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Microbiological Studies on Naturally Present Bacteria in Camel and Buffalo Milk

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ABSTRACT

The aim of current study was to isolate and identify naturally occurring probiotic *Lactobacillus* species in buffalo milk, camel milk, and camel urine to investigate their susceptibility to antibiotics and their antibacterial activity against pathogenic bacteria. A total number of seven samples which included three milk samples from buffalo, three milk samples from camel, and one urine sample from camel were collected and used in this study. The samples were cultured, and 18 isolated strains were identified by using 16S rRNA multiplex Polymerase Chain Reaction analysis, which was performed following DNA extraction from the isolated bacteria. Buffalo and camel milk were different in their *Lactobacilli* content. All *Lactobacilli* strains that were found in both camel milk and camel urine, were also found in buffalo milk, *Lactobacilli* strains in camel milk and urine were generally more resistant to the antibiotic. *Lactobacilli* isolated from buffalo milk, camel milk, and also camel urine presented variable degrees of antibacterial activity against pathogenic bacteria. Further studies should be conducted with more samples to gain more information in the field of antibacterial activity of probiotic *lactobacilli* and to understand the mechanisms of their activity. Hopefully, they can be used as natural alternatives instead of synthetic antibiotics.

Keywords: Antibacterial, Antibiotics, *Lactobacillus*, Probiotics

INTRODUCTION

Antibiotic resistance is considered as a global health crisis threatening the lives of both humans and animals. Many clinically isolated pathogenic bacteria are becoming increasingly resistant to antibiotics and disinfectants which make infection of these bacteria difficult to treat. During their evolution, bacteria have been developing several sophisticated mechanisms of antibiotic resistance to all types of antibiotics with no exception (Davies and Davies, 2010). The growing threat of antibiotic resistance necessitates the employment of creative approaches towards the discovery of novel alternatives to antibiotics. The use of probiotics is one of the options that is being discussed by the medical community to be used as an alternative to antibiotics (Brunel and Guery, 2017).

Probiotics are living microorganisms which confer health benefits to the host upon their administration in suitable amounts (FAO/WHO, 2011). The beneficial balance of the intestinal microbiota is one of the health-promoting properties that can be presented by probiotic microorganisms. Probiotics have been prescribed for patients with gastrointestinal disease and complaints (Williams et al., 2010). There is a set of cumulative evidence that supports the use of probiotics, both in food products and supplements to provide protection against infectious diseases including respiratory infections (Hao et al., 2011; Ozen et al., 2015). *Lactobacilli*, *Enterococci*, and *Bifidobacteria* are families of Lactic Acid Bacteria (LAB) and they constitute the most frequently used strains of probiotics (Fijan, 2014). The LAB constitute a diverse group of microorganisms that are naturally present in human diet and in both gastrointestinal and urogenital tract of animals (Ruiz Rodriguez et al., 2019) The main objective of the current study was to isolate and identify naturally occurring probiotic *Lactobacilli* in buffalo milk as well as camel milk and urine to investigate their susceptibility to antibiotics as well as their antibacterial activity against representative pathogenic bacterial strains of both Gram-positive and Gram-negative bacteria to assess their potential use as natural alternatives to synthetic antibiotics.

MATERIALS AND METHODS

Ethical approval

Institutional Animal Ethics Committee, local laws and regulations were considered in performing our experiment. All procedures involving the use of the animals were approved by the ethics committee of National Research Centre, Egypt.

Sample collection

A total number of seven samples including three milk samples from three different buffalos, three milk samples from three different camels and one urine sample from a separate camel were collected during the summer of 2016 from

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private, individually owned healthy animals in Giza governorate, Egypt. The samples were collected under aseptic conditions in sterile containers and stored on ice. *Lactobacillus* spp. was isolated from the collected samples by using MRS medium as a selective medium. An amount of 1 ml of each of the milk samples as well as 1 ml of the urine sample was dissolved in 100 ml of MRS broth (pH 6.5) and incubated at 37 °C for 24 h in aerobic condition. The initial cultures were subcultured for seven times at 37 °C under low pH (pH 4.5) and anaerobic condition in the presence of 10% CO_2 to eliminate unwanted bacteria. Single colonies were selected and streaked onto MRS agar media at pH 4.8 to obtain pure colonies. Finally, single pure colonies of *Lactobacillus* were selected for further characterization and identification (Shokryazdan et al., 2014).

Characterization of isolated bacteria

The isolated bacteria were evaluated by different biochemical and molecular tests including Gram stain and Catalase test as well as bacterial morphology. The isolate bacteria were identified as *Lactobacilli* based on being Gram-positive, Catalase-negative and having rod-shape under light microscope. The *Lactobacilli* identification of isolated bacteria were further confirmed by using 16S rRNA multiplex polymerase chain reaction (PCR) analysis.

