Isolation and Identification the Indigenous Lactic Flora From Lighvan, as an Iranian Raw Milk Cheese From Milk to Ripened cheese

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Abstract— Nearly 100 strains isolated from different media for lactic acid bacteria of Lighvan cheese have been identified in four different production stages (milk, curd, fresh cheese and ripened cheese). Selectivity of different media including MRS, MRS+vancomyci, M17 and KAA was evaluated and colony forming unit at different temperatures (30, 37 and 45°C) were determined. Random colonies were selected from each medium corresponding to highest dilution and confirmatory tests revealed Enterococci (38.77%), Lactobacilli (31.66%) and Lactococci (20.4%) as the most frequent genus identified in all stages. Results from API system detected following species in all stages: Lb.plantarum,Lb.brevis,Lb.paracasei ssp.paracasei,Lb.delbrueckiissp.delbreuckii,Lb.fructivorans,Lac.lactis ssp.lactis,Ent.faecium,Ent.faecalis,Ent.durans,Pediococcus.pentosaceus,Leu.lactis and Leu.mesenteroides. The evolution of LAB showed a pattern by the dominance of Lactococci and Lactobacilli in the first stage and substitution of these genuses by Enterococci at the end of ripening. The most predominant strains were Ent.faecium (22.44%), Lac.lactisssp.lactis (20.4%), Lb.plantarum (18.36%) and Ent.faecalis (14.28%) respectively. It seems that these three species play an important role in ripening and production of Lighvan cheese and have potential for application in industrial scale.

***Keywords*—** Lactic acid bacteria, Lighvan, production stage, raw milk cheese.

I. INTRODUCTION

THE impact of lactic flora on the sensory and physical properties of different raw milk cheeses has been investigated by others worldwide. The aim of such investigations is the ability of industrializing the traditional products. No such investigation was performed on Iranian raw milk cheeses.

Lactic acid bacteria (LAB) that were added as a starter culture or present as a indigenous flora in milk play an important role in cheese flavor. On the other hand, some LAB like Enterococci can inhibit the pathogenic bacteria growth because of bacteriocin production. That is why, these bacteria are very important from technological point of view. Some particular LAB ,such as Enterococcus faecalis,Enterococcus faecium, lactobacillus curvatus,lactobacillus paracasei ssp paracasei and Lactococcus lactis ssp. lactis has the ability of bacteriocin production[1].

Industrially, the applications of certain starter cultures cause consistent quality but result in product with limited flavor. On the other hand, the consumers prefer dairy products with original taste. That is why; the exploration of wild strains existing in nature and traditionally fermented foods is being interested for manufacturing of novel dairy products with original taste. Raw milk cheeses produced traditionally have this potential for isolation of new strains for exploiting in dairy industry [2].

Among several raw milk cheeses in Iran, Lighvan cheese presents a most famous and popular traditional Iranian cheese made from raw sheep milk. This popularity is dedicated to its typical piquant flavor [3].

This cheese is categorized as a semi soft cheese with a desired sour taste, cream color and high fat content and crumbly texture. Lighvan cheese is produced in a village with a same name in a region of East Azerbaijan province in some small local dairies [2],[3].No starter is used in the production of this cheese and only natural lamb rennet is added then final coagulum is cut and drained using cheesecloth and pressed. The produced curd is put in brine (22%) for 24h followed by in brine (12%) in containers for long time about at least 3 months. Ripening period takes place in underground caves that naturally have cold temperature. Indigenous Lactic acid bacteria presented in milk have proteolytic, lipolytic and glycolytic activity during ripening and produce different aromatic compounds that related to typical cheese flavor.

Exploration and identification of native starter and wild strains is necessary for producing hygienic Lighvan cheese with typical flavor on an industrial scale.

II. MATERIALS AND METHODS

A. Sampling

Samples were collected from producing region (Lighvan valley, Tabriz city, East Azerbaijan, Iran) from several local dairy factories randomly in summer (2009). Samples were taken at 4 different stages (milk, curd, and fresh cheese 1-day old, ripened cheese 3-months old).Then samples were transferred to microbiology laboratory under hygienic conditions.

