**Microbial ecology of Gorgonzola rinds and occurrence of different biotypes of Listeria monocytogenes**

**Microbial Ecology of Gorgonzola Rinds and Occurrence of Different Biotypes of *Listeria monocytogenes***

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**Running title.** Gorgonzola rind ecology and *L. monocytogenes* prevalence

**Abstract.**

In this study we investigated the microbiota of Gorgonzola rinds and maturing shelve swabs collected in 5 different maturing cellars in the Northwest part of Italy, in association with the detection and characterization of *Listeria monocytogenes*.

Culture–dependent and –independent methods were performed in order to profile the main microbial populations present on the rinds and in the maturing shelves and species-specific PCR and Pulsed Field Gel Electrophoresis (PFGE) were used to identify and type *L. monocytogenes* isolates. The microflora was predominated by lactic acid bacteria and coagulase negative cocci, while enterococci and yeasts were very variable between the samples. *Arthrobacter* sp., *Carnobacterium* sp., *Staphylococcus* sp. and *Brevibacterium linens*, as bacteria, and *Debaryomyces hansenii*, as yeast, were detected by Denaturing Gradient Gel Electrophoresis (DGGE). Cluster analysis of the DGGE profiles clearly highlighted a cellar-specific microflora. *L. monocytogenes* was isolated in 11.1% of the rinds and 29.4% of the swabs and the molecular characterization of the isolates suggests a route of contamination from the maturing shelves to the rinds. No correlation was found between DGGE profiles and presence or absence of *L. monocytogenes*.

*Key-words:* Gorgonzola, rind microbial ecology, DGGE, *Listeria monocytogenes*; PFGE; cluster analysis.

## 1. Introduction

According to Gripon and Hubert (2002), Gorgonzola is a blue-veined, mould-ripened cheese, in which *Penicillium roqueforti* develops as an internal blue-green mould. Its tradition is very long in Italy and the first evidences of Gorgonzola production date back to 879 a.c. in Gorgonzola, nearby Milan. It is made of pasteurized cow's milk inoculated with *Lactobacillus* and *Streptococcus* starter cultures, together with *P. roqueforti*. In 1996, it was awarded with the Protected Designation of Origin (PDO) from the European Commission (Commission Regulation No 1107/96).

Recently, several reports were published on the detection, identification and characterization of *Listeria monocytogenes* in Gorgonzola cheese (Carminati et al., 2004b; Manfreda et al., 2005; Lomonaco et al., 2009). Interestingly, the contamination seems to be limited to the surface and even if rinds are contaminated, the internal part does not necessarily contain the pathogen (GOLIS project, 2004). Thereby cross contaminations, from the environment or during handling, are responsible for the presence of *L. monocytogenes* on the Gorgonzola rinds in post-production.

Several strategies have been proposed in order to control *L. monocytogenes* in Gorgonzola cheese and more generally in soft cheeses. High-pressure processing (Carminati et al., 2004a) and washing with high-pressure water spray (Mucchetti et al., 2008) were studied as physical treatment for decontamination, while biological interventions with bacteriophages (Schellekens et al., 2007) or by using antimicrobial producer strains as surface starter cultures (Carminati et al., 1999; Siafaras et al., 2008) were published.

The application of molecular methods in the field of food microbiology has significantly improved the comprehension of complex microbial ecosystems,

including the study of their ecology (Cocolin and Ercolini, 2008) and the behavior of foodborne pathogens, such as *L. monocytogenes* (McLauchlin et al., 2004).

Since *L. monocytogenes* contamination in Gorgonzola is most frequently associated with the cheese rind, in this study we wanted to investigate i) the ecology of Gorgonzola rinds coming from five different maturing cellars located in the northwest part of Italy in order to assess the influence of the processing environment on the diversity of the microbiota and ii) to associate rind ecology with the presence and characteristics of the *L. monocytogenes* isolated from the samples considered in this study. Rinds and maturing shelve swabs were examined by culture–dependent and –independent methods for the definition of their microbial ecology and for detection, identification and characterization of the *L. monocytogenes*.

## 2. Materials and methods

### *2.1 Maturing cellars and sample collection*

Five maturing cellars (A, B, C, D and E ) located in the Novara area (Piedmont region, North West of Italy) were selected for this study. The characteristics of the maturing cellars as well as the number of rinds and swabs collected are reported in Table 1. A total of 18 rinds and 17 swabs were collected in one sampling campaign in winter 2007. Rinds of selected Gorgonzola loafs were scratched with sterile scrapers and 10 $cm^2$ of the shelf, on which the sampled loaf was accommodated, were swabbed and pads were placed in sterile bags.

