Microbial dynamics in suckling lamb "Lechazo de Castilla y León" packaged under different modified atmospheres by DGGE

Osés S.M.[1], Gómez-Rojo E.M.[1], Wilches-Perez D.[1], Melero B.[1], Diez A.M.[1], Jaime I.[1] Luning, P. A.[2], and Rovira, J.[1]

[1] Burgos University, Department of Biotechnology and Food Science, Burgos, Spain
[2] Wageningen University, Department of Agrotechnology and Food Sciences, The Netherlands

Abstract— **Fresh lamb "Lechazo de Castilla y León" was packed under four different atmospheres: air (A) and three MAP (modified atmospheres packaging) 15%O_2 and 30%CO_2 (C), 70%O_2 and 30%CO_2 (O), and 15%O_2 and 85%CO_2 (H), and monitored during 18 days. Denaturing Gradient Gel electrophoresis (DGGE) was used in order to know the microbiota evolution.**

From a general point of view in control samples (A) appear more microbial species than in MAP samples (C, O, H), being the high CO_2 one (85%) the one where less species appear. *Pseudomonas* spp. dominated in atmosphere A, while LAB dominated in atmospheres C, O and H.

Keywords— **Suckling lamb, DGGE, MAP**

I. INTRODUCTION

The consumption of suckling lamb (reared exclusively on ewe's milk) is appreciated by consumers due to its paler and more delicate flavour [1, 2]. Since 1999, in "Castilla y León", the EU has recognized this meat as Protected Geographical Indication "Lechazo de Castilla y León" (Commission Regulation EEC No 2107/1999).

Nowadays, fresh lamb meat needs to be commercialized in smaller pieces that require a longer shelf life, due to changing consumer purchase habits that involve weekly shopping trips to supermarkets and smaller family sizes. This has led to the study of different strategies to increase shelf-life while maintaining food safety. One such strategy is to use a Modified-Atmosphere Packaging (MAP) environment. However, the quality of fresh lamb meat varies according to storage conditions and atmospheres, which affect properties that are likely to influence its purchase, such as colour, flavour and texture [3] as well as microbial quality [4, 5].

DGGE has previously been used to track spoilage processes in fresh meat [6, 7]. However, to the best of our knowledge, no study has examined fresh suckling lamb meat packaged under different atmospheres. It must be taken in account that due to the pink pale colour of this kind of meat, it can not be considered as typical red meat, as it is the one coming from older animals (lamb or sheep).

Thus, the aim of this study is assesses the shelf life of "Lechazo de Castilla y León" suckling lamb under different modified atmospheres, evaluating microbial dynamics throughout the storage life of the product using DGGE.

II. MATERIALS AND METHODS

A. Meat samples

Hind legs of suckling lamb were provided, 24h after slaughter, by a local meat processing company. All the lambs had been raised on the same farm and passed through the same slaughterhouse. They had all been suckled on ewe's milk until slaughter after which the carcasses that weighed between 5 and 6.5 kg were chilled, and within a 24h period, the hind legs and forelegs were removed.

B. Packaging and storage conditions

The hind legs were immediately placed in 150 x 260 x 80 mm white semi-rigid trays (Sanviplast, Barcelona, Spain) made of Polyethylene/Ethylene Vinyl Alcohol/Polystyrene (PE/EVOH/PS), which have oxygen and CO_2 transmission rates of 0.99 cm^3 m^{-2} day^{-1} atm^{-1} and 0.55 cm^3 m^{-2} day^{-1} atm^{-1}, respectively, at 25ºC and 75% relative humidity (RH). They were covered with a Polyethylene Tereftalato coating with Polyvinylidene chloride/Polyethylene (PETPVdC/PE) film (Amcor Flexibles, Burgos, Spain), which has oxygen and CO_2 transmission rates

