Phenotypic and genetic heterogeneity of lactic acid bacteria isolated from "Alheira", a traditional fermented sausage produced in Portugal

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A B S T R A C T

The aim of this study was to evaluate the phenotypic and genetic heterogeneity of lactic acid bacteria (LAB) isolated from "Alheira", a fermented sausage produced in Portugal.

LAB were identified to genus and species level by phenotypic characteristics, using genus or species-specific primers and sequencing of the gene encoding 16S rRNA. Two-hundred and eighty-three isolates were grouped into 14 species. *Lactobacillus plantarum* was isolated from all sausages and *Enterococcus faecalis* from most of the samples. Low numbers of *Lactobacillus paraplantarum*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus zeae*, *Lactobacillus paracasei*, *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, *Weissella cibaria*, *Weissella viridescens* and *Enterococcus faecium* were recorded. The genetic heterogeneity of *L. plantarum* and *E. faecalis* strains were determined by numerical analysis of DNA banding patterns obtained by RAPD–PCR. Strains of *L. plantarum* and *E. faecalis* were different from different producers. This study forms the basis from which starter cultures could be selected for production of "Alheira".

Introduction

"Alheira" is a fermented sausage typical of the Northern regions in Portugal (Trás-os-Montes). The product is produced by boiling meat (pork and/or poultry) in lightly salted and spiced water. Slices of bread are then added to a level of approximately 25% of the total raw material. Fine cut portions of meat, spices and olive oil or fat is then added. The paste is stuffed into cellulose-based casings and allowed to ferment naturally (i.e. without the addition of starter cultures), during a smoking process. Additional salt and spices are added according to taste, the sausages are bent into a horse-shoe shape (approximately 15 cm long and 6 cm in diameter) and smoked for a maximum of eight days at temperatures below 37 °C at uncontrolled humidity. The shelf life of "Alheiras" is about 1 month stored at 4 °C in air or longer if the sausages are packed under modified atmosphere. "Alheiras" are cooked before consumption; either by frying, grilling or boiling. The taste is described as being pleasant, lightly smoked, very particular, with a hint of garlic. The lightly smoked aroma is described as *sui generis*.

Although the aroma and flavour characteristics of "Alheira" and other fermented meat sausages are mainly influenced by the quality and origin of the raw materials and the ripening process, the composition of the microflora, especially lactic acid bacteria (LAB), plays a key role (Moretti et al., 2004; Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2003). *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* are the dominant species in fermented meat sausages, but seldom reach numbers in excess of 10^7 cfu/g (Talon, Leroy, & Lebert, 2007). Apart from the production of lactic acid and antimicrobial compounds such as bacteriocins (Bacus, 1986), LAB produce a number of other antimicrobial and organoleptic compounds, e.g. acetic acid, ethanol, acetoin, carbon dioxide and pyruvic acid (Bacus, 1986).

In general, sausages that undergo a short fermentation, without starter cultures, have a higher pH and thus also a larger population of *Enterococcus* spp. (Dellapina, Blanco, Pancini, Barbuti, & Campanini, 1994). The proportion of enterococci versus other LAB in fermented dry sausages is thus important. However, only a few papers have been published on the microbial composition of "Alheira" (Ferreira, Barbosa, Vendeiro et al., 2006; Ferreira, Barbosa, Silva et al., 2007) and even less is known about the role of enterococci in "Alheira".

The use of selected starter cultures is important to produce the desired flavour and aroma compounds and extend the shelf life of the product (Bacus, 1986). In most studies, LAB in fermented sausages have been identified based on simple physiological, biochemical and chemotaxonomic methods (Montel, Talon,

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Table 1
Species- and genus-specific primers used in PCR reactions (* = genus-pecific primers).

