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Isolation and Identification of Lactic Acid Bacteria in Sudanese Fermented Camel Milk (*Gariss*)

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Abstract

This study was done in order to isolate, identify and determine the acidifying abilities of lactic acid bacteria present in traditional Sudanese fermented camel milk (*Gariss*). Eighteen samples of *Gariss* were collected during the period from September to November 2011 from nomadic camel herders in Butana region (Allahhween tribe), four of them from Al Gadarif state at Alshuack area (Gapat Alfeel), the rest (fourteen) were obtained from the nomads around Alsubag area. The study isolated 36 lactic acid bacteria which classified into five species. Acid production from different species in camel milk after 12 hours incubation were 0.52 % for *Lactococcus lactis*, 0.50% for *Streptococcus thermophilus*, 0.42% for *Lactobacillus rhamnosus*, 0.23% for *Lactobacillus paracasei* and 0.18% for *Lactobacillus helveticus*

Key words: Gariss, isolated bacteria, Lactic acid bacteria, acidifying abilities, camel milk Fermented Camel's milk.

المستخلص

أُجريت هذة الدراسة بغرض عزل وتعريف وتقدير قدرة البكتيريا المنتجة لحمض اللاكتيك الموجودة فى لبن الابل المتخمر السودانى (القارص) على أنتاج الحمض. جمعت تسعة عشرعينة لبن إبل متخمر تقليدى (القارص) فى الفترة من شهر سبتمبر الى نوفمبر 2011 من أصحاب قطعان الإبل البدويين فى منطقة البطانة (قبيلة الحلاويين)، أربعة منهم من ولاية القضارف فى منطقة الشوك (غابة الفيل)، البقية (أربعة عشر) حُصل عليها من البدوويين حول منطقة الصباغ. عزلت فى هذة الدرسة 36 بكتيريا حمض اللاكتيك حيث صنفت الى خمس أنواع. انتاج الأنواع المختلفة من حمض اللاكتيك فى لبن الإبل بعد 12 ساعة تحضين كانت (0.52 %) من *Lactococcus lactis* و (0.5 %) من *Streptococcus thermophiles* و (0.42%) من *Lactobacillus* و (0.23%) من *Lactobacillus paracasei* و (0.18%) من *Lactobacillus helveticus*.

Introduction

Lactic acid bacteria (LAB), particularly those belonging to beneficial and nonpathogenic genera (Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, and Streptococcus) have traditionally been used in the food industry (Jose, 2007). Today, Lactic acid bacteria (LAB) are a focus of intensive international research for their essential role in most fermented food, for their ability to produce various antimicrobial compounds promoting probiotic properties (Temmerman *et al.,* 2002) including antitumoral activity (De Vuyst and Degeest, 1999 and Ostlie *et al.,* 2003), reduction of serum cholesterol (Desmazeaud, 1996 and Jackson *et al.,* 2002), alleviation of lactose intolerance (De Vrese *et al.,* 2001), stimulation of the immune system (Isolauri *et al.,* 2001), stabilization of gut microflora (Gibson *et al.,* 1997). LAB strains that produce exopolysaccharide (EPS) are employed in the manufacture of fermented milk to improve its texture and viscosity (Curk *et al.,* 1996 and Ruas- Madiedo *et al.,* 2002). Some LAB strains are known to produce mannitol, which is claimed to have several health promoting effects (Wood and Holzapfel, 1995 and Wisselink, *et al.,* 2002).

In Sudan, there are many popular traditionally fermented milk products that produced mainly in the rural areas. For example Rob is milk product fermented in a traditionally way on household levels. Milk surplus to the consumption of the family is collected in a container, inoculated with a starter from the fermentation of the previous day, and left to ferment overnight. It is then churned early in the morning (Abdelgadir *et al.,* 2001). Laban-rayeb is another traditional Sudanese fermented product which can be produced from cow's, goat's or sheep's whole milk and it is made by the same method of rob but without churning.

