1   **Diversity and dynamics of antibiotic-resistant bacteria in cheese as determined by**

2   **PCR denaturing gradient gel electrophoresis**

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10   Short title: DGGE analysis of antibiotic resistance in cheese

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17

Abstract

18

19   This work reports the composition and succession of tetracycline- and erythromycin-

20   resistant bacterial communities in a model cheese, monitored by polymerase chain

21   reaction denaturing gradient gel electrophoresis (PCR-DGGE). Bacterial 16S rRNA

22   genes were examined using this technique to detect structural changes in the cheese

23   microbiota over manufacturing and ripening. Total bacterial genomic DNA, used as a

24   template, was extracted from cultivable bacteria grown without and with tetracycline or

25   erythromycin (both at 25 µg ml^{-1}) on a non-selective medium used for enumeration of

26   total and viable cells (Plate Count agar with Milk; PCA-M), and from those grown on

27   selective and/or differential agar media used for counting various bacterial groups; i.e.,

28   lactic acid bacteria (de Man, Rogosa and Sharpe agar; MRSA), micrococci and

29   staphylococci (Baird-Parker agar; BPA), and enterobacteria (Violet Red Bile Glucose

30   agar; VRBGA). Large numbers of tetracycline- and erythromycin-resistant bacteria

31   were detected in cheese samples at all stages of ripening. Counts of antibiotic-resistant

32   bacteria varied widely depending on the microbial group and the point of sampling. In

33   general, resistant bacteria were 0.5-1.0 Log$_{10}$ units fewer in number than the

34   corresponding susceptible bacteria. The PCR-DGGE profiles obtained with DNA

35   isolated from the plates for total bacteria and the different bacterial groups suggested

36   *Escherichia coli, Lactococcus lactis, Enterococcus faecalis* and *Staphylococcus* spp. as

37   the microbial types resistant to both antibiotics tested. This study shows the suitability

38   of the PCR-DGGE technique for rapidly identifying and tracking antibiotic resistant

39   populations in cheese and, by extension, in other foods.

40

41   Key words: Antibiotic resistance; cheese; denaturing gradient gel electrophoresis;

42   DGGE; lactic acid bacteria; non-starter lactic acid bacteria

43

1. Introduction

Antibiotic resistance increases the cost of treatment of infections and can be the
cause of therapeutic failure (Anderson and Hughes, 2010). The spread of antibiotic
resistance to human and animal pathogens is therefore of great concern. The food chain
is thought to be one of the main routes via which such resistance spreads (Rossi et al.,
2014). The transfer of genes from resistant to susceptible bacteria may occur during
food manufacture or during transit through the gastrointestinal tract (Rossi et al., 2014;
Gazzola et al., 2012). Fermented foods, such as cheese, in which several bacterial types
grow to high cell densities, are key players in the transmission of antibiotic resistance
between beneficial/commensal and pathogenic bacteria (Nawaz et al., 2011). Complex
bacterial communities composed of the natural cheese microbiota plus an array of
environmental microorganisms develop and change in fermented foods over time,
particularly in starter-free, raw-milk cheeses (Flórez and Mayo, 2006).

Cheeses made from raw milk have been reported to sometimes contain high
antibiotic resistance gene loads (Flórez et al., 2014; Manuzon et al., 2007). The
characterization of the bacterial species involved via conventional, culture-dependent
analysis can, however, be difficult due to the intrinsic limitations of this approach (it is
time consuming, expensive, and has a high manpower demand, etc.) (Devirgiliis et al.,
2013; Gazzola et al., 2012; Nawaz et al., 2011). To overcome this, a number of culture-
independent molecular methods have been developed in recent decades, including
conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR)
amplification, temporal temperature gel electrophoresis (TTGE), denaturing gradient gel
electrophoresis (DGGE), and the construction and analysis of metagenomic libraries
(Devirgiliis et al., 2014; Flórez et al., 2014; Manuzon et al., 2007).

68   Since its first use in Microbial Ecology research in the early 90s (Muyzer et al.,

69   1993), DGGE analysis of rRNA-encoding genes amplified by PCR (PCR-DGGE) has

70   become a widely used tool for investigating the microbial diversity of food ecosystems,

71   including milk, cheese and other dairy products [for recent reviews see Cocolin et al.

72   (2013) and Quigley et al. (2011)]. Given the source of the nucleic acids used in PCR-

73   DGGE, the technique can be performed in two ways: DNA or RNA can be extracted

74   directly from the food matrix (direct PCR-DGGE), or be purified from cultivable

75   bacteria harvested from non-selective and/or selective/differential media (indirect PCR-

76   DGGE). The technical aspects, advantages and biases of these two alternatives have

77   been discussed elsewhere (Ercolini, 2004). Direct PCR-DGGE has already been applied

78   to analyse the polymorphism of tetracycline resistance genes in dairy and environmental

79   samples (Flórez et al., 2014; Aminov et al., 2001; Chee-Sanford et al., 2001), as well as

80   for tracking antibiotic resistant transconjugants in an experimentally-inoculated food

81   (Gazzola et al., 2012). The indirect PCR-DGGE method has been successfully used for

82   studying the microbiology of water buffalo Mozzarella- and Gouda-type cheeses

83   (Ricciardi et al., 2014; van Hoorde et al., 2010; Ercolini et al., 2004).

84   The present work examines the diversity and dynamics of antibiotic-resistant

85   bacterial communities of a traditional cheese during the manufacture and ripening, using

86   the PCR-DGGE technique. Tetracycline and erythromycin were selected as the target

87   antibiotics since resistance to them is widely spread among lactic acid bacteria species

88   (Ammor et al., 2007), thus having the highest risk of horizontal transfer. Cabrales, a

89   Spanish traditional, blue-veined cheese made from raw milk without the use of starter

90   and ripening cultures (Flórez and Mayo, 2006) was selected as the cheese model. The

91   PCR-DGGE technique was used after harvesting cultivable bacteria grown on a non-

92   selective medium and on selective/differential media (for counting bacteria of different

93 groups). To our knowledge, this is the first study in which indirect PCR-DGGE has

94 been used to characterise the bacterial populations involved in antibiotic resistance in

95 cheese along manufacturing and ripening stages. The technique was found valuable for

96 identifying, quantifying and tracking tetracycline- and erythromycin-resistant

97 communities.

98

99 **2. Material and methods**

100 *2.1 Cheese samples*

101   One batch of Cabrales cheese was made following the traditional manufacturing

102 procedure, which involves the use of raw milk but no commercial starter culture or

103 mould spores. Samples were taken from the cheese at days 3, 7, 15, 30 and 60 of

104 ripening. Cubes of 10 g from the cheese core were cut aseptically and homogenized at

105 45ºC for 1 min in a Colworth Stomacher 400 (Seward Ltd., London, UK) with 90 ml of

106 a sterile 2% (w/v) sodium citrate solution.

107

108 *2.2 Enumeration of total and antibiotic-resistant bacteria*

109   Ten-fold dilutions of homogenized cheese samples were prepared with sterile

110 Ringer's solution (VWR International). Aliquots (100 µl) were plated in duplicate on

111 non-selective and selective/differential solid media: total aerobic mesophilic bacterial

112 counts were determined on Plate Count agar with Milk (1%) (PCA-M) (VWR

113 International) after incubation at 32ºC for 48 h; lactic acid bacteria (LAB) counts were

114 made on de Man, Rogosa and Sharpe agar (MRSA) (VWR International) after

115 incubation at 32ºC for 48 h; Baird-Parker agar (BPA) (VWR International) was used to

116 enumerate staphylococci and micrococci after incubation at 37ºC for 48 h; and

117 enterobacteria were counted on Violet Red Bile Glucose agar (VRBGA) (VWR

118   International) after incubation at 37ºC for 24 h. The respective antibiotic-resistant

119   populations were enumerated on the same media as above supplemented with 25 µg ml⁻

120   ¹ of either tetracycline or erythromycin.

