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Isolation and Identification of Yeasts and Lactic Acid Bacteria from Local Traditional Fermented Camel Milk, Chal

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Abstract

The yeasts and lactic acid bacteria in samples of the Iranian traditional fermented camel milk, *Chal* were identified on the basis of physiological and morphological properties. Yeast species were identified including *Pichia. anamala, Pichia. jadinii, Debaryomyces. hansenii, Pichia. guilliermondii, Kluyvermyces. marxianus, Candida. fermentati, Pichia. ciferrii, Torulospora. delbrueckii, Candida. versatilis, Kluyvermyces. lactis, Candida. kefir, Saccharomyces. pastorianus, Saccharomyces. serevisiae, Candida. friedrichii, Kluyvermyces. polysporus, Rhodotorula. musilaginosa, Candida. lipolytica* and *Candida. lusitaniae*. All of them could assimilate the glucose and liquefacted the gelatin, but could not production starch, tolerated 1% acetic acid, growth in the presence of Nacl 16% (except *Debaryomyces. hansenii*), anassimilateed the nitrat (except *Rhodotorula. musilaginosa*). Within the yeasts species, *Kluyvermyces. lactis* (8.57%) and *Kluyvermyces. marxianus* (8.57%) were the predominant. Also 93 different lactic acid bacteria species were identified including 64 bacilli as *lactobicallus* and *wieselia*, 8 cocci, *leuconostoc*, 11 coccobacili, *leuconostoc, lactococcus* and *wieselia*, 2 streptococci, *streptoccus* and 8 tetrad cocci shapes, *pediococci*. All isolates fermented galactose, except *Lactobacillus delbrueckii subsp. Bulgaricus*, *Lactobacillus kefir, Lactobacillus viridescens*. All the isolates could growth at 37°C, only *Leuconostoc mesenteroides subsp. Cremoris* and *Leuconostoc paramesenteroides* could not growth at 30°C. This study revealed Chal contained a wide variety of yeasts and lactic acid bacteria.

Keywords: Chal; Fermented Camel Milk; Yeast; Lactic acid bacteria; Identification

Introduction

In many societies, milk is traditionally consumed predominantly in the form of fermented milk. In many arid areas, camels play a central role as milk suppliers and they are either home-consumed or sold [1].

Chal is a white sparkling beverage that has a sour flavour [2]. The chal is prepared by first souring camel milk in a skin bag or ceramic jar. Previously soured camel milk is added to the fresh milk. It is well mixed and each day, for 3 to 4 days, fresh milk is added to the mixture. Eventually the end product must have 3 to 5 times the original volume of chal that was initially added. This is the best ratio for the chal. The ambient temperature is normally between 25 and 35°C [3]. In many regions, camel milk and chal are used to treat some diseases and to combat health problems such as dropsy, jaundice, tuberculosis, asthma, anaemia and piles [4]. Due to acidic pH, dairy products are favorable environment for growth of yeasts and LAB [5,6]. The yeasts play usefull effect upon the bacteria due to change in pH and secretion of biological substance such as vitamins, enzymes, amino acids etc [7]. The yeasts isolated from dairy products are different, but the most frequently described strains belong to the genera *Saccharomyces, Kluyveromyces, Debaryomyces, Issatchenkia, Galactomyces*, *Fellomyces*, *Pichia* and *Yarrowia* [8-12]. The yeasts occurr in many dairy related products [13,14] and in the human gastrointestinal tract [15,16] and have probiotic potential. *Saccharomyces boulardii* is the yeast commercialized as probiotic in human medicine [17], but also in several studies strains such as *Kluyveromyces lactis*, *K. marxianus*, *Isaatchenkia orientalis*, *S. cerevisiae* and *Debaryomyces hansenii* have shown antifungal, antibacterial, anti-inflammatory and antitumoral activity [18,19].

