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Antibacterial Activity of Camel Milk and Whey in Combination with some Antibiotics Against Vancomycin Resistant *Staphylococcus Aureus*

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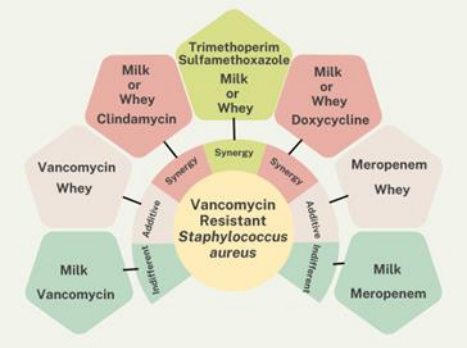
Abstract: The main goal of this research is to observe the antibacterial activity of camel milk and whey in combination with clindamycin, doxycycline, meropenem, trimethoprim sulfamethoxazole and vancomycin antibiotics against Vancomycin resistant Staphylococcus aureus (VRSA) and Staphylococcus aureus (ATCC 6538P). Micro-broth dilution method was used to measure the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for all antibiotics, milk and whey samples. For further antibacterial investigation, the fractional inhibitory concentration (FIC) was also determined. The interpretations of the activity of camel milk and whey combined with the five examined antibiotics produced remarkable synergistic activity in most combinations against both VRSA and S. aureus. It is noticeable that camel milk showed a synergistic effect with clindamycin, doxycycline and trimethoprim sulfamethoxazole against VRSA and S. aureus. Moreover, camel whey exhibited a synergistic effect with clindamycin and doxycycline against the two isolates. Based on the obtained results, it is concluded that there was an additive or indifferent effect rather than synergism when combining camel milk and whey with antibiotics that targeted bacterial cell wall synthesis. Otherwise, when combining camel milk and whey with antibiotics targeting protein synthesis or targeting folate synthesis a synergistic effect was observed. The present research clearly showed that camel milk is considered a promising natural product that plays an important role in elevating the susceptibility of bacteria to some antibiotics.



Keywords: Antibacterial Activity, Camel Milk, Camel Whey, Antibiotics, Vancomycin Resistant Staphylococcus Aureus.

Introduction

Camel milk is an important source of nourishment since it has a number of qualities that make it a good choice for treating a variety of diseases in different parts of the world [1]. It has always represented an important food source for nomadic people in arid parts of the world. Recently, camel milk has attracted great attention as a possible replacement for dairy cow's milk because of its therapeutic effects [2]. Camel milk has been shown to have antibacterial, anticarcinogenic, antioxidant, anti-hypertensive, and anti-diabetic properties [3]. Moreover, camel milk protection proteins may have a function in boosting the immunological defense mechanism [4]. Among these proteins are whey proteins, which have anti-tumor and anti-carcinogenic properties [5]. Furthermore, camel milk has significant concentrations of immunoglobulins, lactoferrin, lysozyme, and other defense proteins [6]. Most of these proteins have been found to be efficient antibacterial agents. In this aspect, native or recombinant forms of camel lactoferrin have previously been shown to reduce the infectivity of the hepatitis C virus [7, 8, 9]. A previous study analyzed the synergistic potential of camel lactoferrin when paired with antibiotics against methicillin-resistant S. aureus (MRSA) isolate in comparison to human lactoferrin. The combinations of camel lactoferrin with oxacillin or vancomycin enhanced the antibacterial activity against MRSA three times higher than human lactoferrin [10].

With the rise in multidrug-resistant infections, many patients are searching for alternatives to traditional antibiotics and antimicrobial treatments. Camel milk is at the top of the list of alternative therapies used by those patients in the Arabian and other developing regions [11-15].

