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1 **Development and validation of a multiplex PCR-based DNA**
2 **microarray hybridisation method for detecting bacterial antibiotic**
3 **resistance genes in cheese**

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18 **Abstract**

19

20 The aim of this study was to develop a method for detecting antibiotic resistance
21 (AR) genes in cheese based on a combination of multiplex PCR and a DNA microarray
22 hybridisation system. Twenty oligonucleotide probes were designed targeting 10
23 common AR genes, namely *aac(6')-Ie-aph(2'')-Ia, aadE, aphA-3, ermB, tet*(L), *tet*(M),
24 *tet*(O), *tet*(S), *vanA* and *vanB*. Specificity of the probes was tested by hybridising
25 against DNA from *Enterococcus* strains harbouring known AR genes. DNA was
26 labelled through two multiplex PCR reactions with fluorescence nucleotides and
27 specific primers flanking the probe sequences. Sensitivity of the microarray was
28 assessed by contamination of a cheese with an *Enterococcus faecium* strain carrying
29 *vanA* gene. Two tetracycline resistance genes, *tet*(M) and *tet*(S), proved to be present in
30 a series of retail cheeses, while genes *aadE*, *aphA3*, *ermB*, *tet*(L) and *tet*(O) were
31 occasionally detected. This method is envisioned as a valuable tool for identification of
32 AR genes in foods.

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34 **1. Introduction**

35

36 The heavy use of antibiotics in human and veterinary medicine has resulted in
37 the appearance of antibiotic resistance (AR) that has spread between bacterial
38 populations. Resistance genes have been the subject of intense investigation since they
39 can be acquired horizontally via conjugation, transformation or transduction, and can be
40 transmitted in groups on plasmids, transposons or integrons (Tenover, 2006). The
41 spread of AR is a critical, global public health concern since the efficacy of drugs for
42 treating infections is reduced when AR genes are present (Levy & Marshall, 2004). The
43 recent findings of AR gene pools in commensal and beneficial bacteria isolated from
44 many retail products indicate AR dissemination to have occurred in food production and
45 processing environments (Duran & Marshall, 2005; Garofalo et al., 2007; Wang et al.,
46 2006). Although the true magnitude of the AR reservoir in food ecosystems is yet to be
47 learnt, the possibility of the transfer of resistance (either during food manufacturing or
48 during passage through the gastrointestinal tract) to opportunistic and pathogenic
49 microorganisms clearly exists (Netherwood et al., 1999). This argues for the
50 development of simple, rapid and reliable assays for the detection of AR genes directly
51 in food matrices. The determination of AR gene loads in foods would help to reveal the
52 types and levels of resistances that already exist in different food ecosystems.
53 Nowadays, PCR is the most common method used for studying the carriage of
54 AR genes in food systems, and several reports have described the use of different
55 molecular approaches based on PCR techniques, including quantitative Real Time PCR,
56 for detecting resistance determinants and for monitoring antimicrobial-resistant bacteria
57 in foods (Devirgiliis, Caravelli, Coppola, Barile, & Perozzi, 2008; Garofalo et al., 2007;
58 Manuzon et al., 2007). However, if every possible resistance gene had to be

59 independently assessed, the amount of work required would be large and time
60 consuming. Fortunately, multiplex PCR allows multiple gene analyses to be performed
61 at the same time. In addition, DNA microarray technology can be used for screening for
62 the presence of a wide diversity of genes, and has already been successfully used for AR
63 gene detection (Cassone et al., 2006; Perreten et al., 2005).

64 The aim of the present study was to develop a multiplex PCR-based DNA
65 microarray for detecting AR genes in cheese. The detection of AR determinants in
66 oligonucleotide microarray analyses employing fluorescently labelled PCR fragments
67 has been previously described (Grimm et al., 2004; Volokhov, Chizhikov, Chumakov,
68 & Rasooly, 2003; Yu, Susa, Knabbe, Schmid, & Bachmann, 2004). However, studies
69 dealing with the identification of AR genes using microarray technology are scarce,
70 except for those dealing with pathogenic bacteria.

71 This paper describes the development and validation of a microarray method for
72 determining the presence of several AR genes directly in cheese. To the best of our
73 knowledge, this is the first report on the use of a microarray system to establish the
74 resistance gene pool in a food matrix.

75

76 **2. Materials and methods**

77

78 *2.1. Bacterial strains*

79

80 Eight *Enterococcus* strains carrying known resistance determinants were used as
81 a control to test the specificity and sensitivity of the developed microarray-based
82 hybridisation system. The strains *Enterococcus faecalis* MN1 (*aadE*, *aphA-3, ermB*,
83 *vanB*), *E. faecalis* Jtet [*tet*(O)], *E. faecalis* ET35 [*aadE*, *aac(6')-Ie-aph(2'')-Ia, aphA-3,*