LAB find increasing acceptance as probiotics which aid in stimulating immune responses, preventing infection by enteropathogenic bacteria, and treating and preventing diarrhea [20]. These bacteria have long been used for changing the aromatic and textural properties of food and for extending the shelf-life of various products such as dairy products [21]. The primary role of LAB is to ferment available carbohydrates and causing a decrease in pH. The combination of low pH and organic acids (mainly lactic acid), diacetyl, hydrogen peroxide, and bacteriocins or bactericidal proteins are the main preservation factor in fermented dairy products [22,23]. Bacteriocins are considered as safe natural preservatives or biopreservatives.

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. This certainly holds true for lactic acid bacteria (LAB), which are used throughout the world for manufacture of a wide variety of traditional fermented foods. Since they are involved in numerous food fermentations, known to man for millennia, it is assumed that most representatives of this group do not pose any health risk to man, and are designated as GRAS (Generally Recognized as Safe) organisms.

The aim of this study was isolation and identification of yeasts and lactic acid bacteria from the traditional fermented camel milk, *Chal* in Golestan province of Iran and use this isolates in later studies for operational aims.

Materials and Methods

Sampling

A total of 9 sour camel milk (Chal) samples (A to I) were randomly collected from different house holds and retail markets with different

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sanitation levels in the original container from Golestan province. The areas under investigation were Gonbad, Aghghala and Bandar Torkman cities.

Compositional Analysis

The Chal samples were analyzed for Fat [24], ash [25], pH, Nacl, Alcohol (after 48 h fermentation) [26], Protein, TS and Acidity content [27]. All the experiments carried out in three repetitions and means reported.

Isolation and Identification of Yeasts

One milliliter from each samples was serially diluted in distilled water and spread- plated onto YGC agar (Mirmedia, Iran) to isolate the yeasts. The plates were incubated at 25°C for 5 days. Colonies with distinct morphological differences such as colour, shape and size were picked and purified by streaking. The purified isolates were stored on YGC agar slant at 4°C under liquid parafin before identification. The colonies were tested and described based on morphological characteristic on Yeast- Mold Agar (YMA) and YM broth as following:

Yeast fermentation base medium containing 2% one of Carbohydrates (glucose, fructose, galactose, maltose, lactose, sucrose, xylose, arabinose, trehalose, manitol, melezitose and rafinose) as a sole carbon source for testing the assimilation of carbon sources for a month, gas production from carbohydrates 5% (glucose, fructose, galactose, maltose and lactose) by using durham tube for three weeks, production of extracellular starch compounds, urease test for hydrolyzation activity, growth in the presence of D-glucose (50 and 60%) and NaCl (10 and 16%), gelatin liquefaction and tolerance of 1% acetic acid according to Suh et al. [28], and investigation of the assimilation of nitrat, growth in the presence of cycloheximide (0.1% and 1%) with modified method according to Kerry et al. [29] using disk inoculum- solid medium method were achiened. The isolate were identified according to protocol described by Suh et al. [28].

Isolation and Identification of Lactic acid bacteria

Lactic acid bacteria count in MRS agar media (Liofilchem, 610024, Italy) determined according to the method described in Compendium of Methods for the Microbiological Examination of food [30]. The LAB isolated on MRS agar after suspending the sample (10 ml) in 90 mL distilled water and making serial dilutions. The medium was sterilised by heating at 121°C for 15 min. the plates were incubated at 37°C for 2 days. Colonies with distinct morphological differences such as colour, shape and size were picked and purified by streaking at least three times. The purified isolates were stored on MRS agar slant at 4°C in liquid parafin untile required for identification.

The cultures were tested and described for colony and cell morphology on MRS agar and MRS broth, Gram stain and catalase test [31], growth at 15, 30, 1nd 42°C anaerobically and at 37 aerobically [32,33], urea and argenin hydrolysis test [34], production of acid from diferent Carbohydrates using media containing 0.8% yeasts extract, 0.8% trypton, 1.2% pepton (from milk), 0.1% tween, 0.0004% bromophenol blue as indicator and 2% carbohydrates [35], Gas production from 2% glucose and sodium gluconate using durham tube [36], and growth in the presence of 2, 4, 6, 8 and 10% Nacl carry out according to De Man et al. [37]. In all tests sensitive compounds to heat such as carbohydrates, cycloheximide, yeasts nitrogen base medium, yeasts fermentation base medium were sterilized using 0.23 µ Millipore filter apparatus.

