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

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Abstract

The aim of this study was to develop a method for detecting antibiotic resistance (AR) genes in cheese based on a combination of multiplex PCR and a DNA microarray hybridisation system. Twenty oligonucleotide probes were designed targeting 10 common AR genes, namely aac(6’)-Ie-aph(2’’)-Ia, aadE, aphA-3, ermB, tet(L), tet(M), tet(O), tet(S), vanA and vanB. Specificity of the probes was tested by hybridising against DNA from Enterococcus strains harbouring known AR genes. DNA was labelled through two multiplex PCR reactions with fluorescence nucleotides and specific primers flanking the probe sequences. Sensitivity of the microarray was assessed by contamination of a cheese with an Enterococcus faecium strain carrying vanA gene. Two tetracycline resistance genes, tet(M) and tet(S), proved to be present in a series of retail cheeses, while genes aadE, aphA3, ermB, tet(L) and tet(O) were occasionally detected. This method is envisioned as a valuable tool for identification of AR genes in foods.
The heavy use of antibiotics in human and veterinary medicine has resulted in the appearance of antibiotic resistance (AR) that has spread between bacterial populations. Resistance genes have been the subject of intense investigation since they can be acquired horizontally via conjugation, transformation or transduction, and can be transmitted in groups on plasmids, transposons or integrons (Tenover, 2006). The spread of AR is a critical, global public health concern since the efficacy of drugs for treating infections is reduced when AR genes are present (Levy & Marshall, 2004). The recent findings of AR gene pools in commensal and beneficial bacteria isolated from many retail products indicate AR dissemination to have occurred in food production and processing environments (Duran & Marshall, 2005; Garofalo et al., 2007; Wang et al., 2006). Although the true magnitude of the AR reservoir in food ecosystems is yet to be learnt, the possibility of the transfer of resistance (either during food manufacturing or during passage through the gastrointestinal tract) to opportunistic and pathogenic microorganisms clearly exists (Netherwood et al., 1999). This argues for the development of simple, rapid and reliable assays for the detection of AR genes directly in food matrices. The determination of AR gene loads in foods would help to reveal the types and levels of resistances that already exist in different food ecosystems.

Nowadays, PCR is the most common method used for studying the carriage of AR genes in food systems, and several reports have described the use of different molecular approaches based on PCR techniques, including quantitative Real Time PCR, for detecting resistance determinants and for monitoring antimicrobial-resistant bacteria in foods (Devirgiliis, Caravelli, Coppola, Barile, & Perozzi, 2008; Garofalo et al., 2007; Manuzon et al., 2007). However, if every possible resistance gene had to be
independently assessed, the amount of work required would be large and time consuming. Fortunately, multiplex PCR allows multiple gene analyses to be performed at the same time. In addition, DNA microarray technology can be used for screening for the presence of a wide diversity of genes, and has already been successfully used for AR gene detection (Cassone et al., 2006; Perreten et al., 2005).

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

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

2. Materials and methods

2.1. Bacterial strains

Eight Enterococcus strains carrying known resistance determinants were used as a control to test the specificity and sensitivity of the developed microarray-based hybridisation system. The strains Enterococcus faecalis MN1 (aadE, aphA-3, ermB, vanB), E. faecalis Jtet [tet(O)], E. faecalis ET35 [aadE, aac(6’)-Ie-aph(2’’)-Ia, aphA-3,
Enterococcus faecium EO5 [aadE, aphA-3, ermB, tet(M)], E. faecium ET51 [ermB, tet(L), tet(S)], E. gallinarum ET15 [tet(M), tet(S)] and Enterococcus mundii ET39 [ermB, tet(L), tet(M)] were previously characterized by Rizzotti et al. (2005) while the strain E. faecium FAIR-E 132 [aac(6’)-Ie-aph(2’’)-Ia, ermB, vanA] was analysed by Vancanneyt et al. (2002). Enterococci were grown overnight at 37 °C in Brain Heart Infusion (BHI, Fluka BioChemika, Buchs, Switzerland). Genomic DNA was isolated from 2 mL overnight bacterial cultures using the Gen Elute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis MO, USA). DNA was quantified using an Eppendorf BioPhotometer Plus apparatus (Eppendorf, Hamburg, Germany).

