Research Article

Efficiency of Lactoferrin to Eradicate Multidrug Resistant *Staphylococcus aureus* Isolated from some Dairy Products

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Abstract | This investigation was done to detect the percent of multidrug resistant (MDR) *Staphylococcus aureus (S. aureus)* in some dairy products as well as to assess the effectiveness of lactoferrin (LF) as a bio preservative for yogurt. 150 dairy product samples from yogurt, ice cream and Damietta cheese (50 for each) were collected from Assiut city, Egypt. Antimicrobial susceptibility against antibiotics commonly utilized in human and animals was tried using the disc diffusion method; PCR was applied on (MDR) *S. aureus* isolates for discovering of *blaz, mecA* and *VanA genes.* 38% of yogurt samples had the highest prevalence of *S.aureus* followed by Damietta cheese (30%) and ice cream (14%). *S.aureus* isolates appeared high resistance to tetracycline, penicillin, oxacillin, ampicillin, streptomycin, amoxicillin/clavulanate and neomycin, in different percentages. *blaz, mecA* and *VanA genes* were detected at 60% for *blaz* gene, 40% for *mecA* gene and 20% for *vanA* gene. Lactoferrin has a satisfactory antibacterial activity Minimum Inhibitory Concentration (MIC) at 10mg/ml and Minimum Lethal Concentration (MLC) at 40mg/ml. The results revealed that 40mg/ml LF in yogurt could inhibit MDR *S. aureus* at 2nd day while, 20mg/ml at the 4th day. The study concluded that LF can be used as a bio preservative in yogurt due to its highest antimicrobial activity and acceptable sensorial properties.

Keywords | MDR, Antibiotic resistance, Sensory evaluation, Yogurt, Ice cream, Damietta cheese

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INTRODUCTION

Staphylococcus aureus is a significant and costly public health concern since it may enter the human nourishment chain and usually causing foodborne illness (Liu et al., 2022). Milk and milk products are known to be a source of *S. aureus* contamination whether they are collected from dairy animals enduring mastitis or from food handlers carrying the organism because of poor individual cleanliness (Bingol et al., 2012).

Moreover, the huge extent of Multidrug Resistant (MDR) *S. aureus* isolates may represent an open wellbeing chance due to the spread of drug-resistant zoonotic *S. aureus* (Gebremedhin et al., 2022).

The development of such resistant strains plays a fundamental position in restorative disappointment in each human and animal disease. The uncontrolled utilization of antibiotics in human and animals, in conjugation with terrible demonstrative methods and improper endorsing by way of unfit doctors, exacerbates the problem and constitutes a top-notch mission for the avoidance and control of this pathogen (Kimang'a, 2012). So, the consumers wanted to take natural compounds, an effective and non-antibiotic antimicrobial agent. Lactoferrin (LF) proved to be the goal of this concept since, it is a multifunctional protein to inhibit the growth of planktonic MDR *S. aureus* form (Sinha et al., 2013, Reznikov et al., 2018) or its quorum form (biofilm formation) (Ammons and Copié, 2013, Quintieri et al., 2020) rather than modulating the host immune status. Thus, it is recommended to be as a food additive (Duran and Kahve, 2017) especially those dairy foods.

So, the reason for this study was to detect MDR *S. aureus* in some dairy products. As well as, to assess the antibacterial activity of lactoferrin and its sensorial properties as a bio preservative in yogurt.

MATERIALS AND METHODS

Collection of samples

150 samples of dairy products items (yogurt, ice cream and Damietta cheese) 50 of each were collected from general stores and dairy shops in Assiut city, Egypt in sterile partitioned tubes, named and carried on ice box to be exchanged with the least delay to the research laboratory for bacteriological examination.

Identification and Enumeration of *Staph. aureus*:

From each sample, 1 gm was inoculated into 9 ml saline, and shacked well to adopt 10-fold serial dilution process, then a loopful was streaked on Baird Parker (Oxoid) agar for enumeration (ISO 6888-1: 2021) and suspected colonies (golden yellow with hallow zone) were picked up and sub cultured onto both blood (Difco) and mannitol salt (HiMedia) agar. According to Quinn et al. (2011), the suspected colonies were subjected to the biochemical affirmation (catalase, hemolysin, and coagulase tests).

Antimicrobial susceptibility test

It was carried out by Kirby-Bauer disc diffusion method (CLSI, M100 2020) utilizing Muller Hinton agar where each strain was tried against 10 antimicrobial discs; penicillin (PEN) ampicillin (AMP), oxacillin (OXA), amoxicillin /clavulanate (AMC), tetracycline (TET), neomycin (N), streptomycin (S), marbofloxacin (MAR), cefotaxim (CTX)and vancomycin (VA) (Oxoid,). The breadth of the inhibitory zone was measured with a caliper, and the comes about were recorded and deciphered using CLSI criteria.