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B. Cheese Manufactures

Raw whole sheep milk was collected and transferred to pilot plant dairy factory. Then through passing cloth, milk was sieved and poured into the metallic big containers. At this step milk samples were collected aseptically in to the pre-sterilized glass bottles. In next step, with the help of putting the water container in to the milk container, the milk temperature was reduced to 25°C and at this temperature the commercial rennet was to the milk for coagulation. This process took place about 2 hours. Sampling from curd was performed at this step. The resulting curd was cut into pieces and the main part of whey was removed at this step. The curd pieces were covered with cheesecloth and whey drainage lasted for 5 hours. The curd was cut into the blocks and placed in 24% brine for 24h followed by salting on the surface with coarse-grained salt for 24 h. Fresh cheese samples were taken at this stage. On the following day, cheese was placed in tins with 12% brine and transferred to under-ground caves (8-10°C) and ripened at least for 3 months. Ripened cheese samples were collected after this period.

C .Isolation and Identification of Isolates

1. Preparation of Milk, Curd and Cheese Samples

For milk samples, decimal dilutions were prepared directly in 0.1% sterile peptone water and regarding the curd and cheese samples , 25g sample was added to 225 ml sterile Na-citrate solution(2%w/v)and this mixture was transferred to stomacher bag and were homogenized in a Stomacher homogenizer(Type 400,Seward,UK).This solution was used as a 0.1 dilution and for following dilution,0.1% peptone water was used. Isolation and colony counts were made on the following media: MRS Agar(Merck) for isolation of lactobacilli,M17 for lactococci, MRS Agar+vancomycin(20µg/mL) for leuconostocs, KAA (Kanamycin aesculin azid agar) for Enterococci. Experiments were conducted duplicates. Finally, inoculated plates incubated anaerobically(Gas-pack system) at 30,37 and 42°C temperatures for mesophilic and thermophilic bacteria. Incubation period varied between 24-48h and 72 h depending on the bacteria. Counting plates was conducted only for those plates contained 30-300 colonies and from plates corresponding to highest dilution,4-5 different colonies(according to shape, size and color) were selected randomly. Then, these colonies purified 2 or 3 times on the same media for purification. Purified, single colonies from each plate were examined by Gram–staining and catalase production and microscopic observations. Finally, only Gram-positive, catalase-negative isolates were considered and stored in MRS broth containing 20% glycerol and were freeze-dried. Totally, about 100 isolates from different samples (milk, curd, fresh and ripened cheese) were subjected to biochemical and confirmatory tests.

2. Biochemical and Confirmatory Tests

Firstly, Gram-staining and catalase production were performed on each single and purified colony. After confirming the gram positive and catalase negative, morphology and bacilli or cocci was determined by microscopic observation. Then, Growth at 10 1nd 45 °C temperatures, growth at 6.5% salt, pH=9.6, L-argentine hydrolysis with Nessler's reagent, aesculin hydrolysis, production of Co2 from glucose in MRS broth(with Durham tubes),citrate utilization in Simon citrate Agar, Vogous-proskoar test in MR-VP medium were conducted as confirmatory tests.

Finally, Gram-positive, catalase-negative homofermentative cocci capable of growing at 10°C and pH9.6 but not at 45°C or in 6.5% NaCl were considered as Lactococci. Argenine hydrolysis, acetoin production was also carried out as confirmatory tests. For typing to species level, API 50 CH with medium 50 CHL (Biomeriux, France) was used.

Gram-positive, catalase negative and homofermentative cocci which can grow at 10 and 45°C and in 6.5% salt and pH9.6 were considered as Enterococci. For typing to species level, API 20 STREP (Biomeriux, France) was used. Gram-positive, catalase-negative and homofermentative cocci with tetrad morphology were considered Pedicocci and Gram-positive, catalase-negative but heterofermentative cocci which could not hydrolyze argenine were considered as Leuconostocs. Gram-positive, catalase-negative bacilli were tested using growth at 10 and 45°C and gas production from glucose in Durham tubes for hemo and heterofermentative. Finally, after identification all isolates at genus level with biochemical and confirmatory tests, carbohydrates fermentation tests was performed using API 50 CH and API 20 STREP for Enterococci according to manufacture's procedure.

