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Food Microbiota Diversity

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1. Introduction

Meat fermentation and fermented sausage manufacturing are ancient processes in Europe, and typical preparations based on fermentations driven by indigenous microflora are still produced by local meat factories or artisanal producers without the use of starter cultures (Rantsiou et al., 2005a; Urso et al., 2006).

LAB (Lactic Acid Bacteria) and CNC (Coagulase Negative Cocci) are the two main groups of bacteria with technological value determining the several biochemical and physical reactions taking place during the fermentation and ripening of sausages (Rantsiou & Cocolin 2006).

Interest in preserving the biodiversity of microorganisms involved in the fermentation of food products has been reported (EC 1999; Aymerich et al., 2006). As a consequence, and also taking into consideration the technological and economic importance of these microorganisms, the availability of methodologies able to unequivocally characterise single species, potentially right up to strain level, is called for. Identification of species, particularly within the genus *Lactobacillus*, using phenotypic methods such as sugar fermentation or other biochemical traits, may produce ambiguous results and be complicated because of the presence of several LAB species with similar characteristics (Quere et al., 1997).

Molecular methods are nowadays increasingly employed to clarify the taxonomy of microbiota from sausages. The largest number of works is based on RAPD (Random Amplified Polymorphic DNA), (Rebecchi et al., 1998; Rantsiou et al., 2005a), 16S rDNA sequencing (Rantsiou et al., 2005a), PCR-DGGE (Denaturing Gradient Gel Electrophoresis) analysis (Cocolin et al., 2001a; Cocolin et al., 2001b; Comi et al., 2005; Urso et al., 2006), and REA-PFGE (Restriction Endonucleases Analysis - Pulsed Field Gel Electrophoresis) analysis (Psoni et al., 2006).

The above DNA-based methodologies, with the exception of REA-PFGE, are easy, fast, and provide an accurate identification at species level, but are not as informative when it comes to characterisation of the bacterial population at strain level. Random amplification of polymorphic DNA is useful for performing identification at strain level, but the lack of reproducibility and the lack of complex band patterns, are the main limiting factors. Restriction endonucleases analysis and pulse field gel electrophoresis is a powerful method

for strain typing, but it is laborious and expensive, and only a limited number of samples can be analysed simultaneously.

AFLP (Amplified Fragment Length Polymorphisms analysis, Vos et al., 1995) is a powerful fingerprinting methodology originally developed for plants but later applied to the taxonomy of several microbiological DNA samples (Antonyshin et al., 2000; Thompson et al., 2001; Vancanneyt et al., 2005; Alter et al., 2006). The AFLP technique is particularly interesting with regard to the characterisation of the isolate to single strain level as it is more reproducible than RAPD; the complex band pattern can be modulated, thus allowing for the identification of single strains.

Among the advantages of AFLPs analysis, is that the process is amenable to full high speed automation and precise analysis, which allows for the simultaneous comparison of hundreds of colonies isolated from the same or different sources permitting an estimation of the microbial biodiversity.

The most frequently isolated LAB from fermented sausages are *Lactobacillus sakei*, *L. curvatus*, and *L. plantarum*, while among the CNC it is *Staphylococcus xylosus* that is most usually found (Cocolin et al., 2001a; Rossi et al., 2001; Aymerich et al., 2003; Cocolin et al., 2004; Rantsiou et al., 2005b).

Concerning the typology of sausages that have been analysed, several reports only consider the microbiota isolated from "Salame friulano" (Cocolin et al., 2001a; Cocolin et al., 2001b; Comi et al., 2005; Rantsiou et al., 2005a; Rantsiou et al., 2005b; Rantsiou & Cocolin 2006; Urso et al., 2006).

In the present study we have used fluorescent AFLP to analyse the genetic variability of cultured bacteria isolated from populations present in traditional North Italian fermented 'salami' in order to: (i) define the biodiversity of cultured bacteria involved in traditional meat fermentation of high quality products produced without using starter cultures; (ii) define the utility of AFLP in characterising, at strain level, the indigenous sausage microflora; (iii) collect the different isolates and identify strain specific electropherograms usable as markers for traceability of the product.

2. Material and methods

2.1 Sampling and microflora collection

Eighteen different artisanal single sausages of fermented "salami" were collected from different local manufacturers in northern Italy, in the provinces of Mantova, Cremona, Verona, Reggio Emilia and Parma, during the years 2007 and 2008. Sausage preparation, maturation time and parameters were typical of the chosen areas and differed for each considered artisanal manufacturer. Due also to the natural seasoning process taking place in specific cellars, it was difficult to ascertain these parameters precisely. In general the fermentation temperature ranged from 16 to 20 °C over a period of 90-120 days.

The isolation of bacteria was carried out as reported in Cocolin et al. (2001a). After growth on selected media (MRS - de Man, Rogosa and Sharp, and BP – Baird-Parker) for 48 h at 30 °C, twenty presumed lactobacilli and up to fifteen presumed staphylococci single colonies were selected for each sausage and two single colony isolation steps, on the same media and under the same conditions for growth, were performed. Single colonies were then inoculated into tubes containing 15 ml of liquid broth and kept at 30 °C for 48 h without (MRS) and with shaking (BP) at 125 rpm. After growth 3 ml of each culture was centrifuged

in a DNA extraction tube, and the remaining 12 ml centrifuged and re-suspended in 4 ml of sterile MRS or BP plus 25% glycerol in order to prepare four tubes for liquid N₂ storage.

All colonies were named for insertion in our general collection with the mark CCCF (Culture Collection Corrado Fogher) followed by a number starting from 4,000 to 4,499 for LAB and from 4,500 to 4,999 for CNC. In this paper, the letters before the number of the strain in the dendrograms refer to the different manufacturers and will be eliminated from the collection name after analysis.

Lactobacillus sakei DSM20100; *Lactobacillus plantarum* DSM20205; *Lactobacillus curvatus* DSM20010; *Lactobacillus casei* LC1; *Staphylococcus xylosus* ATCC35663; *Staphylococcus carnosus* ATCC51365 were used as control strains.

