IJMPR 2021, 5(1), 139-147

International Journal of Modern Pharmaceutical Research

www.ijmpronline.com

ISSN: 2319-5878
IJMPR
Research Article

SJIF Impact Factor: 5.273

ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED CAMEL MILK AND ITS EFFICACY AS PROBIOTIC

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Received on: 20/12/2020
Revised on: 10/01/2021
Accepted on: 30/01/2021

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ABSTRACT

Lactic acid bacteria (LABs) are important microorganisms that are becoming widely used as probiotics. They have a positive effect on human health and are commonly used in food industry. Camel milk is believed to have the ability to treat many diseases, and is considered an important source for LABs. This study aimed to identify LABs isolated from fermented camel milk and investigate its antagonist effect towards other bacteria. Twenty four samples of camel milk were obtained from different camel farms. Milk samples were allowed to ferment naturally and then isolation of LAB was carried out using MRS medium. The isolates were then subjected to different biochemical tests for identification. The tolerance of the isolates towards different temperature, pH, NaCl and bile salt concentration was also tested. The antagonist effect of the different isolates was tested against Salmonella spp, *E. Coli*, and *Staphylococcus aureus* The identified isolates were: *Lactobacillus spp*. (29.4%), *Lactococcus spp* (35.3%) and Enterococcus (17.6%) *Leuconostoc mesenteroides (5.9%)* and *Pediococcus spp (11.8*). Two isolates namely: *Lactobacillus brevis* and *Enterococcus. faecalis* were found to have the best antagonist effect against the tested bacteria. This study revealed that LAB bacteria isolated from camel milk has the potential to be used as probiotics.

KEYWORDS: Lactic acid bacteria, camel milk, antagonist efficacy, Sudan.

1. INTRODUCTION

Lactic acid bacteria (LAB) are one of the beneficial microorganisms that dominate in fermented food (Khedid *et al.*, 2009). Various metabolic and enzymatic activities of LAB lead to production of volatile substances, which contribute to flavor, aroma and texture development of fermented products (Kleerebezemab et al., 2000).

LABs are being widely used as probiotics (Temmerman *et al*., 2002) especially with the increasing misuse of antibiotics in animal farms which eventually results in the presence of antibiotic residues in the various animal products such as meat, eggs and milk (Adil *et al.*, 2012, Hind *et al.*, 2014) and thus development of drug-resistant microorganisms in humans.

Food and Agriculture Organizations and the World Health Organization, defines probiotics as live microorganisms that confers health benefits on their hosts when ingested in an adequate concentration (Hills *et al.*, 2014) LAB also have the ability to stimulate the immune system (Kalliomäki *et al*., 2001), reduce serum cholesterol level (Jackson *et al.*, 2002), inhibit the growth of other food borne pathogens and spoilage microorganism such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium tyrobtyricum* (Alegria *et al.*, 2010). Among the main LAB strains used as probiotics are *Lactococcus*, *Lactobacillus* sp, *Bifidobacterium* sp., *Enterococcus* sp, *Pediococcus*, *Leuconostoc*, *Streptococcus*, and *Tetragenococcus* (Liu *et al.*, 2011, Lavanya *et al.*, 2011). In order to be used as a probiotic, the LABs should possess certain features such as tolerance to pH, bile and have an antagonist effect towards other pathogenic bacteria (Reuben *et al*., 2019) Sudan has one of the largest populations of camel. Pastoralists consume camel milk either in its raw state or after it turns sour (fermented) which is called G*ariss in* Sudan, *suusac* or *Itit*u in in Kenya, Somalia and Ethiopia (Lore *et al*., 2005, Abdelgadir *et al*., 2001, Ashmag, *et al*., 2009, Seifu *et al*., 2012). Milk fermentation can be carried out naturally or by using different types of starters. Traditional methods of fermentation involve storing raw milk at room temperature for 12-24 hours to allow spontaneous fermentation by the natural bacteria present in the milk (Seifu *et al.*, 2012). Camel milk differs from other ruminant's milk being low in cholesterol, sugar and protein but high in minerals (sodium, potassium, iron, copper, zinc and magnesium), vitamins A, B2, C and E and contains a high concentration of insulin and

immunoglobulin (Kamal et al., 2007, Al-Hashem, 2009). It has been reported that camel milk is widely used in stomach and intestinal disorders, Diabetes type I and, food allergy (Shehadeh, 2016).

