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Full Length Research Paper

A rapid PCR based method to distinguish between *Enterococcus* species by using degenerate and species-specific *sodA* gene primers

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Thirty of thirty-seven cocci isolates from traditional 36 h-old fermented goat's raw milk were characterised phenotypically and genotypically in order to assess the biodiversity within this wild microbiol population. Selective SB media, genus and species-specific tests, based on the manganese-dependent superoxide dismutase A encoding gene *sodA*, were used for the identification of enterococci species. All 30 isolates were characterised at strain level and 28 of them could be identifyed as belonging to the genus Enterococcus. In addition, by using Efm1/Efm2, Efs1/Efs2 and Eh1/Eh2 primers, ten different genotypes were recognised. *Enterococcus faecium* was the dominant biotype followed by *E. faecalis.* The results suggest that wild bacterial populations should be preserved in order to protect the traditional lactic fermentation and for product innovation.

Key words: *sodA*, PCR, enterococci, genus primer, species-specific primer, fermented milk, *Enterococcus*.

INTRODUCTION

Algerian traditional fermented goat's milk is a dairy product obtained after spontaneous curdling of fresh raw milk within 24 to 36 h at ambiante temperature. It has rarely been subjected to microbiological genotypic investigation. The identification of the isolates at strain level is of great importance not only in epidemiolagical and phylogenetic studies (Kühn et al., 1995; O'Sullivan and Fitzgerald, 1998) but also for ecological and industrial purposes (Coroler et al., 1998; Fitzsimons et al., 1999; Tynkkynen et al., 1999; Bensalah et al., 2003).

Enterococci are lactic acid bacteria that occur in a wide range of habitats including the gastrointestinal tract of animals, soil, surface water and on plants (Franz et al., 1999). However, they can be found in several other naturals habitats and processed foods. They were also associated with a number of European fermented foods such as dry-fermented sausages or traditional cheeses product in Mediterranean countries from pasteurised or raw milk and are considered to play an essential role in repening and aroma development in a variety of artisanal cheeses. Therefore, they have been proposed as components of straters cultures (Litopoulou-Tzanetaki, 1990; Cogan et al., 1997; Franz et al., 1999).

In the last decade, it has been noted that enterococci play also an important role in human clinical microbiology, mainly because of their frequent occurrence as nosocomial infectious agents as well as their increasing acquired antibioc resistance (Murray 1990 ; Facklam et al., 1989, 1999). *Enterococcus faecalis* is responsable for the majority of resgistred cases, followed by *E. faecium*, but an increasing incidence was reported for infections with *E. durans* and *E. hirae* as causative agents (Franz et al., 1999; Perlada et al., 1997; Descheemaeker et al., 2000). Most taxonomic studies have been based on strains isolated from clinical sources

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Abbreviation: PCR, polymerase chain reaction; E, *Enterococcus*.

or municipal waters (Valdivia et al., 1996; Laukova and Juris, 1997). In contrast, few investigations focus on environment-colonising strains, which are often phenotypically very heterogeneous and different from the strains isolated from human clinical materials (Devriese et al., 1993; Devriese and Pot, 1995; Ulrich and Muller, 1998). In fact, phenotypic classification alone has been repeatdly shown to be unrealible. This is especially true of the genus *Enterococcus* (Devriese et al., 1995; Texteira et al., 1996; Bascom and Manafi, 1998).

In recent years, several studies using similar modern taxonomical concepts are needed for classification and species identification in dairy enterococci. These methods based on the analysis of bacterial DNA have been successfully applied (Marino et al., 2003; Delgado and Mayo, 2004 ; Mannu and Paba, 2002). PCR with species-specific primers is a valuable method, and, this can replace complex molecular clustering techniques and conventional microbiological tests necessary to identify species hard to distinguish by phenotypical approaches (Dutka-Malen et al., 1995; Danbing et al., 1999; Knijff et al., 2001; Marino et al., 2003; Jackson et al., 2004).

This study was carried out mainly to determine a way of rapidly and unequivocally identifying Algerian enterococci isolated from artisanal fermented goat's raw milk from which novel strains or properties might be selected. On the other hand, this work should be praticable in further ecological and clinical studies. A selective SB medium and genus/species-specific primers from *sodA* gene test for the identification of three species of raw milk fermentation importance, *E. faecalis*, *E. faecium* and *E. hirae* are described.

