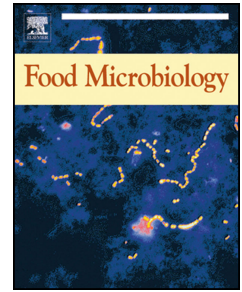


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Title: Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions

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

3

4 **Running title:** LAB strains spoilage dynamics

5

6

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ABSTRACT

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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% CO₂ - 30% O₂ - 30% N₂ in the presence MAP (+) and absence MAP (-) of oregano essential oil. Sequencing of their 16S rRNA gene along with presence of the *katA* 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*, *Weissella 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

51

INTRODUCTION

52 Food spoilage microbiota has been the subject of several studies conducted so far; the
53 ones focused on meat and meat products were based on the identification and/or
54 characterization of the dominant microbiota at different storage conditions. The concept of
55 ‘succession’ of spoilage-related microbial groups i.e. ephemeral / specific spoilage organisms
56 (E/SSO), was only recently, taken into consideration (Ercolini et al. 2006; Chenoll et al.
57 2007; Nychas et al. 2008).

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

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

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

90 The use of conventional phenotypic methods does not always allow efficient
91 characterization of the microbiota at species level (Holzapfel 1998; Stanbridge and Davies
92 1998). On the contrary, molecular identification and characterization tools are far more
93 consistent, rapid, reliable and reproducible and can discriminate even between closely related
94 groups of species, which are otherwise indistinguishable on the basis of their phenotype. The
95 advances in molecular techniques are expected to widen the knowledge of spoilage-related
96 bacterial succession during storage of foods (Chenoll et al. 2003; Ercolini et al. 2006).
97 Several molecular typing techniques have been developed during the past decade for the
98 identification and classification of bacteria at strain level. Among them, Pulsed-Field Gel
99 Electrophoresis (PFGE) of DNA fragments resulting from the digestion of whole genomic
100 DNAs with rare-cutting restriction endonucleases has proved to be reliable for bacterial

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

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

113

114

MATERIALS AND METHODS

115 **Sample preparation and storage conditions.** Minced beef was purchased from the central
116 market of Athens and prepared according to Argyri et al. (submitted for publication). Briefly,
117 minced beef samples were stored at 0, 5, 10 and 15°C, aerobically or under modified
118 atmospheres packaging (MAP) consisting of 40% CO₂ - 30% O₂ - 30% N₂ with MAP (+) or
119 without MAP (-) the application of volatile compounds of oregano essential oil (2% v/w).

120 **The samples were placed on Styrofoam trays; all trays were performed to allow the diffusion**
121 **of the volatile compounds of the essential oil with both side of the samples. In the case of the**
122 **treated samples (MAP +), the essential oil was distributed on a whatman paper that was**
123 **placed on the bottom side of the tray.**

124

125 **Sampling of the meat.** Minced beef was sampled at appropriate time intervals, depending on
126 storage temperature; the incubation lasted 650, 482, 386 and 220 hours at 0, 5, 10 and 15°C,
127 respectively and all samples were analysed in duplicate. A detailed description of the
128 methodology employed for the enumeration of the total viable counts, *Pseudomonas* spp., *Br.*
129 *thermosphacta*, LAB, *Enterobacteriaceae*, yeasts and molds in this work is presented
130 elsewhere (Argyri et al. submitted for publication). Briefly, LAB counts were determined on
131 MRS agar (Biolife, Italiana S.r.l., Milano, Italy) (pH = 5.8) overlaid with the same medium
132 and incubated at 30 °C for 72 h. LAB were isolated from the highest dilution from three
133 different time points (initial, middle and final stage of storage) for further analysis; 10% of
134 the colonies (6 to 10 colonies) derived from plate culture of the highest sample dilution. They
135 were randomly selected and purified by successive subculture on MRS agar at 30°C. Gram
136 positive, catalase and oxidase negative isolates were stored at -80°C in MRS broth (Biolife,
137 Milano, Italy) supplemented with 20% (v/v) glycerol (Merck, Darmstadt, Germany) until
138 further use. Before experimental use each strain was grown twice in MRS broth at 30°C for
139 24 and 16h respectively. Purity of the culture was always checked on MRS agar plates before
140 use.

