JOURNAL OF FOOD QUALITY & HAZARDS CONTROL
SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES & HEALTH SERVICES

Journal of Food Quality and Hazards Control 6 (2019) 66-72

Dominant Lactic Acid Bacteria in Naturally Fermented Milks from Messinese Goat's Breed

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HIGHLIGHTS

- Levels of coccus- and rod-shaped LAB were in the range of 1.78-7.10 log and 1.00-7.09 log colony forming unit/ml, respectively.
- Among 12 identified strains, the most numerous one was *Enterococcus hirae* (n=4) followed by *E. faecium* (n=3).
- Risk assessment of pathogenic enterococci species is needed for the consumers of traditional fermented dairy products.

Article type
Original article

Keywords
Milk
Goats
Lactobacillales
Cultured Dairy Products
Food Microbiology

Article history
Received: 18 Jan 2019
Revised: 27 Mar 2019
Accepted: 11 Apr 2019

Acronyms and abbreviations
LAB=Lactic Acid Bacteria
RAPD=Random Amplification of Polymorphic DNA
PCR=Polymerase Chain Reaction
CFU=Colony Forming Unit
MRS=de Man-Rogosa-Sharpe
M17=Medium 17 agar
FGM=Fermented Goat's Milk

ABSTRACT

Background: Lactic Acid Bacteria (LAB) are an important group of microorganisms responsible for the fermentation dairy products. This study was done to identify the dominant lactic acid bacteria in naturally fermented milks from Messinese goat's breed.

Methods: Eighteen individual raw milk samples collected from Messinese goat's breed were acidified at pH 5.20 and left to spontaneously ferment at 37 °C for 4 days. All samples were analyzed for rod- and coccus-shaped LAB. Also, all presumptive LAB were isolated and differentiated according to their phenotypic properties and genetic polymorphisms and then identified by sequencing the 16S rRNA gene. Data were statistically analyzed using SAS 9.2 software.

Results: Levels of coccus- and rod-shaped LAB were in the range of 1.78-7.10 log and 1.00-7.09 log colony forming unit/ml, respectively. The microbiological counts on the two different growth media were significantly (p<0.05) different among the samples. Among 12 identified strains, the most numerous one was *Enterococcus hirae* (n=4), followed by *E. faecium* (n=3), while the other species (*E. durans*, *E. faecalis*, *E. lactis*, *Lactococcus lactis*, and *Leuconostoc lactis*) included one strain each.

Conclusion: The major group identified in this study was mainly represented by members of *Enterococcus* genus. Although *Enterococcus* spp. are related to the typicality of some traditional fermented dairy products, this study highlights the need for risk assessment of pathogenic enterococci species for the consumers.

Introduction

Lactic Acid Bacteria (LAB) are an important group of microorganisms responsible for the fermentation of a large variety of foods, including those derived from dairy products (Gaglio et al., 2014a; Gaglio et al., 2019a). LAB are able to improve the final quality of dairy productions and in particular their flavour, texture, and nutritional value (Gaglio et al., 2016a; Guarrasi et al., 2017; Leroy and De Vuyst, 2004). Furthermore, they have the ability

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To cite: Palmeri M., Mancuso I., Barbaccia P., Cirlincione F., Scatassa M.L. (2019). Dominant lactic acid bacteria in naturally fermented milks from Messinese goat's breed. *Journal of Food Quality and Hazards Control*. 6: 66-72.

DOI: 10.18502/jfqhc.6.2.957 Journal website: http://www.jfqhc.com

to determine the rapid acidification of the raw materials and inhibit the growth of spoilage and pathogenic microorganisms (Guarcello et al., 2016; Macaluso et al., 2016; Scatassa et al., 2017; Settanni et al., 2013).

The use of starter LAB to ferment milk with particular nutritional properties (e.g. goat's milk), represents one of the technological strategy to develop a large variety of new dairy functional foods and beverages (Minervini et al., 2009). Goat's milk is well recognized as "the king of milk" for its high digestibility and nutritional value as well as for the lower allergenic compound content than cow's milk (Ribeiro and Ribeiro, 2010). Furthermore, goat's milk is one of the matrices most closed to human milk and plays an important role in healthy diet of children and elderly people (Haenlein, 2004).

