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Article *in* Italian Journal of Animal Science · April 2010

DOI: 10.4081/ijas.2007.1s.595 · Source: DOAJ

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Molecular characterization of dominant bacterial population in "Vastedda della Valle del Belice" cheese: preliminary investigation

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ABSTRACT: The sensory characteristics of raw-milk cheeses are linked to the cheese-making process, to the environmental factors as animal feeding and to the biochemical and microbiological composition of the milk. In this report we temped to characterize the microflora in the typical Sicilian historical cheese as Vastedda della valle del Belice.

Each cheese was previous subjected to microbial isolation on specific media (M17 and MRS). The colony obtained on the solid medium were subject to biochemical tests and DNA extraction. The microbial diversity occurring in the strains was evaluated by PCR, RFLP and sequencing targeted on 16S ribosomal DNA. A number of closest relatives species of lactic and contaminating bacteria were identified in a total of 18 cheeses. The more represented genus were: *Enterococcus, Streptococcus, Lactobacillus, Lactococcus and Pediococcus*. Moreover it was found only one strain *Lactococcus lactis* for producing bacteriocines. The different bacteria species probably could play a key role in the maturation of the cheese. The preliminary obtained data show the optimized method is usefully to detect and characterize the bacteria having implications in the fermentation process as well as preservation of traditional products.

Key words: PCR, Sequence, Lactic bacteria, Vastedda della valle del Belice cheese.

INTRODUCTION – The "Vastedda della valle del Belice" cheese is an unripened cheese obtained from raw ovine milk of the Valle del Belice breed ewes reared in the Belice area. This historical cheese is the only *pasta filata* cheese produced in Italy with raw sheep milk. The metabolic activity of the lactic microflora in the raw milk determine the dropping of pH in the curd, that happens after 6-48 h of curd maturation according to different environmental temperatures. Near to pH 5.5-5.2, the curd is stretched using water or whey at 90-95°C. The stretching is made manually and the shape to the Vastedda cheese is due to ceramics plates. The characteristics of a typical product arise mainly from the specific raw materials employed, the environmental conditions, the area of production, the animal feeding, the traditional tools. The microflora of the cheese is responsible for the biochemical transformation leading the development of the typical aroma and taste. Further knowledge is needed to understand the relationships between microbiological and biochemical properties of the various bacteria which lead to the development of typical textures and flavors.

No published data have reported on the structure of the microbial community for this Sicilian historical cheese, although this may represent important knowledge for improving process conditions in order to enhance the quality of the final product while preserving its typical nature. In the present study a combined microbiological and molecular approach was used to evaluate the microbial diversity occurring in the cheese via analysis 16S rDNA variable regions by PCR and sequencing.

MATERIAL AND METHODS – A total of 18 samples of Vastedda cheese, produced in different dairies located around the Belice river, were collected. The samples were analysed by plating appropriate ten-fold dilutions onto specific media for lactococci (M17 medium) and lactobacilli (acidified MRS agar). Two series of M17 agar plates were inoculated and incubated under aerobic conditions at 22°C for 48 h and 44°C for 72 h, moreover two series of acidified MRS agar plates were inoculated and incubated under micro-aerobic conditions (5% CO_2) at 37°C for 72 h. Results were calculated as the means of two determinations. A representative number of colonies were randomly taken from each batch of cheese. After microscopic observation the colonies were sub-cultured to purity on M17 or MRS media. The Gram positive and catalase negative strains were analysed for PCR identification.

Purity strains were used for the DNA extraction as described below. The investigation has been lead on a total of 31 strains. The bacteria were recovered by the surface of the medium and resuspesed in TE 0,1X. A total of 2 µl of the mixture was employed directly for the PCR targeted to the ribosomal intergenic regions (ITS). The amplification of a portion of approximately 350-bp of the variable regions of 16S rDNA has been realized using primer homologues to conserved regions of rDNA (Muyzer *et al*., 1993; Schwieger *et al*., 1998). Polymerase chain reaction conditions consisted of 25 cycles (1 min at 94°C, 1 min at 55°C, 4 min at 72°C) plus one additional cycle with a final 5 min chain elongation. The presence of specific PCR products was controlled by agarose (2% w/v) gel electrophoresis at 80 V. The obtained fragments have been purified, sequenced by Big Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) and visualized on genetic analyzer AB 310 (Applied Biosystems). The data were elaborate by BLAST2 software to determine the closest known relatives of the partial 16S rDNA sequences obtained. Our results were searched in public data libraries (GenBank) to establish the homology degree. Moreover, in the isolated strains were investigated the power of synthesis for inhibitor factors. The inhibitory ability was detected by cultivating the identified lactic species, togheter to other bacteria as *Listeria monocytogenes*, *Salmonella enteriditis*, *Staphylococcus aureus*. The secreted molecules, have been subjected to the action of factors which pH (pH 3, 5, 7), temperature (25, 60, 80, 100°C), and enzyme action (trypsina, chimotrypsina, pronase, pepsina), in order to evaluate the chemical nature and the levels of activity.

