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Activity of Casein Extract from Fermented Goat Milk against E. coli

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بسم الله الرحمن الرحيم

فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ ۗ
وَلَا تَعْجَلْ بِالْقُرْآنِ مِنْ قَبْلِ أَنْ يُقْضَىٰ إِلَيْكَ
وَحْيُهُ ۖ وَقُلْ
رَبِّ زِدْنِي عِلْمًا ﴿١١٤﴾
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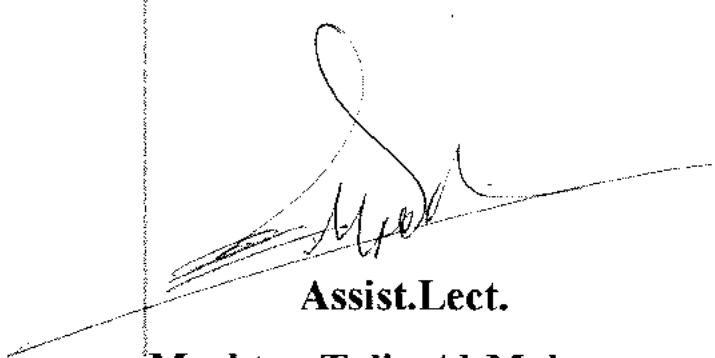
SUPERVISORS CERTIFICATE

We certify that this paper was prepared by **Laith Hassan Mahdy** entitled (**Activity of Casein Extract from Fermented Goat Milk against *E. coli***) under our supervision at the College of Veterinary Medicine / Al-Qadissiyia university in partial fulfillments of requirement for the degree of bachelor of Science in Veterinary Medicine and Surgery.



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Certificate of Instructor

We certify that ***laith Hassan mahdy*** has completed the fulfillment of her graduation project entitled Activity of Casein extract from fermented goat milk against *E. coli* for the year 2015/2016 under our construction.

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April 2016

Dedication

I dedicate my research work to my family and many friends. A special feeling of gratitude to my loving mother, whose words of encouragement and push for tenacity ring in my ears.

Laith 2016

First and foremost, I have to thank God and I would like to extend thanks to the Al-Qadissyia University and Microbiology department of Veterinary Medicine College, who so generously contributed to the work presented in this thesis .

Special mention goes to my enthusiastic supervisor, Assist. lecturer Moshtaq Talip Hussein My study has been an amazing experience and I thank my friends wholeheartedly, not only for their tremendous academic support, but also for giving me so many wonderful opportunities .

Similar, profound gratitude goes to Assist Prof. Dr. Mansor Jada'an, who has been a truly dedicated mentor. I am particularly indebted to Akram for his constant faith in my lab work. I have very fond memories of my time there .

Finally, but by no means least, thanks go to mum and my family for almost unbelievable support. They are the most important people in my world and I dedicate this thesis to them.

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Summary

Summary

The present study was aimed to prepare fermented goat milk riches with low molecular weight peptides using lactic acid bacteria as mixture culture. For such aim, pasteurized milk sample were inoculated with5% bacteria before incubation at 42^0C for various periods of incubation. Peptide concentration and antibacterial activity of the whole fermented milk were determined *in vitro*, then bioactive peptides were separated and purified by the gel filtration of Sephadex G25 column. Peptide concentration was determined in each fraction. Fraction with higher peptide concentration was chosen to estimate of its antibacterial activity *in vitro*.

Gel filtration was gave four fractions. Peptides concentration of each fraction were determinated. The peptides concentration 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 than the forth fraction.

The antibacterial activity of the whole fermented milk and third fractions were estimation. *E.coli* showed the high sensitivity toward antibacterial peptide. antibacterial peptides of fermented milk gave the good result in treatment of clinical cases caused by pathogenic *E. coli*.