Gram staining

A prepared smear of 24 h cultured bacteria was heat fixed on a slide. Gram staining based on standard technique was then performed and then slides were observed under light microscope (Bergey et al., 1994).

Catalase test

Fresh liquid cultures which contained overnight grown cultures from selected single colonies were used for Catalase test. An amount of 3% hydrogen peroxide solution was dropped onto 1 ml of the culture. The formation of gas bubbles was considered as positive Catalase test and these samples were neglected while the other samples with negative Catalase test were selected since *Lactobacilli* are known to be Catalase-negative.

Molecular identification of probiotic strains

The DNA was extracted from the isolated bacteria and *Lactobacillus* strains were confirmed by using 16S rRNA multiplex PCR analysis (Kwon et al., 2004). The reaction mixture (25 µl) contained 12.5 µl of PCR Master Mix, 5 µl primer mixture comprising 50 pmol of each primer, 4.5 µl of water, and 3 µl of DNA template. PCR amplification was performed in Applied Biosystem 2720 thermal cycler, and DNA fragments were amplified as follows. Initial heating at 94 °C for 2 min, 35 cycles consisting of denaturation at 94 °C for 20 sec, annealing at 51 °C for 40 sec, extension at 68 °C for 30 sec, and final extension step in 7 min at 68 °C. The PCR products were separated on 1.5% agarose gel by electrophoresis and analyzed by RedSafe Nucleic Acid Staining Solution (Intron Biotechnology, Korea).

Antibiotic susceptibility of *Lactobacilli*

A wide panel of 14 antibiotic disks was tested against 7 mixed cultures of probiotic *Lactobacilli* isolated from both buffalo and camel samples (Figures 2-8). Antibiotic susceptibility test was performed by using the disk-diffusion method with some modifications (ISO, 2010). *Lactobacilli* activated cultures were swabbed on MRS agar plates instead of Muller Hinton Agar plates. Fourteen different antibiotic disks were used for the susceptibility test including Trimethoprim/sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Cefuroxime (CXM-30), Amoxicillin with clavulanic acid (AmC-30), Cefotaxime (CTX-30), Cefaclor (CEC-30), Rifampicin (RD-5), Erythromycin (E-15), Vancomycin (Va-30), Amikacin (AK-30), Ampicillin with sulbactam (SAM-20), Cefadroxil (CFR-30), Azithromycin (AZM-15), and Doxycycline (DO-30). All plates were incubated for 24 h at 37^{o}C and inhibition zones were measured.

Antibacterial activity of *Lactobacilli*

The ability of the seven mixed cultured of isolated probiotic *Lactobacilli* to inhibit the growth of pathogenic bacteria was investigated against nine pathogenic standard strains of both Gram-positive and Gram-negative bacteria (Figures 9-15). Gram-positive strains were represented by *Staphylococcus aureus* (ATCC 26923), *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pneumoniae* (ATCC 29619), and *Enterococcus faecalis* ATCC (29212). Gram- negative strains were represented by *Pseudomonas aeruginosa* ATCC (27853), *Escherichia coli* ATCC (25922), *Escherichia coli* ATCC (10536), and *Klebsiella pneumoniae* ATCC (700603). Antibacterial activity of probiotic *Lactobacilli* was tested by using Agar-well diffusion method with some modifications (Bauer et al., 1966; Sgouras et al., 2004). Wells of 7 mm diameter were made on Muller-Hinton agar plates. Each plate was swabbed with the respective test pathogen. From each probiotic *Lactobacillus* strain which previously incubated under anaerobic conditions for 24 h at 37^{o}C, 70 µl of MRS liquid culture were placed in the respective wells. After 24 h of incubation at 37 ^{o}C, the inhibition zones were measured and recorded in cm.

Statistical analysis

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The *in vitro* antibacterial activity was conducted in triplicate. All the data were then subjected to SPSS Version 21 (IBM, New York, US). Statistical analysis was performed using two-way ANOVA followed by Duncan's Multiple Range Test to determine significant difference. The given values represented mean ± Standard Deviation (SD). A probability value $P<0.05$ was taken as significant difference (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Lactobacilli isolated from buffalo milk, camel milk, and camel urine were subjected to characterization and identification by using different biochemical and molecular identification methods. A total number of seven samples were collected including three milk samples and one urine sample from camel and three milk samples from buffalo. *Lactobacilli* were isolated by growing the bacterial contents of the samples on MRS medium as selective medium. The bacterial colonies were initially identified as *Lactobacilli* based on being Gram-positive and Catalase-negative as well as being rod-shaped under the microscope. Mixed colonies of each sample in MRS broth medium were used to extract DNA for molecular identification using 16S rRNA multiplex PCR analysis. The mixed colonies of each sample were also used to test antibiotic susceptibility and antibacterial activity of the isolated strains.