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84 *ermB, tet*(M)], *Enterococcus faecium* EO5 [*aadE*, *aphA-3, ermB, tet*(M)], *E. faecium*
85 ET51 [*ermB, tet*(L), *tet*(S)], *E. gallinarum* ET15 [*tet*(M), *tet*(S)] and *Enterococcus*
86 *mundtii* ET39 [*ermB*, *tet*(L), *tet*(M)] were previously characterized by Rizzotti et al.
87 (2005) while the strain *E. faecium* FAIR-E 132 [*aac(6')-Ie-aph(2'')-Ia, ermB, vanA*]
88 was analysed by Vancanneyt et al. (2002). Enterococci were grown overnight at 37 °C
89 in Brain Heart Infusion (BHI, Fluka BioChemika, Buchs, Switzerland). Genomic DNA
90 was isolated from 2 mL overnight bacterial cultures using the Gen Elute Bacterial
91 Genomic DNA kit (Sigma-Aldrich, St. Louis MO, USA). DNA was quantified using an
92 Eppendorf BioPhotometer Plus apparatus (Eppendorf, Hamburg, Germany).
93
94 *2.2. Multiplex PCR amplification and fluorescent labelling*
95
96 Two multiplex PCR reactions were designed for amplifying 10 common AR
97 genes: *aac(6')-Ie-aph(2'')-Ia*, *aadE, aphA-3, ermB, tet*(L), *tet*(M), *tet*(O), *tet*(S), *vanA*
98 and *vanB*. These genes were selected for their wide distribution or clinical importance.
99 These PCR reactions were based on previously published primers (Table 1) and served
100 to label the target DNA via the incorporation of modified fluorescent nucleotides (Cy5-
101 dCTPs, Amersham GE Healthcare, Little Chalfont, UK). The multiplex PCRs were
102 named "M5AR_55", referring to the reaction amplifying the genes *aac(6')-Ie-aph(2'')-*
103 *Ia, aadE, ermB, tet*(M) and *tet*(S), and "M5AR_60", referring to the reaction amplifying
104 the genes *aphA-3*, *tet*(L), *tet*(O), *vanA*, and *vanB*. Both PCR mixtures contained GoTaq
105 Colorless Flexi Buffer at 1.5× in the reaction (Part number M890A, Promega, Madison,
106 USA), 1.5 Units of GoTaq Flexi DNA Polymerase (Promega), 3 mM $MgCl_2$, 100 μM of
107 dATP and dGTP, 90 μM of dTTP and dCTP, and 10 μM of Cy5-dCTP and dUTP
108 (Epicentre Biotechnologies, Madison, USA). All primers were used at a final

109 concentration of 0.3 μM with the exception of primers *van*A and *van*B which were
110 included in the M5AR_60 mixture at 1 μM and 0.5 μM respectively. The reactions were
111 prepared in 20 μL volumes containing 30 ng of template DNA. Amplifications were
112 performed in a GeneAmp PCR System 2400 thermocycler (PerkinElmer, Waltham,
113 USA). Thermocycling was performed using an initial denaturing step of 95 °C for 5 min
114 followed by 35 cycles of 95 °C for 45 s, annealing (see Table 1 for temperatures) for 45
115 s, 72 °C for 45 s, and a final extension step of 72 °C for 10 min. Labelled amplicons
116 were then subjected to a fragmentation process with 0.1 Units of uracyl glycosylase
117 (Epicentre) for 20 min at 37 °C.
118
119 *2.3. Probe design and array printing*
120
121 Based on the regions amplified with the multiplex PCRs, highly conserved
122 sequences of the AR genes were selected to design 20 oligonucleotide probes, two
123 probes for each gene. Sequences corresponding to the AR genes of different
124 microorganisms were retrieved from the GenBank database and aligned using ClustalX
125 software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). For the design
126 of the oligonucleotide probe set, BioEdit 5.0 (Hall, 1999) and MEGA 2.1 (Kumar,
127 Tamura, Jakobsen, & Nei, 2001) programs were used. In silico analyses of the probes
128 were performed using Clone Manager Suite 7. 0 software (Sci Ed Central, North
129 Carolina, USA). The melting temperature (Tm) of each probe was calculated using the
130 Nearest Neighbour method and the thermodynamic parameters of Santa Lucia (1998).
131 The analysis was performed using online TMCHECK software available at
132 http://www.bioinformatics-toolkit.org/Web-TmCheck/. The designed probes were 17-
133 18 nucleotides in length. All had a similar Tm and GC content (Table 2).

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134 The probes were synthesized with a 5’ amino modification and a 12-carbon
135 spacer (Sigma-Genosys, Haverhill, UK) and were spotted in five replicates onto the
136 surface of activated e-surf glass slides (Life Line Lab, Rome, Italy) using a SpotArray
137 24 system (PerkinElmer) with a four-pin printhead configuration. The resulting spots
138 had an average diameter of 200 μm. The probes were imprinted in 300 mM sodium
139 phosphate buffer pH 8.5 at a concentration of 50 μM. In each subarray a control probe
140 consisting of an oligonucleotide sequence of soy lectin (LectSoy) (NH_2-C12-
141 ATTGACGTGAACTCGAT) was printed as a reference of position and as a positive
142 hybridisation control. The LectSoy probe was complementary to a labelled
143 oligonucleotide target (5’-Cy5-ATCGAGTTCACGTCAAT -3’), which was included in
144 the hybridisation solution.

145 Following manufacturer’s instructions for an effective cross-linking, the spotted
146 slides were left overnight in a sealed in-house humidification chamber containing a
147 saturated NaCl solution which generates an environment with 75% relative humidity.
148 Residual reactive groups were blocked by immersion of the slides in a 50 mM
149 ethanolamine, 0.1M Tris-HCl, pH 9.0, solution at 50 °C for 15 min with gentle agitation.
150 Subsequently, slides were washed twice in water followed by two washes at 50 °C for
151 15 min in 4× saline-sodium citrate (SSC) buffer (600 mM NaCl, 60 mM sodium citrate,
152 pH 7.0) containing 0.1% sodium dodecyl sulphate (SDS) and given two final rinses in
153 water. The spotted slides were air dried and maintained dry at room temperature. The
154 quality of the spots was checked with the commercial SpotCheck kit (Genetix, New
155 Milton, UK).