Chapter One

Chapter Three

Materials and Methods

3- Materials and Methods:

3-1: Materials:

3-1-1: Instruments and Equipments:

The instruments and equipments used in the present study are listed in Table (3-1) below:

Table (3-1): Instruments and Equipments used in the Study

Equipment	Manufacturing company/ country
Autoclave	Gallen Kaamp / England
Centerfuge	Hettich/ Germany
Compound Light microscope	Olympus/ Japan
Electric oven	Gallen Kaamp / England
ELISA Reader	Bio Tek/ France
Incubator	Gallen Kaamp/ England
Laminair flow safty cabinet	Labtech / South Korea
Mixer vortex	Gallen Kaamp
Platinum wire-loop	John Bolten/ England
Spectrophotometer	Gallen Kaamp
Water bath	Memmert/Garmany
Water distillater	Fisons/ Japan

3- 1-2 : Chemical and biological materials:

The chemical and biological materials used in this work were listed in Table (3-2).

Table (3-2): Chemical and biological materials with their remarks.

Materials	Manufacturers company/ state
B- mercaptoethanol	BDH
Beef extract	Himedia/ India
Coomassie brilliant blue G-250	BDH
Ethanol 95%	BDH
Ethanol(absolute 99%)	BDH
Glutathione	BDH
Gram stain	Oxiod/UK
Hydrochloric acid	BDH
Hydrogen Peroxide (H_2O_2)	BDH
Kovac' s reagent	Himedia
Methanol	BDH
O-phthaldiadehyde	BDH
Sephadex G-25	Sigma/UK
Sodium chloride (Nacl)	Merk/ Germany
Sucrose	Difco/ USA
Sulfaniolic acid	BDH
Sulpheric acid (H_2SO_4)	BDH

3-1-3: Culture media

Culture media used in this study were listed in table (3-3). They were prepared according to the manufacturer's instruction on their containers and sterilized according to the suitable method.

Table (3-3): Culture media used with their remarks.

Media	Company/state
Blood base agar	Himedia/ India
MRS agar	Himedia
Muller-Hinton agar	Oxoid/ England
Nutreint broth	Himedia/ India
Peptone water medium	Himedia
Simmon citrate agar	Himedia

3-2: Methods:-

3-2-1: Stains, reagents and solutions:-

3-2-1-1: Staining method

3-2-1-1-1: Gram staining:

The isolated bacteria were examined by using gram staining . According to Collins *et al.,* technique, and were observed under light microscope.

3-2-1-2: Reagents:

3-2-1-2-1: Nitrate reduction reagent:

A. **Sulfanilic acid solution (Reagent A):** Eight gram of sulfanilic were dissolve acid in 1 litre (5N) acetic acid and stored at room temperature for 3 months, in dark.

B. **α-Naphthylamine solution (Reagent B):** six gram of N,N-Dimethyl-1-naphthylamine were dissolve in 1 litre (5N) acetic acid and stored at 2 to 8°C for 3 months, in dark.

3-2-1-3: Solutions:

3-2-1-3-1: Physiological normal saline (0.85 NaCl%):

It was prepared by dissolving 8.5g NaCl in 1000 ml distilled water and sterilized by autoclave (Collee *et al.*, 1996).

3-2-1-3-2: Preparation of NaCl solution in various concentration used for determination of LAB tolerance to NaCl:

A. NaCl solution: (2%)

Tow grams of NaCl were dissolved in 100 ml of distilled water to form 2% NaCl solution.

B. NaCl solution:(4%)

Four grams of NaCl were dissolved in 100 ml of distilled water to form 4% NaCl solution.

C. NaCl solution: (6%)

Six grams of NaCl were dissolved in 100 ml of distilled water to form 6% NaCl solution.

3-2-1-3-3: Preparation of 0.5 McFarland standards:

It was prepared according to the method of and Andrews and Wise. (2002):

Solution A: 1,175g of aqueous barium chloride ($BaCl_2.H_2O$) has been dissolved in 100 ml of sterilized distilled water.