Multiplex PCR analysis

The results from 16S rRNA multiplex PCR analysis have been demonstrated in figure 1. A total number of 18 isolated bacteria from buffalo milk, camel milk, and camel urine were identified as *Lactobacilli*. *Lactobacillus* species were identified based on the size of the PCR product (Kwon et al., 2004). The results indicated that buffalo and camel milk were different in their *Lactobacilli* content. There were also differences in *Lactobacilli* content of different milk samples collected from the same species. The results indicated the presence of *L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum*, *L. gasseri* and *L. delbrueckii* in buffalo milk samples. Meanwhile, both camel milk and camel urine samples expressed the presence of *L. casei*, *L. acidophilus* and *L. plantarum*.

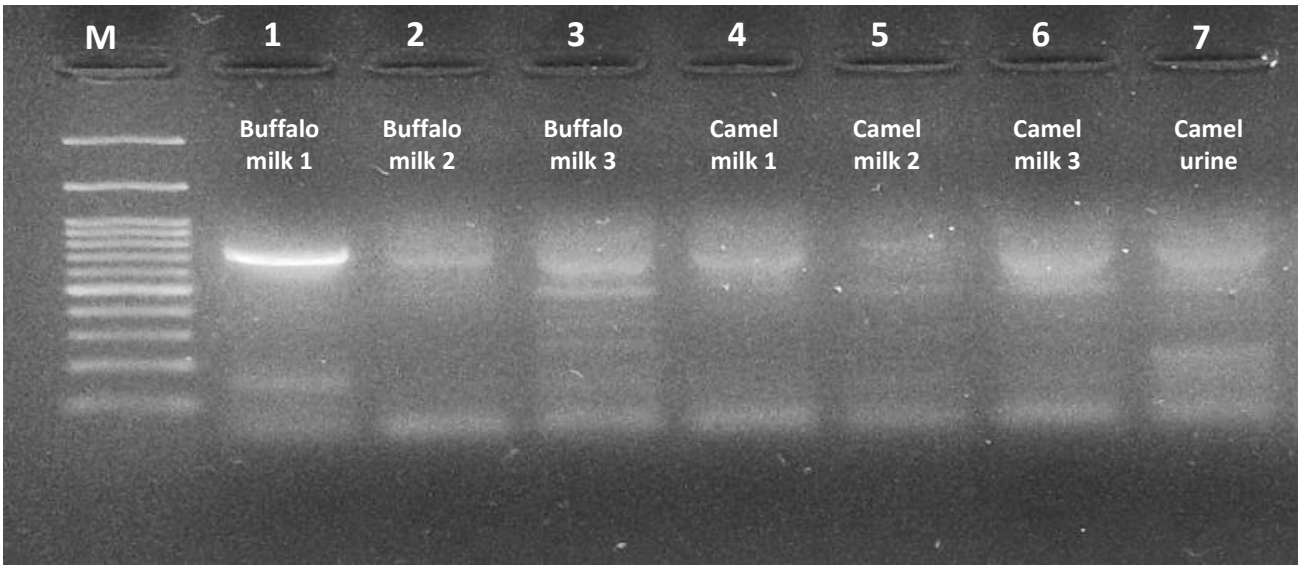

Figure 1. Agarose gel electrophoreses of PCR products from multiplex PCR assays. Multiplex PCR assays were performed with a mixture of seven species-specific or group-specific primers for L. acidophilus, *L. bulgaricus* (same as *L. delbrueckii* subsp. *bulgaricus*), *L. casei*-group *L. gasseri*, *L. plantarum*, *L. reuteri* and *L. rhamnosus* and two bacterial conserved primers. Lanes 1–7 designate the PCR product from each genomic DNA extracted from single or mixed cell suspension isolated from representative host used as PCR template. Lane 1: *L. casei*, *L. delbrueckii*; lane 2: *L. casei*; lane 3: *L. casei*, *L. acidophilus*, *L. rhamnosus*, *L. plantarum* and *L. gasseri*; lane 4: *L. casei*; lane 5: *L. plantarum*; Lane 6: *L. plantarum*; lane 7: *L. plantarum*; lane M: 100 bp-DNA ladder.

