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Isolation and Identification of Lactic Acid Bacteria from Raw Cow Milk in Khartoum State, Sudan

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ABSTRACT

A total of 8 samples of cow raw milks were collected from different areas of Khartoum state were analyzed for bacterial load. A total of 23 strains of lactic acid bacteria were isolated, out of which 12 strains were cocci and 11 strains were facultatively heterofermentative lactobacilli. Lactic acid bacteria were identified on the basis of phenotypic characters as *Lactococcus lactis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus*.

Key words: Raw milk, human diet, lactic acid bacteria

INTRODUCTION

In the history, milk played a major role as nutritional source and since 1900's the start of golden era of industrial microbiology. It was also economically significant because larger quantity of milk was being processed daily in factories for the fermented food products. LAB were first isolated from milk (Carr *et al*., 2002; Metchnikoff, 1908; Sandine *et al*., 1972) and have since been found in such foods and fermented products as meat, milk products, vegetables, beverages and bakery products (Aukrust and Blom, 1992; Caplice and Fitzgerald, 1999; Harris *et al*., 1992; Gobbetti and Corsetti, 1997; Jay, 2000; Liu, 2003; Lonvaud-Funel, 2001; O'Sullivan *et al*., 2002). Lactic acid bacteria are widely distributed in the nature. They could be isolated from soils, waters, plants, silages, waste products and also from the intestinal tract of animals and humans (Axelsson, 1998). The genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are recognized as LAB (Jay, 2000). The objectives of this study were to collect a variety of milk samples from different areas of Khartoum state and to determine the predominant lactic acid bacteria groups (isolation and identification of microorganisms from cow's milk).

MATERIALS AND METHODS

Samples: The samples of cow's milk were collected from various areas of Khartoum state. They were obtained under good conditions from a healthy animal, to avoid any contamination which can influence the lactic flora. The samples were collected in sterile bottles and then transported quickly to the laboratory to be analyzed. The analysis was done at the laboratory of the Department of Food Microbiology, Food Research Centre, Khartoum North, Sudan. Sterilization, serial dilution and preparation of the media were done according to Harrigan and MacCance (1976).

Isolation of lactic acid bacteria from raw milk: Ten milliliter of milk sample were vigorously homogenized with 9 volumes of sterile diluents [0.1% (w/v) bacteriological peptone, 0.85% (w/v)

[NaCl] and serial 10-fold dilutions (10^{-1} to 10^{-8}) were prepared using the same diluents. One milliliter of these dilutions was pour-plated in the media for lactic acid bacteria, M17 (Terzaghi and Sandine, 1975) and MRS (De Man *et al*., 1960) adjusted to pH 5.5. After incubation anaerobically (BBL Gas pak plus Anaerobic System) at 37°C for 3 days, representative strains of lactic acid bacteria were obtained from M17 and MRS plates of highest sample dilutions. Colonies were either randomly picked up or when the plate contained less than 10 colonies (Leisner *et al*., 1997). The purity of the isolates was checked by streaking again to fresh agar plates, followed by macroscopic and microscopic examinations. The strains displaying the general characteristics of lactic acid bacteria were chosen from each plate for further studies. The strains of lactic acid bacteria were stored without appreciable loss of properties in skimmed milk at -20°C. Working cultures were also kept on MRS agar or M17 agar slant at 4°C and streaked every 4 weeks (Samelis *et al*., 1994; Herrero *et al*., 1996).

