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Different Methods For Testing The Microbial Effects Of Camel's Milk Against Some Pathogenic Bacteria

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ABSTRACT

Overuse of antibiotics is causing some bacteria to become resistant to antibiotics, in addition to their price hikes. Therefore, this study was designed to investigate the effect of camel milk as a safe natural product to overcome the pathogens caused by Staphylococcus aureus, Escherichia coli, and Salmonella typhi and to test their effect on them by several ways, in vivo, in vitro, and in histology. In addition to the use of an electron microscope, 48 rats were used and they were divided into eight groups, the first group was considered a negative control. For two consecutive weeks, the second group gets the milk. And by intraperitoneal injection of E, coli and S, aureus group 3 and 4, respectively, Group 5 supplemented the infection by oral administration of Salmonella typhi The sixth, seventh and eighth groups were supplemented with camel milk for two consecutive weeks, then intraperitoneal injection of either E. coli or S. aureus and Salmonella typhi bacteria by mouth, and the spleen was collected after 3 weeks to isolate the bacteria. The isolation rate of S. aureus. E coli, and Salmonella typhi was higher in rats in groups 3, 4, and 5 compared to groups 6, 7, and 8 and with milk diluents (10-100). By the agar diffusion method used to test for antibacterial efficacy, 80% dilution achieved the best result, and by using a transmission electron microscope to detect damage to the bacterial cell wall and disturbance in the cellular protein content. The results indicate that camel milk has an effect on the internal structure and cell wall also in the pathological anatomy. It was found that the pathogenic bacteria had fewer effects in the liver and kidneys in the groups treated with camel milk compared to the groups infected with bacteria only, and therefore we conclude that the camel milk Promising anti-bacterial properties.

INTRODUCTION

Desert Bedouins rely on camels as a source of meat and milk, they also use it as a transportation vehicle and since ancient times they have used it to treat many diseases [Abdel Galiletal ,2016], Camels are characterized by their abundant production of milk, which they produce for a longer period of time in arid regions and harsh environment compared to other ruminants, whose daily production of milk ranges from 3 to 10 kg during the lactation period [Gizachew and Birhanu ,2014 ,Ahmed and Eissa,2015].

Camels can survive well under difficult conditions, therefore, they play important role in local herdsmen's production, life, and economic structure Camel milk has high nutritional value and unique functional characteristics. It not only has low sensitization, but also contains various immune active factors with high medicinal value Wang,etal 2020,camel produces a nutritious milk for human consumption. It is also evident that the taste and quality of milk is directly affected by the amount of water drunk, and the amount and quality of feed eaten. Jonan ,2020

Several studies have been conducted in the world on camel milk composition, physicochemical properties, and functionality (Al Haj and Al Kanhal, 2010; Khalil et al., 2011; Rahli et al., 2013; Ismaili, et al., 2019), Also, Camel milk is called desert white gold and it is the most similar milk to breast milk, and it contains high minerals and vitamin C, low in cholesterol. When compared to other types of milk [Kappeler etal, 1999 Kumar etal, 2015], and its properties can be preserved for a longer period compared to cow's milk when cooled, and even with the heat of the desert it does not spoil soon [Thiagarajan, 2008], Camel milk is reported to have a

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more marked inhibitory system, compared with cow milk [El Agamy ,etal1992], as well as on its microbiological quality and prevalence of some bacterial pathogens (Adjaine andAmiri, 2013), Benmechernene et al. 2014 and Rahmeh,etal 2019 demonstrated the antimicrobial activity of a bacteriocin-producing Leuconostoc LAB, mesenteroides strain against other such as Lactobacillus sp., Lactococcus sp., and against several pathogenic bacteria, such as Escherichia coli, Staphylococcus aureus and Listeria spp. The excessive use of antibiotics resulted in the emergence of types of bacteria resistant to these antibiotics, the most famous of which are salmonella and Staphylococcus aureus. Which causes hemorrhagic colitis and hemolytic uremic syndrome in humans and animals [Cimolai, ,2008 ,Welinder and Kaijser,2005].

On the other hand, Salmonellosis is a type of bacterial food poisoning caused by Salmonella spp. The habitual reservoirs for this bacterium include domestic and wild animals. Salmonella spp are transmitted into these reservoirs via the feces of animals, their feces can contaminate food or water in the environment [Braden,2014].