Objectives

The objective of this study was to identify Lactic acid bacteria isolated from fermented Camel milk, to investigate its tolerance profile toward different temperature, pH, NaCl and bile salt concentrations and to detect the antagonistic activity towards pathogenic bacteria.

2. MATERIAL AND METHODS

2.1 Study area

Camel milk samples were randomly collected from two different areas in Sudan: Khartoum and Al Fasher State during 2017. These two areas have a large population of camel. Samples were collected aseptically in sterile containers then transported to the Microbiology laboratory - University of Bahri for further studies.

2.2. Isolation and Identification of LAB

Milk samples were allowed to ferment spontaneously for four days at room temperature. Fermented camel milk was analyzed microbiologically for the presence of LABs.

2.2.1 Primary Isolation and Purification

Samples of fermented camel milk were cultured in de Man, Rogosa and Sharpe agar (MRS) media (Oxoid Ltd, England), which is a selective culture medium designed to favor the luxuriant growth of Lactobacilli.

Using a sterile pipette, one ml of the fermented camel milk was suspended in nine ml sterile MRS broth and then incubated at 37°C for 24 hours. MRS agar plates were inoculated with sterile full loop from the overnight culture and were incubated anaerobically at 37°C for 48 to 72 hours in anaerobic jar. Primary isolates were sub-cultured on MRS agar to obtain pure cultures. The process was repeated till pure isolates were obtained and kept in MRS broth with 15% glycerol at – 20° C for further identification.

2.2.2 Identification and characteristics of isolated bacteria

Identification of purified isolates was performed according to the methods of (Barrow and Felltham, 2003). The isolates were identified using staining, motility and physiological characteristics and various biochemical tests.

2.2.2.1 Fermentation of sugars

The peptone water sugars were inoculated with test organism, incubated at 37º C and examined daily for acid production, which causes change in color to pink. Durham's tubes were also examined for the presence of gas production.

2.2.2.2 Biotolerance of LAB

The growth of the bacteria at different temperature, NaCl and bile salt concentrations, as well as different pH was studied at different time intervals. The LABs were cultured and inoculated at 1% in MRS broth.

2.3 Antagonist activity detection

The antagonist activity of the isolated LAB was carried out using disc diffusion assay. Muller Hinton agar (MHA) (Oxoid) plates were swabbed with the respective broth culture of the organisms used namely *Salmonella typhimurium*, *Escherichia Coli* and *Staphylococcus aureus* (diluted to 0.5 McFarland Standard with normal saline) and allowed for absorption to take place. Then, sterile 6 mm diameter filter paper discs impregnated with two LAB isolates namely: *Lactobacillus brevis* and *Enterococcus faecalis* were mounted onto MHA. The plates were incubated at 37 °C for 18- 48 hours. The antimicrobial activity was evaluated by measuring the inhibition against test organism. (Church *et al.,* 2002).

3. RESULTS

3.1 Isolation and Identification of LAB

A total of 24 milk samples were obtained from Khartoum and Alfasher State. Following four days fermentation period, 17 isolates (71%) were initially chosen based on their growth appearance on MRS agar media (Fig.1) Plates with no visible growth were re-incubated and examined daily for up to seven days before they were regarded as negative.

Fig. 1: Growth of LABs on MRS.

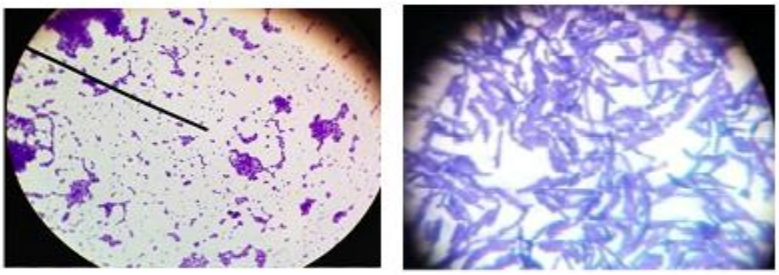
All the isolates grew at 37 °C under anaerobic conditions showing optimum growth just below the surface of the media. The isolates were Gram positive, non-motile, microaerophilic and catalase negative. The isolates showed different reaction regarding producing gas from glucose, all isolates did not produce gas except three isolates. In addition the reaction of the isolates for blood hemolysis showed variable results (Table 1).

Table (1): Characteristics of LAB isolated from fermented camel milk.

