Research Article

AL- ANBAR JOURNAL OF VETERINARY SCIENCES

Vol. 17 Issue:2, (2024) ISSN: P-1999:6527 E-2707:0603

Antibacterial Activity of Chitosan, Trisodium Citrate and Acetic Acid Against *Staphylococcus Aureus* Bacteria Isolated From Raw Milk In Baghdad City

Zina Saab Khudhir

College of Vet. Medicine/ University of Baghdad, Baghdad, Iraq.

*Corresponding Author: zena.s@covm.uobaghdad.edu.iq 0000-0002-5606-8923

Doi: https://doi.org/10.37940/AJVS.2024.17.2.11

Received: 8/2/2024 Accepted:18/10/2024

This article is licensed under a CC BY (Creative Commons Attribution 4.0) http://creativecommons.org/licenses/by/4.0/.

Abstract

Staphylococcus aureus bacteria is cause of dangerous food poisoning through contamination of raw milk; therefore, this study aimed to determine the prevalence of *S. aureus* in raw milk sold in local markets of Baghdad city and the antibacterial activity of chitosan, trisodium citrate and acetic acid against the pathogenic bacteria, the antibacterial susceptibility profile of isolates was performed by using (13) antibacterial agents using Kirby–Bauer disc diffusion technique. the results showed that (3) samples out of 25 samples (12%) were isolated from raw milk, all isolates were positive to the experimental tests characterized as S. *aureus* depending on the cultural, biochemical, serological and molecular examinations. The PCR technique used to verify the positive isolates by targeting the (*nuc*) gene, the molecular size (181 bp), the antibacterial activity was done by broth and agar dilution method with detection the bacterial counts. the Initial counting (mean) log CFU/ ml of isolates was 4.623 CFU/ml. The minimum inhibitory concentration of chitosan and acetic acid against *Staphylococcus aureus* by macro-broth dilution method was 20and 1,25 mg /ml respectively, while trisodium citrate had no inhibitory activity against the exanimated bacteria. The antibiotic susceptibility profile for positive isolates showed that all isolates were resistant to (ampicillin, methicillin, Cefixime and Cefoxitin),in conclusion comparing the effects of the three antibacterial agents against *S. aureus* causes contamination of cow raw milk sold in Baghdad city, 1.25 mg/ml of the acetic acid was found effective with higher antibacterial activity, followed by chitosan 2 mg /ml, while trisodium citrate showed no antibacterial effect.

Keywards: Chitosan; *Staphylococcus aureus*; trisodium citrate, acetic acid, raw milk

النشاط المضاد للبكتيريا للكيتوزان ,سترات ثلاثي الصوديوم وحامض الخليك ضد بكتريا المكورات العنقودية الذهبية المعزولة من الحليب الخام في مدينة بغداد

تسبب بكتيريا المكورات العنقودية الذهبية تسمماً غذائياً خطيراً من خلال تلوث الحليب الخام، هدفت هذه الدراسة إلى تحديد مدى انتشار بكتيريا المكورات العنقودية الذهبية في الحليب الخام المباع في الأسواق المحلية لمدينة بغداد والنشاط المضاد للبكتيريا للكيتوزان وسترات ثلاثي الصوديوم وحمض الخليك, جري فحص الحساسية للعزلات البكتيرية باستخدام (13) عاملاً مضاداً للبكتيريا باستخدام تقنية الانتشار Kirby-Bauer. أظهرت النتائج أن (3) عينات من أصل 25 (12%) عزلت من عينات الحليب الخام كانت إيجابية لجميع الاختبارات التي شخصت على اساسها المكورات العنقودية الذهبية وذلك بالاعتماد على فحوصات الزرع على الاوساط الزرعية الفحوصات الكيموحياتية والمصلية والجزيئية، استخدمت تقنية تفاعل البلمرة المتسلسل للكشف عن العزلات الإيجابية. لوجود جين (nuc) ذو حجم جزيئي (181 زوج أساس)، اجريت الفعالية المضادة للبكتيريا للعوامل المضادة للبكتيريا بطريقة التخفيف الدقيق وحساب أعداد وحدة تكوين المستعمرة البكتيرية. كان العد الأولي (المتوسط) 4.623 وحدة تكوين المستعمرة لكل مل واحد من الحليب . كان أقل تركيز تثبيطي للكيتوزان وحامض الخليك ضد المكورات العنقودية الذهبية بطريقة التخفيف بالوسط البروث 20 و1,25 ملغم/مل للكيتوسان وحامض الخليك على التوالي بينما لم يكن لسترات ثلاثي الصوديوم أي نشاط تثبيطي ضد البكتيريا المفحوصة. فحوصات الحساسية للمضادات الحيوية للعزلات الإيجابية أظهرت أن جميع العزلات كانت مقاومة لـ (الأمبيسيلين، الميثيسيلين، السيفيكسيم والسيفوكسيتين). نستنتج من مقارنة تأثيرات العوامل المضادة للبكتيريا الثلاثة ضد المكورات العنقودية الذهبية المسببة لتلوث حليب البقر الخام المباع في مدينة بغداد، أن 1.25 ملغم/مل من حمض الأسيتيك فعال مع نشاط مضاد للجراثيم أعلى، يليه الكيتوزان 2 ملغم/مل، في حين أن لم تظهر سترات ثلاثي الصوديوم أي تأثير مضاد للجراثيم.