Antibiotic susceptibility of *Lactobacilli*

Antibiotic susceptibility of *Lactobacillus* strains was tested by using a panel of 14 antibiotics (Figures 2-8). It was clear that all samples had extremely significant resistant ($p < 0.0001$) to Cefadroxil (CFR-30) with inhibition zones of 0.0 cm. Cefaclor (CEC-30) exclusively did not present any inhibition to the growth of one of buffalo milk samples (buffalo milk 2) and all camel milk and urine samples. While Vancomycin (Va-30) did not cause any inhibition to only one of buffalo milk samples (buffalo milk 1). The rest of antibiotics exclusively presented no inhibition to camel samples which included Cefuroxime (CXM-30), Cefotaxime (CTX-30), Erythromycin (E-15), Ampicillin with sulbactam (SAM-20), and Azithromycin (AZM-15). Furthermore, camel urine sample was the only one to be totally resistant to (AmC-30)

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with 0.0 cm growth inhibition. On the other hand, all samples were sensitive to Trimethoprim/sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Rifampicin (RD-5) and Doxycycline (DO-30) with varying degrees of inhibition.

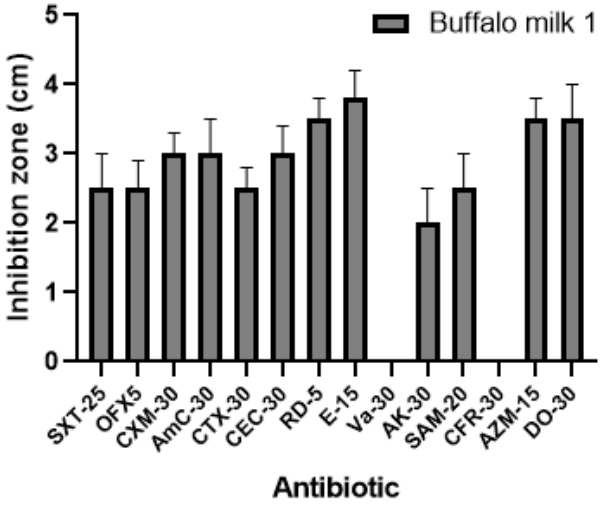
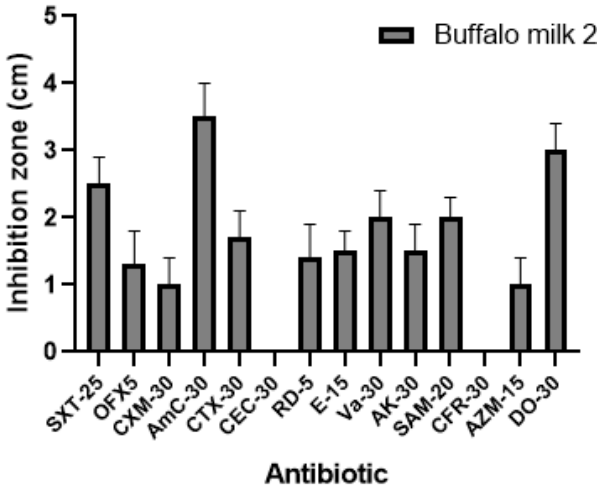


Figure 2



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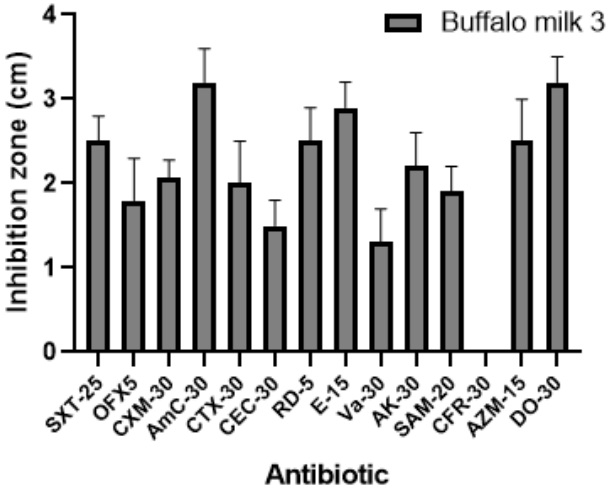

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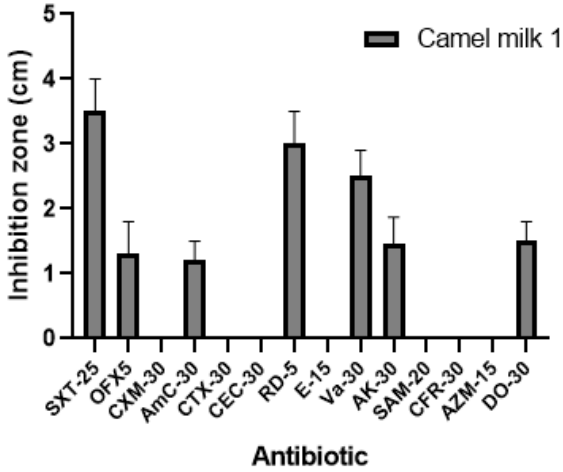