Characteristic Isolates No.	Gram stain	Morphology	Catalase test	Motility	Gas production from glucose	Blood hemolysis
1	+	Diplococci	-	-	+	+
2	+	Large cocci in tetra	-	-	+	V
3	+	Large cocci in tetra	-	-	+	V
4	+	Large coccobacilli	-	-	-	-
5	+	Large coccobacilli	-	-	-	-
6	+	Large coccobacilli	-	-	-	-
7	+	Bacilli (a)	-	-	-	V
8	+	Bacilli(a)	-	-	-	V
9	+	Bacilli(b)	-	-	-	-
10	+	Bacilli(b)	-	-	-	-
11	+	Bacilli(b)	-	-	-	-
12	+	coccobacilli	-	-	-	-
13	+	coccobacilli	-	-	-	-
14	+	coccobacilli	-	-		-
15	+	coccobacilli	-	-	-	-
16	+	coccobacilli	-	-	-	-
17	+	coccobacilli	-	-	-	-

(+): Positive reaction; (-): Negative reaction; (v): Variable

The isolates were further grouped based on their form, cell arrangements, Gram reaction, catalase production, motility, spore formation, and gas production from glucose. Under microscopy, the cells had different shapes and they were grouped as coccobacilli, cocci and bacilli, forming small chains of varying length, pairs or in clusters (Fig.2).



a- cocci b- bacilli

Fig. 2: LAB morphology under microscope.

Twelve isolates (70.5%) were found to be circular in shape and were identified as cocci. Two of them were large cocci, found as single or tetrads cell arrangement known as *Pediococci.* Five isolates (29.4%) were rod-shaped with long and rounded ends mostly appeared as chains of 4-5 cells, pairs or single cells and these could presumptively be determined as derivatives of the genus *Lactobacillus*.

3.2 Carbohydrate fermentation

Regarding the carbohydrate fermentation tests the different isolates showed variable reaction (Table 2).

Table 2: Carbohydrates fermentation results.

No	Microscopic examination	Types of sugar							
		Lactose	Mannitol	Maltose	Trehalose	Arabinose	Raffinose	Sucrose	Fructose
1	Diplococci	+	V	+	v	+	v	+	+
2	Large cocci in tetra	+	-	+	+	+	-	-	+
3	Large cocci in tetra	+	-	+	+	+	-	-	+
4	Large coccobacilli	+	+	+	+	-	-	+	-
5	Large coccobacilli	+	+	+	+	-	-	+	-
6	Large coccobacilli	+	+	+	+	-	-	+	-
7	Bacilli (a)	+	+	+	+	-	-	+	+

8	Bacilli (a)	+	+	+	+	-	-	+	+
9	Bacilli (b)	+	-	+	-	+	+	v	+
10	Bacilli (b)	+	-	+	-	+	+	v	+
11	Bacilli (b)	+	-	+	-	+	+	v	+
12	Coccobacilli(a)	+	-	-	-	-	-	-	+
13	Coccobacilli(a)	+	-	-	-	-	-	-	+
14	Coccobacilli(b)	+	-	+	+	-	-	-	+
15	Coccobacilli(b)	+	-	+	+	-	-	-	+
16	Coccobacilli(b)	+	-	+	+	-	-	-	+
17	Coccobacilli(b)	+	-	+	+	-	-	-	+

(+): Positive reaction; (-): Negative reaction; (v): Variable

1 Leuconostoc mesenteroides,
2-3 *Pediococcus spp*,
4-6 *Enterococcus faecalis*,
7-8 *Lactobacillus plantrum*
9-11 *Lactobacillus brevis*,
12-13 *Lactococcus lactis subsp. cremoris*,
14- 16 *Lactococcus lactis subsp. lactis*,
17: *Lactococcus lactis subsp. Lactis*

3.3. Isolated bacterial species

The main isolated bacteria identified was *Lactococcus lactis* (23.5%) followed by *Lactobacillus brevis* and *Enterococcus faecalis* (17.6%).The isolates were further identified as *Leuconostoc mesenteroides, Pediococcus spp, Enterococcus faecalis, Lactobacillus plantrum, Lactobacillus brevis, Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. Lactis*, according to physical and biochemical tests, their growth at different temperature, pH, NaCl and bile salt concentrations (Table 3 and Fig. 3).

Table 3: Biotolerance of isolated LABs towards Temperature NaCl and Bile.