Introduction:

Staphylococcus. aureus is an indicator for heat efficacy and hygienic practices through the food preparation, handling conditions and food production (1). It is zoonotically transmitted through direct contact with animals or their products such as contaminated raw milk and milk products and this pathogen is the most important cause for the bovine mastitis (2). Decontamination solutions used for food application should be free from adverse effects on food workers' or when consumption by consumers, decontamination systems, based on some chemical agents, are approved as "Generally Recognized as Safe" (GRAS) listed by the FDA (3). Multi- antibiotics resistant microorganisms are characterized by their fast growth and multiplication represent a concern to human life, currently reports are become focus on using the natural antimicrobial to hinder different kinds of human pathogens and treated the microorganism-causing diseases (4). Chitosan is a polysaccharide extracted from the insects, crab, shrimp, and some fungi, many factors interfere with the antibacterial activity of chitosan such as purity, physical kinds, molecular weight, and degree of deacetylation (DD), this natural antibacterial has been used for elimination and /or inactivation of undesirable microorganisms with no toxic effect and environmentally friendly without any effect on food composition (5). Trisodium citrate (TSC) is widely used as a food preservative and antimicrobial agent, it has been shown that (4%) of TSC can be inhibited the biofilm formation by different kinds of bacteria as S. aureus, Pseudomonas aeruginosa and pathogenic Escherichia coli (6). Acetic acid, is an organic compound, with antibacterial activity used in food applications, characterized by acidic properties, the activity of acetic acid as antibacterial substance is attributed to its non-ionized form with ability to penetrate the bacterial cell wall and disrupt the normal physiology action for many types of bacteria (7). Organic acids are either present naturally in the foods or synthesized by chemical methods used as direct or indirect application, while other kinds formed during the fermentation process of carbohydrates in foods (8). The main aim of this study was to evaluated the antibacterial activity of chitosan, trisodium citrate and acetic acid against Staphylococcus aureus isolated from local raw milk.

Materials and methods:

Sampling of Raw milk:

Twenty-five raw milk samples were purchased from the local markets in Baghdad city, the samples processed immediately upon arrival, in which (100) mL of well mixed samples were placed in the sterile labeled bottles and transported to the Veterinary Public Health laboratory, College of Vet. Medicine/ University of Baghdad, Baghdad.

Isolation and identification of S.aureus

The first step of this study includes isolated Staphylococcus aureus bacteria from raw milk using cultural, biochemical and serological tests.

identifying the antibacterial effects of chitosan, trisodium citrate and acetic acid against S. aureus. As well as counting the bacteria on the Baird Parker (BP) agar according to (9), morphological and biochemical identification was done according to (10). isolation the bacteria from raw milk samples was done through homogenized one ml of raw milk with 9 ml of (0.1%) sterile peptone water, 10-fold

dilutions were prepared, 1 ml of 10–4, 10–5, up to 10–6 dilutions were cultured on the Baird Parker Agar (Hi media) supplemented with egg yolk emulsion, this test done in duplicate and the plates were incubated at 370C for 24hr

,typical coagulase positives staphylococci colonies were appearing as black or grey, surrounded by a clear zone, recovered selected colonies were streaked on the plates of blood agar TSA with (5% sheep blood), incubated at 37 °C for 18–24 h. staphylococcus aureus colonies which appears with opaque halo were recorded as coagulase positive staphylococci, these colonies were subjecting to further identified as catalase assay, slide coagulase test , staphylococci colonies showed the typical coagulase positive on the BP agar are black, shining and convex surrounded by a clear zone. The Presumed isolates were reconfirmed as Staphylococcus aureus using gram staining, catalase, oxidase, hemolysis pattern, fermentation and growth on the mannitol salt, DNA- ase test, further analyzed by positive agglutination for Pastorex Staph Plus detection of clumping factor and the presence of coagulase using the slide agglutination test kit (Staphaurex® kit, Oxoid).

Detection of S. aureus by using PCR

The Primers used in the current study is manufactured by Bioneer, Korea for species-specific targeting thermostable nuclease (nuc) gene of Staphylococcus aureus isolates, Extracted DNA was amplified to detect the nuc gene with the molecular weight of 181 bp (11 12), The PCR procedure was performed according (13) with some modification in the annealing time at 65ºC for 1 min.

Preparation chitosan, trisodium citrate and acetic acid solutions

Chitosan in powder from (shrimp shells) was obtained from (hi-media) with molecular weight 3800-20000 and (≥75 %) deacetylation degree, 2% w/v of chitosan solution was prepared as 2g of chitosan were dissolved in 100 mL of 1% (v/v) acetic acid at 5.8 pH purity 99% (Italy) the solution was stirred for 12 hrs at 50 ºC to achieved complete dissolution of chitosan powder and filtrated through 0.2 µm membrane syringe filter. acetic acid solution (2%) ,was prepared by well mixing 2ml of acetic acid with 100 mL of sterilized distilled water , trisodium citrate solution (4%) was prepared by dissolved (4g) of TSC were dissolved in the 100 mL sterile distal water sterilized by filtering through 0.2 µm membrane syringe filter and kept at 4 °C, for determination of the MIC of antibacterial solutions broth and agar dilution methods were used ,S aureus with the standardized number are seeded on the surface of Muller-Hinton agar with the lowest concentration of antibacterial agents, the results recorded as colony forming units (CFU) after 24-48 h of incubation compared to the initial bacterial counts., broth microdilution, Agar dilution and disk diffusion procedure are standard technique recommended by Clinical and Laboratories Standards Institute (CLSI ,2009) for determined the antimicrobial activities of chitosan against different kinds of microorganisms (14) MICs of antibacterial agents were regarded as the lowest concentration of agents that completely inhibit S. aureus bacterial growth.