156

157 *2.4. Microarray hybridisation*

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159 Oligonucleotide-printed slides were prepared by marking out the hybridisation
160 region with a gas-tight reaction chamber (In situ Frames, Eppendorf) and pre-hybridised
161 with 10 mg mL^{-1} of bovine serum albumin (BSA, Fluka) at room temperature for 10
162 min immediately prior to hybridisation. 0.5 µL of a 5 nM solution of Cy5-labelled
163 LectSoy was added to the fluorescent targets and sterile water incorporated to a volume
164 of 43 µL. The mixtures were heated at 99 °C for 10 min to denature the DNA and then
165 chilled on ice immediately. A hybridisation buffer was then added consisting of 5.5 µL
166 of 20× SSC (Sigma), 5.5 µL of SDS 1% and 1.1 µL of 10 mg mL^{-1} BSA. The mixtures
167 were denatured for 2 min, cooled on ice, applied to the pre-hybridised slides, and
168 covered with the supplied plastic cover slip (Eppendorf). Hybridisation was performed
169 over 4 h at 42 °C in a humid hybridisation chamber (Thermomixer Comfort, Eppendorf)
170 with rotation at 1,400 rpm. After hybridisation, slides were washed twice in 2× SSC,
171 0.1% SDS at 42 °C for 5 min each time, then once in 0.2× SSC for 2 min at room
172 temperature, before receiving a final wash in 0.1× SSC for 2 min at room temperature.
173 The slides were then air dried and kept in the dark.
174
175 *2.5. Detection procedure and data acquisition*
176
177 After hybridisation the microarray slides were scanned for Cy5 detection using a
178 ScanArray 4000XL apparatus (PerkinElmer) equipped with a 633 nm laser. The settings
179 used for the scanner were laser power 75-80% and photomultiplier tube (PMT) 80-85%.
180 Analysis of the fluorescent signals was performed using ScanArray Express 4.0
181 software (PerkinElmer). For each spot, the mean pixel intensity was assessed and the
182 background signal subtracted. The mean fluorescence intensity of the five replicate
183 spots and the standard deviation (intra-probe standard deviation) were calculated for

184 each probe. A probe was confirmed as switch-on only if the value of its fluorescence
185 intensity was at least two fold higher than that of the neighbouring background (Lehner
186 et al., 2005).
187
188 *2.6. Cheese samples*
189
190 To study the response of the microarray in a food matrix, an industrial cheese
191 (sample M, Table 3), which was shown to be free of the *vanA* gene by specific PCR,
192 was artificially inoculated with the vancomycin-resistant strain *E. faecium* FAIR-E 132
193 ($vanA^{+}$). For the inoculation, the strain was grown overnight in broth medium and its
194 concentration calculated by plate counting and OD measured at 600 nm using a UV/VIS
195 spectrophotometer (ATI-Unicam, Cambridge, UK). The culture was serially diluted in
196 saline solution (0.9% NaCl) and spiked with a sterile syringe into the matrix of 5 g
197 portions to reach four different concentrations: 10^4, 10^5, 10^6 and 10^7 colony forming
198 units (cfu) g^{-1} of cheese. Duplicate contaminated samples and unspiked cheese were
199 then subjected to total DNA extraction. Additionally, four artisanal cheeses from
200 Northeast Italy (Table 3) were also analysed for the presence of indigenous AR genes.
201
202 *2.7. Extraction of total microbial DNA from cheese*
203
204 Cheese samples were homogenised 1/10 in 2% sodium citrate using a Stomacher
205 (Seward, Worthing, UK) at 260 rpm for 1 min. Initially, bacterial biomass was extracted
206 from the samples over a series of purification steps with ammonium hydroxide, absolute
207 ethanol, petroleum ether, 10% SDS, 6 M urea and 3 M sodium acetate following the
208 protocol of Drake, Small, Spence and Swanson (1996). The DNA was then purified

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209 from the cells using the Gen Elute Bacterial Genomic DNA kit. The amount of DNA
210 recovered was determined using the BioPhotometer. Purified DNA (~ 20 ng) from
211 cheeses was used as a template for multiplex PCR and the labelled amplicons were
212 subjected to microarray analysis.
213
214 *2.8. Capillary electrophoresis of multiplex PCR amplicons*
215
216 To confirm the results obtained with the microarray and the effectiveness of the
217 method, multiplex PCR amplicons were further analysed by capillary electrophoresis in
218 an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto CA, USA). Aliquots of
219 the multiplex PCR products (1 μL) were placed on a DNA microchip (LabChip 7500,
220 Agilent) and analysed according to the manufacturer's instructions. LabChip technology
221 and the Bioanalyzer 2100 allow for the separation of small DNA fragments by capillary
222 electrophoresis better than that achieved though 2% conventional agarose gel
223 electrophoresis (Nachamkin et al., 2001). In addition, the software automatically
224 calculates the size and DNA concentration of each fragment in relation to internal and
225 external standards.
226
227 *2.9. Processed data and reproducibility*
228
229 The reproducibility of the complete PCR-based microarray method was
230 evaluated by means of different approaches; in each hybridisation experiment the
231 presence of a particular AR gene was evaluated with two specific probes, each of which
232 was spotted in five replicates. The mean fluorescence intensity and the standard
233 deviation of each probe were calculated in every assay. Labelling and microarray