III. Results and Discussion

Isolation and identification of LAB of Lighvan cheese was performed at 4 different stages: milk, curd, fresh cheese (1-day old cheese), ripened cheese (90-days old cheese).Lactic acid bacteria were isolated from MRS, MRS+vancomycin, M-17 and KAA agar media. The strains isolated were identified using morphology, colony pigmentation, production of carbon dioxide from glucose, growth at 4 and 40°C, salt tolerance, starch hydrolysis, and sugar fermentation with the API system methods.

TABLE I. DISTRIBUTION OF LAB ISOLATED FROM DIFFERENT MEDIA DURING PROCESSING FROM MILK TO RIPENED LIGHVAN CHEESE.

	Media				
Genus	MRS	MRS+vancomycin	M17	KAA[a]	Total
Lactobacillus	22	8	1	-	31
Lactococcus	3	1	14	2	20
Enterococcus	4	-	10	24	38
Pedicoccus	3	-	1	-	4
Leuconostoc	1	1	-	-	2
ND*	2	-	1	-	3
total	35	10	27	26	98

*ND= Not determined strains

a, Kanamycin aesculin azid agar

The distribution of LAB isolated from different media for Lighvan(at four steps) is presented in Table1.This table gives us some information about the selectivity and suitability of different media for LAB.MRS agar has been suitable for Lactobacilli and this genus was significantly dominated(22/35).Gurses, M et al.(2008) also presented similar results for Tulum cheese[3].On the other hand, other genus such as Lactococcus(3/35), Enterococcus(4/35) and Pediococcus(3/35) and also Leuconostoc(1/35) were found in this medium. Lopez-Diaz et al. (2000),also had founded Lactobacillus and lactococcus more than other genera in this medium, which confirms the usefulness of this medium for isolation of these genera[4].More than half(22 out of the 35) of the isolates on MRS medium were lactobacilli, the others were coccal-shaped cells. MRS medium allows the growth of different genera of coccal-shaped LAB due to low degree of selectivity of this medium [1].

Fox et al. (2000) reported the selectivity of M17 agar for isolation of Lactococci [5]. In our study, also the majority of strains isolated from M17 were lactococci(14/27).In contrast to our results, Navidghasimizad , S. et al.(2009) showed that the most of the strains isolated from M17 medium were Enterococci[2].Almost all of the 27 isolates on M17 agar were coccal-shaped LAB, including Lactococcus(14/27),Enterococcus(10/27) and Pediococcus (1/27).The remaining 1 isolate was Lactobacillus(1/27).The usefulness of this medium has been proven by other researchers[4].

KAA showed high selectivity for Enterococci spp. (24/26.) Gurses,M and Erdogan , A (2006) isolated the majority of Enterococci from PCA agar[3].

MRS in addition to vancomycin is a suitable and selective medium for Leuconostoc spp. due to high resistance of Leuconostocs against this antibiotic. However, other lactic acid bacteria like Lactobacilli and Lactococci can grow on this medium (Table1)[5].3 out of isolates collected from all media were not determined.

TABLE II .LOG COLONY FORMING UNIT(CFU/ML AND LOG CFU/G) AND STANDARD DEVIATION OF LIGHVAN CHEESE AT FOUR DIFFERENT STAGES(MILK, CURD, FRESH AND RIPENED CHEESE).

		Product			
Media	°C	Milk	Curd	Fresh cheese	Ripened cheese
MRS	30	6.825±0.02[a]	7.685±0.3	6.505±0.04	5.925±0.03
	37	7.085±0.12	7.91±0.01	7.86±0.01	6.625±0.03
	45	4.655±0.04	5.82±0.02	5.865±0.24	<1
MRS+ vancomycin	30	6.105±0.09	5.57±0.04	7.12±0.07	5.645±0.06
	37	6.28±0.15	5.74±0.07	7.365±0.06	6.07±0.09
	45	<1*	<1	<1	<1
M17	30	6.43±0.05	7.58±0.15	6.43±0.05	7.085±0.12
	37	6.565±0.07	7.2±0.14	7.085±0.12	7.385±0.12
	45	<1	<1	6.885±0.16	7.625±0.03
KAA	30	6.555±0.06	6.67±0.02	6.245±0.1	uncountable
	37	5.885±0.16	6.235±0.09	5.795±0.14	uncountable
	45	6.005±0.23	6.44±0.04	6.525±0.02	5.085±0.12

