BF-1516
[1-6]

RESEARCH ARTICLE Indian Journal of Animal Research

Characterization and Molecular Identification of Bacteria Isolated from Raw Camel's Milk in Al-Ahsa, Saudi Arabia

Ashraf Khalifa[1,2], Mohammed Almalki[1]

10.18805/IJAR.BF-1516

ABSTRACT

Background: Camel's milk has many benefits on human health, however, drinking fresh untreated camel's milk may pose a serious health concern. Therefore, the current study aimed to determine the bacteriological quality of raw camel's milk by isolating, characterizing and identifying bacterial strains from the untreated milk in Al-Ahsa region, Saudi Arabia.

Methods: Three raw milk samples were collected under aseptic conditions and bacterial counts were determined using the serial dilution plate method. Discrete colonies were picked based on morphological differences. Five representative bacterial strains were characterized by phenotypic analysis, biochemical tests using the API 20E system and sequencing of 16S rRNA genes.

Result: The results showed that the total bacterial counts in camel's milk reached 3×10^7 CFU mL^{-1}, which exceeds the limit specified by the Saudi Food and Drug Authority. The isolates formed colonies that were rounded, entire, convex and elevated from the surface of the agar. The diameter of the colonies ranged from 1 to 4 mm after a 48-h incubation at 30°C on the M17 medium. The isolates were able to metabolize 12 (60%) to 15 (75%) out of 20 different compounds as growth substrates present in the API 20E test system. Additionally, BLAST analysis of the 16S rRNA sequences of the five bacterial isolates revealed that they were closely related to various known bacterial genera (*e.g., Bacillus*, *Kocuria* and *Pseudomonas*), indicating the diverse composition of the microflora in raw camel's milk. Unlike the other four bacterial isolates, a clear zone was noted around the growing one bacterial isolate, CMK1, highlighting a remarkable degradation of the hemoglobin in the erythrocytes, highlighting the potential existence virulence determinants. Good hygiene practices during milk production and handling are recommended to ensure high raw milk quality and avoid health risks. This study sheds light on the bacterial diversity of raw camel's milk that has a direct impact on public health and the economy.

Key words: *Bacillus,* Dairy, Diversity, Identification, *Pseudomonas.*

INTRODUCTION

Camels play a pivotal role in the social culture of Saudi Arabia. Additionally, they own unique qualities that make them superior to other domesticated animals under harsh desert conditions. According to the FAO estimates, the global camel population is about 35 million heads and the camel population in the Kingdom of Saudi Arabia is about 1.6 million heads (FAO, 2019).

Camel's milk contains proteins, fats and considerably high amounts of minerals and vitamins; for this reason, it can stand as a complete diet (Aqib *et al*. 2019), for nomads for weeks. Camel's milk has multiple benefits of on human health. It has been reported that drinking camel's milk improves liver function in hepatitis patients and helps treat juvenile diabetes and improves histopathological parameters in cardiovascular and hepatorenal patients (Hassani *et al*. 2022). In addition, camel's milk has antimicrobial activity against some bacterial pathogens such as *Staphylococcus aureus* and *Escherichia coli* (Ayyash *et al*. 2020). Additionally, it has been reported that raw camel's milk is inhabited by diverse bacterial species such as probiotic strains *Enterococcus*, *Streptococcus, Lactobacillus* and *Bifidobacterium* (Suez *et al*. 2020). Such benefits contribute in increasing camel's milk demand (Mohan *et al*. 2020).

Despite the presence of beneficial bacteria, potentially pathogenic species can contaminate camel's milk. It has been reported that raw camel's milk in Taif, Saudi Arabia, harbors potentially pathogenic species, such as *Serratia nematodiphila* (Samy *et al*. 2017), *Brucella* (Hirad *et al*. 2018) posing a serious health concern. Consumption of untreated camel's milk has been reported as a mode of transmission of the Middle East respiratory syndrome-related coronavirus (Nooh *et al*. 2020) and hence poses a potential health concern for both humans and animals. Desert camping is a common tradition among Saudis, where they drink fresh untreated camel's milk. Few reports address camel's milk from the bacteriological viewpoint in Al-Ahsa. Therefore, this study aimed to assess the bacterial biodiversity of camel's milk in Al-Ahsa, Saudi Arabia. The specific aim of the project was to identify isolated strains using genotypic techniques.