Multiple Antibiotic resistant Index (MARI)

Resistance was calculated to decide the MARI that was defined as a/b, where (a) spoken to the number of antibiotics to which the isolated strain was resistant and (b) spoken to the number of all tested antibiotics. (Kumar et al., 2012). The isolate that appeared resistant to three or more distinctive classes of antimicrobials was considered multi drug resistant (MDR) (Magiorakos et al., 2012). Isolates with MARI values of more than 0.2 were considered exceedingly resistant.

Molecular genotyping study

Was done on isolated MDR *S. aureus* strains for the detection of *23S rRNA*, *blaz*, *mecA* and *vanA* genes in the Reference Research Laboratory for veterinary quality control on poultry production in Animal Health Research Institute, Dokki, Giza, Egypt. DNA extraction from samples was performed utilizing the QIAamp DNA scaled-down unit (Qiagen, Germany, GmbH) with adjustments from the manufacturer's suggestions. Primers used were supplied from Metabion (Germany) and are recorded in Table (A).

Detection of minimum inhibitory concentration (MIC) and minimum lethal (MLC) concentration of LF

Against the different isolated strains were carried out using the broth dilution method (Stephen, 2005). Pure Lactoferrin was purchased from Canada, lactovegetarian, EN: 131947, item number AOR 04110. The following concentrations of lactoferrin solution were prepared, (40, 20, 10, 5, 2.5, 1.25 & 0.65 mg/ ml w/v) in distilled water and sterilized by 0.45 mm filter and freshly used. Genotyped strains of MDR *S.aureus* were sub cultured onto 5% sheep blood agar plates and brooded aerobically at 37 °C for 24h. Chosen 3-4 colonies and inoculated in tryptic soy broth then incubated at 37 °C for 2-6 h. Suspensions turbidity was balanced to coordinate with 0.5 McFarland standards and then diluted to obtain a last concentration of 10^5 CFU /ml approximately. Bi-fold serial dilution of lactoferrin was prepared separately using sterile Muller Hinton broth. Each tube was injected with a suspension of 100 µL from CFU/ml. The inoculated tubes together with the control positive tube (tubes contained broth only) and negative control (non-inoculated either MDR *S. aureus* or LF) were brooded aerobically at 37 °C for 24h. The MIC of LF was identified as the most reduced concentration of LF that inhibits the growth of the organism with a lack of visible turbidity. To determine the MLC, 100 µL from each clear tube (no visible growth) was spread onto sterile Muller Hinton agar (Oxoid, UK) for 24 hours of incubation. MLC was detected as the lowest concentration of LF that killed the tested MDR *S. aureus* organisms (no growth on the plate). The mean MIC and MLC were recorded from triple readings in each test.

In vitro agar well diffusion testing of LF anti MDR *S. aureus* activity

MDR *S. aureus* was spread uniformly on the dried surface of a Muller Hinton Agar plate by utilizing a sterile cotton swab. Multiple wells of 6 mm were made within the agar plate by using sterile cork pourer 50 µL of LF were inoculated in each of the wells containing distinctive concentrations. The plates were incubated for 24 h at 37°C ± 1°C,

Table A: Primers arrangements, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Ref.
				Secondary denaturation	Annealing	Extension		
S. aureus 23S rRNA	ACGGAGTTACAAAGGACGAC AGCTCAGCCTTAACGAGTAC	1250	94°C 5 min.	94°C 30 sec.	55°C 1 min	72°C 1.2 min.	72°C 12 min.	Bhati et al., 2016
blaZ	TACAACTGTAATATCGGAGGG CATTACACTCTTGGCGGTTTC	833	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 50 sec.	72°C 10 min.	Bagcigil *et al.* 2012
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310	94°C 5 min.	94°C 30 sec.	50°C 30 sec.	72°C 30 sec.	72°C 7 min.	McClure *et al.*, 2006
vanA	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	885	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 50 sec.	72°C 10 min.	Patel *et al.*, 1997

under aerobic conditions. After incubation inhibition of the bacterial growth was measured in mm. The tests were made in triplicate (Elsherif and Ali, 2019).

In vivo testing of LF anti MDR S. aureus activity:

Bacterial suspension inoculation: A fresh culture of MDR *S. aureus* isolate was adjusted to MacFarland 0.5 standard, where the growth density was adjusted to match (4.5 log) to be standard inoculum strain (Ruparelia et al., 2008).

Yogurt preparation: Yogurt was prepared according to (Zakaria et al., 2020) with slight modification, Raw buffalo's milk used for yogurt manufacturing was purchased from local markets in Egypt. Lyophilized starter cultures containing rise to blend of *Streptococcus thermophilus* and *Lactobacillus delbrueckii sub spp bulguricus* (YoFlex® Express 2.0 Chr-Hansen, Denmark), which in customary utilize in dairy plants, were used. Crude buffalo's milk was pasteurized at 85 °C for 5 min in a stainless steel two fold coat holder some time recently being cooled to inoculation temperature (42°C). After cooling, one ml of arranged standard inoculum of MDR *S. aureus* was mixed well and divided into suitable jugs. One of them was cleared out to be as control positive and LF was added to the others with 10, 20 and 40 mg/ml. Then the starter culture was inoculated at a concentration of 1:1000 and the blends were poured into Polyethylene yogurt cartons (200 gm capacity) and kept at 42 °C in an incubator. Then kept at the refrigerator at 4° C. A negative control made up of buffalo milk samples without the addition of LF was made in parallel. The inoculated jars have been examined bacteriologically for the existence of viable inoculated MDR *S.aureus* by streaked a loopful onto Baird-Parker plates at 37°C for 24-48h as time zero experiment, after curdling and every 2 days until the cease of the experiment (6th day).

