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Isolation and Identification of Leuconostocs from Traditional Yoghurt in Tribes of Kazerun

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Abstract: Morphological, cultural, physiological and biochemical characteristics were employed to identify Leuconostocs isolated from yoghurt in different areas in Kazerun city of Fars province in Iran. From 15 yoghurt samples a total of 34 Leuconostoc were determined. Additionally, our biochemical tests and API kit showed that all of them were *Leuconostoc mesenteroides* ssp. cremoris. The current study constitutes the first step in the designing process of LAB starter cultures in order to protect the typical organoleptic characteristics of traditional yoghurt. However in the future, we can consider genetical characterization and selection of the most desirable strains which can assess their potential as starter cultures for commercial use.

Key words: Isolation, identification, Leuconostoc, traditional yoghurt, Kazerun, Iran

INTRODUCTION

Interest in microorganisms as a component of biological diversity has been renewed in recent years (Guessas and Kihal, 2004). The interest in microorganisms occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains (Leisner *et al*., 1999).

Lactic Acid Bacteria (LAB) are widely distributed in nature and occur naturally as indigenous microflora in raw milk, yoghurt, etc.

They are gram positive bacteria that play an important role in many food fermentation processes. Some species of the genus Leuconostoc (Ln.) are included in this group. The lactic acid fermentation has long been known and applied by humans for making different food stuffs. For many centuries, LAB have been an effective form of natural preservation.

In addition, they strongly determine the flavor, texture and frequently, the nutritional value of food and feed products. However, the application of well-studied starter cultures has been established for decades (Lee, 1996; Tserovska *et al*., 2002).

In the country, there are different kinds of traditional dairy products which are produced from sheep and goat milk such as drinking yoghurt, yoghurt, kashk, ghara-ghooroot, cheese, etc. In comparison with the commercial species, composition of lactic acid bacteria is more varied and inconstant in these products. The aim of the present study was isolation and identification of Leuconostocs from yoghurt in order to constitute an original collection of Fars province Leuconostoc strains.

MATERIALS AND METHODS

Yoghurt samples: During the spring of 2009, a total of 15 yoghurt samples were collected from the tribes of Kazerun. The samples were collected in sterile universal tubes and kept cool until they could be taken to the laboratory where they were kept at 4°C for further use.

Isolation of Leuconostocs: The samples were aseptically weighted and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10 fold serial dilution. 0.1 mL from each dilution was then subcultured in duplicate into the Acetate agar (Merck, Germany) supplemented with Tuween 80 (Merck, Germany). To prevent the growing of yeasts, the media were then supplemented with 100 mg L^{-1} of cycloheximide before being incubated at the appropriate temperatures (35°C) for 2 days (Beukes *et al*., 2001; Kalavrouzioti *et al*., 2005). MRS agar plates were incubated anaerobically using the Gas Pack

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system (Merck Anaerocult type C) at 35°C for 2 days in order to provide an optimal temperature for growing Leuconostocs. To perform the counts, the higher dilutions were used. Colonies were randomly selected and streak plating was then used to purify the strains which were subsequently kept in two different conditions including at 4°C for Acetate agar plates and at -20°C for MRS broths supplemented by 20% glycerol for further use (Mathara *et al*., 2004).

Identification of the bacterial strains: All strains were initially tested for gram reaction, catalase production and spore formation (Harrigan and MaCance, 1976). Colonies were characterized on Acetate agar. Strains with gram positive and catalase negative reactions were finally used for further identification (Sharpe, 1979). Growth at different temperatures (10, 15, 37, 40 and 45°C) for 5 days, resistance to 60°C for 30 min (Sherman test), growth in the presence of 2, 3, 4 and 6.5% NaCl and different pHs (4.5 and 6.5) were considered to identify the strains. Hydrolysis of arginine and asculin, utilization of citrate, production of acetone, gas formation from glucose and dextran production from sucrose were also determined (Samelis *et al*., 1994). All strains were also tested for fermentation of L-arabinose, D-xylose, galactose, D-fructose, sorbitol, lactose, melibiose, saccharose, D-raffinose, melezitose, mannose and glucose (Tserovska *et al*., 2002).