Statistical analysis

Data were analyzed using SPSS version 10.05-computer program.

Results and Discussion

Compositional analyses

Chemical properties of chal given in Table 1.

Our results revealed that the pH was ranged from 3.8 to 4.5. Although lower pH has inhibitory effect on the vegetative cells of pathogenic microorganisms, but it is favorable for growth of yeasts and lactic acid bacteria [38].

Results in Table 2 revealed yeast and moulds and LAB counts in chal samples are high, it can attributed to acidic medium of chal that is favourable for growth of yeasts and lactic acid bacteria.

Identified yeasts

Yeasts commonly associated with traditional fermented dairy products and have been reported in several studies [39-41]. All the identified strains shown in Figure 1.

Septate hyphae were formed by none of them and *Kluyvermyces marxianus*, *Torulospora delbrueckii, Kluyvermyces lactis, Candida lipolytica* and *Candida lusitaniae* formed ascospores. A part of the detected strains belonged to order *Ascomycotina* and the rest to order *Deuteromycotina* – Imperfect yeasts.

The most yeast isolates utilized galactose, fructose and maltose and most of them fermented glucose and fructose. All of isolates assimilated the glucose and liquefacted the gelatin, but starch production, tolerance to 1% acetic acid, growth in the presence of NaCl 16%, septate hyphae and nitrat assimilation were not seen for all, except *Debaryomyces hansenii* which grown in the presence of NaCl 16% and *Rhodotorula musilaginosa* assimilated the nitrate. Urea hydrolysis was negative for all isolates except the isolate identified as *Rhodotorula musilaginosa.* Isolates identified as *Pichia anamala, Kluyvermyces marxianus, Candida fermentati, Pichia jadinii, Debaryomyces hansenii, Pichia guilliermondii, Pichia ciferrii, Torulospora delbrueckii, Candida friedrichii, Candida lusitaniae* and *Candida versatilis* could grow in the presence of Glucose 50% and 60%, and for rest of them were negative. *Candida fermentati, Pichia jadinii, Pichia guilliermondii, Candida friedrichii, Candida Kefir, Rhodotorula musilaginosa, Candida lipolytica* and *Candida lusitaniae* could grow in the presence of Cyclohex 0.1% and 1%, but for rest of them were negative. Ascospore production for *Kluyvermyces. marxianus, Torulospora. delbrueckii, Kluyvermyces. lactis, Candida lipolytica* and *Candida lusitaniae* were positive.

From the Chal samples, 35 yeasts species including, 8 *Pichia* (22.85%), 8 *Kluyvermyces* (22.85%), 10 *Candida* (28.57%), 2 *Debaryomyces* (5.71%), 2 *Torulospora* (5.71%), 4 *Saccharomyces* (11.42%)and 1 *Rhodotorula* (3.50%) were identified. Among the yeasts species, *Kluyvermyces lactis* (8.57%) *Kluyvermyces marxianus* (8.57%) were the predominant (Figure 1).

Yeast species including *Pichia anamala, P. jadinii, P. guilliermondii, Deb. hansenii, Kluyveromyces polysporus*, *Kluyveromyces marxianus* which were isolated from samples of Chal, were also isolated from dairy products in the previous study [42,43]. However *Candida fermentati* was isolated from Chal only and was n't found previously in dairy products. *Kluyveromyces marxianus* has previously been isolated from indigenous fermented milk products such as Gariss, suusac, koumiss and kefir [44,45]. In the present study, *Kl.marxianus* was frequently

Sample Code	A	B	C	D	E	F	G	H	I
Protein%	1.0	1.0	1.2	1.1	1.5	2.2	1.3	1.5	1.4
Fat%	2.2	2.1	2.0	1.7	1.8	1.6	2.0	1.9	1.6
TS%	4.0	3.9	4.1	3.7	4.0	4.6	4.1	4.0	3.8
Ash%	0.23	0.31	0.32	0.27	0.32	0.38	0.31	0.32	0.29
Acidity (D*)	32.0	33.0	31.0	34.0	31.0	34.5	35.0	35.5	31.0
pH	4.0	4.2	4.5	4.4	4.5	4.0	3.9	3.8	4.5
NaCl%	0.80	0.75	0.90	0.85	0.70	0.80	0.76	0.60	0.80
Alcohol% (w /v)	0.80	0.70	0.40	0.70	0.40	0.50	0.80	0.60	0.60

* Dornic

Table 1: Compossition of chal samples.

