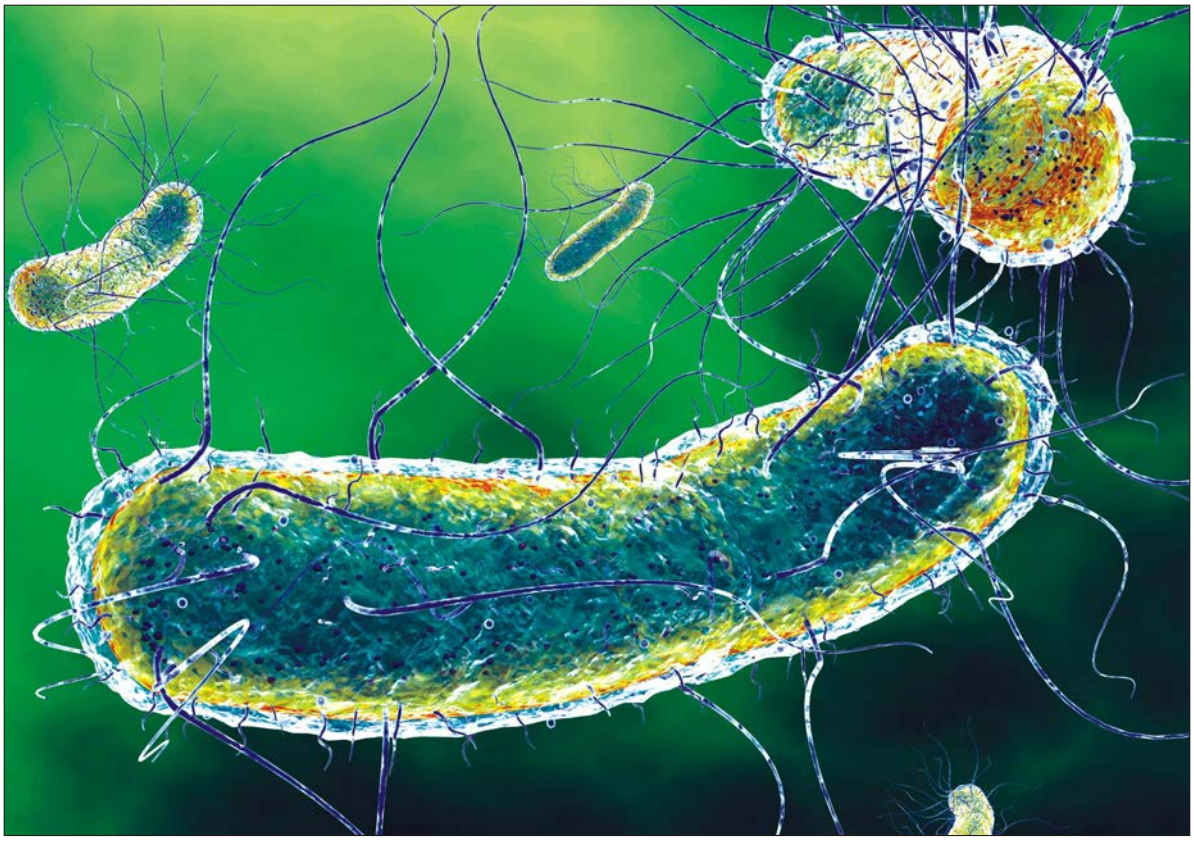

Current Research in Bacteriology

ISSN 1994-5426

science alert

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Current Research in Bacteriology 3 (2): 84-88, 2010
ISSN 1994-5426

Isolation and Phenotypic Characterization of *Lactobacillus* Species from Various Dairy Products

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Abstract: This study was carried out to identify *Lactobacillus* species isolated from different kinds of traditional and local cheeses and yoghurts of Basmenj zone in Iran. The identities of the isolates were based on morphological, cultural, physiological and biochemical characteristics of lactobacilli as presented in the Bergey's Manual of Systematic Bacteriology. Thirty seven lactic acid bacteria were isolated, according to all of the identification tests twenty seven of them belonged to the genus *Lactobacillus*, consisted of *L. rhamnosus* (69%), *L. paracasei* (15%) and *L. fermentum* (16%). The rest of isolates belonged to lactic acid cocci. Based on phenotypic characteristics of lactic acid bacteria, determination of original Iranian collection of local *Lactobacillus* species was carried out.

Key words: *Lactobacillus*, Basmenj zone, lactic acid bacteria, identification

INTRODUCTION

The lactic acid bacteria (LAB) came into view around 3 billion years ago, probably before the photosynthetic cyanobacteria. Their spreading has really begun with the apparition of milk producing mammals, over 65 million years ago (Champomier-Vergés *et al*., 2002). Lactic acid fermentation plays an indispensable role in the production of all dairy products and is involved in the production of many other foods and drinks, sausages, pickles, boza etc. They should possess permanent fermentation characteristics and should be resistant to bacteriophages (Lee, 1996).