Staphylococcus aureus (S. aureus) is a gram-positive bacteria, which causes many infections in humans and animals, *S. aureus* can survive for hours to weeks, and even months, on dry environmental surfaces (Cenci-Gogaetal 2003). Similar to *S. aureus, Escherichia coli (E. coli)* is a gram-negative microorganism, which led to severe pathogenicity to the infected host. It has been confermed that camel milk exhibits bacteriostatic effects against *E. coli* and *Listeria monocytogenes* (Noreddine etal 2004). Camel milk is also considered to have medicinal

properties against particular pathogens in the Middle East (Kappeler etal 1999)

The Bedouins of the Sahara relied on camel milk in the treatment of many diseases and this information between uncertainty and certainty. Therefore, The purpose of this study was to confirm or deny the effectiveness of camel milk on the bacteria, whether positive or negative and confirm this in vivo and vitro knowledge How the milk resisted the bacteria by using a Transmission electron microscope.

MATERIALS AND METHODS

Bacterial strains

Staphylococcus aureus (ATCC: 25923), *Escherichia coli* (ATCC: 25922) and *Salmonella typhi* (ATCC: 14028),They are the strains that were used in this research after they were cultured on Nutrient agar and incubated at 37°C [Cheesbrough,2000].

The experimental animals

Forty –eight adult male Wister rats (150-200 g each) were put in good ventilated cages in humidity and temperature controlled on a 12 h day/night cycle provided by rodent diet and water.

Camel's Milk collection

Samples of camel's milk in the early stage of lactation are collected daily early in the morning from camels taken from Bedouins from Matrouh Governorate and kept in special containers until they reach the laboratory

Confirmation the Sterility of examining Camel milk: -

Isolation of bacterial pathogens was carried out following aseptic sampling techniques [EL-Fakharany etal, 2012]. A loopful of milk sample was streaked on blood agar and incubated at 37 degrees to check for bacteria that grow after 24, 48 and 72 h to exclude slow-growing microorganisms, and sub cultured on blood agar at 37°C for 24 h until you reach to pure culture.

screening for the camel milk antibacterial activity

by using agar well diffusion method, milk with dilutions (10-100%) put in Wells of size 6 mm in Muller-Hinton agar plates by using gel puncture, 50 µl of the camel milk were poured into wells on all plates. Of each concentration by using micropipette, then incubated at 37°C for 24 h. the antibacterial activity measure by determine the diameter of the zones of inhibition around each well three time and take the mean [Cheesbrough,2000].

Experiment Design

Fourty-eight rats were used in the present study and were classified into eight groups (six rats each) as follows, Group I, which served as the control group; group II, in which the rats were drank 100 ml per 6 rats based on previous study [Althnaian etal ,2013], group III in which the rats were injected intraperitoneally (IP) dose of *E. coli*

bacteria of 2 X 10¹⁰ / ml [Cirioni,etal 2006],group IV which the rats were given an oral dose of Salmonella bacteria 1 ml of bacteria 2 X 10¹⁰ / ml , GroupV in which the rats were injected intraperitoneally (IP) dose of S. aureus bacteria of 1×10^8 /ml [Yasser etal,2016], GroupVI E. coli In addition to camel milk, group VII Salmonella typhi plus camel milk, group VIII S. aureus plus camel milk, camel milk were pre-administered for two weeks prior to injection of pathogens. The groups are monitored for 21days, then the rats are sacrificed, and the liver and kidney for histopathology examination, spleen was collected for each group separately, They were weighed and then homogenized with phosphatebuffered saline (PBS), and were serially diluted 10 times and streaked onto HiCrome™ E. coli Agar, , XLD Agar and Baird Parker plates (Oxoid, Basingstoke, Hampshire, UK), 37 ° C. The results and incubated for 24 hour under were expressed the bacterial load, And expressed as log (CFU/gm) of the spleen [Barquero etal, 2013].