The determination of the Antibiotics susceptibility of S. aureus isolates:

The susceptibility of isolated S. aureus against commercial antibiotic was studied using Kirby and Bauer's achieved by discs diffusion technique, standardized bacterial inoculum equivalent to (0.5 McFarland) was swabbed on surface of muller Hinton plate. The inhibition zone was measured after incubation at 37°C for 18-24 hrs and the results were recorded in millimeters. The antibiotics used in the current study were 13 commercially antibiotics discs (OXOID) include Doxycycline (D030µg), Levofloxacin (Lev 5µg), Ampicillin (Amp 10µg), Gentamicin (Gen 10µg), Amikacin (AK 30µg), Methicillin (Met 5µg), Clindamycin

(CLI 2 µg) , Cefixime (CEF 5µg), Rifampicin (Rif 50 µg), Chloramphenicol (CHL 30 µg), Ciprofloxacin (CIP 5µg) Tetracycline (TCN 30µg) and (Cefoxitin30µg) .

Minimum inhibitory concentration (MIC) of antibacterial agents against S. aureus using broth and agar dilution method

The MIC values were determined by using broth and agar dilution assay. All the chitosan, trisodium citrate and acetic acid stocks were serially diluted as two-fold to concentrations ranged of 20mg/ml to 0.6 mg/ml for both of chitosan and acetic acid while ranged from 40mg/ml to 0.6 mg/ml for trisodium citrate 0.1 ml of each concentration was added to 9ml of sterile nutrient broth containing 0.1ml of standardized S. aureus. Negative control was inoculated bacteria without antibacterial agents. Test tubes were incubated for 24hrs to evaluated the presence or absence of visible turbidity in the broth. The lowest concentration or highest dilution of the antibacterial agent that prevented the appearance of bacterial growth turbidity was represented as the (MIC). Positive control (nutrient broth inoculated with bacteria) and negative control (nutrient broth with the antibacterial solution). The concentration of the antibacterial solution at which no growth of the organism was observed was regarded as the minimum inhibitory concentration (MIC). The results were expressed as growth of organisms (+) or inhibition of growth (-) (15).

Statistical analysis

Data analysis of was performed using SAS (Statistical Analysis System - version 9.1). Two-way ANOVA with interaction and least significant differences (LSD) post hoc test were performed to assess significant differences among means. $P < 0.05$ is considered statistically significant.

Results and discussion

Staphylococcus. aureus is frequently found in the raw milk the decrease in drug efficacy against infection S. aureus represents a looming threat to public health to increase the morbidity and mortality levels, all suspected isolates that subjecting to the phenotyping test has been shown that all positive isolates were gram-positive, catalase and coagulase-positive (Table1). The results of isolation percentage showed that 3 isolates out of 25 (12%) as shown (Table 2). The genotypic test of the positive isolates revealed that the (3) samples were amplified the nuc gene detected by the conventional PCR.

Staphylococcus produces a thermostable extra¬cellular nuclease which is encoded (nuc gene), represented a virulence factor, the nuc gene is used as a specific target for iden¬tifying S. aureus by the technique of polymerase chain reaction (PCR). The current study illustrates that no inhibitory activity of trisodium citrate at concentration 4% against Staph aureus bacteria and there is no significantly reduction log, while the tested bacteria shown significant reduction in the population counts after subjecting to the antibacterial activity of acetic acid and chitosan as shown in Table 7. The MIC of chitosan that conducted by broth dilution methods against tested bacteria was 1.25 mg/ml., chitosan solution has antibacterial activity against gram-positive bacteria while aqueous solution of acetic acid has high antibacterial activity against S. aureus. Antibacterial activity of chitosan depends on various factors such as deacetylation degree, temperature, and molecular weight, chitosan solution with low molecular weight can enters the microbial cells and disturbs the metabolism of the cell, all the positive samples, that were positive to the nuc gene of (100%) by PCR Figure 2, Acetic acid as decontaminator solution was exhibited strong antibacterial

activity against staph aureus bacteria compared with other solution that used in current study. concentration of acetic acids that needed to complete reduction of this bacteria needed to farther studies, especially when used in combination with other natural substances, S. aureus isolates had the highly resistance to Ampicillin , methicillin , Cefixime and Cefoxitin as shown in (Tables 4 and 5). The antibacterial activity of decontamination agent against S. aureus represented by MIC values by dilution methods .

