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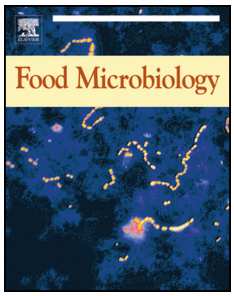

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Title: Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions

Running title: LAB strains spoilage dynamics

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ABSTRACT

A total of 266 lactic acid bacteria (LAB) have been isolated from minced beef stored at 0, 5, 10 and 15°C aerobically and under modified atmosphere packaging consisting of 40% CO_2 - 30% O_2 - 30% N_2 in the presence MAP (+) and absence MAP (-) of oregano essential oil. Sequencing of their 16S rRNA gene along with presence of the *kat*A gene demonstrated dominance of the LAB microbiota by *Leuconostoc* spp. during aerobic storage at 5, 10 and 15°C, as well as during MAP (-) and MAP (+) storage at 10 and 15°C; *Lactobacillus sakei* prevailed during aerobic storage at 0°C, as well as at MAP (-) and MAP (+) storage at 0 and 5° C. The sporadic presence of other species such as *Leuconostoc mesenteroides*, *Weisella viridescens*, *Lactobacillus casei* and *Lactobacillus curvatus* has also been determined. Pulsed-Field Gel Electrophoresis of high molecular weight genomic DNA revealed the dynamics of the isolated LAB strains. Prevalence of *Leuconostoc* spp. was attributed to one strain only. On the other hand, packaging conditions affected *Lb. sakei* strain spoilage dynamics.

Key words; Lactic acid bacteria, meat preservation, PFGE, essential oils, microbial ecology

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INTRODUCTION

Food spoilage microbiota has been the subject of several studies conducted so far; the ones focused on meat and meat products were based on the identification and/or characterization of the dominant microbiota at different storage conditions. The concept of 'succession' of spoilage-related microbial groups i.e. ephemeral / specific spoilage organisms (E/SSO), was only recently, taken into consideration (Ercolini et al. 2006; Chenoll et al. 2007; Nychas et al. 2008).

Lactic acid bacteria (LAB) for instance are considered to be the Specific Spoilage Organisms (SSO) that contribute to the meat spoilage stored under packaging conditions in which the concentration of carbon dioxide is increased (Axelsson 1998; Holzapfel 1998; Nychas and Skandamis 2005). *Lactobacillus*, *Leuconostoc* and *Carnobacterium* are among the most frequently encountered genera on vacuum or modified atmosphere packaged meat and play an important role in the spoilage of refrigerated raw meat (Shaw and Harding 1984; Dainty and Mackey 1992; Hugas et al. 1993; McMullen and Stiles 1993; Rovira et al. 1997; Holzapfel 1998; Labadie 1999; Parente et al. 2001; Nychas and Skandamis 2005). Species of *Leuconostoc* sp. and *Lb. sakei* have been associated with the spoilage of vacuum or modified atmosphere packed meat stored at chill temperatures (Champomier – Verges et al., 2001; Yost and Nattress 2002; Ercolini et al. 2006). The lack of consistency e.g. why these two species were not always found at the end of storage period even if the conditions were similar can be possibly attributed not only to the limitation of the applied methodologies used but also to the potential effect of the man imposed preservation system on the development of the microbial association, e.g., Ephemeral Spoilage Organism (Stanbridge and Davies 1998; Nychas et al. 2008; Vasilopoulos et al., 2010). In this case the word 'ephemeral' does describe the situation where these specific spoilage bacteria contribute to meat spoilage for a very short period of time till the next climax population is established. The identification and

characterization of these ESOs in raw meat under different storage conditions remains still to be elucidated (Jones 2004; Ercolini 2006, 2009; Fontana 2006; Vasilopoulos et al. 2010).

Oregano essential oil, as a potential `hurdle', was found to affect the contribution of spoilage microorganisms to the microbial association as well as to the physico-chemical changes of the minced meat (Skandamis and Nychas 2001, Burt 2004). Skandamis and Nychas (2002) that reported the oregano essential oil effect on microbial population, including LAB, on active packaging conditions. Axelsson (1998) concluded that the addition of oregano essential oil influenced the metabolic activity of LAB. More specifically, the initial heterofermentative microbiota was substituted by an homofermentative one at the end of storage. However, despite the antimicrobial action of essential oil on biota, there is less information about the effect of such compounds on the microbial diversity of the LAB isolated from meat at species and strain level. The only information available relates the essential oil effect on growth of meat spoilage bacteria such as *Lb. sakei*, *Lb. curvatus* and *Carnobacterium piscicola* (Ouattara et al. 1997).

The use of conventional phenotypic methods does not always allow efficient characterization of the microbiota at species level (Holzapfel 1998; Stanbridge and Davies 1998). On the contrary, molecular identification and characterization tools are far more consistent, rapid, reliable and reproducible and can discriminate even between closely related groups of species, which are otherwise indistinguishable on the basis of their phenotype. The advances in molecular techniques are expected to widen the knowledge of spoilage-related bacterial succession during storage of foods (Chenoll et al. 2003; Ercolini et al. 2006). Several molecular typing techniques have been developed during the past decade for the identification and classification of bacteria at strain level. Among them, Pulsed-Field Gel Electrophoresis (PFGE) of DNA fragments resulting from the digestion of whole genomic DNAs with rare-cutting restriction endonucleases has proved to be reliable for bacterial

typing. This method has been used to differentiate members of several genera including *Lactococcus* (Tanskanen et al. 1990), *Clostridia* (Hiel et al. 1998), *Streptomyces* (Leblond et al. 1990), probiotic lactobacilli (Yeung et al. 2004), and to compare the genomic restriction patterns of five *Bifidobacterium breve* strains (Bourget et al. 1993). It is considered to be a discriminating and reproducible method to differentiate strains of intestinal bacteria (O' Sullivan 1999) and for chromosome size estimation in *Lb. acidophilus* (Roussel et al. 1993, Sanders et al. 1996), *Lb. plantarum* (Daniel 1995), and other LAB (Tanskanen et al. 1990). Furthermore, PFGE in association with PCR-based methods are commonly used for strain monitoring (Singh et al. 2009).