[1]Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa, Saudi Arabia.
[2]Department of Botany and Microbiology, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt.

Corresponding Author: Ashraf Khalifa, Biological Sciences Department, College of Science, King Faisal University, Al-Ahsa, Saudi Arabia. Email: akhalifa@kfu.edu.sa

How to cite this article: Khalifa, A. and Almalki, M. (2022). Characterization and Molecular Identification of Bacteria Isolated from Raw Camel's Milk in Al-Ahsa, Saudi Arabia. Indian Journal of Animal Research. DOI: 10.18805/IJAR.BF-1516.

Submitted: 16-03-2022 **Accepted:** 21-05-2022 **Online:** 15-06-2022

MATERIALS AND METHODS

Collection of milk samples

Three samples of raw camel's milk (~200 ml each) were collected under aseptic conditions in sterile bottles directly from the udder of apparently healthy camels, in 9th March, 2019, in Al-Ahsa. To avoid microbial contamination, the first three streams of the milk were discarded. Samples were transferred immediately to an icebox and transported to the laboratory for analysis. The experimental work was conducted in the laboratories of Biological Sciences Department, College of Science, King Faisal University, Al-Ahsa, Saudi Arabia.

Bacterial isolation and enumeration

Briefly, 10 mL of fresh camel's milk was diluted with 90 mL of sterilized saline solution (8.5 g/L, NaCl w/v). The suspension was shaken for 5 min before it was subjected to serial dilutions up to 10^{-8} by transferring 1 mL into 9 mL of the sterile saline solution in sterile tubes. One milliliter from each dilution was pour-plated on M17 agar medium (Frantzen *et al*. 2016). The dried plates were incubated at 37°C for 48 h. After the incubation period, the formed colonies were counted and reported as CFU per liter of milk. Discreet colonies were picked and restreaked onto fresh plates containing the M17 medium to obtain pure isolates.

Phenotypic characterization of bacterial isolates

The bacterial isolates were screened visually for colour, shape, margin and elevation of the formed colonies. To avoid redundancy, five different bacterial isolates representing different morphological colonies were selected for further characterization.

Biochemical characterization of bacterial isolates using the API 20E kit

The ability of the five bacterial isolates to metabolize 20 different biochemical substrates was investigated using the commercially available API 20E strips (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. After inoculation, the strips were incubated at 30°C and the results were recorded after 24 h.

Hemolytic activity

The isolates were checked for hemolysis using the blood agar lysis technique. The isolates were inoculated into blood agar medium, supplemented with 5% sheep blood and incubated at 30°C for 48 h. After incubation, the plates were checked for formation of a hemolytic zone around the bacterial colonies.

Catalase activity assay

The ability of the isolates to produce catalase was determined as previously described (Khalifa and Almalki, 2015). Aliquots of the actively growing bacterial cultures were were flooded with hydrogen peroxide (5%). Positive results were recorded when gas bubbles evolved within a few seconds after the addition of the reagent.

Genotypic identification of bacterial isolates using 16S ribosomal RNA gene sequencing

Extraction of genomic DNA

Total DNA of the bacterial isolates at the mid-exponential growth phase was extracted by boiling the bacterial suspension in the presence of InstaGene Matrix (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene

PCR amplification of the 16S rRNA gene was carried out in a 20 μL reaction using the universal primers 27F 5′ -AGA GTT TGA TCM TGG CTC AG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ as outlined by Khalifa *et al*. (2015). PCR products were purified using the Clean-up kit (Millipore, ThermoFisher Scientific, Loughborough, UK) according to the manufacturer's protocol.

