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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)

صدق الله العلي العظيم

سورة طه..الآية١١٤

Dedication

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

Rokyaye 2017

Acknowledgement

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

ROKYAYE 2017

CERTIFICATE

We certify that Rokyaye Baseem was complete the fulfillment of the her graduation project for the year 2015-2016 under our constrictions.

Head of department of Microbiology and Parasitology

Assist. Prof. Dr. Moneer Abed Al-Ameer

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16/3/2017

SUPERVISORS CERTIFICATE

We certify that this paper was prepared by **Roqaya Baseem** 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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Summary

The present study was aimed to prepare fermented goat milk riches with antibacterial peptides using lactic acid bacteria as mixture culture. For such aim, pasteurized milk sample were inoculated with 5% bacteria before incubation at 42^0C for various periods of incubation. Peptide concentration and antibacterial activity of the casein and whey were determined *in vitro*.

The peptides concentration were determination of each whey and casein (0.902 and 0.632) respectively. Whey has high peptides concentration

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

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Introduction

Chapter one …… ……..…….. **Introduction**

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Introduction

Milk is a whitish food yield by the mammary secretory cells of females during lactation period; it was one of the specific characteristics of mammals. Udder had the glands that produced the milk. Milk secreted in the early period after parturition was colostrum. (Kebchaoui, 2012).

Milk is a complex biological fluid produced by the mammary gland which has three main functions: (i) nutritional; (ii) immunological; and (iii) physiological. Recently, the physiological role of milk has been emphasized in research, and consumer demand for high-quality products has increased. Consequently, the transformation industry has started to market 'high-quality milk' with particular nutritional/dietary characteristics. In industrialized countries, milk proteins represent 30% of total dietary protein intake and 75% of Ca intake (Restani *et al*., 1999). Milk has two main protein fractions: caseins, which are predominant, and whey proteins (Law and Brown, 1994). The qualitative and quantitative content of each protein fraction depends on many factors: (i) physiological (lactation stage, lactation order); (ii) environmental (climate, hygiene); (iii) genetic (breed, genealogy); and (iv) nutritional (Polidori *et al*., 1991).

Milk composition of mammalian species varies widely with reference to genetic, physiological, nutritional factors and environmental conditions. The use of milk proteins to give food desirable organoleptic or textural properties is strongly influenced by their functional properties. Functionality is defined as "any property of a food, or a food ingredient, except its nutritional ones, that affect its utilization. Some scientists propose a more accurate definition by

classifying functional properties of proteins into three major groups: i. Properties depending on the behavior of proteins in water. ii. Properties depending on interactions between macromolecules. iii. Properties depending on interactions with amphiphilic molecules or with a gas phase. Whey proteins make up approximately 20% of the protein in milk, by weight. Lactoglobulin is the most common whey protein by a large margin (McGee H. 2004)..

Casein is a globular protein, present in milk as a colloidal suspension, having the aspect of micelles. It is characterized by a hydrophobic core and a charged polar hydrophilic part. The anionic regions of the polar part are responsible for sensitivity to Ca2+ and for some physicochemical properties of this protein. Moreover, caseins are relatively hydrophobic, and under ionic conditions, such as those present in milk, they tend to associate tightly into their typical colloidal form (micelles). (Jaubert *et al.*, 1999**)**

Antibacterial activity was distinguished in old times in 1930, when Jones and Simms (1930) reported that milk had anti-streptococcal activity in vitro. In addition, it had been presented for a long time that breast-feeding of infants gave defence from many enteric and respiratory diseases. (Yolken *et al*., 1992). Colostrum immunoglobulins were responsible for the most of the milk antibacterial activity.(Pakkanen and Aalto,1997).

The importance of goats as providers around the world of essential food in meat and dairy products has been discussed and documented in many recent proceedings of national and international conferences (Gruner and Chabert, 2000). This importance is also reflected in the largest animal number increase for goats during the last 20 years (FAO, 2001) (Table 1) and the largest increase in goat milk production tonnage compared to other mammalian farm animals. Milk production of goats is likely to be much greater than in these official statistics, because of the large amounts of unreported home consumption, especially in developing countries.