The aim of the present study was to systematically monitor the microbial diversity of LAB, isolated from meat stored at different temperatures and under different packaging e.g. aerobic or MAP conditions, at strain level, by using modern molecular tools.

MATERIALS AND METHODS

Sample preparation and storage conditions. Minced beef was purchased from the central market of Athens and prepared according to Argyri et al. (submitted for publication). Briefly, minced beef samples were stored at 0, 5, 10 and 15^{o}C, aerobically or under modified atmospheres packaging (MAP) consisting of 40% CO_2 - 30% O_2 - 30% N_2 with MAP (+) or without MAP (-) the application of volatile compounds of oregano essential oil (2% v/w). The samples were placed on Styrofoam trays; all trays were performed to allow the diffusion of the volatile compounds of the essential oil with both side of the samples. In the case of the treated samples (MAP +), the essential oil was distributed on a whatman paper that was placed on the bottom side of the tray.

Sampling of the meat. Minced beef was sampled at appropriate time intervals, depending on storage temperature; the incubation lasted 650, 482, 386 and 220 hours at 0, 5, 10 and 15^{o}C, respectively and all samples were analysed in dublicate. A detailed description of the methodology employed for the enumeration of the total viable counts, *Pseudomonas* spp., *Br. thermosphacta*, LAB, *Enterobacteriaceae*, yeasts and molds in this work is presented elsewhere (Argyri et al. submitted for publication). Briefly, LAB counts were determined on MRS agar (Biolife, Italiana S.r.l., Milano, Italy) (pH = 5.8) overlaid with the same medium and incubated at 30 ˚C for 72 h. LAB were isolated from the highest dilution from three different time points (initial, middle and final stage of storage) for further analysis; 10% of the colonies (6 to 10 colonies) derived from plate culture of the highest sample dilution. They were randomly selected and purified by successive subculture on MRS agar at 30°C. Gram positive, catalase and oxidase negative isolates were stored at -80˚C in MRS broth (Biolife, Milano, Italy) supplemented with 20% (v/v) glycerol (Merck, Darmstadt, Germany) until further use. Before experimental use each strain was grown twice in MRS broth at 30°C for 24 and 16h respectively. Purity of the culture was always checked on MRS agar plates before use.

Pulsed-Field Gel Electrophoresis (PFGE). PFGE was performed according to Kagkli et al. (2007). Briefly, cells were harvested by centrifugation at 10,000 x *g* for 5 min and washed with 10 mM Tris-HCl (pH 7.6) containing 1 M NaCl; resuspended in 100 μL of the same solution, heated at 37°C for 10 min and mixed with an equal volume of 2% (w/v) low melting-point agarose (Bio-Rad, Hercules, CA, USA) in 0.125 M EDTA pH 7.6 before letting them to solidify in moulds (Bio-Rad). The cells were lysed *in situ* in a solution containing 10 mg mL^{-1} of lysozyme in EC buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM EDTA, 1% (w/v) Sarkosyl, pH 7.6) for 16 h at 37°C. The lytic treatment was repeated with

the same solution containing 2U mL^{-1} mutanolysin. After treatment with proteinase K (0.5M EDTA containing 1% sarkosyl, pH 8) for 24h at 55°C, the agarose blocks were washed twice for 1 h with 1 mM phenylmethylsulfonyl fluoride (PMSF) in 10 mM Tris-HCl containing 1 mM EDTA, (pH 8.0) at 37°C and then stored at 4°C in 10 mM Tris-HCl containing 100 mM EDTA (pH 8.0) until further use.

The agarose blocks were cut with sterile coverslips and slices (1 - 2 mm thick) of the blocks were washed three times at room temperature in 10 mM Tris-HCl containing 0.1 mM EDTA (pH 8.0) for 30 min with gentle agitation. The restriction enzymes *Apa*I and *Sma*I (10U) (New England Biolabs, Ipswich, MA, USA) were initially selected to digest the slices of a limited number of strains. The enzyme that resulted in the production of clearer and sharper PFGE digestion profile was used for the digestion of all isolates. Digestions were performed according to the recommendations of the manufacturer.

Following digestion, slices were loaded into wells of a 1% PFGE grade agarose gel (Bio-Rad) and the gel was run in 0.5 mM Tris-Borate buffer (45 mM Tris–HCl, 45 mM Boric acid, 1 mM EDTA) using a CHEF-DRII PFGE apparatus and cooling module (Bio-Rad) at 6 Volt cm^{-1} for 16h, with a pulse time ramped from 1 to 10s. Gels were then stained with ethidium bromide (0.5 μg ml^{-1}) in water for 1 h and destained for 2 h before being photographed using a GelDoc system (Bio-Rad). Conversion, normalization and further analysis were performed using the Pearson coefficient and UPGMA cluster analysis with Gel compare software, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium; kindly provided by E. Tsakalidou, Dairy Laboratory, Agricultural University of Athens).