Gariss is a special kind of fermented camel milk popular among the nomads of Sudan; prepared by fermenting the camel milk in large skin bags or si'ins, which contains a large quantity of a previously soured product (Dirar, 1993).

Most of the camel milk is consumed as fermented milk; the milk is usually allowed to ferment naturally at ambient temperature without any heat treatment. The microflora of fermented camel's milk is unique and different from other fermented products due to variations of processing technology and localities where they have been produced (Hassan *et al.*, 2008; Ahmed *et al.*, 2010 and Suliman and El Zubeir, 2014). There for the use of starter cultures could improve the quality of fermented camel milk (Brasca *et al.,* 2008).

The objectives of present study were to isolate, identify and determine the Acidifying abilities of lactic acid bacteria present in Sudanese fermented camel milk (*Gariss*).

Materials and methods

Source of *Gariss* samples

Eighteen samples of traditional fermented camel milk (Gariss), were collected during the period from September to November 2011 from nomadic camel herders in Butana region (Allahhween tribe), four of them from Al Gadarif state at Alshuack area (Gapat Alfeel), the rest (fourteen) were obtained from the nomads around Alsubag area. The samples were collected in sterile bottles and were kept at 4-5^{o}C by using of an ice box brought to the Department of Dairy Production, Faculty of Animal Production, University of Khartoum, and were stored in the laboratory under refrigeration at 4^{o}C until used.

Isolation of lactic acid bacteria After mixing the samples, from each one, a serial dilution (10^{-1}- 10^{-7}) was subsequently made using sterilized distilled water. From the different dilutions, 0.1 ml surface plated on De Man Rogosa Sharpe (MRS) agar (DeMan *et al.,* 1960), (Hi media) and on Elliker agar (Elliker *et al.,* 1956). Plates were then incubated at 37°C for 24- 48 h under anaerobic conditions using anaerobic jars. MRS medium's Final pH was 6.5±0.2 at 25°C. The medium was sterilized at 121°C for 15 minutes and the Elliker agar medium was buffered by addition of 0.4 % disodium phosphate (Barach, 1979). Final pH after the medium was buffered was 7.0±0.1 at 25ºC.

Purification and preservation of LAB isolates

Individual isolates from MRS and Elliker agar plates were picked, representing all morphologically distinct colonies. The bacterial isolates were further tested for Gram reaction, catalase production and cell morphology. Only Gram- positive, catalase negative isolates were purified (3-4 times) by sub-culturing through successive streaking on the appropriate agar medium before being subjected to preliminary identification, and the pure cultures were maintained in MRS and Elliker broth at 4°C or at -20°C in sterile reconstituted skim milk (10% w/v).

Preliminary identification

The Gram stain's test was done according to (Barbara *et al.,* 2000). Catalase test was carried out by using 3% hydrogen peroxide (Harrigan, 1998). The motility was tested according to Tittsler and Sandholzer (1936).

Identification of isolates to species level

Growth at 10°C and 45°C was tested according to Holt *et al.* (1994). The salt toleration ability of the isolates was tested according the method described by Waitkins *et al.* (1980). Gases produced during the fermentation of glucose process were detected by using Durham tube within the liquid culture of MRS medium (Harley *et al.,* 2002). Survive at 60°C for 30 minutes was done as described by Aysrs and Johnson (1914) and the fermentation of carbohydrates was determined in MRS broth (DeMan, *et al.,* 1960) modified by omission of glucose and meat extract, addition of (0.005%) bromocresol purple as pH indicator and supplemented with (1%) by one of following sugars: glucose; lactose; maltose; fructose; manitol; sucrose; raffinose; rhamnose; melizitose (Barrow and Feltham, 2009).

Titratable acidity

Titratible acidity was determined according to Foley *et al.* (1974).