Table 1
World numbers of mammalian farm animals (millions) since 1980 and annual milk production (1000 MT) (FAO, 2001)

	1980	1999	Change (%)
Animal numbers			
Goats	458	710	+55
Buffaloes	122	159	+30
Pigs	796	913	+15
Cattle	1216	1338	+10
Sheep	1096	1069	−3
Milk production			
Goats	7720	12161	+58
Buffaloes	44296	60334	+36
Cattle	423034	480659	+14
Sheep	7887	8026	+2

The nutritional and health benefits of goat milk are related to a number of medical problems of people, foremost being food allergies with cow milk proteins the dominant food cause (Walker, 1964). Goat milk proteins are similar to the major cow milk proteins in their general classifications of α,β, κ caseins, βlactoglobulin, α lactalbumin, but they differ in genetic polymorphisms and their frequencies in goat populations (Grosclaude, 1995). The presence of the α-s-1-casein trait has been studied much in recent years, when it was discovered that it has six different types, A, B, C, E, F and “null” in goat milk.

Chapter Two

Literature Review

2: Literature Review:

2-1: Milk:-

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. Udder had the glands that produced the milk. Milk secreted in the early period after parturition was colostrum. (Kebchaoui, 2012).

Milk contained lactose, protein, lipid minerals in percentages 5% 3.1%, 4% and 0.7%, respectively. 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, produced for neonate feeding and development. It composed of a various types of materials; 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 dairy products was a part of the human food for thousands of years, as cheese being made more than 7,000 years ago. (Lucey, 2009).

2-2: **Milk Protein:**

Milk proteins were considered the most essential source of bioactive peptides and an increasing number of bioactive peptides had been identified in milk protein hydrolysates and fermented dairy products, as reviewed in many articles (Silva and Malcata, 2005). 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).

The major role of milk proteins was to supply amino acids and nitrogen to the young mammals and constitute an important part of dietary proteins for the adult. Intact milk proteins had also specific functions such as micelle formation. Furthermore, milk proteins had physiological importance, they facilitate uptake of several important nutrients such as trace elements and vitamins and contain a group of proteins which perform a protective function. This means that milk proteins are highly functional substances. During the last two decades it has become clear that milk proteins are a source of biologically active peptides. Milk

proteins are a rich source of biologically active peptides such as antihypertensive, antithrombotic, opioid, immune-stimulating, antimicrobial, mineral carrying and cholesterol lowering-peptides(Shah, 2000).

Bioactive fragments liberation occurred by proteolytic enzymes and can form an ingredient of food products or can be isolated by highly industrial separation methods (Korhonen and Pihlanto, 2007). Bioactive peptides can also be produced by chemical (in an almost non-aqueous phase) and enzymatic

synthesis, and with the use of recombinant DNA methods. (Minkiewicz and Dziuba 2009).

2-2-1: Caseins

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).

In all mammals, casein proteins were a family of phosphoproteins. Their presence in milk was as complex micelles of the proteins and mineral calcium phosphate. About 80% of total milk proteins are casein proteins in bovine, ovine, caprine, and buffalo milk. αs1- and αs2-caseins (CN), ß-CN and κ-CN are the principal casein fractions. (Fox *et al.*, 2001).

Moreover, secondary proteins were found in bovine caseins due to limited plasmin proteolysis. The plasmin activity on αs1-CN and β-CN lead to produce λ-caseins and γ-caseins and proteose peptones, respectively (Fox and McSweeney, 1997). The casein isoelectric point was 4.6. In milk, casein had a negative charge at pH 4.6. The purified protein was not water soluble. Though it is insoluble in neutral salt solutions as well, with dilute alkalis and salt solutions such as sodium oxalate and sodium acetate, it is readily dispersible (from, http://en.wikipedia.org/wiki/Casein, 2010).

Casein was kouwn as a phosphor-protein which was sediment at the temperature of 20°C from raw skimmed milk acidified to the pH of 4.6. Based on the results of electrophoretic separation and evaluations of amino acid sequence homology in different casein fractions, four major families of casein had been identified: αs1, αs2, β and κ.

2-2-2-: Whey protein :

Casein and whey proteins formed the protein system of milk. Whey protein molecules had a globular composition with a closely high content of helicalcomposition and an equal distribution of basic amino acid residues and acidic and hydrophobic and hydrophilic amino acid residues along the polypeptide chain (Madureira *et al*., 2007).

