ORIGINAL ARTICLE

Characterization of microbial population of 'Alheira' (a traditional Portuguese fermented sausage) by PCR-DGGE and traditional cultural microbiological methods

H. Albano[1], I. Henriques[2], A. Correia[2], T. Hogg[1] and P. Teixeira[1]

1 Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal
2 CESAM and Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro, Portugal

Abstract

Aims: This study evaluates the microbial ecology of 'Alheira' by traditional microbiological analysis and a PCR-denaturing gradient gel electrophoresis (DGGE) protocol.

Methods and Results: Total microbial DNA from 'Alheiras' was extracted directly from the products and subjected to PCR using Eubacterial primers for 16S rDNA. The amplicons were separated by DGGE. The results demonstrated that different products of the same batch display identical profiles, whereas products from different batches of the same producer could display different DGGE profiles. 'Alheiras' from different producers were distinguishable based on the respective DGGE profiles. The obtained sequences from prevalent phylotypes affiliated with order *Lactobacillales* and order *Bacillales* and class *Gammaproteobacteria*. The same samples were subjected to traditional microbiological analysis. In both methods, lactic acid bacteria were dominant and were present together with other organisms, mainly members of the family *Micrococcaceae*.

Conclusions: The approach explored in this study allowed the description of the microbial community present in 'Alheira' in particular the diversity of lactic acid bacteria.

Significance and Impact of the Study: This can be useful for the microbiological characterization of traditional products in order to develop new methods of quality control capable of supporting a standardization of the processes, while preserving their typical traits.

Introduction

The microbiology of fermented sausages is complex. The type of microflora that develops is often closely related to the ripening technique utilized. The manufacture of fermented sausages has a long history in Portugal where there is a wide variety of typical preparations.

'Alheira' is a traditional fermented meat sausage typical of the North of Portugal (Trás-os-Montes). 'Alheira de Mirandela' is a much appreciated product and represents an important resource for this region where more than 500 tons are produced annually. 'Alheira de Mirandela' is in the process of name registration as 'Traditional Guaranteed Speciality'. The specific characteristics of the final

product mainly arise from the raw materials employed, the agro-ecosystem of the area of production and the traditional technology of manufacture. 'Alheira de Mirandela' is produced from pork and poultry meat, lard and pork fat, wheat bread and olive oil. Ingredients are mixed with salt, garlic and spices until they form a paste which is then stuffed into natural or artificial casings (horseshoe-shaped sausages approximately 15 ± 60 mm long) and submitted to a smoking process, at low but uncontrolled temperature (generally <40°C) and uncontrolled humidity, for no longer than 8 days. The shelf life of 'Alheiras' is about 1 month if stored at 4°C in air or longer if the sausages are packed under vacuum or modified atmosphere. 'Alheiras' are cooked before consumption either

by frying, grilling or boiling, according to regional traditions or consumer preferences. A wide variety of microorganisms has already been isolated from 'Alheiras' by traditional methods. These are mainly lactic acid bacteria (LAB), *Micrococcaceae* and some pathogens, such as *Listeria* spp., *Salmonella* and *Staphylococcus aureus* (Ferreira *et al.* 2006; Esteves *et al.* 2008).

Genetic fingerprinting techniques that use DNA, generated by polymerase chain reaction (PCR) procedures, applied DNA extracted directly from environmental samples, can be used to provide profiles representing the phylogenetic diversity of the microbial community in the environment being studied. This approach eliminates the necessity for traditional culture-dependent methods like selective cultivation and strain isolation, thereby negating the potential biases inherent in such procedures. Moreover, studies which have employed such direct analyses have repeatedly demonstrated considerable variance between cultivated and naturally occurring species, thereby dramatically altering the perception of the true microbial diversity present in various habitats (Head *et al.* 1998; Hugenholtz *et al.* 1998; Cocolin *et al.* 2002a). Denaturing gradient gel electrophoresis (DGGE) is perhaps the most commonly used among the nonculture-dependent fingerprinting techniques. It is based on the separation of PCR amplicons of the same size according to their different melting temperatures. PCR-DGGE of ribosomal DNA was introduced into microbial ecology by Muyzer *et al.* (1993). Despite the wide range of applications, this technique has been introduced into food microbiology no longer than 10 years. The great potential shown in analysing samples from natural environments without employing a culture-based isolation, has stimulated food microbiologists to investigate the suitability of PCR-DGGE to study microbial fermentations in food and food-related ecosystems. In the last few years, several papers have been published reporting the use of DGGE for LAB strain identification (Cocolin *et al.* 2000; Ercolini *et al.* 2001a; Ogier *et al.* 2002), for the study of the ecology of fermented foods and the microbial changes in LAB populations during these food fermentations and to profile pathogens directly in food samples (Cocolin *et al.* 2002b). In particular, direct PCR amplification of different regions of the 16S rRNA gene and subsequent analysis by DGGE has been used to study the ecology of the microbial processes involved in the production of Mexican fermented maize dough (ben Omar and Ampe 2000), Mozzarella cheese (Coppola *et al.* 2001), fermented sausages (Cocolin *et al.* 2001a,b, 2004; Fontana *et al.* 2005; Rantsiou *et al.* 2005; Aquilanti *et al.* 2007), malt whisky (van Beek and Priest 2002), artisanal Sicilian cheese (Randazzo *et al.* 2002), buffalo Mozzarella and Stilton cheese (Ercolini *et al.* 2001b, 2003) and sourdough

