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Detection of the Antibacterial Activity of Bioactive Peptide Isolated from Fermented Buffalo Milk in vitro

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Abstract: *The present study aims to prepare fermented buffalo milk rich with low molecular weight peptides by using a mixture of lactic acid starters. Skim milk sample was inoculated with 5% of the starter. The growing number of starter and anti-bacterial activity were studied after 24 hours of incubation. Protein and peptide concentration were determined before and after fermentation, then biological active peptides were isolated or separated and purified by gel filtration column of Sephadex G25. Finally antibacterial activity of isolated peptides was study in vitro. The results of chemical analysis of fresh and fermented milk showed that the concentration of protein were 0.817mg/ml and 0.501mg/ml before and after fermentation, respectively either peptide concentration were 0.4mg/ml before fermentation and 0.805mg/ml and after fermentation.*

The number of starter was determine during the fermentation process after 6, 12, 18 and 24 hours of incubation and found an increase in the number of lactic acid bacteria . The initiation number was 105 but after the 24 hours the number increased of up to 1,3 × 106. Number of lactic bacteria decreased after 24 hours with the increase in the concentration of lactic acid combined with low pH value.

Colonies of lactobacilli were isolated from fermented buffalo milk and was characterized by the typical characteristics for the purpose of a rating based on morphological and cultural characters. gel filtration gave Seventy-eight fractions. And depending on the absorbency on wavelength 280 were obtained four peaks, each peak represents a fraction. peptide concentration was determined in each fraction, these concentrations were (0 and 0243 and 0902 and 0632) mg / ml of fraction1, 2, 3 and 4, respectively. Fraction three contained a high concentration of peptide. The antibacterial activity of the fraction three . The results showed that the bioactive peptides of fermented milk have good antibacterial activity in vitro. E. coli was more effective than other bacteria.

I. Introduction

Milk was a whitish food yield by the mammary secretory cells of females during lactation period; it was one of the specific characteristics of mammals.(**Kebchaoui, 2012).** Milk and its dairy products were a part of the human food for thousands of years, as cheese being made more than 7,000 years ago **(Lucey, 2009).** Milk as a complete food for infants consists of necessary nutritive elements including lactose, fat and proteins, required for their growth and development. . (**Fox**, **2001**).

Milk contained lactose, protein, lipid minerals. Milk components supplied the essential nutritional elements, immunological protection, and biological active substances to both adults and neonates. **(Cross and Gill, 2000).** Milk was a much various liquid. The main components were fat, water, lactose, organic acids, minerals, proteins, as well as many secondary milk compounds, such as hormones, vitamins, antibodies, enzymes and miscellaneous compounds. **(Fox, 2009).**

Milk was a much source of proteins which were generally divided into caseins and whey proteins. Caseins and whey proteins composed approximately 80% and 20%, respectively, of total proteins of milk. **(Haque and Chand, 2006).**

Caseins had percent about 80% of the total protein, which was identified chemically as proteins of milk that precipitate at pH 4.6. The remaining 20% were whey proteins or milk serum proteins and were soluble at this pH. Milk is a good source of bioactive materials with beneficial effects for mans**.(Mils et al., 2011).**

II. Materials and Methods:

1- **Sample Collection**

Five hindered milliliter of Iraqi buffalo milk were collected. Milk was immediately cooled and kept at 5^{o}C until transportation to the laboratory of the Veterinary Medicine College in Al-Qadissiya University. The fresh whole milk was sterilized in 500 ml at 80^{o}C for 15 min in a water bath then cooled to 5^{o}C in a freezer until used.

2- Skimmed milk preparation:

Skimmed milk was produced by centrifuging the milk samples at 5000 rpm for 15 minutes under 4°C**.(Hassan et al., 1987).**

3- :**Preparation of fermented milk** :

The milk samples were incubated for 1h at the fermentation temperature (43ºC) in a water path before inoculation with the lactic acid starter cultures. Milk was inoculated with 5% (105 cfu/ml) of mixed starters culture. The mixture were gently mixed after inoculation and incubated at 43oC for 6h. After 6 h of incubation, samples of 50 ml volume were colleced in sterile cylinder and used for microbiological and biochemical analysis and sensory estimation.
(Abdel-Rahman et al., 2009).