121   Bacterial cells from plates showing semi-confluent colonies were harvested by

122   vigorous washing in sterile phosphate-buffered saline (PBS). Cells were then suspended

123   in Brain Heart Infusion broth (BHI) (VWR International) supplemented with 25%

124   glycerol (Merck) and maintained at -80ºC until use.

125

126   *2.3 DNA extraction from cultivable bacteria*

127   Total DNA from cultivable bacteria (susceptible and/or resistant) was isolated from

128   180 µl of the BHI-glycerol suspensions. Cell pellets were collected by centrifugation

129   and suspended in the same volume of a lysis buffer containing 20 mg ml^{-1} lysozyme

130   (Merck), 200 U mutanolysin (Sigma-Aldrich), 50 µg ml^{-1} lysostaphin (Sigma-Aldrich),

131   20 mM Tris-HCl (pH 8.0), 2 mM EDTA and 1.2% Triton-X-100 (Merck). This lysis

132   suspension was then incubated at 37ºC for 1 h and the DNA extracted using the DNeasy

133   Blood and Tissue kit (Qiagen), according to the manufacturer's protocol. The DNA

134   concentration was adjusted to 100 ng µl^{-1} and stored at -20ºC until analysis. Genomic

135   DNA was also extracted and purified from well-identified bacteria strains, which have

136   been previously isolated from Cabrales cheese (Flórez and Mayo, 2006). PCR

137   amplicons from these strains were used as DGGE standards.

138

139   *2.4 PCR-DGGE amplification and electrophoresis conditions*

140   Purified total and genomic DNA was used as a template for the amplification of the

141   V3 region of the bacterial 16S rRNA gene by PCR using two universal primers: 357F

142   (5' CCTACGGGAGGCAGCAG 3') and 518R (5' GTATTACCGCGGCTGCTGG 3').

143   A GC clamp of 40 nucleotides

144   (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was attached to

145   the 5' end of the forward primer (357F-GC), as described by Muyzer et al. (1993). The

146   PCR reaction mixtures contained 3 µl of total DNA, 25 µl of Taq Master Mix

147   (Ampliqon), 1 µl of each of the primers (10 µM) and 20 µl of H_2O in a total volume of

148   50 µl. The PCR amplification conditions were as follow: an initial cycle at 95ºC for 5

149   min, 30 cycles at 95ºC for 30 s, 56ºC for 30 s, 72ºC for 1 min, and a final extension step

150   at 72ºC for 10 min.

151   DGGE was performed in a DCode apparatus (Bio-Rad) using 8% polyacrylamide

152   gels with denaturing ranges of 40-60%. Electrophoresis ran at 60ºC and 75 V for 17 h.

153   The resulting gels were stained in an ethidium bromide solution (0.5 µg ml^{-1}) for 15

154   min, rinsed with water, and photographed under UV light using a G-Box system

155   (Syngene).

156

157   *2.5 Identification of PCR-DGGE bands*

158   PCR-DGGE bands were identified by comparing their migration with that of

159   amplicons from standards. Further, DNA from the bands was extracted by cutting out

160   part of each using a micropipette tip and allowing their contents to diffuse out overnight

161   at 4ºC into sterile, nuclease-free water (Sigma-Aldrich). The resulting DNA solution

162   was used as a template for re-amplification, using the same pair of primers but without

163   the GC clamp, and under the same PCR conditions as above. The presence of

164   amplification products was checked in 1% agarose gel after ethidium bromide staining.

165   Amplicons were then purified to remove any unincorporated primers and nucleotides

166   using the ATP Gel/PCR Extraction kit (ATP Biotech). The purified amplicons were

167   sequenced using the 357F primer, and the resulting sequences compared with those

168   deposited in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and Ribosomal

169   Database Project (http://rdp.cme.msu.edu/index.jsp) databases. As reported elsewhere

170   (Stackebrandt et al., 2002), sequences sharing a percentage identity of 97% or higher to

171   those in the databases were considered to belong to the same species.

172

173   **3. Results**

174   *3.1 Counting of bacterial populations in cheese*

175   Large numbers of antibiotic-resistant bacteria were detected in samples at all stages

176   of ripening. Both tetracycline- and erythromycin-resistant counts varied widely (from

177   10^2 to $>10^8$ cfu g^{-1}) depending on the bacterial group in question and the sampling point

178   (Fig. 1). The presence of antibiotics caused a significantly decrease in counts ($p \leq 0.05$;

179   Student's t-Test) of all bacterial groups. In general, counts of resistant bacteria were

180   around 1.0 Log_{10} units fewer in number than those corresponding to susceptible

181   bacteria. The numbers of erythromycin-resistant enterobacteria were, however, equal to

182   those recovered from PCA-M plates without the antibiotic. Resistant and susceptible

183   populations reached a maximum on about day 3, and decreased slowly afterwards -

184   except for the LAB community, among which the antibiotic-susceptible bacteria

185   reached their maximum at day seven while the antibiotic-resistant bacteria did so on day

186   15.

187

188   *3.2 Analysis of the diversity and dynamics of cheese populations by PCR-DGGE*

189   The PCR-DGGE profiles of the total, tetracycline-resistant and erythromycin-

190   resistant cultivable bacteria differed over ripening (Figs. 2-5). As a general trend, either

191   in the absence or in the presence of antibiotics, the PCR-DGGE patterns obtained for

192   day 3 samples showed the largest numbers of bands (largest diversity); this number fell

193 from that point onwards as ripening progressed. In total, 105 bands were obtained, of

194 which 99 were identified at either the genus or species level following excision from the

195 gel, DNA purification, re-amplification, sequencing and comparison with sequences in

196 databases. A summary of the identification results of sequences from PCR-DGGE

197 bands and their closest relatives in databases is provided in Supplementary Table 1.

198 Further, representative nucleotide sequences from the bands are depicted in

199 Supplementary Figure 1.

200

201 *3.2.1 PCR-DGGE profiles for total mesophilic bacteria*

202   The PCR-DGGE profiles for the total cultivable bacteria grown on the PCA-M plates

203 showed 4-10 bands depending on the ripening time (Fig. 2A, 2B and 2C). Some bands

204 were present in all samples both in the presence and absence of antibiotics, while others

205 were only present in certain samples. For example, bands i and j were only observed in

206 the 30- and 60-day old cheese samples in the presence of tetracycline (Fig. 2B, line 4

207 and 5); these bands were assigned to the genus *Enterococcus*. In contrast, a prominent

208 band matching the sequence of *Lactococcus lactis* (band b) was observed in all but one

209 cheese sample (Fig. 2A, 2B and 2C). In the absence of antibiotics (Fig. 2A), the most

210 complex profile was observed for the day 60 sample (Fig. 2A, line 5), at which time

211 several *Enterococcus* and *Staphylococcus* spp. were shown to accompany the bands of

212 *L. lactis* (band b) and *Lactobacillus plantarum* (band c). The complexity of the PCR-

213 DGGE profiles in the presence of tetracycline (Fig. 2B) increased with ripening (Fig.

214 2B, lines 4 and 5). The patterns for tetracycline- and erythromycin-resistant bacteria

215 reflected the same species as before (Fig. 2A), except that the band corresponding to *Lb.*

216 *plantarum* (band c) was not present. Numerous and diverse bands for species of

217 Enteroboacteriaceae (bands a) were identified in the cultures from the PCA-M plates

218   with and without antibiotics, including a prominent band in all gels corresponding to

219   *Escherichia coli* (band d). Abrupt changes in this latter band were detected in

220   consecutive samples; the same was seen in the presence of tetracycline (Fig. 2B, lines 2,

221   3 and 4) and erythromycin (Fig. 2C, lines 1 and 2).