Species	Primer pair/sequence (5′–3′)	20 μl reaction[a]	PCR-cycle	Origin (product size)	Reference strain used as positive control	Reference
L. plantarum	planF (F) CCG TTT ATG CGG AAC ACC TA REV (R) TCG GGA TTA CCA AAC ATC AC	5.0 μl DNA, 0.25 μl PlanF, 0.5 μl REV, 2.0 μl buffer 10x, 1.6 μl dNTPs, 0.1 μl Taq	[b]94 °C/ 30 s 56 °C/10 s 72 °C/30 s 30 cycles	*rec*A gene (318 bp)	*Lb. plantarum* ATCC 14917[T]	Torriani, Felis, and Dellaglio (2001)
L. paraplantarum	ParaF (F) GTC ACA GGC ATT ACG AAA AC REV (R) TCG GGA TTA CCA AAC ATC AC	5.0 μl DNA, 0.5 μl ParaF, 0.5 μl REV, 2.0 μl buffer 10x, 1.6 μl dNTPs, 0.1 μl Taq	[b]94 °C/ 30 s 56 °C/10 s 72 °C/30 s 30 cycles	*rec*A gene (107 bp)	*Lb. paraplantarum* ATCC 700211[T]	Torriani et al. (2001)
L. rhamnosus	*Y2 (F) CCC ACT GCT GCC TCC CGT AGG AGT* *Rham (R) TGC ATC TTG ATT TAA TTT TG*	5.0 μl DNA, 2 μl Y2, 2 μl Rham, 2.0 μl buffer 10x, 1.6 μl dNTPs, 0.1 μl Taq	[b]94 °C/ 45 s 48 °C/45 s 72 °C/ 1 min 30 cycles	16S rRNA gene: V1 region (290 bp)	*Lb. rhamnosus* ATCC 7469[T]	Ward and Timmins (1999)
L. sakei	16 (F) GCT GGA TCA CCT CCT TTC Ls (R) ATG AAA CTA TTA AAT TGG TAC	5.0 μl DNA, 0.6 μl 16, 0.6 μl Ls, 2 μl $MgCl_2$, 2.0 μl buffer 10x, 2 μl dNTPs, 0.1 μl Taq	[c]94 °C/ 1 min 47 °C/30 s 72 °C/ 1 min 35 cycles	16S rRNA gene (222 bp)	*Lb. sakei* DSMZ 20117	Berthier and Ehrlich (1998)
L. zeae	LCZ (F) TTG GTC GAT GAA C *LBL R1 (R) CCA TGC ACC ACC TGT C*	5.0 μl DNA, 2 μl LCZ, 2 μl LBLR1, 2.0 μl buffer 10x, 1.6 μl dNTPs, 0.1 μl Taq	[c]94 °C/ 30 s 40 °C/ 30 s 72 °C/ 1 min 35 cycles	16S rRNA gene (985 bp)		Roy, Sirois, and Vincent (2001)
L. brevis	*Br1 (F) CTT GCA CTG ATT TTA ACA* *Br2 (R) GGG CGG TGT GTA CAA GGC*	5.0 μl DNA, 0.4 μl Br1, 0.4 μl Br2, 2.0 μl buffer 10x, 1.6 μl dNTPs, 0.1 μl Taq	[c]94 °C/ 1 min 52 °C/30 s 72 °C/ 1 min 25 cycles	16S rRNA gene (1340 bp)	*Lb. brevis* ATCC 8287	Guarneri, Rossetti, and Giraffa (2001)
Ln. mesenteroides	Lmes-f (F) AAC TTA GTG TCG CAT GAC Lmes-r (R) AGT CGA GTT ACA GAC TAC AA	5.0 μl DNA, 1 μl Lmes-f, 1 μl Lmes-r, 4 μl $MgCl_2$, 2.0 μl buffer 10x, 4 μl dNTPs, 0.1 μl Taq	[d]94 °C/ 1 min 57 °C/ 1 min 72 °C/ 2 min 30 cycles	16S rRNA gene (1150 bp)	*Lb. mesenteroides* subsp. *mesenteroides* NCDO 523[T]	Lee, Park, and Kim (2000)

Enterococcus spp.*	EntF (F) TACTGACAAACCATTCATGATG EntR (R) AACTTCGTCACCAACGCGAAC	5.0 µl DNA, 0.4 µl EntF, 0.4 µl EntR, 2 µl $MgCl_2$, 2.0 µl buffer 10x, 1.6 µl dNTPs, 0.1 µl Taq	[e]94 °C/ 30 s 55 °C/30 s 72 °C/30 s 35 cycles	16S rRNA gene (112 bp)	*Ec. faecalis* ATCC 29212	Ke et al. (1999)
E. faecalis	Ef0027R (F) GCCACTATTTCTCGGACAGC Ef0027F (R) GTCGTCCCTTTGGCAAAT	4.0 µl DNA, 2 µl Ef0027F, 2 µl Ef0027R, 4 µl $MgCl_2$, 2.0 µl buffer 10x, 1.6 µl dNTPs, 0.2 µl Taq	[e]94 °C/ 30 s 55 °C/30 s 72 °C/30 s 35 cycles	Putative transcriptional regulator gene (518 bp)	*Ec. faecalis* ATCC 29212	Dutka-Malen, Evers, and Courvalin (1995)
E. faecium	Enf1 (F) ATTACGGAGACTACAATTTG Ent2 (R) TAGCGATAGAAGTTACATCAAG	4.0 µl DNA, 0.4 µl Enf1, 0.4 µl Ent2, 4 µl $MgCl_2$, 2.0 µl buffer 10x, 1.6 µl dNTPs, 0.2 µl Taq	[e]94 °C/ 30 s 55 °C/30 s 72 °C/30 s 35 cycles	16S rRNA gene (300 and 400 bp)	*Ec. faecium* LMG 8149	Dutka-Malen et al. (1995)
P. acidilactici	PacF (F) CGAACTTCCGTTAATTGATTAT PuR (R) ACCTTGCGGTCGTACTCC	5.0 µl DNA, 2 µl PacF, 2 µl PuR, 4 µl $MgCl_2$, 2.0 µl buffer 10x, 1.6 µl dNTPs, 0.1 µl Taq	[e]94 °C/ 30 s 67 °C/30 s 72 °C/ 1 min 35 cycles	16S rRNA gene (872 bp)		Mora, Fortina, Parini, and Manachini (1997)
P. pentosaceus	PpeF (F) CGA ACT TCC GTT AAT TGA TCA G PuR (R) ACC TTG CGG TCG TAC TCC	5.0 µl DNA, 2 µl PpeF, 2 µl PuR, 4 µl $MgCl_2$, 2.0 µl buffer 10x, 1.6 µl dNTPs, 0.1 µl Taq	[e]94 °C/ 30 s 67 °C/30 s 72 °C/ 1 min 35 cycles	16S rRNA gene (872 bp)	*Ped. pentosaceus* NCDO 813[T]	Mora et al. (1997)
Weissella spp.*	WeiF (F) CGT GGG AAA CCT ACC TCT TA WeiR (R) CCC TCA AAC ATC TAG CAC	5.0 µl DNA, 2 µl WeiF, 2 µl WeiR, 0.1 µl $MgCl_2$, 2.0 µl buffer 10x, 1.6 µl dNTPs, 0.1 µl Taq	[e]94 °C/ 30 s 55 °C/30 s 72 °C/30 s 35 cycles	16S rRNA gene (725 bp)	*W. hellenica* ATCC 51523[T]	Jang et al. (2002)