### *2.2 Microbiological analysis*

Five grams of rinds were diluted in 45 ml of Ringer solution (Oxoid, Milan, Italy) and mixed for 1 min and 30 s in a Stomacher machine (PBI, Milan, Italy). Pads were squeezed in a plastic sterile bag and 1 ml was subsequently used. In both cases serial

dilutions were prepared and the following microbiological examinations were carried out on duplicate agar plates: a) total viable count on Plate Count Agar (Oxoid) incubated for 48 – 72 h at 30°C; b) lactic acid bacteria (LAB) on M17 agar (Fluka, Buchs SG, Switzerland) for mesophilic cocci incubated aerobically, and MRS agar (Oxoid) for mesophilic lactobacilli incubated with a double layer, both at 30°C for 48 h; c) coagulase negative cocci (CNC) on Mannitol Salt Agar (Oxoid) incubated at 30°C for 48 h; d) enterococci on Kanamycin Aesculin Azide Agar (Fluka) at 37°C for 48h; e) yeasts and moulds on Malt Extract Agar (Oxoid) supplemented with tetracycline (1 μg/ml, Sigma, Milan, Italy) incubated at 25°C for 48-72h. Detection of *Listeria* spp. and *L. monocytogenes* was performed following the procedure UNI EN ISO 1129-1 (2005). The samples, enriched in Fraser broth (Oxoid), were plated onto selective and Chromogenic media (PALCAM and OCLA, Oxoid) and for each plate, two suspect colonies, if present, were chosen. Identification of *L. monocytogenes* was performed through a specific PCR assay, amplifying a 274 bp fragment of the *pfrA* gene (D'Agostino et al., 2004). Positive PCR colonies were further characterized via serotyping and Pulsed Field Gel Electrophoresis (PFGE).

*2.3 Direct DNA extraction and PCR amplification*

One ml of the rind homogenates and 1 ml obtained from the swabs were subjected to direct DNA extraction as previously described (Dolci et al., 2009). DNA was subsequently quantified by measuring the absorbance at 260 nm using the NanoDrop instrument (Celbio, Milan, Italy) and 50 ng were subjected to PCR amplifications.

DNA was amplified with primers 338f and 518r, spanning the V3 region of the 16S rRNA gene, as described by Cocolin et al. (2001) to study the bacterial ecology, and with primers NL1 and LS2, targeting the D1-D2 loop of the 26S rRNA gene, as previously reported by Cocolin et al. (2000) to monitor the yeast ecology. A GC-

clamp (5' –CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G- 3') was attached to the 5' end of primer 338f and NL1 for DGGE analysis.

*2.4 DGGE analysis*

DGGE was carried out with the Dcode Universal Mutation Detection System (Bio-Rad, CA, USA) using the conditions described by Cocolin et al. (2007) for the bacteria and by Cocolin et al. (2000) for yeasts. Images were digitally captured and analyzed under UV light by using UVI pro Platinum 1.1 Gel Software (Eppendorg, Hamburg, Germany) for the recognition of the bands present. Comparisons of DGGE fingerprints obtained from the different samples were performed using the pattern analysis software package, Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium). Calculation of similarity in the profiles of bands was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992). DGGE analyses were performed at least twice. After the DGGE run, blocks of polyacrylamide gels, containing selected bands, were punched using sterile pipette tips and transferred in 50 µl sterile water. The DNA of the bands was let to diffuse overnight at 4°C and 2 µl were used for the re-amplification. The PCR products were cloned in pGEM-T Easy vector (Promega, Milan, Italy) and the resulting colonies were checked by DGGE using previously amplified DNA extracted directly from the samples as a control. Only clones migrating as a single band and at the same position with respect to the control were sequenced by a commercial facility (MWG Biotech, Ebersberg, Germany) and sequences were aligned in GeneBank using the Blast program (Altschul et al., 1997) for identification purposes.

*2.5 Serotyping of L. monocytogenes isolates*

Serotyping was performed following the scheme established by Seeliger and Hohne (1979). Strains were serotyped using antisera against somatic (O) and flagellar (H) antigens according to manufacturer instructions (Denka Seiken, Tokyo, Japan) with minor modifications: for determination of H antigens, isolates were passed three to four times through semi-solid BHI broth containing agar 0.2% w/v at 25°C.