Solution B: 1 ml of concentrated sulpheric acid (H_2SO_4) has been added to 99 ml of sterilized distilled water.

From solution (A) 0.5 ml was added of to 99.5 ml of solution (B). It was used for determination density of bacterial

suspension that was used in determination of the initiation number of LAS and sensitivity test.

3-2-2: Laboratory preparation of culture media:

Culture medium listed in table (3 - 3) were prepared according to the manufacture instructions, autoclaved at 121 °C for 15 min, and used in appropriate tests.

3-3: Standard bacteria:

1. *Escherichia coli* ATCC 25922

3-4: Lactic acid bacterial isolates:

Lactic acid bacteria (LAB) were isolated from local dairy products. Serial dilutions of the product were made by using normal saline. A portion of 0.1 ml of each dilution, was spread on MRS agar and incubated at 42C° for 24h. The isolated bacteria were purified by selection of single pure colony grow on MRS agar plates and sub-cultured on MRS broth. (Gardiner *et al.*, 2002).

3-4-1: Identification of lactic acid bacteria:

3-4-1-1: Catalase test:

A pure bacterial colony was taking by germs loop conveyor and placed on a clean glass slide, one drop of hydrogen peroxide 3 %were added to it. Formation of gas bubbles indicate positive result (Nelson and George,(1995).

3-4-1-2: Oxidase Test:

An isolated colony has been placed with a wooden stick on a clean piece of filter paper, then two to three drops of oxidase

reagent was added. Purple- colored production of colonies within 10-30 seconds referred to positive result (Baron and Fingold, 1990).

3-4-1-3: Carbohydrate fermentation test:

Carbohydrate fermentation broth was inculcated with bacterial growth and incubated for 1-2 days at 37° C. Fermentation test was considered positive result when color of broth be changed to yellow (Collee *et al.*, 1996).

3-4-1-4: Growth test at 45C°:

Lactic acid starter (1%) was inoculated into MRS broth and incubated for 24h at 45C° , then growth was checked after incubation (Holt *et al*,. 1986).

3-4-1-5- Growth test at 15C°:

Lactic acid starter (1%) was inoculated into MRS broth and incubated for 24h at 45C°, then growth was checked after incubation. (Holt *et al*,. 1986).

3-4-1-6: Growth at different concentrations of sodium chloride:

The tolerance of isolates to various concentrations of sodium chloride was determined according to the procedure of Vinderola *et al.*(2002). MRS broth with different concentrations of NaCl (2, 4 and 6%) was inoculated with isolates, all tubes were incubated at 37°C for 24-48 hrs and the growth was observed by measuring the optical density at 620 nm (OD620) using spectrophotometer.

3-4-1-7: Nitrate Reduction:

Lactic acid and suspected isolates were incubated of at 42 °C for 24hrs in nitrate broth. After incubation, the reagent was added (0.5 ml of sulphanilic acid 0.8%), in 5N Acetic acid) and α-naphthylamine (0.5%, in 5N Acetic acid) into the tubes. Red or pink color represented the positive result of nitrate reduction .

3-4-1-8: Citrate utilization test:

Simmons citrate agar was inoculated with the isolates and incubated at 37 °C for 24 h. After incubation, the blue coloration referred to the positive result of citrate utilization.

3-4-1-9: Indol test:

Peptone water medium was inoculated with new bacterial colony for 24-48h at 42 °C, and then drops of Kovac's reagent were added. The positive result is representing of formation of red ring (Collee *et al.*, 1996).

3-5: Goat milk Samples Collection:-

Goat milk samples obtained from Iraqi goat .These samples were immediately kept at 4 C during transporting to the laboratory . Each fermentation batch(500 ml) were equilibrated for 1 h at the fermentation temperature (40C) in a water path before inoculation with the starter bacterial cultures as mixed culture. These bacterial isolates prepared from College of Veterinary Medicine in Baghdad.