of 7 cm^3 m^{-2} day^{-1} atm^{-1} and 20 cm^3 m^{-2} day^{-1} atm^{-1}, respectively at 23ºC and 65% RH. One hundred samples were divided into four equal batches (25 samples) and packaged under the following gas mixtures: control (air, A) covered with a conventional polyethylene film (kitchen cling-film for domestic use), a commercial atmosphere (C) of 15%/30%/55% (O_2/CO_2/N_2), an atmosphere rich in oxygen (O) 70%/30% (O_2/CO_2) and an atmosphere rich in CO_2 (H) 15%/85% (O_2/CO_2). Gas mixtures were prepared using a mixer machine WITT-Gasetechnik (WITT-Gasetechnik GmbH & Co KG, Witten, Germany). The samples were stored in the dark under refrigeration (4 ± 0.5ºC) for 18 days. Each batch was studied by DGGE at 0, 4, 7, 11, 14 and 18 days of storage. The analysis at day 0 was prior to packaging and they are the same samples for all batches.

C. DGGE analysis

DNA extraction: Duplicate analyses were performed on three different packages on each pre-defined day. Twenty-five grams of sterile weighted meat was diluted in 225 ml of peptone water buffer (BPW, AES Chemunex, Bruz, France), and homogenized for 120 s in a lab blender (PBI, Milan, Italy). For the extraction of DNA, approximately 50 ml of the homogenate was transferred from each sample to a sterile tube and the debris was allowed to settle for about 5 min. A further 1/10 dilution in Ringer solution (Oxoid) was performed, of which 1 ml was subjected to centrifugation at 13,400 rpm for 10 min at 4ºC. The resulting pellet was placed in storage at -70ºC while awaiting extraction. DNA extraction was done follow the protocol describes by Diez et al. [8] for the bulk cells.

DNA amplification: Primers 338f, 5' ACT CCT ACG GGA GGC AGC AG 3' and 518r 5' ATT ACC GCG GCT GCT GG 3' [9, 10] were used for DNA amplification in a reaction mixture containing 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), 1.25 U of Taq polymerase (Applied Biosystems, Alcobendas, Madrid, Spain) and 0.2 µM concentrations of each primer. Five microlitres of template DNA (50 ng total) were added to the mixture. Amplifications in a Mastercycler gradient (Eppendorf, Madrid, Spain) were performed in a final volume of 50 µl by using an amplification characterized by one cycle at 95ºC for 5 min, then 30 cycles of denaturation at 95ºC for 1 min, annealing at 46ºC for 1 min and extension at 72ºC for 1.5 min. A final extension at 72ºC for 5 min ended the amplification cycle. Agarose gel electrophoresis (2% w/v) in TBE buffer containing 0.5 µg/ml ethidium bromide (Sigma) was used to assess the presence of the PCR product. A GC clamp (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') was added to primer 338f (gc338f) when used for the DGGE analysis [11].

DGGE analysis: This analysis was performed follow the protocol described by Diez et al. [8] but using a polyacrylamide-bisacrilamide gel with a denaturant gradient from 30 to 70% (100% corresponded to 7M urea and 40% [wt/vol] formamide) increasing in the direction of the electrophoretic run. Gels were subjected to a constant voltage of 120V for 6h at 60ºC. Finally, the eluted DNA was purified with the QIAquick PCR Purification kit (QUIAGEN GmbH, Hilden, Germany) and sent for sequencing to a commercial facility (MWG, Biotech, Germany). The BLAST program was used for the analysis of the DNA sequences.

III. RESULTS

The DGGE profiles of the packed fresh lamb samples are shown in Figure 1. Band identification by 16S rRNA gene sequencing is reported in Table 1. From a general point of view, more microbial species appear in control samples (A) than in the modified atmospheres (C, O, H), atmosphere H (85% CO_2) having the fewest species. The atmospheres with a CO_2 (30%) medium (C and O) and different O_2 concentrations (C: 15%; O: 70%) appear to follow similar behaviour. Therefore, it appears that the oxygen concentration did not modify the microbial population in the fresh lamb, while the CO_2 is responsible for the decrease in the microbial population. *Carnobacterium divergens* and *Brochotrix thermosphacta* are the species that always appear throughout the study regardless of the atmosphere, and *Lactobacillus sakei* is also very common. On the contrary, atmospheres with at least 30% of CO_2 (C, O, H) lead to the disappearance of *Pseudomonas* spp. and *Jeotgalicoccus* spp. Higher concentrations of CO_2

(85%) also makes *Psychrobacter* spp disappear. However, 30% CO_2 (C, O) leads to the appearance of other species such as *Acinetobacter* spp. and *Bacillus* spp., although *Bacillus* spp. disappears when the concentration of CO_2 is higher (85%).