Sensory evaluation: Control yogurt jars (free from the preceding microorganism however inoculated with lactoferrin concentrations of 10, 20 and 40mg/ml respectively) had been prepared as already said and each one was once subjected to the going before medications. Thirty panelists had been chosen in different ages and instruction to style the trials. Different concentrations of lactoferrin were once studied with recognize to three one-of-a-kind attributes (odor, taste and over all acceptability (OAA) (Fernandes et al., 2008). The arrangement of settlement utilized to be scored as percentages.

Statistical Analysis

The factual examination was performed utilizing programs GraphPadPrism 5.04 (GraphPad, Inc., San Diego, USA) and measurable 12.0 (Dell, Inc., Tulsa, USA). The bacterial count was represented by mean ±SE. The data was represented by utilizing the Microsoft Excel Spreadsheet.

RESULTS

As shown in Table (1) *S. aureus* could be detected in Yogurt, Ice cream and Damietta Cheese samples at percentages of 38, 14 and 30% respectively. The *Staphylococcus aureus* count ranged from 2.6 to 4.3 with a mean value of 3.5±2 log $_{10}$cfu/ g in yogurt samples while in ice cream samples ranged from 1.6 to 3.3 with a mean value 2.9±1.5 log $_{10}$cfu/ g and in Damietta Cheese samples it extended from 1 to 4.3 with a mean value 3.2±2.1 log $_{10}$cfu/ g.

Table 1: Statistical analytical results of *S. aureus* examination of examined dairy products.

Samples types	Results of *S. aureus* counts (log $_{10}$cfu/ g)				
	Positive Samples		Min.	Max.	Mean ±SE
	No./50	%			
Yoghurt	19	38	2.6	4.3	3.5±2
Ice Cream	7	14	1.6	3.3	2.9±1.5
Damietta Cheese	15	30	1	4.3	3.2±2.1
Total	41	27.3	5.2	11.9	9.6±5.6

Table (2) declared that the resistance rate was 100%, 95.1%, 92.7%, 87.8%, 85.4%, 80.5% and 73.2% for tetracycline, penicillin, ampicillin, amoxicillin/clavulanate, streptomycin, oxacillin and neomycin, respectively. Resistance rates were observed against marbofloxacin, ceftiofur and vancomycin was (0.00%, 2.4% and 7.3%), respectively. Determination of the multiple antibiotic resistance index (MARI) of the isolates shows that most of isolates were extremely resistant to three or more antibiotics with over all mean value about 0.54.

Suspected isolates which show multi drug resistance phynotyically were examined by PCR for genotypically assessments of *23S rRNA gene, blaz gene, mecA gene* and *vanA gene*. Our results clarified that all tested isolates were harbored *23S rRNA ,blaz, mecA* and *VanA genes* at 100, 60, 40 and 20% as in Photo 1, 2, 3 and 4.

At Table 3, Lactoferrin proved to have antibacterial activity against MDR *S. aureus* at 10 mg/ml for MIC and 40 mg for MLC. Zone of inhibition for 40 mg was (29± 1.7 mm diameter) followed by 20 mg and 10 mg as 22± 2.1mm and 13.4±2.7 mm, respectively.

Table 2: Drug resistance of *S. aureus* strains isolated from dairy products (n = 41).

Antibiotic	Resistant		Sensitive	
	No.	%	N0.	%
Tetracycline TE 30 mcg	41	100	0	0
Penicillin P 10 mcg	39	95.1	2	4.9
Ampicillin AMP 10 mcg	38	92.7	3	7.3
Amoxicillin/clavulanate AMC 20/10 mcg	36	87.8	5	12.2
Streptomycin S 10 mcg	35	85.4	6	14.6
Oxacillin OXA 1mcg	33	80.5	8	19.5
Neomycin N 30 mcg	30	73.2	11	26.8
Vancomycin VAN 30 mcg	3	7.3	38	92.7
Ceftiofur XNL	1	2.4	40	97.6
Marbofloxacin MAR	0	0	41	100
MDRI	0.54			

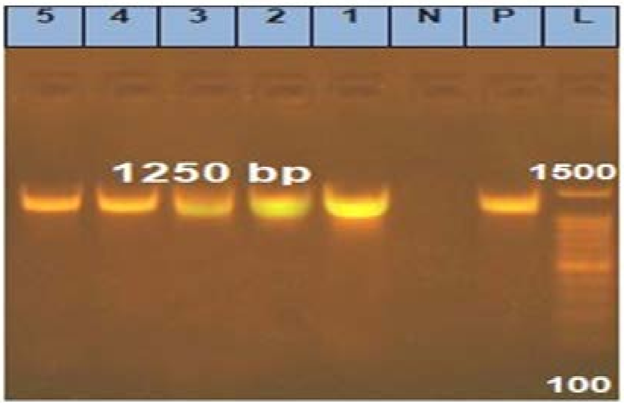

Photo 1: The amplified *S.aureus* 23S rRNA gene (1250 bp) recovered from multidrug resistant *S.aureus* (MDR) isolates. **Lane L:** Molecular marker; **Lane P:** Positive control; **Lane N**: Negative control; **Lane 1, 2, 3, 4, 5**: positive isolates.