MATERIALS AND METHODS

Bacterial strains

All strains analysed in this study were isolated during microbiological analysis of the traditional fermented milk and were provided from LMA (Laboratoire de Microbiologie Appliquée) collection. The reference strains used in this work are the type strains of the respective species and were obtained from INRA collection (Jouy-En-Josas, France). *Enterococcus faecalis* JH2-2, *E. faecium* 63/4, *E. hirae* LMG 6399T and *Lactococcus lactis* spp. *Lactis* IL1403.

Phenotypic characterization

Some of the procedure for testing the phenotypic features was carried out according to previously published identification schemes (Devriese et al., 1993; Teixeira et al. 1997). The different isolates from 36-h old goat's raw milk were tested from their ability to grow in M17 broth at 10 and 45°C with 6.5% NaCl or at pH value adjusted to 9.6. Catalase activity was tested by placing a loopful of cells in a 10% (v/v) H_2O_2 solution and their capacity for hydrolising arginine was analysed. To select enterococci, the isolates were spread on membrane filter agar according to Slanetz and Bartley (1957). In addition to growth, reduction of triphenyl-tetrazolium chloride was assessed by the colony color (pink or dark indicate *Enterococcus*). The plates were incubated under aerobic conditions for 2 days at 42°C.

Taxonomic identification

After microscopic examination, Gram-positive and catalase-negative isolates were considered as presunmptive enterococci. This preliminary approach was followed by a polymerase chain reaction (PCR) identification analysis with *Enterococcus* genus-specific primers *sodA* d1 and *sodA* d2, as previously described (Poyart et al., 2000) and with internal part of *sodA Enterococcus* species-specific primers Efm1/Efm2, Efs1/Efs2 and Eh1/Eh2 respectively from *E. faecium*, *E. faecalis* and *E. hirae* (e.g., O. Firmesse, Centre Nationale de la Recherche Agronomique, INRA-Jouy-En-Josas, France, personal communication, 2003).

PCR using degenerate primers

Bacterial genomic DNA was extracted from 100 µl of overnight cultures in M17 or MRS broth. Cells were pelleted in a microcentrifuge at 8000 X g for 5 min, resuspended in 20 µl of genereleaser™ (Bio Ventures, Inc.) and submited to PCR program as follows: 65°C for 30 s, 8°C for 30 s, 65°C for 1min , 97°C for 3 min, 8°C for 1 min, 65°c for 3 min, 97°C for 1 min, 65°c for 1 min and 80°C for 5 min. A second PCR procedure with primers *sodA* d1 and *sodA* d2 (Table 1) were used to amplify an internal fragment representing 85% of the *sodA* gene of the bacterial strains (Poyart et al., 1995, 2000). Amplification was performed in 25 µl reactions mixtures containing: 2.5 µl 10X PCR buffer (Takara), 2 µl dNTP (Takara), 0.5 µl of each primer, and 0.25 µl of Taq DNA polymerase (Takara). DNA procedure was carried out in a thermal cycler (Applied Biosystem), programmed as follows: an initial denaturation step of 95°C for 3 min, 30 cycles of amplification (60 s of annealing at 37°C, 60 s of elongation at 72°C, and 30 s of denaturation at 95°C) and 7 min at 72°C for the last elongation. 5 µl of the PCR products were examined in a 1% agarose gel at 100 V. The expected size of the amplicon was 438 pb from *sodA* gene. 200 pb DNA Smartladder (Eurogentec) was used as molecular size marker.

Enterococcus-specific PCR

An other approch was followed by PCR identification analysis with *Enterococcus* species-specific primers from *E. faecium* 64/3, *E. faecalis* JH2-2 and *E. hirae* LMG 6399T. The cellular lysates were obtained with the action of genereleaser™ (bio Ventures, Inc.) from an overnight cultures as describrd above, and primers Efm1/Efm2, Efs1/Efs, Eh1/Eh2 (Table 2) were used to identify respectively *E. faecium, E. faecalis* and *E. hirae* within indigenous flora isolates. The program icluded a denaturation step of 94°C for 5 min and then subjected to 30 cycles of amplification (94°C for 30 s, 50°C for 30 s, 72°C for 1 min) with a final soak at 4°C. Fragments DNA were determined by comparing the control-positive bands with amplified fragments of wild enterococci. The expected bands of the amplicons were 190, 209 and 263 pb from *sodA* gene in *E. faecium, E. faecalis* and *E. Hirae*, respectively. Reference strains of *Enterococcus* and *L. lactis* were used as controls. PCR products were visualised in a 2% gel at 80 V, and DNA Smartladder (Eurogentec) of 200 pb was used as molecular size marker.