222

223   *3.2.2 PCR-DGGE profiles for lactic acid bacteria*

224   Marked differences were observed between the profiles of LAB cells recovered in

225   MRSA without antibiotics (Fig. 3A) and cultures supplemented with tetracycline (Fig.

226   3B) and erythromycin (Fig. 3C). Though MRS is a strongly selective medium for

227   lactobacilli, *L. lactis* and other LAB species could still grow. In the absence of

228   antibiotics, a very intense band corresponding to *L. lactis* (band f) was observed at day 3

229   (Fig. 3A, line 1). However, this band was barely observed in subsequent samples in the

230   absence of antibiotics (Fig. 3A, lines 2 to 5); in fact, a prominent band for *Lb.*

231   *plantarum* (band b) appeared instead. Surprisingly, the communities recovered in

232   MRSA in the presence of tetracycline (Fig. 3B) and erythromycin (Fig. C) were

233   composed of *L. lactis* (band f) and *Enterococcus faecalis* (band h). The latter species

234   became dominant at the end of ripening (in 30- and 60-day old cheese samples).

235

236   *3.2.3 PCR-DGGE profiles for micrococci and staphylococci*

237   The profile obtained on day 3 for bacteria recovered from the BPA plates in the

238   absence of antibiotics showed six bands (Fig. 4, line 1). This number was reduced to

239   one in day 7-15 samples, but increased again to five-six in the day 30 and day 60

240   samples. The sequence of the most intense bands matched that of *Ent. faecalis* (band b)

241   both in the presence and absence of antibiotics. This suggests again that, although

242   Baird-Parker is a selective medium for counting micrococci and staphylococci, it does

243   not restrict the growth of *Enterococcus* spp. In fact, a majority of staphylococci-related

244   bands (allocated to the *Staphylococcus epidermidis-Staphylococcus aureus* species-

245   group) was only identified in the absence of antibiotics on day 3 (Fig. 4A, line 1). Bands

246   belonging to other staphylococcal groups were also observed at the end of ripening in

247   the absence of antibiotics (Fig. 4A, lines 4 and 5), as well as in the presence of

248   erythromycin (Fig. 4C).

249

250   *3.2.4 PCR-DGGE profiles for Enterobacteriaceae*

251   As for the other bacterial groups, different PCR-DGGE patterns were observed for

252   the enterobacteria recovered from the VRBGA plates both in the absence (Fig. 5A) and

253   presence of tetracycline (Fig. 5B). As expected, the profiles recorded for these bacteria

254   when grown in the presence of erythromycin were identical to those when no antibiotic

255   was present (Fig. 5C). Due to the high nucleotide identity of the 16S rRNA genes

256   shared by the species of this family, most enterobacterial bands could only be identified

257   at the family level. The exceptions were those corresponding to *E. coli* (band b) and

258   *Enterobacter cloacae* (band c). The profile of the cultures from the day 3 cheese sample

259   grown in the absence of antibiotics showed 14 bands (Fig. 5A, line 1), suggesting this

260   group to be diverse in raw milk at the beginning of ripening. Of these bands, only five

261   were seen at later times during ripening, with *E. coli* the most prominent from day 7

262   onwards (Fig. 5A, lines 2 to 5). The profiles for tetracycline resistant enterobacteria

263   showed a single band belonging to the species *E. coli*, except at 30 days of ripening

264   (Fig. 5B). This indicates that *E. coli* was dominant among the enterobacteria resistant to

265   tetracycline throughout the ripening process.

266

267   **4. Discussion**

268 Large numbers of antibiotic-resistant bacteria and genes have been repeatedly

269 reported in foods, including dairy products (Florez et al., 2014; Devirgiliis et al., 2013;

270 Wang et al., 2006). The development of suitable methods for examining the diversity

271 and dynamics of resistant populations would contribute towards the characterization of

272 the microbes carrying antibiotic resistances. This knowledge is pivotal in the proposal

273 and assessment of strategies aimed at reducing antibiotic resistance loads and limiting

274 the spread of antibiotic resistance genes via the food chain.

275 The presence of large numbers of resistant species mostly in cheese made from raw

276 milk might correlate with the presence of high levels of antibiotic residues in the milk

277 environment (Brunton et al., 2014), as reported for other ecosystems (Gao et al., 2012;

278 Fan and He, 2011). The lower numbers of resistant populations as compared to those

279 susceptible do not appear to be associated with an intrinsic antagonist effect of

280 antibiotic-containing media in growth. Indeed, enterobacteria counts with and without

281 erythromycin were identical (Figure 1). It is said that the biological cost of the

282 resistances is not very high because resistant bacteria increase fitness by compensating

283 mutations (Sousa et al., 2012). It has also been speculated that environmental conditions

284 during fermentation and/or ripening might contribute to the maintenance of antibiotic

285 resistance in food-borne bacteria (Rossi et al., 2014). However, the present counts

286 suggested that the wild-type, non-resistant variants grow faster and reach higher cell

287 densities under cheese manufacturing conditions.

288 Marked differences were seen between the PCR-DGGE profiles obtained in the

289 presence and absence of antibiotics for all bacterial groups analysed in this study. The

290 appearance and disappearance of majority (more intense) bands reveals sharp changes

291 in the composition and structure of the microbial communities. The reduction in the

292 number of bands correlates with a reduction in microbial diversity, suggesting that most

293 of the dominant bacterial populations of this cheese are susceptible to both antibiotics.

294 The PCR-DGGE profiles of the tetracycline- and erythromycin-resistant populations

295 were composed mainly of *L. lactis* and *Enterococcus* spp. bands, which agrees well

296 with results reported elsewhere regarding the large number of strains of these species

297 showing resistance to tetracycline and erythromycin and the carriage of dedicated

298 resistance genes (Pesavento et al., 2014; Devirgiliis et al., 2010; Flórez et al., 2008). In

299 particular, *Enterococcus* species from different food environments have been found

300 highly resistant to these antibiotics (Pesavento et al., 2014; Valenzuela et al., 2008).

301 Species of this genus are thought to act as a reservoir of antibiotic resistance genes,

302 from which they might be transferred to food-borne pathogens (EFSA, 2014). However,

303 the abundance and intensity of *L. lactis* bands obtained in media with tetracycline and

304 erythromycin argue for this species forming the majority antibiotic-resistant population

305 in the present cheese model. Under the actual concern of spreading antibiotic resistances

306 via the food chain (Rossi et al., 2014), it is unacceptable the presence in cheese of

307 technologically-relevant species carrying antibiotic resistances, which argues for the

308 establishment of improved hygienic practices in traditional cheese manufacture.

309 Mesophilic lactobacilli have been shown to form subdominant populations in many

310 cheese varieties (Wouters et al., 2002). Although lactobacilli harbouring tetracycline

311 and erythromycin resistance genes have been reported (Comunian et al., 2010; Feld et

312 al., 2009), the presence of PCR-DGGE bands belonging to *Lactobacillus* species were

313 never seen for cultivable bacteria growing on antibiotic-containing media. Thus, the

314 populations of lactobacilli in the present cheese type seem to have no resistance to either

315 antibiotic. The present data do not guarantee the complete absence of antibiotic-resistant

316   lactobacilli, however, since the larger numbers of other resistant groups (such as

317   lactococci and enterococci) might mask their detection.

