostridium* contamination samples detected the presence of 3-metil-1-butanol; Dimetil

dissulfito; Hexanal; ácido acetic (Table 2). None of the samples evaluated in this study could detect the presence of *C. estertheticum*, *C. estertheticum laramiense* and *C. gasigenes* using at least three restriction enzymes.

Samples	Peack height					Bacteria strains
	HaeIII	**HhaI**	**MseI**	**MspI**	**RsaI**	
1	592;198	2289;678	703; -	840;232	2961;-	*H. alvei; S. liquefaciens*
2	81	-	648	-	1294	*H. alvei*
3	140	2013	1194	-	106	*H. alvei*
4	147	4875	-	86	1894	*H. alvei*
5	105	4227	-	1310	-	*L. sakei*
6						-
7	-	4645;2873	1021;3388	5599;4977	1453;-	*H. alvei; S. liquefaciens*
8	7781/537/ 128	3185/4407/ 2013	931	170/697	2933;182	*C. algidicarnis/ C. putrefaciens/ S.liquefaciens*
9	2515/2515	3039/3039	959	1402/794	2249/2249	*C. algidicarnis/ C.putrefaciens*
10	2414/2414/ 1345	2578/2578/ 244	1381	875/1064	1970/ 1970/65	*C. algidicarnis/ C. putrefaciens/ S. liquefaciens*

Table 1: Height peaks corresponding to the strains patterns in five restriction enzymes obtained from the analysis with the program Peak Scanner v1.0. - Unidentified peak

Sample	Gas composition (% v/v)				Volatile composition	T-RFLP detected Microorganism
	H_2	**O_2**	**N_2**	**CO_2**		
1	5,3	< 0,5	7,1	75,0	S-metil tioacetate; 3-metil-1-butanol; Dimetil dissulfite; Hexanal; 3-metil-butanal; 1-propanol	*H. alvei; S. liquefaciens*
2	24,1	< 0,5	7,3	85,2	Hexanal	*H. alvei*
3	30,2	< 0,5	5,2	78,6	1-butanol	*H. alvei*
4	26,5	< 0,5	< 0,5*	79,8	Dimetil sulfite; 1-butanol; 3-metil-1-butanol	*H. alvei*
5	20,5	0,1	3,4	37,5	1-propanol; 2- butanone	*L. sakei*
6	19,1	0,1	4,9	44,0	2- butanone; 1-pentanol; acetic acid	-
7	24,4	1,3	21,6	45,3	Dimetil sulfite; 1-butanol	*H. alvei; S. liquefaciens*
8	0,1	0,1	7,8	91,1	Dimetil sulfite; acetic acid; Tetradecane;Pentadecane; Hexadecan	*C. algidicarnis/ C. putrefaciens/ S.liquefaciens*
9	0,1	0,1	8,6	91,3	1-butanol	*C. algidicarnis/ C.putrefaciens*
10	13,5	0,07	4,4	76,7	Ethanol; 2-butanona; tetradecane; pentadecane.	*C. algidicarnis/ C. putrefaciens/ S. liquefaciens*

Table 2: Headspace gas and volatiles composition in microorganism detected by T-RFLP. - Unidentified microorganism