Parameter Isolate No.	**Growth at diff. temp (° C)**						**Growth at different pH**				**Growth in NaCl**				**Growth in bile salt**			
	10		**40**		**45**		**4.5**		**6.5**		**4.5%**		**6.5%**		**3%**		**6%**	
	2h	**4h**	**2h**	**4h**	**2h**	**4h**	**2h**	**4h**	**2h**	**4h**	**2h**	**4h**	**2h**	**4h**	**2h**	**4h**	**2h**	**4h**
1	+	+	-	-	-	-	+	v	-	-	-	-	-	-	+	+	v	v
2	-	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+
3	-	-	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+
8	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	+	+
9	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	-	-	-	-	+	+	+	+	-	-	-	-	v	V	v	v
13	+	+	-	-	-	-	+	+	+	+	-	-	-	-	v	V	v	v
14	V	V	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+
15	V	V	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+
16	V	V	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+
17	V	V	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+	+

(+): Positive reaction; (-): Negative reaction; (v): Variable

1 Leuconostoc mesenteroides,
2-3 Pediococcus spp,
4 -6 Enterococcus faecalis,
7 -8 Lactobacillus plantrum,
9-11 Lactobacillus brevis,
12-13 Lactococcus lactis subsp. cremoris,
14-16 Lactococcus lactis subsp. lactis,
17: Lactococcus lactis subsp. Lactis.

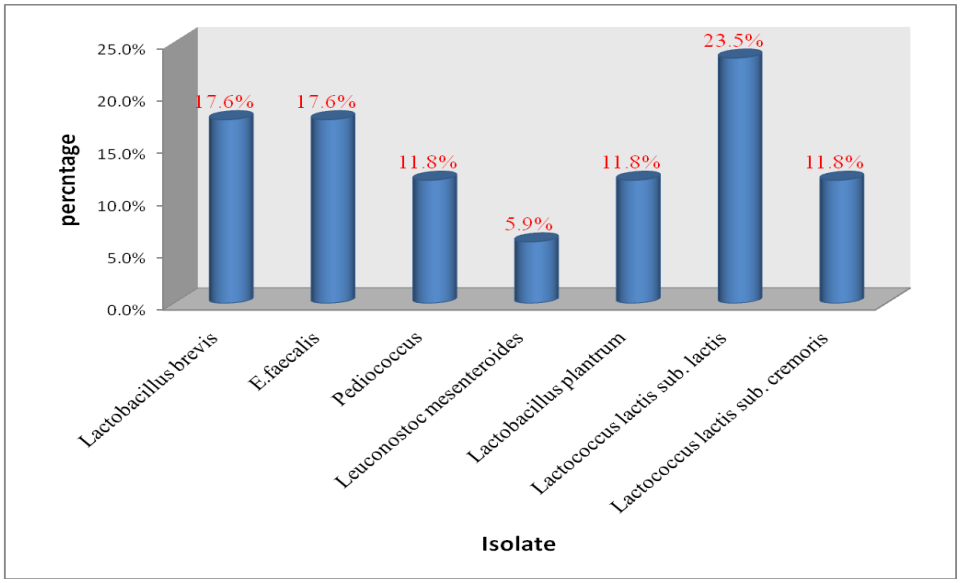


Fig. 3: Percentage of isolated LAB.

3.4 Antagonist effect

All seven isolates were tested for their inhibitory effect against three bacteria *Salmonella typhimurium*, *E. Coli* and *Staphylococcus aureus* The isolates showed variable inhibition zones (Fig.4 and Table 4). The *Staphylococcus aureus* showed resistance to all LAB isolates.

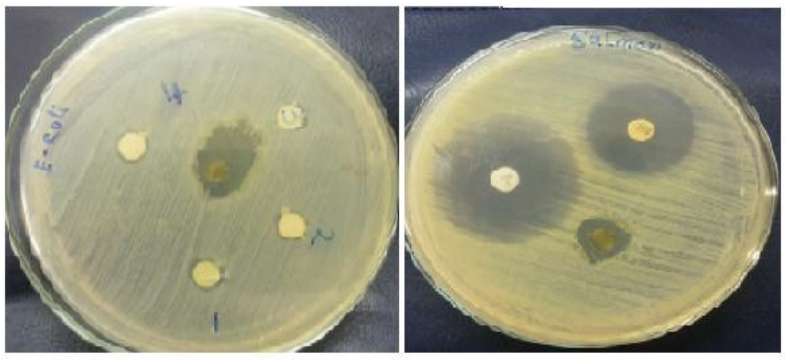


Fig. 4: Inhibition of LAB against *E. Coli* and *Salmonella spp.*

Table 4: Inhibitory zones of LAB isolates.