[a] 10 mM each primer (MWG Biotech AG, Ebersberg, Germany); 5 mM $MgCl_2$, 2.5 mM dNTPs, Taq DNA polymerase (TaKaRa Ex TaqTM, TAKARA, BIO INC. Japan).
[b] Initial denaturation at 95 °C for 3 min and final extension of the amplified product at 72 °C for 5 min.
[c] Initial denaturation at 95 °C for 5 min and final extension of the amplified product at 72 °C for 5 min.
[d] Initial denaturation at 95 °C for 5 min and final extension of the amplified product at 72 °C for 10 min.
[e] Initial denaturation at 95 °C for 5 min and final extension of the amplified product at 72 °C for 5 min.

Fournaud, & Champommier, 1991). Although valuable from a practical point of view, results obtained by these methods are not always sufficient to characterize strains to species level, especially within the genera *Lactobacillus* (Ammor et al., 2005; Aquilanti, Zannini, Zocchetti, Osimani, & Clementi, 2007) and *Enterococcus* (Velasco et al., 2004). SDS–PAGE of whole cell proteins (Samelis, Tsakalidou, Metaxopoulos, & Kalantzpopulos, 1995), restriction fragment length polymorphism (RFLP) of 16S rRNA (Sanz, Selgas, Parejo, & Ordrhez, 1998), hybridization with rRNA probes (Nissen & Dainty, 1995), PCR with species-specific primers (Yost & Nattress, 2000), temperature gradient gel electrophoresis (TGGE) of PCR products (Cocolin, Manzano, Cantoni, & Comi, 2000), denaturing gradient gel electrophoresis (DGGE) (Cocolin, Manzano, Cantoni, & Comi, 2001) and randomly amplified polymorphic DNA (RAPD)–PCR analysis (Berthier & Ehrlich, 1999), have been reported to be more accurate.

In this paper, the phenotypic and genotypic diversity of LAB isolated from "Alheira" produced in seven different processing plants was studied.

Material and methods

Identification of lacticacid bacteria

Origin and sampling of isolates

"Alheiras", unwrapped, sealed in modified atmosphere packages or vacuum-packed, were collected from different retail stores. Samples collected from seven production plants in North-East Portugal were labelled Ef, Tp, Tx, Gr, Ag, Am and PV.

Twenty-five grams of each sample were added to 225 ml of sterile buffered peptone water (Merck, Darmstadt, Germany), homogenized in a stomacher for 2 min and serial dilutions were plated onto De Man, Rogosa Sharpe (MRS) Agar (LabM, Bury, UK) and M17 agar (Merck). Plates were incubated under microaerophilic conditions for 72 h at 30 °C. Colonies were randomly selected, based on colony morphology, from plates having between 15 and 150 colonies and cultured in MRS or M17 broth for 48 h at 30 °C. Isolates were purified by repeated streaking onto the respective growth media. All isolates were tested for Gram reaction, oxidase and catalase production. Gram-positive, catalase-negative and oxidase-negative isolates were selected and stored at −80 °C in growth medium, supplemented with glycerol (30%, v/v, final concentration).

Phenotypic and biochemical tests

Isolates were sub-cultured twice in MRS or M17 broth at 30 °C for 24 h before cell morphology was observed with an optical light microscope. All isolates were tested for CO_2 production from glucose in MRS broth adjusted to pH 7.0, fitted with Durham tubes (Müller, 1990). Incubation was for 48 h at 30 °C. Isolates were considered heterofermentative if gas had been formed. Growth at 10 °C and 45 °C was tested by incubating the isolates in appropriated media (pH 7.0) for 7 and 2 days, respectively. Growth was recorded by an increase in turbidity.

Acid production from D-glucose, D-fructose, galactose, sucrose, lactose, maltose, mannitol, rhamnose, ribose, trehalose and D-xylose was determined using microtitre plates (Parente, Griego, & Crudele, 2001). Filter-sterilized sugar (1 ml of a 100 g/l solution) was added to 9 ml basal medium (MRS without glucose and meat extract and with 0.16 g/l bromocresol purple, pH 7.0) and 180 μl dispensed into each well. Cells harvested from 16-h-old broth cultures (6000*g*, 5 min, 20 °C) were suspended in sterile saline and 20 μl inoculated into each well. The microtitre plates were incubated under microaerophilic conditions at 30 °C for 48 h. A colour change from purple to yellow was regarded as a positive reaction. Hydrolysis of esculin was tested by adding 2 g/l esculin (Sigma Diagnostics, St. Louis, MO, USA) and 5 g/l ferric ammonium citrate (Sigma) to the basal medium. Incubation was as described before. A black colouration of the medium was regarded as a positive reaction. Growth in the presence of 4% and 6.5% (w/v) NaCl, and at pH 4.0 and pH 9.6 was determined in MRS broth, adjusted with 1 N HCl or NaOH before autoclaving and supplemented with bromocresol purple. Microtitre plates were inoculated as described before. A colour change to yellow was recorded as growth. Arginine hydrolysis was tested by inoculating the isolates into MRS broth, supplemented with arginine (3 g/l) and ammonium citrate replaced by sodium citrate. Incubation was at 30 °C for 4 days, as described before. Production of ammonia was detected by using Nessleŕs reagent (Carlo Erba, Rodano, MI, Italy). All tests were performed in triplicate.