Heat treatments for camel milk

The camel milk was obtained from the Camel Research Centre Farm, University of Khartoum. The Camel's milk was divided into conical flasks (250 ml). The milk in the flasks was heat treated; using a water bath; to 90° C for 10 minutes. Then the milk were cooled to 45° C and the milk in the flasks were cultured with the five isolated species bacteria (best isolate from every species) in duplicate. After the milk in the flasks was well stirred, the flasks were incubated at 37°C for 12 h, except for *Streptococcus thermophilus* and *Lactobacillus rhamnosus*, which were incubated at 45°C.

The same procedure where used for the camel milk which was used to determine the acidifying abilities of the mixtures which composed from the isolated strains.

Inoculation percentage for every mixture was 2% and the ration between the strains in the same mixtures was 1:1. The camel milk in the flasks which was inculcated with the mixture of *Lactobacillus rahmnosus* and *Lactococcus lactis* were incubated at 37°C and the camel milk in the flasks which was inculcated with the mixture of *Lactobacillus rhamnosus* and *Streptococcus thermophilus* were incubated at 45°C.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) soft ware program, version 11.5. Two ways analysis of variance were used to determine the effect of different factors (bacteria species and time of incubation) on the acidity development. When significant different among the means were exist, Duncan's multiple range test (DMRT) was used to separate the means.

Results and discussion

Identification of the isolates from *Gariss*

Table 1 showed the general properties of the 36 LAB strains isolated from *Gariss*. Among these LAB isolates, rods were accounted for 6 isolates (16%), while cocci were 30 isolates (84%). The isolates were classified into three genera according to De Vos *et al.* (2009):

1. Lactococci: 28 of the isolates were cocci that occur singly, in pairs, or in short chains, Gram's stain positive, non motile, catalase negative, grow at 10°C but not at 45°C, didn't grow in 4% (w/v) NaCl and produce lactic acid without gas (Co_2). All isolates of this genus were able to ferment glucose, lactose, fructose and raffinose and produce acid. They can't produce acid from maltose, manitol, melezitose, rhamnose, sucrose. So the 28 isolates were considered to be *Lactococcus lactis supsp lactis*.

2. Streptococci: two of coccal shaped isolates were Gram's stain positive, non motile, catalase negative, grow at 10°C and at 45°C, didn't grow in 4% (w/v) NaCl and produce lactic acid without production of gas (Co_2). All isolates in this genus ferment glucose, lactose, and fructose and produce lactic acid. They can't produce acid from maltose, manitol, melezitose, raffinose, rhamnose, sucrose. So the 2 isolates were considered to be *Streptococcus thermophilus*.

3, Lactobacilli: six rod shaped isolates were also considered to relate to genus Lactobacillus. They were non motile, Gram's stain positive, catalase negative, grow at 10°C and at 45°C, unable to grow in 4% (w/v) NaCl and they produce lactic acid without gas (Co_2). The bacilli shape isolates were subdivided to three species:

a. Lactobacillus rhamnosus: one of the rods shaped isolates was capable to produce acid from lactose, fructose and raffinose, but not from maltose, manitol, melezitose, rhamnose, sucrose.

b. Lactobacillus paracasei: two of these isolates were capable to produce acid from glucose, lactose, fructose, maltose, manitol, melezitose, sucrose, but not from raffinose and rhamnose.

c. Lactobacillus helveticus: The last isolate was able to produce acid from glucose and lactose and unable to produce acid from fructose, maltose, manitol, melezitose, raffinose, rhamnose.