The growth of bacterial strains at 10, 15, 37, 40 and 45°C was visually confirmed by the changes in turbidity of MRS broth after 24, 48 and 72 h of incubation. The tolerance of microorganisms to the different levels of salt, pH and heat (60°C) was also visually evaluated (Harrigan and MaCance, 1976). Arginine dihydrolase agar and asculin azid agar (Merck, Germany) were employed to perform the hydrolysis tests. For evaluation of citrate utilization and acetone production, citrate and MR-VP agars (Merck, Germany) were used. MRS or M17 broths containing inverted durham tubes were used for evaluation of gas production and the production of dextran from sucrose was done in MRS agar (Mayeux *et al*., 1962).

In order to assess the fermentation of sugars a medium with the following composition was employed (g L^{-1}): bovine extract, 10.0; neopepton, 10.0; yeast extract, 5.0; K_2HPO_4, 2.0; $CH_3COONa + 3H_2O$, 5.0; diamonium citrate, 2.0; $MgSO_4$, 0.2; $MnSO_4$, 0.05; brom-cresol-purple, 0.17; tween 80, 1 mL. Carbon sources were added individually to this medium as filter-sterilized solutions to a final concentration of 1%. Carbohydrate utilization was assessed at the 24 and 48th h and on the 7th day of the growth at the corresponding temperature (Tserovska *et al*., 2002).

Furthermore, sugar fermentation patterns of 50 strains were also tested by use of API (bioMerieux, France) 50 CH strips and API CHL medium. The tests were done according to the instructions of the manufacturer. Anaerobiosis in the inoculated strips was obtained by overlaying with sterile paraffin oil and incubated at 36°C and the results were read after incubation of the strains for 1-3 days. Identification was done by the computerized database program (version 5.1) provided by the manufacturer.

RESULTS AND DISCUSSION

All 34 g positive, catalase negative and non spore-forming isolates were further characterized as mesophilic heterofermentative cocci. The microorganisms in this group were closely related to *Leuconostoc mesenteroides* ssp. cremoris which represented a reduced fermentative profile, unable to hydrolyze arginine, producing gas from glucose with citrate and acetoin positive and dextrane negative reactions (Table 1). These microaerophilic organisms were also characterized by the fermentation metabolism of lactose, glucose and galactose (Server-Busson *et al*., 1999; Hemme and Foucaud-Scheunemann, 2004).

Table 1: Physiological and biochemical characteristics of isolated strains

Characteristics of the srains	Types
Gram stain reaction	+
Catalase activity	-
Glucose fermentation	+
NH_3 from arginine	-
Growth at temperature (°C)	
10	+
15	+
37	V
45	-
Growth in a medium with NaCl (%)	
2	-
3	-
4	-
6.5	V
Growth at pH	
4.5	-
6.5	+
Production of CO_2 from glucose	+
Dextran production	-
Acetoin production	+
Citrate hydrolysis	+
Heat resistance 63.5°C for 30 min	-
Arabinose	-
Esculin	-
Fructose	-
Galactose	+
Glucose	+
Lactose	+
Mannose	-
Melezitose	-
Melibiose	-
Raffinose	-
Sorbitol	-
Sucrose	V
Xylose	-

V = Variable

It was noted that the 34 isolates were identified as *Leuconostoc mesenteroides* ssp. cremoris. The low number of these lactic acid cocci is probably due to their inability to compete with lactic acid bacilli in mixed cultures (Teuber and Geis, 1981; Togo *et al*., 2002). The low percentage of Leuconostoc strains isolated from the samples could partly be explained by their complex nutritional requirements (Medina *et al*., 2001) but probably also by their lower adaptation to milk and milk products. Leuconostoc species generally show a weak competitive ability during fermentation of milk (Wood and Holzapfel, 1995; Mathara *et al*., 2004). Beukes *et al*. (2001) found *Ln. mesenteroides* as one of the dominant microorganisms of South African traditional fermented milks.

CONCLUSION

Isolation and identification of Kazerun traditional yoghurt has been conducted for the first time. There is no record in the literature to demonstrate the isolation and identification of the Kazerun traditional yoghurt, so far. There is however, a big economic loss due to the import of yoghurt starters, annually. Because of increased demands for traditional fermented products, the results of the present study might be able to launch a considerable native achievement in the production of yoghurt. The identified isolates are used to establish the production of volatile compounds and to assess their potential as starter cultures for their commercial uses.

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