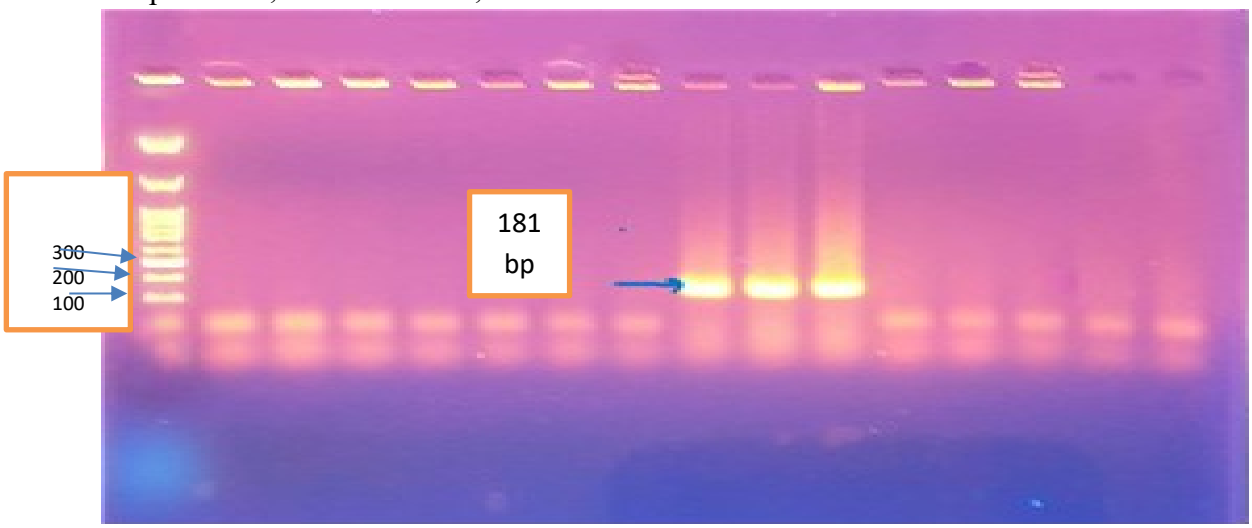

Figure 1 The stained gel by ethidium bromide indicated the typical banding patterns, PCR amplification of the *nuc* gene of *S. aureus* with primers generating 181bp

Table 1 Cultural, Biochemical and stereological tests used for *staph. aureus*

	Cultural		Biochemical tests						Serological tests
									Staphaurex® Latex agglutination
	Mannitol salt agar	Baird Parker supplemented with egg yolk	Gram Stain	Catalase test	Coagulase test	DNAse agar	hemolysins	Oxidase	Dry spot *staph. aureus*
Staph aureus	Yellow colonies surround yellow zones	Colonies with grey to black color	Gram positive	visible bubbles	coagulase-positive	Halo zone	β-hemolysins	Negative Result	agglutination

Table 2 Isolation and enumeration of *Staph aureus* isolated from raw milk samples using Conventional tests.

Sample	number	positive samples	Prevalence (%)	Initial counting (Mean) log CFU/ ml
Raw milk	25	3	12	4.623

Table 3 primers used for amplifying the *nuc* gene.

Specific gene	Sequence (5´-3´)	Size of product
nuc	F- GTGCTGGCATATGTATGGCAATTGT	181 bp
	R- TACGCCGTTATCTGTTTGTGATGC	

Table 4 **Zone of inhibition of antimicrobial dices against *Staphylococcus aureus* isolates**

(Diameter of inhibition zone) mm means													
Isolates	D0 30µg	Lev 5µg	**Amp 10µg**	Gen 10µg	AK 30µg	**Met 5µg**	CLI 2 µg	**CEF 5µg**	Rif 50 µg	CHL 30 µg	Cip 5 µg	TCN 30µg	**Cefoxitin 30µg**
No1	34	30	**10**	35	40	**10**	22	**14**	18	22	17	33	**12**
No2	31	33	**9**	35	43	**12**	22	**18**	25	23	17	34	**14**
No3	33	32	**9**	33	42	**12**	24	**14**	20	25	19	35	**13**

Table 5. Resistance percentage% against *Staphylococcus aureus* isolates isolated from raw milk (N0 of positive isolates =3).

Antibacterial discs	Code and Potency(µg) of antibacterial discs	Resistance percent %	Sensitive percent %
1-Doxycycline	D0 30µg	0 (100)	3 (100)
2-Levofloxacin	Lev 5µg	0 (100)	3 (100)
3-Ampicillin	**Amp 10µg**	**3 (100)**	0 (100)
4-Gentamicin	Gen 10µg	0 (100)	3 (100)
5-Amikacin	AK 30µg	0 (100)	3 (100)
6-Methicillin	**Met 5µg**	**3 (100)**	0 (100)
7-Clindamycin	CLI 2 µg	0 (100)	3 (100)
8-Cefixime	**CEF 5µg**	**3 (100)**	**0 (100)**
9-Rifampicin	Rif 50 µg	0 (100)	3 (100)
10-Chloramphenicol	CHL 30 µg	0 (100)	3 (100)
11-Ciprofloxacin	CIP 5 µg	0 (100)	3 (100)
12-Tetracycline	TCN 30µg	0 (100)	3 (100)
13- Cefoxitin	**CEF 30µg**	**3 (100)**	**0 (100)**

Table 6 Minimum inhibitory concentration (MIC) against *Staph aureus* bacteria by broth dilution technique

Antibacterial agents	Minimum inhibitory concentrations (mg/ml)					
Chitosan	20	10	5	2.5	1.25	0.6
	-	+	+	+	+	+
Trisodium citrate	40	20	10	5	2.5	1.25
	+	+	+	+	+	+
Acetic acid	20	10	5	2.5	1.25	0.6
	-	-	-	-	-	+

+ =Growth - = No growth

Table 7 Minimum inhibitory concentration (MIC) against *Staph aureus* bacteria by agar dilution technique

Antibacterial agent Concentrations	Initial counting (Mean) log CFU/ ml (control)	Counting (Mean) log CFU/ ml after adding the antibacterial agent
Chitosan	A 4.62±0.00a	B 2.37±0.06 b
Trisodium citrate	A 4.62±0.00a	A 4.40±0.05 a
Acetic acid	A 4.62±0.00a	B 1.27±0.01 c
LSD	0.11	

Means with a different small letter in the same column are significantly different (PL<0.05)

Means with a different capital letter in the same row are significantly different (PL<0.05)

The isolation percentage of isolated S. aureus were (3) samples out of 25(12%), contamina¬tion raw milk and dairy products by S. aureus occurs through the unhygienic handling of the raw milk and dairy products, occurs through different stages of animals handling, milking, contact with diseased farmers (2). Other studies reported that prevalence of S. aureus that isolated from raw milk was with high present (16; 17). The contamination rate of raw milk and their dairy products with S. aureus could be depended of bad personal hygiene of workers, poor processing methods, lack of sterilization that should be used for dairy equipment. Adnan and zina (18) reported high prevalence levels of Staph aureus isolates from raw local Iraqi dairy products, the isolation prevalence and isolation percentage of cow's soft cheese samples were 8/10(80%) followed by both ewe's and buffalo's soft cheese samples 7/10 (70%) respectively. While Abed Rabba and Khudhir, (19) reported that the isolation percentage of quality control bacteria as total, fecal coliform, Escherichia coli and Staphylococcus aureus from raw milk were 82, 69, 54 and 42%, respectively, while the isolation percentage from raw soft cheese were 90, 74, 60 and 45%, respectively.