DNA extraction and species identification. DNA was extracted according to the protocol described by the manufacturer of GenElute Bacterial Genomic DNA Kit (Sigma, Chemical Co., St. Louis, Mo. USA). Representative number of isolates per distinct PFGE

cluster were selected and subjected to species identification by sequencing the V1-V3 variable region of the 16S rRNA gene as described previously (Paramithiotis et al. 2008). PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (http://www.macrogen.com). The results were aligned with those in GenBank using the BLASTN program in order to determine their closest known relatives of the partial 16S rRNA gene sequence (Altschul et al. 1997). The GenBank/EMBL/DDBJ accession numbers for the 16S-rRNA gene sequences are GU998850 to GU998881 (Table 2).

Detection of the heme - dependent catalase (*kat*A) gene. All isolates were screened by PCR for the presence of the *kat*A gene, encoding heme-dependent catalase (Knauf et al. 1992; Hertel et al. 1998). For this purpose the specific primers 702-F (5'-AATTGCCTTCTTCCGTGTA-3', position 551–536) and 310-R (5'-AGTTGCGCACAATTATTTTC-3', position 127–139) were used.

RESULTS

LAB were found to be the dominant biota in samples stored under MAP supplemented (+) or not (-) with Essential Oil (EO) (results not shown – Argyri et al. submitted for publication). Table 1 summarizes the lactic acid bacterial counts of the initial biota and of the three different time points (initial, middle and final stage of storage) for each of the storage conditions tested. In case of samples stored under MAP (+), the counts of LAB were lower at two times points (middle, end) than the ones stored in air and under MAP (-).

A total of 266 LAB isolates were recovered throughout the storage period; 99 isolates from aerobic storage, 89 isolates from MAP (-) and 78 isolates from MAP (+). The 99 isolates from aerobic storage were subjected to PFGE to determine the strain diversity during storage. For the aforementioned isolates, high molecular weight genomic DNA was digested with two different restriction enzymes (*Apa*I and *Sma*I). *Apa*I restriction generated better distributed bands than *Sma*I allowing a more reliable analysis of the generated profiles (Fig 1). Therefore, *Apa*I was chosen to digest the 89 and 78 isolates from minced beef stored under MAP (-) and MAP (+), respectively.

A large diversity regarding strain occurrence at the different packaging and temperature conditions was revealed (Fig 1, Table 3). The dendrogram obtained after image analysis of the different PFGE patterns, resulted into 32 different profiles, nine of which were obtained from aerobic storage, while 15 and 17 from MAP (-) and MAP (+), respectively (Table 3). Each strain present in Figure 1 was subjected to 16S rRNA gene sequencing. Strains B 225, B 251, B 226, B 236, B 248, B 253, B 228, B 237, B 229, B 255, B 227, B 239, B 230, B 238, B 250, B 252, B 254 and B 249 were assigned to *Lb. sakei*; strains B 245 and B 246 to *Lb. curvatus*; strain B 247 to *Lb. casei*-group; strains B 234 and B 235 to *Ws. viridescens*; strains B 242 and B 243 to *Ln. mesenteroides*-group and strains B 232, B 241, B 258, B 244, B 233, B 240 and B 231 to *Leuconostoc* spp. All isolates were also screened for the presence of the *kat*A gene, specific for *Lb. sakei*. The *kat*A amplification results were in accordance with the ones already described. Two fingerprints (B 232 and B 233 assigned to *Leuconostoc* spp.) were common for all packaging conditions, two (B 226 and B 227 assigned to *Lb. sakei*) were shared between air and MAP (-), one fingerprint (B 230 assigned to *Lb. sakei*) was shared between air and MAP (+), and two fingerprints (B 242 and B 243 assigned to *Ln. mesenteroides*) were shared between MAP (-) and MAP (+).

From the initial stage of storage, two different strains (B 232 and B 233) were recovered, which were assigned to *Leuconostoc* spp. Strain B 233 was the most common isolate, since it was recovered at a percentage of 83.33% of the isolates recovered from the initial stage of storage.

From the 99 isolates from the aerobic storage of minced beef, the largest group was attributed to *Leuconostoc* spp. The corresponding fingerprints were B 231, B 232 and B 233 , with the latter being the most common isolate, representing the dominant biota during storage at 5, 10 and 15°C. The rest of the fingerprints (B 225, B 226, B 227, B 228, B 229 and B 230) were attributed to *Lb. sakei*, which were recovered from 0 and 5°C, but one (B 228) from 10°C. At 0°C, *Lb. sakei* (B 226) was the prevalent one at the final stage of storage. The frequency of isolation and prevalence of the aforementioned isolates obtained from minced beef regarding aerobic storage at 0, 5, 10 and 15°C is shown in Table 3.

Fifteen different fingerprints were detected during storage of minced beef under MAP (-); they were assigned to *Lb. sakei* (B 226, B 227, B 236, B 237, B 238 and B 239), *Leuconostoc* spp. (B 232, B233, B 258, B 240 and B 241), *Ws. viridescens* (B 234 and B 235), and *Ln. mesenteroides* (B 242 and B 243). Table 3 presents the frequency of isolation and prevalence of the isolates regarding the storage temperature. At 10 and 15°C, *Leuconostoc* spp. (B 233) represented the dominant biota, whilst *Ws. viridescens* (B 234), *Lb. sakei* (B 237), *Ln. mesenteroides* (B 243) and *Leuconostoc* spp. (B 240) were also recovered. At chill temperatures (0 and 5°C), the strain diversity was increased, since 11 different fingerprints were recovered. In both temperatures, *Lb. sakei* (B 237) was the prevalent strain at the final stage of storage. Moreover, *Lb. sakei* (B 236, B 238 and B 239), and *Leuconostoc* spp. (B 233 and B 258) were also recovered from storage at 0°C, whilst *Ws. viridescens* (B 235), *Lb. sakei* (B 226, B 238 and B 227), *Leuconostoc* spp. (B 233 and B 241) and *Ln. mesenteroides* (B 242) were recovered from storage at 5°C.