LAB isolate	Inhibition diameter zone (mm)*		
	Inhibition diameter zone (mm)*	*Salmonella spp*	*E.Coli*
Lactobacillus brevis	**Inhibition diameter zone (mm)***	23 mm	33 mm
Enterococcus faecalis	**Inhibition diameter zone (mm)***	18 mm	18 mm
Lactobacillus plantrum	**Inhibition diameter zone (mm)***	15 mm	11 mm
Lactococcus lactis subsp. Cremoris	**Inhibition diameter zone (mm)***	10 mm	07 mm
Lactococcus lactis subsp. Lactis	**Inhibition diameter zone (mm)***	16 mm	14 mm
Leuconostoc mesenteroides	**Inhibition diameter zone (mm)***	10 mm	10 mm
Pediococcus spp	**Inhibition diameter zone (mm)***	12 mm	14 mm
Antagonistic activity	**Inhibition diameter zone (mm)***	**Sensitive**	**Sensitive**

4. DISCUSSION

The aim of this study was to isolate and identify Lactic acid bacteria from fermented camel milk and evaluate their efficacy as probiotics. After growth on MRS media and identification using conventional methods, the dominant genus were *Lactococcus* (35.3 %) followed by *Lactobacillus* (29%).

Lactic acid bacteria has been isolated from various local Sudanese fermented food including Roob (Abdelgadir *et al*., 2001) and Kisra (Ashman and Muna, 2009). The latter study reported the presence of *Lactobacillus fermentum, Lactobacillus amylovorus* and Lactobacillus *brevis* from both traditionally and laboratory prepared fermented dough used for preparation of Sudanese Kissra .Several studies reported similar results (Ayad et *al*.,2004, Khedid *et al*.,2009, Seifu *et al*.,2012). Camel milk from Saudi Arabia showed the presence of *Lactococcu*s, *Lactobacillus a*nd *Enterococcus spp* (Mutlag *et al.*, 2013). *Lactococcus* spp. and *Lactobacillus* spp were also reported in raw milk from other animal species such as goat and Hu sheep (Zahra *et al*, 2019, Chen *et al.*, 2020).

Other studies reported the dominance *of Lactobacillus* species which constituted 58% to 74% of the total lactic acid bacteria isolated from fermented milk such as Situ and Gariss (El-Hadi *et al*. 2006, Ashmag *et al.*, 2009, Seifu *et al*., 2012).

In this study, the lactococcus genera were further identified as *Lactococcus lactis sub. species lactis* and *Lactococcu*s *lactis sub species cremoris*. The former was able to ferment lactose, maltose, trehalose and fructose. Other results showed that these subspecies were able to ferment maltose, mannitol, lactose, arabinose, salicin, glucose, mannose and fructose (Cheriguene *et al*., 2006, Seifu *et al.*, 2012). These subspecies did not ferment mannitol, arabinose, raffinose and sucrose which was also observed by Cheriguene *et al*. (2006). The other subspecies identified was *Lactococcus lactis subspecies cremoris*. This group only fermented lactose and fructose but did not ferment mannitol, maltose trehalose arabinose, raffinose and sucrose. Cheriguene *et al*. (2006) also reported that *Lactococcus lactis* subspecies cremoris did not ferment raffinose, sorbitol and arabinose *Lactococcu*s *lactis subspecies lactis* were more dominant than *Lactococcus lactis subspecies cremoris* (23% vs 11.6%). Other studies also reported the predominance of *Lactococcus lactis subspecies lactis* isolated from Ititu, Dahi, butter and raw milk (Padmanabha-Reddy *et al*., 1994, Khedid *et al.*, 2009).

The *Lactococcus lactis subsp. lactis* showed higher tolerance to salt and bile concentration than *Lactococcu*s *lactis subsp. cremoris*. It was also observed that *Lactococcus lactis subsp. cremoris* tolerated only low salt concentration when compared to *Lactococcus lactis biovar. diacetylactis* which showed high salt tolerance (4–6.5%) than *S. salivarius subsp*. thermpo-philus which tolerate less than 2% (Sandine, 1998). This resistance is important especially during dairy industry because some dairy products require exposure to high salt level. Camel milk is also known by its salted taste under dehydration effect (Khedid *et al*., 2009).