Genotypic tests

DNA isolation : Two DNA extraction methods were used. Isolates collected from M17 agar were grown to mid-log phase in M17 broth (to an optical density of 1.4 at 600 nm), harvested (8000*g*, 5 min, 4 °C), rinsed twice with sterile saline (0.9%, w/v, NaCl), suspended in 50 μl sterile distilled water and boiled for 15 min. The DNA of isolates collected from MRS plates was extracted with phenol–chloroform, as described by Dellaglio, Bottazzi, and Trovatelli (1973). Both sets of DNA extracts were frozen at −20 °C.

PCR withs pecies-and genus-specific primers and 16Sr RNA gene sequencing. Isolates within each group were identified to genus and species level by using genus or species-specific primers. Representative strains were selected and the gene encoding 16S rRNA sequenced. The DNA primers and PCR conditions used are

Table 2
Physiological and biochemical tests used to classify lactic acid bacteria isolated from "Alheira". The figures refer to the number of strains positive in each test.

Morphology	CO_2 from Glucose	Growth at 10 °C	Growth at 45 °C	Growth in the presence of 4% NaCl	Growth in the presence of 6.5% NaCl	Growth at pH 4.4	Growth at pH 9.6	NH_3 from Arginine	Esculin Hydrolysis	Preliminary classification[a]	Group
Rods	0	90	85	87	69	90	0	3	90	Homofermentative or facultatively Heterofermentative lactobacilli	**G1 (90)**[b]
Rods	21	21	0	16	6	21	0	16	0	Obligately heterofermentative lactobacilli or *Weissella* spp.	**G2 (21)**
Cocci	0	159	159	159	159	159	159	104	120	*Enterococcus* spp.	**G3 (159)**
Coccobacilli	4	4	0	3	2	2	0	1	0	*Leuconostoc* spp.	**G4 (4)**
Cocci in tetrads	0	9	6	9	4	9	3	6	9	*Pedioccocus* spp.	**G5 (9)**

[a] Preliminary classification was based on Bergey's Manual of Determinative Bacteriology (Holt, Krieg, Sneath, Staley, & Williams, 1994) and "The Prokaryotes" (Ballows, Trüper, Dworkin, Harder, & Schleifer, 1992).
[b] Total number of strains isolated.

Table 3
Sugar fermentation reactions recorded for lactic acid bacteria isolated from "Alheira". The numbers in superscript refer to the number of negative strains in each test.

Group	Biochemical profile	Esculin hydrolysis	Fructose	Galactose	Glucose	Lactose	Maltose	Mannitol	Rhamnose	Ribose	Sorbitol	Sucrose	Trehalose	Xylose	NH_3 from arginine	Phenotypic identification[a]	Genotypic identification[b]
G1	**(90)**	G1.1. (76)	+	+	+	+	+	+[1]	+	–	+	+	+	+	–	–	*L. plantarum; L paraplantarum; L. rhamnosus; L. paracasei; L. pentosus*
	L. plantarum (70); *L. rhamnosus* (3); *L.*															*paraplantarum* (1); *L. paracasei* (2)	
	G1.2. (4)	+	–	+[1]	+	–	+	+ [1]	–	+	+	+	+	–	–	*L. plantarum; L. paraplantarum; L. rhamnosus; L. paracasei; L. pentosus*	*L. paraplantarum* (2); *L. plantarum* (1); *L. paracasei* (1)
	G1.3. (4)	+	+	–	+	+	+	+	–	+	+	+	+	–	–	*L. plantarum; L. paraplantarum; L. rhamnosus; L. paracasei; L. pentosus*	*L. rhamnosus* (3); *L. paracasei* (1)
	G1.4. (3)	+	nd	+	+	+	–	+	–	+	–	+	+	–	–	*L. plantarum*	*L. zeae* (2); *L. plantarum* (1)
G1.5. (3)	+	+	+	+	+	+	–	+	+	–	+	+	–	+	*L. sakei*	*L. sakei* (3)	
G2	**(21)** *L. brevis* (7); *L. plantarum* (1)	G2.1. (8)	–	+	+[2]	+	+	+[1]	–	–	+	–	+	+	+	+	*L. brevis*
	G2.2. (11)	–	nd	+	+	+	+	–	nd	–	–	+	–	–	+	*Weissella* spp.	*Weissella* spp. (6); *W. cibaria* (3); *W. viridescens* (1); *L. mesenteroides* (1)
G2.3. (2)	+	+	+	+	+	+	+	–	+	+	+	+	–	–	Not	determined	*L. plantarum* (2)
G3	**(159)**	G3.1. (101)	+	+[3]	+	+	+/–	+	+	+/–	+	+	+[2]	+	–	–	*Enterococcus* spp.
																Enterococcus spp. (32); *E. faecalis* (43); *E. faecium* (26)	
	G3.2. (44)	+	+	+[2]	+	+	+	+	+/–	+	+	+	+	–	–	*E. faecalis*	*E. faecalis* (44)
G3.3.	(14)	+	+	+	+	+/–	+	+	–	+	–	+	+	–	+	*E. faecium*	*E. faecium* (12); *P. pentosaceus* (2)
G4 (4)	G4. (4)	+	+	+	+	–	+	–	–	+	–	+	+	+	–	*Ln. mesenteroides*	*Ln. mesenteroides* (3); *P. pentosaceus* (1)
G5 (9)	G5. (9)	+	+	+	+	+	+[3]			+	–	–	+	+	+	*Pediococcus* spp.	*P. acidilactici* (3); *P. pentosaceus* (6)

nd, not determined.
[a] Preliminary classification, based on Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and "The Prokaryotes" (Ballows et al., 1992).
[b] Using genus or species-specific primers, and 16S rRNA gene sequencing to confirm results.

listed in Table 1. Samples without genomic DNA were used as negative controls. Amplified fragments were separated on agarose gels at a constant 100 V. Tris–acetate (TAE) was used as buffer. Gels were stained with 0.5 μg/ml ethidium bromide (Sigma). A 100-bp DNA ladder (BioRad Laboratories, Richmond, CA) was used as molecular weight marker when the expected product size was less than 400 bp; otherwise Lambda DNA, digested with *Eco*R1 and *Hin*dIII (Roche, Indianapolis, USA) was used.