Table 1: **Primary and secondary confirmatory testes results of the isolates from the samples**

	Lactococcus lactis (n= 28)	*Streotococcus thermophilus* (n= 2)	*Lactobacillus rhamnosus* (n= 1)	*Lactobacillus paracasei* (n=4)	*Lactobacillus helveticus* (n=1)
Sample locations	13 from Alsubag, 15 from Gapat alfeel	Gapat Alfeel (Gadarif state)	Gapat Alfeel (Gadarif state)	Gapat Alfeel (Gadarif state)	Gapat Alfeel (Gadarif state)
Characteristics					
Morphology	Cocci	Cocci	Bacilli	Bacilli	Bacilli
Gram's stain	+	+	+	+	+
Catalase test	-	-	-	-	-
gas production (from Glucose)	-	-	-	-	-
Motility test	-	-	-	-	-
Growth at 4% NaCl	-	-	-	-	-
Growth at 6% NaCl	-	-	-	-	-
Growth at 10^{o} C	+	+	+	+	+
Growth at 45^{o}C	-	+	+	+	+
Survive for 30 minutes at 60^{o}C	+				
Acid production from :					
Fructose	+	+	+	+	-
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	-	-	+	+	-
Manitol	-	-	+	+	-
Melezitose	-	-	+	+	-
Raffinose	-	+	-	-	-
Rhamnose	-	-	+	-	-
Sucrose	-	-	+	+	-

The isolated LABs were clearly dominated by the genus Lactococcus (77.8%), followed by the genus Lactobacillus (16.7%) compared to streptococcus (5.6%). The high level of *Lactococcus lactis* in *Gariss* showed that the natural nomad's starter culture was predominated with *Lactococcus lactis* and that may be due to the high level of *Lactococcus lactis* in raw camel milk since the nomads ferment camel milk without any heat treatment. Brasca *et al.* (2008) found that *Lactococcus lactis subsp lactis* is dominant in raw camel milk. Also Khedid *et al.* (2009) found that the dominating species was *Lactococcus lactis subsp. lactis* (17.5%) among the strains of lactic acid bacteria isolated from raw dromedary milk in Morocco. Also *Streptococcus thermophilus* were present (5.5%) which indicated that the *Streptococcus thermophilus* has limited contribution in *Gariss* fermentation and this result is in agreement with Dirar (1993) who found that the numbers of streptococcus are limited in *Gariss*. This limitation may be due to the temperature in which the nomads ferment the camel milk (room temperature), which is less than the temperature preferred by *streptoccous thermophilus*; 40^{o}C to 45^{o}C (Bylund, 1995).

About 16.7% of the isolates were Lactobacilli, which mean that the lactobacillus have a good contribution in *Gariss* fermentation. The importance of lactobacillus in fermentation of

Gariss may be is due to the ability to grow in camel milk and in the temperature under which the nomads ferment camel milk. Hassan *et al.* (2006) studied the effect of pasteurization and storage conditions (25°C and 37°C) on the microbial count of *Gariss* made in the laboratory. They found that the higher increase was observed in mean log *Lactobacillus* spp. count of *Gariss* samples made from non pasteurized milk that incubated at 37°C and the lower rate was recorded for *Gariss* sample made from pasteurized milk and incubated at 25°C. Similarly Sulieman *et al.* (2006) characterized the LAB flora of *Gariss* samples from two regions in Sudan using phenotypic methods and found that *Lactobacillus paracasei subsp. paracasei* was the dominant LAB with *Lb. fermentum, Lactobacillus plantarum, Lactococcus lactis* and *Enterococcus spp.*

Acidification abilities of isolates

Acidifying abilities of lactic acid bacteria species isolated from fermented camel milk are presented in Figure 1 and Table 2. The amount of lactic acid produced by the different species was increased with the increase in fermentation time. Highest production was obtained by *Lactococcus lactis* (0.52 %) followed by *Streptococcus thermophilus* (0.42%).

