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Isolation, Phenotypic and Molecular Identification of *Lactococcus lactis* Isolates from Traditionally Produced Village Cheeses

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Abstract: A total of 58 Village cheese samples produced from raw milk without using starter culture in Aydin province have been used for isolation of *Lactococcus lactis* isolates. For this purpose, a typical colony from each cheese sample representing lactococcus phenotype was confirmed by PCR using specific primers designed for conserved 16S rDNA regions. The number of bacteria growing on M17 agar plates containing nalidixic acid ranged from 1.1×10^{7}-1.7×10^{9} cfu g^{-1} suggesting that lactococci is widespread in Village cheeses. Catalase activity, microscopy, growing at 10 and 45°C and in the presence of 2, 4 and 6.5% of NaCl were used for phenotypical characterization of isolates. By phenotypical characterization 49 isolates represented lactococcus profile. All isolates were then analyzed by PCR and 29 of them were identified as *L. lactis* sp. *lactis*, while only 4 were *L. lactis* sp. *cremoris*. About 16 isolates exhibiting lactococcal profile with phenotypic tests did not give any amplification band with the tested primers however, 2 isolates having atypical phenotype were identified as sp. *lactis* by PCR. The results of the present study suggest that Lactococcus is the widespread in the flora of village cheeses and would have important role in the formation of desired flavour and textural properties.

Key words: Lactococcus, village cheese, isolation, characterization, DNA region, catalase activity

INTRODUCTION

Lactococci is Gram positive, non-motile, catalase negative cocci belonging to lactic acid bacteria group. With an optimum growth temperature of 30°C, they can survive at 10°C but not able to grow at 45°C. They are generally members of Group N in the Lancefield serologic classification (Schleifer *et al*., 1985). This genus comprises of five species; *L. lactis*, *L. garvieae*, *L. piscium*, *L. plantarum* and *L. raffinolactis* and recently a new species named *L. chungangensis* took place in the nomenclature (Cho *et al*., 2008).

Among these species, *L. lactis* sp. *lactis* and *L. lactis* sp. *cremoris* are frequently used as starter cultures in the dairy industry, notably in cheese technology (Cogan and Hill, 1993). Understanding the importance of the microfloras in the production of traditional cheeses requires discrimination of strains in a mixed population. Classical identification methods, such as physiological and biochemical tests can not be efficient in some case to differentiate organisms at the species and subspecies levels. The *L. lactis* sp. *lactis* can be discriminated from the *L. lactis* sp. *cremoris* by arginine hydrolysis and differences in their growth characteristics at pH 9.2, 40°C and at 4% saline concentration in the medium (Salama *et al*., 1995). Molecular approaches developed in the last decades facilitated the characterization of bacterial species (Pu *et al*., 2002). Specific primers designed to differentiate *L. lactis* sp. *lactis* and *cremoris* subspecies have been using by a simple PCR assay (Beimfohr *et al*., 1997; Ward *et al*., 1998; Garde *et al*., 1999; Basaran *et al*., 2001).

It is generally accepted that cheeses made from raw milk have more intense flavour than cheeses made from pasteurised milk (Beuvier *et al*., 1997; Demarigny *et al*., 1997; Albenzino *et al*., 2001). Combination of starter and Non Starter Lactic Acid Bacteria (NSLAB) originating from raw milk may have role in the formation of typical

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flavour of these cheeses (Moreno *et al*., 2006). Thus, the biodiversity of LAB involved in cheese production is considered as a key factor for quality of artisanal products. Village cheese is a widespread traditionally produced cheese variety in Turkey. Since it is produced from raw milk and consumed freshly, attentions had been mainly paid on the pathogenic microorganisms and food borne diseases related to the consumption of this cheese (Gumussoy and Gonulalan, 2005; Little *et al*., 2008).

Nevertheless, although Village cheese is preference because of its desired aroma and sensory characteristics there are no detailed data in the literature on its lactic acid flora and mainly lactococcal profile. The aim of this study is to isolate Lactococcus bacteria from Village cheeses and identify isolates by phenotypic tests and PCR at the subspecies level. The isolates may be used in the further studies for better understanding the role of lactococcci in Village cheeses.

MATERIALS AND METHODS

Cheese sampling: About 58 village cheese samples produced from raw milk were collected from various markets and bazaars in Aydin province and transported to the laboratory for analysis.