3-6: Preparation of fermented milk :

A milk sample was inoculated with the starter culture at mixed cultures. These fermented samples were incubated at 40° C for

72 hrs.The casein fraction was obtained by adjusted the pH of fermented milk to 4.6 with (1 N HCl).Then it was centrifuged at 10,000 xg for 20 min .The sediment was adjusted to pH 8.3 with (1N NaOH) .Then centrifuged again at 10,000 xg for 10 min .The final sediment was used as the casein fraction (Shua *etal.,* 2008).

3-7: Measurement of Peptide Content :-

Peptide content in casein fraction was quantified using modified method of Anders *etal.,* 2002. A reagent solution was prepared by mixing 25ml of 0.1 mo L-1 borax , 2.5 ml of 20% (w/v) sodium dodecylsulfate , 270 ml of thiolactic acid , and 1.25 ml of Ophthaldiadehyde (EL –Zahar *et al.*, 2003).

3-7-1: Preparation of the standard curve of the Glutathione:

Several glutathione concentrations were prepared (in duplicate) as table (3 -6), then OPA reagent was added to the mixture of 100ul of Glutathione concentration .The mixture was shake and left for 5 minutes at room temperature and the absorbance was measured at wave length 343nm.The blank was prepared by mixing 100ul of distal water and OPA reagent .The standard curve was plotted between the peptide amount and the corresponding absorbance of the standard protein as showing in fig (3- 2).

Table (3-6): Preparation of serial Glutathione concentrations.

No. of tube	Volume of stock Glutathione solution (ml)	Volume of distal water (ml)	Glutathione concentration (mg/ml)
Blank	0	10	0
1	0.5	9.5	0.2
2	1	9	0.4
3	1.5	8.5	0.6
4	2	8	0.8
5	2.5	7.5	1
6	3	7	1.2
7	3.5	6.5	1.4
8	4	6	1.6

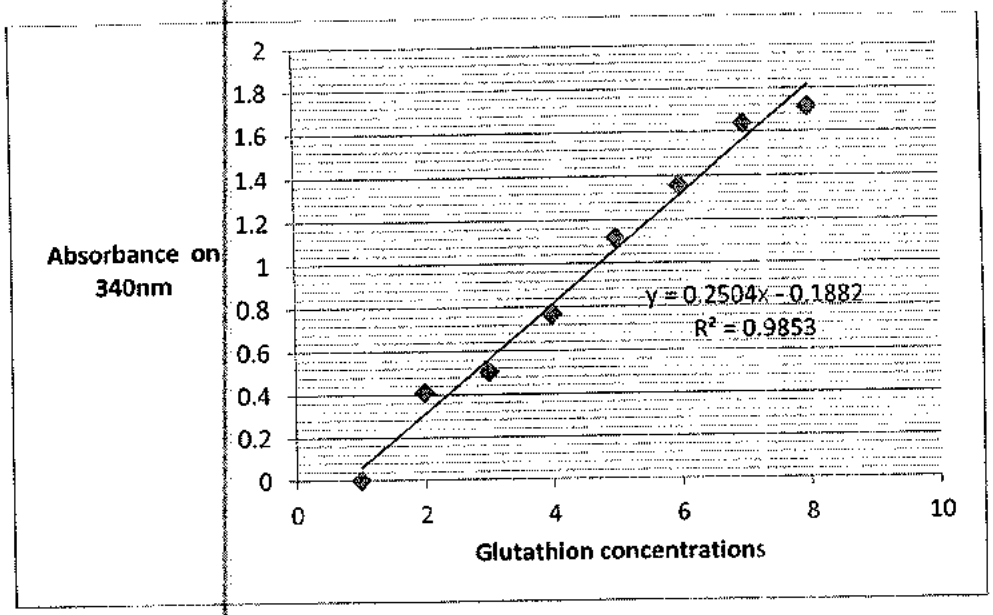

Figure (3- 2): Standard curve of glutathione concentrations.