As shown in Figure 1 we used three concentrations of lactoferrin 40, 20 and 10mg/ml. 40 mg/ml reduced the count of *S.aureus* at 1st day and completely inhibit its growth at 2nd day but 20 and 10mg/ml inhibit *S.aureus* growth at 4th and 6th day, respectively.

Figure 2 represented that fortification of milk with lactoferrin did not interfere with yogurt manufacture and the sensory properties of produced yogurt were acceptable.

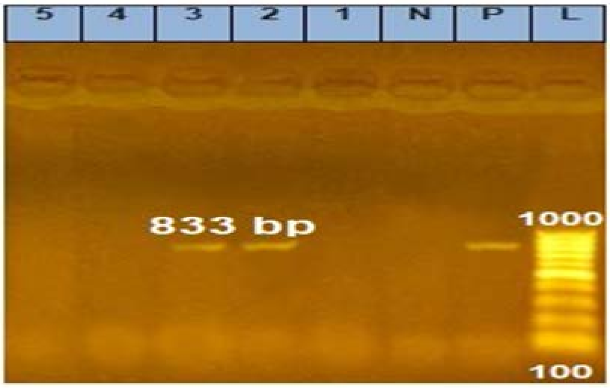

Photo 2: The amplified *blaz* gene (833 bp) of *S. aureus* for detection of penicillin resistance recovered from multidrug resistant *S. aureus* (MDR) isolates. **Lane L:** Molecular marker; **Lane P:** Positive control; **Lane N**: Negative control; **Lane 1, 4, 5:** negative isolates for resistance to penicillin; **Lane 2 and 3**: positive isolates for resistance to penicillin

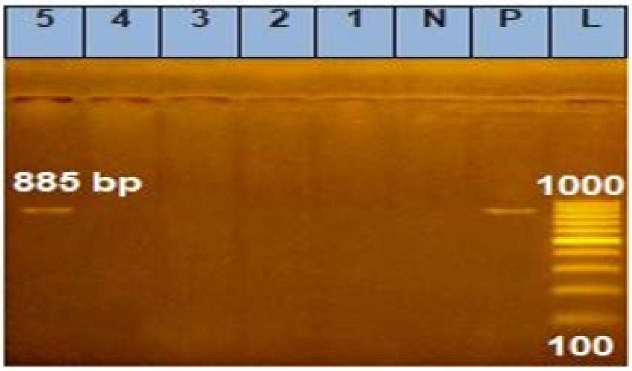

Photo 3: The amplified *mecA* gene (310 bp) of *S. aureus* for detection of methicillin resistance recovered from multidrug resistant *S. aureus* (MDR) isolates. Lane L: Molecular marker; Lane P: Positive control; Lane N: Negative control; Lane 1, 2 and 3: positive isolates for resistance to methicillin. Lane 4 and 5: negative isolates for resistance to methicillin.

Photo 4: The amplified *vanA* gene (885 bp) of *S.aureus* for detection of vancomycin resistance recovered from multidrug resistant *S.aureus* (MDR) isolates. **Lane L:** Molecular marker; **Lane p:** Positive control; **Lane N:** Negative control; **Lane 5**: positive isolate for resistance to vancomycin; **Lane 1,2,3 and 4**: negative isolate for resistance to vancomycin

Table 3: The inhibitory effect of different concentrations of lactoferrin on MDR *S. aureus* isolates:

Concentrations	Zone of inhibition (mm)
40mg/ml	29± 1.7
20mg/ml	22± 2.1
10mg/ml	13.4±2.7
5mg/ml	0
2.5mg/ml	0
1.25mg/ml	0
0.65mg/ml	0

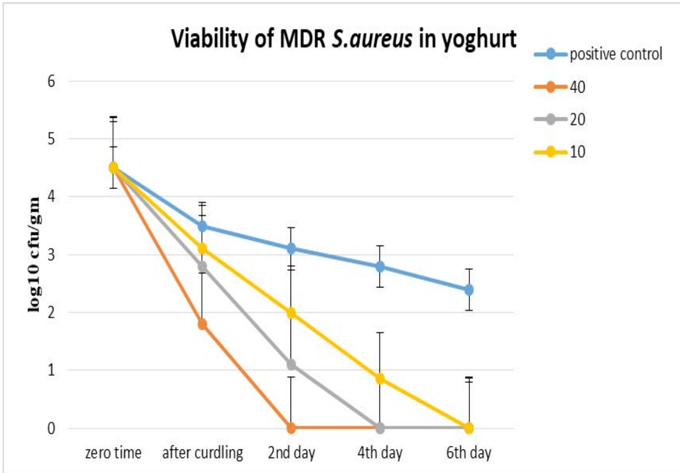

Figure 1: Anti-microbial properties of different concentrations of lactoferrin on MDR in yoghurt.