To confirm results, representative strains within each group were subjected to 16S rRNA gene sequencing, as described by Felske, Rheims, Wolterink, Stackebrandt, and Akkermans (1997). The following primers were used: 8 F (5′-CAC GGA TCC AGA CTT TGA TYM TGG CTC AG-3′) (Y = C + T; M = A + C) and 1512 R (5′-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3′). PCR products were purified with the GFX PCR DNA and Band Purification kit (GE HealthCare, Amersham Biosciences, Amersham, UK) and used as templates. The ABI PRISMs BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA) was used. Sequences obtained from an automatic DNA sequencer (ABI PRISMs 310 Genetic Analyser, PE Applied Biosystems) were aligned with sequences in Genbank using the BLAST program (Altschul et al., 1997).

Random amplified polymorphic DNA (RAPD) analyses : Strains identified as members of the genera *Enterococcus* and *Lactobacillus* were selected for further studies. The genetic heterogeneity of isolates that grouped within the most prevalent species was determined by numerical analysis of DNA profiles obtained by RAPD–PCR.

DNA primers M13 (5′-GAG GGT GGC GGT TCT-3′) and D8635 (5′-GAG CGG CCA AAG GGA GCA GAC-3′) of Huey and Hall (1989) were used. RAPD–PCR was performed on total (genomic and plasmid) DNA, as described by Andrighetto, Zampese, and Lombardi (2001). The 25 μl reaction volume contained 0.99 mM primer M13, 1 × PCR buffer (MBI Fermentas, Mundolsheim, France), 2.5 mM $MgCl_2$ (MBI Fermentas), 0.15 mM dNTP (Abgene, Surrey, UK) and 1 U Taq DNA polymerase (MBI Fermentas). The second amplification contained 0.88 mM primer D8635, 1 × PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP and 1 U Taq DNA polymerase. Amplification was in a DNA thermal cycler (My Cycler™ Thermal Cycler Firmware, BioRad Laboratories) by using the following program: Initial denaturation at 94 °C for 2 min, 35 cycles of 1 min per cycle at 94 °C, and 1 min at 46.9 °C, followed by an increase to 72 °C over 90 s. Extension of the amplified product was at 72 °C for 10 min. Amplified products were separated by electrophoresis in 1.2% (w/v) agarose gels in 1 × TAE buffer at 80 V for 2 h. Gels were stained in TAE buffer containing 0.5 μg/ml ethidium bromide. A 100-bp DNA ladder (BioRad Laboratories) was used as molecular weight marker.

Banding patterns were analysed using Gel Compare, Version 4.1 (Applied Maths, Kortrijk, Belgium). Similarities were calculated by using the Dice product–moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin & Vauterin, 1992).

Results and discussion

Based on a few key biochemical characteristics, 283 isolates were separated into five phenotypic groups (Table 2). All isolates in groups G1 and G2 were rod-shaped. Because none of the isolates in group G1 produced CO_2 from the fermentation of glucose, they were tentatively classified as homofermentative (group I *Lactobacillus*) and facultatively heterofermentative (group II *Lactobacillus*). Most of the isolates (85 out of 90) grew at 45 °C, suggesting that