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234 hybridisation of the eight enterococcal strains were performed twice in independent
235 experiments. Moreover, for a particular probe we compared the signals obtained with
236 the different AR positive strains, i.e., probes for *aac(6')-Ie-aph(2'')-Ia* gene, strains
237 ET35 and FAIR-E 132; probes for *aadE* gene, strains MN1, ET35 and EO5; probes for
238 *aphA-3* gene, strains MN1, ET35 and EO5; probes for *ermB* gene, strains MN1, ET35,
239 EO5, ET51, ET39 and FAIR-E 132; probes for *tet*(L) gene, strains ET51 and ET39;
240 probes for *tet*(M) gene, strains ET35, EO5, ET15 and ET39; probes for *tet*(S)gene,
241 strains ET51 and ET15. Finally, contaminated and non-contaminated cheese samples
242 were analysed in duplicate.
243
244 **3. Results**
245
246 *3.1. Validation of the AR probes*
247
248 To validate the probes, a total of eight *Enterococcus* strains of different species
249 and origin, all of which harbour known resistance determinants, were screened using the
250 microarray system. The purified DNA, amplified and fluorescently labelled in the
251 multiplex PCR reactions, was used to verify the specificity of the microarray probes
252 targeting the AR genes. Fig. 1 shows the hybridisation images of representative strains.
253 Spots of LectSoy probe served both as a positive control for the hybridisation and as a
254 reference for positioning all other spots on the grid. Quantification of the hybridisation
255 signals revealed that the specific probes designed (two probes per AR gene) showed
256 fluorescence values of >1,000 intensity units and were at least 10 times higher than the
257 background signal, with consistency within both the five replicate spots. Most probes
258 (at least one for each gene) showed fluorescence values ranging from 2,000 to 8,000

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259 intensity units in the two replicates (Fig. 2) and in all the assays with the different AR
260 positive strains. It should be noted that the response was quite variable when comparing
261 signals obtained after hybridisation with different strains (Fig. 3). The probe tetM_142R
262 was the only one showing a recurrent low efficiency, indicating that it should be re-
263 designed.

264

265 *3.2. Cheese analysis using the microarray*

266

267 Based on the results obtained with the enterococcal strains, a threshold limit of
268 1,000 intensity units was established by calculating the mean of unspecific fluorescence
269 signals plus three standard deviations. The presence of a particular AR gene in a cheese
270 was considered positive if at least one of the two specific probes had a fluorescence
271 intensity higher than this value in two replicates.

272 The detection limit of the developed microarray was estimated with a
273 contaminated cheese (cheese M, Table 3), following the entire proposed experimental
274 procedure, i.e., purifying DNA from the cheese, labelling the DNA by multiplex PCR,
275 and hybridisation. The cheese was artificially contaminated at different concentrations
276 with the vancomycin-resistant strain *E. faecium* FAIR-E 132, which carries the *van*A
277 gene. The absence of this gene in the original cheese was verified by specific PCR
278 before inoculation of the strain. With an inoculum of 10^7 cfu g^{-1} of the *vanA* positive
279 strain the signal intensity of both *vanA* probes in the microarray was around 10 times
280 higher than the established cut-off. With smaller inocula (10^6 cfu g^{-1} and 10^5 cfu g^{-1}),
281 the intensity of the *vanA* probes was similar to that obtained with the DNA from a pure
282 culture of the strain and within the detection limit. However, when the contamination
283 level was 10^4 cfu g^{-1}, the signal of both probes was low (below 1,000 intensity units).

284 The microarray was then used to analyse the presence of AR determinants in
285 four other Italian cheeses including soft and ripened varieties made from raw or
286 pasteurized milk (Table 3).Table 4 summarises the genes detected and in Fig. 4 are
287 displayed some of the microarray hybridisation patterns obtained. The genes *tet*(M) and
288 *tet*(S) were found in all cheeses. In addition, the *tet*(O) gene was identified in all
289 artisanal cheeses, while the presence of *tet*(L) was revealed only in the industrial cheese.
290 The gene *aphA-3* was evidenced in the two cheeses made from raw milk, which in
291 addition harboured *aadE* and *ermB* genes (one in each sample). The genes *vanA* and
292 *vanB*, encoding vancomycin resistance, and the *aac(6')-Ie-aph(2'')-Ia* gene, responsible
293 for high-level gentamicin resistance, were not detected.
294
295 *3.3. Cheese analysis by capillary electrophoresis*
296
297 To assess the effectiveness of the microarray, the results obtained were
298 compared with those achieved by analysing the multiplex PCR products of the samples
299 using the Agilent 2100 Bioanalyzer. Fig. 5 shows the electrophoretograms obtained and
300 the gel-like images generated from the different samples. The peaks in the
301 chromatograms correspond to the amplification products of the different AR genes
302 detected, except for the 50 bp and 10,380 bp peaks which correspond to two internal
303 reference markers. The results proved to be consistent with the AR gene content
304 identified by the microarray, although amplicons obtained from some cheeses could not
305 be easily distinguished by this methodology (Fig. 5; samples 10 and 11 corresponding
306 to cheeses S and C). In addition, non-specific products generated by the multiplex PCRs
307 were occasionally revealed, such as the third peak of sample 2 (cheese B).
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309 **4. Discussion**

310

311 The direct labelling of total bacterial DNA by random amplification is the
312 method of choice when using microarrays (Vora, Meador, Stenger, & Andreadis, 2004)
313 since it allows any target sequence present in the genomes to be labelled. Unfortunately,
314 a large amount of target DNA is necessary for this type of labelling, which limits its use
315 in complex samples such as cheese. Thus, the use of a target-specific PCR strategy to
316 label the genes was more convenient to identify the presence of AR determinants in
317 cheese.

318 The PCR-based microarray system was validated firstly using DNA from
319 enterococcal cultures. Duplicate analysis of the positive strains demonstrated the high
320 specificity of the designed oligonucleotide probes. Although varying within a certain
321 range of fluorescence values, the results were quite reproducible by analysing the same
322 strain in two replicates and unequivocal specific patters of distinct positive spots were
323 recorded. When hybridising different strains the variability in fluorescence was higher,
324 probably due to differences in the location of AR genes (plasmid or chromosomally
325 encoded genes) and/or to the presence of different AR genes.