Although several works are available on the characterization of LAB from goat's cheeses and fermented milks in literature (Meng et al., 2018; Minervini et al., 2009), only a few works focused on the characteristics of the raw milks before processing. In order to develop *ad hoc* starter cultures for a given product, the characterization of the populations associated with raw materials is of relevance. The particular flavor and typical organoleptic properties of products processed from raw milk are related not only to the race and nutrition of animals, but also to the natural microbiota responsible for fermentation (Franciosi et al., 2008). With this in mind, the present work was carried out to identify the autochthonous LAB composition of naturally Fermented Goat's Milks (FGM) in order to characterize the dominant microbial populations.

Materials and methods

Sample collection, acidification, and incubation of goat's milk

Eighteen individual milk samples of Messinese goat's breed were collected from a farm located in Palermo province, Italy. Just after sampling, all samples were placed into a portable fridge and transferred to the Laboratory of Centro Latte e Lotta alle Mastiti (Istituto Zooprofilattico Sperimentale della Sicilia "Adelmo Mirri", Palermo, Italy) where they were immediately subjected to the acidification procedure; in order to allow exclusively the growth of LAB, each milk sample was added with 5 M lactic acid (BDH Prolabo Chemicals, Singapore) until reaching the pH 5.20 evaluated electrometrically by pH meter HI3220-02 (Hanna Instruments, Woonsocket, RI, USA). All samples were incubated at 37 °C for 4 days. The milk samples from each goat were collected in duplicate at two-week intervals.

Microbiological analyses, LAB isolation, and phenotypic grouping

All 18 FGM samples (FMG1-FMG18) were serially diluted in Ringer's solution (Sigma-Aldrich, Milan, Italy). Cell suspensions were then subjected to plate count for the enumeration of rod- and coccus-shaped LAB after pouring on de Man-Rogosa-Sharpe (MRS) agar acidified with 5 M lactic acid to pH 5.4 incubated anaerobically at 37 °C for 48 h and on Medium 17 (M17) agar incubated aerobically at 37 °C for 48 h, respectively (Aureli et al., 2008). Incubation of rod-shaped LAB was occurred in anaerobiosis using the AnaeroGen AN25 (Oxoid, Milan, Italy) in jars closed hermetically. Both MRS and M17 were purchased from Oxoid®, UK.

After growth, colonies of various shapes of Gram-positive and catalase negative bacteria were randomly picked from count plates considering all different colours, edges, and elevations, then transferred into the corresponding broth media. All different morphologies were considered in order to evaluate total LAB diversity. The isolates were purified by successive subculturing and stored in MRS or M17 broth media containing 20% glycerol (v/v) at -80 °C until further analysis.

All presumptive LAB isolates from FGM were subjected to a phenotypic characterization on the basis of cell morphology, cell disposition, growth at 15 and 45 °C, resistance at 60 °C for 30 min, NH_3 production from arginine, aesculin hydrolysis, acid production from the carbohydrates (arabinose, ribose, xylose, fructose, galactose, lactose and sucrose), and CO_2 production from glucose (Di Grigoli et al., 2015; Gaglio et al., 2014b). The coccus-shaped isolates were further grouped by their ability to grow at pH 9.2 and in the presence of NaCl (6.5 g/l) to separate enterococci from other dairy cocci.

Genotypic differentiation and identification of bacteria

DNAs from LAB cultures were extracted using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Cells were harvested after overnight growth in MRS or M17 broths at 37 °C and washed in distilled H_2O; then the crude cell extracts were used as templates for Polymerase Chain Reactions (PCRs).

The differentiation of the isolates at strain level was performed by Random Amplification of Polymorphic DNA (RAPD)-PCR analysis in a 30 µl reaction volume with the primers M13, AB111, and AB106 used singly as reported by Gaglio et al. (2017). PCR mixture included 62.5 ng of target DNA, 2.5 µl of PCR buffer (Fermentas, MMedical, Milan, Italy), 2.5 mM of $MgCl_2$, 250 µM of each dNTP (Life Technologies Monza, Italy), 0.2 µM of each primer, 2.5 U of Taq DNA polymerase

(Thermo Fisher Scientific, Monza, Italy), and Milli-Q water to reach the final reaction volume. The PCR program applied for all primers comprised 40 cycles of denaturation for 2 min at 94 °C, annealing for 20 s at 40 °C, and extension for 2 min at 72 °C; the cycles were preceded by denaturation at 94 °C for 2 min and followed by extension at 72 °C for 5 min. The amplifications were performed using a Thermal cycler (Bioer, Hangzhou, China) and the amplified products were separated by electrophoresis, visualized and acquired by Gel Doc™ XR+and ChemiDoc ™ XRS+Imaging Systems (Bio Rad, Hercules, CA, USA). The analysis of the RAPD patterns was performed with the Gelcompar II software, version 6.5 (Applied-Maths, Sint-Martens-Latem, Belgium).