Transmission electron microscope examination

To visualize the damage that occurred in the cell wall and the cytoplasm, transmission electron microscopy (TEM) is used [Hammer etal 2010]. At the ultrastructural level, a simple negative staining for TEM of bacterial cells can give evidence on the mechanism of membrane disruption by antimicrobial proteins and peptides [Torrent **etal 2008**]. The highly affected bacterial strain was scanned to define the effect of camel milk on the bacterial structure. Ultrathin sections of ~75–90 µm thickness was prepared and stained with uranyl acetate and lead citrate. Stained sections were examined using a JEOL-JEM 1010 Transmission Electron Microscope at 80 KV at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University [John **etal 1999**].

Histopathological examinations

Samples were taken from the liver and kidneys of rats with 5 μm thick prepared then stained with hematoxylin and eosin according to Tucker, etal 2016

Ethics: - The work was done with the respect recommended by WHO*for the animal welfare .

RESULTS

All examined milk samples were free of microbial contamination when cultured to isolate bacterial contaminants. These samples were then ready to examine its antibacterial effect against the three tested bacterial strains

The agar well diffusion method was carried out to investigate the effect of the camel milk against the tested bacterial strains, The camel's milk effective on all strains, where 80 % concentrations of camel's milk where the highly effective concentrations against all strains so we used this concentration of milk in our research (Table 1)..

Bacterial strains	Milk di	Milk dilutions [inhibition zone diameter (mm)]								
concentration	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Escherichia coli	16	16	17	17	17	17	19	23	21	20
Salmonella typhi	15	15	15	16	16	16	17	19	18	18
Staphylococcus	12	12	12	13	12	12	12	14	13	13
aureus										

Table 1 Determination of the effect of different dilution of camel's milk on the tested bacterial strains

Measuring the effect of camel milk on Total bacterial Count in organs in Wistar rats After Challenge

In the liver the total isolation rate of E. coli, S. aureus and salmonella bacteria were, 17.5×10^5 , 23×10^5 and 11×10^5 CFU / g, respectively, but group in which camel milk

taken and then injected pathogens , 9 x 10^5 , 20 x 10^5 , and 7 x 10^5 cfu / g, respectively. As shown in Table 2.

	Total bacterial counts /gram tissue								
	Bacteria injected rats Organ liver	Camel milk supplemented and bacteria injected rats							
E.coli	17.5 x10 ⁵	9 x10 ⁵							
S. aureus	23x10 ⁵	20x10 ⁵							
salmonella	11 x10 ⁵	7 x10 ⁵							
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Table 2. Effect of camel milk on total *E. coli,S. aureus and salmonella* count in liver of Wistar rat after bacterial challenge

In the kidney showed that the total isolation rate for Escherichia coli, Staphylococcus aureus, and Salmonella 120×10^{5} , 170×10^{5} , 270×10^{5} respectively, and

with camel milk then injected pathogen the rats $60x10^5,75x10^5$ and $90x10^5CFU$ / gram, respectively from the above it is clear that the use of camel milk leads to a significant decrease in the bacterial count in the liver and kidney comparing with injecting mice with bacteria alone. Table 3

	Total bacterial counts /gram tissue								
	Bacteria injected rats	Camel milk supplemented and bacteria							
	Organ kidney	injected rats							
E.coli	120x10 ⁵	60x10 ⁵							
S. aureus	170x10 ⁵	75x10 ⁵							
salmonella	270x10 ⁵	90 x10 ⁵							

Table 3. Effect of camel milk on total *E. coli,S. aureus and salmonella* count in kidney of Wistar rat after bacterial challenge

Transmission electron microscope examination

All bacteria were scanned using TEM before and after treatment with camel's milk to see the effect of camel milk on the bacterial cell structure. Before (a) and after



Fig2 Transmission electron microscope for salmonella before and after treatment with camel milk (a) salmonella control, (b) salmonella treated with camel

treatment (b) camel milk has an effect on internal and cell wall structure of all scanned organisms

Fig1 Transmission electron microscope for Escherichia coli before and after treatment with camel milk (a) *Escherichia coli* control, (b) *E. coli* treated with camel milk camel milk,camel milk has effect on internal and cell wall structure



milk,camel milk has effect on internal and cell wall structure

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Fig3 Transmission electron microscope for staph before and after treatment with camel milk: (a) staph control, (b)



Histopathological examinations\

Figure 4 On the other hand tissue sections from the liver infected by Escherichia coli untreated group (a) showed showing vacuolar degeneration of hepatocyte and area of coagulative necrosis of liver, but treated groups camel milk + Escherichia coli liver (d) showed slight hepatic sinusoid dilatation and beginning to return to normal,