Acetic and lactic acid are organic acid widely used in the preservation of food products due to their antibacterial properties, classified as safe substances (GRAS) as recorded by the organization (20) .in the current study acetic acid showed the higher antibacterial activity against Staph aureus this results agreement with result of (21) , pointed that lactic with concentration (1% and 3%) and acetic acid at (1% and 2%), against Salmonella enteritidis in the poultry meat , the reducing activity showed variation depending on the type and concentrations of organic acid , kind of food and combination the bacterial activity of organic acid with other treatment .Organic acids and their salts widely researched for the antimicrobials properties as bio-preservatives in food such as Lactic, acetic, and citric acids , with the inhibiting activity against both of spoilage and pathogenic microorganisms, large antimicrobial action against Gram-positive, Gram-negative bacteria , fungi and yeasts, increasing the sensory ,functional properties , stabilization of food color , flavor and regulation the acidity, the action of weak organic acids can be attributed to the undissociated molecules that are lipophilic and therefore are able to easily cross the lipidic membrane of microorganisms and penetrated into their cytoplasm, leading to destruction the bacterial cell , the application of weak organic acid in the food bio-preservation can exhibit to the stronger antimicrobial activities than the highly dissociating inorganic acids at the identical pH level contribute to their amount of undissociated molecules and the capacity of penetration (22)

Chitosan, shows antimicrobial activity against both Gram-positive and negative bacteria depending on the structure bacterial cell membrane specially the lipopolysaccharides and proteins (23),

Ying-Chien., (24) described that chitosan exhibited the stronger bactericidal activity against Gram-negative bacteria, while other author reported that a more bactericidal action against Gram-positive bacteria (25). Chitosan usually influencing by concentration, molecular weight and deacetylation degree, the higher degree of deacetylation of chitosan, leads to antibacterial activity is increased (26). Despond et al., (27) and Hernandez-Lauzardo et al., (28) were recorded that chitosan with low molecular weight has a higher antimicrobial activity. Number of amines groups (NH2) involved in the chitosan substances, greatly effect on the ability of chitosan as an antimicrobial agent, the antimicrobial mechanism of chitosan due to interferes with bacterial cell wall morphogenesis directly leads to hinder the microbial growth. (29). Chitosan contains amine group with positively charged these amines capable to binding to the negative charge on the microbial cell surface and alert the bacterial cell wall permeability and intracellular leakage of the important substances as electrolytes, proteins many kinds of amino acids, glucose and enzymes finally inhibited the bacterial growth (30). One study conducted that the antibacterial activity against Staphylococcus aureus, E. coli, Bacillus cereus and Klebsiella pneumoniae achieved as the lower of the molecular weight (LMW) of chitosan, higher the antibacterial effect (31). For the bactericidal effect of chitosan is required 10 kDa as the minimum molecular weight (32). Khudhir , (33) recorded that the Chitosan (CH) exhibited the antimicrobial activity against the Coliforms, E. coli O157: H7, yeasts and molds at concentration of (5%) when dipping the cheese the contact time (6hrs) at refrigeration temperature (4ºC),the result indicated that the concentration of chitosan 5% was the optimum concentrations as the disinfected washing solution to reduce the contamination the local Iraqi cheese.

Acetic acid used in the current study shown higher antibacterial properties, while trisodium citrate had no antibacterial activity against Staph aureus bacteria isolated from raw milk, this result disagreement with another study who indicated that biofilm-forming ability of bacteria became weaker after using trisodium citate (34). Weijmer et al. (35) who studied the efficacy of four concentrations of trisodium citrate (2.2, 7.5, 15 and 30%), the results indicated that trisodium citrate at high concentration (30%) could be killed many kinds of micrograms as Pseudomonas. aeruginosa E. coli S. aureus and Candida albicans. The results of another study illustrated that trisodium citrate inhibited the biofilm formation of K. pneumoniae and the inhibitory effect as low 4% (34). Khayat et al., (36) showed significant ability of sodium citrate in vitro at concentrations of 4% and 5% to mitigate the bacterial virulence, inhibiting the formation of biofilm, motility activity of the protease enzyme and reducing the pyocyanin pigment that production by P. aeruginosa. This result disagreement with results of this current study.