16S rRNA sequencing

The purified 16S rRNA gene of the bacterial isolates was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA) on an Applied Biosystems 3730xl DNA Analyzer. The obtained sequences were deposited in the NCBI database.

Phylogenetic analysis

The 16S rRNA sequences of the bacterial isolates were identified by the BLASTn alignment against the GenBank nr/nt database (BLASTN) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Phylogenetic relationships were inferred using the maximum likelihood method based on the Tamura-Nei model suing MEGA5.02 (Tamura *et al*. 2011).

RESULTS AND DISCUSSION

Camel's milk is the main source of food for nomads in Saudi Arabia. A popular habit among Saudi people is to drink fresh untreated camel milk, which could be contaminated with virulent microbes. Therefore, the current study aimed to provide in-depth insights into the bacterial species inhabiting raw camel milk and the quality of milk from a bacteriological viewpoint.

Bacterial isolation and enumeration

The average total bacterial count of raw camel's milk from Al-Ahsa was 3×10^{7} CFU mL^{-1} and thus exceeded the allowed upper limit recommended by the Saudi Food and Drug Authority (Saudi Arabian Standards 2019). The finding that the total bacterial count of raw camel's milk from Al-Ahsa exceeded the allowed upper limit recommended by the Saudi Food and Drug Authority was in agreement with previous studies in Taif (Samy *et al*. 2017) studies in Qassim (El-Ziney and Al-Turki, 2007) where high bacterial loads were found in raw camel's milk. The total viable counts of bacteria reported in this study were also higher than those reported in raw camel's milk in Taif (Samy *et al*. 2017). Additionally, a high incidence of pathogenic Brucella has been reported in raw camel's milk in Riyadh (Hirad *et al*. 2018). This result suggests poor hygiene practices during milking that can be

explained by inadequate handwashing and improper udder preparation. This finding also uncovers potential health and economic concerns.

Phenotypic characterization of bacterial isolates

Five morphologically different bacterial isolates, called CMK1 to CMK5, were selected for further characterization. The colonial appearance of the isolates is presented in Table 1. The isolates formed colonies that were rounded, entire, convex and elevated from the surface of the agar. The diameter of the colonies ranged from 1 to 4 mm after a 48-h incubation at 30°C on the M17 medium. The colour of the colonies varied among the bacterial isolates: CMK2, milky; CMK1, yellowish white; CMK4, white; CMK3, faint orange; and CMK5, yellow (Table 1). All isolates except CKM4 were gram-positive cells. While three isolates (CKM1, CKM2 and CKM3) were rod-shaped, two (CMK3 and CMK5) were spherical-shaped. The morphological differences among the bacterial isolates were remarkably similar to those described for the relevant strains (*Bacillus, Pseudomonas* and *Kocuria*), as will be discussed later.

Genotypic identification of bacterial isolates using 16S ribosomal RNA gene sequencing

A milestone tool for the identification and classification of bacterial and archaeal taxa is 16S rRNA gene sequencing. The widespread presence and high variability of the 16S rRNA gene in prokaryotes enable efficient discrimination of the assigned taxa. The 16S rRNA sequences of the bacterial isolates were obtained and deposited in the NCBI database under the following accession numbers: CMK1 (MT084027), CMK2 (MT084031), CMK3 (MT084030), CMK4 (MT084032) and CMK5 (MT084034). BLAST analysis of the 16S rRNA sequences of the five bacterial isolates revealed that they were closely related to various known bacterial genera (*e.g., Bacillus, Kocuria* and *Pseudomonas*). CMK1 and CMK2

Table 1: Characterization of the bacterial isolates obtained from Camel's milk.