2.2 DNA extraction

DNA extraction was performed using the commercial kit GenElute Plant Genomic DNA miniprep kit (Sigma) following standard protocol with a few modifications limited to the preparation of the samples. Cells were grown for 48 h in 15 ml of MRS or BP medium then 3 ml was harvested by centrifugation (5 minutes (min.), 10,000 rpm). The pellet was resuspended in the provided lysis solutions, glass-pearls were added and each sample was shacked by vortexing for 1 minute to aid the disruption of cell wall, then cells were incubated at 65 °C for 20 min. The DNA was visualised and quantified by agarose gel (0.8 %) electrophoresis.

2.3 AFLP analysis

The AFLP reactions were performed as described previously (Vos et al., 1995) with minor modifications of the AFLP plant mapping protocol (Applied Biosystems). Approximately 100 ng of DNA for each sample were used for AFLP reaction. Restriction-ligation (RL) was performed in a final volume of 11 µl containing: genomic DNA (100 ng), 1X T4 *ligase* buffer, 0.05M NaCl, 0.05 µg BSA (Bovine Serum Albumine), 5 pmol *EcoRI* adapter, 50 pmol *MseI* adapter, 1 U T4 DNA *ligase* (Promega), 1 U *MseI* (New England Biolabs), 5 U *EcoRI* (New England Biolabs). The reaction was incubated for 2 h 30 min. at 37 °C. RL was diluted ten fold and 4 µl of the diluted RL reaction were considered for Preselective amplification.The pre-selective PCR (72 °C for 2 min.; 30 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min.; 60 °C for 30 min.) with *EcoRI*+A and *MseI*+C primers was performed in a 20 µl reaction volume consisting of 4 µl 10-fold diluted RL DNA, 5 pmol pre-selective primers and AFLP Core Mix (Applied Biosystems) to the final volume. The selective PCR (94 °C for 2 min.; 1 cycle of 94 °C for 20 s, 66 °C for 30 s, and 72 °C for 2 min., followed by 10 cycles of 1 °C reduced annealing temperature each cycle and 25 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 2 min.; 60 °C for 30 min.) with *EcoRI*+AXX and *MseI*+C selective primers was carried out in a 10 µl volume consisting of 2 µl of 20-fold diluted pre-amplified DNA, 0.25 pmol of *EcoRI* primer, 1 pmol of *MseI* primer and 7 µl of AFLP Core Mix. The *EcoRI* selective primers were fluorescent dye-labeled and the following primer combinations employed: *EcoRI*+ACA / *MseI*+C, *EcoRI*+AGC / *MseI*+C, *EcoRI*+ACT / *MseI*+C. Amplified products from selective amplification were loaded and run on the ABI PRISM 3100 genetic analyzer (Applied Biosystems) according to AFLP plant mapping protocol and analyzed, considering a threshold of 100 rfu, using GeneScan Analysis software version 3.7 (Applied Biosystems).

A similarity matrix among genotypes was constructed applying the Jaccard coefficient and the corresponding dendrogram was obtained using the UPGMA method. The closeness of fit between the original similarity matrix and the respective dendrogram was evaluated by calculating the cophenetic correlation coefficient r (Mantel 1967) between the similarity matrix and the cophenetic matrix of the UPGMA clustering (1000 permutations). A bi-dimensional visualization of the relationships between the different clusters have been obtained using the Principal Components Analysis (PCA) method. All the analyses were performed using different modules from the NTSYSpc ver 2.1.

2.4 Ribosomal DNA analysis

Two universal primers; fD1, 5'AGAGTTTGATCCTGGCTCAG3', (Weisburg et al., 1991) and 805R, 5'GACTACCAGGGTATCTAATCC3', (Tanner et al., 1998) for 16S ribosomal DNA were used for the amplifications. PCR reactions were performed in a final volume of 20 µl containing: 10 ng of genomic DNA, 1 X PCR buffer (buffer A, InCura), 2 mM MgCl2, 150 µM dNTPs, 5 pmol of each primer and 1 U *Taq* DNA polymerase (InCura). PCR amplifications were carried out with an initial denaturing step of 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 57 °C, 1 min of extension at 72 °C and a final extension step of 5 min at 72 °C. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Then fragments were direct sequenced using the BigDye v3.1 sequencing kit according to the manufacturer's instructions, and the sequences were loaded on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

3. Results

Using MRS and BP selective media 494 colonies were recovered of which 271 presumed *Lactobacillus* spp. and 223 presumed *Staphylococcus* spp.. The two groups were analysed separately and 215 out of 271 and 132 out of 223 colonies were randomly selected for molecular analyses.

Genetic relationships were determined by means of fluorescent AFLP. The *MseI* primer used for the selective amplifications was the preselective one, with an extra base (C). This was decided after preliminary trials, because the use of *MseI* selective primers with three bases gave too few peaks (usually fewer than ten, data not shown).

The use of three primer combinations was sufficient to detect several clear polymorphisms (at least two hundred for each group) and to recognise unequivocally the high number of strains considered. Only a few monomorphic bands were found and scored, and this reflects the great variability present not only between species but also within species. The use of some standard strains was useful to anchor the dendrograms and to verify the cluster-species correspondence (Table 1).

Strains isolated from all the sausage samples were considered for the analysis. The presumed LAB dendrogram (Figure 1a, 1b) and the similarity matrix were highly correlated (matrix correlation r = 0.97445; t = 52.3474, and Prob. (random Z < obs. Z) p = 1.0000). The tree was characterised by the presence of five well-defined clusters (I to V). The highest number of strains (131, 60.9 %) was clustered in group I, 15 (7 %) of the strains were in group II, 43 (20 %) in group III, 21 (9.8 %) in group IV and 5 (2.3 %) in group V. A bi-dimensional visualization of the sample set has been obtained by means of principal components analysis (Figure 2).