One of the most serious threats from point of the global health and food safety is the resistance bacteria to antibiotics (37). Penicillin is the first choice to treat S. aureus in man and mastitic animals' treatment, evolution of S. aureus and the abuse used of antimicrobial can leads to increase the antibiotic resistance (38; 39)., in current study ampicillin, methicillin, Cefixime and Cefoxitin had the resistance rate, Shiluli et al. (40) reported that percentage of (67%) of isolated S. aureus were showed resistant to ampicillin, amoxicillin, oxacillin, ceftazidime and vancomycin. The ability of S. aureus isolate for the production of β-lactamase enzyme make it resist to the β-lactam agents (41) .The explanation of the S. aureus resistance mechanism is complex, especially in the Methicillin-resistant Staphylococcus aureus (MRSA), Staphylococcus aureus is resistant to different types of commercial antibiotics developed today (42).Evaluation of the antibacterial activity of antibacterial solutions were performed by using the minimum inhibitory concentrations (MIC) through the broth macro-dilution and agar technique according to guidelines of Clinical and Laboratory Standards Institute ,2022 (43), the antibacterial activity of chitosan solutions can be affected by different factors such as bacterial species different in the molecular weight and degree of deacetylation, , pH of the solution and the presence of metal ions also have an effect on the bacterial inhibitory effect of chitosan. (30). lower molecular weight can effected the antibacterial activity of chitosan this attributed to the higher distribution of amino groups at the surface of chitosan (44).These results of current study agreement with (45) who reported that antibacterial solutions that used for decontamination of different bacteria can helpful to increase the antibacterial activity of brine solution as a method to reduce the potential pathogenic bacteria as E. coli O157:H7 that contamination the local Iraqi cheese. The sensitivity test of the 13 commercial antibiotics showed that the S. aureus isolates had the highly resistance to ampicillin, methicillin, Cefixime and Cefoxitin. Antibacterial activity of acetic was studied when MRSA is resistant bacteria but is still inhibited by the acetic acid when diluted to concentration of 0.625%, acetic acid has good antibacterial activity and is inexpensive non-toxic nonirritant and naturally broken down in both body and the environment (46). Strong odor of acetic also study this issue can overcome by used this antibacterial agent at less than 4% or lower by different mode as spraying or dipping of different parts of animal's cuts after slaughtering (47).

Conclusion:

in vitro Comparing the effects of the three antibacterial agents against S. aureus bacteria that implication of contamination of raw milk sold in Baghdad city, 1.25 mg/ml of the acetic acid was found effective with higher antibacterial activity, followed by chitosan 2 mg /ml, while trisodium citrate showed no antibacterial effect against this pathogenic bacterium.

Acknowledgments:

Author is thankful to the Department of Veterinary public health University of Baghdad / Iraq for helping due proved the bacteriology laboratory. The author did not receive any funds for the current study.

Conflict of interest: The authors declare that there is no conflict of interest.

References:

1- da Silva Malheiros P, dos Passos CT, Casarin LS, Serraglio L, Tondo EC. Evaluation of growth and transfer of Staphylococcus aureus from poultry meat to surfaces of stainless steel and polyethylene and their disinfection. Food Control. 2010 Mar 1;21(3): 298-301.DOI: 10.1016/j.foodcont.2009.06.008

2- Juwita S, Indrawati A, Damajanti R, Safika S, Mayasari NL. Genetic relationship of Staphylococcus aureus isolated from humans, animals, environment, and Dangke products in dairy farms of South Sulawesi Province, Indonesia. Veterinary World. 2022 Mar;15(3): 558.Doi: www.doi.org/10.14202/vetworld.2022.558-564.

3- Sallam KI, Abd-Elghany SM, Hussein MA, Imre K, Morar A, Morshdy AE, Sayed-Ahmed MZ. Microbial decontamination of beef carcass surfaces by lactic acid, acetic acid, and trisodium phosphate sprays. BioMed Research International. 2020 Nov 3;2020.. Doi :https://doi.org/10.1155/2020/2324358.

4- Hilmi B, Bustami Y, Trongsatitkul T, Hamid ZA. The effect of natural antimicrobial agents on Staphylococcus aureus and Escherichia coli growth. Journal of Physical Science. 2019; 30:55-63. Doi :https://doi.org/10.21315/jps2019.30.s2.

5- Fan W, Sun J, Chen Y, Qiu J, Zhang Y, Chi Y. Effects of chitosan coating on quality and shelf life of silver carp during frozen storage. Food chemistry. 2009 Jul 1;115(1):66-70. DOI: 10.1016/j.foodchem.2008.11.060

6- Takla TA, Zelenitsky SA, Vercaigne LM. Effectiveness of a 30% ethanol/4% trisodium citrate locking solution in preventing biofilm formation by organisms causing haemodialysis catheter-related infections. Journal of antimicrobial chemotherapy. 2008 Nov 1;62(5):1024-6. DOI: 10.1093/jac/dkn291

7- Ji QY, Wang W, Yan H, Qu H, Liu Y, Qian Y, Gu R. The Effect of Different Organic Acids and Their Combination on the Cell Barrier and Biofilm of Escherichia coli. Foods. 2023 Aug 10;12(16):3011.10.3390/foods12163011 DOI:

8- Stratford M, Eklund T. Organic acids and esters. In Food preservatives 2003 Jul 31 (pp. 48-84). Boston, MA: Springer US. DOI: 10.12691/ajmr-4-5-2

9-FDA "Food and Drug Administration".

Evaluation and definition of potentially hazardous foods. Analysis of microbial hazards related to time/ temperature control of food for safety. Department of Health and Human Services. Food and Drug Administration 2001 Chapter 4:1-19. DOI:03317_8c9081bdb06f14c9b86ff1a8db22b9b2.

10-Markey, B.K, Leonard, F.C,Archambault, M,Cullinane, A. and Maguire, D. Clinical Veterinary Microbiology. 2nd Ed., MOSBY. Elsevier Ltd., Edinburgh, London, New York, Oxford, Philadelphia, St. Louis, Sydney, Toronto 2013.

11- Hegde R. Rapid identification of bacterial pathogens causing subclinical bovine mastitis with special reference to Staphylococcus aureus Escherichia coli and predominant Streptococcal species by molecular methods. PhD Dissertation, Karnataka Veterinary, Animal and Fisheries Sciences University, India. 2011.

