Iranian Journal of Veterinary Research, Shiraz University, Vol. 10, No. 3, Ser. No. 28, 2009

Identification of lactic acid bacteria isolated from traditional drinking yoghurt in tribes of Fars province

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(Received 23 Oct 2008; revised version 23 May 2009; accepted 24 May 2009)

Summary

Morphological, cultural, physiological and biochemical characteristics were employed to identify lactic acid bacteria (LAB), isolated from drinking yoghurt in different areas in Fars province, Iran. From 18 drinking yoghurt samples a total of 673 LAB positives were determined, in which 117 (17.38%) and 556 (82.62%) were identified as lactic acid cocci and lactic acid bacilli, respectively. Additionally, our biochemical tests showed the occurence of 52 (44.44%) *Lactococcus lactis subsp. cremoris* and 65 (55.56%) *Leuconostoc mesenteroides subsp. cremoris* among lactic acid cocci, and in the case of lactic acid bacilli, *Lactobacillus helveticus* 85 (15.3%); *Lactobacillus plantarum* 124 (22.3%); *Lactobacillus brevis* 117 (21%); *Lactobacillus casei subsp. casei* 86 (15.5%) and *Lactobacillus delbrueckii subsp. bulgaricus* 144 (25.9%). Among lactic acid cocci and bacilli, *Leuconostoc mesenteroides subsp. cremoris* and *Lactobacillus delbrueckii subsp. bulgaricus* were found to be the more dominant species, respectively. 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 drinking 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: Lactic acid bacteria, Lactobacillus, Lactococcus, Leuconostoc, Drinking yoghurt

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, drinking yoghurt, etc. They are gram positive bacteria that play an important role in many food fermentation processes. Some species of the genus Lactobacillus (Lb.), Lactococcus (Lc.) and 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 flavour, 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 our 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 a large

number of lactic acid bacteria from drinking yoghurt in order to constitute an original collection of Fars province LAB strains.

Materials and Methods

Drinking yoghurt samples

During the spring of 2007, a total of 18 drinking-yoghurt samples were collected from the tribes of four geographical regions of Fars province (north, south, east and west), migrating to the suburbs of Darab (2 samples), Firoozabad (3 samples), Abadeh (1 sample), Jahrom (2 samples), Kazeroon (3 samples), Farashband (2 samples), Beyza (1 sample), Kavar (2 samples) and Sarvestan (2 samples). The samples were collected in sterile universal tubes and kept cool until they could be taken to the laboratory, where they were kept at 4ºC for further use.

Isolation of lactic acid bacteria

The samples were aseptically weighted and homogenazied. 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 M17 and MRS agars (Merck, Germany) used for isolating LAB (Badis *et al*., 2004a; Guessas and Kihal, 2004). To prevent the growing of yeasts, the media were then supplemented with 100 mgL^{-1} of cycloheximide before being incubated at the appropriate temperatures (42ºC, 35ºC and 30ºC) for 2-3 days (Beukes *et al*., 2001; Kalavrouzioti, 2005). MRS agar plates were incubated anaerobically using the Gas Pack system (Merck Anaerocult type A) at 42ºC, 35ºC and 30ºC for 3 days, in order to provide an optimal temperature for growing thermophilic lactobacilli, mesophilic lactobacilli and Leuconostoc, respectively. M17 agar plates were also incubated aerobically at 30ºC for 2 days, in order to set up an optimal temperature for growing lactococci. To perform the total 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 MRS and M17 plates and at -20°C for M17 and 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 McCance, 1976). Colonies were characterized on MRS and M17 agar. Strains with gram positive and catalase negative reactions were finally used for further identification (Sharpe, 1979). Growth at different temperatures (10ºC, 15ºC, 37ºC, 40ºC and 45ºC) for 5 days, resistance to 60ºC 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 or M17 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 McCance, 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 (gL^{-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 24th and 48th h and on the 7th day of the growth at the corresponding temperature (Tserovska *et al*., 2002).

Results

All 673 gram positive, catalase negative and non spore-forming isolates, were further characterised as follows:

1- Mesophilic homo-fermentative cocci, 52 isolates

This group was represented by $ADH^{(-)}$ (arginine dihydrolase) (negative arginine hydrolysis), $citrate^{(-)}$ (negative citrate utilization) and $acetoin^{(-)}$ (negative acetoin production) isolates, which were identified as *Lactococcus lactis subsp*. *Cremoris* (Table 1). In this group, the microorganisms were spherical or ovoid in shape, occurring in pairs and short chains with non motile, facultative anaerobic fermentative metabolism (Holt *et al*., 1994).

2- Mesophilic heterofermentative cocci, 65 isolates

The microorganisms in this group were closely related to *Leuconostoc mesenteroides subsp*. *cremoris* which represented a reduced fermentative profile,

Table 1: Physiological and biochemical characteristics of isolated strains

Characteristics		Strains						
		1	2	3	4	5	6	7
Gram stain reaction		+	+	+	+	+	+	+
Catalase activity		-	-	-	-	-	-	-
Glucose fermentation		+	+	+	+	-	+	+
NH3 from arginine		-	-	+	-	-	-	-
Growth at temperature (ºC)	10	-	+	+	-	-	+	+
	15	-	+	+	+	-	+	+
	37	+	+	-	-	+	-	-
	45	+	-	-	-	+	-	-
Growth in a medium with NaCl (%)	2	+	+	+	+	+	+	-
	3	+	+	-	+	-	-	-
	4	+	+	-	+	-	-	-
	6.5	+	-	-	-	-	-	-
Growth at pH	4.5	+	+	+	+	+	-	-
	6.5	-	+	+	+	+	+	+
Production of CO_2 from Glucose		-	-	+	-	-	-	+
Dextran production		-	-	-	-	-	-	-
Acetoin production		-	+	-	-	-	-	+
Citrate hydrolysis		-	+	-	-	-	-	+
Heat resistance 63.5ºC for 30'		+	-	-	-	-	-	-
Acid production from								
	Arabinose	-	+	+	-	-	-	-
	Esculin	-	+	-	+	-	-	-
	Fructose	-	+	+	+	+	-	-
	Galactose	+	+	+	+	-	-	+
	Glucose	+	+	+	+	-	+	+
	Lactose	+	+	+	+	+	+	+
	Mannose	-	+	+	+		-	-
	Melezitose	-	+	-	+	-	-	-
	Melibiose	-	+	+	+	-	-	-
	Raffinose	-	+	+	+	-	-	-
	Sorbitol	-	+	+	+	-	-	-
	Sucrose	-	+	+	+	-	-	-
	Xylose	-	+	+	+	-	-	-