2.2. Multiplex PCR amplification and fluorescent labelling

Two multiplex PCR reactions were designed for amplifying 10 common AR genes: aac(6’)-Ie-aph(2’’)-Ia, aadE, aphA-3, ermB, tet(L), tet(M), tet(O), tet(S), vanA and vanB. These genes were selected for their wide distribution or clinical importance. These PCR reactions were based on previously published primers (Table 1) and served to label the target DNA via the incorporation of modified fluorescent nucleotides (Cy5-dCTPs, Amersham GE Healthcare, Little Chalfont, UK). The multiplex PCRs were named “M5AR_55”, referring to the reaction amplifying the genes aac(6’)-Ie-aph(2’’)-Ia, aadE, ermB, tet(M) and tet(S), and “M5AR_60”, referring to the reaction amplifying the genes aphA-3, tet(L), tet(O), vanA, and vanB. Both PCR mixtures contained GoTaq Colorless Flexi Buffer at 1.5× in the reaction (Part number M890A, Promega, Madison, USA), 1.5 Units of GoTaq Flexi DNA polymerase (Promega), 3 mM MgCl₂, 100 µM of dATP and dGTP, 90 µM of dTTP and dCTP, and 10 µM of Cy5-dCTP and dUTP (Epicentre Biotechnologies, Madison, USA). All primers were used at a final
concentration of 0.3 µM with the exception of primers \textit{vanA} and \textit{vanB} which were included in the M5AR\_60 mixture at 1 µM and 0.5 µM respectively. The reactions were prepared in 20 µL volumes containing 30 ng of template DNA. Amplifications were performed in a GeneAmp PCR System 2400 thermocycler (PerkinElmer, Waltham, USA). Thermocycling was performed using an initial denaturing step of 95 °C for 5 min followed by 35 cycles of 95 °C for 45 s, annealing (see Table 1 for temperatures) for 45 s, 72 °C for 45 s, and a final extension step of 72 °C for 10 min. Labelled amplicons were then subjected to a fragmentation process with 0.1 Units of uracyl glycosylase (Epicentre) for 20 min at 37 °C.

2.3. \textit{Probe design and array printing}

Based on the regions amplified with the multiplex PCRs, highly conserved sequences of the AR genes were selected to design 20 oligonucleotide probes, two probes for each gene. Sequences corresponding to the AR genes of different microorganisms were retrieved from the GenBank database and aligned using ClustalX software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). For the design of the oligonucleotide probe set, BioEdit 5.0 (Hall, 1999) and MEGA 2.1 (Kumar, Tamura, Jakobsen, & Nei, 2001) programs were used. In silico analyses of the probes were performed using Clone Manager Suite 7.0 software (Sci Ed Central, North Carolina, USA). The melting temperature (Tm) of each probe was calculated using the Nearest Neighbour method and the thermodynamic parameters of Santa Lucia (1998). The analysis was performed using online TMCHECK software available at http://www.bioinformatics-toolkit.org/Web-TmCheck/. The designed probes were 17-18 nucleotides in length. All had a similar Tm and GC content (Table 2).
The probes were synthesized with a 5’ amino modification and a 12-carbon spacer (Sigma-Genosys, Haverhill, UK) and were spotted in five replicates onto the surface of activated e-surf glass slides (Life Line Lab, Rome, Italy) using a SpotArray 24 system (PerkinElmer) with a four-pin printhead configuration. The resulting spots had an average diameter of 200 μm. The probes were imprinted in 300 mM sodium phosphate buffer pH 8.5 at a concentration of 50 μM. In each subarray a control probe consisting of an oligonucleotide sequence of soy lectin (LectSoy) (NH₂-C₁₂-ATTGACGTGAACTCGAT) was printed as a reference of position and as a positive hybridisation control. The LectSoy probe was complementary to a labelled oligonucleotide target (5’-Cy5-ATCGAGTTCACGTCAAT -3’), which was included in the hybridisation solution.