Staphylococcus aureus is found in healthy people's nostrils, upper respiratory systems, and skin without causing any problems. Virulent S. aureus strains, on the other hand, release toxins that cause local skin degradation, fever, and severe consequences [16, 17]. The emergence of methicillin-resistant S. aureus (MRSA) strains has complicated the treatment of staphylococcus infections because methicillin is the first line of defense against S. aureus infections and resistance to methicillin indicates resistance to all β-lactam medications. MRSA has become one of the most common causes of death among hospitalized patients worldwide [18-21]. After MRSA outbreaks for decades, vancomycin displayed a high level of success until the first report of vancomycin-resistant S. aureus (VRSA) in 2002, which followed by an increased level of VRSA around the

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world [22]. The pathogenesis and resistance patterns of S. aureus significantly pose a threat to human health worldwide. In the absence of effective therapeutic solutions, MRSA, VRSA and VISA (vancomycin intermediate-resistant S. aureus) are bacteria with the potential to cause significant mortality and destruction [23]. Besides that, there are limited options for effective medications against VRSA. It is noteworthy to mention that there are several promising therapeutic options in research and development phases [24].

Our research group studied the role of camel milk and whey in combination with some common antibiotics (clindamycin, doxycycline, meropenem, trimethoprim sulfamethoxazole and vancomycin) that are usually used to treat S. aureus infections in order to explore and understand why camel milk can be utilized as an alternative medication for the treatment of various diseases.

Materials and Methods

Equipments

Microplate reader (Labtech, UK), sterile 96-well microtiter plates (Thermo Fisher Scientific Inc, USA). Freez-dryer (Millrock freeze dryer). Centrifuge (Sorval lynx 160)

Chemicals and reagents

Muller Hinton broth (HIMEDIA, USA), Nutrient agar (HIMEDIA, USA). Rennin (Hansens®). Antibiotics (Laboratorio, Brazil).

Milk collection and whey immunoglobulins preparation

For milk preparation, a skimmed milk sample that was previously collected by a veterinary specialist from a female camel was directly lyophilized into powder. For whey preparation, another milk sample was collected and prepared according to Brüssow et al. method [25]. In order to obtain sufficient crude contraction, casein was precipitated from the pooled skimmed milk samples using rennin. The coagulated milk was then incubated at 56°C for 10 min. The casein was then separated from the lacto-serum via filtering. The lacto-serum was centrifuged once more for 30 minutes at 10,000 rpm and 4°C to make sure the whey sample was completely clear. Whey immunoglobulin pool powder was created by microfiltering the resulting supernatant and then freeze-drying it.

Antibacterial activity assay

Studied bacteria

Two bacterial isolates, *Staphylococcus aureus* (ATCC 6538P), obtained from the American Type Culture Collection (ATCC) and vancomycin resistant *Staphylococcus aureus* (VRSA) clinical isolate obtained from the Biotechnology Laboratory at An-Najah National University, were used to test the *in vitro* antibacterial activities of camel milk and whey and examined antibiotics.

Studied antibiotics

For the determination of the antibacterial profile of the two bacterial isolates, the following 19 antibiotics were used: Amikacin, Aztreonum, Cefazidime, Cefoxitin, Cefepime, Cephalothin, Chloramphenicol, Ciproflaxin, Clindamycin, Doxycycline, Erythromycin, Fusidic Acid, Gentamicin, Imipenem, Meropenem, Methicillin, Oxacillin, Trimethoprim Sulfamethoxazole and Vancomycin.

Agar disk diffusion assay

The disk diffusion method was employed to determine the antibacterial activity of the 19 antibiotics against two bacterial isolates using a standard method [26].

Micro-broth dilution assay

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for camel milk, whey and the examined antibiotics (clindamycin, doxycycline, meropenem, trimethoprim sulfamethoxazole and vancomycin) were determined by the micro-broth dilution standard method [27].

Checkerboard assay

Combinations of clindamycin, doxycycline, meropenem, trimethoprim sulfamethoxazole, and vancomycin with camel milk or whey were tested against VRSA and *S. aureus* using the microdilution checkerboard technique as described by Bellio et al [28]. Briefly, a final inoculum of 1×106 CFU/mL of VRSA or S. aureus was added to 96-well microtiter plates containing twofold diluted milk or whey in vertical wells and the antibiotic in horizontal wells in Muller Hinton broth. The combined antibacterial effects of milk or whey with antibiotics were diluted to reach concentrations ranging from 20 mg/mL to 0.0625 mg/mL for milk or whey, from 4 µg/mL to 0.125 µg/mL of clindamycin, from 0.5 µg/mL to 0.015625 µg/mL of doxycycline, from 2 µg/mL to 0.0625 µg/mL of meropenem, from 20/4 µg/mL to 0.625/0.125 µg/mL of trimethoprim sulfamethoxazole, and from 16 µg/mL to 0.5 µg/mL of vancomycin. These concentration ranges were chosen based on the previously obtained MIC data for each antibiotic. The fractional inhibitory concentration (FIC) was derived from the lowest concentration of antibiotic and milk or whey combination, permitting no visible growth of the test organisms on the plates. The FIC index for each agent was calculated using the following formula:

FIC (antibiotic) = MIC of antibiotic in the combination / MIC of antibiotic alone

AFIC (milk or whey) = MIC of milk or whey in the combination / MIC of milk or whey alone

Combinations were classified as synergistic, if the FIC indices were < 0.5, additive if the FIC indices were between 0.5-1, indifferent if the FIC indices were between 1 and 4 and antagonistic if the FIC indices were > 4.

Results and Discussion

The widespread use of antibiotics has led to a rise in infections in the environment and a rise in infectious microbes that are less vulnerable to the antibacterial effect. As a result of this resistance, the treatment of infections has become more challenging. Moreover, conventional antibiotic therapies have limited efficacy against infections like *S. aureus* [29]. The best weapons against staphylococci are penicillin and closely related medicines from the β-lactam class. However, the widespread use of these antibiotics has resulted in a significant rise in infections that might produce β-lactamase, an enzyme that inactivate β-lactam antibiotics and causes microbial resistance [30]. Therefore, there is an urgent need for novel antimicrobials to treat bacterial diseases [31]. The agar disk diffusion assay revealed that the two examined bacterial isolates were multi-drug resistant, as indicated by the inhibition zone measurement. The vancomycin resistant *S. aureus* (VRSA) isolate was susceptible only to 6 antibiotics and resistant to 13 antibiotics. while *S. aureus* (ATCC 6538P) was susceptible to 11 antibiotics and resistant to all other tested antibiotics (Table 1).

Table (1): Susceptibility testing of vancomycin resistant *S. aureus* (VRSA) and *S. aureus* (ATCC 6538P) to 19 antibiotics by the agar disk diffusion method (R; resistant, S; sensitive, I; intermediate).

Antibiotics	Vancomycin resistant *S. aureus*	*S. aureus* (ATCC 6538P)
Amikacin	S	S
Aztreonum	R	R
Cefazidim	R	R
Cefoxitin	R	R
Cefepime	R	R
Cephalothin	S	S
Chloramphenicol	S	S
Ciproflaxin	R	R
Clindamycin	S	S
Doxycycline	I	S
Erythromycin	R	R
Fusidic acid	R	R
Gentamicin	R	S
Imipenem	S	S
Meropenem	R	R
Methicilin	R	S
Oxacilin	R	I
Trimethoperim	R	S
Sulfamethoxazole		
Vancomycin	R	I

It is well known that certain nutritional substances can reduce the risk of human diseases. In this regard, camel milk and other functional foods are currently being utilized as an adjuvant or alternative for chemotherapy, particularly in the management and prevention of human diseases and to maintain their optimum health [32]. In the current research, results demonstrated that all examined samples (the five antibiotics, camel milk and whey) are bacteriostatic agents with variation among their effective concentrations (Table 2). The resumption of bacterial growth after spreading the wells with no-growing bacteria on agar plates implied that both meropenem and vancomycin were bacteriostatic agents with no bactericidal effect. However, the others can be identified as bactericidal agents against VRSA and *S. aureus*.

Table (2): Antibacterial activity of camel milk (mg/mL), whey (mg/mL) and five antibiotics (µg/mL) against vancomycin resistant *S. aureus* (VRSA) and *S. aureus* (ATCC 6538P) using micro-broth dilution assay.

