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Therapeutic aptitude of fermented camel milk: case of antimicrobial activity of whey proteins

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Abstract.

The objective Whey proteins (WP) are highly functional elements containing bioactive peptides. The aim of this study is to evaluate the effect of spontaneous fermentation on the antimicrobial activity of WP in camel milk. Samples of the milk are analyzed in the raw state and after the fermentation. The pH and acidity are determined as well as the enumeration of lactic acid bacteria (LAB). The antimicrobial activity was carried out by the agar well diffusion assay, adapting the good method. The results shows that the fermentation process has shown that the pH value is decreasing and LAB show a significant increase during fermentation. The antimicrobial activity gave a very significant difference between fermented camel whey proteins (FCWP) and raw camel whey proteins (RCWP). Then the FCWP gives a zone inhibition (ZI) of 17, 16.5, 13.25 and 10.5 mm respectively for *S. aureus, P. aeruginosa, K. pneumoniae* and *E. coli.* In addition, the RCWP gives a ZI of 16, 9.5 mm respectively for *S. aureus* and *P. aeruginosa*, and 0 mm for *K. pneumoniae* and *E. coli*. These results indicate that the fermentation process induced by the development of LAB favors the proteolysis of WP, which increases the antimicrobial activity.

Key words: Biological activity, dromedary, fermentation, lactic acid bacteria, whey proteins.

Introduction

Camel's Milk (*Camelus dromaderius*) is a highly identity product for populations raising camels, it has important and balanced basic nutrients (proteins, fat, lactose and vitamins). It plays an important role in the diet of nomads and the people of southern Algeria. It is little known in other parts of the country [1]. Its composition is significantly different depending on the season and the types of plants grazed in the camel's pathways. Milk productivity is directly related to the floristic component and the individual genetic performance [2].

The camel milk like other types of milk can be contaminated by germs saprophytic of the pie and by the hands of the milker... However, this milk is characterized by the ability to inhibit certain pathogenic microorganisms including halophilic by whey proteins such as lysozyme, Lactoperoxidase, Lactoferrin, Lysozyme, immunoglobulins and free fatty acids [3, 4].

Fermented milk is a major food component of traditional food in many parts of Africa. Due to the limitation of cold storage means in many rural areas in African countries. The milk is stored at room temperature, allowing them to ferment quickly by the natural lactic flora. Sometimes the fermentation process occurs spontaneously by inoculation of raw milk with a small amount of fermented milk previously elaborated. Therefore, the product obtained leads to the domination of the lactic strains best adapted [5].

Fermented camel milk is rich in LAB that boost their antimicrobial properties against pathogenic germs like *Bacillus, Pseudomonas, Mycobacterium, Staphylococcus, Salmonella* and *Escherichia* [6].

LAB use lactose to produce many metabolites with antimicrobial properties such as organic acids, hydrogen peroxide, carbon dioxide, reuterin, diacetyl, and bacteriocins. Bacteriocins are antimicrobial peptides inhibiting the growth of altered or pathogenic bacteria [7].

In this context, and in the framework of expansion of camel milk uses as a valuable national economic source, the present work aims at the following two objectives:

- Assessment of the microbiological quality of camel milk during fermentation.

- Know the effect of fermentation on improving the antimicrobial activity of camel WP.

Material and methods

Preparation of WP samples

Fermented camel milk is prepared by storing milk samples in an ambient temperature (25 °C) for 120 hours (05 days) without adding any lactic ferment. Fermentation is spontaneous by the development of the endogenous lactic flora (Streptococcus and Lactobacillus) [8, 9].

After skimming and separating whey from caseins, the WP are lyophilized and stored in the refrigerator (+ 4°C) for subsequent use in the analyses [10].

Physicochemical analyzes

The pH value is measured at + 22 °C using a pH meter and the Dornic Acidity is assayed by titration using N/9 sodium hydroxide solution in the presence of phenolphthalein [11].

Microbiological analyzes

A dilution series is performed by a Tryptone Salt Diluent from the parent solution (raw or fermented camel milk) for cultivation and enumeration of indigenous and exogenous flora. The culture media and enumeration modes are summarized in table 01 [12].

The analyses either physicochemical or microbiological are performed at the arrival of the samples in the laboratory (T0) and after 120 hours of spontaneous fermentation.