*2.6 Characterization by pulsed field gel electrophoresis (PFGE) of the L. monocytogenes isolates*

Genomic DNA was included in agarose plugs prior to digestion, following the method described by Graves and Swaminathan, (2001). Subsequently DNA cleavage was achieved by restriction with *Asc*I and *Apa*I (New England Biolabs, Beverly, MA). Restricted samples were electrophoresed on 1% SeaKem Gold agarose in 0.5X TBE at 6 V/cm on a Chef DR III system (Bio-Rad, Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C were applied for 21 h. Fingerprints (pulse-types) were analysed with Bionumerics software (Applied Maths). Dice coefficient was used for the calculation of the similarity between fingerprints, which were compared using an optimization value of 1.2% for *Asc*I and 1.4% for *Apa*I, and a band position tolerance of 1.4% for both enzymes. The results were then combined and a dendrogram was generated by the Complete Linkage algorithm (Aldenberger and Blashfield, 1984). Isolates sharing 100% similarity were considered belonging to the same pulse-type.

## 3. Results and discussion

The majority of the studies focusing on the microbial ecology of cheese rinds have been carried out by culture–dependent methods, and only recently molecular methods have been applied in order to investigate this microbial consortium (Dolci et al.,

2009). Data for Gorgonzola cheese rinds is lacking. In this paper we analyzed rind samples and maturing shelves swabs coming from different maturing cellars and in parallel isolation, identification and characterization of *L. monocytogenes* was performed.

The results obtained by plate counts are shown in Figure 1. Considering the rinds (Fig. 1A), LAB, namely lactococci and lactobacilli, dominated the microbial ecology, with counts above 9 $Log_{10}$/g for almost all samples. On the other hand, enterococci presented more diverse counts within the rinds analyzed with variation between 3 and 8 $Log_{10}$/g. In the case of the total aerobic counts on PCA and yeasts and moulds on AMT, the results underlined a great heterogeneity within the samples analyzed. For PCA, rinds showed counts from less than 3 $Log_{10}$/g to more than 9 $Log_{10}$/g, while the counts on AMT spanned from 5 to more than 9 $Log_{10}$/g. In this last case, yeasts represented the dominant flora on the plates. CNC on the rinds followed the same trend as the yeasts. Similarly, the counts of the swabs (Fig.1B) were dominated by LAB on MRS and M17, while enterococci had lower counts from less than 3 to 7 $Log_{10}/cm^2$. As described above total aerobic counts, yeasts and CNC were extremely diverse including counts from less than 3 to more than 9 $Log_{10}/cm^2$.

The bacterial DGGE gels, obtained after direct DNA extraction and PCR amplification, are shown in Figure 2. Panel A refers to the rinds, while panel B to the swabs. Moreover, the results obtained by sequencing the selected bands in the DGGE gel are reported in Table 2. Complex DGGE profiles were observed in both rinds and swabs. One band was always detected for all the samples analyzed in this study (bands 1 and 7), that was identified as *Arthrobacter* sp. In the upper part of the gels, several bands were differentiated. They mainly belonged to *Staphylococcus* species (bands 4, 9 and 14), *Carnobacterium* sp. (bands 3 and 11) and *Phychrobacterium* sp.

(bands 6 and 13). Moreover, in one sample from the maturing cellar D, the band 8 was identified as an uncultured *Proteobacterium* (Fig. 2A and 2B). For all the samples, the central part of the gels was characterized by faint multiple - doublet bands. Several attempts were carried out in order to recover all the signals present in the gel, but after several trials only band 2 was sequenced. However, it was identified as a not culturable bacterium. It is worth to notice that the samples, both rinds and swabs, coming from the maturing cellar E presented cellar-specific bands, positioned at the lower part of the gels. After sequencing, it was determined that bands 5 and 10 belonged to *Brevibacterium linens*. Surprisingly, no lactic acid bacteria band was identified. This evidence is once more highlighting the difference in the results obtained by analyzing the ecology of fermented foods by culture – dependent or – independent methods, as previously reported (Cocolin and Ercolini, 2008). Regarding the yeast ecology all the rinds and swabs, independently of the maturing cellar, were characterized by two unique bands that were identified as *Debaryomyces hansenii* (data not shown). The results obtained by PCR-DGGE are in good agreement with previously published studies on smear-ripened cheeses, such as Fontina (Dolci et al., 2009) and Tilsit and Chaumes (Bockelmann and Hoppe-Seyler, 2001) cheeses.

When the bacterial DGGE profiles were digitalized and subjected to cluster analysis the dendrograms presented in Figure 3 were obtained. For the rinds (Fig. 3A), three distinct clusters were obtained. The cluster composition was in agreement with the origin of the rinds. The first cluster contained rinds from the cellar D and C, the second mainly from cellar E, while the last contained all the samples from the maturing cellar A plus the rind from the cellar B. In the case of the swabs (Fig. 3B) a clear distinction between samples from cellar D and all the others was highlighted. It

is important to underline that the DGGE profiles of the swab samples showed a higher similarity (>95%) when compared to the samples of rinds.