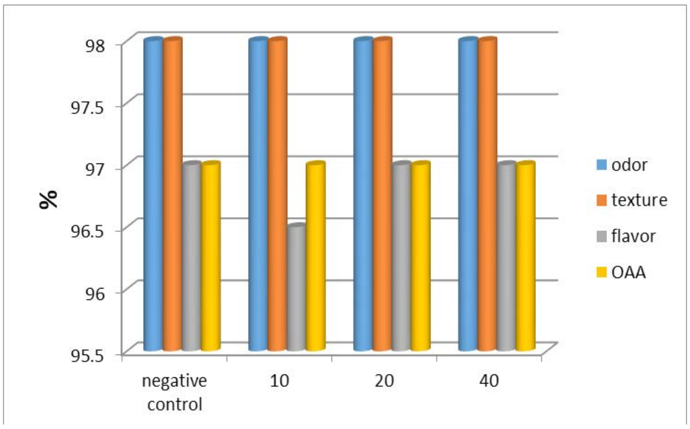

Figure 2: Sensory evaluation of yoghurt after addition of different concentrations of lactoferrin.

DISCUSSION

S. aureus is one of the foremost important food-borne pathogens universally because it produces different heat stable toxins (Ahmed et al., 2019) and invasive enzymes (Dai et al., 2019). Indeed post pasteurization, this micro-organism or its enterotoxins might still stay in pasteurized milk (Rall et al., 2008, Dai et al., 2019) threatening their human consumers. The present work showed that *S. aureus* was recognized in yogurt, Damietta cheese and ice cream (Table 1). Our comes about were concurred with those detailed by Ibrahim et al. (2015), Ahmed et al. (2019) and Zeinhom and Abed, (2021). But lower than Hanaa and Jehan (2015) and higher than Sasidharan et al., (2011), Mashael and Ashwag (2021).The undesired existence of *S. aureus* in Egyptian dairy products was recognized by a few thinks about (Hanaa and Jehan 2015, Ibrahim et al., 2015, Ahmed et al., 2019, Zeinhom and Abed, 2021) declaring environmental pollution, cross contamination between the milk and each other or poor handling during transportation.

The human potential hazard will be maximized when the contaminant is MDR *S. aureus*, so the display investigation was outlined to think about the MDR design of the isolated *S. aureus* strains. The study showed in (Table 2) the distinctive resistance percentage for tetracycline, penicillin, ampicillin, amoxicillin/clavulanate, streptomycin, oxacillin and neomycin and MDRI were with an overall mean of 0.54. Since, usually nearly agreed with past work of Bakheet et al. (2017) and Sineke et al. (2021) who cited that MARI was 0.61 and 0.48 , respectively. On the other hand lesser file was detailed by Amoako et al. (2019) and Aliyu et al. (2020) as 0.23 and 0.3, respectively. MDRI value just ≥ 0.2 is considered high and might be originated from environments with abuse of antibiotics where resistance developed and spread (Subramani and Vignesh 2012). It is curiously to note that resistance rates were watched against marbofloxacin, ceftiofur and vancomycin was low. These comes about were similar with that gotten by Firouzi et al. (2010), Kroemer et al. (2012), Idriss et al. (2014) and Aliyu et al. (2020). The high efficacy of these antibiotics may be attributed to their recent use and expensive costs in veterinary medicine. The isolated MDR *S.aureus* were public health hazardous pathogens.

The *23S rRNA* sequencing was a powerful instrument for the recognition of *S. aureus* isolated from the examined samples which clarified that all isolates harbored both *23S rRNA* and *nuc* genes. Bands with approximate size of 1250 bp were detected for *23S rRNA* gene (Photo 1).
S. aureus produces an extracellular thermo stable nuclease that encoded by *nuc* gene; one of the foremost effective recognizing characteristics for *S. aureus* from other *Staphylococcus spp.* Therefore, *nuc* gene was cautioned as a unique marker gene (Sahebnasagh et al., 2014).

Penicillin used to be notably very positive in opposition to most staphylococcal infections, but *S. aureus* started out producing β-lactamase enzyme in the mid1940s, which destroys the penicillin β-lactam ring. Later, more than 90% of *S. aureus* strains had been penicillin resistant. (Khan et.al., 2013). So, to distinguish the resistance to penicillin t hrough the production of β-lactamase due to the presence of *blaz* gene, Bands with approximate size of 833 bp were detected (Photo 2).