3-8:Column chromatography purification:

Fermented milk was centrifuged at 10,000 xg for 30 min .The supernatant was subjected to gel filteration (cephadex G25 at 1.5 cm diameter X 80 cm length) .The antibacterial activity of the fraction were evaluated . The fractions (100 droops) were collected manually.The samples were concentrated by evaporation and it's absorbance were evaluated at (340nm). One milliliter fractions were collected and evaporated to dryness before measuring of its antibacterial activity against test organisms mentioned previously.

3-9: In vitro, determination of antibacterial activity of fermented milk and third fraction peptide:-

In this study, well diffusion assay was used. Bacterial cultures of *E. coli, Klebsiella pneumonia* and *Staph. aureus* were grown independently in nutrient broth at 37°C. After 24 h of incubation, an inoculum size of 10^6 cfu/ml from each

bacteria was spread on Mueller-Hinton agar plates surface. Wells was made on the surface of each inoculated plate by using sterile borer (6 mm in diameter).. The plates were incubated at 37°C for 24 h and the zone of inhibition was measurement I (mm) and the experiment was repeated for triple. (Tome *et al.*, 2006).

Chapter Four

Results and Discussion

4: Results

4-1: Isolation and identification of *Lactobacillus* spp. from fermented goat milk:

Culture characteristics of suspected bacterial isolate colonies produced after growth on MRS agar small, white with entire margin. These characteristics came accoodoma with Agrawall and Prakash (2013) regarding Lactobacilli , were isolated from fermented milk on MRS agar initially.

Result showed that after the suspected isolates were subjected to the microscopic examination by Gram staining, their all were formed to be gram positive rode mainly grouped in chains and non-spore forming. Such characteristics are similar to those describe by

The common bacteria were *Lactobacillus* spp. and colonies were isolated from fermented goat 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 and 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 lactose. This result was resample to that obtained by Kandler *and* Weis (2005) Table (4-1).

Table(4- 1): Biochemical characterization of Lactobacilli spp. from fermented goat milk

Biochemical tests		Results
Indol production		-ve
Citrate utilization		-ve
Nitrate reduction		-ve
Growth at 45 °C		+ve
Growth at 15 °C		+ve
Gram stain		+ve
Growth in NaCl concentration	**2%**	+ve
	4%	+ve
	6%	-ve
Colonies shape and characters		white, small with entire margin colonies
Acid production		+ve

4-2: Fractions of gel filtration with its low molecular weight peptide:

These peaks were determined according to absorbency of each fraction that was measured on wavelength 280nm. The first peak of the figure (4- 1) extend from 16th tube to the 22th tube, with any peptide which mean no inhibitory activity against any type of pathogenic bacteria. It was represent the first fraction. The second peak contained peptide concentration of (**0.243**) mg / ml and had given the effectiveness of inhibitory action against each type of pathogenic bacteria. This peak

extended from 32th tube to 43th tube. Figure (4- 1). The third peak contained a higher peptide concentration (**0.902**) mg / ml. which extended from 51th tube to the 57th tube. 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. It extended from 63th tube to the 68th tube. Figure (4- 1)