Whey proteins are also not phosphorylated and inactive to Ca+2 (Fox, 2001). They had the most interested biological role, such as transporting of calcium, zinc, copper, iron and phosphate ions in the body. They also play a biological activity as an interested source of a number of different bioactive peptides (Korhonen, 2009). Lactoferricin, one of the multifunctional peptide shows antimicrobial, antifungal, antitumor, and antiviral properties due to tryptophan/arginine rich portion of the peptide, and an anti-inflammatory and immunomodulating characters due to its positive charged region of the molecule (Fox, 2001). The application of lactoferricin derivatives in oral caring and as food preservative had also been suggested by Expósito and Recio in their review. (Lòpez-Expòsito *et al*., (2006).

2-2: Benefit of fermented milk proteins and products:-

For many years, Milk fermentation by LAB had been known to store milk for prolonged storage. As well as the storage role from spoilage, fermented milk had been distinguished to have other functionalities for human health.

During fermentation process, degradation of milk proteins was a good means to change for the better their nutritional value for both humans and animals (Kilpi *et al.,* 2007). A Great attention had been paid to milk protein hydrolysis as strong ingredients to health-promoting functional foods targeting diet-related chronic diseases, such as diabetes mellitus type 2, cardiovascular disease and obesity (Tudor *et al*., 2009).

Milk proteins are a rich source of biologically active peptides such as antihypertensive, antithrombotic, opioid immune-stimulating, antimicrobial, mineral carrying and cholesterol lowering-peptides. (Shah, 2000). These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. According to Kamau *et al*.,(2010), many bioactive peptide fragments can be obtained through hydrolysis of whole milk or precursor protein by digestive enzymes. This powerfully hypothesizes the existence of such peptides in the GIT after consumption of milk. The quantity and composition of milk, presence of additional food, instance, pH and enzymatic action utter the type and destiny of peptides released in digestive. system. These factors are influenced by age, genetic makeup, nutritional patterns and health status of the consumer. A healthy diet is thus a prerequisite for attaining full benefits of these peptides.

2-3:Antimicrobial peptides:

Antibacterial activity was distinguished in old times in 1930, when Jones and Simms (1930) reported that milk had anti-streptococcal activity in vitro. In addition, it had been presented for a long time that breast-feeding of infants gave defence from many enteric and respiratory diseases. (Yolken *et al*., 1992). Colostrum immunoglobulins were responsible for the most of the milk antibacterial activity.(Pakkanen and Aalto,1997).

Carrasco- Castilla *et al.,* (2012) explained different methods for estimation of the five kinds of peptides activity liberated by protein hydrolysis. Antimicrobial peptides and foods containing like peptides might be used as antibacterial, antiviral and antifungal agents. Their activity was determined by identifying little concentrations that inhibit the growth of microorganisms. (Najafian and Babji, 2012).

The antimicrobial activity of milk is mainly associated with minor whey proteins, namely lactoferrin. This protein has bacteriostatic and bactericidial properties attributed to its ability to chelate iron or to bind to bacterial surfaces. Tomita *et al*., (1994) found out that pepsin digestion of bovine lactoferrin produces potent bactericidial peptide, and that the antimicrobial potency of hydrolysate was higher than that of undigested lactoferrin. Dionysius and Milne, (1997) have identified two peptides from the N-terminal of lactoferrin which displayed antimicrobial activity toward a number of pathogenic and food spoilage microorganisms. According to the research of Lahov and Regelson. (Lahov and Regelson, (1996), the bactericidial mechanism is independent of iron because the identified peptides are distinct from the iron-binding site of the molecule. It is possible that the active peptides have an affinity for the bactericidal cell surface and act by disrupting the essential membrane functions. No effect has been detected against bifidobacterium; therefore lactoferrin derived peptides may positively affect the intestinal flora. αs-casein f1-f23

obtained from chymosin hydrolysis has been shown to have antibacterial activity against *Satphylococcus aureus* and *Candida albicans.* (Lahov and Regelson, 1996).

2-4: Mechanisms of production of antibacterial peptides:

Milk-derived antibacterial peptides were formed from 2-20 amino acids and became active only when they were liberated from the inactive protein where they were coded.

Different mechanisms could liberated the coded antibacterial peptides from the

inactive proteins. (Korhonen and Pihlanto, 2003).

1. *In vivo,* during gastrointestinal digestion by the action of digestive enzymes or of the microbial enzymes of the intestinal *flora*;
2. During milk processing (e. g. milk fermentation, cheese production) by the action of microbial enzymes appeared by the microorganisms used as starter;
3. During milk processing by the action of a single purified enzyme or a mixed of selected enzymes.