Sample NO.	1	2	3	4	5	6	7	8	9
Yeast and moulds	4.11	4.04	3.90	4.04	3.95	4.00	4.08	4.05	4.02
Lactic acid bacteria	4.58	4.51	4.44	4.14	4.04	4.46	4.32	4.08	4.25

Table 2: Yeast and moulds and Lactic acid bacteria counts (log Cfu/ml).

isolated and constitutes the dominant yeast microflora of Chal. Yeast growth in Chal is thus probably positively influenced by the metabolic activities of the LAB present. Lactose fremneting *Kl.lactis* was found to be the predominant yeast species; the precise role of yeasts in Chal requires further study (Figures 2 and 3).

Identified LAB

LAB strains isolated, purified and further identified and differential tests were applied including morphological and physiological characteristics which facilitate the opportunity for identification of the LAB. The identification results were confirmed by the carbohydrates fermentation and assimilation profile obtained in correlation with bergeys manual [46] and also using the manuals of Sharpe [47]; Holt et al. [48]; Garrity et al. [49]; Hardie [50]; Kandler and Weiss [51]; Mundt [52] and Sneath et al. [32].

From the collected samples a total of 93 isolates randomly picked, after the original characterization. The selected isolate gave blue-purple color with gram staining; hence they are all gram positive and catalas negative. 64 of them were found to be bacilli with long/ short and rounded ends, pairs or single cells and these could determine as derivatives of the genus *Lactobacillus* and *Wieselia*. The rest of the isolates were cocci with spherical, round or ovoid morphology and appeared mostly as pairs, sigle or forming chains and tetrad shapes, therefore they referred to *Lactococcus*, *Streptococcus, Leuconostoc, Wieselia* and *Pediococcus* genus. Results showed in Figures 2 and 3.

Elgadi et al. [53] showed in cows and goats milk, the counts of streptococci on M17 were higher than those of lactobacilli, whereas the lactobacilli were higher in the camel milk and the ewes milk contained almost the same counts of lactobacilli and streptococci. The results of this study confirmed it as lactobacillus were higher than other lactic acid bacteria species.

Results showed *Lactobacillus reuteri*, *Lactobacillus fermentum*, *Lactobacillus salivarius, Lactobacillus curvatus, Pediococcus urinaeequi* and *Lactobacillus animalis* could growth in the presence of 10% Nacl, while all the strains showed proper growth in 2% NaCl except isolates identified as *Leuconostoc mesenteroides subsp. Cremoris* and *Lactobacillus helveticus*. It was also observed that all isolates fermented galactose, except *Lactobacillus delbrueckii subsp. Bulgaricus*, *Lactobacillus kefir* and *Lactobacillus viridescens*. Growth at 37^{0}Ċ for all the isolates were positive, while *Leuconostoc mesenteroides subsp. Cremoris* and *Leuconostoc paramesenteroides* could not growth at 30^0 Ċ. Production of Co_2 from sodium gluconate for *Lactobacillus curvatus* and *Lactobacillus fermentum* were variable and positive respectively, and for rest of them were negative. Mannose, lactose and xylose assimilated by the most of the isolates, whereas only *Lactobacillus divergens* could assimilate glycerol. There was variation in the utilization of other the carbohydrates sources for different lactic acid bacteria isolated from *chal* samples. None of the isolates could hydrolize urea. These results are similar to the Bergey's manual of determinative bacteriology [54,55].