Identification of lactic acid bacteria to the genus level: For identification of lactic acid bacteria, overnight cultures of each isolate in MRS broth (Oxoid) were used. All isolates were initially tested for Gram reaction, catalase enzyme and production of acid from glucose in Hugh and Leifson medium by oxidation or fermentation reaction (Harrigan and MacCance, 1976). Only Gram positive bacteria with catalase negative reactions were observed (Schillinger and Lucke, 1987; Garvie, 1986; Kandler and Weiss, 1986) and the representative isolates were purified by successive streaking onto the same agar substrate. For the Gram-positive, catalase negative rods, growth at various temperatures 10°, 15° and 45°C, tolerance of different salt levels (2, 4 and 6.5% w/v NaCl, hetero- and homo-fermentative activity (using MRS broth) with inverted Durham tubes in MRS broth were determined. Twenty isolates from raw cow milk were then selected based on the above tests for further identification. The isolates were stored at -40°C in MRS broth containing 10% glycerol. The bacteria were characterized by microscopic and by conventional biochemical and physiological tests. The cultures were examined for colony and cell morphology, motility, Gram stain and production of acid from glucose (Harrigan and MacCance, 1976), in addition to the oxidation and fermentation test according to Hugh and Leifson (1953). These preliminary tests make it possible to classify the isolates in genus on the basis of characteristic and tests of identification mentioned by Harrigan and MacCance (1976), Hammes *et al*. (1992), Holzapfel and Schillinger (1992) and Dicks *et al*. (1993).

Identification of lactic acid bacteria to the species level: After their microscopic examination, Gram +ve and catalase –ve lactobacilli were tested for their sugar fermentation pattern, production of ammonia from arginine in addition to their ability of growth at 15 and 45°C according to Harrigan and MacCance(1976). The carbohydrate fermentation profiles of the selected 20 isolates were investigated using API 20A and API CHL medium according to manufacture's instructions (API System, Bio-Merieux, France). The bacteria identified by the use of a computer programme, API LAB PLUS, version 3.2.2 software (BioMerieux) and reference to Bergey's Manual of Systematic Bacteriology.

RESULTS

Identification of isolates: The physiologic characteristics of the strains are shown in Table 1. Out of a total of 37 isolates obtained from raw cow milks from different areas of Khartoum state, twenty-three strains showed positive Gram reactions, absence of mobility, absence of spore formation,

Table 1: Phenotypic characteristics of lactic acid bacteria isolated from cow's raw milks

Gram	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Morphology	Cocci	Cocci	Cocci	Cocci	Rods	Rods	Rods
Production of CO_2 from glucose	-	-	-	-	-	-	-
Growth at							
10°C	+	+	+	+			
15°C	+	+	+	+	+	+	+
37°C	+	+	+	+			
45°C	-	+	+	+	+/-	-	+
Growth at							
2% NaCl	+	+	+	+			
3% NaCl	+	+	+	+			
4% NaCl	+	+	+	+			
6.5% NaCl	-	+	+	+	-	-	-
Acid from							
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Sorbitol	-	+	-	-	+	+	+
Melibiose	-	-	+	-	-	+	-
Raffinose	-	-	-	-	-	+	-
Xylose	+	-	-	-	-	+	-
Sucrose	+	+	+	-	+	+	-
Arabinose	-	+	+	-	-	+	-
Melezitose	-	-	-	-	+	+	+
Rhamnose	-	+/-	-	-	-	-	+
Maltose	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+
Mannitol	+	+	+	-	+	+	+
Trehalose	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+
Identified as	*Lc. lactis* subsp. *lactis* (n = 5)	*Ec. faecalis* (n = 3)	*Ec. faecium* (n = 3)	*Ec. durans* (n = 1)	*Lb. paracasei* (n = 2)	*Lb. plantarum* (n = 5)	*Lb. rhamnosus* (n = 4)

+: Positive reaction, -: Negative reaction, -/+: Variable reaction

absence of catalase activity, cocci which produced no gas from glucose (12 isolates) and/or rods (11 isolates). Among the cocci, 5 isolates were able to grow at 10 and 37°C, but none at pH 9.6 broth and 45°C. In addition, they did not survive at 60°C for 30 min. All of the 5 strains could grow in 4%, but not in 6.5% NaCl broth and produced NH_3 from arginine. The strains formed acid from lactose and ribose but acid production from mannitol, sucrose and xylose was strain dependent.