326 Based on the fluorescence values obtained with the enterococci, and following
327 comparable criteria to those reported in similar studies (Cassone et al., 2006; Frye et al.,
328 2006; Vora et al., 2004), a cut-off value of 1,000 intensity units was established for the
329 analysis of AR genes in cheese. When a cheese was inoculated with a vancomycin-
330 resistant strain at a concentration of 10^5 cfu g^{-1} or higher, the fluorescence signal
331 obtained with the developed microarray was over the threshold limit, and unambiguous
332 microarray images with switch on *vanA* spots were obtained in both duplicates.
333 Consequently, we consider that for reliable detection in cheese, AR gene-carrying

334 bacteria should be above this concentration. Notwithstanding, when using culture-
335 independent microbial techniques, such as this microarray system, it is certainly
336 difficult to ensure the absence of DNA extraction biases, since not all bacterial groups
337 present within the cheese matrix can be released in the same way (Mayo, Marzotto,
338 Flórez, & Torriani, 2008).

339 On the other hand, AR gene-carrying bacteria in cheese can be alive, dead or
340 even on a viable-but-non-culturable (VBNC) state. Analysis of resistances by culturing
341 in media-containing antibiotics will neither reveal those carried by VBNC cells nor
342 those present in dead bacteria. However, under appropriate conditions, all these AR
343 determinants could be transferred to harmful microbes in food and in the
344 gastrointestinal tract (Netherwood et al., 1999), thus contributing to their spread.

345 Compared to capillary electrophoresis, the microarray system provided distinct
346 advantages in the detection and differentiation of AR genes, and was shown to be a
347 good, practical tool for assessing the incidence of important AR genes in cheese
348 matrices. In fact, the microarray technology allows for the simultaneous detection of
349 several PCR products, even though they may have similar dimensions. Moreover, the
350 hybridisation step introduces the possibility of detecting only the desired PCR products.
351 Also, the sensitivity of the microarray technology is potentially greater than
352 electrophoresis and might be even improved by enhancing the ratio between Cy5 and
353 dCTPs in the PCR reactions or by increasing the settings of the scanner. With a
354 threshold of 1,000 intensity units any risk of false positive results is avoid, even in the
355 case of a high background signal. It was only because of this very restrictive condition
356 that the hybridisation signal obtained with cheese inoculated with 10^4 cfu g^{-1} was
357 considered negative. In the near future it is likely that the detection cut-off of the system

358 will be improved by optimising the experimental procedures and increasing the
359 hybridisation efficiency of the probes.

360 In this study, the presence of different AR genes was detected by the microarray
361 in both soft and ripened cheese samples of different varieties made from raw or
362 pasteurized cows' milk, including one industrial cheese. Antibiotic-resistant bacteria
363 have been shown to be present in a large variety of fermented foods. Moreover, up to
364 10^7 cfu of antibiotic-resistant bacteria per gram of food have been reported in studies
365 examining several Cheddar-type retail cheeses (Wang et al., 2006). In the present work,
366 tetracycline resistance genes were shown to be abundant in the Italian cheeses analysed,
367 with *tet*(M) and *tet*(S) as the most prevalent. Tetracycline is a broad spectrum antibiotic
368 and bacterial resistance to it is rather common. These two particular tetracycline
369 resistance genes have been reported widespread in different food products of animal
370 origin (Garofalo et al., 2007; Manuzon et al., 2007; Wang et al., 2006) and have been
371 associated not only with enterococci but also with commensal lactic acid bacteria
372 usually present in cheese such as *Lactobacillus plantarum* (Gevers et al., 2003),
373 *Streptococcus thermophilus* (Ge et al., 2007) and *Lactococcus lactis* (Flórez, Ammor, &
374 Mayo, 2008).

375 Although the use of microarrays has been recommended as the most suitable
376 technique for the determination of resistance genes (Holzman, 2003; Perreten et al.,
377 2005), to date their use has been restricted to the analysis of individual bacterial isolates
378 (Cassone et al., 2006; Frye et al., 2006; van Hoek, Scholtens, Cloeckaert, & Aarts, 2005;
379 Volokhov et al., 2003; Yu et al., 2004). However, in microbial complex samples such as
380 food matrices powerful genetic tools - such as that proposed in this work - are necessary
381 to identify the presence of antimicrobial resistance determinants. Recently Patterson,
382 Colangeli, Spigaglia and Scott (2007) used macroarrays to investigate the distribution of

383 AR genes in environmental samples (bacterial DNA extracted from soil and faeces), but
384 to the best of our knowledge the present study is the first to report the use of a
385 microarray method for detecting an unknown resistance gene pool directly in a food
386 system.
387
388 **5. Conclusions**
389
390 The experimental procedure developed here offers a useful way of rapidly
391 collecting data on the load and distribution of AR genes in cheese samples. Although
392 the repertoire of probes for AR detection is not yet complete, the proposed multiplex
393 PCR-based DNA microarray should be of use in assessing the presence of AR genes in
394 other food samples in which the presence of antibiotic resistant bacteria is suspected.
395
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397
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Table 1.

Primers used in this study for the two multiplex PCR reactions.

Primer	Gene	Sequence (5' to 3')	Amplicon size (bp)	Annealing T (ºC)	Reference
aph-1 aph-2	*aac(6')-Ie-aph(2'')-Ia*	GAGCAATAAGGGCATACCAAAAATC CCGTGCATTTGTCTTAAAAAACTGG	480	55	Kao et al.(2000)
ant(6´)-F ant(6´)-R	*aadE*	ACTGGCTTAATCAATTTGGG GCCTTTCCGCCACCTCACCG	597	55	Clark, Olsvik, Swenson, Spiegel, & Tenover (1999)
aphA3-F aphA3-R	*aphA-3*	GCTGCGTAAAAGATACGGAAGG CCCAATCAGGCTTGATCCCC	698	60	Derbise, Aubert, & El Solh (1997)
ermB-I ermB-II	*ermB*	GAAAAGGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	639	55	Sutcliffe, Grebe, Tait-Kamradt, & Wondrack (1996)
tetL-up tetL-down	*tet*(L)	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAACTAATGACAATGAT	1,077	60	Trzcinski, Cooper, Hryniewicz, & Dowson (2000)
tet(M)-F tet(M)-R	*tet*(M)	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	406	55	Ng, Martin, Alfa, & Mulvey (2001)
tet(O)-F tet(O)-R	*tet*(O)	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	515	60	Ng et al. (2001)
tet(S)-F tet(S)-R	*tet*(S)	ATGTTTTTGGAACGCCAGAG CATAGACAAGCCGTTGACC	667	55	Ng et al. (2001)
vanA1 vanA2	*vanA*	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	60	Dutka-Malen, Evers, & Courvalin (1995)
vanB1 vanB2	*vanB*	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	60	Dutka-Malen et al. (1995_

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Table 2.