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

2.4. Microarray hybridisation
Oligonucleotide-printed slides were prepared by marking out the hybridisation region with a gas-tight reaction chamber (In situ Frames, Eppendorf) and pre-hybridised with 10 mg mL\(^{-1}\) of bovine serum albumin (BSA, Fluka) at room temperature for 10 min immediately prior to hybridisation. 0.5 µL of a 5 nM solution of Cy5-labelled LectSoy was added to the fluorescent targets and sterile water incorporated to a volume of 43 µL. The mixtures were heated at 99 °C for 10 min to denature the DNA and then chilled on ice immediately. A hybridisation buffer was then added consisting of 5.5 µL of 20× SSC (Sigma), 5.5 µL of SDS 1% and 1.1 µL of 10 mg mL\(^{-1}\) BSA. The mixtures were denatured for 2 min, cooled on ice, applied to the pre-hybridised slides, and covered with the supplied plastic cover slip (Eppendorf). Hybridisation was performed over 4 h at 42 °C in a humid hybridisation chamber (Thermomixer Comfort, Eppendorf) with rotation at 1,400 rpm. After hybridisation, slides were washed twice in 2× SSC, 0.1% SDS at 42 °C for 5 min each time, then once in 0.2× SSC for 2 min at room temperature, before receiving a final wash in 0.1× SSC for 2 min at room temperature. The slides were then air dried and kept in the dark.

### 2.5. Detection procedure and data acquisition

After hybridisation the microarray slides were scanned for Cy5 detection using a ScanArray 4000XL apparatus (PerkinElmer) equipped with a 633 nm laser. The settings used for the scanner were laser power 75-80% and photomultiplier tube (PMT) 80-85%. Analysis of the fluorescent signals was performed using ScanArray Express 4.0 software (PerkinElmer). For each spot, the mean pixel intensity was assessed and the background signal subtracted. The mean fluorescence intensity of the five replicate spots and the standard deviation (intra-probe standard deviation) were calculated for
each probe. A probe was confirmed as switch-on only if the value of its fluorescence intensity was at least two fold higher than that of the neighbouring background (Lehner et al., 2005).

2.6. Cheese samples

To study the response of the microarray in a food matrix, an industrial cheese (sample M, Table 3), which was shown to be free of the vanA gene by specific PCR, was artificially inoculated with the vancomycin-resistant strain *E. faecium* FAIR-E 132 (vanA<sup>+</sup>). For the inoculation, the strain was grown overnight in broth medium and its concentration calculated by plate counting and OD measured at 600 nm using a UV/VIS spectrophotometer (ATI-Unicam, Cambridge, UK). The culture was serially diluted in saline solution (0.9% NaCl) and spiked with a sterile syringe into the matrix of 5 g portions to reach four different concentrations: 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup> and 10<sup>7</sup> colony forming units (cfu) g<sup>-1</sup> of cheese. Duplicate contaminated samples and unspiked cheese were then subjected to total DNA extraction. Additionally, four artisanal cheeses from Northeast Italy (Table 3) were also analysed for the presence of indigenous AR genes.

2.7. Extraction of total microbial DNA from cheese

Cheese samples were homogenised 1/10 in 2% sodium citrate using a Stomacher (Seward, Worthing, UK) at 260 rpm for 1 min. Initially, bacterial biomass was extracted from the samples over a series of purification steps with ammonium hydroxide, absolute ethanol, petroleum ether, 10% SDS, 6 M urea and 3 M sodium acetate following the protocol of Drake, Small, Spence and Swanson (1996). The DNA was then purified
from the cells using the Gen Elute Bacterial Genomic DNA kit. The amount of DNA recovered was determined using the BioPhotometer. Purified DNA (~ 20 ng) from cheeses was used as a template for multiplex PCR and the labelled amplicons were subjected to microarray analysis.

2.8. Capillary electrophoresis of multiplex PCR amplicons

To confirm the results obtained with the microarray and the effectiveness of the method, multiplex PCR amplicons were further analysed by capillary electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto CA, USA). Aliquots of the multiplex PCR products (1 µL) were placed on a DNA microchip (LabChip 7500, Agilent) and analysed according to the manufacturer’s instructions. LabChip technology and the Bioanalyzer 2100 allow for the separation of small DNA fragments by capillary electrophoresis better than that achieved though 2% conventional agarose gel electrophoresis (Nachamkin et al., 2001). In addition, the software automatically calculates the size and DNA concentration of each fragment in relation to internal and external standards.