318   PCR-DGGE bands belonging to *Staphylococcus* spp. appeared mostly at the end of

319   ripening, suggesting that staphylococci develop when the cheese matrix approaches the

320   neutrality (pH 7.0). Antibiotic resistances in *Staphylococcus aureus*, coagulase-negative

321   staphylococci and other staphylococci species has been repeatedly reported (Jamali et

322   al., 2014; Spanu et al., 2014; Wang et al., 2012; Resch et al., 2008). However, the PCR-

323   DGGE findings of this study suggest that the major carriers of antibiotic resistance

324   proved to be members of the species groups *Staphylococcus simulans* and

325   *Staphylococcus saprophyticus*.

326   The intrinsic nature of erythromycin resistance in enterobacteria (Liu et al., 2010)

327   was the cause for identical counts and the same PCR-DGGE patterns of this group in

328   the absence of antibiotics and in the presence of erythromycin. *E. coli* was the only

329   enterobacterial species resistant to tetracycline. Its resistance to this antibiotic has been

330   extensively documented, particularly in pathogenic strains of animal and human origin

331   (Karczmarczyk et al., 2011; Guerra et al., 2003). The abrupt changes in the band of *E.*

332   *coli* from consecutive samples may be a reflection of a heterogeneous spatial

333   distribution for *E. coli* within the cheese matrix. As mentioned earlier, the presence of

334   enterobacteria-related bands in the profiles for total cultivable bacteria grown with

335   erythromycin was due to intrinsic resistance.

336

337   **5. Conclusion**

338   In this study we report on the use of the indirect PCR-DGGE technique for studying

339   antibiotic-resistant bacterial communities in a model cheese made from raw milk

340   without commercial starters. This method was shown to be suitable for identifying and

341   tracking antibiotic-resistant bacteria in cheese. Conceivable, it could also serve for

342   similar purposes in other foods. Tracking resistant bacteria and the genes they encode

343   might be accomplished in the near future through the use of Next Generation

344   Sequencing techniques. In our cheese model, *E. coli, L. lactis, Ent. faecalis,* and

345   *Staphylococcus* spp. were the microbial types involved in antibiotic resistance. The

346   discovery of large numbers of resistant populations in cheese advocates for the

347   establishment of stronger hygienic practices in cheese manufacture. Pasteurisation of

348   milk and/or the use of well-characterized, starter and adjunct cultures devoid of

349   resistance determinants could further help to control antibiotic resistance spread.

350

357

358   **Conflict of interest:** The authors declare that they have no conflict of interest.

359

360   **References**

361   Aminov, R. I., N. Garrigues-Jeanjean, and R. I. Mackie. 2001. Molecular ecology of

362   tetracycline resistance: development and validation of primers for detection of

363   tetracycline resistance genes encoding ribosomal protection proteins. Applied and

364   Environmental Microbiology, 67, 22-32.

365   Ammor, M.S., A. B. Flórez, and B. Mayo. 2007. Antibiotic resistance in non-

366   enterococcal lactic acid bacteria and bifidobacteria. Food Microbiology, 24, 559-570.

367   Andersson, D. I., Hughes, D., 2010. Antibiotic resistance and its cost: is it possible to

368   reverse resistance? Nature Review Microbiology, 8, 260-271.

369   Brunton, L. A., Reeves, H. E., Snow, L. C., Jones, J. R., 2014. A longitudinal field trial

370   assesing the impact of feeding waste milk containing antibiotic residues on the

371   prevalence of ESBL-producing *Escherichia coli* in calves. Preventive Veterinary

372   Medicine, 117, 403-412.

373   Chee-Sanford, JC., R. I. Aminov, I. J. Krapac, N. Garrigues-Jeanjean, and R. I. Mackie.

374   2001. Occurrence and diversity of tetracycline resistance genes in lagoons and

375   groundwater underlying two swine production facilities. Applied and Environmental

376   Microbiology, 67, 1494-1502.

377   Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., Rantsiou, K., 2013. Culture

378   independent methods to assess the diversity and dynamics of microbiota during food

379   fermentation. International Journal of Food Microbiology, 167, 29-43.

380   Comunian, R., Daga, E., Dupre, I., Paba, A., Devirgiliis, C., Piccioni, V., Perozzi, G.,

381   Zonenschain, D., Rebecchi, A., Morelli, L., De Lorentiis, A., Giraffa, G., 2010.

382   Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains

383   isolated from traditional Italian fermented foods. International Journal of Food

384   Microbiology, 138, 151-156.

385   Devirgiliis, C., Barile, S., Caravelli, A., Coppola, D., Perozzi, G., 2010. Identification of

386   tetracycline- and erythromycin-resistant Gram-positive cocci within the fermenting

387   microflora of an Italian dairy food product. Journal of Applied Microbiology, 109,

388   313-323.

389   Devirgiliis, C., Zinno, P., Perozzi, G., 2013. Update on antibiotic resistance in

390   foodborne *Lactobacillus* and *Lactococcus* species. Frontiers in Microbiology, 4, 301.

391   Devirgiliis, C., Zinno, P., Stirpe, M., Barile, S., Perozzi, G., 2014. Functional screening

392   of antibiotic resistance genes from a representative metagenomic library of food

393   fermenting microbiota. BioMed Research International, ID 290967.

394   EFSA (European Food Safety Authority)., 2014. European Union summary report on

395   antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and

396   food 2012. EFSA Journal, 12, 3590.

397   Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of

398   microbes in food. Journal of Microbiological Methods, 56, 297-314.

399   Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G., Coppola, S., 2004. PCR-DGGE

400   fingerprints of microbial succession during a manufacture of traditional water buffalo

401   mozzarella cheese. Journal of Applied Microbiology, 96, 263-270.

402   Fan, C.A., He, J.Z., 2011. Proliferation of antibiotic resistance genes in microbial

403   consortia of sequencing batch reactors (SBRs) upon exposure to trace erythromycin

404   or erythromycin-H_2O. Water Research, 45, 3098-3106.

405   Flórez, A.B., Mayo, B., 2006. Microbial diversity and succession during the

406   manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as

407   determined by PCR-DGGE. International Journal of Food Microbiology, 110, 165-

408   171.

409   Flórez, A. B., Alegría, A., Rossi, F., Delgado, S., Felis, G., Torriani, S., Mayo, B., 2014.

410   Molecular identification and quantification of tetracycline and erythromycin

411   resistance genes in Spanish and Italian retail cheeses. BioMed Research

412   International, ID 746859.

413   Gazzola, S., Fontana, C., Bassi, D., Coccocelli, P. S., 2012. Assessment of tetracycline

414   and erythromycin resistance transfer during sausage fermentation by culture-

415   dependent and –independent methods. Food Microbiology, 30, 348-354.

416   Gao, P., Munir, M., Xagoraraki, I., 2012. Correlation of tetracycline and sulfonamide

417   antibiotics with corresponding resistance genes and resistant bacteria in a

418   conventional municipal wastewater treatment plant. Science of the Total

419   Environment, 421-422, 173-183.

420   Guerra, B., Junker, E., Schroeter, A., Malorny, B., Lehmann, S., Helmuth, R., 2003.

421   Phenotypic and genotypic characterization of antimicrobial resistance in German

422   *Escherichia coli* isolates from cattle, swine and poultry. Journal of Antimicrobial

423   Chemotherapy, 52, 489-492.

424   Jamali, H., Radmehr, B., Ismail, S., 2014. Short communication: prevalence and

425   antibiotic resistance of *Staphylococcus aureus* isolated from bovine clinical mastitis.

426   Journal of Dairy Science, 97, 2226-2230.

427   Karczmarczyk, M., Walsh, C., Slowey, R., Leonard, N., Fanning, S., 2011. Molecular

428   characterization of multidrug-resistant Escherichia coli isolates from Irish cattle

429   farms. Applied and Environmental Microbiology, 77, 7121-7127.