Oligonucleotide probes designed for their inclusion in the microarray.

Probe	Target gene	Sequence (5´to 3´)	Length	Tm[a] (°C)	% GC
aacAaph_72	*aac(6')-Ie-aph(2'')-Ia*	CCTCGTGTAATTCATGTT	18	45.4	38.9
aacAaph_218		CACACTATCATAACCACT	18	44.7	38.9
aadE_269R	*aadE*	GTATGATGATTGCTGCA	17	45.0	41.2
aadE_233		ATCAGTCGGAACTATGT	17	44.6	41.2
aphA3_295	*aphA-3*	GCATACAGCTCGATAAT	17	44.9	41.2
aphA3_337		CAATCCGATATGTCGAT	17	43.8	41.2
ermB_495R	*ermB*	CACAGATGTTCCAGATAA	18	44.3	38.9
ermB_557R		GAGAATATCGTCAACTGT	18	44.5	38.9
tetL_121	*tet*(L)	CTACAACCATTACGAGT	17	44.2	41.2
tetL_689R		TTGATAGAAGAGGTCCTT	18	44.6	38.9
tetM_117	*tet*(M)	GTCTATGATGTTCACCTT	18	44.3	38.9
tetM_142R		GCAGAAGTATATCGTTCA	18	45.3	38.9
tetO_264R	*tet*(O)	AGACGGAGCAGTATTAT	17	45.1	41.2
tetO_217		CTGGCGTATCTATAATGT	18	45.4	38.9
tetS_96	*tet*(S)	TCCAGGAGTATCTACAAT	18	44.7	38.9
tetS_215		CTAAGTGCATGGAATAGT	18	45.1	38.9
vanA_145	*vanA*	GATCCATCTTCACCTGA	17	44.8	47.1
vanA_244		GCAACGATGTATGTCAA	17	45.4	41.2
vanB_245	*vanB*	TGTAAGAATGTAGGCCA	17	45.5	41.2
vanB_490R		CGAGGATGATTTGATTGT	18	44.8	38.9

[a] Melting temperature of the probes calculated with the Nearest Neighbour method and the thermodynamic parameters of Santa Lucia (1998).

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Table 3.

Italian cheeses analysed for the detection of antibiotic resistance genes.

Sample code	Cheese	Geographical region	Stage of ripening	Origin
A	Malga	Friuli-Venezia-Giulia	Ripened	Artisanal from raw milk
B	Malga	Trentino Alto-Adige	Ripened	Artisanal from raw milk
M	Montasio	Friuli-Venezia-Giulia	Ripened	Industrial from pasteurized milk
S	Stracchino	Trentino Alto-Adige	Fresh-soft	Artisanal from pasteurized milk
C	Caciotta	Trentino Alto-Adige	Fresh-soft	Artisanal from pasteurized milk

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Table 4.

Antibiotic resistance genes detected in the cheese samples with the microarray.

Multiplex PCRs[a]	Cheese sample				
	A	B	M	S	C
M5AR_55	*ermB*, *tet*(M), *tet*(S)	*aadE*, *tet*(M), *tet*(S)	*tet*(M), *tet*(S)	*tet*(M), *tet*(S)	*ermB, tet*(M), *tet*(S)
M5AR_60	*aphA-3*, *tet*(O)	*aphA-3*, *tet*(O)	*tet*(L)	*tet*(O)	*tet*(O)

[a] M5AR_55, PCR reaction amplify *aac(6')-Ie-aph(2'')-Ia*, *aadE*, *ermB*, *tet*(M) and *tet*(S) genes; M5AR_60, PCR reaction amplify *aphA-3*, *tet*(L), *tet*(O)*, vanA*, and *vanB* genes.

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1 **Figure legends**

2

3 **Fig. 1.** Microarray hybridisation images of different enterococcal strains obtained by
4 labelling through multiplex PCR for the following antibiotic resistance genes *aac(6')-*
5 *Ie-aph(2'')-Ia, aadE, ermB, tet*(M) and *tet*(S): A) *E. faecalis* ET35 [*aac(6')-Ie-aph(2'')-*
6 *Ia*$^+$, *aadE*$^+$, *ermB*$^+$, *tet*(M)$^+$]; B) *E. gallinarum* ET15 [*tet*(M)$^+$, *tet*(S)$^+$]; C) *E. faecium*
7 FAIR-E 132 [*aac(6')-Ie-aph(2'')-Ia*$^+$, *ermB*$^+$]; D) *E. faecium* EO5 [*aadE*$^+$, *ermB*$^+$,
8 *tet*(M)$^+$]; E) *E. mundtii* ET39 [*ermB*$^+$, *tet*(M)$^+$]. The names of the probes are referred as
9 in Table 2. Spots of the control LectSoy probe are also indicated.