Table 2: Acidifying abilities (T.A %) of lactic acid bacteria isolated from *Gariss*, in camel milk (2% inoculation)

Time (h)	*Lactococcus lactis*	*Streptococcus thermophiles*	*Lactobacillus rhamnosus*	*Lactobacillus paracasei*	*Lactobacillus helveticus*	*Mean*± S.E
0	0.20	0.20	0.19	0.15	0.14	**0.18±0.01^{e}**
2	0.23	0.20	0.20	0.15	0.15	**0.19±.02de**
4	0.28	0.21	0.23	0.15	0.15	**0.20±.02de**
6	0.37	0.3	0.26	0.16	0.16	**0.25±0.04^{cd}**
8	0.45	0.36	0.30	0.18	0.17	**0.29±0.05^{bc}**
10	0.47	0.44	0.36	0.21	0.18	**0.33±0.06^{ab}**
12	0.52	0.50	0.42	0.23	0.18	**0.37±0.07^{a}**
Mean± S.E	**0.36±0.05^{a}**	**0.316±0.05^{ab}**	**0.28±0.03^{b}**	**0.176±0.01^{c}**	**0.161±0.01^{c}**	

*$^{a, b, c, d, e}$ means within the same column or row with different superscripts differ significantly (P≤0.05)

The temperature in which the strains were incubated was 37°C except for *Streptococcus thermophilus* and *Lactobacillus rhamnosus* which was incubated at 45°C.

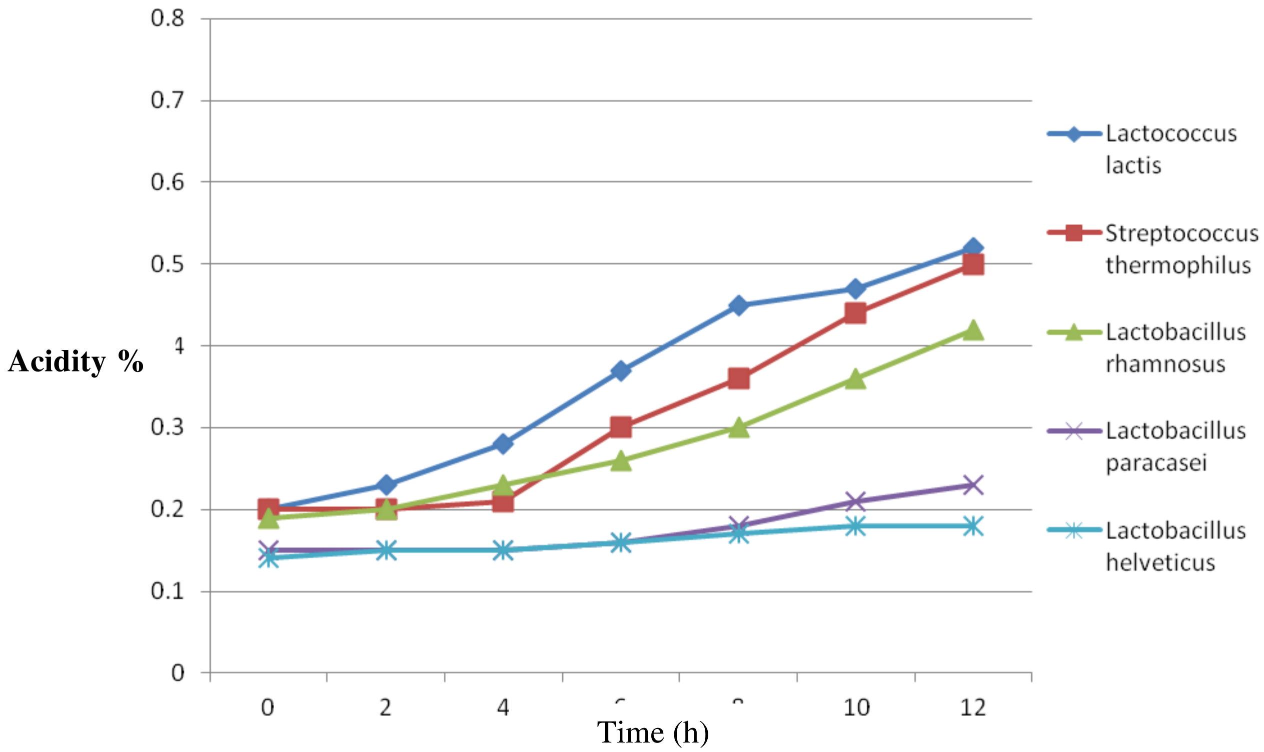

Figure 1: Acidifying abilities of lactic acid bacteria isolated from fermented camel milk *(Gariss)* (2% inoculation)