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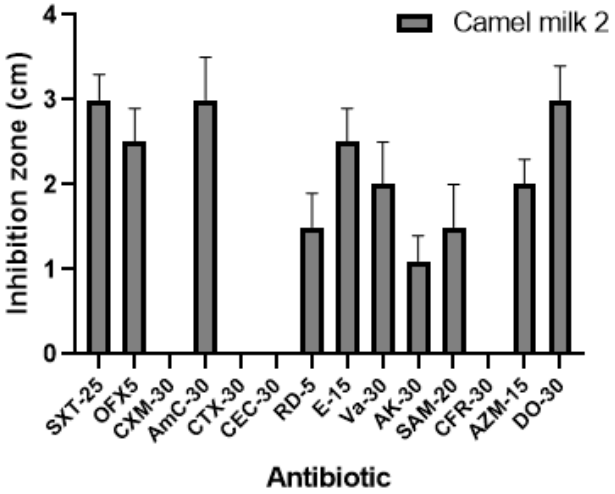

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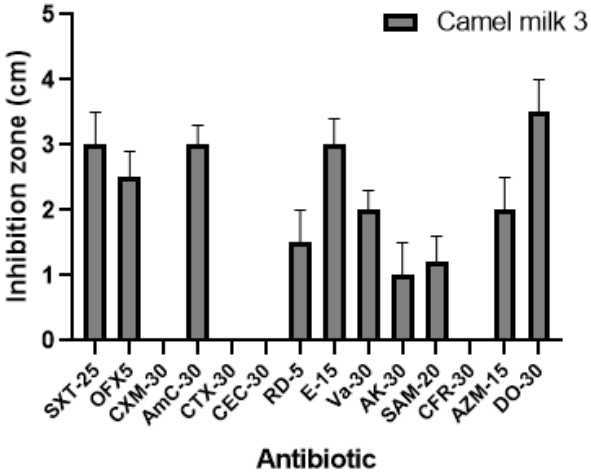

Figure 7

Camel urine

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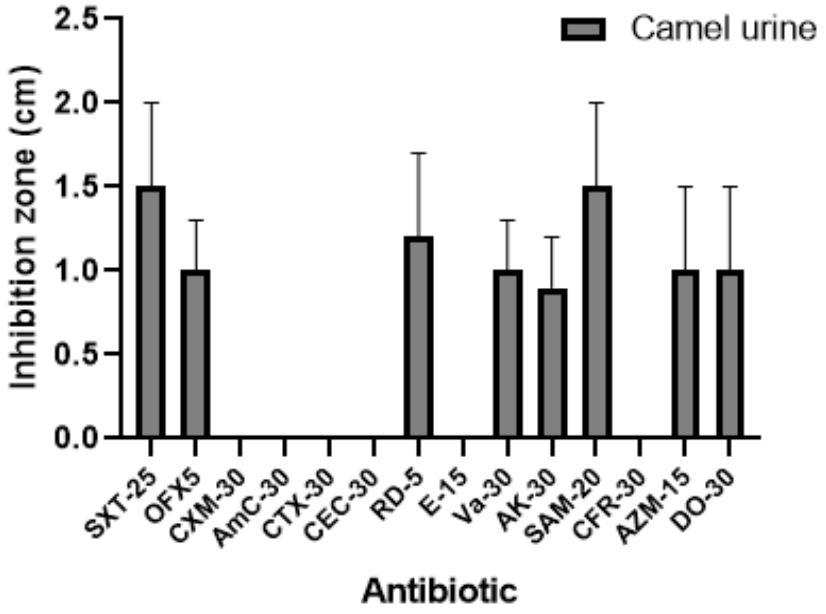

Figure 8

Figures 2-8. Antibiotic susceptibility of the probiotic *Lactobacilli* isolated from buffalo milk, camel milk or camel urine. Fourteen antibiotic disks were used for the susceptibility test including Trimethoprim/sulfamethoxazole (SXT-25), Ofloxacin (OFX5), Cefuroxime (CXM-30), Amoxicillin with clavulanic acid (AmC-30), Cefotaxime (CTX-30), Cefaclor (CEC-30), Rifampicin (RD-5), Erythromycin (E-15), Vancomycin (Va-30), Amikacin (AK-30), Ampicillin with ctam (SAM-20), Cefadroxil (CFR-30), Azithromycin (AZM-15), and Doxycycline (DO-30).