(b) Effect of salmonella untreated group on Liver, rat, showing coagulative necrosis of liver , newly formed bile

duct and hepatic sinusoid dilatation. (e) liver of camel milk+ salmonella infected rats showing apparently normal histological findings of hepatic parenchyma, (c) Effect of *S. aureus on* Liver, rat, showing , eosinophilic cytoplasm, dilated sinusoids, lymphocytic infiltration in the portal and periportal areas ,hepatic sinusoid dilatation and haemorrhage and slight necrosis (f) liver of camel milk+ *S. aureus* infected rats showing hepatic sinusoid dilatation and mild fibroplasia at the portal area all H&E X400



staph treated with camel milk, camel milk has effect on internal and cell wall structure



Figure 5 Kidney (a) Effect of E.col untreated group on Kidney,rat showing apparently healthy renal glomeruli and renal tubules no effects , (d) kidney of camel milk+ *Escherichia coli* infected rats showing congested interstitial blood vessel , mild change , (b) :Effect of salmonella on Kidney,rat, showing sever haemorrhage , necrosis,atrophy of glomerular tuft and increase widness of capsular spase(**e**) kidney of camel milk + salmonella showing vacuolated renal tubular epitheliummild change, (**c**) Effect of *S. aureus on* Kidney,rat, showing sever necrosis in all tubules,edema,increase change in glomeruli (**f**)kidney of camel milk + *S. aureus* Kidneys showing vacuolated renal tubular epithelium (arrows), (mild change)all(H&E X 400).



DISCUSSION

The Bedouins of the desert depend on natural resources to treat many diseases, and a lot of research has been discovered that relying on natural resources and not overusing antibiotics, which in turn stimulates thelactamase enzyme that inhibits the activity of the-lactam antibiotic, thus leading to Mi crobial resistance [Aarestrup and Jensen, 1998], Interest in camel milk used for human nutrition is rising due to its characteristic composition, Sboui, etal 2012, in addition to bad side effects and exorbitant drug prices, all of these factors led to raising the slogan of returning to nature. Therefore, attention has been paid to a natural product such as camel milk which is available in the desert, in addition to its distinctive formula and unique biological functional properties used to protect against the attack of various pathogenic microorganisms. [Kappeler etal, 1999], our Studies deal with antimicrobial properties of camel milk against microorganisms with veterinary importance in vitro and in vivo are as yet restricted, and how to detect the effect of camel milk on the bacteria by using the Transmission electron microscope, in our study, we found that milk has an anti-bacterial effect on the E. coli ,salmonella and S. aureus this result coming with Mwambeteetal 2009 and Narmadha, etal 2011 proved that camel milk was bacteriostatic against the Grampositive strains this due to camel milk contain Lysozyme which is one of the most ubiquitous antibacterial molecules that exert broad-spectrum antimicrobial action Streptococcus, also el Agamy EI etal1992, against etal,2004 Benkerroum and Public Health Association,2012 which has been reported that camel milk has a bacteriostatic effect against E. coli and L. monocytogenes Cardoso and Ponte 2013, reported that camel milk has effect on several bacterial species specially Salmonella spp. this result may be due to the

presence of the high amounts of antimicrobial peptides and protective proteins such as lactoperoxidase. N-acetyl-§-glucosaminidase (NAGase) and short peptidoglycan recognition protein (PGRP) which is found in camel milk, which has been proven to contain anti-bacterial, antifungal, parasitic and antiviral activity, as well as an immune properties ,Mona and Abeer 2010 , Gizachew and Teha 2014, In addition, it contains lysozyme, which is considered one of the most common anti-bacterial molecules that exerts a broad-spectrum anti-microbial effect, whether for positive or negative bacteria, Rossi,2002, Amal,2016 and Magdy etal ,2015, explained that camel milk contain Lactoferrin which act as One of the first defense mechanisms against the microbial agents that invade the organism through the mucous tissue. The growth of Gram-positive or Gram-negative bacteria is affected by lactoferrin and this is due to its ability to bind to free iron, which is one of the basic elements of bacterial growth In addition, the N-terminal region receptors of lactoferrin have been detected on the surface of some microorganisms, which leads to the binding of lactoferrin to these receptors, which leads to cell death, especially Gram-negative bacteria, as a result of a disturbance in the cell wall.

