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Research Article

Lactic Acid Bacteria from Tarkhineh, A Traditional Iranian Fermented Cereal Product Investigated by Cultivation and 16S rRNA Clone Library. A Short Communication.

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Abstract

The present study address to characterize the bacterial community of Tarkhineh, a traditional Iranian fermented cereal product, by conventional culturing and 16S rRNA clone library. Twenty-nine lactic acid bacteria (LAB) from culturing on Man, Rogosa and Sharpe agar were identified by 16S rRNA gene sequencing and 55 clones from the 16S rRNA clone library. Discrepancies regarding the identity of the LAB were encountered by the two methods. By cultivation, Lactobacillus (Lb.) plantarum and Lb. casei were dominant followed by Lb. brevis, Lb. paracasei, Lb. paracasei subsp. paracasei, Lb. pentosus, Lb. zeae and uncultured bacterium clone MS-238. In contrast, construction of the clone library analysis revealed that the dominant clones belonged to Lb. paralimentarius, but Lb. alimentarius, Lb. delbrueckii subsp. bulgaricus, Lb. kefiri, Lb. kefiranofaciens subsp. kefirgranum, Lb. pontis, Lactobacillus sp. and Pediococcus were also detected. In addition to LAB, seven clones belonging to Bacillus and three bacterium clones were also detected.

Keywords: Tarkhineh, Lactic Acid Bacteria, Cultivation and 16S rRNA Clone Library.

Introduction

According to Ozen and Dinleyici, sophisticated technology in molecular archeology, can trace the use of fermented products as early as 8.000 BC [1]. Tarkhineh is the basic part of a traditional Iranian fermented cereal soup in the west mountain area of Iran. It is compost of grind wheat, soak in fermented milk and the local people use this soup in winter to prevent influenza and reduce symptoms. Based on processing of Tarkhineh it seems that the product could be a good source of lactic acid bacteria (LAB) with remedy functions. Some previous studies have investigated the microbial communities in Tarkhineh by cultivation, but culture-dependent methods do not reflect the true bacteriological community, rather the needs of different growth media for cultivation of certain bacteria [2-6]. Therefore, various culture-independent methods are developed during the last decades e.g., including 16S rRNA clone libraries [7, 8]. Knowledge is accessible on the construction of 16S rRNA clone libraries to evaluate the bacterial diversity of fermented food and milk products e.g., [9-13]. The aim of the present study was construction of a 16S rRNA clone library and compare the results with 16S rRNA sequence analysis of culturable bacteria from Tarkhineh.

Materials and Methods

Isolation of Bacterial Strains

Twenty grams of Tarkhineh were inoculated into 200 mL of man, Rogosa and Sharpe (MRS) broth (Fuka, catalogue no. 69966). Isolation of bacteria in tarkhineh was carried out according to the procedure described elsewhere [14]. To avoid time consuming isolation steps and interference with yeast, lactobacilli colonies were selected on MRS agar containing nystatin as suggested elsewhere [15]. Plates were incubated under anaerobic conditions at 37°C for 48 to 72 h and Gram-positive rods and catalase negative were cultured in MRS broth and stored at -80oC until further use.

Purification of bacterial DNA and 16S rRNA gene sequence analysis of culturable bacteria DNA was extracted from 29 randomly selected isolates by a method described elsewhere [16]. The pellet was resuspended in 100 µL deionized water and the concentration of DNA was determined spectrophotometrically at wavelength of 260nm. The DNA was stored at -20°C until further use. The selected strains were identified by 16S rRNA gene sequence analysis as described elsewhere [17].

Construction of 16S rRNA Clone Library

Bacterial DNA was purified with QIAmp DNA Mini kit (Qiagen), according to the manufactures protocol for bacterial DNA. The clone library was constructed as previously described [12, 13]. Of the 98 clones isolated, 69 had an insert and amplified 16S rRNA genes of 55 clones were sequenced. Sequences were individually analyzed and edited in the program Chromas Pro. Edited sequences were subjected to Blast search in the NCBI database and subsequently all samples were aligned with selected reference sequences in the BioEdit program.