Antibacterial activity of *Lactobacilli*

The antibacterial activity of the isolated probiotic *Lactobacilli* was investigated against nine pathogenic standard strains of both Gram- positive and Gram- negative bacteria (Figures 9-15). The results indicated that the antibacterial activity of *Lactobacilli* which were isolated from camel urine was in general extremely significant lower ($p < 0.0001$) than the antibacterial activity of *Lactobacilli* from both buffalo milk and camel milk against all tested bacterial strains. The antibacterial effect of buffalo milk three was significantly higher ($P = 0.0045$) than all other samples against *E. coli* 25922 while the antibacterial activity of camel milk two was significantly higher against *S. aureus* 29213 ($P = 0.0014$), *S. pneumonia* 29619 ($P = 0.0014$) and *E. faecalis* 29212 ($P = 0.0014$) when compared to its effect against *E. coli* 10536.

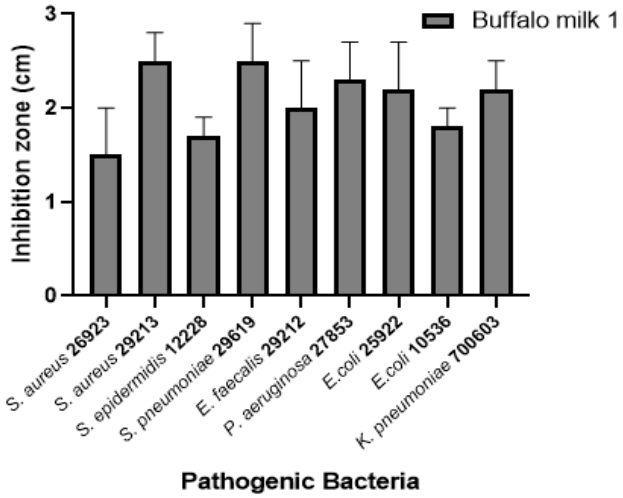

Figure 9

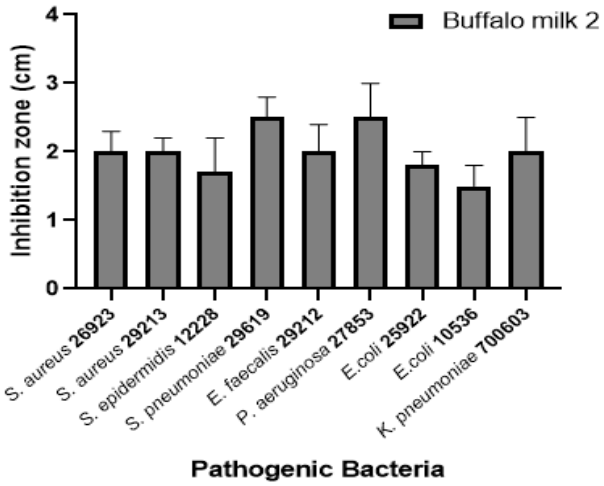

Figure 10

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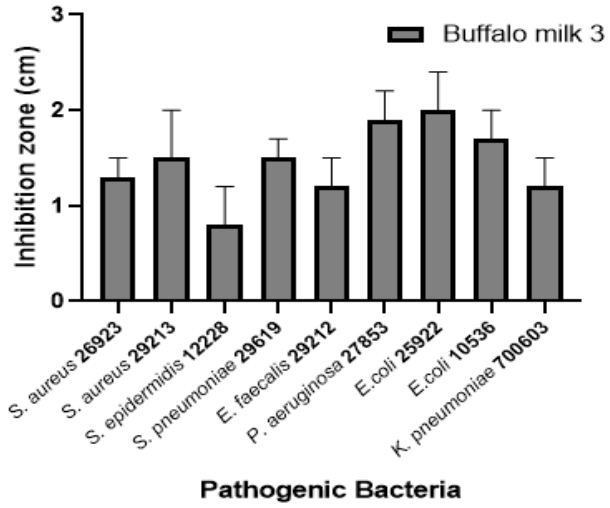
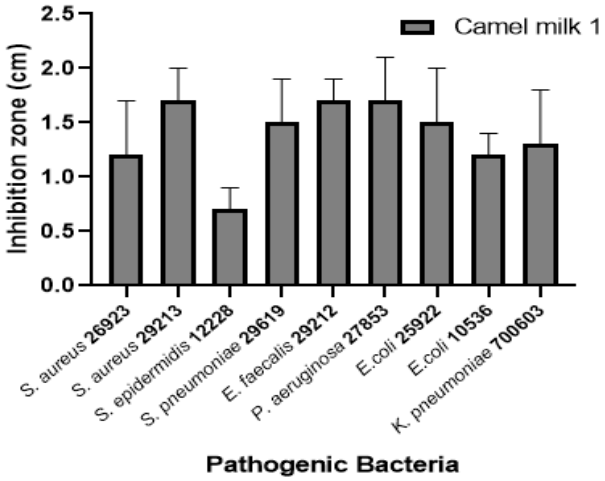