	Vancomycin resistant *S. aureus*		*S. aureus* (ATCC 6538P)	
	MIC*	MBC**	MIC	MBC
Clindamycin	2±0.00	4±0.00	2±0.00	4±0.00
Doxycycline	0.06125±0.00	0.125±0.00	0.030625±0.00	0.125±0.00
Meropenem	4±0.00	-	1±0.00	-
Trimethoperim	20±0.00	40±0.00	5±0.00	40±0.00
Sulfamethoxazole	2±0.00	4±0.00	1±0.00	4±0.00
Vancomycin	16±0.00	-	16±0.00	-
Milk	5±0.00	10±0.00	5±0.00	10±0.00
Whey	10±0.00	20±0.00	20±0.00	20±0.00

Camel milk is gaining popularity as a source of human nourishment due to its unique composition and biofunctional qualities. Camel milk has a synergistic effect with some antibiotics, according to the current research findings. As the interpretations of the activity of camel milk and whey combined with the five examined antibiotics produced remarkable synergistic activity in most combinations against both VRSA and *S. aureus* (Tables 3, 4, 5 and 6). It is noticeable that camel milk and whey showed a synergistic effect with clindamycin, doxycycline and trimethoprim sulfamethoxazole against VRSA and *S. aureus*.

Table (3): Synergistic activity of the camel milk with five antibiotics against vancomycin resistant *S. aureus* (VRSA) using a checkerboard assay.

	FIC antibiotic	FIC milk	FIC index	Interpretation
Clindamycin	0.03	0.125	0.155	Synergistic
Doxycycline	0.127	0.125	0.252	Synergistic
Meropenem	0.125	1	1.125	Indifferent
Trimethoperim Sulfamethoxazole	0.06	0.125	0.185	Synergistic
Vancomycin	0.125	1	1.125	Indifferent

Table (4): Synergistic activity of the camel whey with five antibiotics against vancomycin resistant *S. aureus* (VRSA) using a checkerboard assay.

	FIC antibiotic	FIC milk	FIC index	Interpretation
Clindamycin	0.125	0.06	0.185	Synergistic
Doxycycline	0.127	0.06	0.187	Synergistic
Meropenem	0.5	0.06	0.56	Additive
Trimethoperim Sulfamethoxazole	0.25	0.125	0.375	Synergistic
Vancomycin	0.5	0.06	0.56	Additive

Table 5: Synergistic activity of the camel milk with five antibiotics against *S. aureus* (ATCC 6538P) using a checkerboard assay.

	FIC antibiotic	FIC milk	FIC index	Interpretation
Clindamycin	0.0625	0.125	0.187	Synergistic
Doxycycline	0.25	0.125	0.375	Synergistic
Meropenem	0.125	0.25	0.375	Synergistic
Trimethoperim Sulfamethoxazole	0.25	0.125	0.375	Synergistic
Vancomycin	0.0625	0.125	0.187	Synergistic

Table 6: Synergistic activity of the camel whey with five antibiotics against *S. aureus* (ATCC 6538P) using a checkerboard assay.

	FIC antibiotic	FIC milk	FIC index	Interpretation
Clindamycin	0.125	0.03	0.155	Synergistic
Doxycycline	0.254	0.03	0.284	Synergistic
Meropenem	0.5	0.06	0.56	Additive
Trimethoperim Sulfamethoxazole	0.25	0.03	0.28	Synergistic
Vancomycin	0.125	0.03	0.155	Synergistic

The antibacterial activity of camel milk and whey is in response to the availability of several protective proteins, like casein. In addition to casein, lactalbumin, and lactoglobulin, milk contains antimicrobial peptides such as lysozyme, lactoferrin, lactoperoxidase, immunoglobulins, and short peptidoglycan recognition protein [6]. Lactoferrin, which is considered to be among the first lines of defense against microbial pathogens that enter the body through the mucosal tissues, as it inhibits the growth and proliferation of viruses, protozoa, fungi and bacteria, including Gram-positive and Gram-negative bacteria. A prior investigation revealed that the bacteriostatic impact of lactoferrin is owing to its capacity to bind free iron, which is one of the components required for bacterial development [33]. Moreover, researchers found that there are receptors for the N-terminal region of lactoferrin on the surface of several bacteria. Lactoferrin binds to these receptors, breaking the cell wall and releasing lipopolysaccharide (LPS), which lowers permeability and makes Gram-negative bacteria more susceptible to lysozyme and other antimicrobial agents. Also, the changes in membrane permeability are caused by electrostatic interactions between the positively charged lactoferrin surface and the negatively charged lipid layer, resulting in bactericidal activity against Gram-positive bacteria [34, 35]. The existence of lactoferrin in the prepared milk and whey samples in this study explains their antibacterial activity against the examined isolates [36]. Radwan and his colleagues suggested that lactoferrin might