Camel milk decreased the total bacterial count of S. aureus ,salmonella and E. coli in liver and kidney of rats in camel milk together with pathogen when compare to the same group but inject with pathogen, These counts were not in agreement with those obtained by Magdy etal 2015, found that the counts in liver were around 4.5×10^5 . 3.4×10^5 respectively after and before give milk and around 11 x10⁵, 8.5x 10⁵ in kidney respectively after and before give milk in case of E.coli injection $,7x10^{5},3.6x10^{5}$ in liver, $17.5x10^{5}$ $,7x10^{5}$ in kidney respectively after and before give milk in case of S. aureus injection , But we differ in the number of bacteria that have been isolated from the kidney, and liver after treatment with milk, this possibly due to differences in the pasture, camel age and period of lactation but The possible explanation of such decrease in number of bacteria after treatment may be attributed to high amounts of antimicrobial peptides such as Lysozyme (LZ), lactoferrin (LF), lactoperoxidase (LP), short peptidoglycan recognition protein (PGRP) present in camel milk [Benkerroum, et al 2008, Abbas et al , 2013].

Also, Lysozyme is one of the most ubiquitous antibacterial molecules that exert broad spectrum antimicrobial action. It has lethal effect against Gram positive bacteria and Streptococcus [Mwambete etal, 2009, Narmadha etal, 2011.].

A previous study was conducted by amal ,2016 on the effect of camel milk against E. coli and we add staph and salmonella scanned by means of TEM, which indicated disruption of internal and cell wall structure of both treated strains this result agree with us and this may be due to present of Lysozyme (LZ), lactoferrin which act as antibacterial .In the present study, the liver of the rats infected with the staph aureus, , Salmonella spp. or E. colli showed a degeneration of hepatocyte and apoptosis in and coagulative necrosis, sinusoid dilatation, eosinophilic cytoplasm, lymphocytic infiltration in the portal and periportal areas and haemorrhage, These results were assured by the results obtained by Rahimi et al.2010 and Osman, et al.2016 in rats, and by Ajibade and Famurewa 2012 in rabbits infected with S. typhi bacteria. Cater et al.1887 showed these changes with E. coli. In the liver, hepatocytes were swollen associated with decreased sinusoidal spaces, and widely distributed necrotic foci were seen by Al Zanfely and falh 2011. In rats infected with Salmonella bacteria, degeneration or swelling of the renal tubules accompanied with foci of hemorrhage in the interstitium of the renal tubules were found in rabbits. There were few foci of tubular necrosis and hyaline casts with interstitial cellular infiltration by macrophages were found by Ajibade ,etal 2012 also, in a study occurred by Ajibade and Famurewa, Al Zanfely,etal 2011 found that extensive epithelial swelling with decreased lumen space and generalized necrotic changes in renal tubules with interstitial hemorrhage in the renal cortex . moreover, Kumar etal,2004 confirmed that no specific change in the kidney was observed in E. coliinfected broiler chicks .In addition to most desert Bedouins drink camel milk without boiling or pasteurizing, and In many studies it has been reported that unpasteurized camel milk has distinctive properties, especially its anti-infection effect, and it has been recommended as an alternative to other types of milk Cardoso etal,2013 , and based on these facts we used unpasteurized camel milk, especially since the Bedouins use it without pasteurization

CONCLUSION

Our results provided basic information on the uses of camel milk as a treatment against Escherichia coli, Salmonella and Staphylococcus aureus infection. Camel milk had protective effects against pathogenicity caused by these bacteria, in Wistar mice. Thus it can be considered as an alternative to antimicrobial drugs in the face of developing drug resistance. Moreover, camel milk affects the bacterial cell by destroying the cell wall and disinfecting the internal cell components. This leads to the destruction of the bacteria, and there is a need for further study to extract the chemically active substance present in camel milk and determine its concentration to explain the cause of the effect of some milk concentration and others, and also must be taken into account general hygiene and sanitation for staff, milking, handling of milk and cooling containers and water quality are very important in order to prevent contamination of milk and

its use as a treatment

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