10

11 **Fig. 2.** Quantification of the microarray signals (in fluorescence intensity) of the
12 antibiotic resistance probes for hybridisations with DNA from enterococcal strains
13 labelled by the multiplex PCR in replicate experiments (▨, first replicate; ▨, second
14 replicate): A) *E. faecium* ET51 [*ermB*$^+$, *tet*(S)$^+$] amplified for the resistance genes
15 *aac(6')-Ie-aph(2'')-Ia, aadE, ermB, tet*(M) and *tet*(S); B) *E. faecalis* MN1 (*aphA-3*$^+$,
16 *vanB*$^+$) amplified for the resistance genes *aphA-3*, *tet*(L), *tet*(O), *vanA*, and *vanB*.

17

18 **Fig. 3.** Comparison of microarray signal (in fluorescence intensity) of the 20 antibiotic
19 resistance probes in the different assays with the positive enterococci strains: probes for
20 *aac(6')-Ie-aph(2'')-Ia* gene, two strains; probes for *aadE* gene, three strains; probes for
21 *aphA-3* gene, three strains; probes for *ermB* gene, six strains; probes for *tet*(L) gene, two
22 strains; probes for *tet*(M) gene, four strains; probes for *tet*(S)gene, two strains. Probes
23 for *vanA*, *vanB* and *tet*(O) genes single strains were examined. Data are the average of
24 two independent experiments. The names of the probes are referred as in Table 2.

25

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26 **Fig. 4.** Microarray hybridisation images of cheeses obtained by labelling of the bacterial
27 DNA with the multiplex PCR for the antibiotic resistance genes *aphA-3*, *tet*(L), *tet*(O),
28 *vanA*, and *vanB*. Letters A, B and C illustrate the hybridisation patterns of cheese M
29 [*tet*(L)$^+$], cheese C [*tet*(O)$^+$], and cheese B [*aphA-3*$^+$, *tet*(O)$^+$], respectively.

30

31 **Fig. 5.** Electrophoretograms (upper panel) and simulated gel (lower panel) obtained by
32 capillary electrophoresis in the Agilent 2100 Bioanalyzer. Samples 1, 2, 3, 4, 5 and 6
33 correspond to the amplicons obtained with the multiplex PCR for the antibiotic
34 resistance genes *aphA-3* (698 bp), *tet*(L) [1,077 bp], *tet*(O) [515 bp], *vanA* (732 bp) and
35 *vanB* (635 bp)in the cheeses A [*aphA-3*$^+$, *tet*(O)$^+$], B [*aphA-3*, *tet*(O)$^+$], M [*tet*(L)$^+$], S
36 [*tet*(O)$^+$], C [*tet*(O)$^+$] and cheese M contaminated with 10^5 cfu g^{-1} *van*A$^+$ strain,
37 respectively. Samples 7, 8, 9, 10, 11 and 12 correspond to the amplicons obtained with
38 the multiplex PCR for the antibiotic resistance genes *aac(6')-Ie-aph(2'')-Ia* (480 bp),
39 *aadE* (597 bp), *ermB* (639 bp), *tet*(M) [406 bp] and *tet*(S) [667 bp] in the cheeses A
40 [*ermB*$^+$, *tet*(M)$^+$, *tet*(S)$^+$], B [*aadE*$^+$, *tet*(M)$^+$, *tet*(S)$^+$], M [*tet*(M)$^+$, *tet*(S)$^+$], S [*tet*(M)$^+$,
41 *tet*(S)$^+$], C [*ermB*$^+$, *tet*(M)$^+$, *tet*(S)$^+$] and the strain *E. faecalis* ET35 [*aadE*$^+$, *aac(6')-Ie-*
42 *aph(2'')-Ia*$^+$, *ermB*$^+$, *tet*(M)$^+$], respectively. L, 50 - to 10,380 bp marker. The 50 bp and
43 10,380 bp peaks appear in the electrophoretograms as internal references.

44

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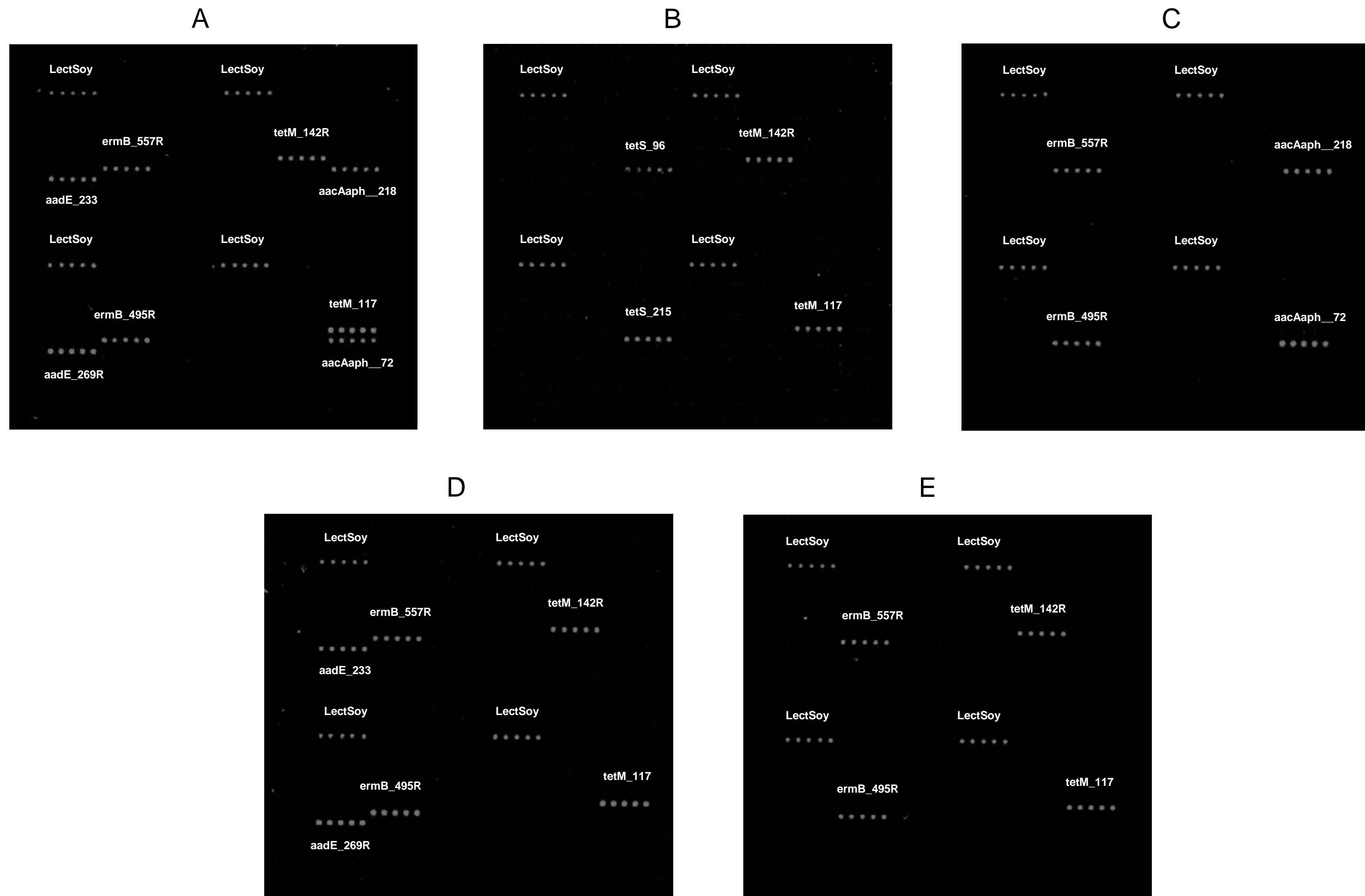