From obtained results observed that *Lactobacillus plantarum* (13%) and *Lb. hilgardii* (9.78%) in bacilli, and leuconostoc species (13%) within cocci shapes are predominant in lactic acid bacteria isolated from *chal* samples.

These results confirm the predominance of LAB in traditionally fermented camel´s milk, as reported by other researchers [56,57] and comparable to those reported in previous studies on fermented milk products in Sudan and neighboring countries [58]. *Weissella hellenicus* was isolated only from one Chal sample meaning that these bacteria are probably present in low numbers. The species is occasionally detected in dairy products [59] and it is believed that their presence is a result of low sanitary conditions during the handmilking and handling.

Elgadi et al. [53] found that the dominant lactic acid bacteria in milk of different animal species were *streptococcus lactis* (34.9%), *lactobacillus fermentum* (20.6%), *Lb. plantarum* (19.1%), *Strep. Cremoris* (14.3%), *Lb. acidophilus* (9.5%) and *Leuconostoc lactis* (1.6%). The homofermentative lactobacilli from cow and camel milk were tentavily isolated as *Lactobacillus plantarum* and *Lb. acidophilus*, whereas the heteroferementative ones from cows, goats and ewes milk found to be *Lb. fermentum*. Also their results revealed the homo fermentative streptococci isolated from all milk samples identified as *streptococcus cremoris* and *streptococcus lactis*, whereas the only heterofermentative strain from camel milk was found to be *Leuconostoc lactis. Lb. fermentum* that isolated from Chal in this study, previously was isolated by Abdel Gadir et al. [19] from cows milk, Although results obtained mostly reported in cereal based fermented dough [60,61].

According to Chandan [62]; Anuradha and Rajeshwari [63]; Gibson and Roberfroid [64], many of the LAB isolated from chal in this study such as *Lactobacillus delbrueckii subsp. delbrueckii, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus plantarum, Lactobacillus gasseri, Lactobacillus acidophilus, Lactobacillus casei subsp rhamnosus, Lactobacillus casei subsp. paracesei, Lactobacillus reuteri, Streptococcus thermophilus, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus helveticus* and *Lactobacillus curvatus* classified in

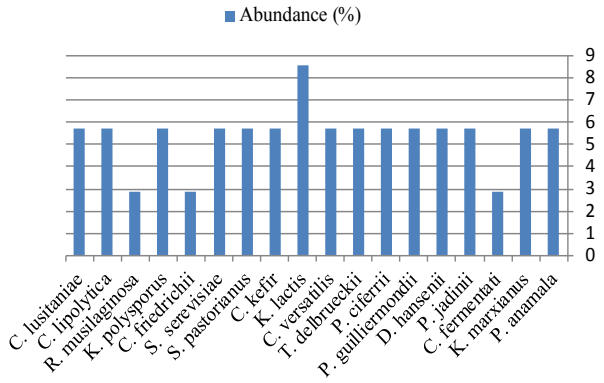

Figure 1: Estimation of yeasts species isolated from Chal samples.

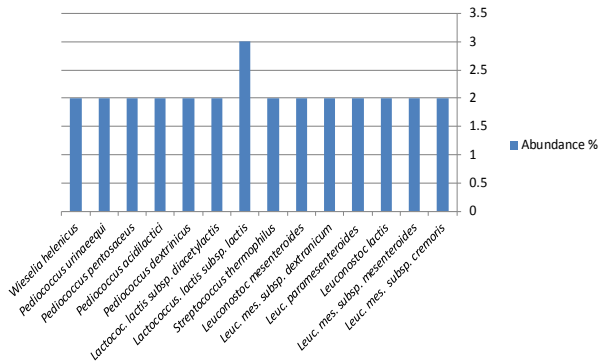

Figure 2: Cocci shape LAB isolated from Chal samples.