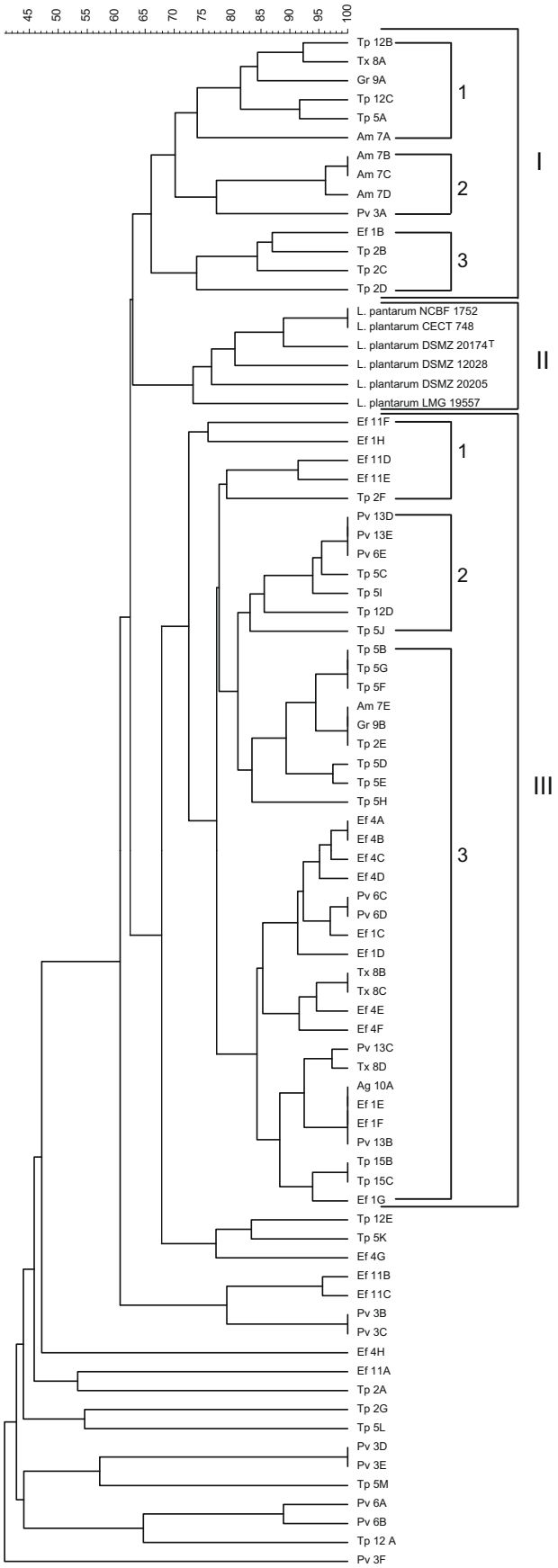

Fig. 1. Numerical analysis of RAPD–PCR profiles obtained for strains of *L. plantarum* isolated from "Alheira" manufactured in seven different plants. Clusters are indicated with Roman numerals. Ef, Tp, Am, Gr, Tx, Ag and PV are the producers.

they belong to the group I – *Lactobacillus*. Five isolates did not grow at 45 °C and were tentatively classified as members of the group II – *Lactobacillus*. Isolates from group G2 produced CO_2 from the fermentation of glucose and were classified as members of group III – *Lactobacillus* or *Weissella*. Isolates collected from M17 medium were coccoid, grew at pH 9.6 and in the presence of 6.5% NaCl (Table 2), and were tentatively classified as *Enterococcus* spp. (group G3, Table 2). Four isolates had a coccobacillus (oval) shape and were tentatively classified as members of the genus *Leuconostoc* (group G4, Table 2). Nine isolates were cocci arranged in tetrads and were tentatively classified as *Pediococcus* spp. (group G5, Table 2).

Sugar fermentation reactions separated the 90 isolates in group G1 into five subgroups (G1.1–G1.5, Table 3). Most of these isolates (84) shared the same sugar fermentation reactions and were classified as *L. plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei* and *Lactobacillus pentosus* (Table 3). Small differences in sugar fermentation reactions separated the strains into subgroups G1.1, G1.2 (fructose and lactose not fermented), and G1.3 (fructose fermented, but galactose not fermented). Three isolates did not ferment maltose and sorbitol and were tentatively classified as strains of *L. plantarum* (subgroup G1.4, Table 3). Another three isolates did not ferment mannitol and sorbitol, but fermented rhamnose and produced NH_3 from arginine, and were tentatively classified as strains of *L. sakei* (subgroup G1.5, Table 3). Isolates in group G2 were rod-shaped and were separated into three subgroups (G2.1–G2.3, Table 3). Eight of the isolates were tentatively classified as strains of *Lactobacillus brevis* (subgroup G2.1) and 11 as strains belonging to the genus *Weissella* (subgroup G2.2). Two isolates remained unclassified (subgroup G2.3). The vast majority of isolates (159) were coccoid and resembled a homogeneous collection of strains, tentatively classified as members of the genus *Enterococcus* (group G3, Table 3). Most of the isolates (101) could not be identified to species level and were tentatively classified as *Enterococcus* spp. (subgroup G3.1). The remaining isolates were classified as *Enterococcus faecalis* (44 strains in subgroup G3.2) and *Enterococcus faecium* (14 strains in subgroup G3.3). Four Isolates with an oval (coccobacillus) morphology were placed in group G4 and were tentatively classified as *Leuconostoc mesenteroides* (Table 3). Nine isolates with a coccoid morphology and arranged in tetrads were placed in group G5 and were tentatively classified as *Pediococcus* spp.

All the isolates were identified to species level by PCR with species- and genus-specific primers. Representative strains from each group were subjected to 16S rRNA sequencing. The majority of strains in group G1 (72 out of 90) were identified as *L. plantarum*, six as *L. rhamnosus*, three as *L. paraplantarum*, four as *L. paracasei*, two as *Lactobacillus zeae* and three as *L. sakei* (Table 3). Strains in Group G2 were genotypically heterogeneous and represented *L. plantarum* (three strains), *L. brevis* (seven strains), *Weissella* sp. (six strains), *Weissella cibaria* (three strains), *Weissella viridescens* (one strain) and *L. mesenteroides* (one strain). The majority of strains (87 out of 159) in group G3 were identified as *E. faecalis* and grouped into subgroup G3.2 as a homogeneous collection of strains. Thirty-eight strains were identified as *E. faecium* and two as *Pediococcus pentosaceus* (Table 3). Thirty-two strains remained unidentified and were regarded as *Enterococcus* spp. (Table 3). Three isolates in group G4 were identified as *L. mesenteroides* and one as *P. pentosaceus* (Table 3). Isolates in group G5 belonged to *Pediococcus acidilactici* and *P. pentosaceus* (Table 3).