Isolate	CMK1	CMK2	CMK3	CMK4	CMK5
Morphological characterization					
Colony shape	Circular	Circular	Circular	Circular	Circular
Colony Colour	Yellowish white	Milky	Faint orange	White	Yellow
Colony diameter	3	3	1	4	2
Colony elevation	Elevated	Elevated	Elevated	Elevated	Elevated
Colony margin	Entire	Entire	Entire	Entire	Entire
Gram reaction	Gram +veRods	Gram +veRods	Gram +vecocci	Gram –veRods	Gram +vecocci
Biochemical characterization					
ONPG *	+	+	-	+	-
Arginine	+	+	+	+	+
Lysine	-	+	-	-	-
Ornithine	+	+	+	+	+
Citrate	+	+	+	+	+
Na thiosulfate	-	-	+	-	+
Urea	-	-	-	-	-
Tryptophan	+	+	+	+	-
Indole	-	-	-	-	-
Na pyruvate	+	+	-	+	-
Charcoal gelatin	-	-	+	-	+
Glucose	+	+	+	+	+
Mannitol	+	+	+	+	+
Inositol	-	-	-	+	-
Sorbitol	+	+	+	-	-
Rhamnose	+	+	+	+	+
Sucrose	+	+	+	+	+
Melibiose	+	+	+	+	+
Amygdalin	+	+	+	+	+
Arabinose	+	+	+	+	+
Genotypic characterization					
Closest strain	*Bacillus sp.*	*Bacillus aryabhattai B8W22 (EF114313*	*Kocuria tytonicola*	*Pseudomonas hunanensis*	*Kocuria marina*
Identity (%)	97.8	99.1	99.8	99.4	99.5
Accession number	MT084027	MT084031	MT084030	MT084032	MT084034

*: ortho-Nitrophenyl-β-galactoside.

displayed a 97.8% and 99.1% homology with *Bacillus* sp. and *B. zanthoxyli*, respectively, while CMK3 and CMK5 shared a 99.8% and 99.5% homology with *K. tytonicola* and *K. marina*, respectively (Table 1 and Fig 1), indicating the composition of the microflora in raw camel's milk. Indeed, many reports have documented the existence of *Bacillus* and *Kocuria* (Wang *et al*. 2018) and *Pseudomonas* (Hirad *et al.* 2018) in in raw camel's milk.

Inferring phylogenetic relationships and evolutionary history among bacterial and archaeal taxa relies on the 16S rRNA genes. Therefore, 16S rRNA gene-based phylogenetic tree among the bacterial isolates from camel's milk and the related genera was generated (Fig 1). Bacterial isolates were clustered within three distinctive clades inferred by the maximum likelihood method27. CMK1 and CMK2 were clustered within the Bacillus clade, while CMK3 and CMK5 clustered within the Kocuria clade. CMK4 formed a monophyletic group within the Pseudomonas clade. Phylogenetic trees generated with the neighbor-joining method using the MEGA7 software were similar to the trees constructed with the maximum likelihood method, thus confirming the robust positioning of the bacterial isolates within the clades (data not shown). CMK2 and CMK5 clustered with the closest bacterial species, *B. aryabhattai* B8W22 (EF114313) and *K. marina* KMM 3905 (AY211385), respectively. Interestingly, CMK1, CMK3 and CMK4 formed outgroups within the *Bacillus* and *Pseudomonas* clades, respectively. This could be because 16S rRNA genes contain highly conserved domains that unable distinction between closely related taxa. Alternatively, the three isolates could be three novel species or subspecies within the bacterial genera. Further studies are hence needed to determine the exact taxonomic position of these isolates; for instance, techniques such as DNA–DNA hybridization and sequencing of other housekeeping genes.