Growth of *Listeria* spp. on agar plates was detected in 13 samples: 7 from maturing shelve swabs and 6 from rinds. Visual inspection of the OCLA plates resulted in 5 swabs and 2 Gorgonzola rinds contaminated with *L. monocytogenes*, while the remaining positive plates were determined to contain *L. innocua* (as determined by 16S rRNA gene sequencing, data not shown). As shown in Figure 3B, the majority of *L. monocytogenes* positive swabs were collected in cellar A, while only 1 in cellar C, whereas the rinds containing *L. monocytogenes* came from cellars A and D (Fig. 3A).

A total of 19 colonies suspected to belong to the species *L. monocytogenes* were isolated and after molecular identification by species-specific PCR all gave the expected signal. The confirmed 19 *L. monocytogenes* isolates were then serotyped as 1/2a (13 isolates), 3a (2 isolates) and 1/2b (4 isolates). It is interesting to notice that a plant-specific serotype distribution was observed: strains 1/2a were all isolated in cellar A, while the 3a and 1/2b strains were all coming from cellar D and C, respectively (data not shown). All the tested isolates were typeable with PFGE, except one colony belonging to serotype 1/2a and isolated from a swab sample. The 18 typed strains generated 11 pulse-types, all joined at 45% similarity. The dendrogram generated by the combination of the results of both enzymes evidenced that the pulse types of isolates collected in cellar A and D were highly similar (72%) and shared lower similarity values (48% and 45%) with those collected from cellar C. On the other hand, the similarity between most of the strains collected from cellar C was lower, being 54%. (Figure 4). It is interesting to notice that biotypes isolated in cellar C showed a higher level of biodiversity when compared with those coming from cellars A and D.

## 4. Conclusions

In agreement with other studies (GOLIS project, 2004), the counts of the Gorgonzola rinds and maturing shelve swabs showed a great heterogeneity, being dominated by LAB. When molecular culture-independent methods were applied to study the microbial ecology of the samples collected in this study, it was determined that species of *Arthrobacter*, *Carnobacterium*, *Staphylococcus* and *B. linens* were the main components of the bacterial consortium, while only one yeast species, namely *D. hansenii* was detected. This is the first time PCR-DGGE is used to study the microbiota of Gorgonzola rinds and the results obtained correlate well with previous published papers investigating smear-ripened cheeses. The cluster analysis of the rind DGGE gels underlined a strict correlation between the rind microflora and the maturation cellar, highlighting once more the influence of the environment on the surface of cheeses as previously reported (Dolci et al., 2009). When presence of *L. monocytogenes*, one of the main concerns for Gorgonzola producers, was taken into consideration, it was determined that maturing shelve swabs were more frequently contaminated (29.4%), while only 2 out of the 18 rinds were positive (11.1%). Interestingly, the majority of the swabs containing *L. monocytogenes* were collected from the maturing cellar A, characterized by the co-presence of Gorgonzola coming from different producers with a fast turnover of the cheeses. The higher frequency of isolation of *L. monocytogenes* in cellar A resulted in one positive rind. From the PFGE analysis of the isolates obtained in this study, a high similarity of patterns was obtained from strains isolated from the maturing shelve swab and from the rind. It can be speculated that strains present on the shelves were responsible for the cheese contamination. It is improbable that the strains isolated came from the loaf and were

then transferred to the shelve, because Gorgonzola cheese is made from pasteurized milk and is placed on the shelve after production. This study contributes in understanding the microbial ecology of Gorgonzola cheese and gives the first insights on the effect of the environmental conditions on the contamination of *L. monocytogenes*. However, a direct correlation between rind microbial ecology, in terms of presence or absence of specific populations, and presence of *L. monocytogenes* could not be determined.

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Table 1. Characteristics and number of samples collected in each maturing cellar considered in this study.

| | | Number of samples | |
|---|---|---|---|
| Maturing cellar | Characteristics | Rinds | Maturing shelve swabs |
| A | modern, automatized, with controlled ventilation and mobile benches in plastic and wood | 6 | 5 |
| B | modern, automatized, with controlled ventilation and mobile benches in plastic and wood | 1 | 1 |
| C | modern, automatized, with controlled ventilation and mobile benches in plastic and inox | 2 | 2 |
| D | modern, automatized, with controlled ventilation and mobile benches only in plastic | 6 | 7 |
| E | traditional and old establishment with static wood benches not automatized | 3 | 2 |
| | Total | 18 | 17 |