Table 01: Culture media and enumeration methods of indigenous and exogenous flora of camel milk

Microorganisms	Culture media	Temperature and incubation time	Observations
lactobacillus	MRS (Man, Rogosa and Sharpe)	30 °C/48h	deep inoculation
TC and TTC	VRBL (Violet Red Bile Lactose Agar)	37 and 45 °C/48h	deep inoculation
Staphylococcus aureus	Baird Parker Agar with egg yolk tellurite supplement	37 °C/24 h	Surface Culture
SRC	meat-liver glucose agar supplemented with iron alum and sodium sulphite	37 °C/24 h	The samples heated for 10 minutes at 80 °C.

TC: total coliforms, TTC: thermos-tolerant coliforms, SRC: sulfite-reducing clostridia.

Antimicrobial activity

Bacterial strains used

For testing the antimicrobial potential of camel WP, four bacterial strains were brought from Algeria Pasteur laboratory, one Gram-positive, *Staphylococcus aureus* (ATCC 25923) and three others are Gram-negative, *Escherichia coli* (ATCC 25922)*, Pseudomonas aeruginosa* (ATCC 49619) and *Klebsiella pneumoniae* (ATCC 13883). These strains used are widely encountered in various humans' pathology.

Principle of the test

An agar well diffusion assay described in the literature [13, 14], was used to test the antimicrobial activity of WP.

The Petri dishes are adhered by the Mueller-Hinton agar and inoculated by 1 ml of inoculum with optical density equivalent to 0.5 Mac Farland Standard (10^8 cfu/ml) of the pathogenic strains used so as to cover the all agar surface. The petri dishes are then dried for 15 minutes at 37 °C.

In each Petri dish corresponding to a strain tested, and using the end of a sterile Pasteur pipette were made wells of 6mm in diameter, each receive 100 µl of RCWP solution or FCWP of different concentration. A Gentamicin disk (10 µg) is placed on the agar surface as a positive quality control of the strains tested. The dishes are incubated at 37 ° C for 18 to 20 hours.

The antimicrobial activity is manifested by the appearance of a halo of inhibition of microbial growth around the well. The result of this activity is expressed as the diameter of the IZ in millimeters (mm).

Statistical analysis

The results of antimicrobial activity were statistically analyzed by one-way analysis of variance (ANOVA) using R software version 3.4.3.

Results and discussion

Results of physicochemical analyzes

The physicochemical parameters (pH, Dornic Acidity) at T0 and after 120H of fermentation are summarized in Table 02.

Table 02: Physicochemical parameters (pH, Dornic Acidity) at T0 and after 120H of fermentation

Parameters	**T0**	**120H**
pH	6.53±0.14	3.85±0.18
Dornic Acidity (D°)	18±2.11	105.3±2.91

The pH value of the samples collected at T0 represents an average value of 6.53 ± 0.14. After 120H of fermentation, we are marked a low value of 3.85 ± 0.18 (table 02). At the same time, the Dornic Acidity has been progressively increased by 18 ± 2.11 at T0 to 105°D at 120H.

These results are clearly due to fermentation, during which the growth of lactic acid bacteria increases the acidity of the medium by lactose metabolism [15, 16].

Results of microbiological analyzes

The lactobacillus present a considerable mean concentration of 3.83 Log10 cfu/ml at T0, after 120H of fermentation undergo a significant increase of the order of 8.06 Log10 cfu/ml (Fig.01).

The lactic flora acidified the medium during its growth and exerts an antagonistic activity against several food contaminants responsible for organoleptic defects or posing health risks, this activity bacteriostatic or bactericidal is a result of production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins [17, 18].

The effect of the lactic flora is clearly evident on the fecal flora represented by TC and TTC. This fecal flora exhibited a decrease of 3.19 and 2.68 Log10 cfu/ml at T0 to 2.36 and 1.36 Log10 cfu/ml at 120H respectively (Fig. 01).

S. aureus and SRC represent low initial values, 1.48 and 0.6 Log10 cfu/ml respectively, and after 120H of fermentation become zero (Fig. 01).

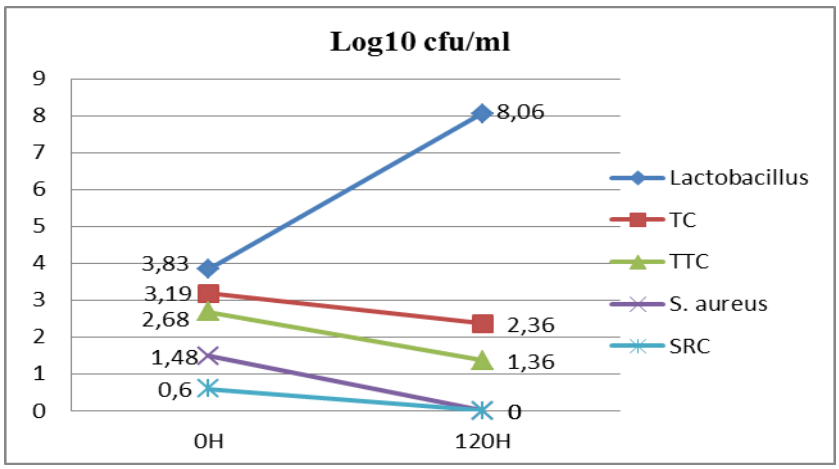

Fig.01: Evolution of growth of Lactobacillus, TC, TTC, *S. aureus* and SRC during fermentation