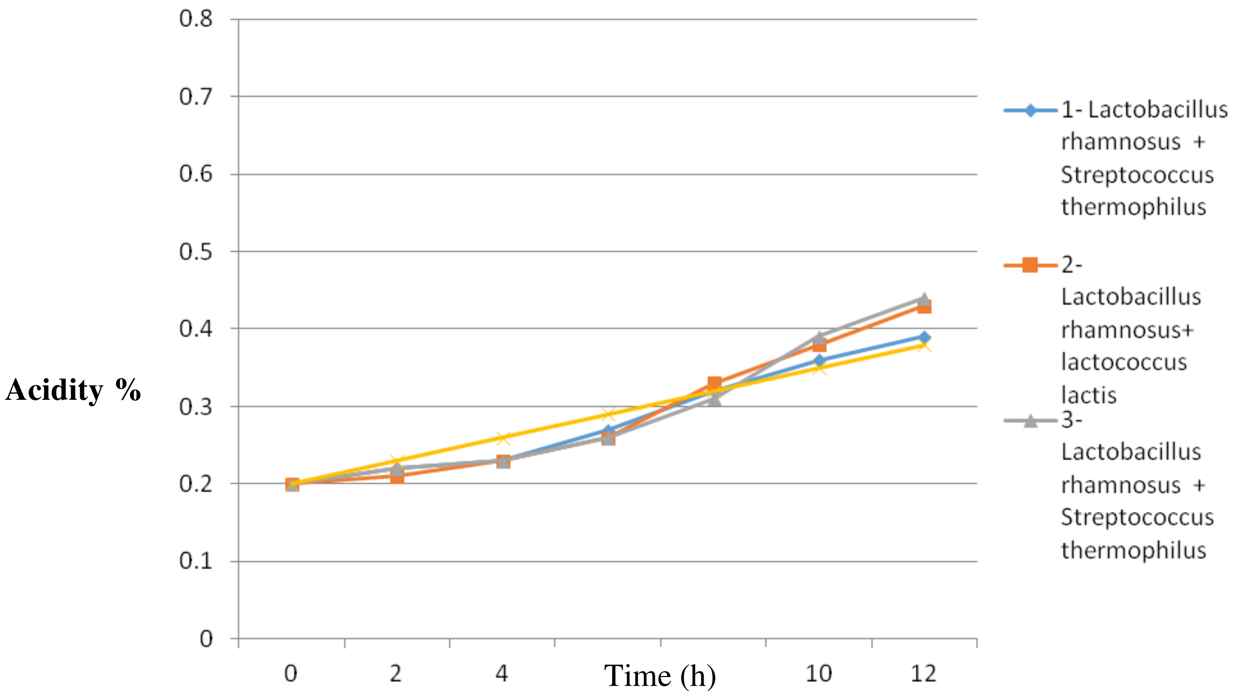

Figure 2: Acidifying activities of mixtures composed from selected strains isolated from *Gariss*, in camel milk

Table 3: Acidifying abilities (T. A%) of mixtures composed from selected strains isolated from *Gariss*, in camel milk

*[a, b, c, d, e] means within the same column or rows with different superscripts differ significantly (P≤0.05)