141
142 **Pulsed-Field Gel Electrophoresis (PFGE).** PFGE was performed according to Kagkli et al.
143 (2007). Briefly, cells were harvested by centrifugation at 10,000 x g for 5 min and washed
144 with 10 mM Tris-HCl (pH 7.6) containing 1 M NaCl; resuspended in 100 µL of the same
145 solution, heated at 37°C for 10 min and mixed with an equal volume of 2% (w/v) low
146 melting-point agarose (Bio-Rad, Hercules, CA, USA) in 0.125 M EDTA pH 7.6 before
147 letting them to solidify in moulds (Bio-Rad). The cells were lysed *in situ* in a solution
148 containing 10 mg mL⁻¹ of lysozyme in EC buffer (6 mM Tris-HCl, 1 M NaCl, 100 mM
149 EDTA, 1% (w/v) Sarkosyl, pH 7.6) for 16 h at 37°C. The lytic treatment was repeated with

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

155 The agarose blocks were cut with sterile coverslips and slices (1 - 2 mm thick) of the
156 blocks were washed three times at room temperature in 10 mM Tris-HCl containing 0.1 mM
157 EDTA (pH 8.0) for 30 min with gentle agitation. The restriction enzymes *ApaI* and *SmaI*
158 (10U) (New England Biolabs, Ipswich, MA, USA) were initially selected to digest the slices
159 of a limited number of strains. The enzyme that resulted in the production of clearer and
160 sharper PFGE digestion profile was used for the digestion of all isolates. Digestions were
161 performed according to the recommendations of the manufacturer.

162 Following digestion, slices were loaded into wells of a 1% PFGE grade agarose gel (Bio-
163 Rad) and the gel was run in 0.5 mM Tris-Borate buffer (45 mM Tris-HCl, 45 mM Boric acid,
164 1 mM EDTA) using a CHEF-DRII PFGE apparatus and cooling module (Bio-Rad) at 6 Volt
165 cm^{-1} for 16h, with a pulse time ramped from 1 to 10s. Gels were then stained with ethidium
166 bromide ($0.5\ \mu\text{g mL}^{-1}$) in water for 1 h and destained for 2 h before being photographed using
167 a GelDoc system (Bio-Rad). Conversion, normalization and further analysis were performed
168 using the Pearson coefficient and UPGMA cluster analysis with Gel compare software,
169 version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium; kindly provided by E.
170 Tsakalidou, Dairy Laboratory, Agricultural University of Athens).

171

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

175 cluster were selected and subjected to species identification by sequencing the V1-V3
176 variable region of the 16S rRNA gene as described previously (Paramithiotis et al. 2008).
177 PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden,
178 Germany) according to the manufacturer's instructions and directly sequenced with an ABI
179 3730 XL automatic DNA sequencer by Macrogen (<http://www.macrogen.com>). The results
180 were aligned with those in GenBank using the BLASTN program in order to determine their
181 closest known relatives of the partial 16S rRNA gene sequence (Altschul et al. 1997). The
182 GenBank/EMBL/DDBJ accession numbers for the 16S-rRNA gene sequences are GU998850
183 to GU998881 (Table 2).

184

185 **Detection of the heme - dependent catalase (*kata*) gene.** All isolates were screened by
186 PCR for the presence of the *kata* gene, encoding heme-dependent catalase (Knauf et al.
187 1992; Hertel et al. 1998). For this purpose the specific primers 702-F (5'-
188 AATTGCCTTCTCCGTGTA-3', position 551–536) and 310-R (5'-
189 AGTTGCGCACAATTATTTTC-3', position 127–139) were used.

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RESULTS

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

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

208 A large diversity regarding strain occurrence at the different packaging and
209 temperature conditions was revealed (Fig 1, Table 3). The dendrogram obtained after image
210 analysis of the different PFGE patterns, resulted into 32 different profiles, nine of which were
211 obtained from aerobic storage, while 15 and 17 from MAP (-) and MAP (+), respectively
212 (Table 3). Each strain present in Figure 1 was subjected to 16S rRNA gene sequencing.
213 Strains B 225, B 251, B 226, B 236, B 248, B 253, B 228, B 237, B 229, B 255, B 227, B
214 239, B 230, B 238, B 250, B 252, B 254 and B 249 were assigned to *Lb. sakei*; strains B 245
215 and B 246 to *Lb. curvatus*; strain B 247 to *Lb. casei*-group; strains B 234 and B 235 to *Ws.*
216 *viridescens*; strains B 242 and B 243 to *Ln. mesenteroides*-group and strains B 232, B 241, B
217 258, B 244, B 233, B 240 and B 231 to *Leuconostoc* spp. All isolates were also screened for
218 the presence of the *katA* gene, specific for *Lb. sakei*. The *katA* amplification results were in
219 accordance with the ones already described. Two fingerprints (B 232 and B 233 assigned to
220 *Leuconostoc* spp.) were common for all packaging conditions, two (B 226 and B 227
221 assigned to *Lb. sakei*) were shared between air and MAP (-), one fingerprint (B 230 assigned
222 to *Lb. sakei*) was shared between air and MAP (+), and two fingerprints (B 242 and B 243
223 assigned to *Ln. mesenteroides*) were shared between MAP (-) and MAP (+).