Table 1: Identification of excised DGGE gel bands by sequencing and alignment in the Gene Bank using the BLAST program. [a] Bands numbered as indicated on DGGE gel shown in Fig. 1.[b] Accession number of closest relative sequence found with Blast search.

Bands[a]	Size (bp)	Closest relative	%Identity	Source[b]
1	164	*Carnobacterium maltaromaticum*	99	FJ656722
2	160	*Lactobacillus sakei*	93	FJ040202
3	154	*Carnobacterium divergens*	100	FJ656716
4	101	*Bacillus* spp.	96	DQ658919
5	162	*Staphylococcus equorum*	99	GQ169122
6	154	*Bacillus cereus*	100	GQ226038
7	167	*Enterobacter hormaechei*	99	FJ608246
8	162	*Psychrobacter* spp.	98	GQ169118
9	62	*Psychrobacter* spp.	100	AY700222
10	146	*Psychrobacter* spp.	99	AF513418
11	166	*Acinetobacter* spp.	100	EU977657
12	164	*Brochothrix thermosphacta*	99	AY543029
13	167	*Pseudomonas* spp.	99	FM161360
14	162	*Pseudomonas fluorescens*	99	AJ971392
15	165	*Pseudomonas* spp.	99	AM421976
16	101	*Pseudomonas* spp.	100	FN377722
17	125	*Ovis aries*	100	AM711877
18	166	*Jeotgalicoccus* spp.	97	GQ304892
19	147	*Escherichia* spp.	100	DQ857009

IV. DISCUSSION

Pseudomonas spp. was the most common genera detected on day 0, and contaminated wool (from soil) appears to be the major source of these psychrotrophic bacteria [12]. *Pseudomonas* spp. was also the dominant microorganism in atmosphere A throughout storage because it is an important potential competitor due to its fast growth at low temperatures [12, 13, 14]. However, in atmospheres C, O and H, LAB were the predominant species as reported by Kennedy et al. [5] and Sheridan et al. [15]. The shelf-life of fresh meat is limited by the growth and biochemical activities of the *Pseudomonas* spp. species [16]. It was observed in this study that atmospheres O and H both delay the growth of these microorganisms. This agrees with work by Kennedy et al. [5] and Sahoo and Anjaneyulu [17]

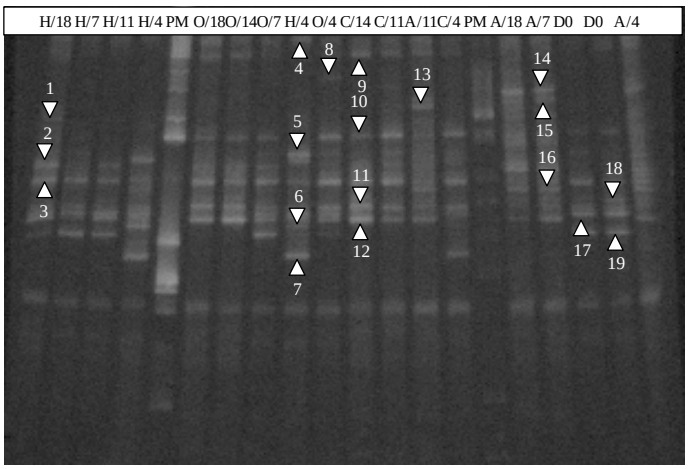

Fig.1 DGGE profiles for the fresh lamb packed at different atmospheres throughout the study (A: control atmosphere (air); C ($15\%O_2/30\%CO_2$); O ($70\%O_2/30\%CO_2$); H ($15\%O_2/85\%CO_2$); PM: patron molecular weight; numbers mean days of storage)

who found that the most effective inhibition of meat spoilage bacteria is achieved at 40%-60% CO_2, although other researchers found that raising CO_2 concentrations over 20% produced little additional inhibition [18].