12- Preethirani PL, Isloor S, Sundareshan S, Nuthanalakshmi V, Deepthikiran K, Sinha AY, Rathnamma D, Nithin Prabhu K, Sharada R, Mukkur TK, Hegde NR Isolation, biochemical and molecular identification, and in-vitro antimicrobial resistance patterns of bacteria isolated from bubaline subclinical mastitis in South India. PLoS One. 2015 Nov 20;10(11):e0142717

Doi: 10.1371/journal.pone.0142717.

13- Sheet OH, Jwher DM, Al-Sanjary RA, Alajami AD. Direct detection of Staphylococcus aureus in camel milk in the Nineveh Governorate by using the PCR technique. Iraqi Journal of Veterinary Sciences, 2021, 35.4: 669-672.

DOI :10.33899/IJVS.2020.127725.1524

14- Clinical and Laboratory Standards Institute. "Performance standards for antimicrobial disk susceptibility tests; approved standard-tenth edition M02-A10." National Committee for Clinical Laboratory Standards, 2009.29.https://doi.org/10.1592/phco.29.11.1326

15- Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens. 2021 Feb 4;10(2):165. Doi: 10.3390/pathogens10020165

16- Wang W, Lin X, Jiang T, Peng Z, Xu J, Yi L, Li F, Fanning S, Baloch Z.Prevalence and characterization of Staphylococcus aureus cultured from raw milk taken from dairy cows with mastitis in Beijing, China. Frontiers in microbiology. 2018 Jun 22;9:1123. DOI: 10.3389/fmicb.2018.01123

17 -Kou X, Cai H, Huang S, Ni Y, Luo B, Qian H, Ji H, Wang X. Prevalence and characteristics of Staphylococcus aureus isolated from retail raw milk in Northern Xinjiang, China. Frontiers in Microbiology. 2021 Aug 9; 12:705947..https://doi.org/10.3389/fmicb.2021.705947

18- Ahmed AJ. Evaluation of the bactericidal effect of Nisin and/or Potassium sorbate and Sodium chloride on the viability of Staphylococcus aureus in soft cheese: Zina

Saab Khudhir and Adnan Jawad Ahmed. The Iraqi Journal of Veterinary Medicine. 2017 Jun 5;41(1):55-9.https://doi.org/10.30539/iraqijvm.v41i1.79 .

19- Al-Shuwaili AR, Khudhir SZ. Evaluation of the bacterial load in the raw dairy products in Baghdad, Iraq.. Institute Razi. Archives, 2022, 77. 6 ..Doi: 10.22092/ARI.2022.358618.2268. eCollection 2022 Dec.

20- Edris A, Amin R, Eleiwa N, Elnagar R. Lactic and acetic acids as bacterial decontaminators on chicken carcasses. Benha Veterinary Medical Journal. 2020 Dec 1;39(2):158-60. DOI :10.21608/BVMJ.2020.50321.1297

21- Coşansu Akdemir S. Effects of Lactic and Acetic Acid on Survival of Salmonella enteritidis During Refrigerated and Frozen Storage of Chicken Meats.. 2012, Food Bioprocess Technol ,5:372–377. DOI 10.1007/s11947-009-0320-x.

22- Ben Braïek O, Smaoui S. Chemistry, safety, and challenges of the use of organic acids and their derivative salts in meat preservation. Journal of Food Quality. 2021 Feb 16; 2021:1-20. https://doi.org/10.1155/2021/6653190

23- Guarnieri A, Triunfo M, Scieuzo C, Ianniciello D, Tafi E, Hahn T, Zibek S, Salvia R, De Bonis A, Falabella P. Antimicrobial properties of chitosan from different developmental stages of the bioconverter insect Hermetia illucens. Scientific Reports. 2022 May 16;12(1):8084.https://doi.org/10.1038/s41598-022-12150-3.

24- Chung YC, Su YP, Chen CC, Jia G, Wang HL, Wu JG, Lin JG. Relationship between antibacterial activity of chitosan and surface characteristics of cell wall. Acta pharmacologica sinica. 2004 Jul 1;25(7):932-6.. http://www.ChinaPhar.com

25- Fernandez-Saiz P, Lagaron JM, Ocio MJ. Optimization of the film-forming and storage conditions of chitosan as an antimicrobial agent. Journal of Agricultural and Food Chemistry. 2009 Apr 22;57(8):3298- 307.DOI: 10.1021/jf8037709.

26- Goy RC, Britto DD, Assis OB. A review of the antimicrobial activity of chitosan. Polímeros. 2009; 19:241-7.https://doi.org/10.1590/S0104-14282009000300013

27- Despond S, Espuche E, Cartier N, Domard A. Barrier properties of paper–chitosan and paper–chitosan–carnauba wax films. Journal of applied polymer science. 2005 Oct 15;98(2):704-10.DOI:10.1002/APP.21754

28 Hernandez-Munoz P, Almenar E, Del Valle V, Velez D, Gavara R. Effect of chitosan coating combined with postharvest calcium treatment on strawberry (Fragaria× ananassa) quality during refrigerated storage. Food chemistry. 2008 Sep 15;110(2):428-

35.. DOI:10.1016/j.foodchem.2008.02.020

29- Ke CL, Deng FS, Chuang CY, Lin CH. Antimicrobial actions and applications of chitosan. Polymers. 2021 Mar 15;13(6):904 https://doi.org/10.3390/polym13060904

30- Ardean C, Davidescu CM, Nemeş NS, Negrea A, Ciopec M, Duteanu N, Negrea P, Duda-Seiman D, Musta V. Factors influencing the antibacterial activity of chitosan and chitosan modified by functionalization. International Journal of Molecular Sciences. 2021 Jul 12;22(14):7449. https://doi.org/ 10.3390/ijms22147449.