Out of 78 isolates, seventeen different fingerprints were obtained during storage of minced beef under MAP (+), indicating the increased diversity of the isolates. The frequency of isolation and prevalence of these isolates regarding storage at 0, 5, 10 and 15°C is shown in Table 3. At 10 and 15°C, *Leuconostoc* spp. (B 233) was the most common isolate, representing the dominant strain, whilst *Lb. sakei* (B 252 and B 255) and *Ln. mesenteroides* (B 243) were also recovered. *Lb. sakei* (B 254) was the dominant strain at the final stage of storage at 5°C, while *Lb. sakei* (B 248 and B 255), and *Leuconostoc* spp. (B 233 and B 244) were also recovered during the storage at 5°C. At the final stage of storage at 0°C, *Leuconostoc* spp. (B 233), *Ln. mesenteroides* (B 242), *Lb. curvatus* (B 246) and *Lb. sakei* (B 249 and B 251) were equally contributed. *Lb. curvatus* (B 245), *Lb. casei* and *Lb. sakei* (B 248, B 250 and B 253) were also recovered during storage at 0°C.

DISCUSSION

Spoilage and spoilage progress of meat and meat products have been the subject of several studies conducted so far (Borch et al. 1996; Stanbridge and Davies 1998; Labadie 1999; Skandamis and Nychas 2002; Nychas and Skandamis 2005; Nychas et al. 2008). Nevertheless, meat spoilage has only been associated with the physicochemical and microbiological analysis of the bacterial loads ignoring the spoilage potential of a specific bacterial species or strain (Skandamis and Nychas 2002). Only recently did research took into consideration the specific characteristics of the spoilage microbiota of the meat products and its contribution to the deterioration of the product (Cocolin et al. 2004; Rantsiou et al. 2005; Ercolini et al. 2006, 2009; Vasilopoulos et al. 2010).

The present study focused on the evaluation of the microbial diversity of LAB isolated from minced beef stored under different storage conditions at strain level. Storage

conditions had an important effect on the diversity of the microbial population, since different strains were recovered during the storage of meat under different conditions. These findings strengthen the opinion that the storage temperature and the modified atmosphere packaging affect the spoilage potential of LAB (Stanbringe and Davies 1998; Ercolini et al. 2006, 2009). Moreover, this observation can be explained by the fact that different metabolic activities occur when different species/strains are present and when meat is stored under specific conditions. Not all species belonging to the same bacterial group, e.g., LAB, necessarily grow at the same temperature. It might therefore be misleading the fact that selective media only are used for the determination of the spoilage biota. Further characterization of the isolates grown on the selective plates should be demanded if a better insight and understanding of the phenomenon is required. This is in accordance with the observations of Ercolini et al, (2006) who reported that different species / strains were isolated from beef although similar counts were determined. Ercolini et al. (2006) also mentioned that the viable counts alone may not be enough to highlight the shifts of the bacterial communities depending on the environmental changes and species that are actually involved in meat spoilage.

Among the species listed in Table 2, several meat associated ones were identified. Holzapfel (1998) reported that more rarely *Lb. plantarum* and *Lb. casei* are associated with meat systems and in lower frequency and numbers than *Lb. curvatus* and *Lb. sakei*; the presence of *Ws. viridescens* in raw meat has been also described. Moreover, *Lb. curvatus, Lb. sakei* and *Leuconostoc* spp. have been found to indicate a mixture community of vacuum packed (vp) beef (Yost and Nattress 2002). *Leuconostocs* have been identified as predominant organisms in beef stored under vp/MAP (Stanbringe and Davis 1998; Yost and Nattress 2002) and their presence in the initial mesophilic bacterial microbiota is very frequent (Borch et al. 1996). *Lb. sakei* has been associated with fresh meat (Champomier – Verges et al. 2001) as well as spoilage of a variety of meat products both under vacuum and

299 modified atmosphere packaging (Ercolini et al. 2006, 2009) and it is known to be among the
300 most psychrotrophic lactobacilli. It has also been found to be the dominant spoilage LAB
301 during storage at chill temperatures (Ercolini et al. 2006; Chenoll et al. 2007).

302 PFGE has also provided important information in relation to the strain distribution of
303 the LAB population which would have not been acquired if strain typing had not been
304 performed. Within the LAB population of the present study, *Leuconostoc* spp. and *Lb. sakei*
305 were identified as significant members of the microbiota at abuse and chill temperatures,
306 respectively. More accurately, *Leuconostoc* spp. (B 233) that was initially present at high
307 levels, dominated eventually the microbiota of the minced beef stored at abuse temperatures
308 at all packaging conditions. Although, it was persistent throughout storage at chill
309 temperatures, *Lb. sakei* strains dominated the LAB population only at the final stage of
310 storage. However, some degree of microbial variability was detected at the final stage of
311 storage of meat at chill temperatures, since different *Lb. sakei* strains were the most prevalent
312 ones at the different packaging conditions. Indeed, *Lb. sakei* (B 226), (B 237) and (B 245)
313 dominated the LAB population at 0°C under aerobic conditions, at 0 and 5°C under MAP (-)
314 and at 5°C under MAP (+). This finding is of great importance since it shows the
315 intraspecies variability of *Lb. sakei* and the ability of certain strains to adapt to the different
316 storage conditions outgrowing the other.