Labioui et *al.,* (2005) [19], and Lafta *al.,* (2014) [20] indicating that fermented milk has a system of protection against Gram-positive bacteria such as Staphylococci and Clostridia. This system is composed of lactoperoxydase and bacteriocins synthesized by lactic acid bacteria.

Antimicrobial activity

The antibacterial activity of the RCWP and FCWP was evaluated by the agar well diffusion assay. The results of the screening are shown in Fig. 2. Gentamicin (10 µg) is used as a quality control antibiotic for strains tested.

The classification of bacterial strains into "susceptible, (s)" or "resistant, (R)" categories to antibiotics is defined by the Antibiogram Committee of the French Microbiology Society (CA-SFM, 2017). The reference values, provided by the CA-SFM, indicate that the strains tested (*E. coli, K. pneumoniae, S. aureus, P. Aeruginosa*) are sensitive to gentamicin (10 µg) if the ZI given by the antibiotic are greater than or equal to 19, 15, 20, 18 mm respectively. The mean inhibition diameters of gentamicin (10 µg) on the strains tested are 24, 16, 21, and 20 mm, respectively. From this result it can be said that the strains tested are susceptible to the action of gentamicin (10 µg) (Fig. 02). The inhibition diameters, generated from RCWP (550 µg/µl) and FCWP, are significantly lower than those produced by gentamicin (10 µg). The antibacterial activity of organic products, which are complex mixtures of bioactive molecules, is generally lower than that exerted by pure antibiotic molecules.

The ZI show various diameters. These diameters are situated between 10 and 15 mm for RCWP and between 10.5 and 16 mm for FCWP (Fig. 02). According to Mohankumar and Murugalatha, (2011) [21], these results obtained by the well diffusion assay are considered positive because the ZI was greater than 8 mm of diameter.

Antimicrobial activity takes several levels of inhibition, Mohankumar and Murugalatha, (2011) [21] have shown that microbial growth inhibition activity is considered very strong if it gives a ZI between 15 and 18mm, strong if it is between 10 and 14 mm, moderate if it is between 6 and 9 mm and no inhibition if the zone of inhibition is equal to 0 mm. So we observe in our study, a very strong inhibition (15-18 mm) for RCWP with *S. aureus* which gave a ZI of 16 ± 1.19 mm and for FCWP with *S. aureus* and *P. aeruginosa*, which gave a ZI of 17 ± 1.19 and 16.5 ± 1.19 mm respectively. A strong inhibition (10-14 mm) for FCWP observed with *K. pneumoniae* and *E. coli*, which gives a ZI of 13.25 ± 1.08 and 10.5 ± 0.70 mm, respectively. A moderate inhibition (6-9 mm) for RCWP observed with *P. aeruginosa*, which gives a ZI of 9.5 ± 0.70 mm and no inhibition was observed for RCWP with *E. coli* and *K. pneumoniae* (0 mm) (Fig. 02).

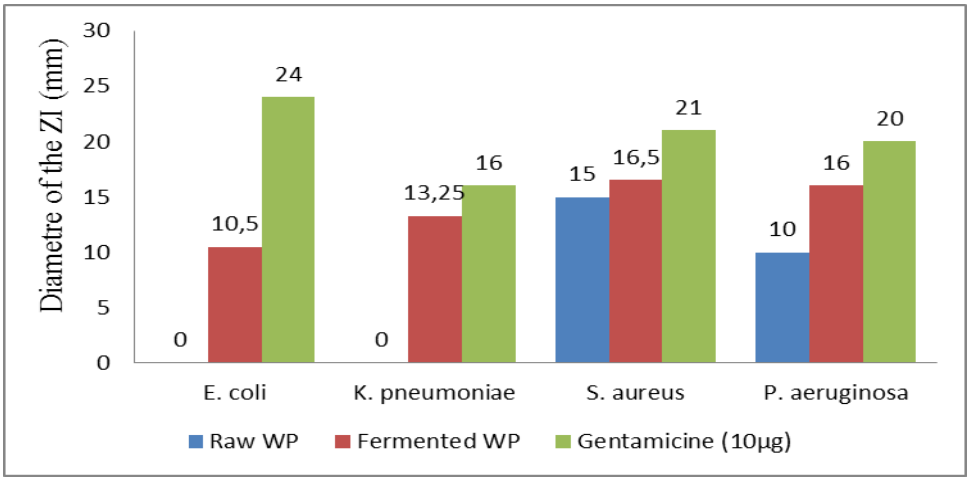

Fig.02: Diameter of the IZ of the raw and fermented WP (550 µg/100 µL) and gentamicin (10 µg) on the tested bacterial strains