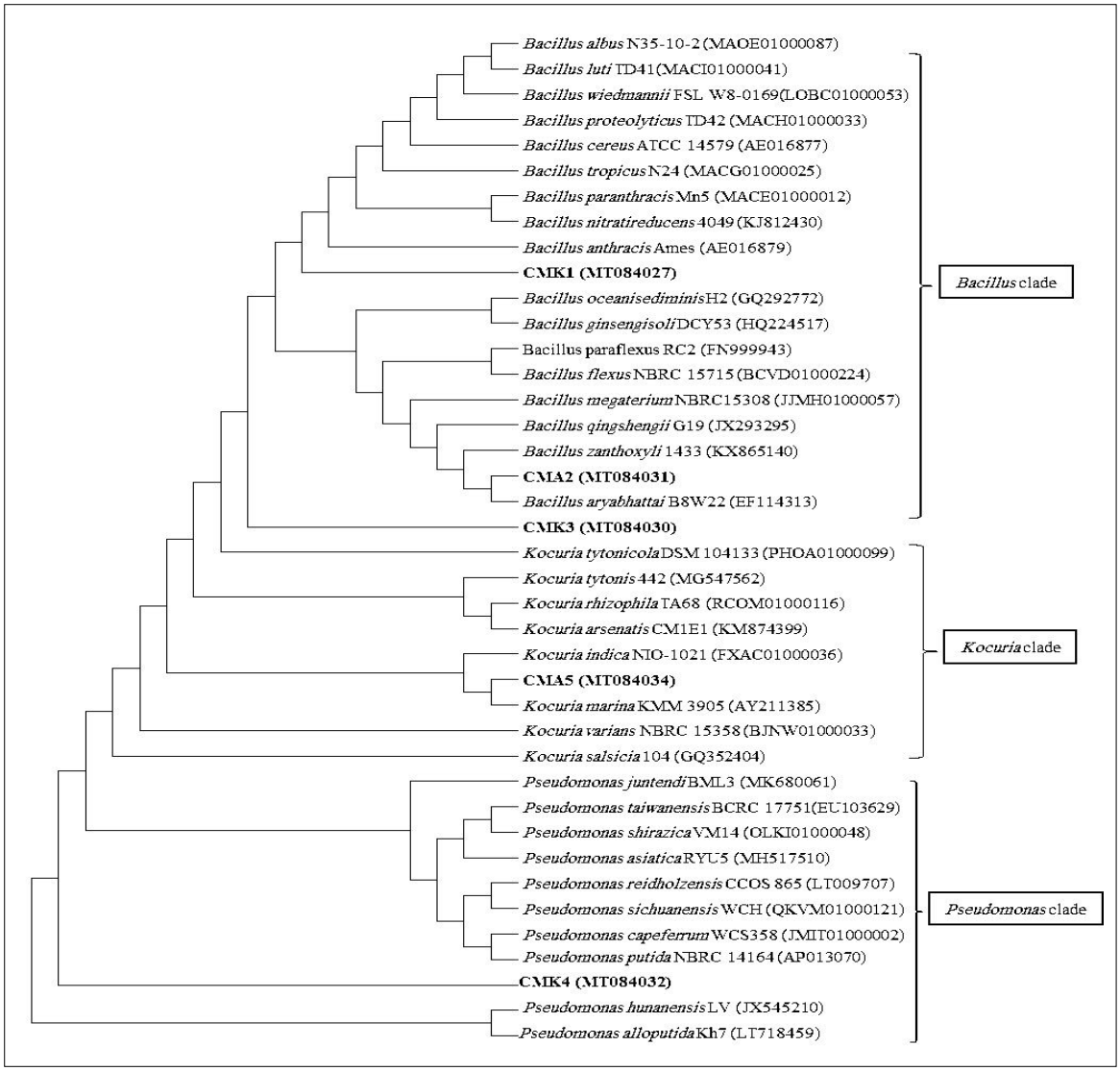

Fig 1: Unrooted maximum likelihood tree showing phylogenetic relationships among five bacterial isolates identified in raw camel's milk and their closely related bacterial species.