4- **Extraction of whey protein**:

Fermented milk was acidified to pH of 4.2 by adding 2N HCl. The solution was centrifuged at a speed of 10000 g at 4°C, for 30 minutes. casein (the sediment) was removed from whey by filtration using No.1 Whatmann filter paper. Whey acid was neutralized to pH 6.8 by addition 2 N NaOH, then centrifuged at 10000g at 4°C for 30 minutes. The whey supernatant obtained was neutral. **(Serkan et al., 2013).**

5- **Estimation of protein concentration**:

The protein concentration of the fresh and fermented milk was determined by the method of Bradford. Bovine Serum Albumin (BSA) was used as standard protein to prepare the standard curve and Coomassie Brilliant Blue G-250 as the reagent. **(Bradford, 1976).**

6- **Determination of peptide concentration methods**:

The peptide concentration of the fresh and fermented milk was determined by the method of OPA. Glutathion was used as standard matter to prepare the standard curve and OPA as the reagent.

7- Separation of low molecular weight bioactive peptide from fermented milk by gel filtration:

Peptides were separated by passing of whey protein of fermented milk through the Sephadex G25 column in dimension 40 X 1.6 cm .The recovered parts of the column were collected in a speed flow 30 ml / hour, the optical absorption of the washing and recovery parts were measured on wavelength 280 nm to determinate the fraction numbers according to the curves then the fractions were concentrate by a rotary evaporator at a temperature of 50 degrees **(Schillinger and Luke, 1989).**

8- Determination of antibacterial activity of bioactive peptide of the third fraction in vitro

Well diffusion assay was used to determination of antibacterial activity of peptide of fermented milk. The bacterial cultures (E. coli, Klebsiella pneumonia and Staph. aureus) were prepared for this test. The number of each microorganism was 106 cfu/ml, was spread on Mueller-Hinton agar plates surface. Wells was made on the surface of each inoculated plate.The well was prepared in the plate by using sterile borer (6 mm in diameter).The plates were incubated at 37°C for 24 h and observed the zone of inhibition. Inhibition zone was made around each well was recorded and the experiment was repeated for three times. **(Tome et al., 2006).**

III. Results and Discussion

Peptide and protein content of Fresh and fermented buffalo milk.

The results also showed the concentration of protein and peptide in fresh milk that estimation by OPA and Bradford methods.

Table (1): the proteins and peptides concentration per ml of a fresh buffalo milk.

Materials	Mg/ml (mean ± SE)	
	Befor fermentation	After fermentation
Protein	0.817	0.501
Peptide	0.4	0.805

This study was showed the means of peptide and protein per ml of fresh buffalo milk and that refer to buffalo milk was higher than cow milk in these concentration due to they depend on many factors as analysis methods, geographical position, feeding, as well as management, age and lactation stage, this is agreement with results of **Khaskhel et al., (2005).**

The viable counts of starter bacteria cultures during fermentation

A) Isolation and identification of Lactobacillus spp. from fermented buffalo milk:

Lactic acid starters (LAS) were isolated from fermented milk on MRS agar initially. All isolates were obtained identification morphologically characterized by the colony characteristics of the isolates obtained, along with their Gram reaction and microscopic examination.

The common bacteria were Lactobacillus spp. and colonies were isolated from fermented buffalo milk with typical characteristics white, small with entire margin were picked and transferred to nutrient broth which was then subjected to classification based on morphological & biochemical characters. All strains were reacting positively to Gram stain. Lactobacilli spp. were long rods sometimes they are coccobacilli. Lactobacillus showed negative result to motility test because they did not possess flagella, citrate and indole were negative and catalase were negative. All isolates were isolated from fermented milk were found to ferment glucose and lactose. This result was resample to that obtained by **Kandler et al.,. (2005).**

Changes in the viable counts of the starter cultures of lactic acid bacteria throughout fermentation are presented in table (2).