In agreement with our study the *blaz* gene was identified in 59.2% to 65 and 97% (Shukla et al., 2004; Naas et al., 2005; Christine et al., 2021; Ahmed et al., 2018). High *blaz* genes might demonstrate the expand utilization, and conceivably misuse, of β lactams in the study farms (Yang et al., 2016). Shifeng et al. (2021) and Schmidt et al. (2017) encoding for the beta-lactamases *blaz* gene in staphylococci at 42.9% and 28.8% of *S.aureus* isolates.

Antimicrobial resistance in methicillin-resistant strains of *S.aureus* (MRSA) is related with the securing of a cell genetic element alluded to as the staphylococcal cassette chromosome *mec*, which carries the *mecA* gene, encoding the low-affinity penicillin-binding protein 2a and confers resistance to the β-lactam antibiotics (Katayama et al., 2000). So, our outcomes revealed the presence of *mecA* gene in (40%) of the examined samples. Bands with approximate size of 310 bp had been detected for *mecA* gene (Photo 3).

Our results were lower than Zeinhom and Abed, (2021) and Christine et al. (2021) who confirmed the presence of *mecA* gene in 66.7 and 75% of MDR *S. aureus* isolates, also (Shukla et al., 2004; Naas et al., 2005) found a really tall extent of methicillin resistant *S. aureus* (MRSA) strains with rates of 77% to 100%. The presence of *mecA* in MDR *S.aureus* have been detailed around the world in numerous previous studies (Kreausukon et al., 2012; Awad et al., 2017; Abed et al., 2018).

Vancomycin has been a viable operator in a restriction to MRSA infections for decades (Yan guang et al., 2020). But in July 2002, the circumstances changed when the Centers for Disease Control and Prevention (CDC) in the USA archived the first sample of *S.aureus* that used to be resistant to both vancomycin and methicillin (CDC, 2002) So, we examined resistance of vancomycin by using *vanA* gene and (20%) of isolates were resist to vancomycin. Bands with approximate dimension of 885 bp had been detected for *vanA gene* (Photo 4).

Antibiotic have been utilized in animal production during the last decades worldwide resulted in emergence and increment resistance bacteria to antibiotics (Abadi et al., 2019), so, getting rid of food MDR *S. aureus* is of extraordinary concern. As antimicrobial peptides (AMPs) are considered as promising approaches leading to novel potential antimicrobial drugs (León-Buitimea et al., 2020), LF which is an iron protein found in human and bovine milk is considered as an AMP and multifunctional glycoprotein of the innate immune system (Zarzosa-Moreno et al., 2020) owing to binding to bacteria and their cell wall products (Kell et al., 2020) that called heparan sulfate proteoglycans (HSPGs), which are cell-surface and extracellular matrix macromolecules (Lang et al., 2011). Within the present study, the *in vitro* broth dilution of LF revealed MIC & MLC values against MDR *S. aureus* as 10 & 40 mg/ml respectively (Table 3). With agar diffusion method different concentrations of lactoferrin (0.65, 1.25, 2.5, 5, 10, 20 and 40mg/ml) against *S.aureus* to decide the suitable concentration to be applied in yogurt manufacturing. The results showed that 40mg/ml was the best one while 10mg/ml was the minimum inhibitory concentration.

Several authors have reported antibacterial activity of lactoferrin in vitro against pathogens and reported that lactoferrin prevented the increase of *S. aureus* population (Murdock and Matthews 2002, Da Silva et al., 2012; Ombarak et al., 2019).

Also, Kutila et al. (2003), illustrated the antibacterial impact of lactoferrin was tried on isolates of *(S. aureus)* originally isolated from bovine mastitis but in lower concentrations 0.67 mg/ml, 1.67 mg/ml, and 2.67 mg/ml.

Subsequently, the *in vivo* study was designed to inoculate fresh prepared yogurt with 40 (MLC dose), 20 and 10 (MIC dose) mg/ml, where the MLC dose reduced the count of MDR *S. aureus* at the 1st day and completely inhibit its growth at the 2nd day, while the (MIC dose) inhibited MDR *S. aureus* development at the 6th day but 20 mg of LF killed MDR *S. aureus* growth at the 4th day, respectively. MDR *S. aureus* strain could survive in the positive control group (induced infected and non LF inoculated) up to the 6th day with a mean count 2.4 $\log_{10}$ cfu/gm, it could be detected but in reduced count and that may be due to increasing the acidity of yogurt, *S.aureus* organisms are the most sensitive bacterial species to acidity (Bergdoll and Lee Wong, 2006) declaring that MLC dose was promising to fight the MDR *S. aureus* in dairy food manufacture and production.

It was once discovered that, as the lactoferrin degree in the item expanded, the bacterial boom reduced dramatically in contrast to the control, thereby growing the shelf life of some dairy products, (Shashikumar and Puranik, 2011).

The antibacterial activity of lactoferrin is mainly via 2 mechanisms, the first involves sequestration of iron in sites of infection, which deprives the bacteria from this nutrient and causes a bacteriostatic effect (Superti, 2020). The second mechanism is the direct interaction between lactoferrin molecule and the infectious microorganism (Latorre et al., 2010).