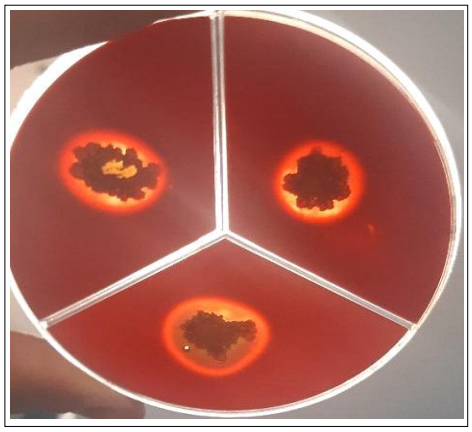

Fig 2: Formation of a clear zone around the growing CMK1 colonies indicating complete degradation of hemoglobin in the erythrocytes.

Hemolytic activity

Unlike the other four bacterial isolates, a clear zone was noted around the growing CMK1colonies highlighting a remarkable degradation of the hemoglobin in the erythrocytes (Fig 2). The finding highlighted potential existence of virulence determinants. Similar findings have been reported in *Bacillus* sp., (Klichko *et al*. 2003). Additionally, six potential membrane-damaging proteins, were existed in the genome of as *Bacillus* sp. Such proteins were highly similar to hemolysins and phospholipases C from *B. cereus* (Klichko *et al*. 2003). More investigations are needed to verify the potential virulence of the strain CMK1.

Biochemical characterization of bacterial isolates using the API 20E kit

To further characterize the isolates, a biochemical analysis with the API E20 test was performed. As shown in Table 1, the strains were able to metabolize 12 (60%) to 15 (75%) out of 20 different compounds as growth substrates present in the API 20E test system. All isolates consumed arginine, ornithine, citrate, glucose, mannitol, rhamnose, sucrose, melibiose, amygdalin and arabinose as growth substrates. No isolate was able to consume all compounds (Table 1). None of the isolates metabolized urea. The biochemical diversity among the bacterial isolates as revealed by the API E20 test, indicating the existence of the enzymatic machinery that enables the isolates to metabolize these substrates. For example, arginine dihydrolase and ornithine decarboxylase are enzymes responsible for the first step in the utilization of arginine and ornithine, respectively. None of the isolates metabolized urea, suggesting the possible lack of urease. These observations are in agreement with those reported for *Bacillus, Kocuria* and *Pseudomonas* (Khalifa and Almalki 2015). Unlike *K. marina,* CMK5 was able to metabolize certain carbohydrates such as glucose, sucrose, melibiose and arabinose as the only carbon source indicating preferences toward different substrates at the species level. Similar phenotypic variations have been reported in Pseudomonas aeruginosa (Chandler *et al*. 2019).

Catalase activity assay

All isolates formed air bubbles upon addition of drops of H_2O_2 indicating the presence of the catalase enzyme. This enzyme splits the toxic hydrogen peroxide into water and oxygen, indicating the protective role of catalase enzyme in preventing the damage to the vital macromolecules existed inside bacterial cells.

CONCLUSION

Collectively, the total bacterial count of raw camel's milk in Al-Ahsa exceeded the allowed upper limit recommended by the Saudi Food and Drug Authority. This finding indicates a lack of proper hygiene practices during the milking process and, consequently, poses potential health and economic concerns. Camel milk is inhabited by morphologically, biochemically and genetically diverse bacteria.Taken together, this study sheds light on the bacterial composition of raw camel's milk that has a direct impact on public health and the economy.

ACKNOWLEDGEMENT

This work was supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Al-Ahsa, Saudi Arabia [Project No. GRANT26 (180115)].

Conflict of interest

There are no conflicts of interest to declare.

REFERENCES

Aqib, A.I., Kulyar, M.F.E.A., Ashfaq, K., Bhutta, Z.A., Shoaib, M. and Ahmed, R. (2019). Camel milk insuline: Pathophysiological and molecular repository. Trends in Food Science and Technology. 88: 497-504.

Ayyash, M., Abu-Jdayil, B., Itsaranuwat, P., Galiwango, E., Tamiello-Rosa, C., Abdullah, H. and Hamed, F. (2020). Characterization, bioactivities and rheological properties of exopolysaccharide produced by novel probiotic *Lactobacillus plantarum* C70 isolated from camel milk. International Journal of Biological Macromolecules. 144: 938-946.