The genotypic relatedness of *L. plantarum* strains isolated from "Alheira", determined by RAPD–PCR, is shown in Fig. 1. Fourteen strains shared similar DNA banding profiles and grouped in Cluster I at 66%. Three subgroups were identified in Cluster I at $\geqslant$70%. Forty-two strains were genotypically closely related and grouped in Cluster III at 72%. Three subgroups were identified within Clus-

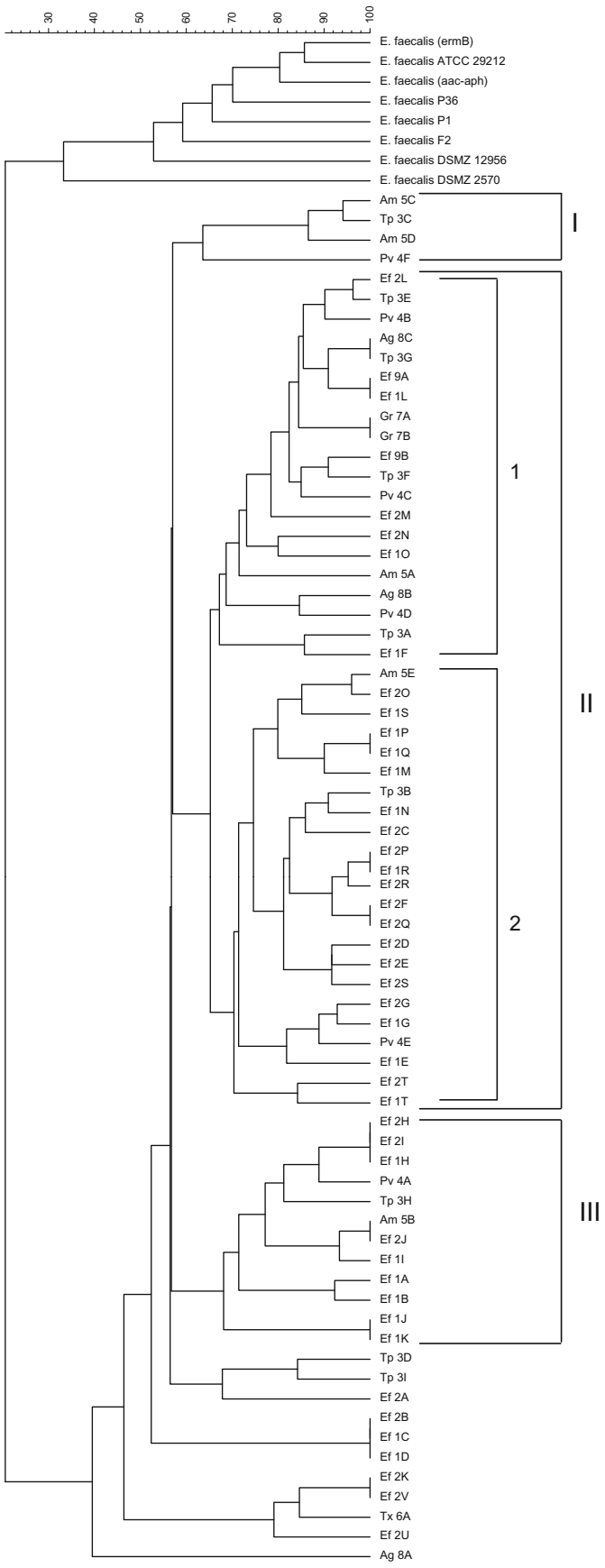

Fig. 2. Numerical analysis of RAPD–PCR profiles obtained from strains of *E. faecalis* isolated from "Alheira" manufactured in seven different plants. Clusters are indicated with Roman numerals. Ef, Tp, Am, Gr, Tx, Ag and PV are the producers.

Table 4
Identification of strains isolated from seven producers of "Alheiras".

Species	Ef	Tp	PV	Am	Tx	Gr	Ag	Total
L. plantarum	21	27	15	5	4	2	1	75
L. paraplantarum	–	2	–	–	1	–	–	3
L. rhamnosus	4	–	–	1	1	–	–	6
L. sakei	–	1	–	–	–	2	–	3
L. zeae	2	–	–	–	–	–	–	2
L. paracasei	4	–	–	–	–	–	–	4
L. mesenteroides	1	2	–	–	1	–	–	4
L. brevis	1	1	2	–	1	–	2	7
Enterococcus spp.	1 + 12[a]	1 + 3[a]	5[a]	1[a]	1 + 1[a]	1[a]	5 + 1[a]	8 + 24[a]
E. faecalis	6 + 44[a]	8 + 9[a]	6[a]	5[a]	1[a]	2[a]	3 + 3[a]	17 + 70[a]
E. faecium	17[a]	1 + 9[a]	1 + 5[a]	–	3[a]	2[a]	–	2 + 36[a]
P. acidilactici	1	–	1	–	–	–	1	3
P. pentosaceus	3	3	–	–	1	–	2	9
Weissella spp.	2	1	3	–	–	–	–	6
W. cibaria	3	–	–	–	–	–	–	3
W. viridescens	–	–	1	–	–	–	–	1
Total	49 + 73[a]	47 + 21[a]	23 + 16[a]	6 + 6[a]	10 + 5[a]	4 + 5[a]	14 + 4[a]	153 + 130[a] = 283

[a] Isolated on M17 agar. All other organisms were isolated on MRS agar.

ter III, each with a specific level of relatedness. Six reference strains of *L. plantarum* grouped in Cluster II at 73% and were genotypically closer related to strains in Cluster I than strains in Cluster III. This suggests that at least 14 strains (grouped in Cluster I) originated from the type strain of *L. plantarum* (DSMZ 20174^T) or closely related strains. It also suggests that 42 strains (grouped in Cluster III) had evolved to become less related to the type strain of *L. plantarum.* Nineteen strains were identified as *L. plantarum*, but were genotypically not closely related to the strains in Clusters I, II and III and formed smaller groups with little intra- and inter-genotypic similarity. Based on these results, *L. plantarum* consists of a heterogeneous collection of strains, similar to findings reported by Duffner and ÓConnel (1995) and Molenaar et al. (2005). More strains of *L. plantarum* will have to be studied to determine the taxonomic status of the species. It is also evident that strains from Cluster III form the core group within the *L. plantarum* strains isolated from "Alheira". Strains within each of the two clusters (I and III) were from different "Alheira" producers, suggesting that specific ingredients, method of production and area did not select for a specific group of strains with specific genetic characteristics.