2.9. Processed data and reproducibility

The reproducibility of the complete PCR-based microarray method was evaluated by means of different approaches; in each hybridisation experiment the presence of a particular AR gene was evaluated with two specific probes, each of which was spotted in five replicates. The mean fluorescence intensity and the standard deviation of each probe were calculated in every assay. Labelling and microarray
hybridisation of the eight enterococcal strains were performed twice in independent experiments. Moreover, for a particular probe we compared the signals obtained with the different AR positive strains, i.e., probes for aac(6')-Ie-aph(2'')-Ia gene, strains ET35 and FAIR-E 132; probes for aadE gene, strains MN1, ET35 and EO5; probes for aphA-3 gene, strains MN1, ET35 and EO5; probes for ermB gene, strains MN1, ET35, EO5, ET51, ET39 and FAIR-E 132; probes for tet(L) gene, strains ET51 and ET39; probes for tet(M) gene, strains ET35, EO5, ET15 and ET39; probes for tet(S) gene, strains ET51 and ET15. Finally, contaminated and non-contaminated cheese samples were analysed in duplicate.

3. Results

3.1. Validation of the AR probes

To validate the probes, a total of eight Enterococcus strains of different species and origin, all of which harbour known resistance determinants, were screened using the microarray system. The purified DNA, amplified and fluorescently labelled in the multiplex PCR reactions, was used to verify the specificity of the microarray probes targeting the AR genes. Fig. 1 shows the hybridisation images of representative strains. Spots of LectSoy probe served both as a positive control for the hybridisation and as a reference for positioning all other spots on the grid. Quantification of the hybridisation signals revealed that the specific probes designed (two probes per AR gene) showed fluorescence values of >1,000 intensity units and were at least 10 times higher than the background signal, with consistency within both the five replicate spots. Most probes (at least one for each gene) showed fluorescence values ranging from 2,000 to 8,000.
intensity units in the two replicates (Fig. 2) and in all the assays with the different AR positive strains. It should be noted that the response was quite variable when comparing signals obtained after hybridisation with different strains (Fig. 3). The probe tetM_142R was the only one showing a recurrent low efficiency, indicating that it should be re-designed.

3.2. Cheese analysis using the microarray

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

The detection limit of the developed microarray was estimated with a contaminated cheese (cheese M, Table 3), following the entire proposed experimental procedure, i.e., purifying DNA from the cheese, labelling the DNA by multiplex PCR, and hybridisation. The cheese was artificially contaminated at different concentrations with the vancomycin-resistant strain *E. faecium* FAIR-E 132, which carries the *vanA* gene. The absence of this gene in the original cheese was verified by specific PCR before inoculation of the strain. With an inoculum of $10^7$ cfu g$^{-1}$ of the *vanA* positive strain the signal intensity of both *vanA* probes in the microarray was around 10 times higher than the established cut-off. With smaller inocula ($10^6$ cfu g$^{-1}$ and $10^5$ cfu g$^{-1}$), the intensity of the *vanA* probes was similar to that obtained with the DNA from a pure culture of the strain and within the detection limit. However, when the contamination level was $10^4$ cfu g$^{-1}$, the signal of both probes was low (below 1,000 intensity units).
The microarray was then used to analyse the presence of AR determinants in four other Italian cheeses including soft and ripened varieties made from raw or pasteurized milk (Table 3). Table 4 summarises the genes detected and in Fig. 4 are displayed some of the microarray hybridisation patterns obtained. The genes \textit{tet}(M) and \textit{tet}(S) were found in all cheeses. In addition, the \textit{tet}(O) gene was identified in all artisanal cheeses, while the presence of \textit{tet}(L) was revealed only in the industrial cheese. The gene \textit{aphA-3} was evidenced in the two cheeses made from raw milk, which in addition harboured \textit{aadE} and \textit{ermB} genes (one in each sample). The genes \textit{vanA} and \textit{vanB}, encoding vancomycin resistance, and the \textit{aac(6')-Ie-aph(2'')-Ia} gene, responsible for high-level gentamicin resistance, were not detected.

3.3. Cheese analysis by capillary electrophoresis

To assess the effectiveness of the microarray, the results obtained were compared with those achieved by analysing the multiplex PCR products of the samples using the Agilent 2100 Bioanalyzer. Fig. 5 shows the electrophoretograms obtained and the gel-like images generated from the different samples. The peaks in the chromatograms correspond to the amplification products of the different AR genes detected, except for the 50 bp and 10,380 bp peaks which correspond to two internal reference markers. The results proved to be consistent with the AR gene content identified by the microarray, although amplicons obtained from some cheeses could not be easily distinguished by this methodology (Fig. 5; samples 10 and 11 corresponding to cheeses S and C). In addition, non-specific products generated by the multiplex PCRs were occasionally revealed, such as the third peak of sample 2 (cheese B).
4. Discussion