Figure 11



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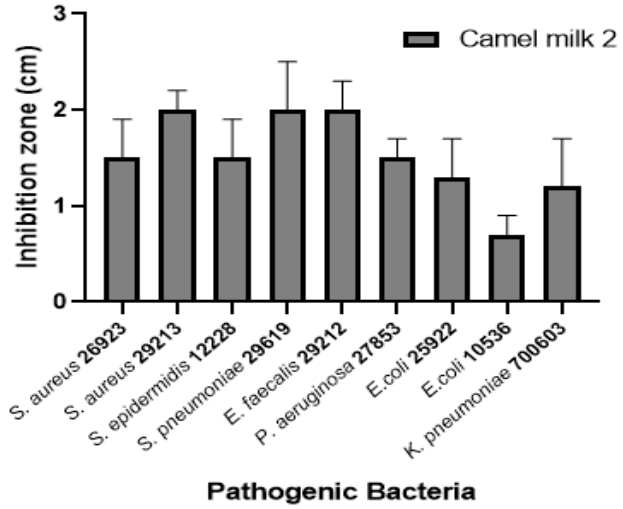

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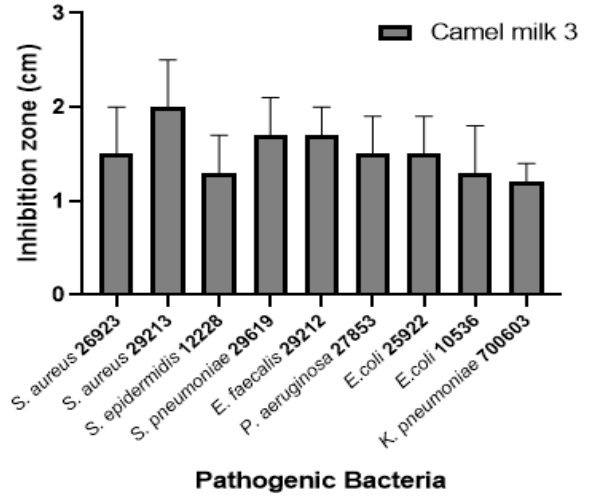

Figure 14

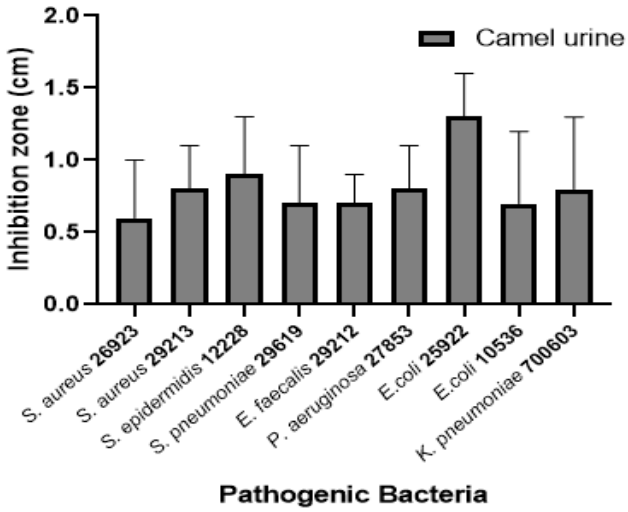

Figure 2

Figures 9-15: The antibacterial activity of the probiotic *Lactobacilli* isolated from buffalo milk, camel milk or camel urine. The antibacterial activity was investigated against both Gram-positive and Gram- negative bacteria using Agar-well diffusion method. Gram-positive strains were represented by *S. aureus* (ATCC 26923), *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228), *S. pneumoniae* (ATCC 29619), and *E. faecalis* ATCC (29212). Gram- negative strains were represented by *P. aeruginosa* ATCC (27853), *E. coli* ATCC (25922), *E. coli* ATCC (10536), and *K. pneumoniae* ATCC (700603).

