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Anti-activity of Sodium Bicarbonate on Biofilm Formation by some bacterial isolates from raw milk

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ABSTRACT

Bacterial biofilm has been considered a major cause of many pathogen outbreaks and antimicrobial resistance. Therefore, the current study aimed to investigate the prevalence of the most significant pathogens that can form biofilm in raw milk, estimation of antibiotic sensitivity, anti-bacterial and anti-biofilm activity of sodium bicarbonate (SB), besides, its effect on the expression level of the virulence genes involved in biofilm formation using real-time PCR, also its effect on organoleptic and chemical constituents of milk. *S. aureus* and *K. pneumoniae* were isolated from 38% and 23% of the total samples, respectively, with a higher rate of biofilm production for *K. pneumoniae* than *S. aureus* which showed the more strong biofilm density. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of SB were 125mg/ml and 500mg/ml for *S. aureus*, respectively while, 125 mg/ml and 250 mg/ml for *K. pneumoniae.* All isolates were sensitive to ciprofloxacin and resistant to β- lactams. SB showed antibacterial and anti-biofilm activity with higher efficacy against *K. pneumoniae* and down-regulated the expression level of biofilm-associated genes (*ica*A in *S. aureus, mar*kA, and *fim*Ao gene in *K. pneumoniae).* SB enhanced the activity of β-lactam, sulfamethoxazole/trimethoprim, and tetracycline, but slightly suppressed the activity of ciprofloxacin and gentamycin. Furthermore, the addition of 0.3 % of $NaHCO_3$ is enough to preserve milk samples for up to 12 hours at room temperature, it was effective chemically and microbiologically by reducing the initial viable cell count, also neutralizing the acids produced by acid-producing bacteria. so, it can be used for short-term preservation of milk.

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INTRODUCTION

Biofilm-producing bacteria are recognized to be the major source of both spoilage and pathogenic microflora in the dairy industry **(Elmoslemany et al. 2009)**. Biofilms have been identified as a major cause of many pathogen outbreaks. Some assessment suggests that more than 80% of microbial infections are caused by biofilms **(Epstein et al. 2011).**

Bacteria that form biofilms may harmfully affect the safety and quality of milk and its products (**Møretrø and Langsrud, 2017**). Biofilm is a community or a collection of microorganisms closed to the surface one to each other, enclosed by a matrix of synthesized extracellular polymeric substances, which demonstrate a change in the phenotype, which is expressed changes in parameters of growth and expression of specific genes **(Gomes et al. 2016)**. Microbes in the biofilm are resistant to antibacterial substances due to the occasional existence of resistant cells known as "persisters" and/or the decreased ability of antibiotics to enter the polysaccharide matrix. With the intention of nutrient and antimicrobial molecules to enter the microbial cells of biofilms, they must be spread through the matrix of biofilm or the mucus produced by the bacterium **(Bengtsson et al. 2009)**.

A large number of food-borne disease outbreaks related to contaminated dairy products demonstrate that S. *aureus* has great public health importance **(McMillan et al. 2016)**. The extracellular matrix of *staphylococcal* biofilms consists of exo polysaccharide, pretentious, and extracellular DNA **(Payne and Boles, 2016).** Exo-poly saccharide materials, which are also called Polysaccharide Intercellular Adhesion (PIA) or Poly-_-1,6-N-acetyl-D-glucosamine (PNAG), the production and secretion of these adhesion materials are created by a protein expressed as the *ica* ABCD gene, an intercellular adhesion (*ica)* operon **(Cramton et al. 1999).** Intercellular adhesion (*ica*A) gene has been reported to be significantly involved in the formation of biofilms of *S. aureus* **(Melo et al. 2013)**. Moreover, a definite species of *S. aureus* may also encode a microbial surface constituent named a biofilm-associated protein (*bap*), that recognizes adhesive matrix substances and confers PIA production and biofilm development independently through cell-to-cell aggregation **(Avila-Novoa et al. 2018).**

Klebsiella sp. is a type of bacteria that is commonly found in milk and its related products. This bacterium is known for being both widespread and potentially harmful to both animals and humans **(Nalini Mohanty et al. 2013).** The ability of Klebsiella to form biofilms has been related to antimicrobial resistance **(Vuotto et al. 2014)**.

Some major virulence factors contribute to biofilm development and formation in *K. pneumoniae* such as the capsular polysaccharides, type 1 and type 3 fimbriae. Genes encoding *fim* and *Mrk*A are well characterized as the most experimentally determined and encode type I and type III fimbriae **(Paczosa and Mecsas, 2016).** Type 3 fimbriae are primarily composed of subunits of the protein that codes as *Mrk*A that activate and initiate the biofilm formation in *K. pneumoniae* **(Chung, 2016)**.

Biofilm-forming bacteria are more expected to sustain aggressive environments and they can be well protected from the action of the host immune system while becoming less sensitive to the antibiotic or disinfectant activity **(Felipe et al. 2017)**. For decades, Sodium bicarbonate has been used and indicated for use as a microbial disinfectant in the food and agriculture industries **(Rutala et al. 2000)**. Sodium bicarbonate (NaHCO3) is a cheap chemical available in the local market, present in powder form, white color, and very easy to handle **(Elmoslemany et al. 2009)**. NaHCO3 affects bacterial biofilm by causing limitation of bacterial growth **(Silhacek and Taake, 2005)**.

The time interval between milk collected from the small farmers to the consumers is most important to ensure fresh, clean, and pure milk, it is very important to adopt some techniques for increasing the shelf life of milk. The presence of different types of microorganisms or undesirable bacteria in milk may cause deterioration of flavor, color, taste, or physical ap-

pearance. The spoilage takes place rapidly due to the formation of excess lactic acid from the breakdown of lactose by lactic acid-producing bacteria. To make milk safe for public health and also to increase its shelf life it is very important to preserve milk scientifically **(Hossain et al. 2011; Nonga and Mtambo, 2015).**

Milk can be preserved for a short time for human consumption by using some chemical substances such as hydrogen peroxide (H202), and sodium bicarbonate **(Rahman et al. 2018)**, or by regulating the temperature where cooling, pasteurization, and boiling. Cooling and pasteurization facilities are not available throughout the small village.

It is urgently needed to develop low-cost short-time milk preservation technology to reduce the spoilage of milk that occurs during transportation. Some studies revealed that the addition of sodium bicarbonate was effective as a short-term preservative of milk **(Hamid et al. 2003; Mahboob, 1992)**. Therefore, the present study aims to estimate the prevalence of some major pathogens isolated from raw milk, the density and the ability of these pathogens to form a biofilm, assess the sensitivity of these bacteria to antibacterial drugs, investigate the antimicrobial sensitivity, antibiofilm activity of Sodium bicarbonate (SB) (NaHCO3) and its combination action with therapeutic antimicrobials. Moreover, this study was conducted to detect the effect of sodium bicarbonate on the chemical and physical characteristics of milk and its usefulness as a short-term preservative of milk.

2. MATERIALS and METHODS

2.1. Sampling

A total of 100 raw milk samples were randomly collected under complete a septic condition from apparently healthy lactating cows in some dairy farms at El-Gharbia governorate. All samples were collected separately on sterile plastic syringes and transported immediately in an ice box to the laboratory for bacteriological examination.