The resistance of the strains tested against the camel WP varied according to their concentration (figs. 03 and 04).

The results of this study indicate that the RCWP has no significant antibacterial effect against *E. coli* and *K. pneumoniae* (as Gram-negative bacteria) with different concentrations used compared to *S. aureus* (as Gram-positive bacteria), it was sensitive from low

concentration of 1mg/ml (Fig. 03), with a highly significant difference (p= 6.14e-08 < 0.001) for concentrations greater than 1mg/ml (table 03).

On the other hand, the FCWP give a remarkable activity from a concentration of 3 mg/ml against *K. pneumoniae* and from a concentration of 4 mg/ml against *E. coli* (Fig. 04), with a highly significant difference (P = 0.0081 < 0.01) for concentrations above 3 mg/ml (table 01). But this inhibition is important with *S. aureus* and *P. aeruginosa*, which were sensitive from low concentration of 1mg/ml (Fig. 04), with a highly significant difference (p= 4.36e-08, 2.13e-10 < 0.001 respectively) for concentrations greater than 1 mg/ml (Table 03).

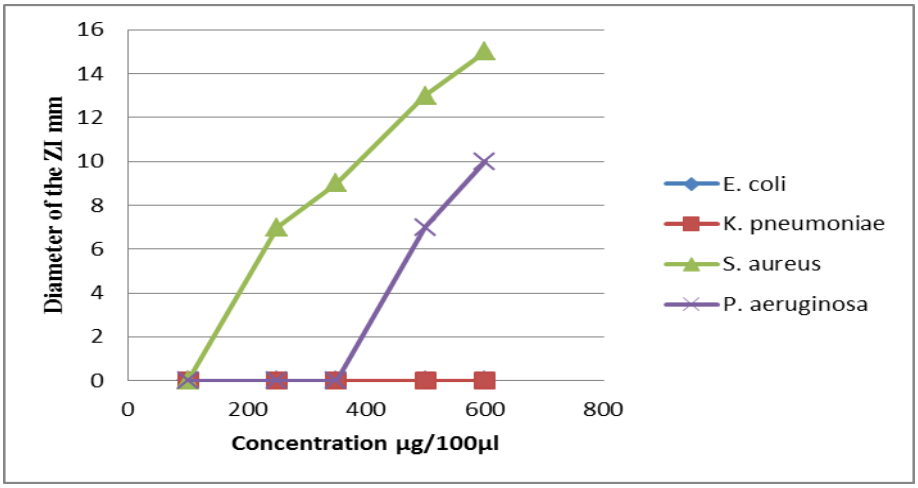

Fig.03: Variation of diameter ZI as a function of the concentration of RCWP on the bacterial strains tested

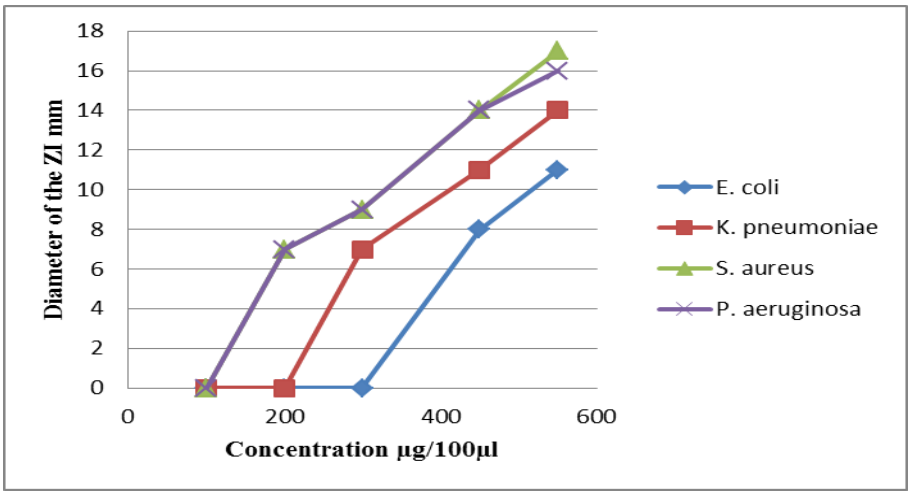

Fig.04: Variation of diameter ZI as a function of the concentration of FCWP on the bacterial strains tested

During the fermentation, the number of lactic acid bacteria increases, at the same time there is the accumulation of primary metabolites such as lactic acid, acetic acid, ethanol and

carbon dioxide CO_2, as well as lactic bacteria are capable of synthesizing antibacterial substances such as formic acid, benzoic acid, hydrogen peroxide and bacteriocins [9].