317 Dominance of *Leuconostoc* spp. at relatively higher temperatures can be partially
318 attributed to the favourable environmental conditions and partially to the shorter generation
319 time (Harris 1998), both of which enabled it to outgrow *Lb. sakei* strains which were indeed
320 detected as a secondary microbiota. On the other hand, dominance of *Lb. sakei* strains at chill
321 temperatures can be attributed partly to its psychrotrophic nature.

322 From the different LAB detected throughout the storage under MAP (-) and MAP (+),
323 a wide range of strains were sporadically present, especially at chill temperatures. This

finding indicates that modified atmosphere packaging resulted in a development of a totally different spoilage ecosystem. It has been previously reported (Jay 2000), that during storage of meat under MAP, the initial heterofermentative microbiota was substituted by a homofermentative one at the end of storage. Moreover, the MAP and the presumed activity of oregano essential oil against heterofermentative LAB species (Axelsson 1998) seem to have provided the latter with an ecological advantage over *leuconostocs*.

The findings of the present study were based on the culture-dependent approach, most frequently applied when storage studies are performed. Selective media have been used for isolation and subsequent characterisation of the microbiota; stressed or injured cells might not have managed to recover and grow, resulting in their non isolation from the plates and giving therefore the impression that they were absence from the system under investigation. A bias is therefore inserted which could have an effect on the description of the microbial community present. Except from the factors mentioned above, random selection of colonies is required to have a representative sample. This is not always possible because it depends on the person performing the task and it is therefore not objective.

A culture-independent approach could have been an alternative to the plates used for the characterization of the different microbiota (Cocolin et al. 2004). Nevertheless, even this approach has drawbacks which lie in the fact that species have to be above the detection limit (10^4 cfu g^{-1}) and very frequently, the dominant species prevents evidence of the less abundant ones. Primer affinity to the target has also an effect on the amplification and therefore on the species identified. In order to clarify possible discrepancies between culture-dependent and independent methods, and to evaluate whether these differences would give a different overview of the ecology of the meat stored at the conditions mentioned, a similar study could be performed applying both approaches in the future. This investigation lies beyond the scope of the present study, which was actually focused on elucidating the effect of the different

storage conditions with or without the presence of essential oil on the dynamics of LAB strains.

The present study did provide an insight of the population dynamics of LAB strains in relation to the temperature and the packaging conditions. It has been clearly demonstrated that certain species and/or strains are present or dominant only under certain conditions. This finding is extremely important since studies conducted so far had only taken into consideration the microbiological counts as an indication of the spoilage process, and had ignored the possibility that different species or strains would prevail under different storage and/or packaging conditions. The qualitative information derived from the microbiological analyses and the characterization of the species or even the strains present were not evaluated previously. It has been shown that storage temperature combined with packaging conditions induced the selectivity of the spoilage LAB microbiota. Moreover, the microbiota recovered from the initial stage of storage was markedly different from that of the final stage of storage at chill temperatures. The above observations are of great importance and, to our opinion, fundamental in understanding the spoilage process and in explaining the presence of different products or by-products that occur during the different dynamic storage conditions (Skandamis and Nychas 2002).

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374 **REFERENCES**

375

376 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and
377 Lipman, D. J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein
378 database search programs. Nucleic Acids Res. 25, 3389-3402.

379 Argyri, A. A., Doulgeraki, A. I., Blana, V. A., Panagou, E. Z. and Nychas G – J. E.
380 (Revised submitted for publication). Potential of a simple HPLC based approach to
381 quantify spoilage of minced beef stored at different temperatures and packaging systems.
382 Revision FM-S-09-00571R1

383 Axelsson, L. T., 1998. Lactic acid bacteria: Classification and physiology. In: Lactic Acid
384 Bacteria Microbiology and Functional Aspects. S. Salminen. and A. Von Wright (eds.),
385 New York, Marcel Dekker. pp. 1-72

386 Borch, E., Kant-Muemans, M.-L. and Blixt, Y., 1996. Bacterial spoilage of meat and
387 cured meat products. Int. J. Food Microbiol. 33, 103-120.

388 Bourget, N., Simonet, J. M. and Decaris, B., 1993. Analysis of the genome of the five
389 *Bifidobacterium breve* strains: plasmid content, pulsed-field gel electrophoresis genome
390 size estimation and rrn loci number. FEMS Microbiol. Letters 110, 11–20.

391 Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in
392 foods—a review. Int. J. Food Microbiol. 94, 223– 253.

393 Champomier-Verges, M.-C., Chaillou, S., Cornet, M. and Zagorec, M., 2001.
394 *Lactobacillus sakei*: recent developments and future prospects. Res. Microbiol. 152, 839–
395 848.

396 Chenoll, E., Macian, M. C. and Aznar, R., 2003. Identification of Carnobacterium,
397 *Lactobacillus*, *Leuconostoc* and *Pediococcus* by rDNA-based techniques. System. Appl.
398 Microbiol. 26, 546–556.