(Meroth *et al.* 2003). The use of DGGE in food microbiology was reviewed by Ercolini (2004).

In this work, the microbial ecology of 'Alheiras', produced in six different plants in North-East Portugal, was studied by using culture-dependent and -independent methods. 'Alheiras' were subjected to traditional microbiological analysis in order to enumerate specific bacterial groups. The aim of this study was also to evaluate if PCR-DGGE could be useful to identify specific motifs in the bacterial community profiles of these products. To accomplish this aim, DNA was extracted directly from the same samples and 16S DNA fragments were amplified by PCR, using 'universal' bacterial primers. DGGE analysis allowed fingerprinting of the microbial populations present in 'Alheira de Mirandela' and determination of the DNA sequence of relevant bands permitted the establishment of the phylogenetic affiliation of bacteria present.

Materials and methods

Sampling

'Alheiras' from six different producers (two or three batches of each), were purchased from retail stores, at storage time, during 2005. Samples were labelled as Ef, Tp, Tx, Gr, Ag and Am to represent the six different producers.

Microbiological analyses by cultural methods

Samples of 'Alheiras' (25 g) were added to 225 ml of sterile buffered peptone water (Merck, Darmstadt, Germany), and homogenized in a stomacher for 2 min. Appropriate decimal dilutions were prepared in sterile Ringer's solution (LabM, Bury, UK) for microbial enumeration: LAB on de Man, Rogosa Sharpe Agar (MRS, LabM) and on M17 (LabM), incubated at 30°C for 72 h; total counts at 30°C according to ISO Standard 4833 (ISO 2003); *Enterococcaceae* on bile esculin azide agar (Biokar Diagnostics, Beauvais, France), incubated at 30°C for 72 h; *Micrococcaceae* on mannitol salt agar (Biokar Diagnostics), incubated at 37°C for 48 h; *Enterobacteriaceae* according to the Portuguese Standard NP 4137 (IPQ 1991). Enumeration of *Listeria* spp. was performed on Palcam Agar (Merck) medium and incubated at 30°C for 72 h. Three independent analyses, using randomly selected pieces, were performed for each sample.

DNA extraction

Ten grams samples were homogenized in a stomacher bag with 10 ml of saline-peptone water for 1 min. After each preparation had settled for 1 min, 1 ml subsamples were

placed in 1·5-ml screw-cap tubes containing 0·3 g of glass beads. Total DNA was extracted from 'Alheira' following the method previously described for fermented sausages (Cocolin *et al.* 2001a), using treatment with vortex instead bead beader.

PCR-DGGE protocol

DNA was amplified with primers 338F (5′-GAC-TCCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTA-CCGCGGCTGCTGG-3′), with a GC clamp attached to the forward primer (5), spanning the V3 region of the bacterial 16S rRNA gene (Muyzer *et al.* 1993). PCR was performed in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) as previously described (Henriques *et al.* 2006). Five microlitres of the product was analysed by standard agarose gel electrophoresis before DGGE analysis.