stimulate cell death and growth inhibition of *S. aureus* and MRSA [10]. In their experiment, vancomycin was demonstrated to cause MRSA cell membrane deterioration. The addition of camel lactoferrin provided a synergistic mode of action as MRSA membrane disorganization dramatically increased. The same observation was noticed in this research, as the combination of vancomycin with both camel milk and whey against *S. aureus* indicated a synergism. While an additive or indifferent effect of these combinations was observed against VRSA. Since the *S. aureus* isolate under investigation is vancomycin resistant. So, the addition of camel milk and whey slightly improves the vancomycin effect.

Lysozyme, the other constituent of camel milk, is a common antibacterial molecule that has a broad spectrum of antimicrobial activity. It inhibits Gram-positive bacteria and Streptococcus with muramidase activity [37, 38].

Additionally, lactoperoxidase is present in saliva, tears, and milk. It has bactericidal effects, mostly on Gram-negative bacteria, and supports the non-immune host defense system [39]. Otherwise, peptidoglycan recognition protein (PGRP) stimulates the immune system's response and has robust antimicrobial activity.

Besides that, the principal protein found in milk is casein, which is present in two essential variants, A1 and A2 beta-casein. Furthermore, the main casein type in camel milk is A2 beta casein [40]. Moreover, Jrad group provided that to suppress certain Gram-positive and Gram-negative bacteria, greater camel casein protein concentrations are required [41]. Previous reports on caseins antimicrobial behavior noted that the native caseins provided low or no antimicrobial activity; they just released several bioactive peptides after their digestion [41, 42]. Indeed, the antibacterial action of camel milk casein was raised by means of pepsin or pancreatin hydrolysis. Thus, these facts supported the research findings, as camel milk showed low bactericidal activity against the studied bacteria.

On the other hand, lactoglobulins, albumin, and immunoglobulin are among the proteins contained in whey [43]. Compared to cow and buffalo milk proteins, camel whey proteins were shown to be more heat resistant. The antibacterial agents lactoferrin and immunoglobulin G are among these heat-resistant proteins [44].

Furthermore, because the whey sample under study was heated, several biofunctional peptides may have lost their activity [45]. Lysozyme was thought to be the primary component responsible for inhibitory action [46]. Unfortunately, lysozyme was heat sensitive, which could explain camel whey's low activity in this study when compared to milk.

The five screened antibiotics through this running experiment affect bacteria in different mechanisms. In general, vancomycin and meropenem affect cell wall synthesis by binding with penicillin binding proteins [47, 48]. While clindamycin and doxycycline inhibit protein synthesis through interaction with ribosomal subunits [49, 50]. Trimethoprim/sulfamethoxazole directly affect the synthesis of folate inside microbes [51].

Conclusion

Based on the obtained results, it is concluded that there was an additive or indifferent effect rather than synergism when combining camel milk and whey with antibiotics that targeted bacterial cell wall synthesis, like vancomycin and meropenem. This is because the same mechanism may be achieved by milk constituents like lactoferrin. So, no additional value was obtained. Otherwise, when combining camel milk and whey with antibiotics targeting protein synthesis, such as clindamycin and doxycycline or targeting folate synthesis, like trimethoprim/sulfamethoxazole a synergistic effect was observed. Since milk and whey components may facilitate the entry of these antibiotics by damaging the bacterial cell wall. In addition to that, as previously mentioned, the bioactive molecules in camel milk and whey exhibit diverse mechanisms of action against bacteria.

Disclosure Statements

- **Ethics approval and consent to participate:** Not applicable.
- Consent for publication: Not applicable.
- **Availability of data and materials:** The raw data required to reproduce these findings are available in the body and illustrations of this manuscript.
- **Author's contribution:** Study conception and design: LA, data analysis and validation, LA, .IS, NZ, RD, MM, MT, MK; draft manuscript preparation: LA, .IS, NZ, RD, MM, MT, MK. All authors reviewed the results and approved the final version of the manuscript.
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