Lactic acid bacteria cause rapid acidification of the raw material by the production of organic acids, especially lactic acid. Also, they possess some important products such as acetic acid, ethanol, aroma compound, bacteriocins, exopolysaccharids and several enzymes. In this way they enhance shelf life microbial safety, improve texture and contribute to the pleasant sensory profile of the end product (Harris, 1998).

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The group of lactic acid bacteria consists of these representatives genus: *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc* (Guessas and Kihal, 2004). Among all LAB types, the genus *Lactobacillus* has some beneficial characteristics which make it useful for industrial applications (Stiles, 1996). The different *Lactobacillus* species are aerotolerant, non pathogenic and do not produce toxic substances or toxins. They can resist weak acids, pH 3.5-6.5 and the yield of lactic acid is 90%. They are highly used in controlled fermentations and there is now a growing interest in their use as probiotics (Falsen *et al*., 1999; Espírito Santo *et al*., 2003; Roos and Katan, 2000).

Probiotics are food or preparations including live microorganisms, traditionally regarded as healthy for human use. When probiotics ingested in sufficient numbers, they play an important role in the control of host intestinal microbiota and preserving of its normal state (Fuller, 1997).

The aim of this study was to isolate and determine the *Lactobacillus* species isolated from different kinds of traditional and local cheeses and yoghurts of Basmenj zone, in order to identify original Iranian collection of lactic acid isolates.

MATERIALS AND METHODS

Isolation of Bacteria

The lactic acid bacteria were isolated form traditional and local cheeses and yoghurts of Basmenj zone in Iran. The isolation was performed in Department of Microbiology, University of Medical Sciences in 2008 by the common microbiological procedure and inoculation on a solid medium. Selective media for lactic acid bacteria were MRS and M17 agar. The samples were spread on the surface of these media. The cultivation was performed at the appropriate temperature (32 and 40°C) from one to five days. Once single colonies were obtained, they were inoculated in MRS broth or M17 broth. The cultures were diluted and tested for purity on the suitable agar medium after growing (Guessas and Kihal, 2004).

Identification of the Bacteria

Determination of the isolates was performed according to their morphological, cultural, physiological and biochemical characteristics by the procedures described in the Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986). The following tests were applied to distinct the morphological, physiological and biochemical characteristics of the isolates:

Initially all of the isolates were examined for Gram staining and catalase production. Then cell morphology and colony characteristics on MRS agar were tested and the isolates were separated into different phenotypic groups. Only the Gram-positive, catalase-negative and rods shape isolates were selected for further studies.

Growth at 30, 37 and 45°C was observed in MRS broth under anaerobic conditions. The development was assessed by reading the optical densities at 570 nm every 2 h.

For pH assay, the cultures were developed on MRS medium at various pH ranges: 3, 5.5, 6.5, 7, 7.5 and 8.

The gas producing capacity (H_2S) was done by culturing the microorganisms on a medium containing (g L^{-1}): casein peptone 20, meat peptone 6.1, sodium thiosulphate 0.2, Fe ammonium sulphate 0.2 and agar 3, incubation for 2 weeks at 35°C.

The starch hydrolysis was performed on MRS agar medium with adding starch 1%, incubation for 1 week at 35°C.

Fermentation capacity of different carbohydrates was determined on MRS-BCP broth medium, by using the carbon source, which was added to the sterile basal medium as filter

sterilized solution to a final concentration of 1%. Carbohydrates utilization was evaluated at the 24 and 48th h. The following 16 sugars were used for fermentation all of the isolates: arabinose, cellobiose, esculin, D-fructose, galactose, D-glucose, maltose, mannose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose and trehalose. For preparing anaerobic conditions, two drops of sterile liquid paraffine were settled in each tube after inoculation.

Gas production from glucose was assessed by inoculation of cultures into 5 mL MRS broth containing inverted Durham tubes and incubating at 35°C for 2 days.

Nitrate reduction was tested by culturing the microorganisms on a medium containing (g L^{-1}): peptone 5, potassium nitrate 1.5 and sodium chloride 6.8.

Urease test was performed by culturing the isolates on a medium containing (g L^{-1}): peptone 1, sodium chloride 5, mono- potassium phosphate 2, glucose 1, urea 20, agar 15 and phenol red 0.1, incubation for 3 days at 34°C.

RESULTS

From the tested samples thirty seven lactic acid bacteria were isolated. After the original characterization, ten of them were determined as representatives of the lactic acid cocci and the rest of isolates were referred to the genus *Lactobacillus*. Only the Gram-positive, catalase-negative, nonspore-forming and rods shape isolates were further identified.

For their identification to species level the morphological, cultural, physiological and biochemical characteristics were determined. After incubation on MRS agar for 24 h, isolates formed round, creamy white colonies. Only five isolates failed to grow at 30-37°C, the rest of them demonstrated the best growth at mentioned temperatures. Optimum pH for all of the isolates was 5.5-6.5. The isolates were tested for biochemical and other physiological characteristics. Their distinguishing features are shown in Table 1 and 2. All isolates were heterofermentative *Lactobacillus*, with negative pattern of H_2S formation, starch hydrolysis, nitrate reduction and urease activity. Fermentation tests distinguished the isolates into three groups (Table 2).