DGGE was performed on a DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories), according to Henriques *et al.* (2006). Briefly, samples were loaded onto 8% polyacrylamide gels (37·5 : 1, acrylamide: bis-acrylamide) in 0·5x TAE buffer (20 mmol l^{-1} Tris-acetate, pH 7·4, 10 mmol l^{-1} sodium acetate, 0·5 mmol l^{-1} Na$_2$EDTA) using a denaturing gradient ranging from 35–50% (100% denaturant contains 7 mol l^{-1} urea and 40% formamide). Electrophoresis was performed at 60°C, initially at 20 V (15 min) and then at 200 V (330 min). The gels were stained in an ethidium bromide solution (5 min) and then rinsed in distilled water (20 min). The image was acquired using a Molecular Image FX apparatus (Bio-Rad Laboratories). Every gel contained two lanes with a standard of eight bands for internal and external normalization and as an indication of the quality of the analysis.

Sequencing of DGGE bands and phylogenetic analysis

DGGE bands were excised with a sterile scalpel and eluted in 20 μl of sterile water, overnight at 4°C. Five microlitres of the supernatant was used for re-amplification with the original primer set. The accuracy of the bands and the position in the gel were checked on DGGE gels together with the original sample. Whenever necessary, bands were processed again as described above.

For sequencing analysis, PCR products were purified with the Jetquick PCR Purification Spin Kit (Genomed, Löhne, Germany) and used as template in the sequencing reactions. Those were carried out using the primer 518R and an ABI Prism$^{®}$ BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). The reaction mixtures were analysed in an automatic DNA sequencer (ABI Prism$^{®}$ 310 Genetic Analyzer, PE Applied Biosystems).

Band sequences were compared to the GenBank nucleotide data library using the BLAST software at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) in order to determine their closest phylogenetic relatives.

Nucleotide sequence accession numbers

The nucleotide sequences determined in the present study have been deposited in the GenBank database under the accession numbers EU555489–EU555518.

Results

'Alheiras' from six different producers, named Gr, Am, Ef, Tx, Ag and Tp (Fig. 1), were sampled during retail display at storage time from retail stores. From each producer, two or three batches ('1', '2' and '3' in Fig. 1) were used and, from each batch, two replicates were taken ('a' and 'b' in Fig. 1 correspond to the same batch). 'Alheiras' were subjected to traditional microbiological analysis and DNA, directly extracted from the samples, was subjected to DGGE analysis from which dominant bands were sequenced.

Microbiological characterization by cultural methods

The results of the plate counts obtained from the six producers and their samples in this study are reported in Table 1. It is clear that there is variability in counts between producers and even between different lots from the same producer. The values of total counts at 30°C were greater than 8·7 log CFU g^{-1} in most samples with LAB being the dominant microflora. Counts on MRS and M17 were, in most cases, >7·5 log CFU g^{-1}, with the exception of one sample from producer Gr (Table 1). In addition, enterococci counts were >6·5 log CFU g^{-1} in most samples. With the exception of one of the samples from producers Gr, Ef and Tx, counts on mannitol salt agar were >log 4·44 log CFU g^{-1}, although values varied between 4·4 log CFU g^{-1} and >6·20 log CFU g^{-1}. *Enterobacteriaceae* counts were >4 log CFU g^{-1} in most samples, with the exception of producer Gr. *Listeria* spp. was isolated in countable quantities in some samples, the highest values coming from two samples from the producer Tx (3·8 CFU g^{-1} and 4·3 CFU g^{-1}).