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
Ethanol 95%	BDH
Ethanol(absolute 99%)	BDH
Glutathione	BDH
Hydrochloric acid	BDH
Hydrogen Peroxide (H_2O_2)	BDH
Methanol	BDH
O-phthaldiadehyde	BDH
Sodium chloride (Nacl)	Merk/ Germany
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
Muller-Hinton agar	Oxoid/ England
Nutreint broth	Himedia/ India
Peptone water medium	Himedia

3-2: Methods:-

3-2-1: Stains 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: Solutions:

3-2-1-2-1: 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).

3-2-1-2-2: 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

3-5: Goat milk Samples Collection:-

Goat milk samples obtained from Iraqi goat .These samples were immediately kept at 4 C during transporting to the laboratory

3-6: Preparation of fermented milk :

milk sample was inoculated with the starter culture at mixed cultures. These fermented samples were incubated at 40^{o} 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 Concentration :-

Peptide content in casein and whey were 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: In vitro, determination of antibacterial activity of casein and whey:-

In this study, well diffusion assay was used. Bacterial cultures of *E. coli* 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
Result and Discussion

4: Results

4- 3: Antibacterial activity of the casein in vitro :

The result showed the antimicrobial activity of casein and whey 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 activity of whey appeared in zones ranged from (9.2-12.7) mm while inhibition activity of casein appeared in zones ranged from 8.7- 10.2) . Table (4-2).figure (4-3).

Table (4-2): Inhibition activity of whey and casein in vitro.

Peptides derived from	Inhibition zone (mm)			mean
	Freq1	Freq 2	Freq 3	
Whey	9.2	10.9	12.7	10.9
Casein	8.7	8.9	10.2	9.2

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

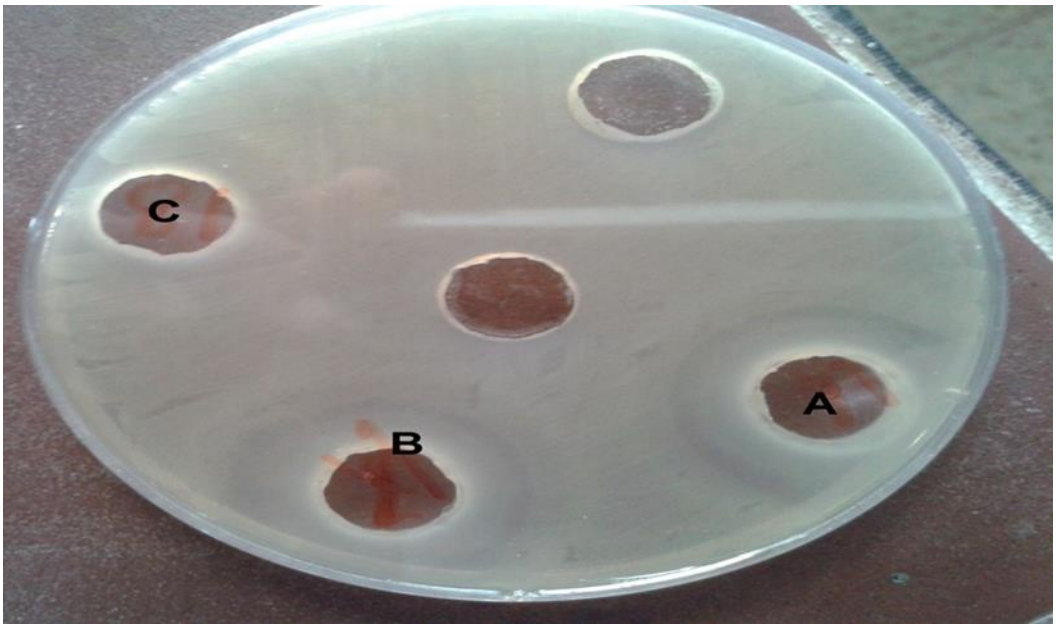

Figure (4-3): Inhibitory activity of whey against *E. coli* in three repetitions.

4- : Relationship of antibacterial activity and peptide concentration

The zone of inhibition had relationship with peptide concentration of each whey and casein . whey showed high concentration of antibacterial peptide with high inhibitory zone. Table (4-4).