*Log Colony forming unit(Log cfu/mL ,Log cfu/g) in these cases was lower than 1.
a Std(standard deviation of two replicates from each observation).

As shown in Table2, in MRS medium, the number of colonies increased from milk to curd and after that decreased until ripened cheese. In MRS+vancomycin medium, was not seen any consistent increasing or decreasing trend from milk to ripened cheese. In M17, there was a increasing trend from milk to curd only in 37°C.In KAA, the increasing trend was seen from milk to fresh cheese and after that this trend decreased to ripened cheese in 45°C.

Table3 shows the biochemical characterization of the lactic acid bacteria isolated from different stages of Lighvan cheese production. Among the lactobacilli, only one species could grow at high temperature and 6.5% salt. All of lactobacilli showed growth in pH9.6 and none of them grew in VP medium. The only heterofermentative lactobacillus was Lb.brevis. Among the cocci, only Enterococci could show growth at high temperatures and at pH9.6.All of the cocci showed growth at 15°C.Lac.lactis ssp. lactis and Leu.lactis did not grow at high concentration of salt (6.5%NaCl).Two heterofermentative species were Leu.lactis and Leu.mesenteroides. All of the cocci could grow at VP medium except for Leu.mesenteroides. Argenine hydrolysis was negative for Leu.lactis and Leu.mesenteroides.

TABLE III. BIOCHEMICAL CHARACTERISTICS OF THE LAB ISOLATED FROM LIGHVAN CHEESE AT DIFFERENT STAGES FROM MILK TO RIPENED CHEESE.

Strain	Growth at 15C	Growth at 45C	Growth at 6.5% salt	Growth at pH 9.6	Co_2 from glucose	VP test	Argenine hydrolysis
Lb.plantarum	+	-	+/-	+	-	-	-
Lb.paracasei ssp.paracasei	+	-	-	+	-	-	-
Lb.brevis	+	-	-	+/-	+	-	+
Lb.delbrueckii ssp delbreuckii	-	+	+	+	-	-	-
Lb.fructivorans	-	+/-	-	+	-	-	-
Lac.lactis ssp lactis	+	-	-	-	-	+	+
Leu.lactis	+	-	-	-	+	+	-
Leu.mesenteroides	+	-	+/-	-	+	-	-
Pediococcus pentosaceus	+	-	+	-	-	+	+
Ent.faecalis	+	+	+	+	-	+	+
Ent.faecium	+	+	+	+	-	+	+
Ent.durans	+	+	+	+	-	+	+

+=positive reaction; -=negative reaction

In order to understand the role of each of these species, firstly we looked at evolution during ripening and at different stages. Figure1. Shows the evolution of each genus during different stages. In milk sample, Enterococci were dominant and after that Lactobacilli and Lactococci followed by Pediococci with lower proportions. During the ripening in next stages, Enterococci and to a lesser extent lactobacilli showed the increasing trend but Lactococci became lower. This phenomenon sounds logical because in most types of cheeses similar trend has been seen.

In first stage of processing, Lactococci which are the responsible for milk acidification are predominant and progress until the formation of curd. In following steps, the other LAB like Enterococci and lactobacilli overcome.