Genus	Species	CCCF[a] strain	Cluster
Lactobacillus	L. sakei	CCCF4161, CCCF4066 CCCF4000, CCCF4033 CCCF4047, CCCF4060 CCCF4044, CCCF4075 CCCF4262, CCCF4271	I
	L. coryniformis	CCCF4142, CCCF4226	II
	L. curvatus	CCCF4016, CCCF4139 CCCF4173	III
	L. plantarum	CCCF4178, CCCF4006 CCCF4032, CCCF4169	IV-1
	L. casei	CCCF4148	IV-2
Leuconostoc	L. mesenteroides	CCCF4059, CCCF4185	V
Staphylococcus	S. xylosus	CCCF4722, CCCF4716 CCCF4723, CCCF4519 CCCF4500, CCCF4707	I-1
	S. carnosus	CCCF4514, CCCF4692	I-2
	S. xylosus / saprophyticus	CCCF4520, CCCF4521 CCCF4714	I-3
	S. equorum	CCCF4556, CCCF4570 CCCF4674, CCCF4661 CCCF4671	II-1 II-2
Bacillus Bacterium		CCCF4560, CCCF4561 CCCF4565, CCCF4525 CCCF4530	II III
Enterococcus	E. faecium, E. durans	CCCF4529, CCCF4708	IV

a: Collezione Ceppi Corrado Fogher.

Table 1. Genus and Species classification of selected isolated strains based on GenBank homology search (homology greater than 98%) for a fragment at the 5' end of the 16S ribosomal DNA gene.
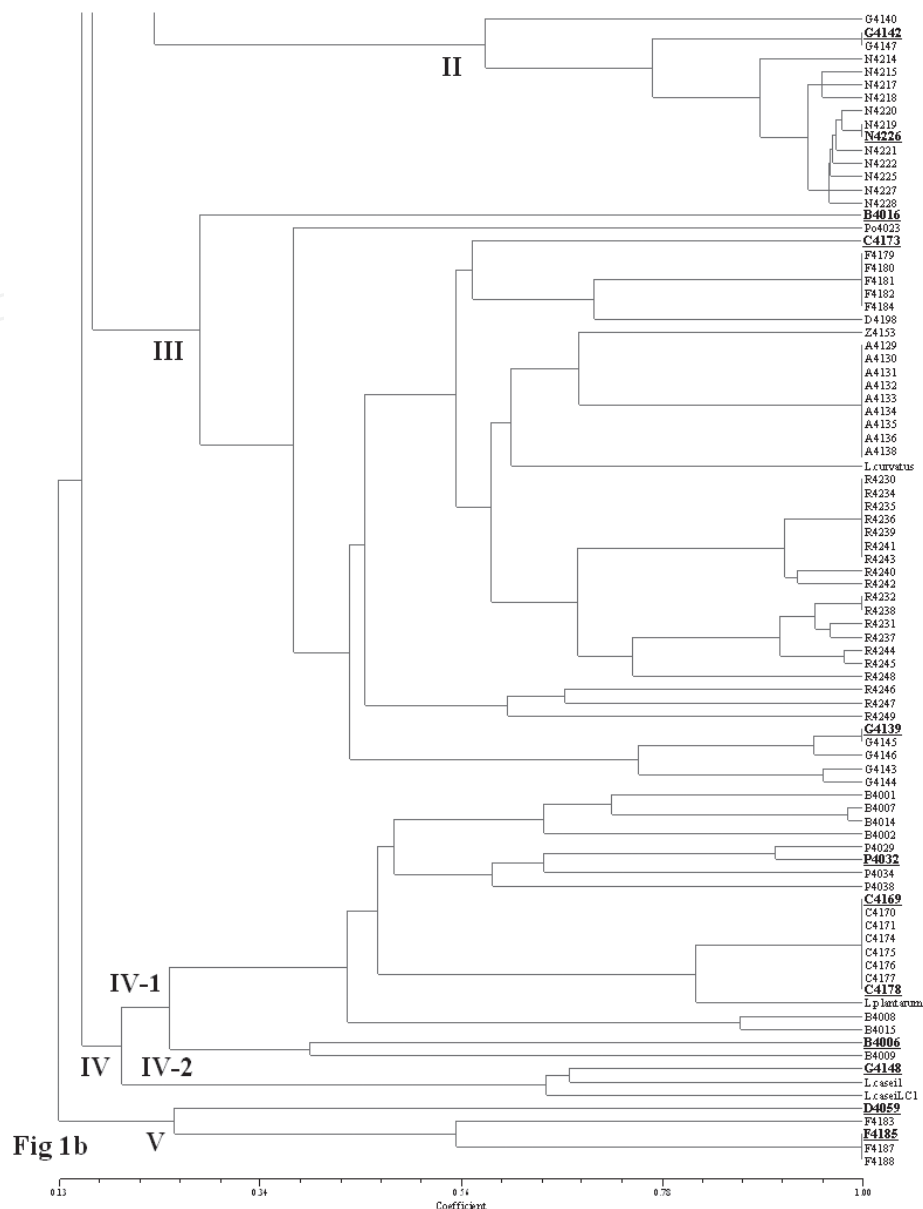
Fig 1a
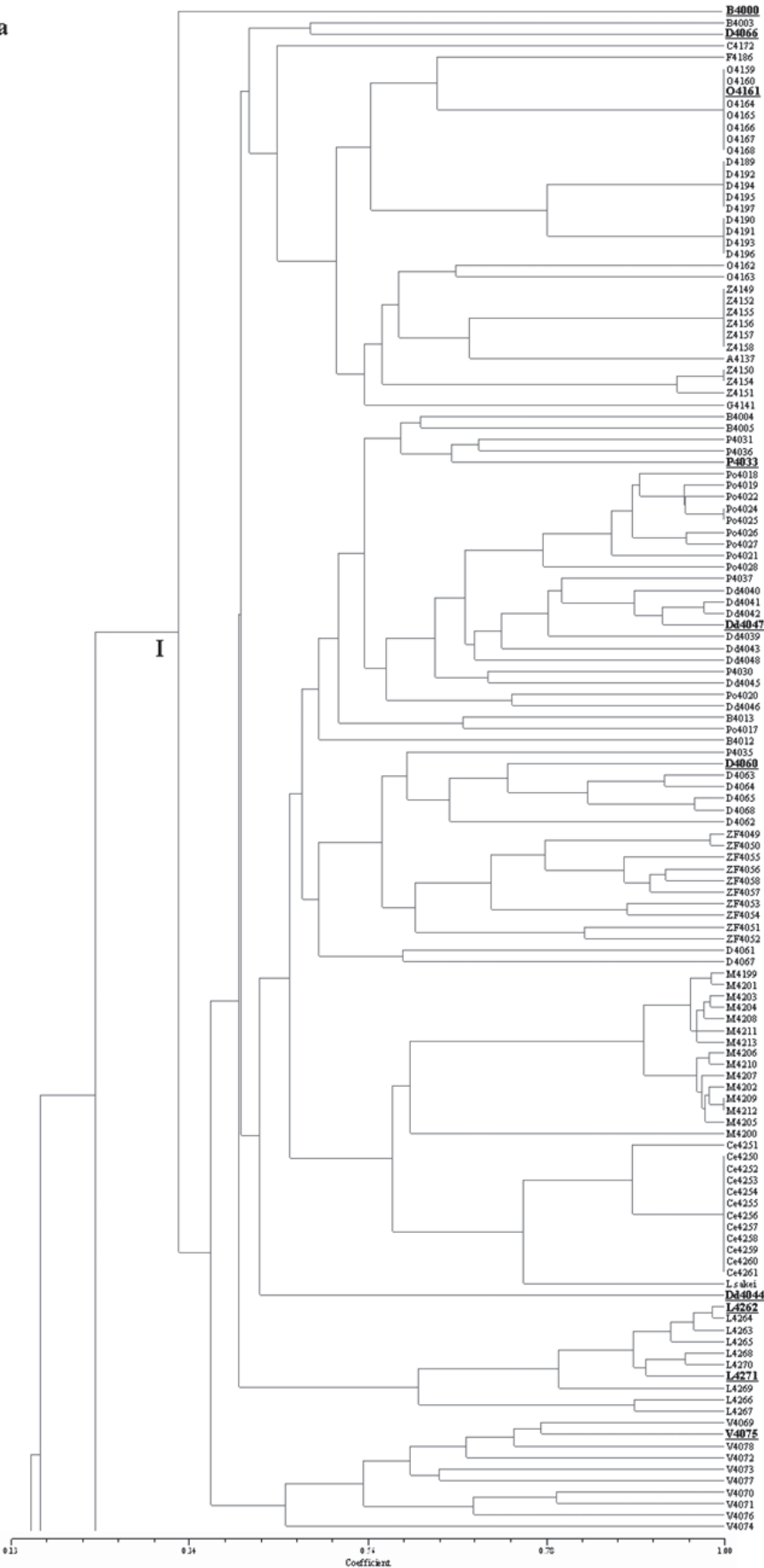
Fig. 1a, 1b. UPGMA phylogenetic tree of the Lactic Acid Bacteria population isolated from fermented sausages. The different clusters are reported. Cluster I: *L. sakei*; Cluster II: *L. coryniformis*; Cluster III: *L. curvatus*; Cluster IV-1: *L. plantarum*; Cluster IV-2: *L. casei*; Cluster V: *Leuconostoc mesenteroides*. The X axis represents the similarity coefficient obtained from the matrix constructed applying the Jaccard coefficient.