All LAB showing different RAPD-PCR profiles were genetically identified at species level by 16S rRNA gene sequencing as described by Weisburg et al. (1991). PCR mixture (50 µl total volume) included 62.5 ng of target DNA, 1×Taq DNA polymerase buffer with 2 mM $MgCl_2$ (Thermo Fisher Scientific, Monza, Italy), 250 µM of each dNTP (Life Technologies, Italy), 0.2 µM of each primer, 2.5 U of Taq DNA polymerase (Thermo Fisher Scientific, Monza, Italy), and Milli-Q water to reach the final reaction volume. PCR program comprised an initial template denaturation step for 3 min at 95 °C followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 45 s at 54 °C, and extension for 2 min at 72 °C. The final elongation step was for 7 min at 72 °C. The PCR products were visualized as reported above and the amplicons corresponding approximately 1600 bp were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Sequencing analyses were performed in an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) and compared using a BLAST search in the GenBank/ EMBL/DDBJ database (http://www.ncbi.nlm.nih.gov).

Statistical analyses

Statistical analyses of microbiological data were subjected to one-way Analysis of Variance (ANOVA) using SAS 9.2 software (Statistical Analysis System Institute Inc., Cary, NC, USA). Pair comparison between means were determined by the post-hoc Duncan test at $p<0.05$. Differences between the samples were analyzed using a Generalised Linear Model (GLM) procedure.

Results

Figure 1 shows the viable counts of the rod- and coccus-shaped LAB harboured on FGM samples. Levels of these microbial groups for each sample were almost comparable. The microbiological counts on the two different growth media were significantly ($p<0.05$) different among the samples. In fact, the levels of coccus-shaped LAB were in the range of 1.78-7.10 log Colony Forming Unit (CFU)/ml while rod-shaped LAB were in the range of 1.00-7.09 log CFU/ml. Samples FGM1, FGM4, FGM9, FGM14, FGM16, FGM17, and FGM18 displayed the highest bacterial density of rod- and coccus-shaped LAB; while the samples FM8 and FM11 showed the lowest levels of these bacterial groups.

A total of 247 colonies were collected from the 18 samples of FGM. After purification and microscopic inspection, all the cultures showed a coccus-shaped morphology. One hundred eighty seven cultures were considered presumptive LAB, being Gram-positive and catalase negative. According to the combination of the phenotypic properties evaluated the 187 presumptive LAB cultures, were separated into five groups (Table 1). The most numerous groups were groups II and III, composed of more than 50 isolates each. LAB cultures included between the groups I to IV showed an obligate homofermentative metabolism while group V showed a heterofermentative metabolism.

Approximately 30% of the isolates from each phenotypic group, forming a total of 56 isolates, were subjected to RAPD-PCR analysis for strain typing. The genotyping differentiation indicated that the cultivable bacterial community associated to FGM in the present study was composed of 12 distinct strains (Figure 2). The dendrogram clearly showed that the strains belonging to the phenotypic groups I to III clustered closely after RAPD-PCR analysis.

Twelve strains were identified by sequencing of the 16S rRNA gene. The sequence comparison within BLAST database identified seven major dominating species. The species with the highest number of strains was *Enterococcus hirae* (n=4) followed by *E. faecium* (n=3), while the other species (*E. durans*, *E. faecalis*, *E. lactis*, *Lactococcus lactis*, and *Leuconostoc lactis*) included one strain each.

Discussion

Some previous works about LAB in raw ewe's and cow's milk showed potential of LAB to drive the fermentation/ripening processes of different dairy products (Gaglio et al., 2014b; Guarcello et al., 2016; Turchi et al., 2011); however, few information are available on goat's LAB from Italian milk products. In the present study, all goat milk samples were dominated by rod- and coccus-shaped LAB, but only a few samples showed levels comparable with those of typical milk based fermented products, such as yogurt (Rezac et al., 2018) with the levels at around 7 log CFU/ml. The presumptive LAB