2.2. Isolation and identification of bacteria

The collected milk samples were inoculated in nutrient broth and incubated aerobically at 37 *°C* for 24 h. for enrichment. A loop full of the inoculated broth was spread onto the surface of Baird-Parker agar as a selective medium for the isolation of *Staphylococcus aureus according to* **da Silva et al. (2017)**. While MacConkey's agar (Oxoid, UK) as well as Eosin Methylene Blue (EMB) agar (Oxoid, UK) for isolation of *Klebsiella pneumoniae.* The inoculated plates were incubated at 37°C for 24-48hs aerobically. After incubation separate pure suspected colonies were identified by microscopic examination of gram staining films and biochemical identification tests according to **Koneman et al. (2012) and Tallent et al. (2020).**

2.3. Detection and evaluation of biofilm production

The biofilm formation ability of isolated strains was evaluated using the tissue culture plate method according to **Vasudevan et al. (2003).** Briefly: fresh colonies from each of the bacterial isolates (*S. aureus* and *Klebsiella pneumonia*) were inoculated separately on 5 mL of Tryptic soy broth (TSB) supplemented with 1% glucose and adjusted to a concentration of cells 10^8 CFU/mL. Then the inoculated broths were incubated aerobically at 37°C for 24 h. After that, the inoculated cultures were then diluted to 1:100 with fresh broth medium, then using sterile 96 well-tissue culture microtiter plate plates, a volume of 200 µL of the diluted cultures was transferred to the individual wells of the plate and incubated at 37oC for 24 h. The inoculated broth medium was then thrown out, and the wells of the plate were washed gently three times with 200 µL of sterile PBS (pH 7.4), left to dry for 20 min, and then stained with 50 µL of crystal violet solution 1% for 15 minutes. Each well was washed away three times with 200 µL of sterile distilled water, then left to dry at 45°C for 20 min; 200 µL of 95% ethanol was then added to each well for 30 min. The absorbance was measured at 630 nm using a micro plate ELISA reader(MR-96 CLINDIAG Device) at the Micro Analysis Unit, Faculty of Science, Tanta University after adjustment to zero with negative control as blank. The cut-off value (ODc) was calculated by the formula:

ODc=average OD of negative control + 3 x standard deviation of negative control). Each strain was tested in triplicate and the OD (optical density) value was averaged and detracted from the cut-off value to get the final OD for each strain biofilm activity. Interpretation of the results was obtained according to the following sets; OD ≤ ODc (Optical density cut-off value) = non-biofilm producer; ODc < OD ≤ 2 x ODc = weak biofilm producer; 2 x ODc < OD ≤ 4 x ODc = moderate biofilm producer; 4 x ODc < OD= strong biofilm producer **(Stepanović et al. 2007).**

2.4. Detection of biofilm virulence genes in *Staph. aureus and kelepsiella* using uniplex PCR.

Three isolates revealed strong biofilm production were selected from each bacterial species and subjected to polymerase chain reaction (PCR) technique for the detection of virulence genes associated with biofilm production. **DNA extraction**: DNA was extracted from the isolates of *S. aureus* and *K. pneumoniae* using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications. Oligonucleotide primers, cyclic conditions and analysis were supplied from Metabion (Germany) and listed in Table (1).

Table 1. Primers sequences, target genes, and cycling conditions for SYBR green rt-PCR

Bacteria	Target gene	Primers sequences	Amplified segment (bp	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
						Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation	
S. aureus	*16S rRNA*	CCTATAAGACTGGGATAACTTCGGG CTTTGAGTTTCAACCTTGCGGTCG		50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.	(Mason et al. 2001)
	icaD	AAA CGT AAG AGA GGT GG GGC AAT ATG ATC AAG ATA	381 bp				49˚C 30 sec.			49˚C 1 min.		(Ciftci et al. 2009)
	bap	CCC TAT ATC GAA GGT GTA GAA TTG GCT GTT GAA GTT AAT ACT GTA CCT GC	971 bp									
K.pneumoniae	*gyrA*	CGC GTA CTA TAC GCC ATG AAC GTA ACC GTT GAT CAC TTC GGT CAG G					55˚C 30 sec.			55˚C 1 min.		(Brisse and Verhoef, 2001)
	fimA	CGGACGGTACGCTGTATTTT GCTTCGGCGTTGTCTTTATC	436 bp				55˚C 30 sec.			55˚C 1 min.		) Alcántar-Curiel et al. 2018)
	mrkA	CGGTAAAGTTACCGACGTATCTTGTACTG GCTGTTAACCACACCGGTGGTAAC	475 bp				55˚C 30 sec.			55˚C 1 min.		

2.5. Antibiotic Sensitivity Test:

In vitro, antibiotic sensitivity of biofilm producers bacterial isolates of *S. aureus* and *K. pneumoniae* was estimated by the disc diffusion (Kirby-Bauer) method using Muller Hinton agar (MHA) and different types of commonly used antibiotics in the veterinary field**.** Adjust the bacterial suspension to a density of 0.5 McFarland, which corresponds to approximately 10^8 CFU/ml. The antibacterial drugs used in the experiment were amoxicillin/ clavulanic acid (AMC; 30 µg/disc), oxacillin (OX; 1 µg/disc), gentamicin (CN; 10 µg/disc), ciprofloxacin 5 µg/disc), sulfamethoxazole/ trimethoprim (SXT; 25 µg/disc), tetracycline (TE; 30 µg/disc), and cefotaxime (CTX 30 µg/ disc). The diameter (in millimeters) of the zone of antibiotic inhibition was measured and the results were interpreted according to the Clinical and Laboratory Standards Institute **(CLSI, 2018).** Resistance of an isolate strain to one antibiotic drug in three or more antibiotic groups was considered as multidrug resistance (**Magiorakos et al. 2012**).

2.6. In vitro evaluation for antibacterial activity of sodium bicarbonate

Sodium bicarbonate was obtained from a local pharmacy, and the antibacterial activity of sodium bicarbonate against biofilm producer's bacterial isolates was performed using the agar well diffusion method according to **Magaldi et al. (2004)** and **Valgas et al. (2007).** Briefly, a volume of the bacterial inoculum spread over the entire surface of Muller Hinton plates with a concentration adjusted to 0.5 McFarland (1–2× 10^8 CFU/ml). A sterile cork borer or a tip of about 6-8 mm in diameter was used to make halls and about 20 µL from sodium bicarbonate freshly prepared solution with a concentration of (125, 250, 500 mg/ml) was introduced into the halls. The plates were incubated at 37°C for 24h, after that, the inhibition zone diameters (IZD) were measured in millimeters (mm).