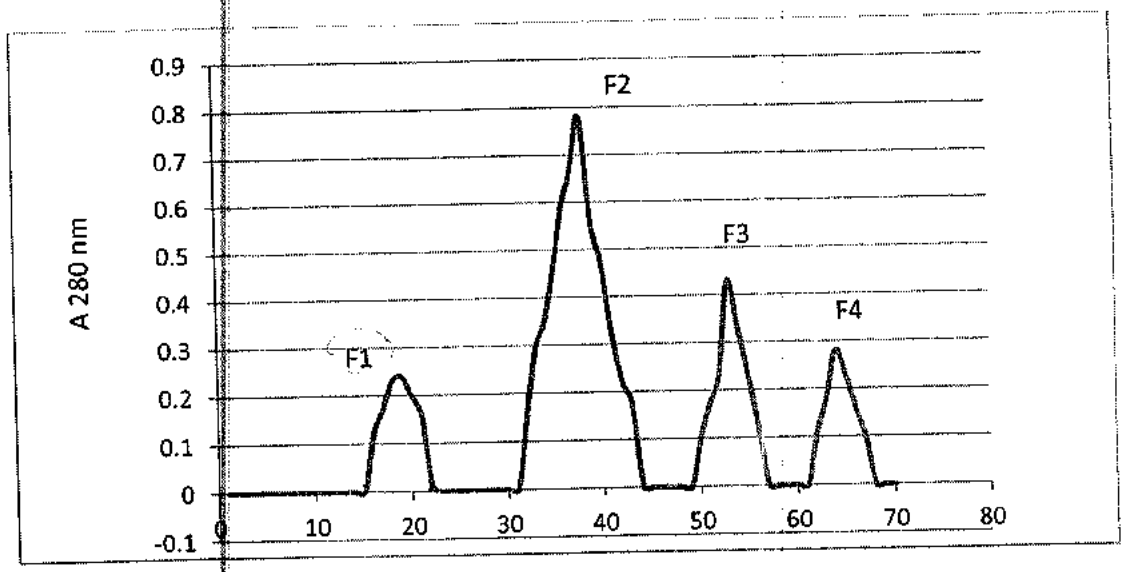

Figure (4-1): Fraction of the gel filtration.

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 (4- 2).

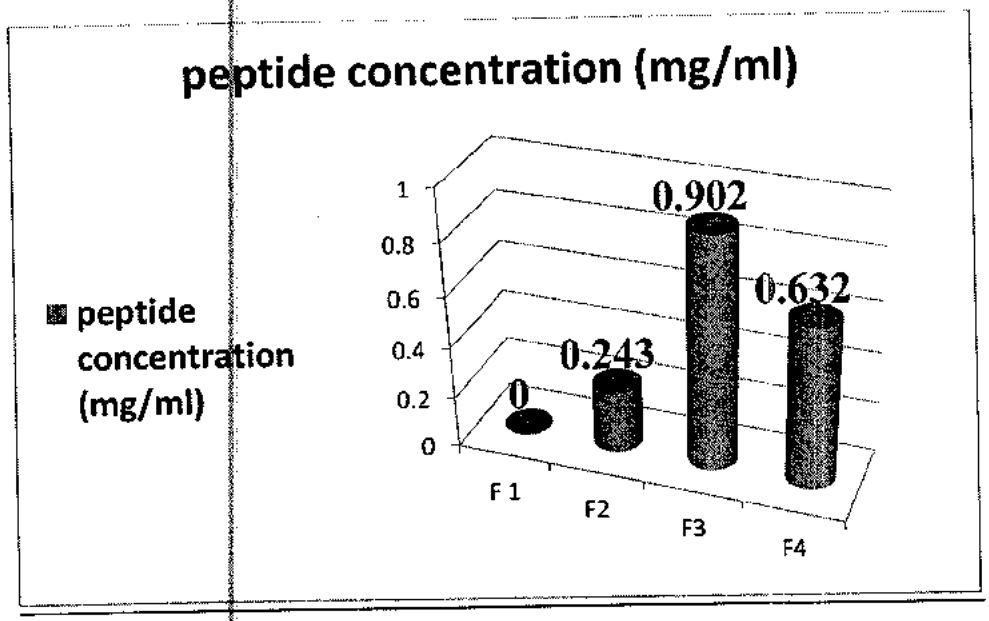

Figure (4-2): Peptides concentration of each fraction (mg/ml)

4- 3: Antibacterial activity of the whole fermented milk in vitro :

The result showed the antimicrobial activity of fermented milk against pathogenic bacteria (*E. coli*). Well diffusion method was used to estimation of this activity. Fermented milk had several types of antimicrobial material release from lactic acid starter or nature materials of fresh milk. Agar diffusion method was not suitable for determination of antibacterial activity of fermented milk peptide.