Table 1: Phenotypic grouping of the LAB isolated from fermented goat's milks

Characteristics	**Clusters**				
	I (n=13)	II (n=62)	III (n=81)	IV (n=19)	V (n=12)
Morphology	C*	C	C	C	C
Cell disposition	SC**	SC	SC	SC	SC
Growth:					
15 °C	+	+	+	+	+
45 °C	+	+	+	-	+
pH 9.2	+	+	+	+	-
6.5% NaCl	+	+	+	-	+
Resistance to 60 °C	-	-	+	+	-
Hydrolysis of:					
Arginine	+	+	+	+	-
Aesculin	+	+	+	+	-
Acid production from:					
Arabinose	+	+	+	-	+
Ribose	+	+	+	+	+
Xylose	+	+	+	-	+
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	+	-	+	+	+
Glycerol	+	+	+	+	+
CO_2 from glucose	-	-	-	-	+

C: Coccus; SC: Short Chain

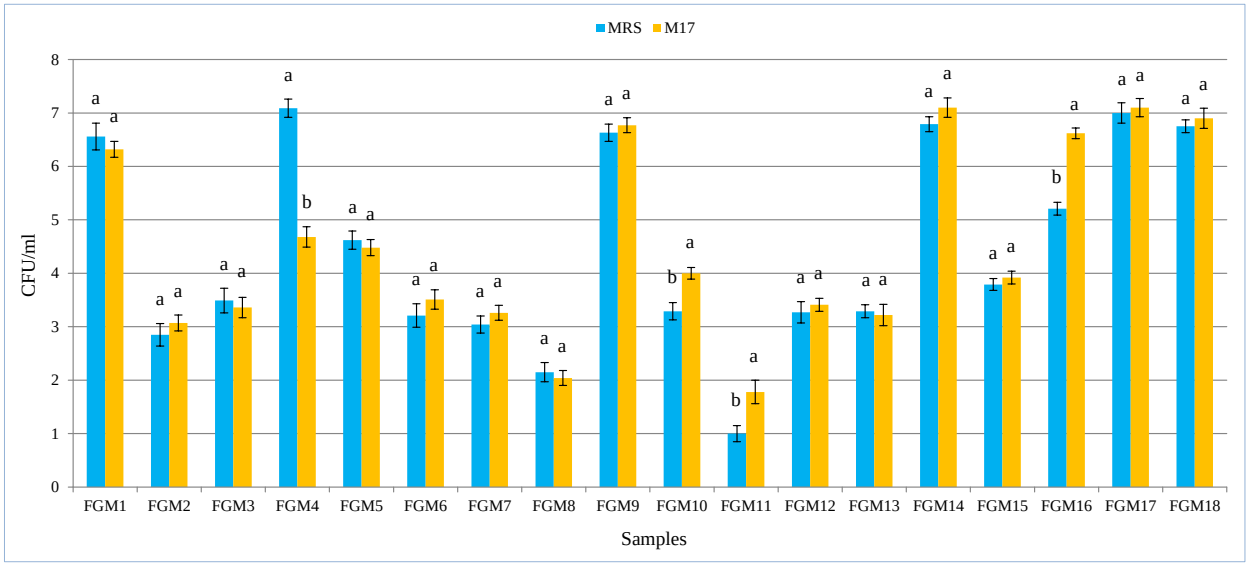
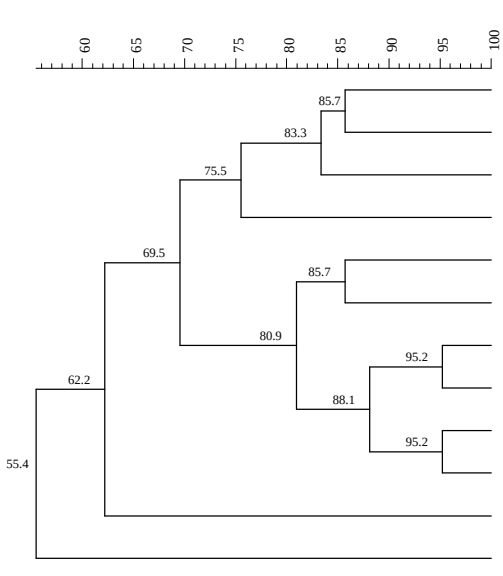


Figure 1: Microbiological concentrations (CFU; Colony Forming Unit/ml) of fermented goat's milk samples. MRS: de Man-Rogosa-Sharpe agar for detection of rod-shaped LAB; M17: Medium 17 agar for detection of coccus-shaped LAB; FGM: Fermented Goat's Milk. Different superscript letters indicate significant differences on microbial loads were performed for each sample according to Duncan test between MRS and M17 media for $p<0.05$