DGGE analysis

A sample-specific fingerprint was obtained from total DNA directly extracted from 'Alheiras', after amplification of the V3 region of 16S rRNA gene followed by DGGE analysis (Fig. 1). Reproducibility of PCR amplification
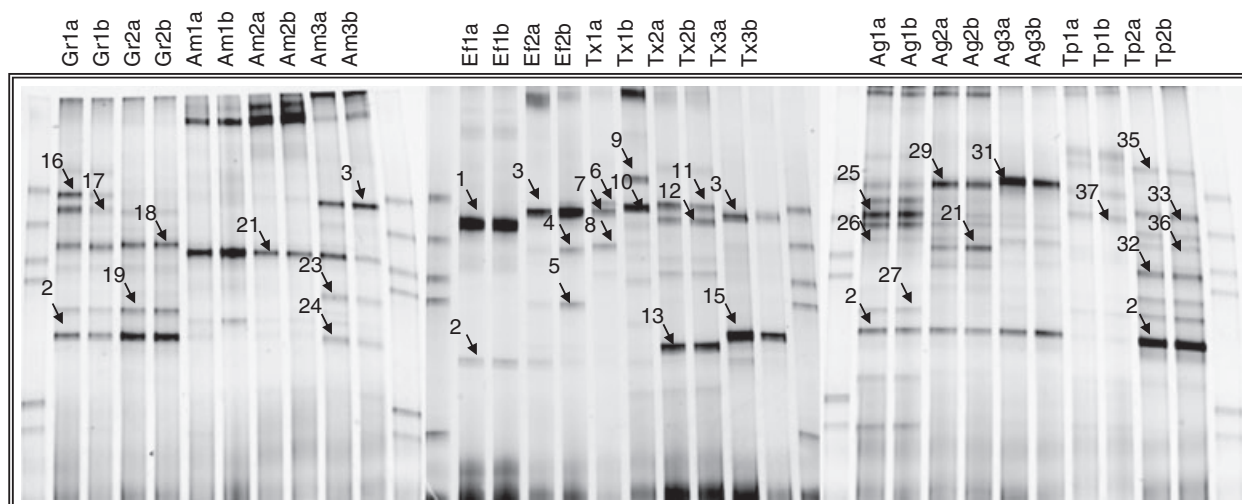
Figure 1 DGGE gels showing PCR-amplified bacterial 16S rRNA gene profiles from 'Alheiras' from six different producers (Ag, Am, Ef, Gr, Tp, Tx; a and b correspond to the same batch; indicated on top of the lanes). Lane M: DGGE marker constructed using previously characterized 16S rRNA gene clones from environmental libraries (Henriques *et al.* 2004). Bands that were excised for sequence analysis are labelled with the same number as in Table 2 and indicated with an arrow.

and DGGE was confirmed by performing three replicate runs, the profiles proving to be highly reproducible between different gels and PCR runs. The total number of band positions detected in the three gels was 30 and the number of DGGE bands per sample varied between 2 and 10 indicating significant differences between samples in terms of their microbial diversity. Samples Gr1, Am3, Tx2, Ag1 and Tp2 displayed the most complex profiles, whereas samples Am1, Am2, Ef1 and Ef3 displayed simpler profiles.

In general, products from the same batch displayed identical profiles, with the exception of sample Tx1,

where products from the same batch displayed different DGGE profiles. On the other hand, for some producers, consistent profiles were evident within different batches of 'Alheira'; certainly this was the case for producers Gr, Am, Ag and Tx. Producers Ef and Tp produced batches which were much less consistent in terms of the microbial ecology present.

Sequencing and identification of DGGE fragments

Thirty-one bands were excised from the three DGGE gels and re-amplified with the primers 338F and 518R

Table 1 Microbiological characterization of 'Alheira de Mirandela' from six different producers (Ag, Am, Ef, Gr, Tp, Tx;): important microbial parameters in fermented products

Producer	Total counts 30°C (log CFU g⁻¹)	Counts on MRS (log CFU g⁻¹)	Counts on M17 (log CFU g⁻¹)	Counts on MSA (log CFU g⁻¹)	Enterococci (log CFU g⁻¹)	Enterobacteria (log CFU g⁻¹)	*Listeria* spp. (log CFU g⁻¹)
Gr1	6·9 ± 0·6	6·0 ± 0·0	6·0 ± 0·3	5·4 ± 0·2	7·7 ± 0·3	2·3 ± 0·1	<1
Gr2	9·7 ± 0·2	8·8 ± 0·6	8·8 ± 0·2	<1	6·6 ± 0·0	2·7 ± 0·3	1·2 ± 0·2
Am1	9·4 ± 0·3	8·6 ± 0·1	9·0 ± 0·8	>6·2 ± 0·3	9·3 ± 0·2	7·1 ± 0·0	<1
Am2	1·0 ± 0·2	8·8 ± 0·2	8·5 ± 0·2	4·4 ± 0·6	7·8 ± 0·5	6·3 ± 0·0	<1
Am3	9·7 ± 0·1	8·5 ± 0·1	7·9 ± 0·1	4·2 ± 0·0	7·2 ± 0·1	6·8 ± 0·2	2·3 ± 0·3
Ef1	9·6 ± 0·2	7·6 ± 0·8	7·7 ± 0·1	<1	4·6 ± 0·2	3·3 ± 0·2	<1
Ef2	8·7 ± 0·4	7·7 ± 0·1	7·9 ± 0·4	5·9 ± 0·1	7·0 ± 0·0	5·6 ± 0·3	<1
Tx1	9·5 ± 0·0	9·8 ± 0·2	9·8 ± 0·3	6·0 ± 0·0	7·1 ± 0·1	4·2 ± 0·3	3·7 ± 0·1
Tx2	9·4 ± 0·3	8·1 ± 0·3	9·4 ± 0·2	>6·2	>7·2	4·3 ± 0·8	<1
Tx3	9·3 ± 0·2	8·0 ± 0·5	9·1 ± 0·2	<1	7·4 ± 0·0	7·2 ± 0·2	4·3 ± 0·2
Ag1	9·8 ± 0·1	8·9 ± 0·1	8·9 ± 0·1	5·8 ± 0·0	>7·2	>7·5	<1
Ag2	>10·5	9·8 ± 0·5	>10·5	>6·2	>7·2	6·9 ± 0·4	<1
Ag3	10·4 ± 0·1	9·9 ± 0·3	>10·5	5·9 ± 0·1	6·8 ± 0·3	4·9 ± 0·2	<1
Tp1	10·4 ± 0·4	9·2 ± 0·0	9·0 ± 0·1	>6·2	>7·2	>7·5	<1
Tp2	9·5 ± 0·1	8·0 ± 0·1	7·7 ± 0·0	5·9 ± 0·2	6·4 ± 0·2	5·5 ± 0·0	2·4 ± 0·3