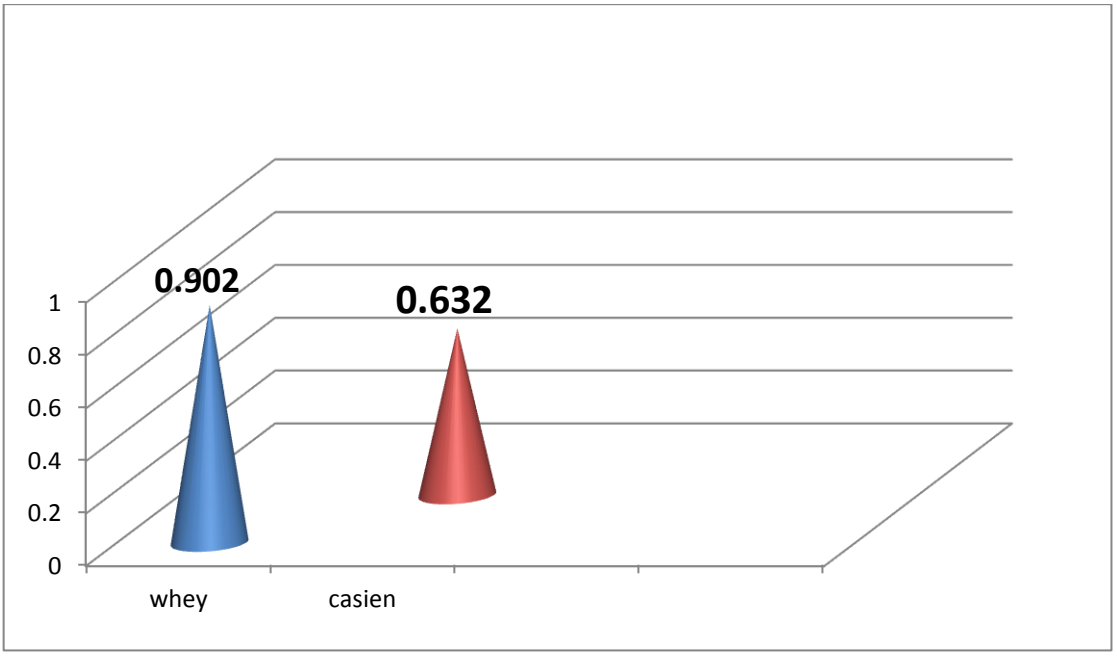

Table (4-4): Relationship of antibacterial activity and peptide concentration.

Fraction No.	Peptide concentration (mg/ml)	Inhibition zone (mm)
Whey	**0.902**	**10.9**
casein	**0.632**	**9.2**

Conclusions
and
Recommendations

1: Conclusions:-

1. Using 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 whey and casein 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 these peptides towards non-bacterial pathogens (viruses, parasites and mycoplasma).
2. Study the amino acid sequence of the bioactive peptides that have antibacterial activity.

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الخلاصة .....

تهدف هذه الدراسة إلى تحضير حليب المتخمرة الغني بالببتيدات منخفضة الوزن الجزيئي باستخدام بكتيريا حامض اللبنيك كخليط زرعي. لهذا الهدف، تم تلقيح عينة الحليب المبسترة بـ ٥٪ بالبكتيريا قبل الحضن عند ٤٢ مئوية ولفترات. تم تحديد تركيز الببتيد والنشاط المضاد للبكتيريا للكازين والشرش في المختبر، تم تحديد تركيز الببتيدات في كل جزء و الفعالية المضادة للبكتيريا في المختبر.

كان تركيز الببتيدات في كل من الشرش و الكازين. (٠.٩٠٢ و ٠.٦٣٢) ملغ / مل على التوالي. وأظهر الشرش اعلى تركيز.

تم تقييم النشاط المضاد للبكتيريا للكازين و الشرش. أظهر الشرش فعالية عالية تجاه الجراثيم. أعطت الببتيدات المضادة للبكتيريا من الحليب المتخمرة نتيجة جيدة في علاج الحالات المرضية التي تسببها الاشريشيا القولونية المرضية.

وزارة التعليم العالي
جامعة القادسية
كلية الطب البيطري

فعالية الكازين المستخلص من حليب الماعز المتخمر ضد بكتريا القولون *E. coli*

بحث مقدم إلى مجلس كلية الطب البيطري كجزء من متطلبات الحصول على درجة البكالوريوس في الطب والجراحة البيطرية

تقدم به الطالب

رقية باسم

إشراف

المدرس الدكتور

مشتاق طالب حسين المحنة

جمادى الثاني ١٣٤٦ اذار ٢٠١٧