Microbiological analysis: About 10 g of cheese was homogenized in 90 mL of 2% sodium citrate using a BagMixers after which 10-fold serial dilutions were prepared in peptone bacteriological solution (0.1% bacteriological peptone and 0.85% NaCl). A volume of 1 mL of appropriate dilutions was used for double layer inoculation on the M17 agar (Merck 1.15108) supplemented with nalidixic acid (40 mg L^{-1}, Sigma N4382) and incubated at 30°C for 48 h. After incubation, a typical colony (white-cream colored, 2-3 mm in diameter) was purified twice by plating on M17 agar and M17 broth (Merck 1.15029). The colonies were then tested for morphology by microscopic examination, Gram staining, catalase production, growth at 10 and 45°C and in the presence of 2, 4 and 6.5% of NaCl. Isolates were stored in M17 broth containing 20% (v/v) glycerol at -80°C.

DNA extraction: The isolates were allowed to grow in liquid M17 lactose medium overnight at 30°C and centrifuged in 2 mL Eppendorf tubes for 5 min at 10.000 rps (Eppendorf mini plus). The supernatant was discarded and the bacterial pellet was washed twice with cold (+4°C) distilled water and re-centrifuged. The DNA was isolated using the genomic DNA isolation kit according to the protocol provided by the manufacturer (Fermentas, FE-K0512). DNA from *L. lactis* sp. *lactis* NRRL-B 1821, *L. lactis* sp. *lactis* CECT 4432 and *L. lactis* sp. *cremoris* NRRL-B 634 obtained from Izmir Institute of Technology were used as positive control.

PCR identification: In order to amplify the 16S rDNA gene, the oligonucleotide primers described by Pu *et al*. (2002) LacreR (19-GGGATCATCTTTGAGTGAT), LacF (19-GTACTTGTACCGACTGGAT) and CreF (19-GTGCTTGCACCGATTTGAA), for the specific amplification of *L. lactis* sp. *lactis, L. lactis* sp. *cremoris*, respectively were used. PCR reactions were carried out by using a Perkin Elmer Thermocycler (Model 3600) in a final volume of 50 mL containing 1.0 μL of template DNA, 5 mL of 10×PCR buffer, 25 mM MgCl2, 25 mM deoxynucleoside triphosphates, each primer at a concentration of 10 pmol μL^{-1} and 5 U of Taq polymerase (Fermentas, FE-EP0602). The amplification cycle was as follows: an initial denaturation of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 58°C for 40 sec and extension at 72°C for 1 min (Pu *et al*., 2002). PCR products were runned by electrophoresis on %1 (w/vol) agarose gels (Fermentas, Basica Le, HS-8012) in TAE buffer (Fermentas, FE-B49) at 90 V for the first 5 min and at 60 V for the remaining 60-75 min (ThermoEC 250-90). Gels were stained by 0.03 μL mL^{-1} of ethidium bromide (Fermentas, ZD-A1152). Pictures of the gels were digitally captured using the Spectroline TC-312E/F UV trans-illuminator, camera Pulnix TM-7ETX and Sony printer.

RESULTS AND DISCUSSION

About 55 microscopically coccal formed isolates were selected from 58 cheese samples (Table 1). Special attention has been paid to select only one typical colony from each of cheese sample. The counts of bacteria grown on M17 plates demonstrated variations and ranged from 1.1×10^{7}-1.7×10^{9} cfu g^{-1} implying that lactococcal flora is widespread in Village cheeses. Bacteria from three colonies were morphologically in rod forms. Out of other 55 isolates, 49 displayed typical lactococcal profile by their ability to grow at 10°C but not at 45°C and 6.5% of NaCl. All the isolates were then subjected to molecular identification at the subspecies level by using lactis and cremoris specific primers. Whilst 29 isolates (~57%) were identified as *L. lactis* sp. *lactis*, 4 isolates (~7%) identified as *L. lactis* sp. *cremoris* by PCR. Although, 16 isolates displayed lactococcus profile by phenotypic tests, any amplification band either with lactis or cremoris primers were observed. Interestingly, two isolates (isolates 4 and 7) growing in media containing 6.5% of NaCl, thus they did not represent lactococcal profile by phenotype

Table 1: Phenotypic characteristics and PCR results of the isolates from village cheeses