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

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

Based on the fluorescence values obtained with the enterococci, and following comparable criteria to those reported in similar studies (Cassone et al., 2006; Frye et al., 2006; Vora et al., 2004), a cut-off value of 1,000 intensity units was established for the analysis of AR genes in cheese. When a cheese was inoculated with a vancomycin-resistant strain at a concentration of $10^5 \text{ cfu g}^{-1}$ or higher, the fluorescence signal obtained with the developed microarray was over the threshold limit, and unambiguous microarray images with switch on $\text{vanA}$ spots were obtained in both duplicates. Consequently, we consider that for reliable detection in cheese, AR gene-carrying
bacteria should be above this concentration. Notwithstanding, when using culture-independent microbial techniques, such as this microarray system, it is certainly difficult to ensure the absence of DNA extraction biases, since not all bacterial groups present within the cheese matrix can be released in the same way (Mayo, Marzotto, Flórez, & Torriani, 2008).

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

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

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

Although the use of microarrays has been recommended as the most suitable
technique for the determination of resistance genes (Holzman, 2003; Perreten et al.,
2005), to date their use has been restricted to the analysis of individual bacterial isolates
(Cassone et al., 2006; Frye et al., 2006; van Hoek, Scholtens, Cloeckaert, & Aarts, 2005;
Volokhov et al., 2003; Yu et al., 2004). However, in microbial complex samples such as
food matrices powerful genetic tools - such as that proposed in this work - are necessary
to identify the presence of antimicrobial resistance determinants. Recently Patterson,
Colangeli, Spigaglia and Scott (2007) used macroarrays to investigate the distribution of
AR genes in environmental samples (bacterial DNA extracted from soil and faeces), but to the best of our knowledge the present study is the first to report the use of a microarray method for detecting an unknown resistance gene pool directly in a food system.

5. Conclusions

The experimental procedure developed here offers a useful way of rapidly collecting data on the load and distribution of AR genes in cheese samples. Although the repertoire of probes for AR detection is not yet complete, the proposed multiplex PCR-based DNA microarray should be of use in assessing the presence of AR genes in other food samples in which the presence of antibiotic resistant bacteria is suspected.

Acknowledgments

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References


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correlation with origin and pathogenicity. Applied and Environmental Microbiology, 68, 1381-1391.


<table>
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<tr>
<th>Primer</th>
<th>Gene</th>
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<td>60</td>
<td>Dutka-Malen et al. (1995)</td>
<td></td>
</tr>
<tr>
<td>vanB2</td>
<td>GTATTCGTTTCCTGCCAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.