Table 2. Sequencing results of the bands cut from the DGGE gels

| Band[a] | Closest relative | %Identity | Source[b] |
|---|---|---|---|
| 1 | *Arthrobacter* sp. | 100 | AB449065 |
| 2 | Not culturable bacteria | 97 | AB441451 |
| 3 | *Carnobacterium* sp. | 100 | FJ151401 |
| 4 | *Staphylococcus* sp. | 98 | FJ006897 |
| 5 | *Brevibacterium linens* | 100 | AY017072 |
| 6 | *Phychrobacterium* sp. | 100 | AB453700 |
| 7 | *Arthrobacter* sp. | 100 | AY177350 |
| 8 | Uncultured *Proteobacterium* | 98 | AJ310687 |
| 9 | *Staphylococcus* sp. | 100 | FJ006897 |
| 10 | *Brevibacterium linens* | 98 | AY017072 |
| 11 | *Carnobacterium* sp. | 100 | FJ151398 |
| 12 | *Cobetia* sp. | 97 | EU828452 |
| 13 | *Phychrobacterium* sp. | 91 | EU016162 |
| 14 | *Staphylococcus lentus* | 100 | EU794392 |

[a]Bands as indicated in Figure 2.
[b]Accession number of sequence of closest relative found with Blast search.

**Figure legends.**

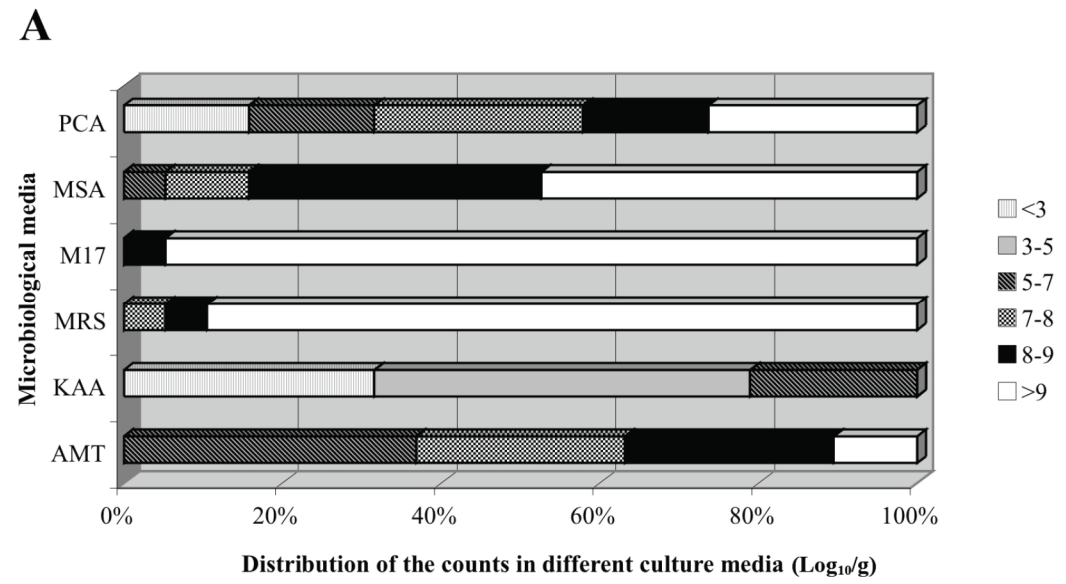
**Figure 1.** Results of the microbiological analysis of rinds (panel A) and swabs (panel B) included in this study. Counts are presented as distribution % for each medium.

**Figure 2.** DGGE profiles of the bacterial microflora in rinds (panel A) and swabs (panel B). Letters A, B, C, D and E represent the maturation cellars considered here (see Table 1 for specification of the characteristics), while M is a migration marker (represented by *Lactobacillus casei* DSMZ 20011). Bands labelled by numbers were excised and the results of their sequencing identification are reported in Table 2.

**Figure 3.** Cluster analysis of the bacterial DGGE profiles obtained from the rinds (panel A) and swabs (panel B). Full circles (●) open circles (○) identify samples containing *L. monocytogenes* and *L. innocua,* respectively.

**Figure 4.** Cluster analysis of the PFGE profiles obtained from the *L. monocytogenes* strains isolated from the rinds and swabs analysed in this study.