Principal component analysis (PCA) is a mathematical procedure that offers an easy visualization of the relationships between the different microbiota finding a set of synthetic variables that summarize decreasing portion of the observed variance. PCA finds those linear combinations that are maximizing the variance within the data and by using the DCENTER (Double Center) module, the Jaccard similarity matrix has been transformed to scalar product so that its eigenvalues and eigenvectors can be computed (resulting in a Principal Coordinates Analysis).
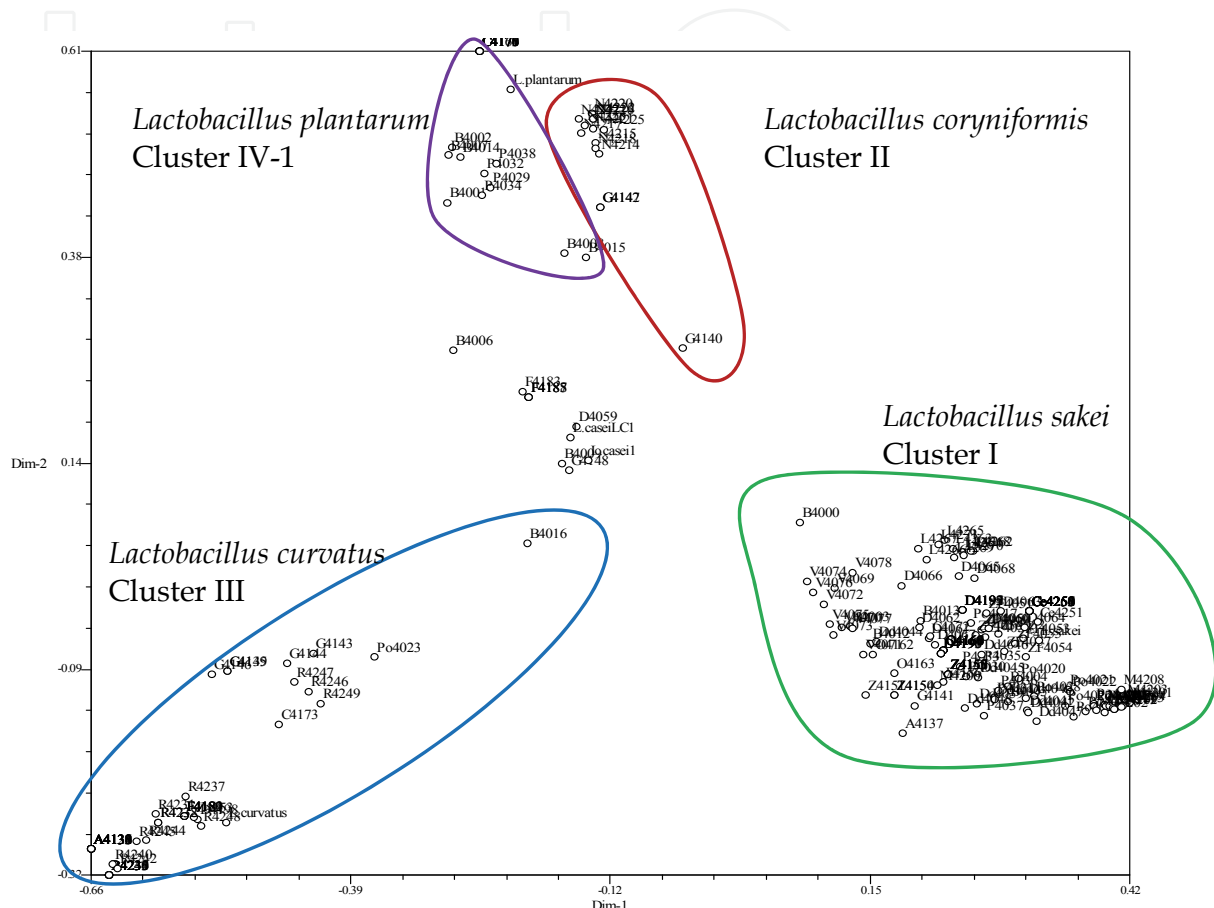
Fig. 2. Bi-dimensional representation of the presumed Lactobacilli main clusters defined by the AFLP analysis.