430   Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, Tran K, Miller JH., 2010. Antibiotic

431   sensitivity profiles determined with an *Escherichia coli* gene knockout collection:

432   generating an antibiotic bar code. Antimicrobial Agents and Chemotherapy, 54,

433   1393-1403.

434   Manuzon, M. Y., Hanna, S. E., Luo, H., Yu, Z., Harper, W. J., Wang, H. H., 2007.

435   Quantitative assessment of the tetracycline resistance gene pool in cheese samples by

436   real-time TaqMan PCR. Applied and Environmental Microbiology, 73, 1676-1677.

437   Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial

438   populations by denaturing gradient gel electrophoresis analysis of polymerase chain

439   reaction-amplified genes coding for 16S rRNA. Applied and Environmental

440   Microbiology, 59, 695-700.

441   Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J.E., Millar, B.C., Xu, J., 2011.

442   Characterization and transfer of antibiotic resistance in lactic acid bacteria from

443   fermented food products. Current Microbiology, 62, 1081-1089.

444   Pesavento, G., Calonico, C., Ducci, B., Magnanini, A., Lo Nostro, A., 2014. Prevalence

445   and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-

446   eat salads, ham, and raw meat. Food Microbiology, 41, 1-7.

447   Quigley, L., O'Sullivan. O., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D.,

448   2011. Molecular approaches to analysing the microbial composition of raw milk and

449   raw milk cheese. International Journal of Food Microbiology, 150, 81-94.

450   Resch M, Nagel V, Hertel C. 2008. Antibiotic resistance of coagulase-negative

451   staphylococci associated with food and used in starter cultures. International Journal

452   of Food Microbiology, 127, 99-104.

453   Ricciardi, A., Blaiotta, G., Di Cerbo, A., Succi, M., Aponte, M., 2014. Behaviour of

454   lactic acid bacteria populations in Pecorino di Carmasciano cheese samples

455   submitted to environmental conditions prevailing in the gastrointestinal tract:

456   evaluation by means of a polyphasic approach. International Journal of Food

457   Microbiology, 179, 64-71.

458   Rossi, F., Rizzotti, L., Felis, G. E., Torriani, S., 2014. Horizontal gene transfer among

459   microorganisms in food: current knowledge and future perspectives. Food

460   Microbiology, 42, 232-243.

461   Sousa, A., Magalhães, S., Gordo, I., 2012. Cost of antibiotic resistance and the

462   geometry of adaptation. Molecular Biology and Evolution, 29, 1417-1428.

463   Spanu, V., Scarano, C., Cossu, F., Pala, C., Spanu, C., De Santis, E.P., 2014. Antibiotic

464   resistance traits and molecular subtyping of *Staphylococcus aureus* isolated from raw

465   sheep milk cheese. Journal of Food Science, 79, M2066-2071.

466   Stackebrandt, E., Frederiksen, W., Garrity, G.M., Grimont, P.A., Kampfer, P., Maiden,

467   M.C., Nesme, X., Rossello-Mora, R., Swings, J., Truper, H.G., Vauterin, L., Ward,

468   A.C., Whitman, W.B., 2002. Report of the ad hoc committee for the re-evaluation of

469   the species definition in bacteriology. International Journal of Systematic and

470   Evolutionary Microbiology, 52, 1043-1047.

471   Valenzuela, A.S., Omar, N.B., Abriouel, H., López, R.L., Ortega, E., Canamero, M.M.,

472   Gálvez, A., 2008. Risk factors in enterococci isolated from foods in Morocco:

473   determination of antimicrobial resistance and incidence of virulence traits. Food and

474   Chemical Toxicology, 46, 2648-2652.

475   van Hoorde, K., Heyndrickx, M., Vandamme, P., Huys, G., 2010. Influence of

476   pasteurization, brining conditions and production environment on the microbiota of

477   artisan Gouda-type cheeses. Food Microbiology, 27, 425-433.

478   Wang, HH., M.Manuzon, M. Lehman M, Wan K, Luo H, Wittum TE, Yousef A,

479   Bakaletz LO. 2006. Food commensal microbes as a potentially important avenue in

480   transmitting antibiotic resistance genes. FEMSMicrobiology Letters, 254, 226-231.

481   Wang, X., Meng, J., Zhou, T., Zhang, Y., Yang, B., Xi, M., Sheng, J., Zhi, S., Xia, X.,

482   2012. Antimicrobial susceptibility testing and genotypic characterization of

483   *Staphylococcus aureus* from food and food animals. Foodborne Pathogens and

484   Disease, 9, 95-101.

485   Wouters, J.T.M., Ayad, E.H.E., Hugenholtz, J., Smit, G., 2002. Microbes from raw milk

486   for fermented dairy products. International Dairy Journal, 12, 91-109.

Figure Captions

Figure 1.- Counts of total (panel A) and representative (panels B through D) microbial groups in cheese at different stages of ripening in media supplemented or not with tetracycline or erythromycin. In parenthesis, it is indicated the counting media utilized for the different target populations.

Figure 2.- DGGE profiles of PCR amplicons of the V3 region of the bacterial 16S rRNA gene using as a template DNA from bacteria recovered from PCA-M plates without antibiotics (Panel A) or from PCA-M plates supplemented with tetracycline (Panel B) or erythromycin (Panel C). Lanes 1 through 5, cheese samples at 3, 7, 15, 30 and 60 days of ripening. Bands with a letter code were identified as follows: a, Enterobacteriaceae; b, *Lactococcus lactis*; c, *Lactobacillus plantarum*; d, *Escherichia coli*; e, *Staphylococcus saprophyticus* species-group (*S. xylosus, S. saprophyticus, S. arlettae, S. equorum, S. succinus*) f, *Enterococcus faecalis*; g, *Staphylococcus simulans* species-group (*S. simulans*, *S. carnosus*, *S. condimenti*, *S. piscifermentans*); h, *Enterococcus casseliflavus;* i, *Enterococcus faecium*; j, *Enterococcus* spp.*;*

Figure 3.- DGGE profiles of PCR amplicons of the V3 region of the bacterial 16S rRNA gene using as a template DNA from bacteria recovered from MRS agar plates without antibiotics (Panel A) or from MRSA plates supplemented with tetracycline (Panel B) or erythromycin (Panel C). Lanes 1 through 5, cheese samples at 3, 7, 15, 30 and 60 days of ripening. Key of the identified bands: a, *Lactobacillus sakei-Lactobacillus curvatus*; b, *Lactobacillus plantarum*; c, *Lactobacillus brevis* ; d,

Streptococcus spp.; e*, Lactobacillus* spp.; f*, Lactococcus lactis*; g, *Enterococcus casseliflavus;* h, *Enterococcus faecalis*; i, uncultured bacterium; j, *Escherichia coli*.

Figure 4.- DGGE profiles of PCR amplicons of the V3 region of the bacterial 16S rRNA gene using as a template DNA from bacteria recovered from Baird-Parker (BP) agar plates without antibiotics (Panel A) or from BPA plates supplemented with tetracycline (Panel B) or erythromycin (Panel C). Lanes 1 through 5, cheese samples at 3, 7, 15, 30 and 60 days of ripening. Key of the identified bands: a, *Staphylococcus aureus*-*Staphylococcus epidermidis* species-group (S. *aureus, S. epidermidis, S. capitis, S. hominis, S. pasteuri*); b, *Enterococcus faecalis;* c, Enterobacteriaceae; d, *Staphylococcus saprophyticus* species-group (*S. xylosus, S. saprophyticus, S. arlettae, S. equorum, S. succinus*) ; e*, Staphylococcus simulans* species-group (*S. simulans, S. carnosus*, *S. condimenti*, *S. piscifermentans*); f, *Enterococcus* spp.