Lactoferrin interacts with lipopolysaccharide to destroy the outer membrane of Gram-negative bacteria (Leitch and Willcox, 1999). In some cases, positively charged amino acids in lactoferrin can interact with anionic molecules on certain bacterial, viral, fungal, and parasite surfaces, causing cell lysis (Gruden and Ulrih, 2021; Kell et al., 2020; Roseanu et al., 2010).

Through the sensory experiment, since the all groups (control negative as well as the three treated groups) did not alter in odor, texture and over all acceptability, LF is recommended to be added. The addition of bovine lactoferrin to yogurt did not considerably influence the physicochemical properties of this fermented product. However, the growth of Lactic Acid Bacteria (LAB) is upgraded with the addition of LF (Franco et.al., 2010). Also, Zakaria et al. (2020) found that fortifying of milk with lactoferrin did not interfere with yogurt manufacture and the sensory properties of produced yogurt was acceptable.

CONCLUSION

The study showed that *S.aureus* is widely prevalent especially in yogurt (38%) which may cause a public health risk due to its widespread consumption and concluded that most of the isolated *S. aureus* in dairy products are MDR against several antibiotic groups with high MAR index and different prevalence rates (yogurt, cheese and ice cream respectively). LF proved to be a good promising food preservative at a dose ≥ 40 mg/ml and it is considered a suitable food preservative in yogurt due to its powerful antibacterial activity and its good sensorial properties.

ABBREVIATIONS

LF: lactoferrin, MRSA: methicillin resistant Staphylococcus aureus, VARSA: vancomycin resistant Staphylococcus aureus, MDR: multi drug resistant.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

FUNDING

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ETHICS APPROVAL

This research did not involve experiments on humans or animals and received the ethical approval of the Animal Health Research Institute, Agriculture Research Center, Egypt.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study were sent to the journal.

AUTHORS' CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dina Nour- Eldin Ali and Sayed Al Habty. The first draft of the manuscript was written by Dina Nour- Eldin Ali and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

REFERENCES

Abed AH, Attia AR, Atia AA (2018). Genotyping of β-lactams resistant staphylococci isolated from bovine subclinical mastitis. Beni-Suef Univ. J. Basic Appl. Sci., 7: 499-504. https://doi.org/10.1016/j.bjbas.2018.05.004

Abadi A, Talebi B, Albert AR, Thomas H, Nataliya LB (2019). World Health Organization Report: Current Crisis of Antibiotic Resistance. BioNanoScience (2019) 9:778–788. https://doi.org/10.1007/s12668-019-00658-4.

Ahmed HA, Al Sayed RAA, Ahmed A (2018). Genotyping of β-lactams resistant staphylococci isolated from bovine subclinical mastitis. Beni-Suef University J. Basic Appl. Sci. 7 (2018) 499–504. https://doi.org/10.1016/j.bjbas.2018.05.004

Ahmed AA, Maharik NMS, Valero A, Kamal, SM (2019). Incidence of enterotoxigenic *Staphylococcus aureus* in milk and Egyptian artisanal dairy products. 2019 Food Control., 104: 20–27. https://doi.org/10.1016/j.foodcont.2019.04.017

Aliyu Y, Abdullah IO, Whong CZ, Olalekan BO , Reuben RC (2020). Occurrence and antibiotic susceptibility of methicillin resistant Staphylococcus aureus in fresh milk and milk products in Nasarawa State, North-Central Nigeri, J. Microbiol. Antimicrob., 12(1): 32-41. https://doi.org/10.5897/JMA2020.0424

Ammons MC, Copié V (2013). Mini-review: Lactoferrin: a bioinspired, anti-biofilm therapeutic. J. Bioadhesion Biofilm Res. 29. 2013 - Issue 4. https://doi.org/10.1080/08927014.2013.773317

Amoako DG, Somboro AM, Akebe LK, Molechan C, Perrett K , Bester L , Sabiha, Y E (2019). Antibiotic Resistance in Staphylococcus aureus from Poultry and Poultry Products in uMgungundlovu District South Africa, Using the "Farm to Fork" Approach, Article in Microbial Drug Resistance• August 2019, https://doi.org/10.1089/mdr.2019.0201

Awad A, Ramadan H, Nasr S, Ateya A, Atwa, S (2017). Genetic characterization, antimicrobial resistance patterns and virulence determinants of Staphylococcus aureus isolated form bovine mastitis. Pak. J. Biol. Sci., 20(6): 298–305. https://doi.org/10.3923/pjbs.2017.298.305

Bagcigil AB, Taponen S, Koort J, Bengtsson B, Myllyniemi A, Pyörälä S (2012). Genetic basis of penicillin resistance of S. aureus isolated in bovine mastitis. Acta Vet. Scandin. 2012, 54:69. https://doi.org/10.1186/1751-0147-54-69

Bhati T, Nathawat P, Sharma SK, Yadav R, Bishnoi J, Kataria AK (2016). Polymorphism in *spa* gene of *Staphylococcus aureus* from bovine subclinical mastitis. Vet. World., 2016;9:421-424. https://doi.org/10.14202/vetworld.2016.421-424

Bakheet AA, Ali MN, Al-Habaty SH, Nasef S (2017). Detection of Disinfectant resistant aerobic bacteria in un hatched chicken eggs. BVMJ -32 (2): 284-259.