	Phenotypic characteristics							
Isolate	Morphology	Growth in 2% NaCl	Growth in 4% NaCl	Growth in 6.5% NaCl	Growth at 10°C	Growth at 45°C	Catalase activity	PCR identification
1	cocci	+	+	-	+	-	-	NI
2	rods	+	+	+	+	+	-	NI
3	cocci	+	+	-	+	-	-	NI
4	cocci	+	+	+	+	-	-	*L. lactis*
5	cocci	+	+	-	+	-	-	*L. lactis*
6	cocci	+	-	-	+	-	-	*L. cremoris*
7	cocci	+	+	+	+	-	-	*L. lactis*
8	cocci	+	+	-	+	-	-	NI
9	rods	+	+	+	+	+	-	NI
10	cocci	+	+	-	+	-	-	NI
11	cocci	+	-	-	+	-	-	*L. cremoris*
12	cocci	+	+	-	+	-	-	*L. lactis*
13	cocci	+	-	-	+	-	-	*L. cremoris*
14	cocci	+	+	-	+	-	-	NI
15	cocci	+	+	-	+	-	-	NI
16	cocci	+	+	-	+	-	-	NI
17	cocci	+	+	-	+	-	-	*L. cremoris*
18	rods	+	+	+	+	+	-	NI
19	cocci	+	+	-	+	+	-	NI
20	cocci	+	+	+	+	+	-	NI
21	cocci	+	+	-	+	-	-	*L. lactis*
22	cocci	+	+	-	+	-	-	*L. lactis*
23	cocci	+	+	-	+	-	-	*L. lactis*
24	cocci	+	+	-	+	-	-	*L. lactis*
25	cocci	+	+	-	+	-	-	*L. lactis*
26	cocci	+	+	-	+	-	-	NI
27	cocci	+	+	-	+	-	-	*L. lactis*
28	cocci	+	+	-	+	-	-	*L. lactis*
29	cocci	+	-	-	+	-	-	NI
30	cocci	+	+	-	+	-	-	*L. lactis*
31	cocci	+	+	-	+	-	-	*L. lactis*
32	cocci	+	+	-	+	-	-	NI
33	cocci	+	+	-	+	-	-	NI
34	cocci	+	+	-	+	-	-	NI
35	cocci	+	+	-	+	-	-	*L. lactis*
36	cocci	+	+	-	+	-	-	*L. lactis*
37	cocci	+	+	+	+	-	-	NI
38	cocci	+	+	-	+	-	-	*L. lactis*
39	cocci	+	+	-	+	-	-	*L. lactis*
40	cocci	+	+	-	+	-	-	*L. lactis*
41	cocci	+	+	-	+	-	-	*L. lactis*
42	cocci	+	+	-	+	-	-	NI
43	cocci	+	+	-	+	-	-	*L. lactis*
44	cocci	+	+	-	+	-	-	NI
45	cocci	+	+	-	+	-	-	*L. lactis*
46	cocci	+	+	-	+	-	-	*L. lactis*
47	cocci	+	+	-	+	-	-	*L. lactis*
48	cocci	+	+	-	+	-	-	NI
49	cocci	+	+	+	+	-	-	NI
50	cocci	+	+	-	+	-	-	*L. lactis*
51	cocci	+	+	-	+	-	-	*L. lactis*
52	cocci	+	+	-	+	-	-	NI
53	cocci	+	+	-	+	-	-	*L. lactis*
54	cocci	+	+	-	+	-	-	*L. lactis*
55	cocci	+	+	-	+	-	-	*L. lactis*
56	cocci	+	+	-	+	-	-	*L. lactis*
57	cocci	+	+	-	+	-	-	NI
58	cocci	+	+	-	+	-	-	NI

NI: Not Identified

but were identified as *L. lactis* sp. *lactis* by PCR. The differentiation between *L. lactis* sp. *lactis* and *L. lactis* sp. *cremoris* is important due to the difference in their characteristics that they possess during the production of cheese. For a long time, the differentiation between the subspecies *lactis* and *cremoris* has been carried out

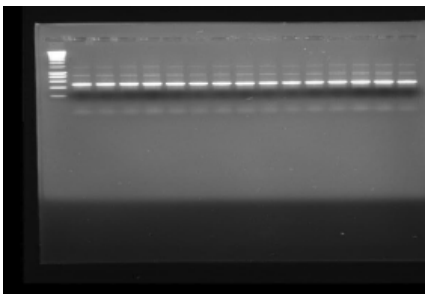

Fig. 1: The specific multi-copy PCR product regions of 161 bp lenght in various strains

based on the ammonia production from arginine test. However, this test was reported not to give reliable results at all times (Garde *et al*., 1999).