The first principal component (PC) is then the linear combination of the variables yielding the largest variance. The second PC explains the largest amount of the remaining variance. Thus, PCs can be sorted naturally by the explained variance and the four main clusters result to correspond to *L. sakei*, *L. coryniformis*, *L. curvatus*, and *L. plantarum*.

A more detailed comparison of the AFLP electropherograms revealed that the strains inside each cluster usually had similar banding profiles, and this made it possible to find some general banding profiles characteristic of the different clusters (Figure 3). Only one exception was present inside cluster IV, where strain CCCF4148 had a profile which was different compared with the profiles of the other strains, and therefore two sub-clusters were defined: IV-1, and IV-2.
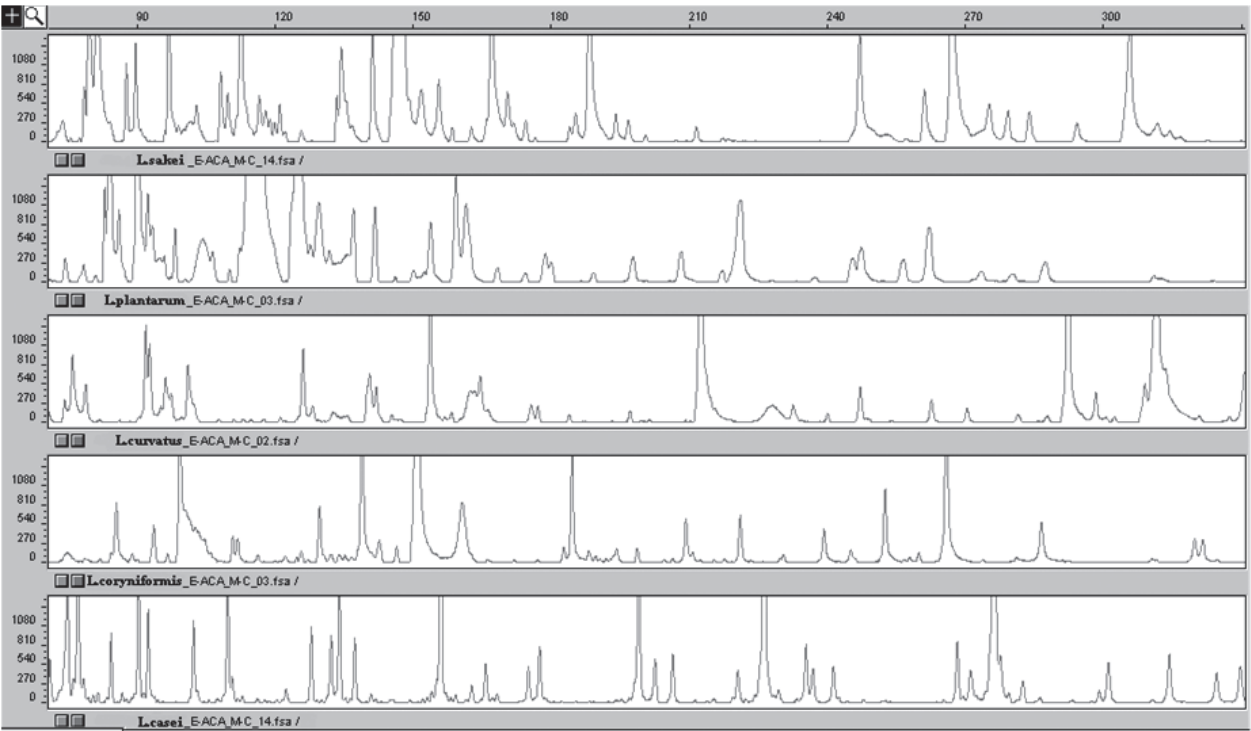
Fig. 3. AFLP electropherograms obtained using the automated genetic analyser. The five profiles are characteristic of different species of the genus *Lactobacillus*: *L. sakei* (strain 4191), *L. coryniformis* (strain 4214), *L. curvatus* (strain 4240), *L. plantarum* (Strain 4169), *L. casei* (strain 4148).