2.7. Anti-biofilm activity and minimum inhibitory concentration (MIC) of SB.

Anti-biofilm activity of SB was assessed using a 96-well microtiter plate according to **Gurunathan et al. (2014).** Three strains evaluated as strong biofilm producers were included in the test as representative samples for each bacterial species isolate (*S. aureus* and *K. pneumoniae*). 180 µl of freshly prepared Muller Hinton broth were placed into the wells of the microtiter plate then, 10 µl of the standard concentration of the tested bacterial culture was added to each well of the plate. After that, 10 µl of freshly prepared SB solution was added to the first well with a concentration of 1000 mg/ml and mixed well. Two-fold serial dilution was done to estimate the MIC of SB. The positive control well contained only bacterial culture without SB while the negative control well included sterile distilled water. The micro titer plate was incubated again for 24 h at 37° C. The growth of bacteria was evaluated based on the visible change of turbidity. MIC was identified as the lowest concentration of $NaHCO_3^-$ at which no visible growth was observed. To determine MBC, specimens from the wells without visible bacterial growth were inoculated onto antibiotic-free agar plates and incubated for 24 h. MBC was defined as the lowest concentration of HCO_3 where no colonies were observed. The biofilm was measured using the crystal violet assay as stated above **(Stepanović et al. 2007)**.

2.8. Combination of sodium bicarbonate with different antibiotics

Sodium bicarbonate was used in combination with different antibiotics and estimate the antimicrobial activity on biofilm-producer strains isolated in the experiment. 0.1 ml of the bacterial strain suspension with turbidity adjusted to 0.5 McFarland was spread above the surface of MHA plates and the selected antibiotic discs were separately saturated with 5 µL of sodium bicarbonate solution with a concentration of 250 mg/ml and distributed on the inoculated agar plates. The plates were incubated overnight at 37°aerobically. The inhibition zone diameters produced were estimated as described by **Ali, (2018)** and **Lo Cantore et al. (2004)**.

2.9. Effect of SB on the relative genes expression of biofilm encoding genes using qRT PCR

Most biofilm producer isolates contained biofilm virulence genes of *staph. aureus* and *K. pneumoniae* were chosen for assessment of the effect of SB on the relative expression and regulation of the biofilm coding genes using qRT-PCR .Oligonucleotide primers used were supplied from Metabion (Germany) and are listed in Table (1). SYBR green rt-PCR. Primers were utilized in a 25- µl reaction containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reaction was performed in a Stratagene MX3005P real-time PCR machine. Analysis of the SYBR green rt-PCR results was determined by the Stratagene MX3005P software. The fold changes of mRNA levels in the treated samples with sub MIC concentration of SB were calculated compared with that of the positive control non-treated and relative to the reference house-keeping gene according to the "ΔΔCt" method stated by **Yuan et al. (2006)**.

2.10. Effect of sodium bicarbonate on physical, microbiological and chemical properties of milk and short-term preservation of milk

Whole milk samples collected from dairy farms were taken to the Laboratory for experimental inspection under hygienic conditions. The milk samples were prepared for the chemical analysis after thoroughly mixing, and were divided into five equal parts; one was kept as whole milk (control) without NaHCO3, and the other four parts were preserved with different concentrations of NaHCO3 **(Thiex, 2009).** The milk samples were as follows: (1) Milk sample without $NaHCO_3$ (control), (2) Milk sample with 0.1% $NaHCO_3$, (3) Milk sample with 0.3% $NaHCO_3$, (4) Milk sample with 0.5% $NaHCO_3$ and (5) Milk sample with 1 % NaHCO3. The parameters used to monitor the physical, and chemical quality of milk were determined initially just before adding NaHCO3 and then after two-hour intervals up to 14 hours and until the milk samples were spoiled. The following tests were performed with each milk sample:

(1) Physical test: a) Organoleptic tests (Color, Flavor, and Texture). b) Clot-on-boiling (COB) test.

(2) Total bacterial count : Total viable bacteria / ml - were determined initially just before adding NaHCO3 (control sample) and then after two-hour of adding NaHCO3.

The sample was serially diluted up to 1:106 and 1:107 then duplicate samples (1ml) were pour plated using 15-20 ml standard plate count agar solution and mixed thoroughly. The plated sample could incubated at 37 °C for 48 hours, counts were made using a colony counter. Results from plates, which contained 30 to 300 colonies per plate were recorded. and the average for each sample were recorded as CFU/ml **(Marth , 1978).**

Chemical test: The chemical analysis of milk samples applied by MilkoScan FT1 equipment used for determining the quality control of the milk to analyze its fat, protein, total solids, solids not fat, casein, lactic acid %, and acidity. The FTIR MilkoScan FT1 equipment can scan the entire middle infrared region with wavelengths between 2.0 and 10.8 µm (5012–926 cm^{-1}), and the spectra were exported and stored in electronic files. Three samples were prepared for each concentration **(Coitinho et al. 2017)**.

Statistical analysis:

Analysis of variance using SPSS VERSION 16 (T-test) was done to find the statistical difference (Significant or not) between the different treatments and in case of significant difference calculated to make a comparison between treatments.

RESULTS

The prevalence rate of bacterial isolation

Based on microbiological and biochemical characterization, *S. aureus* was isolated from the total collected raw milk samples with an isolation rate of 38% (38/100) and *K. pneumoniae* with an isolation rate of 23% (23/100).

Evaluation of biofilm production

Isolated strains of *S. aureus* and *K. pneumoniae* were examined for biofilm production and the results clarified that *K. pneumoniae* was the more biofilm producers than *staph aureus* but *S. aureus* showed a higher percentage of strong biofilm producers

our results showed that 29 isolates of *S. aureus* from the total isolates 76.31% (29/38) were biofilm producers with a different activity. 22/29 (75.8%) of the isolates were strong biofilm producers, 5/29(17.2%) were moderate biofilm producers and (2/29) 6.89% were weak biofilm producers.

In *K. pneumoniae* isolates, 19/23 (82.6%) were biofilm producers. 10/19(52.63%) were strong biofilm producers, 7/19(36.84%) were intermediate biofilm producers and 3/19(15.78%) were weak biofilm producers as shown in Table (3).

Table 2. Evaluation of biofilm production in the tested organisms

Tested organism	Biofilm production							
	strong		Moderate		weak		Total	
	No	%	No	%	No	%	No	%
S. aureus	22/29	75.8%	5/29	17.2%	2/29	6.89%	29/38	76.3%
K. pneumoniae	10/29	52.63%	7/19	36.86%	3/19	15.78%	19/23	82.6%

3.3. Detection of biofilm virulence genes in *S. aureus and K. pneumoniae* using PCR

The results showed that *icaD* gene was detected in all screened isolates of *S. aureus* which gave positive amplification product at 381bp, while the *bap* gene was not detected.

In *K. pneumoniae, mrkA* and *fimA* genes were detected in the screened isolates and gave amplification products at 475 and 436bp respectively. The results are illustrated in Figures (1) and (2).