**Figure 1**


A
PCA
MSA
M17
MRS
KAA
AMT
Microbiological media
0%
20%
40%
60%
80%
100%
Distribution of the counts in different culture media (Log10/g)
<3
3-5
5-7
7-8
8-9
>9

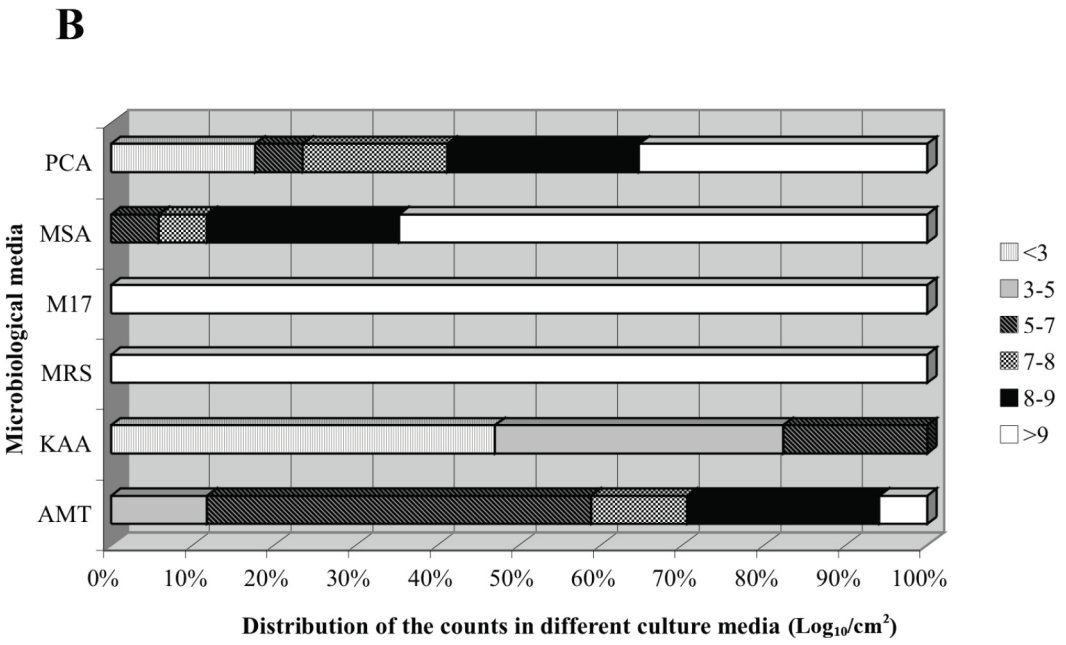
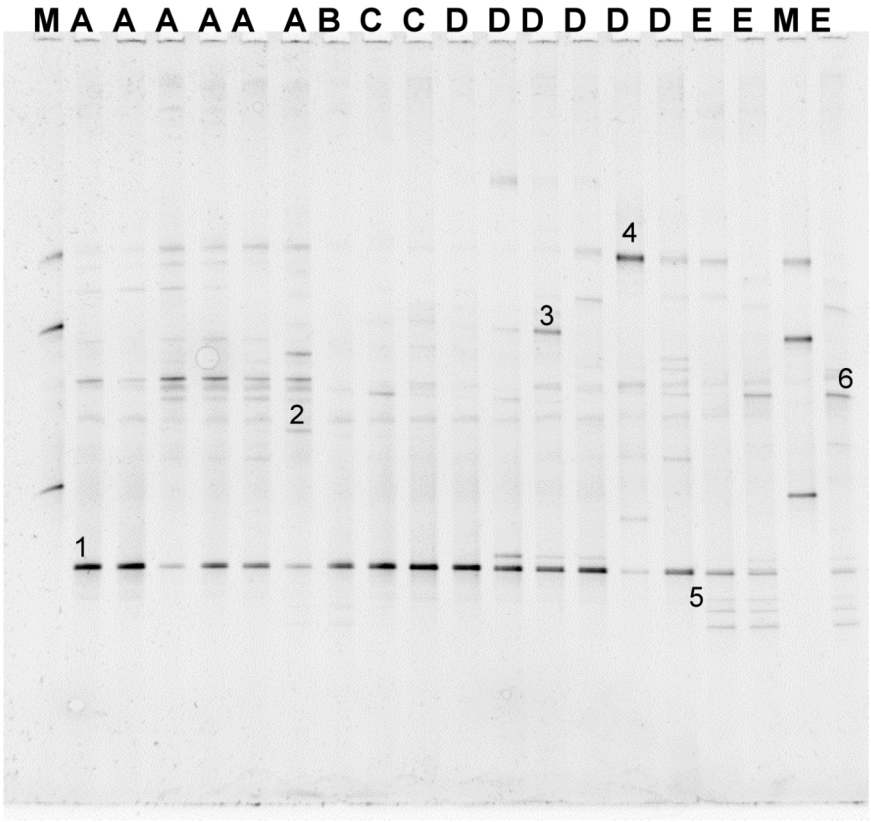