Table 2 Closest relatives of DGGE band sequences

Band no.	Accession number	Sample	Closest relative (accession number)	Similarity (%)
1	EU555489	Ef1a	*Lactobacillus sakei* (EU081017.1)	100
2	EU555490	Ef1a	*Brochothrix thermosphacta* (AY543029.1)	100
3	EU555491	Ef2a	*Leuconostoc lactis* (AB295117.1)	98
4	EU555492	Ef2b	*Streptococcus lutetiensis* (EU163503)	100
5	EU555493	Ef2b	*Macrococcus caseolyticus* (EU048336.1)	100
6	EU555494	Tx1a	Uncultured soil bacterium clone (EU052112.1)	100
7	EU555495	Tx1a	*Lactococcus lactis* (EU080989.1)	99
8	EU555496	Tx1a	*Pseudomonas* sp. (EF028699.1)	99
9	EU555497	Tx1b	*Leuconostoc mesenteroides* (EU099617.1)	99
10	EU555498	Tx1b	*Weissella paramesenteroides* (EF422380.1)	100
11	EU555499	Tx2b	*Enterococcus* sp.(EU157915)	100
12	EU555500	Tx2b	*Enterococcus gallinarum* (EF025908)	98
13	EU555501	Tx2a	*Pediococcus acidilactici* (EF059987.1)	100
15	EU555502	Tx3a	*Lactococcus lactis* BMG 125 (EU080989.1)	99
16	EU555503	Gr1a	Uncultured *Bacilli* bacterium (EF706149.1)	100
17	EU555504	Gr1b	*Pseudomonas aeruginosa* (DQ149582.1)	99
18	EU555505	Gr2b	*Psychrobacter* sp. (EU075120.1)	100
19	EU555506	Gr2a	*Acinetobacter* sp. (AB365066)	100
21	EU555507	Am2a	Uncultured bacterium DGGE gel band (EU200335)	100
23	EU555508	Am3a	Uncultured bacterium (AY511592.1)	99
24	EU555509	Am3a	*Klebsiella* sp. (EU075144.1)	99
25	EU555510	Ag1a	*Lactobacillus curvatus* (EU081014.1)	100
26	EU555511	Ag1a	*Bacillus* sp. (DQ448756.1)	98
27	EU555512	Ag1b	*Psychrobacter* sp. (AJ582399.1)	99
29	EU555513	Ag2a	*Lactobacillus brevis* (EF120367.1)	98
31	EU555514	Ag3a	Uncultured *Bacilli* bacterium (EF706149.1)	98
32	EU555515	Tp2a	Uncultured soil bacterium clone G08 (EU052112.1)	99
35	EU555516	Tp2a	*Hafnia alvei* strain (AM042710.1)	99
36	EU555517	Tp2b	Uncultured *Bacilli* bacterium (EF706149.1)	100
37	EU555518	Tp1b	*Lactobacillus curvatus* (EU081014.1)	100

(Table 2). Figure 1 shows the original gel from which the bands were excised together with band numbers. The sequence obtained from band 33 was of low quality and so not considered for phylogenetic analysis.