RESULTS

Phenotypic characteristics

A total of 30 of 37 Gram-positive and catalase-negative isolates from 36-h old fermented traditional milk were presenmptively identified as enterococci on the basis of

Table 1. Growth SB medium and genotypic patterns of enterococci isolated from fermented milk.

Strains/isolates	Primers d1/d2	Primers Eh1/Eh2	Primers Efm1/Efm2	Primers Efs1/Efs2	SB Agar
Ch4	-	ND	ND	ND	+,dr
Cm11	-	ND	ND	ND	+,dr
SD17	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
La15	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
Sm15	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
Stb4	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
La20	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
Stc14	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
Ch6	*Enteroco.* Spp.	ND	*E. faecium*	ND	+,dr
Cm4	*Enteroco.* spp.	ND	*E. faecium*	ND	+,dr
Cm6	*Enteroco.* spp.	ND	-	ND	+,dr
S5	*Enteroco.* spp.	ND	-	ND	+,dr
BB1	*Enteroco.* spp.	ND	-	ND	+,dr
Stb8	*Enteroco.* spp.	ND	-	ND	+,dr
S2	*Enteroco.* spp.	ND	-	ND	+,dr
16a	*Enteroco.* spp.	ND	ND	*E. faecalis*	+,dr
S28	*Enteroco.* spp.	ND	-	*E. faecalis*	+,dr
L1	*Enteroco.* spp.	ND	ND	-	+,dr
E1	*Enteroco.* spp.	ND	ND	-	+,dr
St1	*Enteroco.* spp.	ND	ND	-	+,dr
Stc10	*Enteroco.* spp.	ND	ND	-	+,dr
Stb7	*Enteroco.* spp.	ND	ND	-	+,dr
G1	*Enteroco.* spp.	ND	ND	-	+,dr
L10	*Enteroco.* spp.	-	ND	ND	+,dr
S8	*Enteroco.* spp.	-	ND	ND	+,dr
G1	*Enteroco.* spp.	-	ND	ND	+,dr
S3	*Enteroco.* spp.	-	ND	ND	+,dr
L44	*Enteroco.* spp.	-	ND	ND	+,dr
St7	*Enteroco.* spp.	-	ND	ND	+,dr
St3	*Enteroco.* spp.	-	ND	ND	+,dr
BR2	-	ND	ND	ND	-,dr
Cm8	-	ND	ND	ND	-,dr
Ch7	-	ND	ND	ND	-,dr
Cm9	-	ND	ND	ND	-,dr
Cm1	-	ND	ND	ND	-,dr
Ch5	-	ND	ND	ND	-,dr
S1	-	ND	ND	ND	-,dr
JH2-2	*E. faecalis*	-	-	+	+,dr
64/3	E. faecium	-	+	-	+,dr
LMG 6399T	E. hirae	+	-	-	+,dr
IL1403	L. lactis	-	-	-	-,dr

dr, dark; ND, no data; +, positive reaction; -, negative reaction; Enteroco. spp., Enterococcus spp.
All strains were Gram-positive catalase-, homofermentative-positive and ovoid or spherical non-motile cocci in pairs or short chains.

phenotypic characterisation. Bacterial cells were ovoid cocci occuring in pairs, short chains or small groups. All strains selected grew well on enterococci selective medium at 10 and 45°C as well as in M17 broth containing 6.5% NaCl or at pH 9.6 (Table 1). The identification of enterococcal species using only phenotypical test is often problematic, and differences in one or few biochemical reaction are often meaningles. DNA PCR amplification was carried out to analyse the isolates.