31- Verlee A, Mincke S, Stevens CV. Recent developments in antibacterial and antifungal chitosan and its derivatives. Carbohydrate polymers. 2017 May 15; 164:268-83. Doi: 10.1016/j.carbpol.2017.02.001

32-Raafat D, Sahl HG. Chitosan and its antimicrobial potential–a critical literature survey. Microbial biotechnology. 2009 Mar;2(2):186-201. Doi: 10.1111/j.1751-7915.2008.00080.x.

33- Khudhir ZS. Antimicrobial activity of chitosan and/or Gum Arabic in the local produce soft and hard cheese in Baghdad city. Biochemical & Cellular Archives. 2021 Apr 2;21. https://connectjournals.com/03896.2021.21. 2727.

34- Chu L, Zhou X, Shen Y, Yu Y. Inhibitory effect of trisodium citrate on biofilms formed by Klebsiella pneumoniae. Journal of Global Antimicrobial Resistance. 2020 Sep 1;22:452-6.https://doi.org/10.1016/j.jgar.2020.04.025

35- Weijmer MC, Debets-Ossenkopp YJ, Van De Vondervoort FJ, ter Wee PM.Superior antimicrobial activity of trisodium citrate over heparin for catheter locking. Nephrology Dialysis Transplantation. 2002 Dec 1;17(12):2189-95https://doi.org/10.1093/ndt/17.12.2189

36- Khayat MT, Ibrahim TS, Khayyat AN, Alharbi M, Shaldam MA, Mohammad KA, Khafagy ES, El-Damasy DA, Hegazy WA, Abbas HA. Sodium citrate alleviates virulence in Pseudomonas aeruginosa. Microorganisms. 2022 May 18;10(5):1046.doi: 10.3390/microorganisms10051046

37- alam MA, Al-Amin MY, Salam MT, Pawar JS, Akhter N, Rabaan AA, Alqumber MA. Antimicrobial resistance: a growing serious threat for global public health. In Healthcare 2023 Jul 5 (Vol. 11, No. 13, p. 1946). MDPI.. DOI: 10.3390/healthcare11131946

38- Song JaeWon SJ, Yang SooJin YS, Shin Sook SS, Seo KeunSeok SK, ParkYongHo PY, Park KunTaek PK. Genotypic and phenotypic characterization of methicillin-resistant Staphylococcus aureus isolated from bovine mastitic milk in Korea.. Journal of food protection, 2016, 79.10: 1725-1732. DOI: 10.4315/0362-028X.JFP-16-067

39- Tenhagen BA, Alt K, Pfefferkorn B, Wiehle L, Käsbohrer A, Fetsch A.Methicillin-resistant Staphylococcus aureus in conventional and organic dairy herds in Germany. Journal of dairy science. 2018 Apr 1;101(4):3380-6. Doi:0.3168/jds.2017-12939. Epub 2018 Feb 1.

40 - Shiluli C, Achok C, Nyaswa P, Ogwai S, Aroko A, Obila J, Koigi G, Ridhwana M, Okwayo B, Wanjiru D, Lukeba L. Antimicrobial sensitivity patterns of Staphylococcus species isolated from mobile phones and implications in the health sector. BMC Research Notes. 2021 Dec; 14:1-5.Doi.org/10.1186/s13104-020-05413-7] [PMID: 33407799].

41- Muñoz-Gallego I, Viedma E, Esteban J, Mancheño-Losa M, García-Cañete J, Blanco-García A, Rico A, García-Perea A, Ruiz Garbajosa P, Escudero-Sánchez R, Sánchez Somolinos M. Genotypic and phenotypic characteristics of staphylococcus aureus prosthetic joint infections: insight on the pathogenesis and prognosis of a multicenter prospective cohort. InOpen Forum Infectious Diseases 2020 Sep (Vol. 7, No. 9, p. ofaa344). US: Oxford University Press. Doi.org/10.1093/ofid/ofaa344] .

42- Lowy FD. Antimicrobial resistance: the example of Staphylococcus aureus. The Journal of clinical investigation. 2003 May 1;111(9):1265-73. 10.1172/JCI18535 43-

CLSI Clinical and Laboratory Standards Institute, Wayne PA .2022. CLSI M100, Performance Standards for Antimicrobial Susceptibility Testing. DOI https://clsi.org/media/rcqcsoad/ast-news-update-volume-7-issue-1-june- 2022.pdf

44- Yaghobi N. Controlling chitosan's molecular weight via multistage deacetylation. Biotechnology and bioprocess engineering. 2012 Aug;17:812-7. Doi: www.doi.org/10.14202/vetworld.2022.558-564.

45- Khudhir ZS. Evaluation the Antibacterial Activity of the Brine, Nisin Solution and Ozonated Water Against E. coli O157: H7 in the Experimentally Local Produced Soft Cheese. The Iraqi Journal of Veterinary Medicine. 2021 Jun28;45(1): 17-21.DOI: https://doi.org/10.30539/ijvm.v45i1.1035

46- Fraise AP, Wilkinson MA, Bradley CR, Oppenheim B, Moiemen N. The antibacterial activity and stability of acetic acid. Journal of Hospital Infection. 2013 Aug 1;84(4):329-31. http://dx.doi.org/10.1016/j.jhin.2013.05.001.

47- United States Department of Agriculture, USDAS. In: Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Products. Directive F., editor. USDA ERS; Washington, DC, USA: 2020. 7120.1 Rev. 55. https://www.fsis.usda.gov/sites/default/files/media_file/2021-09/7120.1.pdf