The study reported here shows that the PCR methodology can be used in efficient differentiation of the two subspecies of the *L. lactis* isolates from the Village cheeses that are traditionally produced without using starter cultures (Fig. 1).

In the present study, 49 isolates were phenotypically identified as lactococci. By PCR assay, 29 of these isolates were confirmed as *L. lactis* sp. *lactis* and 4 isolates as *L. lactis* sp. *cremoris*. Bacteria that are members of this species can be confused with others, especially when they are categorized based on their phenotypic characteristics (Deasy *et al*., 2000). The strains with negative responses might have belonged to other groups of microorganisms of the *Lactococcus* genus.

This shows that the combination of different biochemical and molecular techniques may efficiently be used in the identification lactococcal isolates. Isolates exhibiting atypical profile were also reported by other researchers (Salama *et al*., 1993; Klijn *et al*., 1995; Ward *et al*., 1998; Mangin *et al*., 1999; Fortina *et al*., 2003; Bulut *et al*., 2005; Nomura *et al*., 2006; Ouzari *et al*., 2006). When the two sub-species of interest were taken into consideration, *L. lactis* sp. *lactis* was found to be more dominant in the Village cheese flora than *L. lactis* sp. *cremoris*. *L. lactis* sp. *cremoris* is generally a rare subspecies isolated from cheeses. Researchers have concluded that *L. lactis* sp. *lactis* was more stable in environmental conditions in comparison to *L. lactis* sp. *cremoris* which has less chance of survival in a non-dairy environment (Klijn *et al*., 1995; Corroler *et al*., 1998). From this perspective, the crowded presence of *L. lactis* sp. *lactis* in the environment of cheese production resulted in its dominance over *L. lactis* sp. *cremoris* in natural cheese flora.

CONCLUSION

The results of the current study revealed that lactoccocci are widespread in traditionally made Village cheese but their role in the maturation process and their contribution to flavour development are required to be elucidated. Isolates obtained by the present study may constitute a basis for the future experiments. Cheese manufacturing assays by using identified isolates would be much more interesting to evaluate their role in the ripening process.

REFERENCES

Albenzino, M., M.R. Corbo, S.U. Rehman, P. F. Fox and M. de Angelis *et al*., 2001. Microbiological and biochemical characteristics of Cane Strato Pugliese cheese made from raw milk, pasteurized milk or by heating the curd in hot whey. Int. J. Food. Microbiol., 67: 35-48.

Basaran, P., N. Basaran and I. Cakir, 2001. Molecular differentiation of *Lactococcus lactis* subspecies *lactis* and *cremoris* strains by ribotyping and site-specific PCR. Curr. Microbiol., 42: 45-48.

Beimfohr, C., W. Ludwig and K.H. Schleifer, 1997. Rapid genotypic differentiation of *Lactococcus lactis* subspecies and biovar. Syst. Applied Microbiol., 20: 216-221.

Beuvier, E., K. Berthaud, S. Cegarra, A. Dasen, S. Pochet, S. Olange Buchin and G. Duboz, 1997. Ripening and quality of Swiss-type cheese made from raw, pasteurized or microfiltered milk. Int. Dairy J., 7: 311-323.

Bulut, C., H. Gunes, B. Okuklu, S. Harsa, S. Kilic, H.S. Coban and A.F. Yenidunya, 2005. Homofermentative lactic acid bacteria of a traditional cheese, *Comlek peyniri* from Cappadocia region. J. Dairy Res., 72: 19-24.

Cho, S.L., S.W. Nam, J.H. Yoon, J.S. Lee, A. Sukhoom and W. Kim, 2008. *Lactococcus chungangensis* sp. nov., a lactic acid bacterium isolated from activated sludge foam. Int. J. Syst. Evol. Microbiol., 58: 1844-1849.

Cogan, T.M. and C. Hill, 1993. Cheese Starter Cultures. In: Cheese: Chemistry, Physics and Microbiology, Fox, P.F. (Ed.). Chapman and Hall Publishers, London, England, pp: 193-255.