Strain	Phenotypic group	Species	Acc. No.
GM71	II	*E. faecium*	MK533774
GM52	II	*E. faecium*	MK533775
GM33	II	*E. faecium*	MK533776
GM1	I	*E. faecalis*	MK533777
GM55	III	*E. durans*	MK533778
GM122	III	*E. lactis*	MK533779
GM157	III	*E. hirae*	MK533780
GM5	III	*E. hirae*	MK533781
GM61	III	*E. hirae*	MK533782
GM81	III	*E. hirae*	MK533783
GM101	IV	*Lc. lactis*	MK533784
GM171	V	*Ln. lactis*	MK533785

Figure 2: Dendrogram obtained with combined RAPD-PCR patterns of the LAB strains isolated from fermented goat's milks. Scale bar indicate the percentage of similarity. *E.*: *Enterococcus*; *Lc.*: *Lactococcus*; *Ln.*: *Leuconostoc*

isolated from the FGM samples represented five phenotypic groups of cocci, even though on MRS should preferably develop rod-shaped species. However, despite the high count detected in MRS, our results confirmed that LAB cocci are able to develop on this media commonly used for mesophilic as well as thermophilic rods LAB, maybe due to the lower pH of this media (Settanni et al., 2012).

RAPD-PCR, a technique commonly used to differentiate microorganisms indicated the presence of 12 different strains in our goat milk samples. This biodiversity is quite low compared to that of traditional cheese evidenced by Franciosi et al. (2008), but the explanation for this controversy could be due to the fact that only one farm was analyzed. In a single farm, even if several animals are object of investigation, the environmental factors and the feed sources are the same and this might have reduced biodiversity found among milk samples. The genetic identification resulted in the following LAB species: *E. durans*, *E. faecalis*, *E. faecium*, *E. hirae*, *E. lactis*, *Lc. Lactis*, and *Ln. lactis*. All these species are commonly associated with raw milk and cheeses (Franciosi et al., 2011; Gaglio et al., 2019b) and the equipment commonly used in traditional cheese productions (Cruciata et al., 2018; Scatassa et al., 2015). Among them, *Lc. lactis* and *Ln. lactis* are typical mesophilic starter cultures for dairy use (Settanni and Moschetti, 2010), while the other identified species are commonly shown to be part of the non-starter LAB population implicated in the maturation of several cheeses (Gatti et al., 2014; Settanni and Moschetti, 2014).

In the current research, the high number of strains detected as members of the *Enterococcus* spp. was surprising especially those belonging to the species *E. hirae* and *E. faecium*. Although in other studies, enterococci were not found at consistent levels in FGM, their presence at 10^6–10^8 CFU/g was reported by some researchers who analyzed the traditional ovine and caprine cheeses produced in Greece (Nikolaou et al., 2002; Prodromou et al., 2001; Psoni et al., 2003). It is well known that enterococci are an integral part of the microbial population of different typologies of traditional dairy productions (Nikolaou et al., 2002; Psoni et al., 2003; Suzzi et al., 2000) and due to their proteolytic and lipolytic activities, they are involved in the development of typicality organoleptic characteristics (Giraffa, 2002). On the other hand, it should be highlighted that in the last years, enterococci are considered as emerging pathogens for humans may endangering public health. So, inclusion of enterococci as starter cultures for cheese production needs to be validated by the absence of pathogenic traits, such as antibiotic resistance, virulence and haemolytic activity (Gaglio et al., 2016b; Russo et al., 2018; Settanni et al., 2014).

Conclusion

This work showed the importance of selecting acidifying LAB from raw materials for production of fermented dairy products; since they might ensure the ability of persist during fermentation. The major group identified in this study was mainly represented by members of *Enterococcus* genus. Although enterococci sometimes are related to the typicality of some traditional fermented dairy products, this study highlights the need for risk assessment of pathogenic enterococci species for the consumers.

Author contributions

M.L.S. designed the project of study; P.B., F.C., and M.P. conducted the experiments; M.P. and I.M. analyzed the data; M.L.S. and I.M. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

There was no conflict of interest in this study.

Acknowledgements

This work was financed by the Italian Ministry of Health Research Project RC 06/16 Valutazione del rischio associato alla presenza di enterococchi antibiotico-resistenti nei prodotti lattiero-caseari e sviluppo di metodologie che consentano la preservazione delle produzioni (CUP H76J17000480001). The authors are grateful to Dr. Rossella Calascibetta for their precious collaboration during samples collection.

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