Results

16S rRNA gene sequencing of culturable bacteria

The total viable count of the Tarkhineh sample was between 4.4 x 10^5 – 6.8 x 10^5 CFU g^{-1}. Twenty-nine LAB strains were randomly selected and examined by microscopy after Gram staining, catalase and oxidase reaction and further characterized by 16S rRNA gene sequencing. The LAB strains and their corresponding accession no. revealed that the culturable bacteria were dominated by strains showing high similarities to Lactobacillus (Lb.) plantarum (11 isolates), Lb. casei (six isolates), Lb. brevis (four isolates), Lb. paracasei subsp. paracasei (three isolates), Lb. paracasei (two isolates), one isolate to Lb. pentosus, Lb. zeae (one isolate) and one isolate showing similarity to uncultured bacterium clone MS-238 (Table 1).

Table 1: Identification of Culturable Bacteria Isolated from Tarkhineh with Partial Sequence of 16S rRNA Genes Referenced to Accession no. in GenBank

Strain no.	Closest relative (obtained from Blast search)	Accession no.	Similarity (%)
T1	Lactobacillus plantarum isolate L4-1	AB550299	98
T2	Lactobacillus plantarum strain S2	GU292429	95
T3	Lactobacillus plantarum strain KLDS 1.0725	EU626010	97
T4	Lactobacillus plantarum isolate L4-1	AB550299	98
T6	Lactobacillus plantarum	AB494717	97
T8	Lactobacillus plantarum strain IMAU:10272	GU138600	99
T10	Lactobacillus plantarum KLDS 1.0725	EU626010	91
T16	Lactobacillus plantarum strain IMAU700004	GQ131121	98
T19	Lactobacillus plantarum	AB510751	98
T23	Lactobacillus plantarum	AB494717	90
T26	Lactobacillus plantarum	AB494717	98
T9	Lactobacillus casei	AB494735	95
T13	Lactobacillus casei	AB494735	93
T17	Lactobacillus casei	AB494735	97
T18	Lactobacillus casei	AB494735	97
T20	Lactobacillus casei	AB494735	97
T25	Lactobacillus casei	AB494735	91
T11	Lactobacillus brevis	GU369768	91
T14	Lactobacillus brevis	GU138534	98
T15	Lactobacillus brevis strain JS-7-3	GU369768	94
T24	Lactobacillus brevis	AB494718	96
T5	Lactobacillus paracasei subsp. paracasei	AY773951	94
T12	Lactobacillus paracasei subsp. paracasei	FJ861109	94
T29	Lactobacillus paracasei subsp. paracasei tot. 3	AY773951	96
T21	Lactobacillus paracasei strainKLDS1.0658	FJ607302	96
T28	Lactobacillus paracasei strainKLDS1.0658	FJ607302	95
T7	Lactobacillus pentosus strain N3	GU253891	99
T27	Lactobacillus zeae	AB362765	98
T22	Uncultured bacterium clone MS-238	GQ477897	99

16S rRNA clone library

Fifty-five clones from the clone library were sequenced and revealed that clones belonging to LAB were dominant and they belonged to: Lb. paralimentarius, 31 clones, one clone to Lb. alimentarius, three clones to Lb. delbrueckii subsp. bulgaricus, Lb. kefiri (two clones), Lb. kefiranofaciens subsp. kefirgranum (one clone), Lb. pontis (one clone), six clones to Lactobacillus and one clone to Pediococcus parvulus. In addition, were six cones belonged to Bacillus and three uncultured clones detected (Table 2).