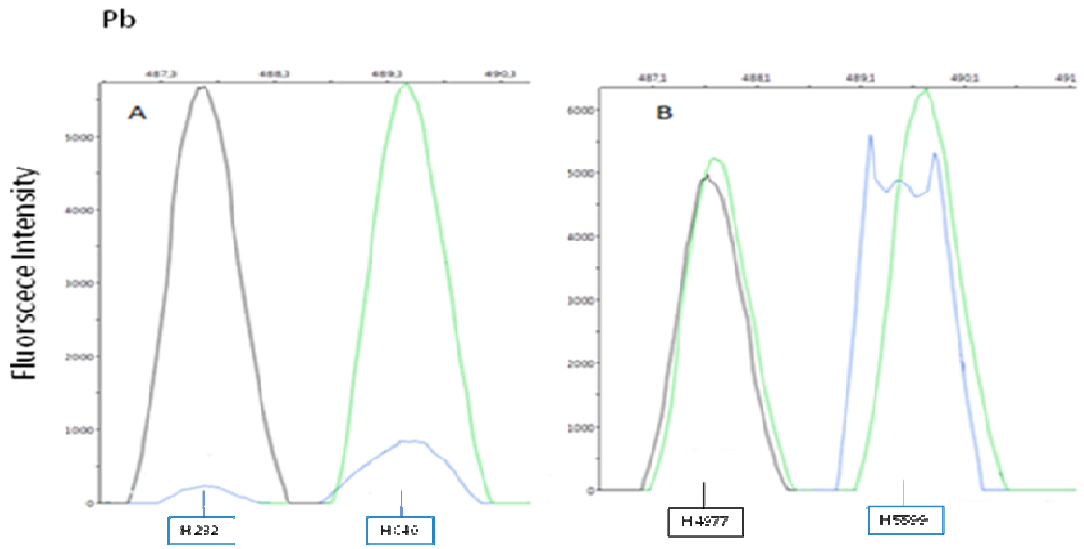

Figure 1: T-RFLP profiles from *Msp*I digestion of the 16S rDNA gene products. Peaks from 1 and 7samples and strain patterns. (A) Blue: Sampe 1, green; H. alvei; black: S. liquefaciens. (B) Blue: H. alvei; green: sample 7; Black: S. liquefaciens.

IV. CONCLUSION

The TRFLP technique has high sensitivity in detecting the main microorganisms involved in the deterioration blown Pack.

Enterobacteria were the main contaminants of the samples studied, suggesting inappropriate processing or post-processing contamination.

The main gas components were found in samples H_2 and CO_2, and CO_2 was predominant in all of them. The volatile compounds found are consistent with bacterial community metabolism detected in the samples.

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REFERENCES

Aznar, R., Chenoll, E. (2006) Intraspecific diversity of Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus sakei, and Leuconostoc mesenteroides associated with vacuum-packed meat product spoilage analyzed by randomly amplified polymorphic DNA PCR. *J Food Prot*.,69:2403-10.

Brightwell , G., Clemens ,R., Urlich, S., Boerema, J. (2007). Possible involvement of psychrotolerant Enterobacteriaceae in blown pack spoilage of vacuum-packaged raw meats. *Int J Food Microbiol. 119*, 334-3339.

Broda, D. M., Bell, R. G., Boerema, J. A., Musgrave, D R. (2002). The abattoir source of culturable psychrophilic Clostridium spp. Causing "blown pack" spoilage of vacuum-packed chilled venison. *J. Appl. Microbiol. 93*, 817-824.

Broda, D.M., Boerema, J.A., Brightwell, G. (2009). Sources of psychrophilic and psychrotolerant clostridia causing spoilage of vacuum-packed chilled meats, as determined by PCR amplification procedure. *J Appl Microbiol. 107*, 178-186.

Burke, D.J., Chan, C.R. (2010). Effects of the invasive plant garlic mustard (Alliaria petiolata) on bacterial communities in a northern hardwood forest soil. *Can J Microbiol. 56,* 81-86.

Chenoll, E., Macián, M.C., Elizaquível, P., Aznar, R. (2007). Lactic acid bacteria associated with vacuum-packed cooked meat product spoilage: population analysis by rDNA-based methods. *J Appl Microbiol. 102,* 498-508.

Jones, R.J., Zagorec, M., Brightwell, G., Tagg, J.R. (2009). Inhibition by Lactobacillus sakei of other species in the flora of vacuum packaged raw meats during prolonged storage. *Food Microbiol. 26*, 876-881.

Kalchayanand, N., Ray, B., Field, R. A., Johnson, M C. (1989). Spoilage of vacuum-packaged refrigerated beef by *Clostridium. Journal of Food Protection 52*, 424-426.

Lawson, P., Dainty, R. H., Kristiansen, N., Berg, J., Collins, M. D. (1994). Characterisation of a psychrotrophic *Clostridium* causing spoilage in vacuum-packed cooked pork: description of *Clostridium algidicarnis* sp. nov. *Lett. App. Microbiol. 19*, 153-157.

Rademaker, J, L, W., Hoolwerf, J. D., Wagendorp, A. A., Te Giffel, M. C. (2006). Assessment of microbial population dynamics during yoghurt and hard cheese fermentation and ripening by DNA population fingerprinting. *International dairy Journal 16,* 457-466.

Sánchez, J. I., Rossetti, L., Martínez, B., Rodríguez, A., Giraffa, G. (2006). Application of reverse transcriptase PCR-based T-RFLP to perform semi-quantitative analysis of metabolically active bacteria in dairy fermentations. *J Microbiol Methods.* 65, 268-277.

Spring, S., Merkhoffer, B., Weiss, N., Kroppenstedt, R. M., Hippe, H., Stackebrandt, E. (2003).Characterization of novel psychrophilic clostridia from an Antartic microbial mat: description of *Clostridium frigoris* sp. nov., *Clostridium lacusfryxellense* sp. nov., *Clostridium bowmanii* sp. nov. and *Clostridium psychrophilum* sp. nov. and reclassification of *Clostridium laramiense* as *Clostridium estertheticum* subsp. *laramiense* subsp. nov. *Int. J. Syst. Evol. Microbiol. 53*, 1019-1029.