Table (2): The total number of bacteria after fermentation (6,12,18 and 24h).

Time	6h	12h	18h	24h
No. of bacteria	6.2×10^5	7.4×10^5	1.1×10^6	1.3×10^6

Lactic acid starter number was decreased after 24h due to increase the lactic acid concentration company with decrease the pH value. Depression in the pH value in the buffalo milk was higher than depression in cow milk due to decrease in the buffering capacity and variation in the milk composition. **(Al- Saleh and Hammed, 1990).**

Determination of fractions as the peak according to the presence of low molecular weight peptide:

These peaks were determined according to absorbency of each fraction that was measured on wavelength 280nm.

Three peaks were obtained. The first peak did not contain any peptide so that it did not have the inhibitory activity against any type of pathogenic bacteria. It was represent the first fraction. The second peak contained peptide concentration (**0.243**) mg / ml and had given the effectiveness of inhibitory action against each type of pathogenic bacteria. The third peak contained a higher peptide concentration (**0.902**) mg / ml. This fraction showed the higher antibacterial activity against bacteria.The 4th peak contained a peptide concentration (**0.632**) mg / ml and had given the effectiveness of inhibitory action.

The peptide concentrations of each fraction were (0 , 0.243, 0.902 and 0.632) mg/ml of fraction 1, 2, 3 and 4 respectively. Fraction three showed the high concentration then the forth fraction. Figure (1).

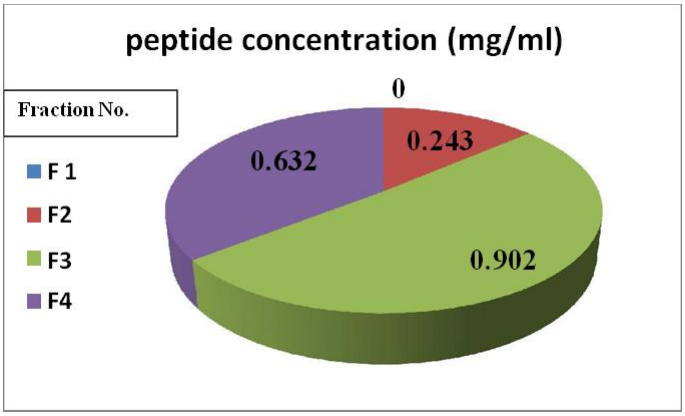

Figure (1): peptide concentration of each fractions.

The results showed the inhibitory activity of the fractions:
In vitro study:

The results showed the inhibitory activity of each fraction that obtained from gel filtration and purified by amicon 10kd and 5 kd. The fraction one (F1) did not show any antibacterial activity against any type of pathogenic bacteria that was used in this study due to it did not have bioactive peptide.

The second peak, was represent the fraction two, showed narrow zone of inhibition against pathogenic bacteria. The means of the diameters of inhibitory zone were (8.5, 4 and 7.3) of E. coli, K. pneumonia and Staph.aureus, respectively. E. coli showed high sensitivity toward bioactive peptides.

Third fraction, that represent the third peak, was the active fraction and appeared large inhibitory zone against each type of pathogenic bacteria due to the peptide concentration was high (0.805mg/ml). E. coli also showed high sensitivity than K. pneumonia and Proteus spp. The rate of inhibitory zone diameters (19.5 mm, 12.3mm and 15.3mm) for E. coli, K. pnumoniae and Staph.aureus, respectively.

Fourth fraction also showed high inhibitory zones in means 15mm, 13.8mm and 12.8mm of E. coli, K. pnumonia and Staph.aureus, respectively. Third fraction was more effective than the forth fraction due to the bioactive peptide concentration of the first one was higher than that of fourth fraction.

Table (3): Means of inhibition zones of fraction three toward each bacteria.

Fraction No.	**Peptide concentration (mg/ml)**	**Rates of inhibition zone(mm)**		
		Staph.aureus	**K. pneumonia**	**E. coli**
F1	**0**	0	0	0
F 2	**0.243**	7.3	4	8.5
F 3	**0.902**	15.3	12.3	19.5
F 4	**0.632**	12.4	10.8	15

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