In Table 2 the closest relatives of DGGE band sequences is presented. In some cases retrieved sequences shared the same value of similarity with several closely related micro-organisms and in such cases only one example of each close relative is presented.

Most of the sequences were similar to 16S rRNA gene sequences reported for cultured bacteria present in food samples such as meat products, packaged fish, spoiled food, fermented vegetables and cheese (Table 2). However, the DNA sequence of some bands affiliated to sequences with their origins in bacteria initially isolated from other sources such as soil, marine sediments, human blood, phyllosphere, animal gut, faeces and ice. Six bands (labelled in Fig. 1 as 6, 16, 21, 23, 31, 32 and 36) affiliated with sequences corresponding to bacteria classified as 'uncultured' in the BLAST database. In the case of DNA sequences from bands 6 and 32, these could not be clearly affiliated to any phylogenetic group, displaying similarity

with 16S rRNA gene sequences from uncultured bacterial clones.

According to Blast results sequences affiliated with LAB, included in order *Lactobacillales*, with micro-organisms included in order *Bacillales* and with *Gammaproteobacteria*. Most of the profiles displayed clearly dominant bands (Fig. 1). Bands affiliated with *Lactobacillus* were found in samples from producers Ef, Ag and Tp; bands affiliated with *Leuconostoc* were found in producers Ef, Am and Tx (Band 3); *Enterococcus* were found only in samples form producer Tx; samples from producer Am have an intense band affiliated with *Firmicutes* (Band 21); samples form producer Tx have intense bands affiliated with *Pediococcus* and *Lactococcus* (Band 13 and 15, Fig. 1); band associated with *Psychrobacter* is intense in producer Gr. Band 2 was detected at the same position in all the six producers. To confirm that matching positions correspond to identical phylotypes, Band 2 was excised and sequenced from all samples. The same nucleotide sequence was obtained for each band. This band position was detected in most of the profiles (exceptions were samples Am3a and Am3b), although it was more

pronounced in samples Gr2a, Gr2b, Tp2a and Tp2b. The sequence from this band was 100% identical to *Brochothrix thermosphacta*, isolated from modified-atmosphere-packed salmon and coalfish. In addition, Bands 7 and 15 were retrieved from different gels as presented in Fig. 1 and confirmed as identical.

Discussion

Studies on the micro-ecology of fermented sausages date back to the 1970s (Lücke 1974). These earlier studies, based on traditional methods, described the microbial populations developing during production and storage. In the last few years, the possibility of using culture-independent molecular approaches has opened up areas of research. In this study we used PCR-DGGE analyses in combination with traditional microbiological analyses for studying the microbial diversity of 'Alheira'. Microbial DNA was sampled directly in order to determine the diversity of the 16S rRNA genes of the most prominent bacteria, which may also reflect the microflora of 'Alheira'.

The most salient observation from Fig. 1 concerns the variability in the microecology between samples from different producers, which is considerable, and that between lots from the same producer which is evident but lesser. Variability between producers is not, in itself, surprising; production methods, recipes and raw materials vary themselves. Other factors probably contributing to the detected inter-producer variability include the manner in which they were stored and distributed ('Alheiras' are sold loose or packaged – vacuum or modified atmosphere) and indeed the time elapsed since production – not all samples were labelled with shelf life information. Indeed, as a largely artisanal production process which is not initiated by starter cultures, these same factors are likely to be responsible for the intra-producer variations. The variations between batches from the same producer are less pronounced which suggests some stability of the microecology although variations in batches of raw materials, process parameters and plant hygiene are likely to contribute some variation.

When the results obtained from both traditional plating and DGGE are analysed (Tables 1 and 2), it becomes evident that LAB constitute the predominant microbial population of 'Alheira'. In DGGE gels, multiple bands were visible in most of the samples, and different phylotypes, most of which related to the order *Lactobacillales*, were identified. In a previous, culture-based study, fourteen species of LAB were isolated from 'Alheira' from MRS and M17 media, such us: *Lact. paraplantarum*, *Lact. brevis*, *Lact. rhamnosus*, *Lact. sakei*, *Leuc. mesenteroides*, *Lact. zeae*, *Lact. paracasei*, *Ped. pentosaceus*, *Ped. acidilactici*, *Weissella cibaria*, *W. viridescens* and *Ent. faecium*

(Albano *et al.* 2007). Albano *et al.* (2007) also observed that *Lact. plantarum* and members of the genus *Enterococcus* were isolated from all samples of 'Alheira' analysed. *Enterococcus*-related phylotypes were identified also in PCR-DGGE sequences.