399 Chenoll, E., Macian, M. C., Elizaquivel, P. and Aznar. R., 2007. Lactic acid bacteria
400 associated with vacuum packed cooked meat product spoilage: population analysis by
401 rDNA based methods. J. Appl. Microbiol. 102, 498-508.
402 Cocolin, L., Innocente, N., Biasutti, M. and Comi, G. 2004. The late blowing in cheese: a
403 new molecular approach based on PCR and DGGE to study the microbia1 ecology of the
404 alteration process. Int. J. Food Microbiol. 90, 83–91.
405 Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C. and Comi, G., 2004. Study
406 of the ecology of fresh sausages and characterization of populations of lactic acid bacteria
407 by molecular methods. Appl. Environ. Microbiol. 70, 1883-1894.
408 Dainty, R. H. and Mackey, B. M., 1992. The relationship between the phenotypic
409 properties of bacteria from chill-stored meat and spoilage processes. J. Appl. Bacterial.
410 Symp. Suppl. 73, 103-114.
411 Daniel, P., 1995. Sizing of the *Lactobacillus plantarum* genome and other lactic acid
412 bacteria species by transverse alternating field electrophoresis. Current Microbiol. 30,
413 243–246.
414 Ercolini, D., Russo, F., Torrieri, E., Masi, P. and Villani, F., 2006. Changes in the
415 spoilage-related microbiota of beef during refrigerated storage under different packaging
416 conditions. Appl. Environ. Microbiol. 72, 4663–4671
417 Ercolini, D., Russo, F., Nasi, A., Ferranti, P. and Villani, F., 2009. Mesophilic and
418 psychrotrophic bacteria from meat and their spoilage potential in vitro and in beef. Appl.
419 Environ. Microbiol. 75, 1990–2001.
420 Fontana, C., Cocconcelli, P. S. and Vignolo, G., 2006. Direct molecular approach to
421 monitoring bacterial colonization on vacuum-packaged beef. Appl. Environ. Microbiol.
422 72, 5618–5622.

Harris, L. J., 1998. The microbiology of vegetable fermentations. In: Microbiology of fermented foods. B. J. B. Wood, (Ed.), London, Blackie Academic Professional, pp. 45-72.

Hertel, C., Schmidt, G., Fischer, M., Oellers, K. and Hammes, W. P., 1998. Oxygendependent regulation of the expression of the catalase gene katA of *Lactobacillus sakei* LTH677. Appl. Environ. Microbiol. 64, 1359–1365.

Hielm, S., Bjorkroth, J., Hyytia, E. and Korkeala, H., 1998. Genomic analysis of *Clostridium botulinum* group II by pulsed-field gel electrophoresis. Appl. Environ. Microbiol. 64, 703–708.

Holzapfel, W. H. 1998. The Gram-positive bacteria associated with meat and meat products. In The Microbiology of Meat and Poultry. R. G. Board and A. R. Davies (eds.), London, U.K., Blackie Academic and Professional. pp.35-84.

Hugas, M., Garriga, M., Aymerich, T. and Monfort, J. M., 1993. Biochemical characterization of lactobacilli from dry fermented sausages. Int. J. Food Microbiol. 18, 107–113.

Jay J. M., 2000. Modern food microbiology. Sixth edition. Gaithersburg, Maryland., An Aspen Publication. pp 288

Jones, R. J., 2004. Observations on the succession dynamics of lactic acid bacteria populations in chill-stored vacuum packaged beef. Int J Food Microbiol. 90, 273–282.

Kagkli, D.-M., Vancanneyt, M, Hill, C., Vandamme, P. and Cogan, T. M., 2007. *Enterococcus* and *Lactobacillus* contamination of raw milk in a farm dairy environment. Int. J. Food Microbiol. 114, 243–251.

Knauf, H. J., Vogel, R. F. and Hammes, W. P., 1992. Cloning, sequence, and phenotypic expression of *kat*A, which encodes the catalase of *Lactobacillus sakei* LTH677. Appl. Environ. Microbiol. 58, 832–839.

448 Labadie, J., 1999. Consequences of packaging on bacterial growth. Meat is an ecological
449 niche. Meat Sci. 52, 299–305.
450 Leblond, P., Francou, F.X., Simonet, J.-M. and Decaris, B, 1990. Pulsed-field gel
451 electrophoresis analysis of the genome of *Streptomyces ambofaciens* strains. FEMS
452 Microbiol. Letters 72, 79–88.
453 McMullen, L. and Stiles, M. E., 1993. Microbial ecology of fresh pork stored under
454 modified atmosphere at - 1, 4.4 and 10°C. Int. J. Food Microbiol. 18, l-14.
455 Nychas, G.-J. E. and Skandamis, P., 2005. Fresh meat spoilage and modified atmosphere
456 packaging (MAP). In Improving the Safety of Fresh Meat. J. N. Sofos (ed.), Cambridge,
457 UK., CRC/Woodhead Publishing Limited. pp. 461–502.
458 Nychas, G.-J. E., Skandamis, P.N., Tassou, C.C and Koutsoumanis, K.P., 2008. Meat
459 spoilage during distribution. Meat Sci. 78, 77-89.
460 Outtara, B., Simard, R. E., Holley, R. A., Piettte, G. J. P. and Begin, A., 1997.
461 Antimicrobial activity of selected fatty acids and essential oils against six meat spoilage
462 organisms. Int. J. Food Microbiol. 37, 155-162.
463 O'Sullivan, D. J. 1999. Methods for analysis of the intestinal microflora, In Probiotics. A
464 Critical Review. G.W. Tannock (ed.), Norfolk, UK., Horizon Scientific Press. p. 23–44.
465 Paramithiotis, S., Kagkli, D.-M., Blana, V. A., Nychas, G.-J. E. and Drosinos, E. H.,
466 2008. Identification and characterization of *Enterococcus* spp. in Greek spontaneous
467 sausage fermentation. J. Food Prot. 71, 1244-1247.
468 Parente, E., Grieco, S. and Crudele, M. A., 2001. Phenotypic diversity of lactic acid
469 bacteria isolated from fermented sausages produced in Basilicata (Southern Italy). J.
470 Appl. Microbiol. 90, 943–952.