Time (h)	*Lactobacillus rhamnosus* + *Streptococcus thermophilus* at 37°C. (2%)	*Lactobacillus rhamnosus* + *Lactococcus lactis at* 37° C. (2%)	*Lactobacillus rhamnosus* + *Strptococcus thermophilus* at 45°C. (2%)	*Lactobacillus rhamnosus* + *Lactococcus lactis at* 45°C. (2%)	*Mean* ± S.E
0	0.20	0.20	0.20	0.20	**0.20 ± 0.00^{a}**
2	0.22	0.21	0.22	0.23	**0.22 ± 0.00^{ab}**
4	0.23	0.23	0.23	0.26	**0.23 ± 0.01^{b}**
6	0.27	0.26	0.26	0.29	**0.27 ± 0.01^{c}**
8	0.32	0.33	0.31	0.32	**0.32 ± 0.01^{d}**
10	0.36	0.38	0.39	0.35	**0.37 ± 0.01^{f}**
12	0.39	0.43	0.44	0.38	**0.41 ± 0.01^{e}**
Mean ±S.E	**$0.28\pm.03^{a}$**	**$0.29\pm.03^{a}$**	**$0.29\pm.03^{a}$**	**$0.29\pm.02^{a}$**	

The temperatures in which the mixtures incubated were as flows:
1. *Lactobacillus rahmnosus* + *Streptococcus thermophilus* at 37°C.
2. *Lactobacillus rahmnosus* + *Lactococcus lactis* at 37°C.
3. *Lactobacillus rhamnosus* + *Streptococcus thermophilus at* 45°C.
4. *Lactobacillus rhamnosus* + *Lactococcus lactis at* 45°C.

Inoculation percentage were (2%) for every mixture (1:1).
The lowest acidity was produced by *Lactobacillus paracasei* (0.23%) and *Lactobacillus helveticus* (0.18%).

The results show no significant difference between *Lactococcus lactis* and *Streptococcus thermophilus* and between *Streptococcus thermophilus* and *Lactobacillus rhamnosus*. These results showed that the ability of the isolated species to produce lactic acid is weak when compared with the 2% yoghurt starter culture which usually reaches 0.80% lactic acid in about 3-4 h. The weakness in acid production may be due to the fact that natural selection of lactic acid bacteria in raw camel milk depends on its ability to resist the inhibitors substances, use the bacterial growth factors found in the milk and grow in the ambient temperature, besides their ability to produce lactic acid.

Interactions with microorganisms originating from environmental exposure during manufacture and ripening, as well as the initial natural diversity of the microbiota present in milk, all play a role in fermentation processes and are important in the final development of traditional dairy products (Jose, 2007).

Acidifying activities of mixtures from isolated selected strains from *Gariss* (2%)

The results showed that there is no significant difference between the mixtures in terms of acid production after 12 h of incubation (Figure 2). Highest production of acid among the mixtures was obtained by *Lactobacillus rahmnosus* + *Lactococcus lactis* at 37°C and *Lactobacillus rhamnosus* + *Streptococcus thermophilus at* 45°C were 0.43% and 0.44%, respectively. These again showed that the isolates are still slow in acid production even when used in form of mixtures. The final acidity of the nomad's *Gariss* is usually high (1.2%), which may be due to the long incubation time and the high percentage of inoculation they use (El Zubeir and Ibrahium, 2009). Usually camel milk used in *Gariss* production is inoculated by previous *Gariss* culture or added to container which contains a large quantity of a previously soured product in the evening and left over night at ambient temperature (Dirar, 1993).

Conclusion

Natural fermentation of camel milk constituted LABs which is predominated by *Lactococcus lactis*. There is a significant difference between the isolated species in their abilities to produce acid. The isolated lactic acid bacteria are weak acid producers in camel milk and the *Lactoccus lactis* and *streptococcus thermophilus* are the best acid producers among the species present in Sudanese fermented camel milk. However more research is needed to determine the acidifying abilities of lactic acid bacteria isolated from *Gariss* in camel milk and artificial selection on the best acid producers among the isolates in order to improve their ability to produce lactic acid in a short time.

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