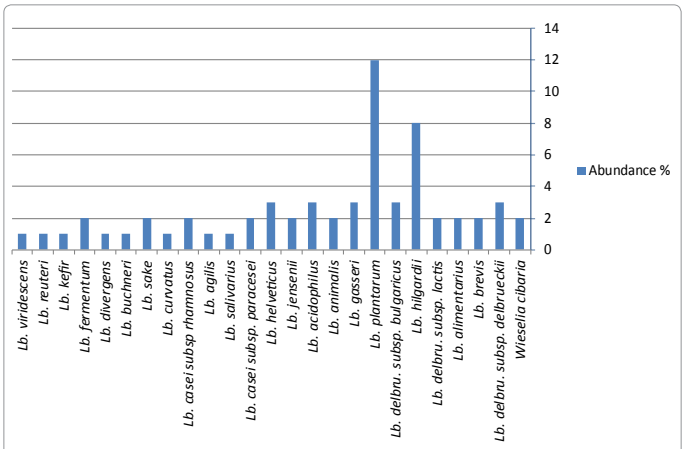

Figure 3: Bacilli shape LAB isolated from Chal samples.

group of probiotic microorganisms, accordingly chal has a potential to considere as a probiotic product.

Ashmaig et al. [9] from Sudanese fermented camel milk (gariss) isolated *Lactobacillus plantarum* (66.6% of the *gariss* isolates), *Lactococcus raffinolactis* (33.3%). *Lactobacillus animalis*, *Lactobacillus brevis*, *Lactobacillus divergens*, *Lactobacillus rhamnosus*, *Lactobacillus gasseri*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Lactococcus alimentarium* and other *Lactobacillus* species. Many of the mentioned isolated above (in gariss samples) isolated from chal in this study.

Mostly, lactic acid producing bacteria that grow in camel milk are Lactobacilli (*Lactobacillus acidophilus*) and Streptococci (*S. cremoris* and *S. lactis*), which are used as starters in dairy products. More growth of *L. acidophilus* was observed in camel milk as compared to others, as in every camel milk sample *L. acidophilus* was found. These findings are in accordance with Abu-Tarboush [13], who reported that camel milk provided support to the growth of *L. acidophilus*.

Although morphological and biochemical characterization based on sugar fermentation pattern is a useful tool for presumptive classification, but may not always provide sufficient basis for the reliable identification of lactic acid bacteria and yeasts, as reported by other researchers [65-67] accordingly, available systems should be combined with genotypic techniques which allowed more accurate identification.

Micro-organisms are important in dairy products. One of the most important groups of acid producing bacteria in the food industry is the Lactic Acid Bacteria (LAB) which are used in making starter culture for dairy products. The proper selection and balance for starter culture is critical for the manufacture of fermented products of desirable texture and flavour. Our results provide some raw data and strain resource for further study involved in probiotic strain selection and starter culture design concerning the industrialization production of traditional fermented milk.

Conclusions

Thirty five yeast species belonging to 18 genera were isolated and identified from samples of chal. Results showed Chal contained a wide variety of yeasts that are predominanted at Chal after the 48 h fermentation. Chal was made from raw camel milk, but the presence of yeasts indicates the contamination of the product by air, water or persons who are engaged in the preparation and transportation. This study recommended that increasing the hygienic level in Chal production be effective to decrease the yeast contamination. The present study has showed that the microflora in Chal comprises a combination of LAB and yeasts. The LAB were represented by Lactobacillus, *Lactococcus*, Leuconostoc, *streptococcus, Pediococcus* and Weissella, with the most frequently isolated LAB being *Lactobacillus plantarum*, *Lb. hilgardii* and leuconostoc species.

Based on the finding of the present study, it is concluded that chal contains wide variety of lactic acid bacteria, but lactobacillus in comparison to other species were predominant. Because of the preparation the chal from raw camel milk, saniting program should be conducted. It is strongly recommended that the behavior and presence of lactic acid bacteria even pathogenic microorganisms studied in raw camel milk and be compare with chal, also genotypic characterization need to conducte for more accurate identification of isolated lactic acid bacteria from chal.

Micro-organisms are important in dairy products. One of the most important groups of acid producing bacteria in the dairy industry is the Lactic Acid Bacteria (LAB) which is used in making starter culture for dairy products. The proper selection and balance for starter culture is critical for the manufacture of fermented products of desirable texture and flavour.

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