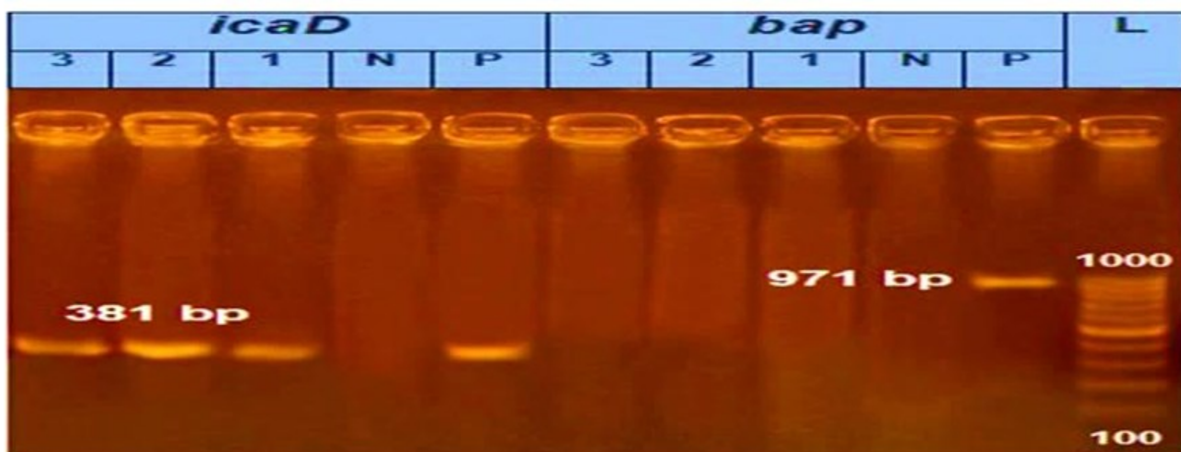


Fig. 1: Agarose gel electrophoresis showed amplification of *ica*D gene of *S. aureus*. lane: 1,2 and 3 show positive amplification of *ica*D gene at 381bp. While negative amplification of *bap* gene at 971bp, L: ladder (100-1000bp) P: positive control; N: negative control

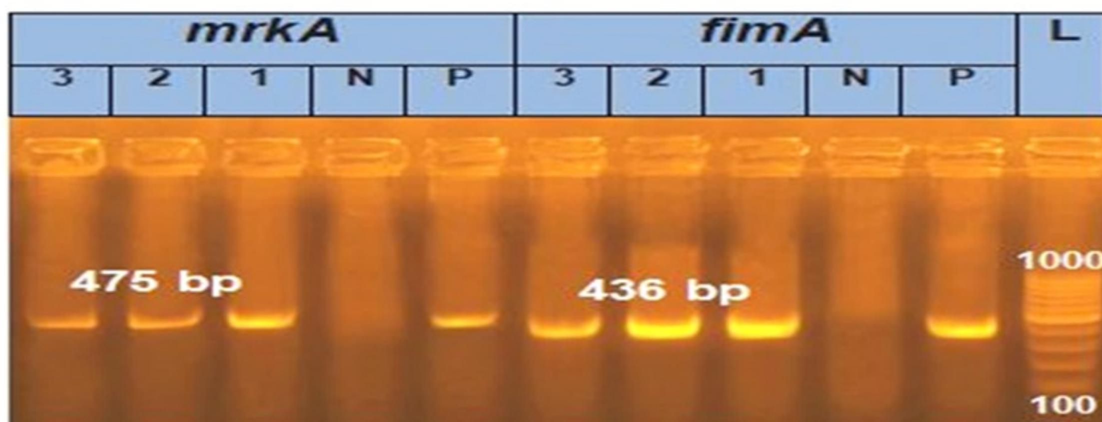


Fig 2. Agarose gel electrophoresis showed amplification of *mrk*A gene of *K. pneumoniae*. lane: 1,2 and 3 show positive amplification of *mrk*A and *fim*A genes at 475bp and 436bp respectively. L: ladder (100-1000bp), P: positive control; N: negative control.

Antibiotic sensitivity of bacterial isolates

After interpretation of the results of inhibition zone diameter for the different used antibiotics according to the Clinical and Laboratory Standards Institute **(CLSI, 2018).** The obtained results clarified that all *S. aureus* isolates were sensitive to ciprofloxacin (CIP), sulfamethoxazole/trimethoprim (SXT) followed by tetracycline (TE), intermediate sensitive to gentamicin (GEN), while all of the *S. aureus* isolates were resistant to β- lactams including Oxacillin (OX), amoxicillin/clavulanic acid (AMC), and cefotaxime (CTX). 10/29 (34%) of isolates showed MDR.

In *K. pneumoniae*, Also, all of the isolates showed high sensitivity to ciprofloxacin (CIP) and sulfamethoxazole /trimethoprim (SXT), intermediate sensitivity to gentamicin (GEN), oxacillin, and tetracycline. Similarly to *S. aureus* all of *K. pneumoniae* isolates were resistant to amoxicillin/clavulanic acid (AMC) and cefotaxime (CTX) and 7/19 (36%) showed MDR.

In vitro evaluation for antibacterial activity of sodium bicarbonate

Antibacterial sensitivity of sodium bicarbonate against *S. aureus* and *K. pneumoniae* clarified that SB displays antibacterial activity with marked inhibition zone diameters against the examined isolate with increasing the used concentration of sodium bicarbonate (125mg/ml, 250 mg/ml, and 500mg/ml), with higher efficacy against *K. pneumoniae*, the results illustrated in figure (3).

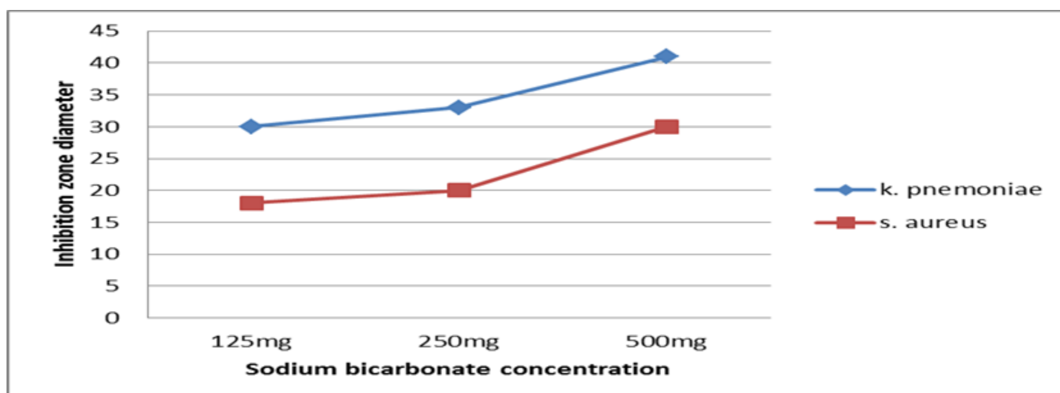


Fig 3. Antimicrobial activity of sodium bicarbonate on *S. aureus* and *K. pneumoniae.*

Anti-biofilm activity of SB and MIC

The obtained result showed that SB inhibits the formation of biofilm and the degree of inhibition depends on the concentration of SB. The minimum inhibitory concentration (MIC) of sodium bicarbonate was 125 mg/ml for *S. aureus* and *K. pneumoniae.* The optical density of biofilm decreased by 62.2% in examined isolates while it decreased by 66.6% in *K. pneumoniae* examined isolates. The minimum bactericidal concentration (MBC) for *S. aureus* was 500 mg/ml for *S. aureus* while it was at a lower concentration (250 mg/ml) for *K. pneumoniae.*

Combination of sodium bicarbonate with the different antibiotics

SB with a concentration of 250mg/ml appeared to enhance the inhibition activity of amoxicillin/clavulanic acid (AMC), cefotaxime (CTX), and oxacillin. Also, SB increases the activity of sulfamethoxazole /trimethoprim (SXT) and tetracycline (TE). However, it slightly suppressed the activity of ciprofloxacin (CIP) and gentamicin, the result was shown in figure (4,5).