Figure 5.- DGGE profiles of PCR amplicons of the V3 region of the bacterial 16S rRNA gene using as a template DNA from bacteria recovered from VRBGA plates without antibiotics (Panel A) or from VRBGA plates supplemented with tetracycline (Panel B) or erythromycin (Panel C). Lanes 1 through 5, cheese samples at 3, 7, 15, 30 and 60 days of ripening. Key of the identified bands: a, Enterobacteriaceae group (*Hafnia* spp., *Citrobacter* spp., *Raoultella* spp.) b, *Escherichia coli*; c, *Enterobacter cloacae.*
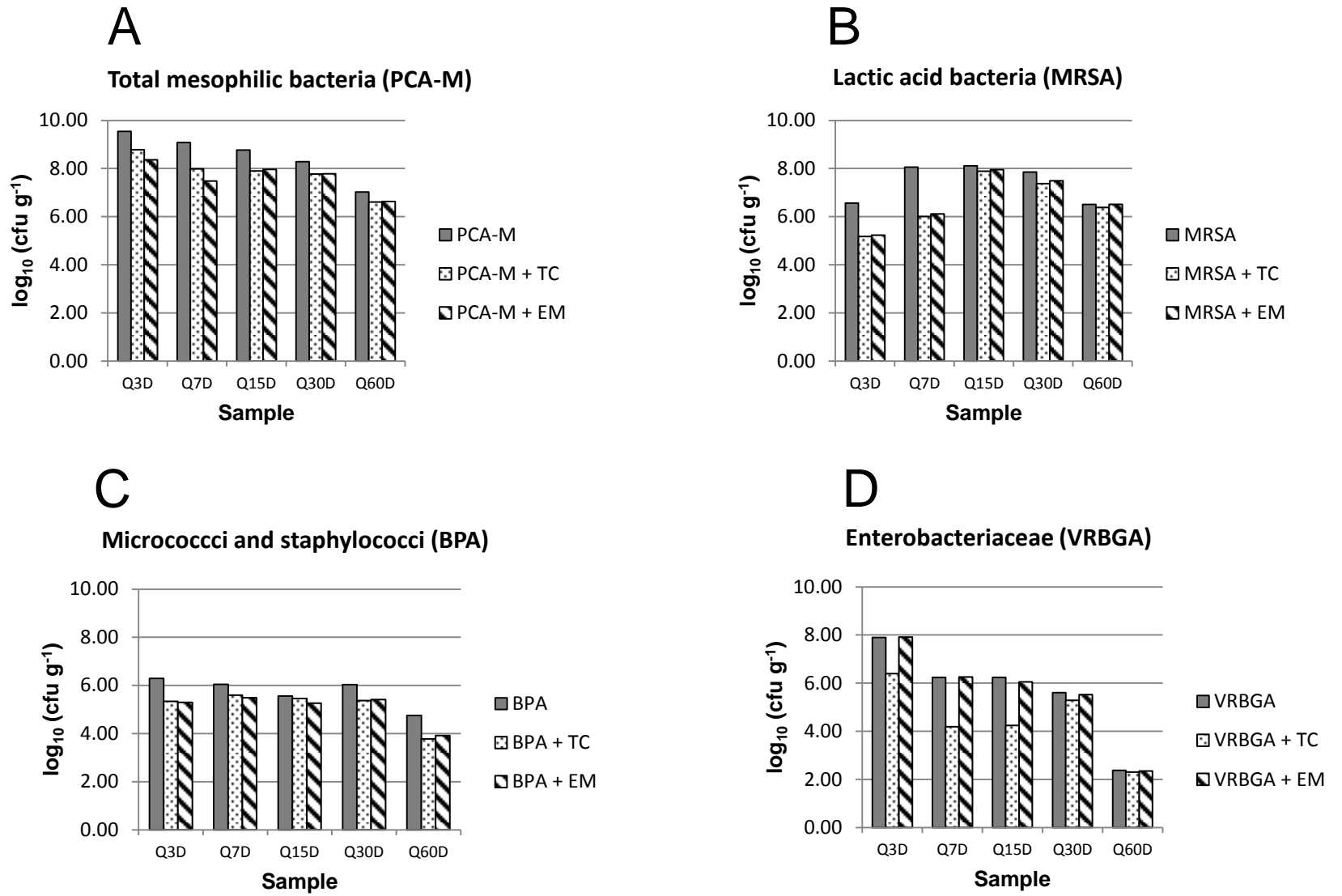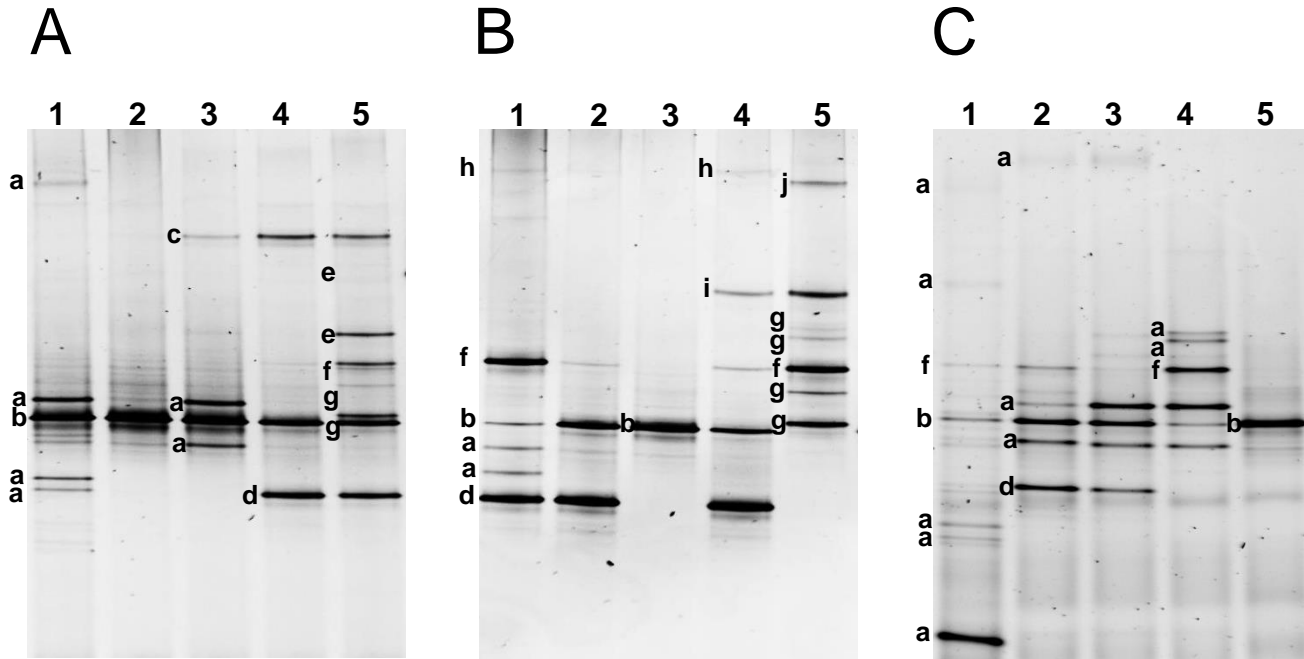
Figure 1

A

Total mesophilic bacteria (PCA-M)

B

Lactic acid bacteria (MRSA)

C

Micrococccci and staphylococci (BPA)