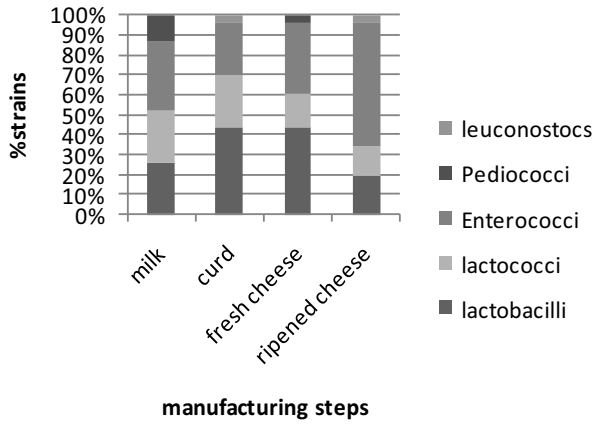

Fig.1 Evolution of genera of LAB during manufacture of Lighvan cheese. Percentage of strains of each genus isolated from media at each stage

In ripened cheese we found high percentage of Enterococci. Due to consistent presence of this genus in different types of cheeses, the impact and influence of Enterococci in manufacturing of cheese has been investigated. There are some contradictory opinions about this group of LAB. Some researchers proved the positive effect of Ent.faecalis var. liqefaciens in the quality of Roquefort cheese [6],[7],[8].They found a beneficial effect of Enterococci on other LAB growth. On the other hand, bacteriocin production (enterocins) by Enterococci has a controlling effect on pathogens in cheese and biogenic amines producing bacteria like some lactobacilli [9]-[12].In contrast, some negative effects on cheese quality and health for Enterococci have been reported. Salvadori(1969) found the development of bitter flavor due to application of Ent.faecalis as a starter in the production of Gorgonzola cheese[13].

Table4. shows the distribution of LAB species found in our cheese samples in different stages of production. Lactobacillus genus (31.63%) comprised the Lb.plantarum (18.36%) the most dominant, followed by Lb.paracasei ssp paracasei(7.14%),Lb.brevis(3.06%),Lb.delbrueckii ssp delbrueckii(2.04%) and Lb.fructivorans(1.02%) in all production stages. Generally, Lactobacilli genus had an increasing trend from milk (26.08%) to curd (43.47%) and thereafter presented the declining trend until the ripened cheese (19.23%). Lb.plantarum was the only species that found in all stages. Lb.brevis did not change significantly during these stages. Gurses , M. and Erdogan, A. showed similar results for Lb.brevis and Lb.curvatus in their research[3]. Leu.mesenteroides and Leu.lactis were detected in curd and ripened cheese respectively. Lac.lactis ssp. lactis was 26.08% in milk and curd samples and after that decreased in ripened cheese. Similar trend was seen by Gurses, M. and Erdogan, A. in Tulum cheese [3].Pediococcus pentosaceus was found only in milk and fresh cheese stages. Finally, a wide variety of Enterococci was found in our samples especially, Ent.faecalis and Ent.faecium were predominant species (18.36%).Ent.faecalis is a common species which isolated frequently from different varieties of cheeses like in blue cheese[4].These genus have been found in different traditional cheeses like Feta, Manchego, Teleme, Comte, Fontina, Serra and Cebreiro [14]-[17].

TABLE.IV. DISTRIBUTION OF LACTIC ACID BACTERIA DURING RIPENING FROM MILK TO RIPENED 3-MONTHS IN LIGHVAN CHEESE.(ACCORDING TO PHENOTYPIC CHARACTERIZATION)

Type of LAB	Production stages				
	Milk(%)	Curd(%)	Fresh cheese(%)	Ripened cheese(%)	Total number(%))
Lactobacillus spp.	6(26.08)	10(43.47)	10(38.46)	5(19.23)	31(31.63)
Lb.plantarum	14(17.39)	5(21.73)	7(26.99)	2(7.69)	18(18.36)
Lb.brevis	1(4.34)	-	1(3.84)	1(3.84)	3(3.06)
Lb.paracasei ssp.paracasei	1(4.34)	3(13.04)	1(3.84)	2(7.69)	7(7.14)
Lb.delbreuckii sspdelbreuckii	-	2(8.69)	-	-	2(2.04)
Lb.fructivorans	-	-	1(3.84)	-	1(1.02)
Leuconostoc spp.	-	1(4.34)	-	1(3.84)	2(2.04)
Leu.mesenteroides	-	1(4.34)	-	-	1(1.02)
Leu.lactis	-	-	-	1(3.84)	1(1.02)
Lactococcus spp.	6(26.08)	6(26.08)	4(15.38)	4(15.38)	20(20.4)
Lac.lactis ssp lactis	6(26.08)	6(26.08)	4(15.38)	4(15.38)	20(20.4)
Pediococcus spp.	3(13.04)	-	1(3.84)	-	4(4.08)
Pediococcus pentosaceus	3(13.04)	-	1(3.84)	-	4(4.08)
Enterococcus spp.	8(34.78)	6(26.08)	8(30.76)	16(61.53)	38(38.77)
Ent.faecium	5(21.73)	4(17.39)	4(15.38)	9(34.61)	22(22.44)
Ent.faecalis	3(13.04)	2(8.69)	4(15.38)	5(19.23)	14(14.28)
Ent.durans	-	-	-	2(7.69)	2(2.04)
Not determined	-	-	3(11.53)	-	3(3.06)
Total	23(100)	23(100)	26(100)	26(100)	98(100)