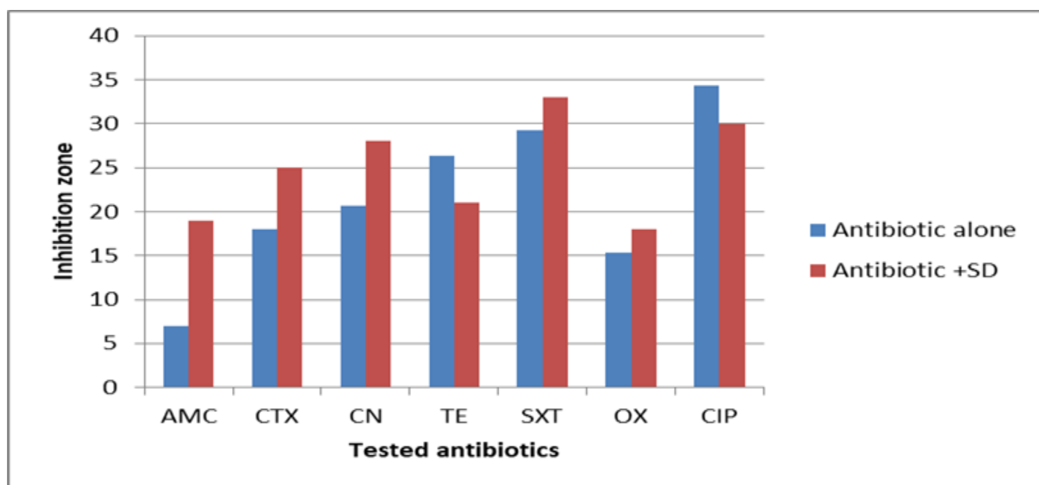


Fig 4. Antimicrobial activity of antibiotic alone or in combination with SB on *S. aureus*

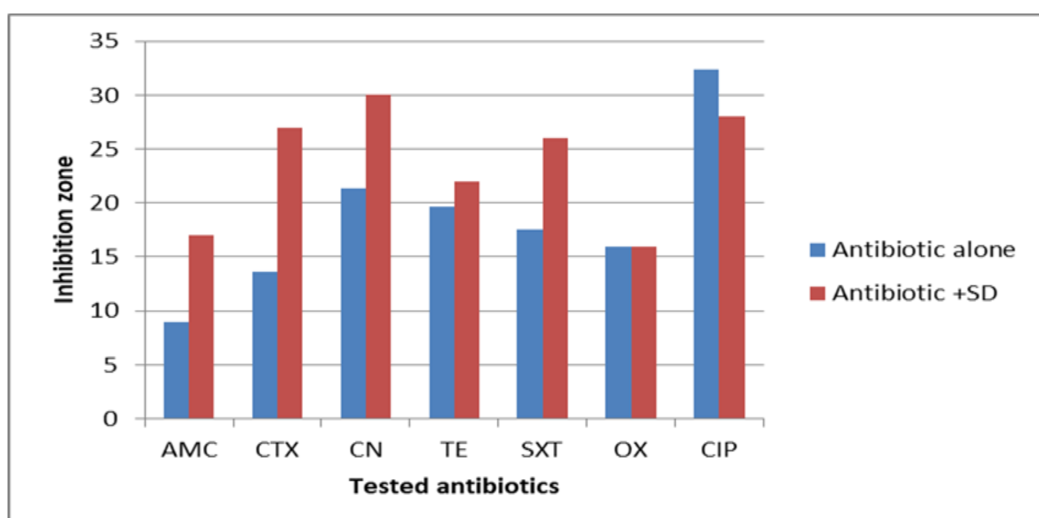


Fig 5. Antimicrobial activity of antibiotic alone or in combination with SB on *K. pneumoniae*

3.8. Effect of SB on the relative genes expression of biofilm encoding genes using qRT PCR

The result clarified that the relative expression level of *ica*A gene in the *S. aureus*-treated sample decreased to 0.1174. The expression level decreased by 88% under the control level, Figure (6). Moreover, the relative expression level of *mar*kA and *fim*A genes in *K. pneumoniae* treated isolate decreased to 0.3186 and 0.2679 respectively. The expression level decreased by 68% and 73% in comparison with the control untreated sample, Figure (7).

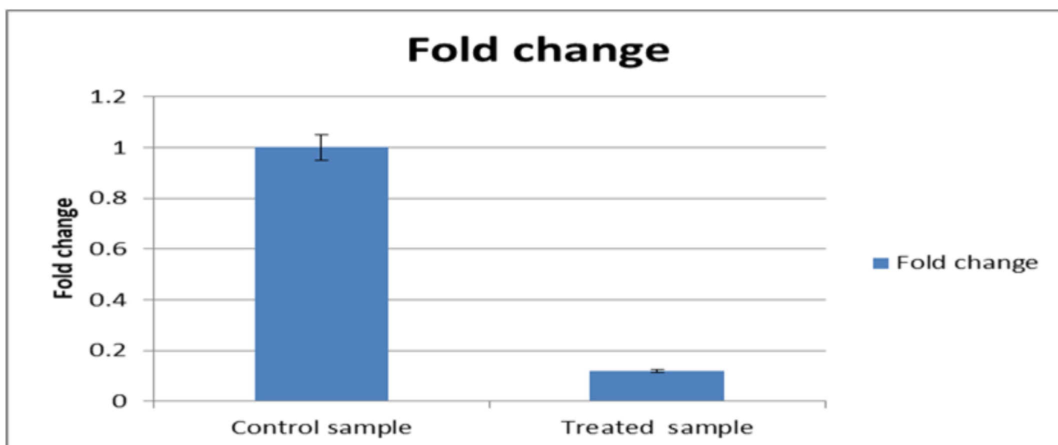


Fig 6. Relative expression (fold change) of *ica*A gene in *S. aureus* treated isolate in comparison to control none treated isolate