DISCUSSION

The phenotypic characteristics of the strains (Table 1) suggest their close resemblance to *Lc. lactis* subsp. *lactis* (Sharpe, 1979; Schleifer *et al*., 1985; Balows *et al*., 1991). Seven isolates of cocci were able to grow at 10 and 45°C in 6.5% NaCl and pH 9.6 broth. They also survive at 60°C for 30 min and form NH_3 from arginine, but not CO_2 from glucose and were characterized as enterococci. Three of them seemed to be *Ec. faecalis*, as suggested by their ability to survive at 60°C

for 30 min and to ferment sorbitol (Sharpe, 1979; Schleifer and Kilpper-Balz, 1984; Devriese *et al*., 1993; Manero and Blanch, 1999). Four enterococci strains were differentiated by their ability to form acid from sugars. Thus, 3 strains producing acid from mannitol and Arabinose were characterized as *Ec. faecium*, one strain is characterized by inability to ferment melibiose and sucrose and unable, in general, to ferment sugars as *Ec. durans* (Schleifer and Kilpper-Balz, 1984; Devriese *et al*., 1993; Manero and Blanch, 1999). The 11 isolates of Gram-positive rods grew at 15°C and did not form either CO_2 from glucose or NH_3 from arginine. These characteristics suggest their classification as facultatively heterofermentative lactobacilli (Sharpe, 1979; Balows *et al*., 1991). Two out of 11 isolates did not form acid from arabinose, melibiose, raffinose and rhamnose and were characterized as *Lb. paracasei* subsp. *pracasei* (Collins *et al*., 1991; Balows *et al*., 1991). A total of 5 isolates of rods were classified as *Lb. plantarum*, as suggested by their sugar fermentations patterns. All these strains fermented arabinose, cellobiose, lactose, maltose, melibiose, raffinose, ribose, sucrose and trehalose (Sharpe, 1979; Balows *et al*., 1991). These strains did not form acid from rhamnose and acid production from sorbitol and xylose was variable and strain dependant. The last 4 strains were unable to ferment melibiose, raffinose, xylose, sucrose and arabinose but were able to form acid from rhamnose and classified as *Lb. rhamnosus*. Lactobacilli isolates represent a significant part among our isolates and are represented by *Lb. plantarum*, *L. rhamnosus* and *Lb. paracasei*. These species are frequently isolated from raw milk and dairy products (Tsakalidou *et al*., 1994). In other works, Mathara *et al*. (2004) and Abdelgadir *et al*. (2001) isolated *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lb. rhamnosus* and *Lb. fermentum* from fermented products and showed that these species represent more than 60% of the isolated lactobacilli. In addition, Medina *et al*. (2001) showed that 8% of *Lactobacillus* isolated from ewe's milk and cheese in North from Argentina belonged to *Lb. acidophilus*.

Generally, the species identified in the present study, were in good agreement with other studies. *Lactobacillus plantarum, Lactococcus lactis* ssp. *lactis, Lactobacillus delbrueckii* subsp. *lactis, Leuconostoc lactis* and *Leuconostoc citreum* were identified in South African traditional fermented milks (Beukes *et al*., 2001). *Lactococcus lactis* ssp. *lactis, Lactobacillus plantarum* and *Lactobacillus delbrueckii subsp. lactis* were identified in Zimbabwean fermented milk (Feresu and Muzondo, 1990). *Lactobacillus plantarum*, *Lactococcus lactis* ssp. *lactis* and *Lactobacillus confus* were identified in Masaï fermented milk in Northern Tanzania (Isono *et al*., 1994).

CONCLUSION

The obtained results demonstrated that there is a diversity of lactic acid bacteria in cow's raw milk from cow in Khartoum state, Sudan. This local raw milk could serve as source for beneficial lactic acid bacteria in future researches.

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