RESULTS AND CONCLUSIONS –The number of the bacteria were comprised in the ranges between 1.5 10^6 ufc/g-3.5 10^8 ufc/g; 7.0 10^5 ufc/g-7.9 10^8 ufc/g; 4.3 10^6 ufc/g-5.7 10^8 ufc/g for lactobacilli, mesophile lactococci, and thermopile lactococci respectively. The GeneBank accession numbers for 16S rDNA partial sequences relative to the closest identified fragment are showed in the table 1. As a result of the sequencing of the rDNA, the Vastedda cheese microflora was found to consist of closest relatives to *Enterococcus, Streptococcus, Lactobacillus, Lactococcus and Pediococcus* genus. The more frequent species were: *Enterococcus faecium* (4/18) and *Streptococcus bovis* (4/18). We observed as to different morphologically colonies corresponded the same species and genotype with the same GeneBank accession number (Table 1). We investigated the ability of the isolated strains to produce the inhibitor factors. It was found only one strain (*Lactococcus lactis* n. 467) producing own bacteriocines with direct bacteriostatic effects against Listeria monocytogenes in experimental procedures. On the basis of the obtained data we can suppose the production of class II bacteriocines. Theese molecules could have a potential effect to control a variety microrganisms, including Gram-negative bacteria and molds. The preliminary results of this study represent a contribution to the knowledge on the microflora of the "Vastedda della valle del Belice" cheese. Moreover, the microbiological and molecular approach may represent a tool of utmost importance in ecological studies looking at specific microflora development in traditional cheese. The preliminary obtained data show the optimized method is usefully to detect and characterize the bacteria having implications in the fermentation technologies as well as preservation of some sensory attributes belonging to these cheeses.

Table 1. List of the identified bacteria in cheese samples. (ID): identification.

ID	Species	Accession number	Alligneament %	Vastedda ID
462	Lactococcus garvieae	AY430484	98	1
463	Lactococcus garvieae	AY430484	93	1
466	Lactococcus lactis	AY675242	97	1
467	Lactococcus lactis	AY675242	90	1
468	Lactococcus garvieae	AY430484	100	1
469	Lactococcus garvieae	AY430484	98	1
470	Lactococcus garvieae	AY430484	98	1
471	Lactococcus garvieae	AY430484	97	1
1014	Lactobacillus casei	AY675252	99	2
1049	Lactobacillus rennini	AY332395	99	2
972	Pediococcus acidilactici	DQ294959	99	3
976	Leuconostoc pseudomesenteroides	AF468002	98	4
982	Streptococcus infantarius	AF177729	99	5
986	Pediococcus acidilactici	DQ294959	99	6
990	Lactobacillus rennini	AY332395	99	7
992	Streptococcus bovis	AB002481	99	8
1024	Lactobacillus delbrueckii	AY773949	98	9
206	Enterococcus faecium	AB062557	99	10
209	Enterococcus faecalis	AY692453	98	10
210	Enterococcus faecalis	AY692453	99	10
231	Enterococcus faecalis	AY692453	99	11
236	Streptococcus infantarius	AF177729	97	11
1024	Lactobacillus delbrueckii	AY773949	98	12
1032	Enterococcus faecium	AY172570	98	13
1042	Enterococcus faecium	AY172570	99	14
1043	Streptococcus bovis	AB002481	99	14
1006	Streptococcus bovis	AB002481	99	14
1062	Streptococcus bovis	AB002481	98	15
1068	Streptococcus bovis	AB002481	98	16
1067	Enterococcus faecium	AJ874342	99	17
1071	Lactobacillus casei	AY675252	98	18

The research was supported by grant APQ Ricerca – Dipartimento Industria, Assessorato Industria Regione Siciliana

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