Regarding *S. aureus* and *P. aeruginosa,* the RCWP was found to have an effective activity against these organisms. For *S. aureus* (as Gram-positive bacteria) the effect is clear from 1 mg/ml and from 3.5 mg/ml for *P. aeruginosa* (as Gram-negative bacteria) and from 3.5 mg/ml against *P. aeruginosa* (as Gram-negative bacteria) (Fig. 03). At the same time, the FCWP shows a highly effective activity against these two organisms from 1 mg/ml (Fig. 04).

These results show that antimicrobial action is more important on Gram-positive bacteria compared to Gram-negative bacteria, several authors have shown that bacteriocins produced by certain lactic strains are more active on Gram-positive bacteria than on Gram-negative bacteria [22, 23].

The bacteriocins act on the Gram-positive bacteria walls by forming pores in the cytoplasmic membrane that lead to disturbances in cell functions, but the outer membrane of Gram-negative bacteria can protect the cytoplasmic membrane and cytoplasm against the action of antimicrobial compounds [22, 24].

Table 03: Mean diameter of ZI values of the RCWP and FCWP on bacterial strains tested

Proteins	RCWP			FCWP		
Strains tested	DZI (mm)	Effective concentrations of RCWP	P value	DZI (mm)	Effective concentrations of FCWP	P value
E. coli	00	---	> 0.05	10.5±0.70	From 4 mg/ml	0.0081*
K. pneumoniae	00	---	> 0.05	13.25±1.08	From 3 mg/ml	0.0081*
S. aureus	16±1.19	From 1 mg/ml	6.14e-08***	17±1.19	From 1 mg/ml	4.36e-08 ***
P. aeruginosa	9.5±0.70	From 4 mg/ml	> 0.05	16.5±1.19	From 1 mg/ml	2.13e-10 ***

***: significant difference. *: highly significant difference

Yateem et al., (2008) [9], showed in their study the bactericidal effect of lactic acid bacteria isolated from fermented camel milk (*Lactobacillus plantarum, Lactobacillus pentosus* and *Lactococcus lactis*) on Gram-negative bacteria (*Salmonella* spp. and *E. coli*) and in the same time showed no bactericidal effect of these lactic strains on Gram-positive bacteria like *Staphylococcus* spp. On the other hand, researchers showed the sensitivity of Gram-positive bacteria and Gram-negative bacteria to bacteriocins produced by lactic strains [21].

During fermentation, lactic bacteria have a bactericidal effect on Gram-positive bacteria more than on Gram-negative bacteria, by synthesizing antibacterial substances such as formic acid, benzoic acid, hydrogen peroxide and BACTERIOCINS [14, 23].

Salami et al., (2010) [25] showed that the RCWP have significantly higher antimicrobial activities than bovine milk. This finding is explained by the high content of camel milk in protective proteins as antimicrobial factors such as lysozyme, lactoferrin and immunoglobulins [26].

The lowering of the pH at the end of fermentation, at 120 hours (table 01) means that the lactic strains have an acidifier effect on the medium, by production of organic acids. This is the main factors of microbial inhibition [27].

In the face to the problems posed by antibiotic resistance in the clinical outcomes. Currently there are new natural antimicrobial products to fight against bacterial pathogens. Constituents of food can be used for reducing the risk of developing or aggravating human diseases [28, 29]. Based on these results, camel milk derivatives can be contains an abundant source of active biomolecules against several infectious diseases.

Conclusion

The spontaneous fermentation of camel milk is ensured by the growth of the lactic flora. The latter inhibits the growth of other germs of contamination and prolongs the shelf life of milk.

CWP exhibits antimicrobial activity against *S. aureus* such as Gram-positive bacteria. After fermentation there is a broadening of the spectrum of action against Gram-negative bacteria like *E. coli* and *K. pneumoniae*. This improvement in antimicrobial activity comes from the action of the lactic flora, which ensures proteolysis of whey proteins and release of bioactive peptides.

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