D

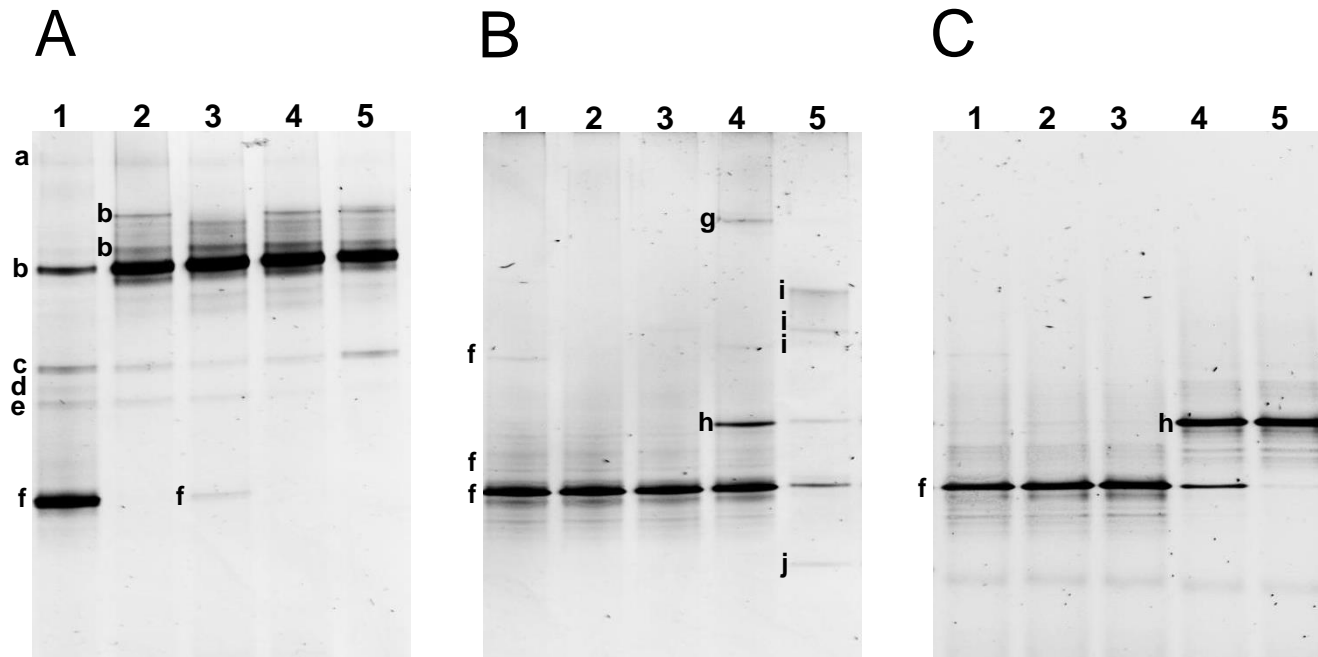## Enterobacteriaceae (VRBGA)
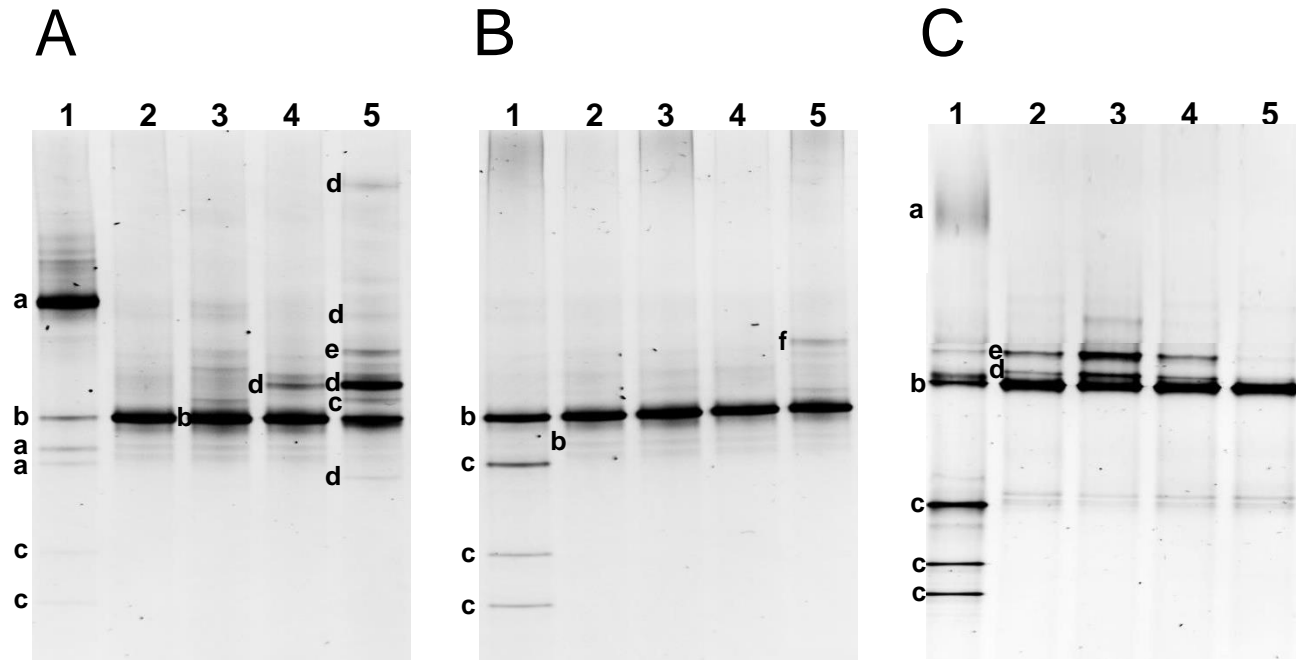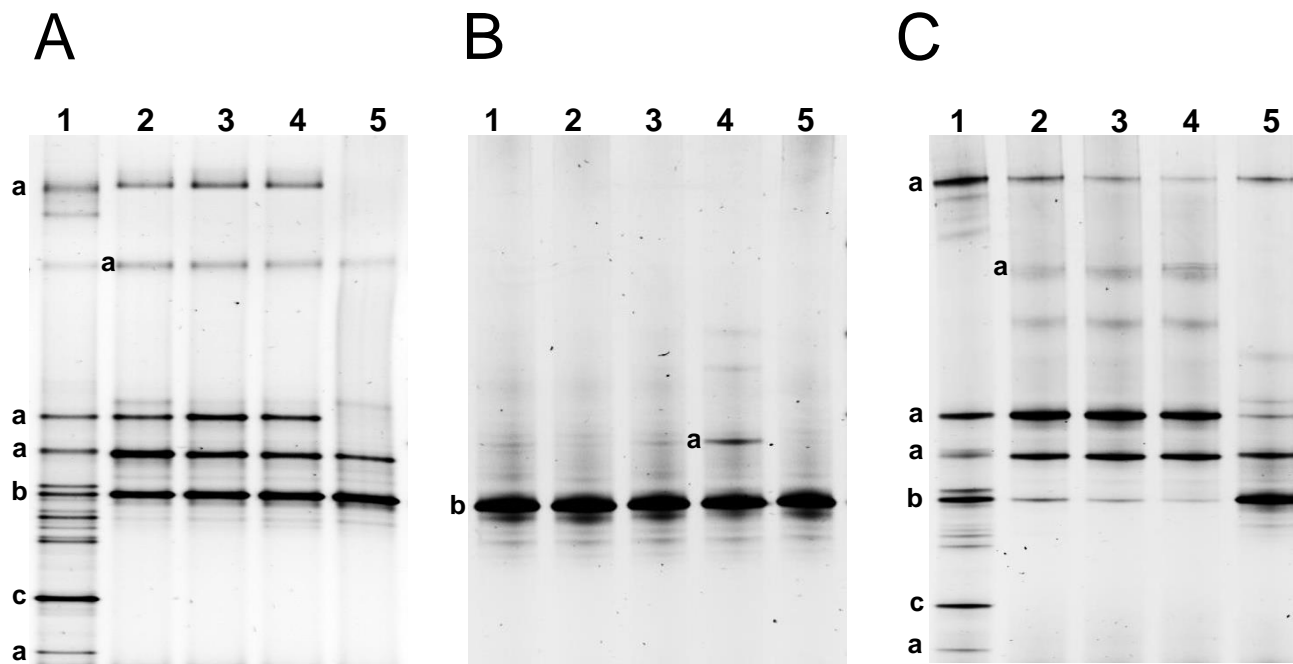
Figure 2

Figure 3

Figure 4

Figure 5

Supplementary Figure 1.- Representative sequences obtained from DGGE bands and their tentative identification by comparison against the databases.