Table 1: Morphological, cultural and physiological characteristics of the isolates

	Groups		
Characteristics	1	2	3
No. of isolates	18	4	5
Taxonomic classification			
Family	*Lactobacillaceae*	*Lactobacillaceae*	*Lactobacillaceae*
Genus	*Lactobacillus*	*Lactobacillus*	*Lactobacillus*
Species	*L. rhamnosus*	*L. paracasei*	*L. fermentum*
Morphological characteristics			
Colour	Cream-white	Cream-white	Cream-white
Shape	Rods	Rods	Rods
Gram stain	+	+	+
Development on solid medium	Smooth round colonies	Smooth round colonies	Smooth round colonies
Physiological characteristics			
pH optimum	5.5 - 6.5	5.5 - 6.5	5.5 - 6.5
Temperature (°C)	30-37	30-37	30- 45
Spores formation	–	–	–
Catalase activity	–	–	–
H_2S formation	–	–	–
Starch hydrolysis	–	–	–
Nitrate reduction	–	–	–
Urease	–	–	–
CO_2 from glucose	–	–	+

+: Positive reaction, -: Negative reaction

Table 2: Biochemical characteristics of the tested isolates by utilization of carbon sources

	Groups		
Carbon sources	1	2	3
Arabinose	+	–	–
Cellobiose	+	+	–
Esculin	+	+	–
D-Fructose	+	+	+
Galactose	+	+	+
D-Glucose	+	+	+
Maltose	+	+	+
Mannose	+	+	–
Melibiose	–	–	+
Raffinose	–	–	+
Rhamnose	–	–	–
Ribose	+	+	+
Salicin	+	+	–
Sorbitol	+	–	–
Sucrose	–	–	+
Trehalose	+	+	–

+: Positive reaction, -: Negative reaction

DISCUSSION

Thirty seven lactic acid bacteria were isolated from traditional and local cheeses and yoghurts, after primary characterization of them the experimental data cleared that ten of them were lactic acid cocci. The other twenty seven lactic acid isolates, which belonged to the genus *Lactobacillus*, were selected for further identification. Based on morphological, cultural, physiological and biochemical characteristics of the studied *Lactobacillus* isolates, they were represented in three groups; the isolates (18 isolates, 69%) in group 1 were mesophilic heterofermentative. They were able to ferment arabinose, cellobiose, esculin, D-fructose, galactose, D-glucose, maltose, mannose, ribose, salicin, sorbitol and trehalose but they didn't hydrolyze melibiose, raffinose, rhamnose and sucrose. None of the isolates exhibited nitrate reduction, H_2S formation, urease activity and starch hydrolysis. During fermentation of glucose acid was produced without gas. The isolates in group 1 could be considered as *Lactobacillus rhamnosus.*

As a result of the taxonomic studies, the tested isolates in group 2 (4 isolates, 15%) were determined as *Lactobacillus paracasei.* They were mesophilic heterofermentative lactobacilli. These isolates produced acid from the following sugars: cellobiose, esculin, D- fructose, galactose, D-glucose, maltose, mannose, ribose, salicin and trehalose but none of the isolates in group 2 were able to ferment arabinose, melibiose, raffinose, rhamnose, sorbitol and sucrose. Acid was produced from glucose while gas was not. All of the studied isolates in this group represented negative pattern of nitrate reduction, H_2S production, urease activity and starch hydrolysis. Generally morphological, cultural and biochemical characteristics of the isolates in group 2 were very similar to those in group 1, but the isolates of group 2 differentiated from group 1 isolates on the basis of ability of group 1 isolates to ferment arabinose and sorbitol while none of the isolates in group 2 were able to ferment arabinose and sorbitol. The studied isolates in the last group (5 isolates, 16%), produced acid and gas from glucose. The phenotypic analysis of the isolates in this group, demonstrated their relativity to *Lactobacillus fermentum.* They were capable to ferment D-fructose, galactose, D-glucose, maltose, melibiose, raffinose, ribose and sucrose however arabinose, cellobiose, esculin, mannose, rhamnose, salicin, sorbitol and trehalose were not hydrolyzed. Nitrate reduction, H_2S formation, urease activity and starch hydrolysis of all the

isolates in this group were negative. These obligate heterofermentative isolates belonged to the group of the thermophilic lactobacilli, which are commonly included in starter cultures during the production of the cheese Gruyere, Gorgonzola, Mozarella (Hammes and Christian, 2006). The identified isolates will undergo further tests for production of bacteriocins and other compounds to evaluate their potential as starter culture to preparing traditional fermented cheese and yoghurt from raw goat milk in Basmenj zones.

ACKNOWLEDGMENT

We thank Laleh Parsa Yeghaneh and Nahid Hosseinzadeh for critical review of the manuscript and invaluable help.

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