In Table5, shows some of the selected colonies which were examined with API 20 STREP phenotypic ally. As seen aesculin hydrolysis and L-argenine hydrolysis for all Enterococci were positive. Acid production from D-ribose, D-lactose and D-trehalose for all of the selected Ent.faecalis, Ent.faecium and Ent.durans were positive. None of the Enterococci could produce acid from inulin and glycogen. For other carbohydrates, different species showed different fermentation profile. Only Ent.faecalis could produce acid from D-sorbitol(5/5) and amidon(4/5)(Table5).Among all of the colonies from different media, one Lac.lactis ssp. cremoris and three Lac.lactis ssp. lactis were detected with API 20 STREP.

TABLE. V. PHENOTYPICAL PROFILES OF SOME ENTEROCOCCI AND LACTOCOCCI ISOLATED FROM DIFFERENT STAGES OF LIGHVAN CHEESE ACCORDING TO API 20 STREP.

Characteristic	Ent. faecalis 5[a]	Ent. faecium 8	Ent. durans 2	Lac.lactis ssp cremoris1	Lac.lactis ssp lactis3
Aesculin hydrolysis	5[b]	8	2	0	3
L-Argenine hydrolysis	5	8	2	0	3
Growth on NaCl 6.5%	5	8	2	0	0
Growth at 45°C	5	8	2	0	0
Acid from:					
D-ribose	5	8	2	1	3
L-arabinose	2	8	0	0	1
D-manitol	5	8	0	1	3
D-sorbitol	5	0	0	0	0
D-lactose	5	8	2	1	3
D-trehalose	5	8	2	1	3
Inulin	0	0	0	0	0
D-raffinose	0	1	0	0	0
Amidon(starch)	4	0	0	0	0
Glycogen	0	0	0	0	0

a Number of strains investigated.
b Number of positive strains.

The importance of LAB in cheese production and ripening has been investigated by many scientists. Among them, Lactococci plays an important role in acidification of milk .Lactobacilli participate in the flavor improvement because of proteolytic and lypolytic activities. Leuconostoc species and Lb.brevis due to their heterofermentative property are responsible for gas production and subsequently, results in making some holes in Lighvan cheese.

CONCLUSION

Among the lactic acid bacteria found in our samples, genus Enterococci, Lactobacilli and Lactococci constituted the main LAB flora during production stages respectively. Enterococci were predominant especially in ripened cheese. Enterococci had increasing trend at the end of ripening and Lactococci showed declining trend. Lac.lactis ssp lactis, Ent.faecium, Ent.faecalis and Lb.plantarum were detected from different stages in higher numbers. This fact implies that these strains or species should play an important role in cheese ripening and production and also in aroma and flavor development. In order to application of these strains in industrial scale, more attention should be paid to identification of these species at subspecies level. To obtain this aim, more accurate and precise techniques like molecular assays are required.

ACKNOWLEDGMENTS

Authors must thank for Razavi Dairy Industry (Khorasan Razavi, Mashhad, Iran) for financial support. We appreciate

from Mr.Pourakrami and Dr. Bazmi regarding to collecting cheese samples from Lighvan village.

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