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DISCUSSION

Probiotic bacteria have been recognized for their beneficial health effects in humans and animals. Their consumption in traditional food was associated with an extended life span and protection against diseases (Kechagia et al., 2013). The mechanisms of their beneficial effects include the protection against infectious disease either by direct competition with pathogenic microorganisms or by the modulation of the immune system and improving the digestion and reduction of metabolic disorders (Azad et al., 2018; Ghosh et al., 2019; Yousefi et al., 2019).

The main source of probiotics is fermented food including fermented milk, cheese and other dairy products. Probiotics are also isolated from both human and animal gastrointestinal tract. Furthermore, probiotic strains have been isolated from non-dairy fermented substrates including meat and fruits. Surprisingly, probiotic strains are also present in both human and animal milk which are originally expected to be sterile (McGuire and McGuire, 2015). These findings are consistent with the findings that breast-fed infants are less affected by gastrointestinal infections and have fewer allergies than formula-fed infants (Fontana et al., 2013). The same is also true about urine which had been thought to be sterile but after the development of sequencing techniques it was found that urine is colonized by normal flora including *Lactobacillus* and *Streptococcus* (Akgul and Karakan, 2018).

The diversity of probiotic *Lactobacilli* which isolated from different animal species has been documented (Abdou et al., 2018;Abdou et al., 2019). This diversity is the result of several factors including nutrition, infections, antibiotics, stress and various disease conditions. The variety of probiotic strains causes different types of benefits for the host.

It was clear from present findings that *Lactobacilli* strains isolated from both camel milk and camel urine were more resistant to the effect of antibiotics than *Lactobacilli* isolated from buffalo milk. This could be useful for restoring the gut microbiota after antibiotic treatment (Gueimonde et al., 2013). Although all *Lactobacilli* strains found in both camel milk and camel urine were also found in buffalo milk, the first two presented more resistance in general to antibiotic. This could be due to the acquisition of plasmids from other bacteria (Gueimonde et al., 2013). Camel milk and urine have been used in traditional medicine for several years to treat many diseases (Hu et al., 2017). In spite of the popularity of buffalo and cow milk and their preference among general public, camel milk is a very important replacement in arid and semi-arid areas where buffalo and cow milk are lacking. The camel milk investigation for bacterial content found it to be rich in LAB (Bin Masalam et al., 2018). In current study buffalo and camel milk were different in their *Lactobacilli* content. This difference might be due to the difference in milk composition (Yoganandi et al., 2014), which may allow the growth of different *lactobacillus* strains. *Lactobacillus plantarum* was isolated from camel milk and it is one of the frequently isolated LAB from raw camel milk (Khedid et al., 2009; Edalati et al., 2019).

Probiotic *Lactobacilli* have the potential to be used as natural alternatives to currently used synthetic antibiotics due to their antagonistic activity against various pathogenic bacteria (Prabhurajeshwar and Chandrakanth, 2017). In current study, it has been indicated that *Lactobacilli* isolated from buffalo milk, camel milk as well as camel urine presented variable degrees of antibacterial activity against pathogenic bacteria. Although present data indicated that isolated *Lactobacilli* from camel urine had the least antibacterial activity when compared to both buffalo and camel milk, the antibacterial, antifungal and antiviral activity of both camel milk and urine were reported previously (Al-Bashan, 2011; Hu et al., 2017). One of the reasons for the least antibacterial activity of camel urine could be using only one sample of it. The antibacterial activities of camel milk and urine in general may be partly due to the presence of different probiotic *Lactobacilli* including *Lactobacillus plantarum* and *Lactobacillus casei* which had been found earlier to represent promising antimicrobial activity (Bin Masalam et al., 2018).

CONCLUSION

The present study indicated the variability in contents of *lactobacillus* strains which isolated from buffalo milk, camel milk, and camel urine. Although some strains were similar among these samples, they presented different susceptibility to antibiotics and had different antibacterial activity against pathogenic bacteria. Further studies should be conducted with more samples to gain more information in the field of antibacterial activity of probiotic *lactobacilli* and to understand the mechanisms of their activity. Hopefully, they will be used as natural alternatives instead of synthetic antibiotics.

DECLARATIONS

Authors' contributions

Amr M. Abdou participated in the molecular identification of probiotic strains, performed the statistical analysis and drafted the manuscript. Riham H. Hedia participated in characterization of isolated bacteria, molecular identification of probiotic strains and antibiotic susceptibility of *Lactobacilli*. Shimaa T. Omara participated in characterization of isolated bacteria, molecular identification of probiotic strains, and antibacterial activity of *Lactobacilli*. Mai M. Kandil

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participated in sample collection and participated in the molecular identification of probiotic strains. M. A. Bakry participated in sample collection and characterization of isolated bacteria. Mohammad M. Effat proposed the idea of current study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declared that they had no competing interests.

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