The presumed *Staphylococcus* dendrogram (Figure 4) and the similarity matrix were highly correlated (matrix correlation r = 0.94801; t = 64.2042, and Prob. (random Z < obs. Z) p = 1.0000). The tree was less defined in comparison with the LAB tree, but some main groups (four from I to IV) were still visible. The highest number of strains (66, 50 %) was clustered inside group II, 45 (34.1 %) of the strains were in group I, 7 (5.3 %) in group III, 10 (7.6 %) in group IV. Four strains (3 %) were located outside the main clusters.
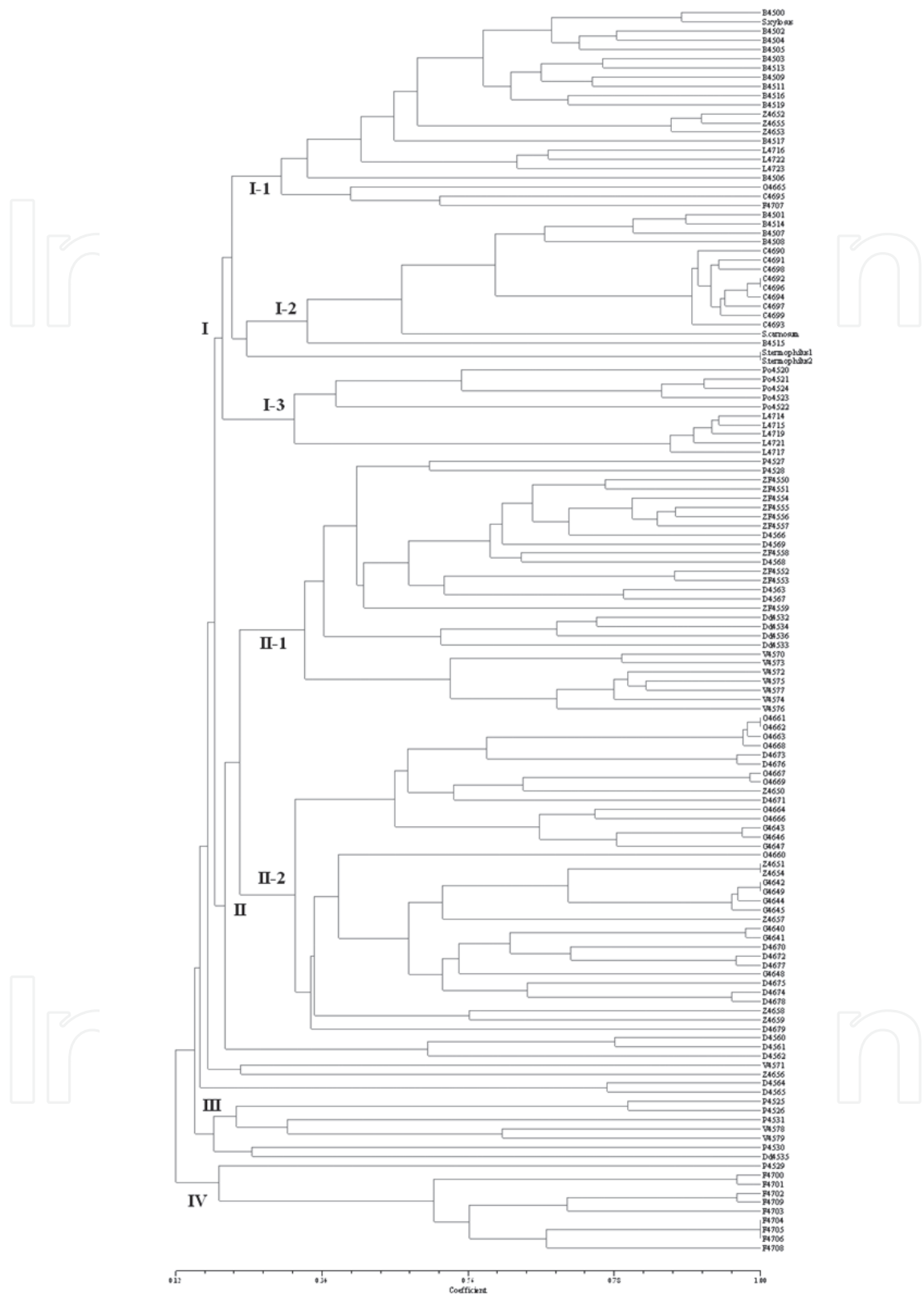
Fig. 4. UPGMA phylogenetic tree of the Coagulase Negative Cocci population isolated from fermented sausages. The different clusters are reported. Cluster I-1: *S. xylosus*; Cluster I-2: *S. carnosus*; Cluster I-3: *S. saprophyticus / xylosus*; Cluster II-1: *S. equorum*; Cluster II-2: *S. equorum*; Cluster III: *Bacillus* and *Bacterium* spp.; Cluster IV: *Enterococcus* spp.

As for the Lactobacilli, a PCA analysis has been obtained also for the presumed Staphylococci. The main clusters are clearly visible with the only exception of cluster I-3 that is not easily distinguished (Figure 5).
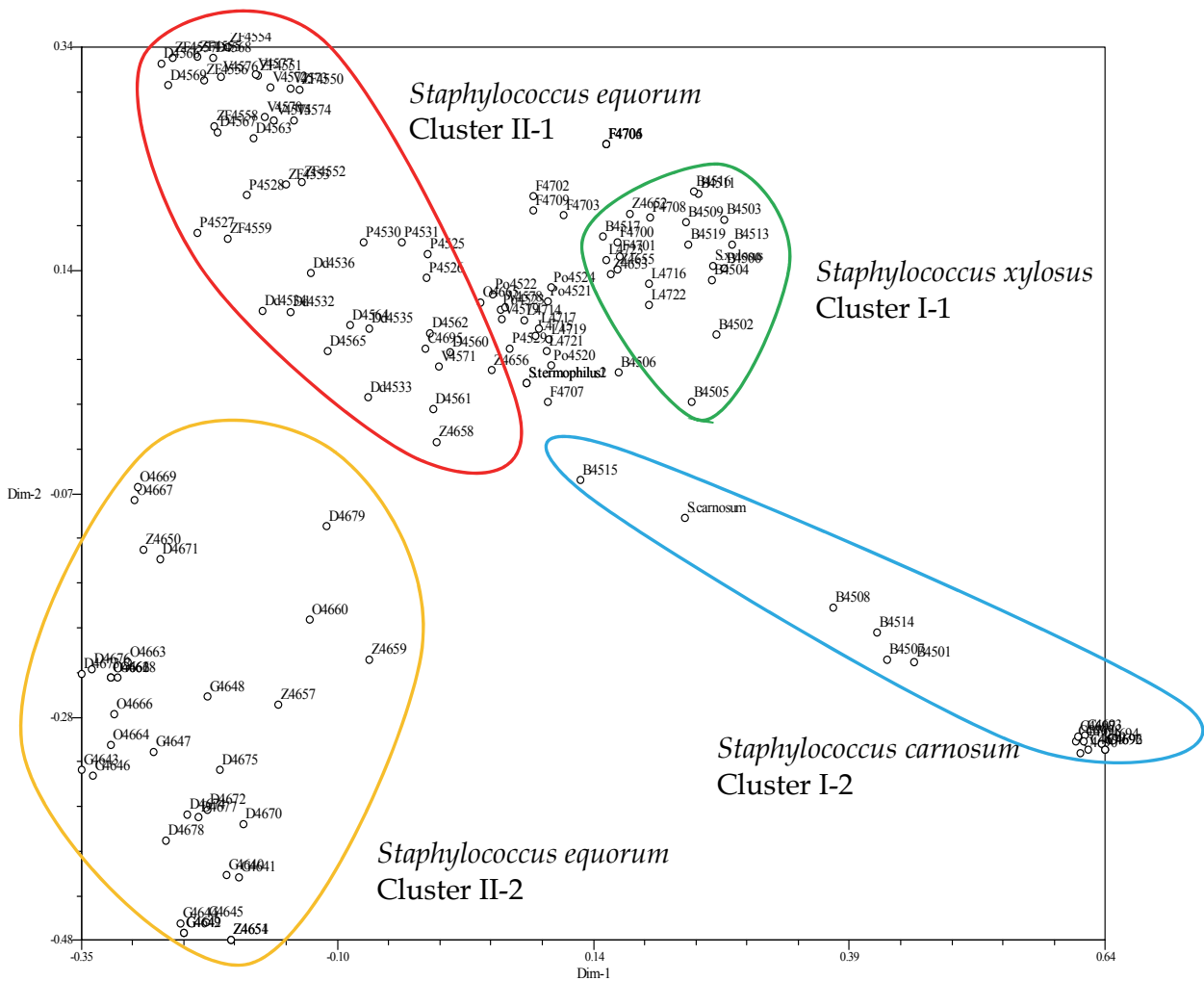