1 = *Lactobacillus helveticus*, 2 = *Lactobacillus plantarum*, 3 = *Lactobacillus brevis*, 4 = *Lactobacillus casei subsp casei*, 5 = *Lactobacillus delbrueckii subsp*. *Bulgaricus*, 6 = *Lactococcus lactis subsp cremoris* and 7 = *Leuconostoc mesenteroides subsp cremoris*

unable to hydrolyse 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 (Busson *et al*., 1999; Hemme and Foucaud-Scheunemann, 2004).

3- Lactobacilli bacteria, 556 isolates

We have divided the Lactobacilli group into three subgroups according to Stiles and Holzapfel (1997), as follows (Table 1):
(1) Mesophilic facultative hetero-fermentative Lactobacilli (210 isolates), which included *Lb. plantarum* (124 isolates, 22.3%) and *Lb. casei subsp. casei* (86 isolates, 15.5%). (2) Thermophilic obligate homo-fermentative Lactobacilli (229 isolates), including *Lb. helveticus* (85 isolates, 15.3%) and *Lb. delbrueckii subsp. bulgaricus* (144 isolates, 25.9%) which had a narrow fermentation profile and was able to ferment lactose and fructose and thus, would likely belong to *Lactobacillus delbrueckii subsp. bulgaricus* (Samelis *et al*., 1994; Guessas and Kihal, 2004; Ammor *et al*., 2005) and (3) mesophilic obligate hetero-fermentative Lactobacilli (117 isolates). *Lb. brevis* (117 isolates, 21%) was included in this group.

Discussion

It was noted that the mesophilic facultative hetero-fermentative lactobacilli group was represented by two species; 124 isolates were identified as *Lb. plantarum* and 86 isolates as *Lb. casei subsp. casei* according to Collins *et al.* (1989). The last species together with *Lb. helveticus* is included in starter cultures during the production of the cheese Gruyere, Gorgonzola and Mozarella (Tserovska *et al*., 2002). The above-mentioned results are in accordance with other research groups, in raw goat milk (Guessas and Kihal, 2004). *Lb. plantarum* was also the major lactobacillus species found in kule naoto, the maasai traditional fermented milk (Mathara *et al*., 2004). For group two, 85 and 144 isolates were identified as *Lb. helveticus* and *Lb. delbrueckii subsp. bulgaricus*, respectively. Moreover, in the last group, a supplementation test for mannose and melizitose fermentation permitted the identification of 117 isolates as *Lb. brevis* (Samelis *et al*., 1994). Olarte *et al.* (2000) noted that the presence of *Lb. plantarum* in the cheese (Cameros) from goat milk decreased the number of the enterobacteriacae and fecal coliforms in the final product. Lactobacilli isolated from household bushera belonged to *Lb. plantarum, Lb. brevis and Lb. delbrueckii subsp. bulgaricus* (Muyanja *et al*., 2003). In the characterization of microflora of homemade semihard white zlatar cheese, *Lactobacillus brevis* was found as one of the main groups (Terzic-Vidojevic *et al*., 2007).

In the cocci group, 65 isolates were identified as *Leuconostoc mesenteroides subsp. cremoris* and 52 isolates as *Lactococcus lactis subsp. cremoris*. The lower number of 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).

As starter cultures, LAB are omnipresent in dairy manufacturing. Specific fermentation processes have been developed in order to encourage the growth of the desired species, some of which are fastidious organisms such as *Lb. delbrueckii subsp. bulgaricus* and *Lb. helveticus* (Bottazzi, 1988).

Isolates belonging to the *Lb. plantarum* group were shown to be the predominant members of the LAB flora of acid-fermented condiment (Tempoyak). In addition, isolates belonging to the *Lb. brevis* group and *Ln. mesenteroides* were also observed (Leisner *et al*., 2001). Beukes *et al*. (2001) found *Lb. plantarum, Lb. delbrueckii, Ln. mesenteroides* and *Lc. lactis* as dominant microorganisms of South African traditional fermented milks.

The most abundant isolated species from raw goat's milk of four Algerian races were *Lb. helveticus, Lb. plantarum, Lb. delbrueckii subsp. bulgaricus, Lb. brevis* and *Lc. lactis subsp. lactis* (Badis *et al*., 2004b). Leisner *et al.* (1999) identified lactic acid bacteria (LAB) of Chili Bo and found *Lb. plantarum* to be the most important predominant organism.

Isolation and identification of Iranian traditional drinking yoghurt has been

conducted for the first time. There is no record in the literature to demonstrate the isolation and identification of the Iranian traditional drinking 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 drinking 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.

Acknowledgements

We would like to thank Dr. S. Sh. Shekarforoosh, Dr. S. Hosseinzadeh, Dr. A. Hosseini, Dr. M. Shah Ahmad Ghasemi and Dr. M. H. Eskandari for their helpful support, and Mr. A. Saeedzade for his technical assistance.

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