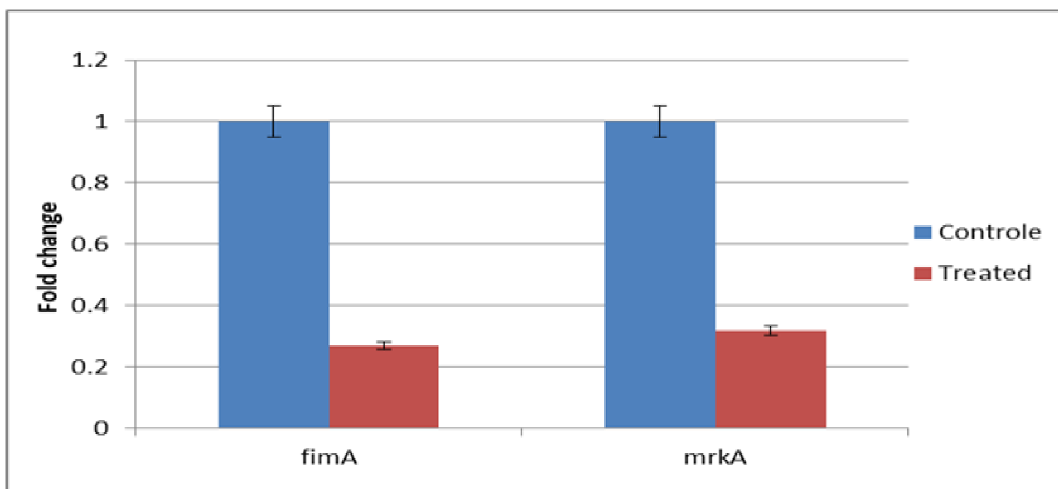
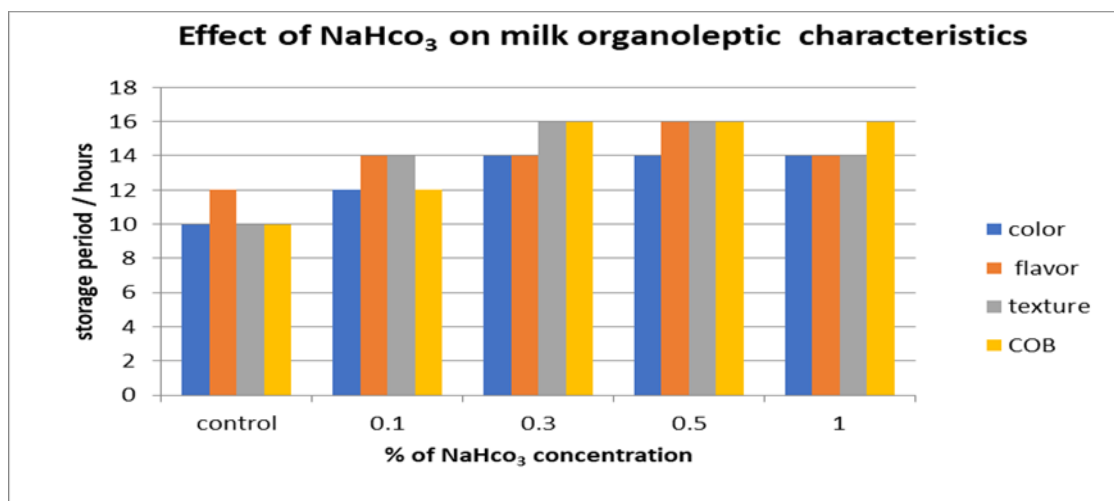


Fig 7. Relative expression (fold change) of *fim*A, and *mrk*A gene in *K. pneumoniae* treated isolate in comparison to control none treated isolate

Effect of sodium bicarbonate on Physical and chemical properties and Short-term preservation of milk

The results cleared that the color appeared normal up to the 8th hour and after which the color became pall yellow at 10 th hour, then the color became whitish at the 12th and 14th hours, Color deterioration was very rapid in whole milk (control) followed by 0.1, 0.3, 0.5 and 1 percent NaHCO3 treated milk samples respectively as shown in Figure (8).



The normal Organoleptic characteristics of the examined milk samples before and after adding $NaHCO_3$

The texture of milk samples was normal up to the 10th and 12th, except the concentration 0.1 of the NaHCO3 treated milk samples became slightly clotted at the 12th hours of study. Texture deterioration was more rapid in control milk samples due to lactic acid production than in treated milk with NaHCO3.For the flavor, the beginning sour flavor of the control milk samples started at The 10th hour while the treated milk samples with NaHco3 continued to be normal till 14 hours. The Clot-on-boiling (COB) test revealed that the whole milk (control) sample clotted earlier than that of NaHCO3-treated milk samples. This was due to the more acid production in control milk samples., NaHCO3 neutralized the acids produced by lactic acid-producing bacteria from the breakdown of lactose. The earlier COB was for the concentration of 0.1 %.

Chemical analysis of Milk constituent before and after adding $NaHCO_3$

Milk constituent measurement by MILKOSCAN is shown in Table(4).

Table 3. Chemical analysis of Milk constituent before and after adding (SB) NaHCO3.

Variables	Control (Befor adding-$NaHCO_3$)	After adding$NaHCO_3$ Mean± SD				P. Value
		0.1%	0.3 %	0.5%	1gm%	
Fat	3.31 ± 0.02	3.88±0.09	3.92±0.01	4.09±0.10	3.84±0.12	0.155
Protein	2.64 ± 0.02	2.88±0.03	2.80±0.00	2.79±0.03	2.76±0.2	0.554
SNF	8.48 ± 0.00	8.18±0.01	6.74±0.00	5.57±0.05	2.69±0.01	0.003
TS	14.85 ± 0.02	15.57±0.07	14.69±0.01	14.40±0.10	13.44±0.13	0.000
Lactose	2.61 ± 0.37	4.91 ± 0.04	4.84 ± 0.01	4.75 ± 0.03	4.51 ± 0.03	0.000
Casein	2.04 ± 0.03	12.73±0.	23.32±0.71	32.18±1.24	67.12±2.32	.001
Lactic acid	0.183 ± 0.016	0.119±0.006	0.087±0.004	0.052±0.003	0.018±0.002	0.000
Acidity	18.31 ± 1.63	11.90±0.56	8.69±0.36	5.20±0.28	-1.76±0.19	0.024

SD= standard deviation SB= sodium bicarbonate

DISCUSSION

Diseased or infected cows with environmental bacteria have the potential to cause contamination in raw milk **(Kongo et al. 2008).** Moreover, **Addis et al. (2016)** suggested that numerous types of microorganisms exist in the mammary gland when it's in a healthy state. Several investigations indicate that antibiotic-resistant pathogens causing outbreaks may primarily originate from unpasteurized milk or dairy products derived from unpasteurized milk **(Ulusoy and Chirkena, 2019).** In the current study, *S. aureus* was isolated from a total of (100) raw milk samples with a prevalence rate 38%. A similar result of isolation (38.88%) for *S. aureus* from raw cow milk was reported by **Pajohesh and Tajbakhsh, (2022),** and a nearly parallel study, by **Hassani et al. (2022a)** who reported that *S. aureus* was isolated from 25% of bovine milk samples. Other authors identified a high prevalence rate of 75% in raw milk samples in Mansoura City, Egypt by **Al-Ashmawy et al. (2016)** and (77.38%) from bovine milk samples **(Ren et al. 2020).** A lower rate (12.79%) was reported in China from dairy farms' milk samples **(Yang et al. 2021).**

Dairy food products are widely known to contain *Klebsiella spp.* **El-Sukhon, (2003)** that reported to have zoonotic importance **(Nalini Mohanty et al. 2013)**. In our study, out of 100 milk *samples, K. pneumoniae* was isolated at a rate of 23%. A greater prevalence rate (45.29%) was detected in India **(Koovapra et al. 2016)** and in Iran 40 % **(Enferad and Mahdavi, 2020)**. In Bangladesh was up to 62.50% **(Salauddin et al. 2019).** While, in China, **Yang et al. (2021)** isolated *Klebsiella* spp. with a lower prevalence rate (9.78%) from raw milk of cows. It has been observed that the combined occurrence of samples confirmed positive for *Klebsiella* detected in 2013 or afterward was greater than the results collected before 2013. Also, the authors found that the rate of isolation of *Klebsiella* spp was higher in developing countries **(Song et al. 2023).** Therefore, the study suggested that the differences in the isolation rate of the bacterial pathogens observed in the study could be attributed to many factors such as hygiene, geographic location, measures taken for protection against diseases or infections, management, and immune system competences.