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

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

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

249 Out of 78 isolates, seventeen different fingerprints were obtained during storage of
250 minced beef under MAP (+), indicating the increased diversity of the isolates. The frequency
251 of isolation and prevalence of these isolates regarding storage at 0, 5, 10 and 15°C is shown
252 in Table 3. At 10 and 15°C, *Leuconostoc* spp. (B 233) was the most common isolate,
253 representing the dominant strain, whilst *Lb. sakei* (B 252 and B 255) and *Ln. mesenteroides*
254 (B 243) were also recovered. *Lb. sakei* (B 254) was the dominant strain at the final stage of
255 storage at 5°C, while *Lb. sakei* (B 248 and B 255), and *Leuconostoc* spp. (B 233 and B 244)
256 were also recovered during the storage at 5°C. At the final stage of storage at 0°C,
257 *Leuconostoc* spp. (B 233), *Ln. mesenteroides* (B 242), *Lb. curvatus* (B 246) and *Lb. sakei* (B
258 249 and B 251) were equally contributed. *Lb. curvatus* (B 245), *Lb. casei* and *Lb. sakei* (B
259 248, B 250 and B 253) were also recovered during storage at 0°C.

260

261

DISCUSSION

262

263 Spoilage and spoilage progress of meat and meat products have been the subject of
264 several studies conducted so far (Borch et al. 1996; Stanbridge and Davies 1998; Labadie
265 1999; Skandamis and Nychas 2002; Nychas and Skandamis 2005; Nychas et al. 2008).
266 Nevertheless, meat spoilage has only been associated with the physicochemical and
267 microbiological analysis of the bacterial loads ignoring the spoilage potential of a specific
268 bacterial species or strain (Skandamis and Nychas 2002). Only recently did research took into
269 consideration the specific characteristics of the spoilage microbiota of the meat products and
270 its contribution to the deterioration of the product (Cocolin et al. 2004; Rantsiou et al. 2005;
271 Ercolini et al. 2006, 2009; Vasilopoulos et al. 2010).

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

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

289 Among the species listed in Table 2, several meat associated ones were identified.
290 Holzapfel (1998) reported that more rarely *Lb. plantarum* and *Lb. casei* are associated with
291 meat systems and in lower frequency and numbers than *Lb. curvatus* and *Lb. sakei*; the
292 presence of *Ws. viridescens* in raw meat has been also described. Moreover, *Lb. curvatus*, *Lb.*
293 *sakei* and *Leuconostoc* spp. have been found to indicate a mixture community of vacuum
294 packed (vp) beef (Yost and Nattress 2002). *Leuconostocs* have been identified as
295 predominant organisms in beef stored under vp/MAP (Stanbringe and Davis 1998; Yost and
296 Nattress 2002) and their presence in the initial mesophilic bacterial microbiota is very
297 frequent (Borch et al. 1996). *Lb. sakei* has been associated with fresh meat (Champomier –
298 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

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

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

339 A culture-independent approach could have been an alternative to the plates used for
340 the characterization of the different microbiota (Cocolin et al. 2004). Nevertheless, even this
341 approach has drawbacks which lie in the fact that species have to be above the detection limit
342 (10^4 cfu g⁻¹) and very frequently, the dominant species prevents evidence of the less abundant
343 ones. Primer affinity to the target has also an effect on the amplification and therefore on the
344 species identified. In order to clarify possible discrepancies between culture-dependent and
345 independent methods, and to evaluate whether these differences would give a different
346 overview of the ecology of the meat stored at the conditions mentioned, a similar study could
347 be performed applying both approaches in the future. This investigation lies beyond the scope
348 of the present study, which was actually focused on elucidating the effect of the different