Another genera identified in the present study was Lactocbacillus that was further identified as *Lactocbacillus plantrum* and *Lactocbacillus brevis*. The former was able to ferment all tested sugars except arabinose and raffinose while the latter fermented lactose, maltose, arabinose, raffinose and fructose. Another study reported the isolation of only *Lactobacillus plantarum* and *Lactobacillus paracasei* from fermented camel milk (El-Hadi *et al*. 2006, Seifu *et al*, 2012). The study of Elvira *et al* (2020) showed the presence of Lactobacillus species– *fermentum, casei, curizae, oryzae, brevis, plantarum, rhamnosus, paracasei.*

Another identified species was *Enterococcus faecalis* which fermented most sugars except arabinose, raffinose and fructose. Similar results were reported by Chingwaru *et al* (2003) who found that these isolates fermented the majority of the tested substrates except arabinose and dolcitol and were too weak to ferment sorbitol and raffinose.

In this study, one isolate was identified as *Leuconostoc mesenteroides* (5.9%). Edalati *et al*, (2018) reported the isolation of *Leuconostoc mesenteroides* in addition to *Lactobacillus plantrum* from raw camel milk, while the study of Bayili *et al.*, (2019), reported *Leuconostoc mesenteroides* being the predominant LAB followed by *Pediococcus pentosaceus and Weissella paramesenteroides* at the onset of milk fermentation, while *Lactococcus lactis* and Enterococcus spp. where the predominant LAB after 7 h Another identified isolated was *Pediococcus* (11.3%) which was also reported in the study of Elvira *et. al.*, (2020) constituting 23% of isolated LAB.

LABs are becoming widely used as probiotics now a days. Since they are usually given orally, they have to pass through the digestive system and tolerate the low pH of the stomach and the bile salts in the small intestine which are an important criteria for selecting the candidate bacteria to be used as probiotics (Olejnik *et al*., 2005, Reuben *et al*., 2019). In this study, *Enterococcus faecalis* was found to be tolerable to various temperature, pH, bile and NaCl concentration while the *Lactobacillus brevis* gave similar results except they did not grow at low temperature. Chidre and Revanasiddappa (2017) also reported a similar tolerance pattern for *Lactobacillus*. Tolerance of the bacteria to the bile is necessary for the metabolic activity of the bacteria in the host and balancing the intestinal microflora (Tambekar and Bhutada, 2010) which is an important function of LABs. In the current study, except for *Leuconostoc* and *Pediococcus* all species were able to grow at different

pH.. Similar results were reported by(Olejnik *et al*., 2005).

An additional important feature of LAB is its antagonist activity towards other pathogenic bacteria. In this study, the isolates were tested against three bacteria, *Staphylococcus aureus, Salmonella spp and E Coli*. The different isolates had different inhibitory effects against *Salmonella spp* and *E. Coli* but the *Staphylococcus aureus* was resistant to all isolates. The inhibitory effect of different LAB isolates against various bacteria such as *E. Coli, Staphylococcus aureus* and *List. Monocytogenes* was also reported by various studies (Aguilar *et al* 2011, Mutalg *et al*., 2013, Edalati *et al*, 2018, Ołdak *et al.*, 2017; Sahar *et al*, 2018, Yateem *et al*, 2018). For example, Lactobacilli are able to produce soluble antimicrobial peptides, called bacteriocins which may show various antimicrobial and cytotoxic effects these bacteria may exert (Ocaña et al., 1999; Mutlag, *et al*, 2013, Bogovič-Matijašić *et al*, 1998; Bozoudi *et al* 2015). This is important in food industry since these bacteriocins also inhibit the growth of several food borne pathogens and spoilage bacteria such as *Listeria monocytogenes Staphylococcus aureus, Enterococcus, E. coli, Listeria, Clostridium, Bacillus cereus, Salmonella Enteritidis and clostridium tyrobtyricum* (Alegria *et al.*, 2010). LAB isolated from Hu milk also showed a high antimicrobial activity against Enterohemorrhagic Escherichia Coli (EHEC), enterotoxigenic Esherichia Coli (ETEC), *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Aeromonas caviae* (Chen *et al*., 2020)

CONCLUSION

This study revealed the presence of LABs in fermented camel milk with the dominance of species *Lactococcus lactis.* Most of the isolates showed tolerance to different temperature, pH, and NaCl and bile salt concentrations which can make them suitable candidate to be used as probiotics. In addition, the isolates were found to have an antagonist effect against *Salmonella spp* and *E. Coli*, while the *Staphylococcus aureus* was found to be resistant to all isolates.

ACKNOWLEDGMENT

The authors would like to thank the Deanship of Academic Research-University of Bahri for funding this research and Ms. Siham Ali for providing the milk samples from AlFasher State.

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