Microorganisms in the form of biofilms allow bacteria to survive for extended periods in cows that act as carriers, and so, these animals become a source of infection **(Horiuk et al. 2019).** Moreover, bacterial species that form a biofilm, frequently display higher virulence **(Wilson et al. 2017),** and a better capacity to show antibiotic resistance **(Chao et al. 2015)**.

Micro-titer plate test is a cost-effective and practical approach to quantitatively determine the essential factors and optimal environments for biofilm formation **(Stepanović et al. 2007).** In our study, the results revealed that *K. pneumoniae* and *Staph aureus* produce biofilm with variable degrees of concentration. *S. aureus* showed a higher percentage of strong biofilm producers. We evaluated 76.31% of the total *S. aureus* isolates as biofilm producers, (75.8%) of the isolates were strong, (17.2%) were moderate and 6.89% were weak biofilm producers. Another study by **Darwish and Asfour (2013)** evaluated 96.3% of *S. aureus* isolates as biofilm producers with variable production levels: strong, moderate, and weak biofilm producers (52.5%, 27.5%, and 20%) respectively. **Fabres-Klein et al. (2015)** stated that 87% of the total *S. aureus* isolated from bovine milk were considered to be biofilm producers**.** In Japan **Thongratsakul et al. (2020)** determined that *S. aureus* isolates from raw milk of cows displayed extensive production of biofilm, they classified (89.1%) as strong, moderate, and weak. Furthermore*, S. aureus* isolates from milk showed strong, moderate to weak biofilm formation ability **(Kim et al. 2022; Wang et al. 2022).**

Concerning the isolated strains of *K. pneumoniae,* (82.6%) were biofilm producers with variable degrees of density as strong, moderate, and weak (52.63%, 36.84%, and 15.78%) respectively. Biofilm production ability was previously detected with a rate of 84% in *Klebsiella* spp. **(Schönborn et al. 2017).** Also, it was reported that all *K. pneumoniae* isolates collected from quarter-milk samples from ap-

parently normal milking cows and clinical mastitis were revealed to be biofilm producers **(Massé et al. 2020).** In Egypt, 91.7% of *K. pneumoniae* isolates from cow milk and humans were able to produce the biofilm, (54.5%, 27.3%, and 18.2%) were moderate, strong, and weak biofilm producers respectively **(Gomaa, 2021).**

The formation of biofilm increased the virulence of *S. aureus.* The genes coding *ic*a are responsible for slime formation in *S. aureus* by controlling PIA production and It can determine the ability of *S. aureus* strains to generate biofilm **(Namvar et al. 2013).** In our study PCR determination of biofilm virulence genes revealed the presence of *ica*A gen in all *S. aureus* tested strains, these results almost agree with other findings reported by **Wang et al. (2018)** who identified *ica* gene in all isolates, and similar results detected by **Ibrahim et al. (2022)** who detected *ica*A gene in 90% of tested isolates. The *bap* gene implicates biofilm formation by promoting primary attachment and adhesion to inert and live surfaces **(Cucarella et al. 2004).** Our results revealed that all tested strains (100%) were negative for the bap gene. According to **(Vautor et al. 2009)** the absence of bap indicates that the *ica*-dependent pathway is predominantly responsible for adhesion and biofilm development, a similar result demonstrated by **Ibrahim et al. (2022)** who reported the absence of the bap gene in all tested isolates. Our results are in agreement with **(Xu et al. 2015)** who were unable to detect the bap gene in *S. aureus* recovered from cow milk.

The formation of biofilms by *K. pneumoniae* is a key player in facilitating the evasion of host defense mechanisms, communication between bacterial cells, and protection against antibiotic action. The bacteria's capability to produce biofilms depends on multiple genetic factors. Therefore, surface components of the bacterial cell that increase the efficiency of biofilm formation are likely to play a major role in the establishment of infection by pathogens **(Jagnow and Clegg, 2003).** Type 1 fimbriae mediate adhesion to mannose-containing structures on host cells and extracellular matrix and are present in many species of *Enterobacteriaceae*. However, there are significant genetic, serological, and functional differences between type 1 fimbria variants in the different species **(Duncan et al. 2005)**. In the current study, the genotypic detection of biofilm genes revealed the detection of *mar*K and *fim*A in all tested isolates of *K. pneumoniae.* Different results were recorded by Kadhim et al. (2020) who detected *mark*A in only 5 (15.15%) of the isolates. Also, **Makhrmash et al. (2022)** detected *fim*A and *mar*K with a percent (87.5%) and (46.4%) respectively, these findings demonstrated the capability of *S. aureus* and *k. pneumoniae* to produce biofilms with changed production density and so, these bacteria may ensure the possibility for multidrug resistance transmission in dairy farms or to humans. Forming bacterial biofilms is one of the survival strategies of bacteria to tolerate antimicrobial agents and other external stress by interfering with the penetration of antimicrobials into the biofilm **(Rabin et al. 2015).**

Therefore, we performed an antibiotic sensitivity test for the bacterial isolates, and the result clarified that all *S. aureus* isolates were sensitive to ciprofloxacin (CIP), sulfamethoxazole/trimethoprim (SXT) followed by tetracycline (TE). 34% of the isolates showed MDR with 100% resistance to Oxacillin (OX) and amoxicillin/clavulanic acid (AMC) followed by cefotaxime (CTX) 33.3% . Parallel results by **Shahid et al. (2021)** who found that all *S. aureus* isolates were sensitive to ciprofloxacin. In Egypt**, Talaat et al. (2023)** reported 70% sensitivity of *S. aureus* isolates to ciprofloxacin, while 73.3% and 96.6% were resistant to tetracycline (TE) and oxacillin respectively. Also, previous results in Iran, Brazil, and Egypt stated high resistance of *S. aureus* isolates from bovine milk to β-lactam antibiotics **(Ameen et al. 2019; Freu et al. 2022; Hassani et al. 2022b and Wang et al. 2022).**

Concerning *K. pneumoniae*, all of the isolates were resistant to amoxicillin/clavulanic acid (AMC), and cefotaxim (CTX) and highly sensitive to ciprofloxacin (CIP). A previous similar study in Egypt by **Gomaa, (2021)** who reported that *K. pneumoniae* isolates from milk and other samples had a high incidence of resistance to ampicillin and amoxicillin /

clavulanate (100%). On the contrary, previous studies did not detect resistance of *kellebsiella* spp., for the first, second, or third-generation cephalosporins **(de Jong et al. 2018; Masse et al. 2020)**. Increased resistance rate to β-lactam antibiotics in *S. aureus* and *kelebsiella* may attributed to the misuse or uncontrolled use of these drugs in the treatment of mastitis.

Our findings demonstrated the high capacity of *S. aureus* and *K. pneumoniae* isolates to produce biofilms with changed production density. Also, increased resistance rate to antibiotics especially β-lactams. So, attention must be paid to determining alternatives and new methods for effective control and treatment.