Primers used in this study for the two multiplex PCR reactions.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Target gene</th>
<th>Sequence (5’ to 3’)</th>
<th>Length</th>
<th>Tm(^{a}) (°C)</th>
<th>% GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>aacAaph_72</td>
<td>aac(6’)-Ie-aph(2’’)-Ia</td>
<td>CCTCGTGTAATTTCATGTT</td>
<td>18</td>
<td>45.4</td>
<td>38.9</td>
</tr>
<tr>
<td>aacAaph_218</td>
<td>aac(6’)-Ie-aph(2’’)-Ia</td>
<td>CACACTATCATAACCACT</td>
<td>18</td>
<td>44.7</td>
<td>38.9</td>
</tr>
<tr>
<td>aadE_269R</td>
<td>aadE</td>
<td>GTATGATGATTGCTGCA</td>
<td>17</td>
<td>45.0</td>
<td>41.2</td>
</tr>
<tr>
<td>aadE_233</td>
<td>aadE</td>
<td>ATCAGTCGAACATGAT</td>
<td>17</td>
<td>44.6</td>
<td>41.2</td>
</tr>
<tr>
<td>aphA3_295</td>
<td>aphA-3</td>
<td>GCATAACAGCTCGATAAT</td>
<td>17</td>
<td>44.9</td>
<td>41.2</td>
</tr>
<tr>
<td>aphA3_337</td>
<td>aphA-3</td>
<td>CAATCGTGATACGTGAT</td>
<td>17</td>
<td>43.8</td>
<td>41.2</td>
</tr>
<tr>
<td>ermB_495R</td>
<td>ermB</td>
<td>CACAGATGTTCCAGATAA</td>
<td>18</td>
<td>44.3</td>
<td>38.9</td>
</tr>
<tr>
<td>ermB_557R</td>
<td>ermB</td>
<td>GAGAATATCGTCAACTGT</td>
<td>18</td>
<td>44.5</td>
<td>38.9</td>
</tr>
<tr>
<td>tetL_121</td>
<td>tet(L)</td>
<td>CTACAACCATTACGAGT</td>
<td>17</td>
<td>44.2</td>
<td>41.2</td>
</tr>
<tr>
<td>tetL_689R</td>
<td>tet(L)</td>
<td>TTGATAGAGAGGTCCTT</td>
<td>18</td>
<td>44.6</td>
<td>38.9</td>
</tr>
<tr>
<td>tetM_117</td>
<td>tet(M)</td>
<td>GTCTATGATGTCACCTT</td>
<td>18</td>
<td>44.3</td>
<td>38.9</td>
</tr>
<tr>
<td>tetM_142R</td>
<td>tet(M)</td>
<td>GCAGAAGTATCGTACG</td>
<td>18</td>
<td>45.3</td>
<td>38.9</td>
</tr>
<tr>
<td>tetO_264R</td>
<td>tet(O)</td>
<td>AGACGGAGCAGTATAT</td>
<td>17</td>
<td>45.1</td>
<td>41.2</td>
</tr>
<tr>
<td>tetO_217</td>
<td>tet(O)</td>
<td>CTGGCGATATCTATAATGT</td>
<td>18</td>
<td>45.4</td>
<td>38.9</td>
</tr>
<tr>
<td>tetS_96</td>
<td>tet(S)</td>
<td>TCCAGGAGTATCTACAAT</td>
<td>18</td>
<td>44.7</td>
<td>38.9</td>
</tr>
<tr>
<td>tetS_215</td>
<td>tet(S)</td>
<td>CTAAGTGATGGAATAGT</td>
<td>18</td>
<td>45.1</td>
<td>38.9</td>
</tr>
<tr>
<td>vanA_145</td>
<td>vanA</td>
<td>GATCCATCTTCACCCTG</td>
<td>17</td>
<td>44.8</td>
<td>47.1</td>
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<tr>
<td>vanA_244</td>
<td>vanA</td>
<td>GCAACGATGATGATCAGA</td>
<td>17</td>
<td>45.4</td>
<td>41.2</td>
</tr>
<tr>
<td>vanB_245</td>
<td>vanB</td>
<td>TGTAAGAATGTTAGGCCA</td>
<td>17</td>
<td>45.5</td>
<td>41.2</td>
</tr>
<tr>
<td>vanB_490R</td>
<td>vanB</td>
<td>CGAGGATGATTTGATTGT</td>
<td>18</td>
<td>44.8</td>
<td>38.9</td>
</tr>
</tbody>
</table>

\(^{a}\)Melting temperature of the probes calculated with the Nearest Neighbour method and the thermodynamic parameters of Santa Lucia (1998).
Table 3.

Italian cheeses analysed for the detection of antibiotic resistance genes.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Cheese</th>
<th>Geographical region</th>
<th>Stage of ripening</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Malga</td>
<td>Friuli-Venezia-Giulia</td>
<td>Ripened</td>
<td>Artisanal from raw milk</td>
</tr>
<tr>
<td>B</td>
<td>Malga</td>
<td>Trentino Alto-Adige</td>
<td>Ripened</td>
<td>Artisanal from raw milk</td>
</tr>
<tr>
<td>M</td>
<td>Montasio</td>
<td>Friuli-Venezia-Giulia</td>
<td>Ripened</td>
<td>Industrial from pasteurized milk</td>
</tr>
<tr>
<td>S</td>
<td>Stracchino</td>
<td>Trentino Alto-Adige</td>
<td>Fresh-soft</td>
<td>Artisanal from pasteurized milk</td>
</tr>
<tr>
<td>C</td>
<td>Caciotta</td>
<td>Trentino Alto-Adige</td>
<td>Fresh-soft</td>
<td>Artisanal from pasteurized milk</td>
</tr>
</tbody>
</table>
Table 4.