This activity was performed before 4-6 h but did not show any activity due to the concentration of antimicrobial peptides was very low while after 6h, inhibition zone appeared where the inhibitory zone against *E. coli* ranged from (9.2-12.7) mm respectively. Table (4-2).figure (4-3).

Table (4-2): Inhibition activity of fermented milk in vitro.

Bacteria	Inhibition zone (mm)			mean s±SE
	Freq1	Freq 2	Freq 3	
E.coli	9.2	10.9	12.7	10.9±1.01

E.coli was showed the high sensitivity to the antibacterial action of the fermented milk. This was in agreement with Manab *et al.*, (2011) and Detha *et al.*,(2013) who reported that *E. coli* had the high zone of growth inhibition.

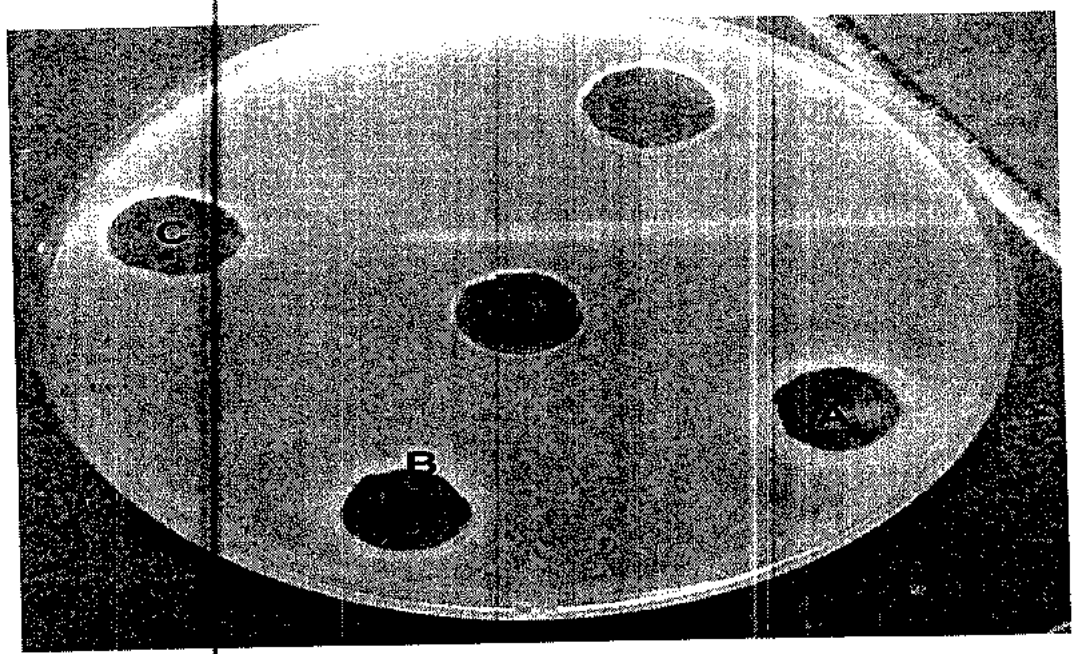

Figure (4-3): Inhibitory activity of whole fermented milk against *E. coli* in three repetitions.

4-4: Inhibitory activity of gel filtration fractions in vitro:

The results showed the inhibitory activity of each fraction that obtained from gel filtration. The fraction one (F1), was represent the peak one , 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)mm of *E. coli*.

Third fraction, that represent the third peak, was the active fraction and appeared large inhibitory zone against pathogenic bacteria due to the peptide concentration was high (0.805mg/ml). *E. coli* also showed high sensitivity . The rate of inhibitory zone diameters (19.5 mm, 12.3mm and 15.3mm) of *E. coli*. Table (4-3), Figure (4-4). Fourth fraction also showed high inhibitory zones in means 15mm, 13.8mm and 12.8mm of *E. coli*. 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 (4-3),Figure (4- 4).