Table 2: Identification of Clones from Tarkhineh with Partial Sequence of 16S rRNA Genes Referenced to Accession no. in GenBank

Closest relative (obtained from Blast search)	Accession no.	Similarity (%)	No. of clones showing high similarity to the closest relative
Lactobacillus paralimentarius strain 412	EU483111	98	30
Lactobacillus paralimentarius strain DSM 13238	AJ417500	97	1
Lactobacillus alimentarius strain M-M3	FJ4157241	97	1
Lactobacillus delbrueckii subsp. bulgaricus ATCC BAA-365	CP000412	96	1
Lactobacillus delbrueckii subsp. bulgaricus strain IMAU20102	FJ845002	96	1
Lactobacillus delbrueckii subsp. bulgaricus strain IMAU40079	FJ749354	96	1
Lactobacillus kefiri strain IMAU450007	FJ749410	96	1
Lactobacillus kefiri strain YIT 0222	AB429371	97	1
Lactobacillus kefiranofaciens subsp. kefirgranum strain IMAU50004	FJ749408	96	1
Lactobacillus pontis strain LMG 114187	AJ422032	98	1
Lactobacillus genome sp. C2	AY278620	98	1
Lactobacillus sp.		<96	5
Pediococcus parvulus strain LA1110	FM878597	98	1
Bacillus cereus Q1	CP000227	96	1
Bacillus subtilis strain E9-1	FJ573171	97	1
Bacillus subtilis strain RC24	FJ263368	96	1
Uncultured Bacillus sp. clone W2	FJ863099	97	1
Bacillus sp.		<96	1
Brevibacillus thermoruber	AB362290	96	1
Uncultured bacterium clone p-2370-55G5	AF371474	97	1
Uncultured bacterium clone p-3083-SwA-3	AF371484	97	1
Uncultured bacterium clone AP07K.42	AM275437	97	1

Discussion

Tarkhineh fermentation occurs mainly by bacteria naturally present in the raw materials included flour, dough, salt, turnip, leaven (mint, red pepper) containing numerous microorganisms including LAB [3, 4]. Due to the popularity of Tarkhineh and its increasing consumption some information is known about the bacterial community of the product [2-6]. In these studies, the bacterial community was evaluated by traditional cultivation. However, as culture-dependent methods provide a rather skewed and restricted presentation of the bacteriological community, we investigated the bacterial community in Tarkhineh by cultivation and 16S rRNA clone library. Of the culturable LAB strains isolated in the present study, were Lb. brevis, Lb. casei, Lb. paracasei, Lb. pentosus and Lb. zeae, of which culturable Lb. brevis, Lb. casei and Lb. pentosus strains previously reported in Tarkhineh [5, 18, 19]. In addition, one of the culturable bacteria isolated in the current study, uncultured bacterium clone MS-238, a strain previously isolated from olive-oil mill wastewater revealing high similarity to Lb. rhamnosus [20].

Clone library analysis of Tarkhineh revealed several LAB strains, Lb. alimentarius, Lb. delbrueckii, Lb. kefiri, Lb. kefiranofaciens, Lb. paralimentarius and Lb. pontis not frequently isolated from Iranian fermented products. The discrepancy determined in the bacterial community determined by culture-dependent and independent methods of Tarkhineh in the present study is a finding previously been reported in variety ecosystems and fermented milk, koumiss from Mongolia [13, 21-25]. 16S rRNA clone library analysis might be more representative of the community in qualitative and quantitative terms especially when the

numbers of clones were enough. Even though relative few bacteria strains were isolated, and few clones investigated, combining partial sequencing of 16S rRNA genes of the culturable bacteria and the 16S rRNA clone library, interesting results were obtained showing a diverse LAB community of the investigated Tarkhineh sample.

Interesting findings reported in previous studies revealing inhibitory effect of LAB isolated from Tarkhineh towards Escherichia coli and Listeria monocytogenes and E. coli, Pseudomonas aeruginosa, Salmonella typhimurium and Staphylococcus aureus, which S. aureus was the most susceptible strain [6, 26]. These studies and some other studies revealing probiotic potential characteristics of LAB indicating that LAB isolated from Tarkhineh can be used as suitable supplements to functional foods [19, 27-29]. In addition to the presence of potential probiotics reported associated to Tarkhineh it is of interest noticing that fermentation of Tarkhineh by Lb. bulgaricus and Streptococcus thermophilus as well as the native probiotics in Tarkhineh binds mycotoxins and thereby reducing the concentrations of aflatoxin M1 and B1 during fermentation [30].

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