The genotypic relatedness of 70 of the original 87 strains of *E. faecalis* is shown in Fig. 2. Three well-separated clusters were identified. Cluster I contained four strains grouped at 64%. Cluster II consisted of 43 strains grouped at 66%, separated into two subgroups. Cluster III consisted of 12 strains, grouped at 68%. The eight reference strains of *E. faecalis* and 11 strains isolated from "Alheira" did not group into any of the three clusters. This suggested that the *E. faecalis* strains isolated from "Alheira" are genotypically not closely related to the type strain and other reference strains included in this study. Clusters I and II contained strains isolated from all producers. Cluster III contained 12 strains, mostly isolated from producer Ef. The genetic diversity could be explained by selective pressure inflicted on the strains in "Alheira". As far as we could determine, this is the first detailed taxonomic study on *E. faecalis* in "Alheira". Further research is needed to determine if the strains are characteristic for "Alheira" from a specific producer. It may also be that the strains represent new species within the genus *Enterococcus*.

The study has shown that "Alheira" contains a large number of LAB. Since these bacteria play an important role in meat fermentation, it is safe to assume they will influence the aroma and taste of the final product. LAB are also known to produce antimicrobial compounds, including bacteriocins, and may be used to extend the shelf life of the product. Identification of the strains is the first step towards the selection of starter cultures. By choosing the correct strains, "Alheira", with unique organoleptic properties, may be developed.

Physiological and biochemical tests proved valuable in the preliminary identification of the lactic acid bacteria (Teixeira et al., 1997). However, in the present study, the identity of some isolates could only be confirmed by PCR with species- and genus-specific primers. To determine the specificity of this technique, representative strains within each group were subjected to 16S rRNA gene sequencing, confirming the results obtained by PCR. RAPD–PCR has indicated that *L. plantarum* and *E. faecalis* isolated from different producers are genetically diverse, suggesting that conditions in "Alheira" from a specific producer do not select for a homogeneous collection of strains. Similar findings have been reported for studies conducted on sausages produced in Greece, Hungary and Italy (Rantsiou, Drosinos, Gialitaki, Urso et al., 2005; Rantsiou, Drosinos, Gialitaki, Metaxopoulos et al., 2006; Urso, Comi, & Cocolin, 2006).

The distribution of isolates between different samples and thus amongst different producers, is indicated in Table 4. It is interesting to note that *L. plantarum* was isolated from all samples of "Alheira" (in total 75 isolates, which represented 27% of all isolates). Similar results have been reported for Greek fermented sausage (Drosinos et al., 2005), Botillo (Fontán, Lorenzo, Martínez, Franco, & Carballo, 2007), Sardinian and regional Friuli-Venezia-Giulia sausage (Aquilanti et al., 2007; Urso et al., 2006). *L. brevis* was found in five different "Alheiras", but at low numbers. A few other isolates were classified as *L. paraplantarum*, *L. rhamnosus*, *L. sakei*, *L. mesenteroides, P. pentosaceus, P. acidilactici, W. cibaria, W. viridescens* and *E. faecium*. *L. zeae* and *L. paracasei* were present in sample Ef. The presence of LAB and such a large variation of strains are not surprising, as different production methods, recipes and raw materials were used (bread, spices). Furthermore, the fermentation process is not controlled, with a low ripening time and a high a_w of the final product. Others factors that may contribute to the variability in strains is the manner in which the final product is stored and distributed.

Enterococcus spp. were isolated from all samples, with *E. faecalis* being the dominant species (87 isolates in total; 20% from MRS medium and 80% from M17 medium). *E. faecium* was the second most dominant (38 strains). Enterococci are frequently isolated from fermented sausages, especially in products with a high pH and in the absence of competitive starter cultures (Hugas, Garriga, & Aymerich, 2003). Apart from the glycolytic, proteolytic and lipolytic activities of enterococci, the role of these organisms in fer-

mented sausages has not been studied in detail (Sarantinopoulos et al., 2001). The ability of enterococci to promote health and illness at the same time is not well understood. They produce antimicrobial compounds, including bacteriocins, that may extend the shelf life of fresh products and they may contribute to the sensorial quality of fermented foods. However, a number of enterococci have been associated with the formation of biogenic amines and contain virulence genes and have been associated with nosocomial and opportunistic infections (Franz, Stiles, Schleifer, & Holzapfel, 2001).

A vast number of LAB were isolated from "Alheira", despite the high temperatures the ingredients were exposed to. It is important to note that "Alheira" is smoked at low temperatures and had high a_w. Unlike other fermented meat products, "Alheira" is exposed to a short ripening period. Many strains of LAB will withstand these conditions. It is thus not surprising to isolate specific groups of LAB from "Alheira". Although *L. plantarum* and *E. faecalis* were the dominant lactic acid bacteria in "Alheira", strains within these species are phenotypically and genetically different and may even represent new species. The strains classified as *E. faecalis* will have to be tested for virulence. This study forms the basis from which safe starter cultures could be selected for production of "Alheira".

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