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

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

366

367

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368

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FIGURES LEGENDS

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516 **Figure 1.** Cluster analysis of PFGE *ApaI* 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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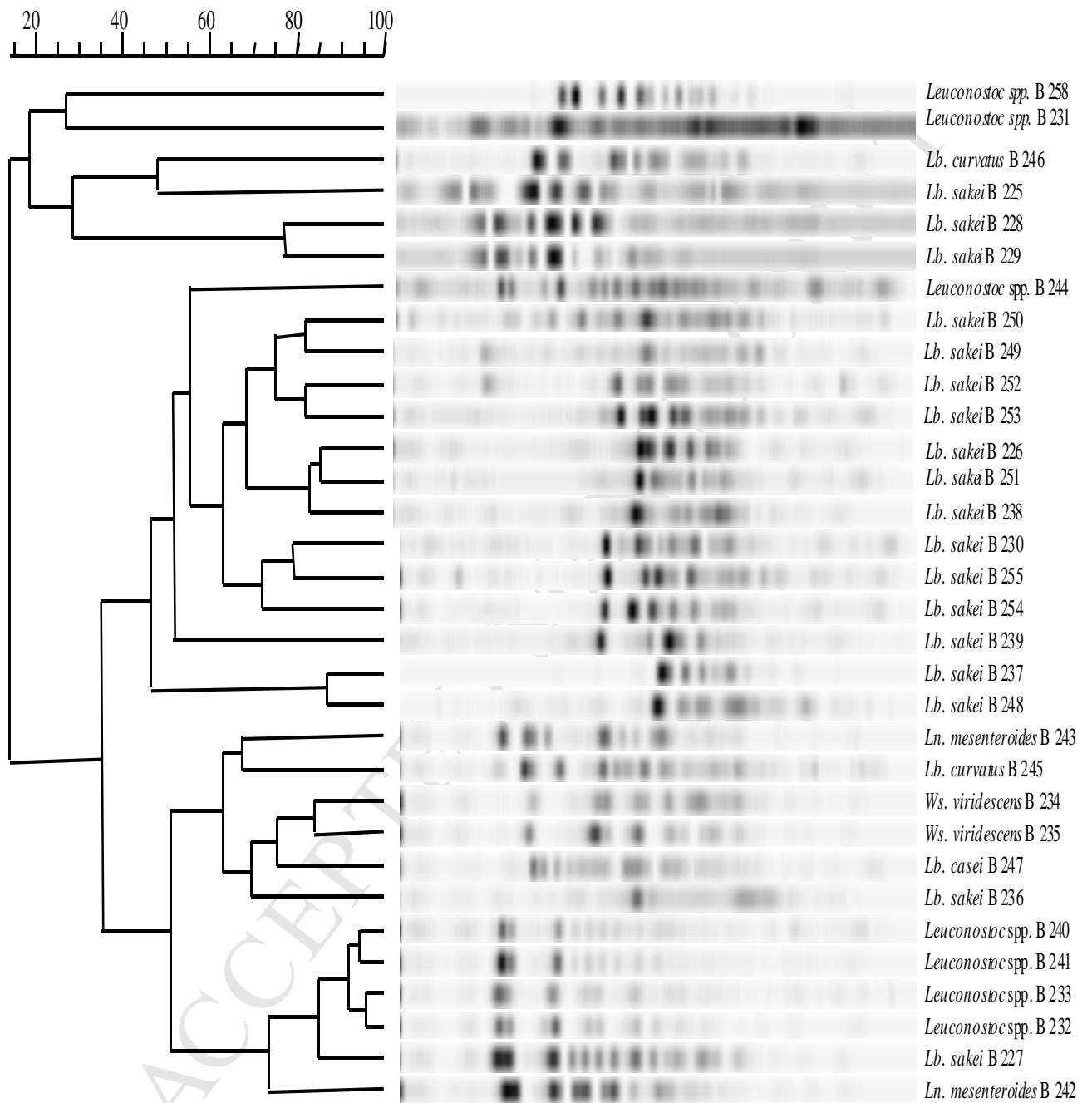
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524 **Figure 1.**

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531 **Table 1.** Lactic acid bacteria populations in minced meat stored under aerobic, MAP (-) and MAP
532 (+) conditions.

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534 **Table 2.** Identity of isolates obtained from minced beef

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536 **Table 3.** Frequency (%) of isolation and distribution of lactic acid bacteria strains isolates
537 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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Lactic acid bacteria population dynamics during minced beef storage under aerobic or modified atmosphere packaging conditions

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