Chandler, C.E., Horspool, A.M., Hill, P.J., Wozniak, D.J., Schertzer, J.W., Rasko, D. A. and Ernst, R.K. (2019). Genomic and phenotypic diversity among ten laboratory isolates of *Pseudomonas aeruginosa* PAO1. Journal of Bacteriology. 201(5): e00595-18.

El-Ziney, M.G. and Al-Turki, A.I. (2007). Microbiological quality and safety assessment of camel milk (*Camelus dromedaries*) in Saudi Arabia (Qassim region). Applied Ecology and Environmental Research. 5(2): 115-122.

FAO-statistics (2019). Available from: <http://faostat.fao.org/>.

Frantzen, C., Kleppen, H.P. and Holo, H. (2016). Use of M17 and a milk-based medium enables isolation of two distinct and diverse populations of *Lactococcus lactis* strains from undefined mesophilic starter cultures. International Dairy Journal. 53: 45-50.

Hassani, M.I., Saikia, D. and Walia, A. (2022). Nutritional and therapeutic value of camel milk. IJAR, 8(4): 01-05.

Hirad, A.H., Ahmad, J., Alkhedhairy, A.A., Bahkali, A.H. and Khan, S.T. (2018). Bacterial isolates exhibiting multidrug resistance, hemolytic activity and high 16S rRNA gene similarity with well known pathogens found in camel milk samples of Riyadh region. APMIS. 126(3): 215-226.

Khalifa, A.Y. and Almalki, M.A. (2015). Isolation and characterization of an endophytic bacterium, *Bacillus megaterium* BMN1, associated with root-nodules of *Medicago sativa* L. growing in Al-Ahsa region, Saudi Arabia. Annals of Microbiology. 65(2): 1017-1026.

Khalifa, A., Lee, C.G., Ogiso, T., Ueno, C., Dianou, D., Demachi, T. and Asakawa, S. (2015). *Methylomagnum ishizawai* gen. nov., sp. nov., a mesophilic type I methanotroph isolated from rice rhizosphere. International Journal of systematic and Evolutionary Microbiology. 65(10): 3527-3534.

Klichko, V.I., Miller, J.A. Wu, S.G. and Popov, K. (2003). Anaerobic induction of *Bacillus anthracis* hemolytic activity. Biochemical and Biophysical Research Communications. 303: 855-862.

Mohan, G., Gupta, V., Raj, A. and Kaur, R. (2020). Consumer acceptance of camel milk in emerging economy. Journal of International Food and Agribusiness Marketing. 32(1): 54-68.

Nooh, H.Z., Alshammary, R.H., Alenezy, J.M., Alrowaili, N.H., Alsharari, A.J., Alenzi, N.M. and Sabaa, H.E. (2020). Public awareness of coronavirus in Al-Jouf region, Saudi Arabia. Journal of Public Health. 1-8.

Samy, M.F., Yasser, E.H., Othman, A.L. and Amer, S.A. (2017). Microbial quality and molecular identification of pathogenic bacterial strains collected from raw camel's milk in taif region. Journal of Camel Practice and Research. 24(1): 89-98.

Saudi Arabian Standards and Metrology Organization. Specifications 43,194 / 1977 and 130/3.1994/756.

Suez, J., Zmora, N. and Elinav, E. (2020). Probiotics in the next-generation sequencing era. Gut Microbes. 11(1): 77-93.

Tamura, K., Peterson, D., Peterson, N., Stecher, G. *et al*. (2011). MEGA5, molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Molecular Biology and Evolution. 28(10): 2731-2739.

Wang, Z., Zhang, W., Wang, B., Zhang, F. and Shao, Y. (2018). Influence of Bactrian camel milk on the gut microbiota. Journal of Dairy Science. 101(7): 5758-5769.