Fig. 1

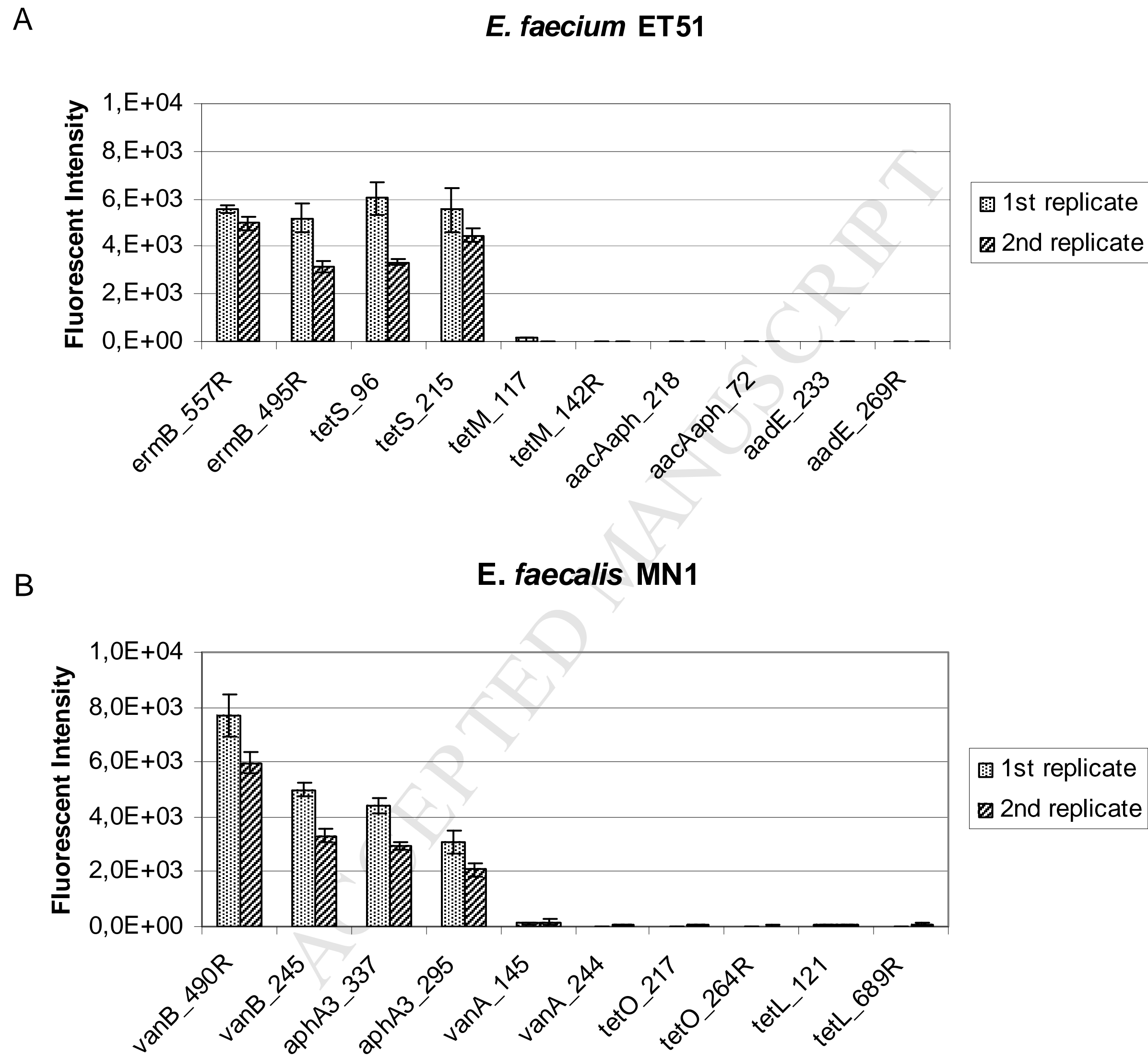

Fig. 2

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