Corroler, D., I. Mangin, N. Desmasures and M. Gueguen, 1998. An ecological study of lactococci isolated from raw milk in the camembert cheese registered designation of origin area. Applied Environ. Microbiol., 64: 4729-4735.

Deasy, B.M., M.C. Rea, G.F. Fitzgerald, T.M. Cogan and T.P. Beresford, 2000. A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus*. Syst. Applied Microbiol., 23: 510-522.

Demarigny, Y., E. Beuvier, S. Buchin, S. Pochet and R. Grappin, 1997. Influence of raw milk microflora on the characteristics of Swiss-type cheeses: II. Biochemical and sensory characteristics. Lait, 77: 151-167.

Fortina, M.G., G. Ricci, A. Acquati, G. Zeppa, A. Gandini and P.L. Manachini, 2003. Genetic characterisation of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the *Toma piemontese*. Food Microbiol., 20: 397-404.

Garde, S., M. Babin, P. Gaya, M. Nunez and M. Medina, 1999. PCR amplification of the gene *acmA* differentiates *Lactococcus lactis* subsp. *lactis* and L. *lactis* subsp. *cremoris*. Applied Environ. Microbiol., 65: 5151-5153.

Gumussoy, G.F. and Z. Gonulalan, 2005. Studies on the presence of *Enterohemorrhagic Escherichia coli* O157:H7 strain in raw cheese sold in public open markets in Kayseri province. J. Health Sci., 14: 13-19.

Klijn, N., A.H. Weerkamp and W.M. de Vos, 1995. Detection and characterization of lactose–utilizing *Lactococcus* spp. in natural ecosystems. Applied Environ. Microbiol., 61: 788-792.

Little, C.L., J.R. Rhoades, S.K. Sagoo, J. Harris and M. Greenwood *et al*., 2008. Microbiological quality of retail cheeses made from raw, thermized or pasteurized milk in the UK. Food Microbiol., 25: 304-312.

Mangin, I., D. Corroler, A. Reinhardt and M. Gueguen, 1999. Genetic diversity among dairy lactococcal strains investigated by polymerase chain reaction with three arbitrary primers. J. Applied Microbiol., 86: 514-520.

Moreno, M.R.F., P. Sarantinopoulos, E. Tsakalidou and L. de Vuyst, 2006. The role and application of *Enterococci* in food and health. Int. J. Food Microbiol., 106: 1-24.

Nomura, M., M. Kobayashi, T. Narita, H. Kimoto-Nira and T. Okamoto, 2006. Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. Applied Environ. Microbiol., 101: 396-405.

Ouzari, H., A. Hassen, A. Najjari, B. Ettoumi and D. Daffonchio *et al*., 2006. A novel phenotype based on esterase electrophoretic polymorphism for the differentiation of *Lactococcus lactis* sp. *lactis* and *cremoris*. Lett. Applied Microbiol., 43: 351-359.

Pu, Z.Y., M. Dobos, G.K.Y. Limsowtin and I.B. Powell, 2002. Integrated polymerase chain reaction-based procedures for the detection and identification of species and subspecies of the Gram-positive bacterial genus *Lactococcus*. J. Applied Microbiol., 93: 353-361.

Salama, M.S., T. Musafijajeknic, W.E. Sandine and S.C. Giovannoni, 1995. An ecological study of lactic acid bacteria: Isolation of new strains of *Lactococcus* including *Lactococcus lactis* subspecies *cremoris*. J. Dairy Sci., 78: 1004-1017.

Salama, M.S., W. Sandine and S.J. Giovannoni, 1993. Isolation of *Lactococcus lactis* subsp *cremoris* from nature by colony hybridization with rRNA probes. Applied Environ. Microbiol., 59: 3941-3945.

Schleifer, K.H., J. Kraus, C. Dvorak, R.K. Balz, M.D. Collins and W. Fischer, 1985. Transfer of *Streptococcus lactis* and related *Streptococci* to the genus *Lactococcus* gen. nov. Syst. Applied Microbiol., 6: 183-195.

Ward, L.J.H., J.C.S. Brown and G.P. Davey, 1998. Two methods for the genetic differentiation of *Lactococcus lactis* sp. *lactis* and *cremoris* based on differences in the 16S rRNA gene sequence. FEMS Microbiol. Lett., 166: 15-20.