Fig. 5. Bi-dimensional representation of the presumed Streptococci main clusters defined by the AFLP analysis.

A more detailed comparison of the AFLP electropherograms of the strains inside clusters II, III, and IV revealed that they usually had similar banding profiles, while cluster I was characterised by three different AFLP profile typologies dividing the cluster into three sub-clusters: I-1, I-2, and I-3 (Figure 6).

In order to gain information about the genus or the specie corresponding to the different clusters, some strains were further analysed at ribosomal DNA level. In each group the strains were selected considering the genetic distances as reported in the similarity matrix, and those strains characterised by low similarity were selected.

The sequence of a fragment approximately 800 bp long was determined for the strains reported in Table 1, the results of a homology search is summarised in the same table.

Based on these results and on the similarities between the AFLP banding profiles, we identified the following species for *Lactobacillus*: *L. sakei* (cluster I), *L. coryniformis* (cluster II), *L. curvatus* (cluster III), *L. plantarum* (cluster IV-1), and *L. casei* (cluster IV-2). Strains inside

cluster V were recognised as belonging to the species *Leuconostoc mesenteroides*. Several strains were recognised unequivocally, while some strains showed an identical peak pattern. Indeed these could be replicates of the same strains. This situation was found mainly in groups I, III and IV-1. Inside each cluster, some sub-clusters grouping the strains isolated from the different producers were clearly visible (strains with the same letter).

The following species were identified within genus *Staphylococcus*: *S. xylosus* (cluster I-1), *S. carnosus* (cluster I-2), *S. saprophyticus / xylosus* (cluster I-3), and *S. equorum* (cluster II-1 and II-2). The strains of cluster III belong to different genera and species of *Bacillus* and *Bacterium*. Finally Strains of cluster IV belong to the genus *Enterococcus*, and the species with the greatest degree of homology were *E. durans*, and *E. faecium*. Some sub-clusters grouping strains from the same producers are visible
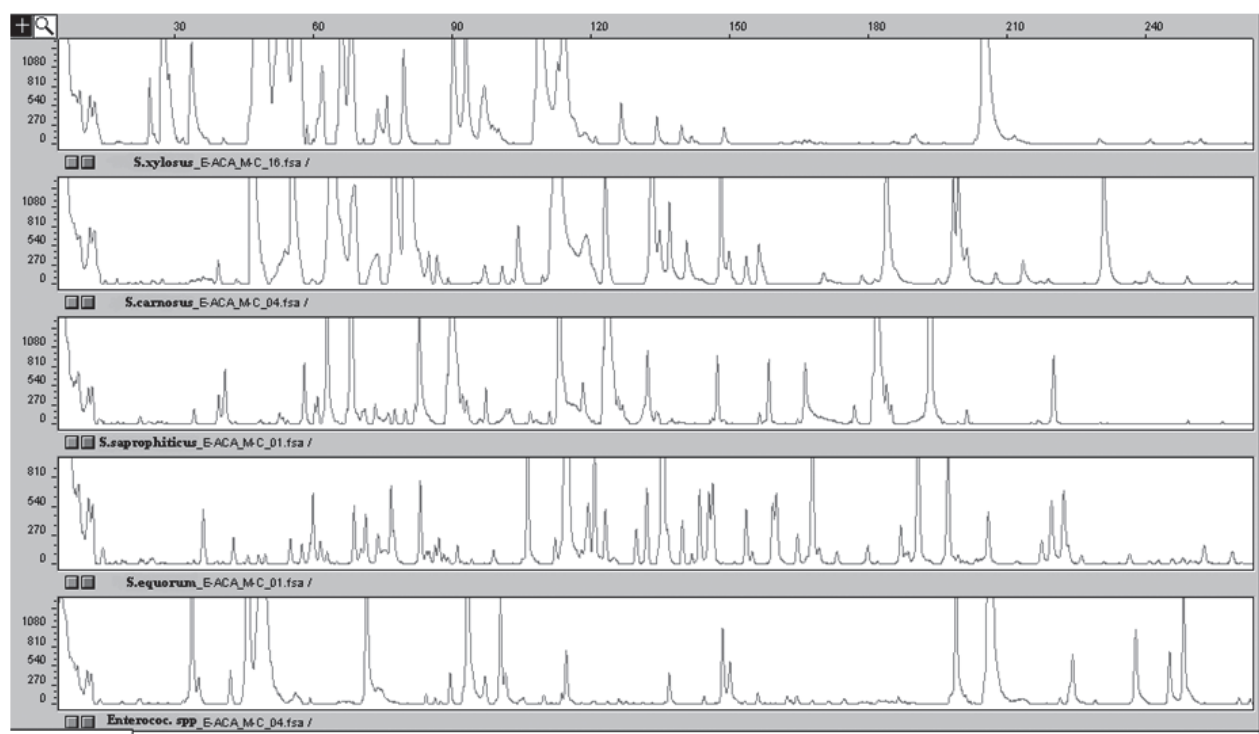

Fig. 6. AFLP electropherograms obtained using the automated genetic analyser. Panels 1 to 4 are characteristic of different species of the genus *Staphylococcus*: *S. xylosus* (strain 4722), *S. carnosus* (strain 4514), *S. saprophiticus* (strain 4714), and *S. equorum* (strain 4676). The last panel reports the profile of a strain (4704) belonging to the genus *Enterococcus*.