Sodium bicarbonate (SB) is good-looking due to its safety, low cost, antimicrobial properties, and bactericidal action as a result of osmotic pressure changes **(Barnes, 1999; McCombs et al. 2001).** Sodium bicarbonate in the media resulted in an alkaline environment, that decreased the growth rate of neutrophilic bacteria **(Maurer et al. 2005).** Bicarbonate was first shown to inhibit the growth of various aerobic and anaerobic microorganisms by **Gutiérrez Huante et al. (2015).** It resulted in bacterial killing via increasing the intracellular bacterial cAMP (Cyclic Adenosin Monophosphate) levels and energy consumption, also, disturbing the pH gradient of the proton motive force through the cytoplasmic membrane of gram-positive bacteria and gram-negative **(Farha et al. 2018).** Moreover, increased intracellular cAMP concentration levels are related to the production of acute virulence factors and reduced biofilm formation **(Almblad et al. 2015).**

In our study, we evaluated the antibacterial activity of SB against *S. aureus* and *K. pneumoniae* isolates from bovine milk and the data showed that it produced antibacterial activity with marked inhibition zone diameters against the tested isolates with increasing the used concentration (125, 250, 500 mg/ml), and a higher efficacy against *K. pneumoniae* than *S. aureus*. Also, it exhibited a destructive activity on the biofilm of both tested bacterial species when used alone or in combination with antibiotics. Another study by **Yassein and Suhail (2018) stated** that SB displayed a significant inhibition of bacterial biofilms of (*S. aureus* and *K. pneumoniae*) at a concentration of 15% and 20% which is equal to (150mg/ml and 200mg/ml respectively) alone or combined with antibiotics. Moreover, **El Badrawy et al. (2018)** concluded that SB at 8.4% (84mg/ml) inhibits bacterial, fungal, and mycobacterial growth. On the contrary, a lower concentration of SB (16mg/ml) inhibits *S. aureus* growth in another study **(Saleh et al. 2022).**

Previous studies reported that high -doses of sodium bicarbonate (100mM) or (120mM) display improved killing ability for *S. aureus* and P. *aeruginosa* **(Pezzulo et al. 2012).** Also, the MIC value of bicarbonate was 125 mmol, and MBCs for the *P. aeruginosa* was 500 mmol l^{-1}, meanwhile *S. aureus* remained alive even at the highest concentration of HCO_3^- (MBC > 1000 mmol l^{-1}) as mentioned by **Dobay et al. (2018)**.

Regarding the action of SB on the tested antibiotic sensitivity, in the current study, we recorded that SB with a concentration of 250mg/ml enhanced the antibacterial activity of β- lactam antibiotics including amoxicillin/clavulanic acid (AMC), cefotaxim(CTX) with a lower degree with oxacillin. Also, SB increases the activity of sulfamethoxazole / trimethoprim (SXT) and tetracycline (TE). A previous study by **Yassein and Suhail (2018)** stated that the addition of cefotaxime to the mixture of bacteria and sodium bicarbonate ($NaHCO_3$) at 20% concentration affected on survival of bacterial cells. Also, Ersoy **et al. (2019)** proposed that $NaHCO_3$ in the media may enhance the activity of β-lactam therapy including oxacillin on MRSA. Also, **Ersoy et al. (2022)** suggested that SB caused inhibition of bacterial cell wall teichoic acid formation and abnormal cell division so, it resulted in sensitization of MRSA to β-lactams. Dissimilar results by **Farha et al. (2018)** stated that SB at a physiological concentration of 25 mM decreases the activity of oxacillin and amoxicillin/clavulanic. Regarding the action of SB with ciprofloxacin (CIP) and gentamicin (GEN), our result showed that SB slightly suppressed the activity of both types of antibiotics. These results may be due to the high concentration of

SB as stated by **Gutiérrez-Huante et al. (2015)** which found that the enhancing effect of SB occurred with gentamicin and kanamycin starting at 5 mmol l^{-1} bicarbonate, kanamycin, while that effect was significantly decreased at a high concentration of bicarbonate than at the lower concentrations. Also, may be compatible with **Farha et al. (2018)** who suggested that SB decreases the act of cell wall active antibiotics whose action requires energetically growing bacteria.

In the present study, the inhibitory activity of sodium bicarbonate, against the regulatory genes *ica*A *of S. aureus and mark*A *and fim*A *of K. pneumonia* were examined using qRT-PCR, and the results showed the effectiveness of sodium bicarbonate treatment in down-regulation of the relative expression of these biofilm-associated genes (*icaA, markA, and fimA*). In proportional to our findings, a previous study by **Saleh et al. (2022)** used sub-inhibitory concentrations (1/8 MIC) of ascorbic acid, dexamethasone, and sodium bicarbonate which reduced the relative expression levels of all the tested genes including *icaA*.

Regarding the effect of sodium bicarbonate on the preservation of milk samples. The result of the organoleptic examination (Fig,8) showed that NaHCO3 is effective for controlling the color, flavor, texture, and COB quality of milk. This was due to lactic acid produced from the fermentation of lactose which occurred due to the growth of acid-producing bacteria being neutralized by $NaHCO_3$ and hence the quality of milk was increased. So $NaHCO_3$ can be used as a short preservative These results were supported by **Barabas, (1995)** and agree with the finding of **Hamid et al. (2003) and Rahman et al. (2018).**

The results in Table (3): showed that there were significant differences ($p \leq 0.003$, 0.000,0.001, 0.000, and 0.024 respectively for the milk constituents SNF, TS, Lactose, Casein, Lactic acid, and the Acidity % after adding $NaHCO_3$ while there are no significance differences for the changes in Fat % and protein % (0.155 and 0.554) respectively, these finding were agreed with the finding of **Hamid et al. (2003)** and **Rahman et al. (2018).** However, **Sarwar et al. (2007)** found that high Sodium bicarbonate added to diet not only increased dry matter water intake, and milk yield but also increased the milk fat %.

It was observed that the addition of 0.3 % of $NaHCO_3$ is enough to preserve milk samples for up to 12 hours at room temperature (32-34°C). It may be concluded that NaHCO3 is an effective microbiologically and chemically for neutralizing the acids produced by acid-producing bacteria and significantly reduced viable cell counts and biofilm formation in a concentration-dependent manner as supported by the finding of **Jaikumpun et al. (2020)** who proved that NaHCO3 increases the permeability of the bacterial cell membrane, thus reducing cell viability. So, NaHCO3 can be used for short-term preservation of milk where scientific cooling or pasteurization facilities are not available, similar findings were reported by **Biswas,(1997).**

CONCLUSION

According to our result, sodium bicarbonate displayed antibacterial and anti-biofilm activity against *S. aureus* and *K. pneumoniae isolated* from milk. Besides it increased the inhibition activity of some antibiotics. So, further studies were recommended to determine if it may be used alone or as an adjuvant with antibiotics especially β- lactam, in the treatment of *K. pneumoniae* and S. *aureus* infection and/or biofilm-related complicated infection in dairy cattle. Moreover, sodium bicarbonate exhibits a successful role in the short -term preservation of milk where scientific cooling or pasteurization facilities are not available.

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