Seq1   DGGE-band a, Figure 2   cheese Enterobacteriaceae (*Hafnia* spp., *Citrobacter* spp., *Raoultella* spp.)

GTACGTCATCACTAAGGTTATTAACCTTAATGCCTTCCTCCTCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAG
GCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGT

Seq2   DGGE-band b, Figure 2   cheese *Lactococcus lactis*

GTAGTTACCGTCACTTGATGAGCTTTCCACTCTCACCAACGTTCTTCTCTACCAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTC
GGTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGT

Seq3   DGGE-band b, Figure 3   cheese *Lactobacillus plantarum*

ATACCGTCATACCTGAACAGTTACTCTCAGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAG
ACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGT

Seq4   DGGE-band b, Figure 5   cheese *Escherichia coli*

TACGTCATGAGCAAGGTATTAACTTTACTCCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGC
TTGCGCCCATTGTGCAATATTCCCCACTGGTGCATCCCGT

Seq5   DGGE-band e, Figure 2   cheese *Staphylococcus saprophyticus* species-group

GTACCGTCAAGACGTGCACAGTTACTTACACATTTGTTCTTCCCTAATAACAGAGTTTTACGAGCCGAAACCCTTCATCACTCACGCGGCGTTGCTCCGTC
AGGCTTTCGCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGT

Seq6   DGGE-band b, Figure 4   cheese *Enterococcus faecalis*

TAGAACCGTCAGGGGACGTTCAGTTACTAACGTCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGG
TCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGT

Seq7   DGGE-band e, Figure 4   cheese *Staphylococcus simulans* species-group

TGATTAGGTACCGTCAGGTGCGCATAGTTACCTACGCACTTGTTCTTCCCTAATAACAGAGTTTTACGATCCGAAGACCTTCATCACTCACGCGGCGTTGC
TCCGTCAGGCTTTCGCCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGT

Seq8   DGGE-band h, Figure 2   cheese *Enterococcus casseliflavus*

GATACCGTCAAGGGATGAACATTTTACTCTCATCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAACCTTCTTCACTCACGCGGCGTTGCTCGGGC
AGACTTTCGTCCATTGCCAAGAATCCCTACTGCTGCCTCCCGT

Seq9   DGGE-band i, Figure 2 cheese *Enterococcus faecium*

GTAGATACCGTCAGGGATGAACAGTTACTCTCATCCTTGTTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCG
GTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGT

Seq10   DGGE-band j, Figure 2 cheese *Enterococcus* spp.

TAGATACCGTCAAGGGATGTACAGTTACTCACGTCCTTATTCTTCTCTAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGG
TCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGT

Seq11   DGGE-band a, Figure 3   cheese *Lactobacillus sakei-curvatus*

TACGTCACTACCTGATCGGTTACTATCAGATATGTTCTTCTCCAACAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCATCAGA
CTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGT

Seq12   DGGE-band c, Figure 3   cheese *Lactobacillus brevis*

ATACCGTCACACTTGAACAGTTACTCTCAAGATGTGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCA
GACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGT

Seq13   DGGE-band d, Figure 3   cheese *Streptococcus* spp.

CGTCACAGTGTGAGCTTTCCACTCTCACACTCGTTCTTCTCTTACAACAGAGTTTTACCAACCCAAAACCTTCTTCACTCCCGCGGCGTTGCTCGGTCAGA
CTTCCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGT

Seq14   DGGE-band e, Figure 3   cheese *Lactobacillus* spp.

ATACCGTCACACTTGAACAGTTACTCTCAAGATGTGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCA
GACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGT

Seq15   DGGE-band i, Figure 3 cheese uncultured bacterium

CGTTAGGTATTTACATTGTACTCATTCCAATTACAAGACCCGAATGGGCCCTGTATCGTTATTTATTGTCACTACCTCCCTGAATTAGGATTGGGTAATTTGC
GCGCCTGCTGCCTCCCGT

Seq16   DGGE-band a, Figure 4   cheese *Staphylococcus aureus-epidermidis* species-group

CTTACACGTTTGTTCTTCCCTAATAACAGAGTTTTACGATCCGAAGACCTTCATCACACACGCGGCGTTGCTCCGTCAGGCTTGCGCCCATTGCGGAAGAT
TCCCTACTGCTGCCTCCCGT

Seq17   DGGE-band c, Figure 5   cheese *Enterobacter cloacae*

ACGTCAATCAGCTGCGGTTATTAACCATAACGCCTTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAG
GCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGT

Supplementary Table 1.- BLASTN homology of the nucleotide sequences from representative PCR-DGGE bands and their closest relatives in databases.

DGGE Band	Closest relative(s)	Nucleotide identity (%)	Accession nº
Total mesophilic bacteria (PCA-M)			
a	Enterobacteriaceae		
	Hafnia spp.	99	KC210872
	Citrobacter spp.		KP972219
	Raoultella spp.		KC211307
b	*Lactococcus lactis*	100	KJ781895
c	*Lactobacillus plantarum*	100	KJ778116
d	*Escherichia coli*	99	JF919875
	Staphylococcus saprophyticus species-group		
e	*S. saprophyticus*	100	KR059863
	S. xylosus		KM586983
f	*Enterococcus faecalis*	99	KP298396
	Staphylococcus simulans species-group		
g	*S. simulans*	99	KP202162
	S. carnosus		KJ862002
h	*Enterococcus casseliflavus*	98	JX045722
i	*Enterococcus faecium*	99	KP275679
j	*Enterococcus* spp.	98	KM388675
Lactic acid bacteria (MRSA)			
a	*Lactobacillus sakei*	98	LN827925
	Lactobacillus curvatus		KR422333
b	*Lactobacillus plantarum*	100	KJ778116
c	*Lactobacillus brevis*	97	KP773479
d	*Streptococcus* spp.	97	GU429575
e	*Lactobacillus* spp.	97	AB894863
f	*Lactococcus lactis*	100	KJ781895
g	*Enterococcus casseliflavus*	98	JX045722
h	*Enterococcus faecalis*	99	KP298396
i	Uncultured bacterium	99	KJ013312
j	*Escherichia coli*	100	JF919875
Micrococci and staphylococci (BPA)			
	Staphylococcus aureus-epidermidis species group		
a	*Staphylococcus aureus*	99	KP033216
	Staphylococcus epidermidis		AY699287
	Staphylococcus hominis		KM392087
b	*Enterococcus faecalis*	99	KP298396
c	Enterobacteriaceae		
	Hafnia spp.	99	KC210872
	Citrobacter spp.		KP972219
	Raoultella spp.		KC211307
	Staphylococcus saprophyticus species-group		
d	*S. saprophyticus*	100	KR059863
	S. xylosus		KM586983
e	*Staphylococcus simulans* species-group	99	
	S. simulans		KP202162
	S. carnosus		KJ862002
f	*Enterococcus* spp.	98	KM388675
Enterobacteriaceae (VRBGA)			
	Enterobacteriaceae		
a	*Hafnia* spp.	99	KC210872
	Citrobacter spp.		KP972219
	Raoultella spp.		KC211307
b	*Escherichia coli*	100	JF919875
c	*Enterobacter cloacae*	98	KF498698