Table (4-3): Means of inhibition zones of third fraction toward each bacteria.

Fraction No.	Inhibition zone of *E. coli*
Fraction 1	**0**
Fraction 2	**8.5±0.208**
Fraction 3	**19.5±0.23**
Fraction 4	**15.2±0.202**

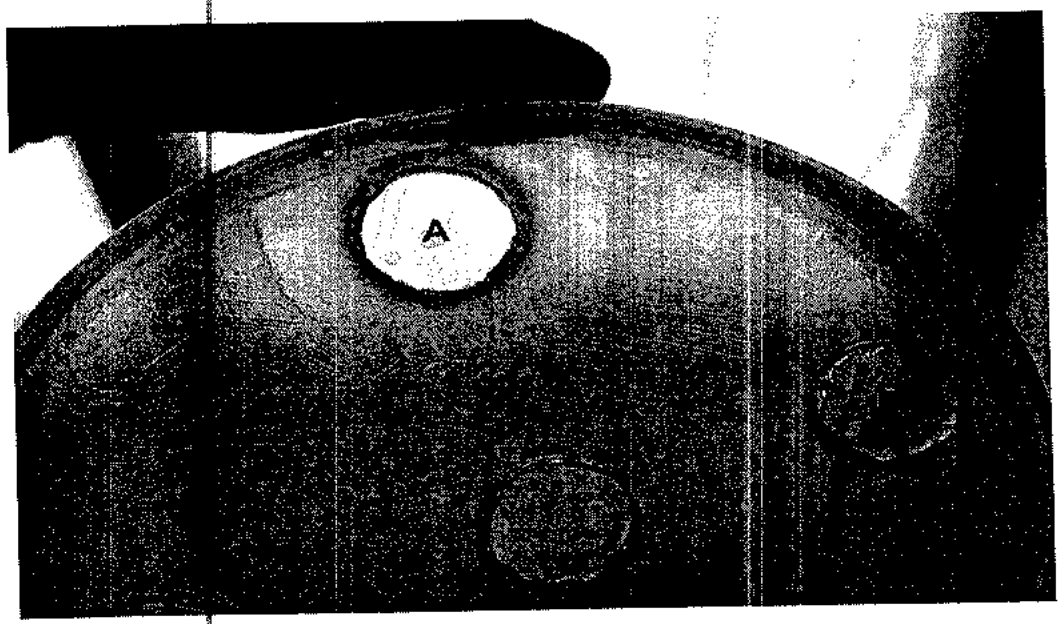

Figure (4-4): Antibacterial activity of the third fraction **(A)** and fourth fraction **(B)** fractions against *E. coli*.

4-4-2-2: Relationship of antibacterial activity and peptide concentration

The zone of inhibition had relationship with peptide concentration of each fraction. Third fraction showed high concentration of bioactive peptide with high inhibitory zone. Table (4-4).

Table (4-4): Relationship of antibacterial activity and peptide concentration.

Fraction No.	Peptide concentration (mg/ml)	Inhibition zone of *E. coli* (mm)
F1	0	0
F 2	0.243	8.5
F 3	0.902	19.5
F 4	0.632	15

Conclusion and Recommendation

1: Conclusions:-

1. Using cultures of LAS mixture led to produce fermented milk rich with the low molecular weight peptides (antibacterial peptides).
2. There was a variation in the peptides concentration of fresh and fermented milk.
3. Fermented milk had the ability to kill or inhibition of bacterial growth.
4. The antibacterial peptides were effective against pathogenic bacteria as *E. coli.*

2-Recommendations:-

1. Study the inhibitory effect of bioactive peptide towards non-bacterial pathogens (viruses, parasites and mycoplasma).
2. Study the amino acid sequence of the bioactive peptides that have antibacterial activity.