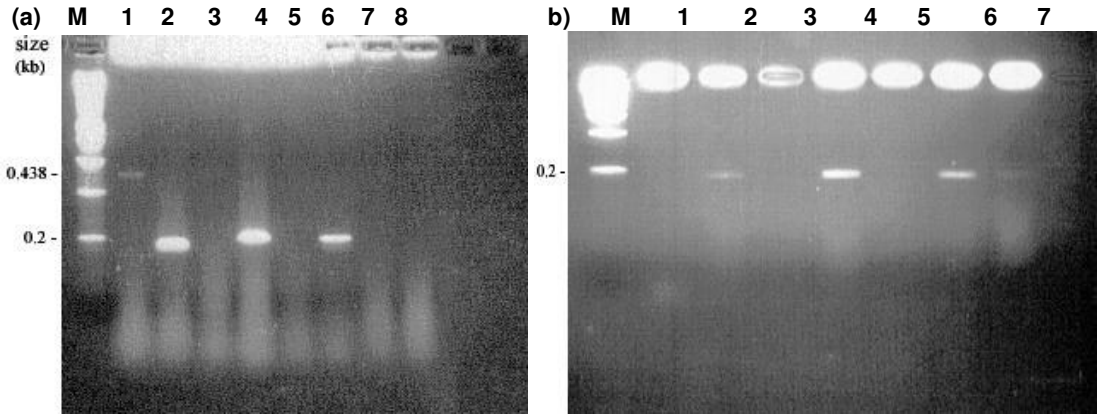

Figure 1. Genus and species-specific PCR amplifications for *sodA* gene. **a**: Genus and species-specific PCR with primer pair d1/d2, Efm1/Efm2 and Efs1/Efs2. Lane M, 200-pb DNA molecular mass marker; lane 1, d1/d2 *Ent. faecalis* (S28) isolate from goat's milk; lane 2, Efm1/Efm2 *E. faecium* 64/3 as positive control; lane 3, d1/d2 *L. lactis* IL1403 as negative control; lane 4, Efs1/Efs2 *Ent. faecalis* JH2-2 as positive control; lane 5, Efs1/Efs2 *E. hirae* LMG 6399T as negative contol; lane 6, Efs1/Efs2 *E. faecalis* (S28) isolate from goats' milk; lane 7, Efs1/Efs2 *L. lactis* IL1403 as negative control; lane 8, Efm1/Efm2 *L. lactis* IL1403 as negative control. **b**: Species-specific PCR with primer pair Efm1/Efm2. Lane M, 200-pb DNA molecular mass marker; lane 1, *E. faecalis* JH2-2 as negative control; lane 2, *E. faecium* 64/3 as positive control; lane 3, *E. hirae* LMG 6399T as negative control; lane 4, *E. faecium* (SD17) isolate from goats' milk; lane 5, *E. faecalis* (S28) isolate from goat's milk as negative control; lane 6, *E. faecium* (La15) isolate from goat's milk; lane 7, *Ent. faecium* (Sm15) isolate from goat's milk.

Table 2. Universal and species-specific oligonucleotides primers used in this study.

Target gene (pb)	Oligonucleotide primer	Sequence (5'-3')[a]	product size (pb)
sodA	*sodA*d1 (universal-F), *sodA*d2 (universal-R)	CCITAYICITAYGAYGCIYTIGARCC ARRTARTAIGCRTGYTCCCAIACRTC	438
sodA	Efm1/Efm2	TKCAGCAATTGAGAAATAC CTTCTTTTATTTCTCCTGTA	190
sodA	Efs1/Efs2	CTGTAGAAGACCTAATTTCA CAGCTGTTTTGAAAGCAG	209
sodA	Eh1/Eh2	AAACAATCGAAGAACTACTT TAAATTCTTCCTTAAATGTTG	263

a I =A,T,G or C; Y=C or T; R=A or G.

Identification of amplified DNA genus products

The identification by using the internal part of *sodA* gene of the gram-positive cocci, which encodes the manganese-dependent superoxide dismutase, fulfills these criteria. Tese universal primers were used to construct a *sodA* database of enterococcal species (Poyart et al., 2000). The specificity of the genus was determined by testing all isolates presumptively identified as enterococcal. Only 28 strains were amplified with the enterococcal genus primers, producing a product of 438 pb, indicating that they members of *Enterococcus*. No significant common band were found from Ch4 and Cm11 isolates and from *Lactococcus lactis* Il1403, used as negative DNA control. These results indicates the discriminating power of the *sodA* d1 and *sodA* d2 PCR described here.