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Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G. and Cocolin, L., 2005. Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. Appl. Environ. Microbiol. 71, 1977–1986.

Roussel, Y., Colmin, C., Simonet, J. M. and Decaris B., 1993. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group (Hansen and Mocquot). J. Appl. Bacteriol. 74, 549–556.

Rovira, A., Nieto, J. C., Rodriguez, A., Reguera, J. I. and Gonzalez, Z., 1997. Characterization and selection of lactobacilli isolated from Spanish fermented sausages. Microbiol. 13, 201–208.

Sanders, M. E., Walker, D. C., Walker, K. M., Aoyama, K. and Klaenhammer, T. R., 1996. Performance of commercial cultures in fluid milk applications. J. Dairy Sci. 79, 943–955.

Shaw, B. G. and Harding, C. D., 1984. A numerical taxonomic study of lactic acid bacteria from vacuum-packed beef, pork, lamb and bacon. J. Appl. Bacterial. 56, 25-40.

Singh, S., Goswami, P., Singh, R. and Heller, K. J., 2009. Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: A review. Food Sci. Techn. 42, 448–457.

Skandamis, P. N. and Nychas, G.-J. E., 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. J. Appl. Microbiol. 91, 1011–1022.

Skandamis, P. N. and Nychas, G.-J. E., 2002. Preservation of fresh meat with active and modified atmosphere packaging conditions. Int. J. Food Microbiol. 79, 35–45.

Stanbridge, L. H. and Davies, A. R., 1998. The microbiology of chill stored meat. In The microbiology of meat and poultry. A. Davies and R. Board (eds.), London, UK, Blackie academic & professional. pp 174 - 219.

ACCEPTED MANUSCRIPT

Tanskanen, E.I., Tulloch, D.L., Hillier, A. and Davidson, B.E., 1990. Pulsed-field gel electrophoresis of *Sma*I digests of lactococcal genomic DNA, a novel method of strain identification. Appl. Environ. Microbiol. 56, 3105–3111.

Vasilopoulos, C., De Maere, H., De Mey, E., Paelinck, H., De Vuyst, L. and Leroy, F., 2010. Technology-induced selection towards the spoilage microbiota of artisan-type cooked ham packed under modified atmosphere. Food Microbiol. 27, 77-84.

Yeung, P. S. M., Kitts, C. L., Cano, R., Tong, P. S and Sanders, M. E., 2004. Application of genotyping and phenotyping analyses to commercial probiotic strain identity and relatedness. J.Appl. Microbiol. 97, 1095–1104.

Yost C. K., and Nattress, F. M., 2002. Molecular typing techniques to characterize the development of a lactic acid bacteria community on vacuum-packaged beef. Int. J. Food Microbiol. 72, 97–105.

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513 **FIGURES LEGENDS**

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516 **Figure 1**. Cluster analysis of PFGE *Apa*I digestion fragments of the lactic acid bacteria
517 isolates calculated by the unweighted average pair grouping method. The distance between
518 the pattern of each strain is indicated by the mean correlation coefficient (r%). Strain identity
519 is indicated by the lower and upper case letters.

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524 **Figure 1.**

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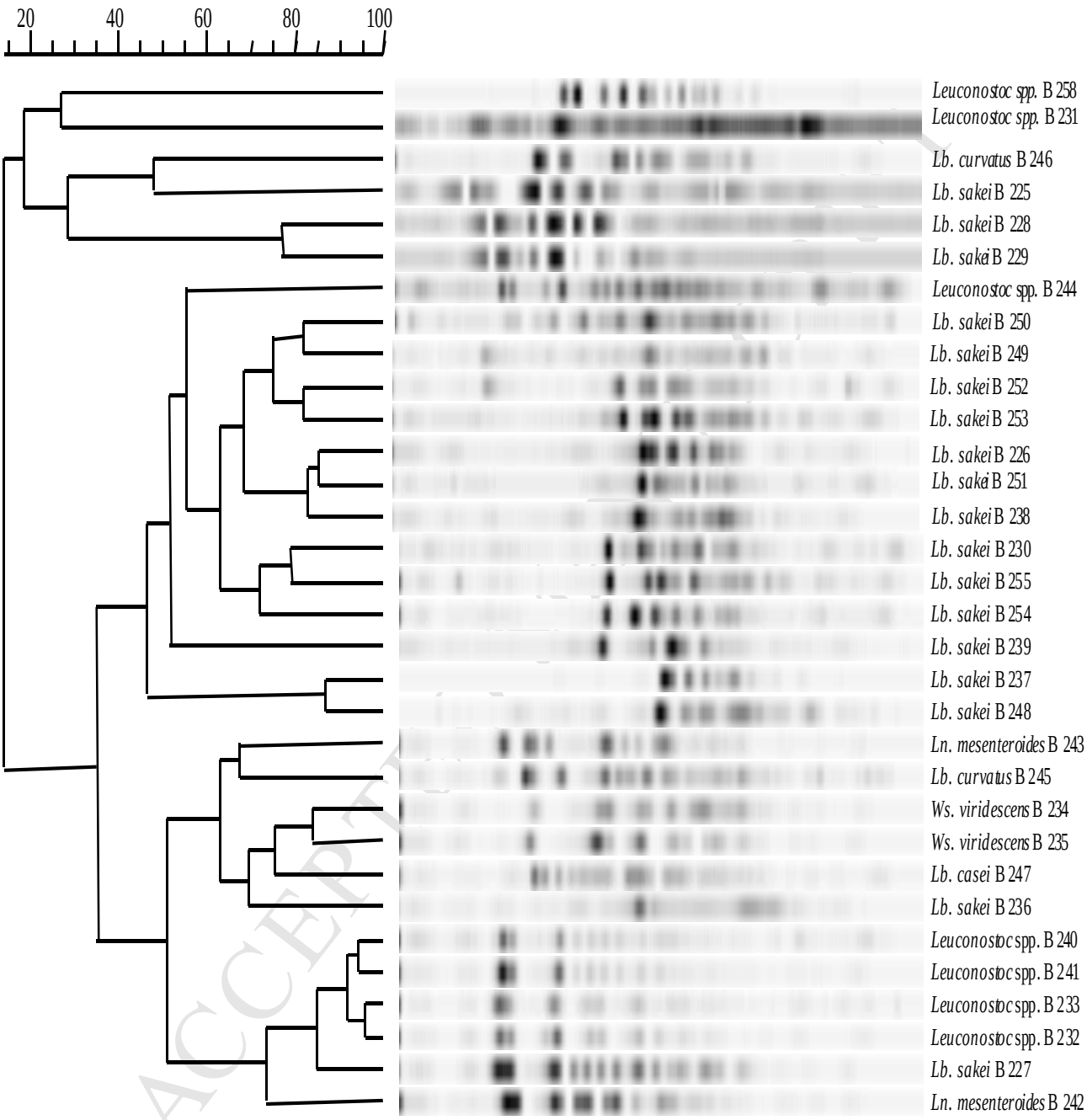