V. CONCLUSIONS

All the atmospheres modified the original microbial diversity that was recorded at day cero, after which the SSO (Specific Spoilage Organisms) responsible for food deterioration took hold. In a comparison of four treatments, atmosphere H shows a longer shelf-life, probably because it inhibits and/or delays the growth of most microorganisms, such as *Pseudomonas* spp. *Acinetobacter* spp., *Bacillus* spp. and *Psychrobacter spp.*

AKNOWLEDGEMENTS

This study forms part of the FOOD-CT-2005-007081 (PathogenCombat) project. The PhD grant of Sandra M. Osés was funded by the "Programa de FPU del Ministerio de Educación y Ciencia". The authors would also like to thank the lamb processing company and Sofia Santillana.

REFERENCES

1. Miguélez E, Zumalacárregui JM, Osorio MT et al. (2006) Carcass characteristics of suckling lamb protected by the PGI "Lechazo de Castilla y León" European quality label: Effect of breed, sex and carcass weight. Meat Sci 73: 82-89
2. Sañudo C, Alfonso M, San Julián R, et al. (2007) Regional variation in the hedonic evaluation of lamb meat from diverse production systems by consumers in six European countries. Meat Sci 75: 610-621
3. Vergara H, Gallego L (2001) Effects of gas composition in modified atmosphere packaging on the meat quality of Spanish Manchega lamb. J Sci Food Agr 81: 1353-1357
4. Berruga MI, Vergara H, Gallego L (2005) Influence of packaging conditions on microbial and lipid oxidation in lamb meat. Small Ruminant Res 57: 257-264
5. Kennedy C, Buckley DJ, Kerry JP (2004) Display life of sheep meats retail packaged under atmospheres of various volumes and compositions. Meat Sci 68: 649-658
6. Ercolini D, Russo F, Torrieri E et al. (2006). Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. Appl Environ Microb 72: 4663-4671
7. Fontana C, Cocconcelli PS, Vignolo G (2006) Direct molecular approach to monitoring bacterial colonization on vacuum-packaged beef. Appl Environ Microb 72: 5618-5622
8. Diez AM, Urso R, Rantsiou K et al. (2008) Spoilage of blood sausages morcilla de Burgos treated with high hydrostatic pressure. Int J Food Microbiol 123: 246-253
9. Ampe F, Ben Omar N, Moizan C et al. (1999) Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. Appl Environ Microb 65: 5464-5473
10. Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. App Environ Microb 59: 695-700
11. Shelfield VC, Cox DR, Lerman LS et al. (1989) Attachment of a 40-base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction resultsin improved detection of single-base changes. P Natl Acad Sci 86: 297-303
12. Prieto M, Garcia-Lopez ML, Garcia-Armesto MR et al. (1993) Factors affecting spoilage microflora succession on lamb carcasses at refrigeration temperatures. J Appl Bacteriol 74: 521-525
13. Drosinos EH, Board RG (1995) Microbial and physicochemical attributes of minced lamb: Sources of contamination with pseudomonas. Food Microbiol 12: 189-197
14. Nychas GJE, Skandamis PN, Tassou CC et al. (2008) Meat spoilage during distribution. Meat Sci 78: 77-89.
15. Sheridan JJ, Doherty AM, Allen P et al. (1997). The effect of vacuum and modified atmosphere packaging on the shelf-life of lamb primals, stored at different temperatures. Meat Sci 45: 107-117
16. Lambert AD, Smith JP, Dodds KL (1991) Shelf life extension and microbiological safety of fresh meat - A review. Food Microbiol 8: 267-297
17. Sahoo J, Anjaneyulu ASR (1995) Modified atmosphere packaging of muscle foods: Technology, shelf-life and safety aspects. Indian Food Ind 14: 28-36
18. Gill CO (1996). Extending the storage life of raw chilled meats. Meat Sci 43: 99-109