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

<table>
<thead>
<tr>
<th>Multiplex PCRs</th>
<th>Cheese sample</th>
<th>A</th>
<th>B</th>
<th>M</th>
<th>S</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5AR_55</td>
<td>ermB, tet(M), tet(S)</td>
<td>aadE, tet(M), tet(S)</td>
<td>tet(M), tet(S)</td>
<td>tet(M), tet(S)</td>
<td>ermB, tet(M), tet(S)</td>
<td></td>
</tr>
<tr>
<td>M5AR_60</td>
<td>aphA-3, tet(O)</td>
<td>aphA-3, tet(O)</td>
<td>tet(L)</td>
<td>tet(O)</td>
<td>tet(O)</td>
<td></td>
</tr>
</tbody>
</table>

*aM5AR_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.*
Figure legends

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

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

Fig. 3. Comparison of microarray signal (in fluorescence intensity) of the 20 antibiotic resistance probes in the different assays with the positive enterococci strains: probes for aac(6')-Ie-aph(2'')-Ia gene, two strains; probes for aadE gene, three strains; probes for aphA-3 gene, three strains; probes for ermB gene, six strains; probes for tet(L) gene, two strains; probes for tet(M) gene, four strains; probes for tet(S) gene, two strains. Probes for vanA, vanB and tet(O) genes single strains were examined. Data are the average of two independent experiments. The names of the probes are referred as in Table 2.
Fig. 4. Microarray hybridisation images of cheeses obtained by labelling of the bacterial DNA with the multiplex PCR for the antibiotic resistance genes \textit{aphA-3}, \textit{tet}(L), \textit{tet}(O), \textit{vanA}, and \textit{vanB}. Letters A, B and C illustrate the hybridisation patterns of cheese M [\textit{tet}(L)⁺], cheese C [\textit{tet}(O)⁺], and cheese B [\textit{aphA-3}⁺, \textit{tet}(O)⁺], respectively.

Fig. 5. Electrophoretograms (upper panel) and simulated gel (lower panel) obtained by capillary electrophoresis in the Agilent 2100 Bioanalyzer. Samples 1, 2, 3, 4, 5 and 6 correspond to the amplicons obtained with the multiplex PCR for the antibiotic resistance genes \textit{aphA-3} (698 bp), \textit{tet}(L) [1,077 bp], \textit{tet}(O) [515 bp], \textit{vanA} (732 bp) and \textit{vanB} (635 bp) in the cheeses A [\textit{aphA-3}⁺, \textit{tet}(O)⁺], B [\textit{aphA-3}, \textit{tet}(O)⁺], M [\textit{tet}(L)⁺], S [\textit{tet}(O)⁺], C [\textit{tet}(O)⁺] and cheese M contaminated with $10^5$ cfu g⁻¹ \textit{vanA}⁺ strain, respectively. Samples 7, 8, 9, 10, 11 and 12 correspond to the amplicons obtained with the multiplex PCR for the antibiotic resistance genes \textit{aac(6)’}-\textit{Ie-aph(2)’’}-\textit{Ia} (480 bp), \textit{aadE} (597 bp), \textit{ermB} (639 bp), \textit{tet}(M) [406 bp] and \textit{tet}(S) [667 bp] in the cheeses A [\textit{ermB}⁺, \textit{tet}(M)⁺, \textit{tet}(S)⁺], B [\textit{aadE}⁺, \textit{tet}(M)⁺, \textit{tet}(S)⁺], M [\textit{tet}(M)⁺, \textit{tet}(S)⁺], S [\textit{tet}(M)⁺, \textit{tet}(S)⁺], C [\textit{ermB}⁺, \textit{tet}(M)⁺, \textit{tet}(S)⁺] and the strain \textit{E. faecalis} ET35 [\textit{aadE}⁺, \textit{aac(6)’}-\textit{Ie-aph(2)’’}-\textit{Ia}⁺, \textit{ermB}⁺, \textit{tet}(M)⁺], respectively. L, 50 - to 10,380 bp marker. The 50 bp and 10,380 bp peaks appear in the electrophoretograms as internal references.
Fig. 1
**E. faecium ET51**

![Fluorescent Intensity Graph for E. faecium ET51]

- **E. faecium ET51**
- **Fluorescent Intensity**
- **1st replicate**
- **2nd replicate**

**E. faecalis MN1**

![Fluorescent Intensity Graph for E. faecalis MN1]

- **E. faecalis MN1**
- **Fluorescent Intensity**
- **1st replicate**
- **2nd replicate**

Fig. 2