4. Discussion

Meat fermentation is a process with a long history in Europe, and many different countries have their own, characteristic long traditions of sausage production. Several studies have been carried out to define the biodiversity and the ecology of indigenous microflora in traditional Italian fermented products, both dry fermented sausages (Rantsiou et al., 2005a; Urso et al., 2006) and fresh sausages (Cocolin et al., 2004). The specific variety of the sausage is not always specified but, as reviewed in Rantsiou & Cocolin (2006), with the exception of a few studies concerning "Soppressata" and "Salsiccia sotto sugna", the greatest number of these reports looking into the identification of microflora by means of molecular methods,

deal with products from northeast Italy mainly 'salame friulano' The existence of different conditions in the preparation and fermentation of sausages, both between different countries and within the same country (Rantsiou et al. 2005a; Urso et al. 2006), can determine the existence of differences in microflora dynamics (Rantsiou et al., 2005a). In this study, natural fermented sausages or 'salami' were sampled from different local manufacturers in north Italy at the end of the fermentation process. The use of reference strains both with the AFLP and the 16S ribosomal RNA gene analysis was considered in order to define the genus and, if possible, the species corresponding to the different clusters.

To assess the reproducibility of the AFLP analyses a panel of seven strains was replicated four times and the profiles consistently showed a good level of correspondence (data not shown) with a percentage of homology usually greater than 95% between the replicates. Considering this data, it is also possible that colonies showing homology greater than 95 % could be considered as replicates of the same strain.

The AFLP banding profiles of the strains grouped in the same clusters or sub-clusters showed similar patterns and the profiles of the single species can be used to rapidly classify new strains.

The potential to run AFLP on automated platforms such as genetic analysers is important since a significant number of isolates have to be characterised in order to better represent the bacterial population present in specific fermented sausages. The availability of automated platforms and the use of different dye-labelled primers provide the possibility to analyse, in a single day, several hundreds of samples. Furthermore, the use of reference strains reduces the need to perform ribosomal DNA analysis in order to define the species or the genus of the different isolates, thus further increasing the rapidity of the process.

As reported in Aymerich et al. (2006) some important points have to be considered for an optimal selection of strains and to define the microbial role in these products: i) rapid classification and identification of unknown isolates, ii) evaluation of genetic diversity among strains, iii) strain typing to assess genetic stability over time. Considering these points and based on the obtained results, fluorescent-AFLPs seem to tick all the boxes and therefore can be considered as an interesting, precise and rapid tool to identify the sausage bacterial microflora in addition to the existing and well-established methods.

Inside the genus *Lactobacillus*, we found the species *L. sakei* (61%) followed by *L. curvatus* (20 %) and *L. plantarum* (10%) to be the most represented. The same species were reported in other works (Cocolin et al., 2004; Rantsiou et al., 2005a; Rantsiou et al., 2006; Urso et al., 2006). Inside each cluster, some sub-cluster grouping strains from the same producer were defined (e.g. Strains CCCF4250 to 4261), and usually the differences between different sampling sites are greater than the differences inside single sites. This could be an important aspect to consider in the collection of microbiota biodiversity of fermented sausages.

The presence of *L. coryniformis* was also reported (Samelis et al., 1994; Rantsiou & Cocolin 2006) and, in agreement with several reports (Cocolin et al., 2004; Rantsiou et al., 2005a; Aymerich et al., 2006; Urso et al., 2006) the presence of *L. casei* is rare. Rare also are strains belonging to the species *Leuconostoc mesenteroides* which represent only 2.3 % of the LAB populations.

Inside genus *Staphylococcus*, *S. equorum* (48 %), followed by *S. xylosus* (21 strains, 47 % inside cluster I and 16 % of total), *S. carnosus* (14 strains, 31 % inside cluster I and 11 % of total), and *S. saprophyticus / xylosus* (10 strains, 22 % inside cluster I and 7.6 % of total) were found.

The same species were also found in other studies, but not in the same proportions. Rantsiou et al. (2005 c) found *S. xylosus*, *S. equorum*, and *S. saprophyticus* while they did not

find *S. carnosus*, and the percentages of the three species were respectively 48 % for *S. xylosus*, 23% for *S. equorum*, and 1.2 % for *S. sapropyiticus*. The presence of *S. carnosus* was also reported by Cocolin et al. (2001a, 2001b).

Bacteria belonging to the genus *Enterococcus* were reported by Rantsiou et al. (2005b) in similar percentages. The presence of enterococci must be evaluated carefully because they can have a role in the production of various traditional fermented foods, but at the same time certain enterococcal strains can cause human disease (Franz et al., 2003).

In this work we have analysed a significant number of typical and naturally fermented sausages produced in north Italy and provided an evaluation of the different cultured species and strains of bacteria present in these artisanal products. We have analysed all the isolates to strain level, obtaining for each one the corresponding AFLP profile demonstrating moreover that AFLP is a robust and useful technique for characterising the strain levels of cultured microbiota.

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As everybody knows, the dynamic interactions between biotic and abiotic factors, as well as the anthropic ones, considerably affect global climate changes and consequently biology, ecology and distribution of life forms of our planet. These important natural events affect all ecosystems, causing important changes on biodiversity. Systematic and phylogenetic studies, biogeographic distribution analysis and evaluations of diversity richness are focal topics of this book written by international experts, some even considering economical effects and future perspectives on the managing and conservation plans.

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