B
PCA
MSA
M17
MRS
KAA
AMT
Microbiological media
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
Distribution of the counts in different culture media (Log10/cm2)
<3
3-5
5-7
7-8
8-9
>9

**Figure 2**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A
1
2
3
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13
14

**Figure 3**

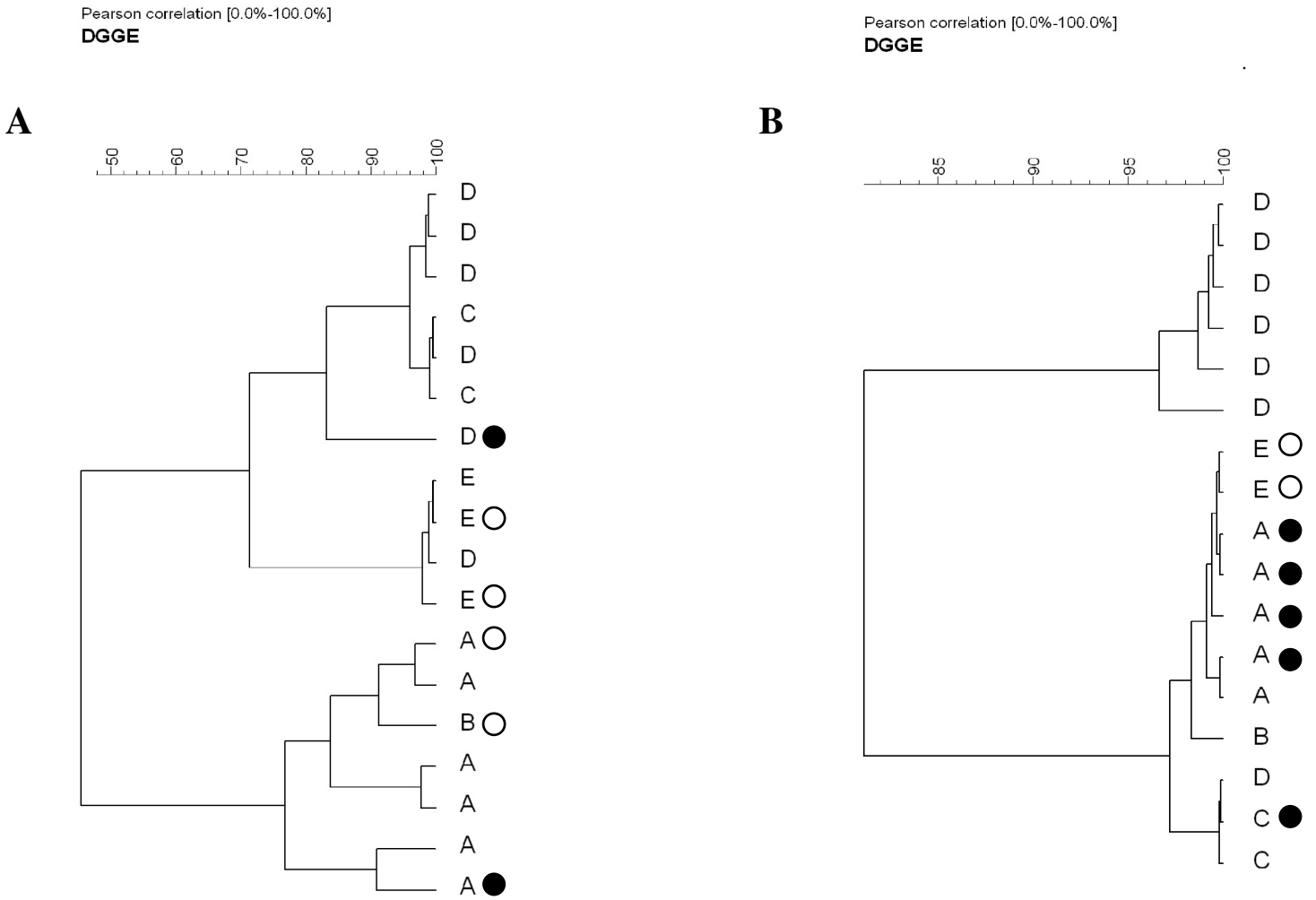
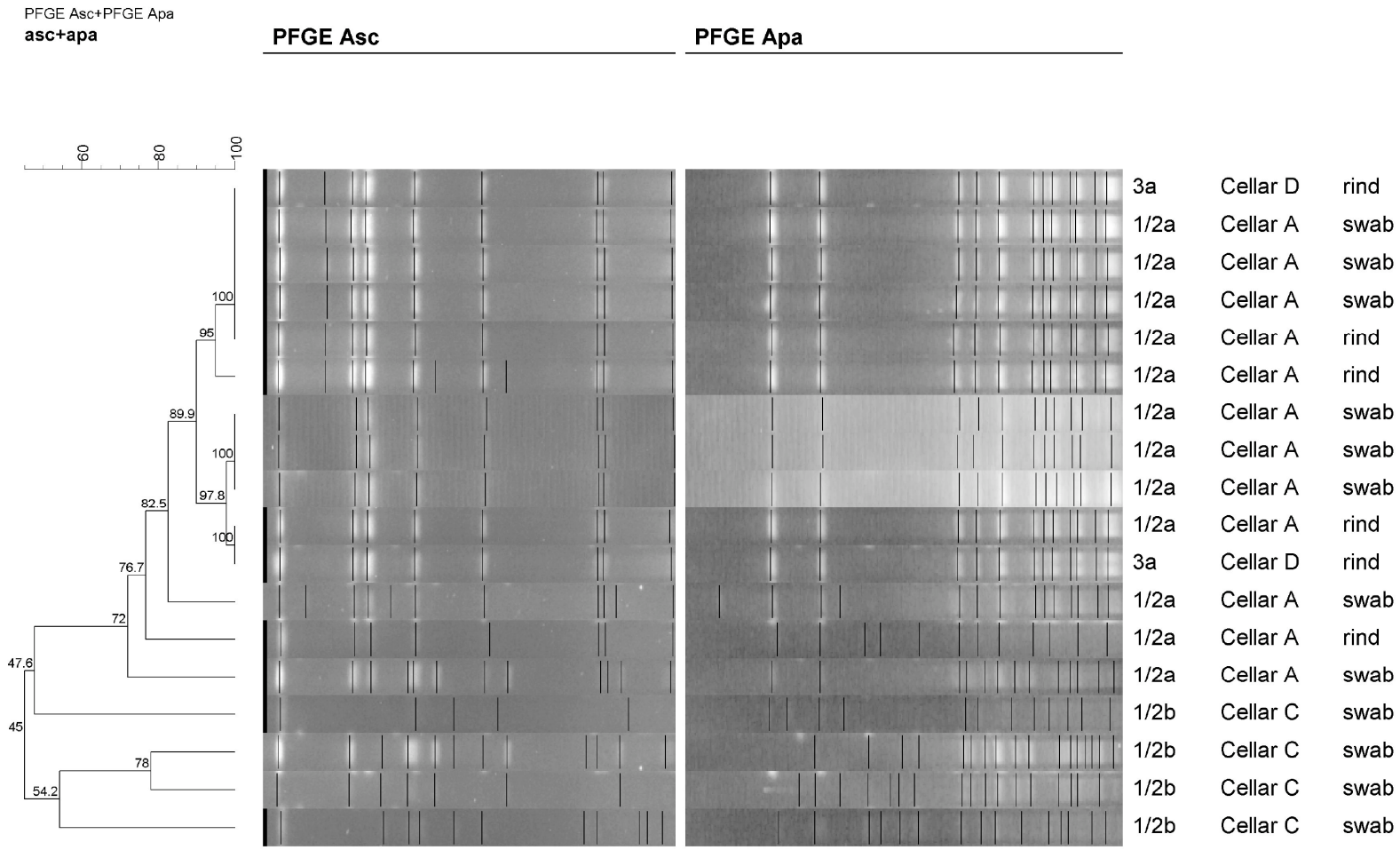

Pearson correlation [0.0%-100.0%]
DGGE
A
50
60
70
80
90
100
D
D
D
C
D
C
D
E
E
D
E
A
A
B
A
A
A
A
Pearson correlation [0.0%-100.0%]
DGGE
B
85
90
95
100
D
D
D
D
D
D
E
E
A
A
A
A
A
B
D
C
C

**Figure 4**


PFGE Asc+PFGE Apa
asc+apa
PFGE Asc
PFGE Apa
60
80
100
100
95
89.9
100
97.8
82.5
100
76.7
72
47.6
45
78
54.2
3a Cellar D rind
1/2a Cellar A swab
1/2a Cellar A swab
1/2a Cellar A swab
1/2a Cellar A rind
1/2a Cellar A rind
1/2a Cellar A swab
1/2a Cellar A swab
1/2a Cellar A swab
1/2a Cellar A rind
3a Cellar D rind
1/2a Cellar A swab
1/2a Cellar A rind
1/2a Cellar A swab
1/2b Cellar C swab
1/2b Cellar C swab
1/2b Cellar C swab
1/2b Cellar C swab

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