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Table1. Lactic acid bacteria populations in minced meat stored under aerobic, MAP (-) and MAP (+) conditions.

Table 2. Identity of isolates obtained from minced beef

Table 3. Frequency (%) of isolation and distribution of lactic acid bacteria strains isolates recovered from minced beef stored under aerobic, MAP (-) and MAP (+) conditions.

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Temperature (°C)	Storage period	Lactic acid bacteria counts (log CFU g^{-1})[1]		
		Air	MAP -	MAP +
	Initial biota	5.26 ± 0.13	5.26 ± 0.13	5.26 ± 0.13
0°C	Initial	5.33 ± 0.30	5.10 ± 0.11	5.07 ± 0.07
	Middle	6.24 ± 0.12	6.31 ± 0.24	5.48 ± 0.01
	Final	7.30 ± 0.18	7.54 ± 0.11	6.54 ± 0.32
5°C	Initial	6.04 ± 0.09	5.60 ± 0.39	5.60 ± 0.39
	Middle	7.21 ± 0.09	6.74 ± 0.37	6.63 ± 0.10
	Final	7.66 ± 0.07	7.24 ± 0.08	7.47 ± 0.01
10°C	Initial	6.01 ± 0.41	5.97 ± 0.42	5.86 ± 0.07
	Middle	7.41 ± 0.05	7.02 ± 0.17	6.52 ± 0.74
	Final	8.50 ± 0.03	8.56 ± 0.15	7.74 ± 0.11
15°C	Initial	5.86 ± 0.16	6.86 ± 0.08	6.38 ± 0.08
	Middle	7.32 ± 0.02	7.17 ± 0.04	6.70 ± 0.12
	Final	8.62 ± 0.02	8.44 ± 0.01	7.62 ± 0.15

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559 [1] Lactic acid bacteria counts are presented as mean ± standard deviation

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Table 3.

			AIR												MAP -												MAP +											
			0 °C				5 °C			10 °C			15 °C			0 °C			5 °C			10 °C			15 °C			0 °C			5 °C			10 °C			15 °C		
Closest species	Code	Initial	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end	initial	middle	end
Lb. sakei	B 225							6.3												6.3																	
Lb. sakei	B 226			9.1	40.0														50.0																	
Lb. sakei	B 227			9.1																12.5																	
Lb. sakei	B 228								9.1																													
Lb. sakei	B 229				10.0																																
Lb. sakei	B 230							6.3																											11.1		
Leuconostoc spp.	B 231					20.0																															
Leuconostoc spp.	B 232	16.7		9.1	20.0			31.3																													
Leuconostoc spp.	B 233	83.3	100.0	72.7	30.0	80.0	100.0	56.3	90.9	100.0	100.0	100.0	100.0	100.0	40.0	62.5		80.0	50.0	25.0	100.0	100.0	80.0	100.0	100.0	75.0	75.0	42.9	20.0	80.0	44.4	33.3	100.0	100.0	77.8	100.0	100.0	88.9
Ws.viridescens	B 234																						10.0														
Ws.viridescens	B 235																	10.0		12.5																	
Lb. sakei	B 236														20.0																						
Lb. sakei	B 237																100.0			25.0						12.5											
Lb. sakei	B 238															25.0				12.5																	
Lb. sakei	B 239														20.0	12.5																					
Leuconostoc spp.	B 240																									12.5											
Leuconostoc spp.	B 241																	10.0																			
Ln. mesenteroides	B 242																			12.5								20.0									
Ln. mesenteroides	B 243																						10.0														11.1
Leuconostoc spp.	B 244																												11.1								
Lb. curvatus	B 245																											14.3									
Lb. curvatus	B 246																												20.0								
Lb. casei	B 247																										25.0										
Lb. sakei	B 248																											14.3		20.0	33.3						
Lb. sakei	B 249																												20.0								
Lb. sakei	B 250																											14.3									
Lb. sakei	B 251																												20.0								
Lb. sakei	B 252																																		11.1		
Lb. sakei	B 253																											14.3									
Lb. sakei	B 254																															66.7					
Lb. sakei	B 255																														11.1						
Leuconostoc spp.	B 258														20.0																						

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Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions

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