In addition to LAB, *Micrococcaceae* counts were >4·2 log CFU g^{-1} in most samples. Intriguingly, although the separation of the V1 and V3 rRNA gene amplicons by PCR-DGGE has been described as a reliable tool to investigate the LAB and CNC diversity in fermented sausages (Cocolin *et al.* 2001a,b), only one band was identified as being part of this latter microbial group (*Macrococcus caseolyticus*, Band 5) in the denaturing gels generated in this present study. This finding might be due to the selective amplification of the most abundant templates, a bias previously recognized when performing PCR-DGGE analysis of heterogeneous DNA template mixtures (Felske *et al.* 1998; Ampe *et al.* 1999; Ercolini *et al.* 2001b).

According to the guidelines for the microbiological quality of fermented meats published by Gilbert *et al.* (2000), most of the samples tested (80%) would be considered unsatisfactory, with *Enterobacteriaceae* counts greater than 4 log CFU g^{-1}. The detection of *Listeria* spp. in some samples points to the possibility of product contamination with the pathogenic specie *L. monocytogenes*. Sequences closely related to *Enterobacteriaceae* were retrieved from DGGE bands. *Brochothrix*-related phylotypes were also detected in almost all the samples, as were close relatives of *Pseudomonas*; in both cases suggesting the presence of potential spoilage strains. Quantitative approaches, such as those using real-time PCR would be needed in order to quantify the presence of specific potentially problematic bacteria. As shown by Rantsiou and Cocolin (2006), the combination of cultivation strategies and DNA-based techniques allows the identification of the dominant microbial species in fermented sausages, since it permits the generation and comparison of characteristic patterns from cultivated isolates and complex microbial communities. However, Ercolini (2004) described possible pitfalls of the whole procedure when applied to food products, like sampling, DNA extraction, DNA purity, PCR conditions, formation of heteroduplex and chimeric molecules, small fragments, between others.

PCR-DGGE applied to template DNA, directly extracted from a food matrix, generates a specific profile of that product in that moment, given the conditions used. DGGE analysis and sequencing of the resultant bands allows the identification of the dominant phylotypes present in all products. The approach explored in this study allowed the description of the microbial community present in 'Alheira' in particular the diversity of LAB. DGGE could be used in monitoring the growth kinetics during the ripening process and the effects of

these on the hygienic quality, allowing tracking of the microbial 'typicity' of the 'Alheira de Mirandela', and collecting of important information for the designing of autochthonous starter cultures.

Acknowledgements

References

Albano, H., van Reenen, C.A., Todorov, S.D., Hogg, T., Dicks, L.M.T. and Teixeira, P. (2007) Ecology of lactic acid bacteria in ''Alheira'', a fermented sausage traditionally produced in Portugal: isolation, identification and molecular characterization. *Personal communication in MICRO'07 – BIOTEC'07-XXXIII*, Lisbon, Portugal, 30 November, 2007–2 December, 2007.

Ampe, F., ben Omar, N., Moizan, C., Wacher, C. and Guyot, J.-P. (1999) Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation independent methods to investigate traditional fermentations. *Appl Environ Microbiol* 65, 5464–5473.

Aquilanti, L., Santarelli, S., Silvestri, G., Osimani, A., Petruzzelli, A. and Clementi, F. (2007) The microbial ecology of a typical Italian salami during its natural fermentation. *Int J Food Microbiol* 120, 136–145.

van Beek, S. and Priest, F.G. (2002) Evolution of the lactic acid bacterial community during malt whisky fermentation: a polyphasic study. *Appl Environ Microbiol* 68, 297–305.

Cocolin, L., Manzano, M., Cantoni, C. and Comi, G. (2000) Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis. *Lett Appl Microbiol* 30, 126–129.

Cocolin, L., Manzano, M., Cantoni, C. and Comi, G. (2001a) Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Appl Environ Microbiol* 67, 5113–5121.

Cocolin, L., Manzano, M., Aggio, D., Cantoni, C. and Comi, G. (2001b) A novel polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) for the identifcation of *Micrococcaceae* strains involved in meat fermentations. Its application to naturally fermented Italian sausages. *Meat Sci* 57, 59–64.

Cocolin, L., Aggio, D., Manzano, M., Cantoni, C. and Comi, G. (2002a) An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *Int Dairy J* 12, 407–411.

Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C. and Comi, G. (2002b) Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. *Appl Environ Microbiol* 68, 6273–6282.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C. and Comi, G. (2004) Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. *Appl Environ Microbiol* 70, 1883–1894.

Coppola, S., Blaiotta, G., Ercolini, D. and Moschetti, G. (2001) Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese. *J Appl Microbiol* 90, 414–420.

Ercolini, D. (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J Microbiol Methods* 56, 297–314.

Ercolini, D., Moschetti, G., Blaiotta, G. and Coppola, S. (2001a) Behavior of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. *Curr Microbiol* 42, 199–202.

Ercolini, D., Moschetti, G., Blaiotta, G. and Coppola, S. (2001b) The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: bias of ''culture dependent'' and ''culture independent'' approaches. *Syst Appl Microbiol* 24, 610–617.

Ercolini, D., Hill, P.J. and Dodd, C.E.R. (2003) Bacterial community structure and location in Stilton cheese. *Appl Environ Microbiol* 69, 3540–3548.

Esteves, A., Patarata, L., Saraiva, C. and Martins, C. (2008) Assessment of the microbiological characteristics of industrially produced Alheira, with particular reference to food-born pathogens. *J Food Safety* 28, 88–102.

Felske, A., Akkermans, A.D. and de Vos, W.M. (1998) Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. *Appl Environ Microbiol* 64, 4581–4587.

Ferreira, V., Barbosa, J., Vendeiro, S., Mota, A., Silva, F., Monteiro, M.J., Hogg, T., Gibbs, P. *et al.* (2006) Chemical and microbiological characterization of alheira: a typical Portuguese fermented sausage with particular reference to factors relating to food safety. *Meat Sci* 73, 570–575.

Fontana, C., Vignolo, G. and Cocconcelli, P.S. (2005) PCR–DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. *J Microbiol Methods* 63, 254–263.

Gilbert, R.J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C.D., Richards, J. and Roberts, D. (2000) Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. PHLS advisory

committee for food and dairy products. *Communicable Dis Public Health* **3**, 163–167.

Head, I.M., Saunders, J.R. and Pickup, R.W. (1998) Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbiol Ecol* **35**, 1–21.

Henriques, I.S., Almeida, A., Cunha, A. and Correia, A. (2004) Molecular sequence analysis of prokaryotic diversity in the middle and outer sections of the Portuguese estuary Ria de Aveiro. *FEMS Microbiol Ecol* **49**, 269–279.

Henriques, I.S., Alves, A., Tacão, M., Almeida, A., Cunha, A. and Correia, A. (2006) Seasonal and spatial variability of free-living bacterial community composition along an estuarine gradient (Ria de Aveiro, Portugal). *Estuar Coast Shelf Sci* **68**, 139–148.

Hugenholtz, P., Goebel, B.M. and Pace, N.R. (1998) Impact of culture-independent studies on emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**, 4765–4774.

IPQ (1991) *Food Microbiology. General Guidance for the Enumeration of Enterobacteriacea Without Ressuscitation. MPN Technique and Colony Count Technique*. Portuguese Standard NP 4137.

ISO (2003) *Microbioloy of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Microorganisms – Colony-Count Technique at 30°C*. International Standard 4833:2003E.

Lücke, F.K. (1974) Fermented sausages. In *Microbiology of Fermented Foods* ed. Wood, B.J.B. pp. 41–49 London: Applied Science Publishers.

Meroth, C.B., Walter, J., Hertel, C., Brandt, M.J. and Hammes, W.P. (2003) Monitoring the bacterial population dynamics in sourdough fermentation process by using PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol* **69**, 475–482.

Muyzer, G., De Waal, E.D. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.

Ogier, J.C., Son, O., Gruss, A., Tailliez, P. and Delacroix-Buchet, A. (2002) Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl Environ Microbiol* **68**, 3691–3701.

ben Omar, N. and Ampe, F. (2000) Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl Environ Microbiol* **66**, 3664–3673.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M. and Vaughan, E.E. (2002) Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. *Appl Environ Microbiol* **68**, 1882–1892.

Rantsiou, K. and Cocolin, L. (2006) New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. *Int J Food Microbiol* **108**, 255–267.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G. and Cocolin, L. (2005) Culture-dependent and -independent methods to investigate the microbial ecology of Italian fermented sausages. *Appl Environ Microbiol* **71**, 1977–1986.