Amplification using enterococcal species primers

In view of the unclear results of the methods described above, species-specific PCR of *En. faecium*, *E. faecalis* and *E. hirae* were carried out to clarify the taxonomic position and to compare the isolates with enterococcal reference species. Specific-primers for *sodA* gene Efm1/Efm2, Efs1/Efs2 and Eh1/Eh2 were used in this study. The expected 190-pb band from *E. faecium* was revealed in 8 strains from 13 tested (Figure 1b). The specific 209-pb band of *E. faecalis* (Figure 1a) was obtained in only two strain (16a, S28) from 8 tested, while

no significant bands were found from the 7 other strains tested with Eh1/Eh2 specific-primers (Table 2).

DISCUSSION

The problem in the taxonomy of enterococci is generally that they are a diverse group of Gram-positive cocci sharing many characteristics with the genera *Streptococcus* and *Lactococcus*. In this study, phenotypic data indicated that the isolated strains belonged to the genus *Enterococcus* with the exception of seven isolates. It is not possible to unequivocally categorise strains, especially from environment samples, into one of these genera by simple physiological tests and heterogeneity in phenotypic features is very high in *Enterococcus* species, regardless of the origin of isolates (Devriese et al., 1993; Leclerc et al., 1996; Ulrich and Müller, 1998). These observations demonstrate the necessity of molecular methods for reliable species identification of unsual enterococcal strains.

Genus and species-specific primers have been previously applied successfully by poyart et al. (2000), Knijff et al. (2001), Marino et al. (2003) and Jackson et al. (2004). By using the degenerate *sodA* gene encoding a manganese-dependent enzyme (Mn-SOD) in isolates identified as enterococci, a single amplification product having the expected size of 438 pb was observed with all (excepted two) strains selected in SB media. It should be noted that growth on SB agar alone is not a criterion for enterococci (Ulrich and Müller, 1998).

On the other hand, we investigated the specificity of the three primer pairs by testing 28 isolates selected as enterococcal. Amplification products with the expected size were obtained with DNA from *E. faecium* and *E. faecalis*. By contrast, the third primer pair did not amplify DNA from *E. hirae* within selected strains**.** Species were identified for 13 strains including 8 *E. faecium* and for 8 including 2 *E. faecalis*. No PCR product was observed with *E. hirae* species-specific DNA from 7 other strains, suggesting that they belong to other enterococcal species.

E. faecium is the species most frequently present in traditional fermented milk followed by *E. faecalis*. Their presence in our study confirm the results obtained by Marino et al. (2003) who investigated indigenous lactic acid bacteria from artisanal Montasio cheese as well as that of Elotmani et al. (2002) and Mathara et al. (2004) in Raib and Kule naoto, an Maroccan and Kenyan traditional fermented milks.

Using the same genus primers and similar species-specific condition to those of Poyart et al. (2000) for identifing medical isolates, we are able to identify food isolates of enterococci. Their role as relevant component of natural cultures involved in the fermentation of artisanal cheeses and traditional lactic fermented milk has been described in detail (Giraffa et al., 1997; Mathara et al., 2004).

Nevertheless, over the last decades, enterococci, formely viewed as organisms of minimal clinical impact, have emerged as important hospital-acquired pathogens in immunosuppressed patients and intensive care units.

Their role in nosocomial infections is probably due to a variety of factors ; antimicrobiol resistance appears to be a primary cause (Wade, 1997). The identification method presented in this study which is simple, reproducible and reliable technique may be applied to Algerian hospital microbiology laboratories for epidemiological purposes and/or diagnosis of enterococcal infections.

Strains of food enterococci have been proposed as strarters (Garg and Mital, 1991; Giraffa et al., 1997) but it should be ensured that they are free of antibiotic resistances and/or other determinants of virulence (Giraffa et al., 1997; Eaton and Gasson, 2001). There is still little information about the strain composition of the natural microbial population colonising the traditional fermented goat's milk in Algeria. This is a preliminary study and further investigation into strain variability within species should be extended in different artisanal lactic femented raw milk. Autochthonous microflora of African traditional lactic fermentation represents a heritage wich has to be protected and conserved.

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