Bergdoll MS, Lee Wong, AC (2006). Staphylococcal intoxication. In: Foodborne Inections and intoxication, (Eds) H.P. Reimann and D.O.Cliver, 523-562. Elsevier. https://doi.org/10.1016/B978-012588365-8/50018-9

Bingol EB, Cetin O, Colak H, Hampikyan H (2012). Presence of enterotoxin and verotoxin in Turkish cheeses sold in Istanbul. Turkish J. Vet. Anim. Sci., 36: 424–432. https://doi.org/10.3906/vet-1105-5

CDC Centers for Disease Control and Prevention (2002). Staphylococcus aureus resistant to vancomycin--United States. MMWR Morb Mortal Wkly Rep. 2002;51:565.

Christine MB, George CG, Paul JP, Benard W, Kulohoma C, Mulei M, Rawlynce B (2021). Antimicrobial Resistance Profiles and Genes of Staphylococci Isolated from Mastitic Cow's Milk in Kenya Antibiotics 2021, 10, 772. https://doi.org/10.3390/antibiotics10070772 https://www.mdpi.com/journal/antibiotics.

CLSI, M100 (2020). Performance standards for antimicrobial susceptibility testing: 30th edition. Informational supplement, Clinical Laboratory Standard Institute, Wayne, PA, USA, 2020.

Dai J, Wu S, Huang J, Wu Q, Zhang F, Zhang J, Wang J, Ding Y, Zhang S, Yang X, Lei T, Xue L, Wu H (2019). Prevalence and Characterization of Staphylococcus aureus Isolated From Pasteurized Milk in China. Front. Microbiol. 10:641. https://doi.org/10.3389/fmicb.2019.00641.

Da Silva AS, Honjoya E R, Cardoso S C, De Souza C H B, De Rezende Costa M, De Santana EHW, Aragon-Alegro LC (2012). Antimicrobial action of lactoferrin on *Staphylococcus aureus* inoculated in Minas frescal cheese. Arch. Latinoam. Nutr. 62(1): 68–72.

Duran A, Kahve HI (2017). The use of lactoferrin in food industry. Academic J. Sci., CD-ROM. ISSN: 2165-6282 :: 07(02):89–94.

Elsherif MW, Ali DN (2019). Antibacterial effect of silver nanoparticles on Antibiotic resistant *E. coli* O157:H7 isolated from some dairy products Bulgarian J. Vet. Med. ISSN 1311-1477; https://doi.org/10.15547/bjvm.2019-0027.

Fernandes JC, Tavaria FK, Soares JC, Ramos OS, Joao Monteiro M, Pintado ME, Xavier MF (2008). Antimicrobial effects of chitosans and chitooligosaccharides, upon Staphylococcus aureus and Escherichia coli, in food model systems. Food Microbiol. 25(7): 922-8. https://doi.org/10.1016/j.fm.2008.05.003

Firouzi R, Rajaian H, Tabaee MI, Saeedzadeh A (2010). In vitro antibacterial effects of marbofloxaciin on microorganisms causing mastitis in cows J. Vet. Res. 65, 1:51-55.

Franco I, Castillo E, Pérez MD, Calvo M, Sánchez L (2010). Effect of bovine lactoferrin addition to milk in yogurt manufacturing. J. Dairy Sci. 93 :4480–4489 doi: 10.3168/ jds.2009-3006 © American Dairy Science Association, 2010. https://doi.org/10.3168/jds.2009-3006

Gebremedhin EZ, Addisu BA, Bizunesh MB, Kebede AK, Nega DT, Lencho MM, Edilu JS (2022). Isolation and Identification of *Staphylococcus aureus* from Milk and Milk Products, Associated Factors for Contamination, and Their Antibiogram in Holeta, Central Ethiopia. Hindawi. Vet. Med. Int. Volume 2022, Article ID 6544705, 13 pages https://doi.org/10.1155/2022/6544705.

Gruden Š, Ulrih NP (2021). Diverse mechanisms of antimicrobial activities of lactoferrins, lactoferricins, and other lactoferrin-derived peptides. Int. J. Molecul. Sci., 22: 11264. https://doi.org/10.3390/ijms222011264

Hanaa MF, Jehan I (2015). Occurrence and Zoonotic Importance of Methicillin-Resistant Staphylococcus aureus in raw Milk and Some Dairy Products at Ismailia City, Egypt. Zagazig Vet. J. 43 (3): 95-104, 2015. https://doi.org/10.21608/zvjz.2015.28446

Ibrahim G, Sharaf OM, Abd El-Khalek AB (2015). Microbiological Quality of Commercial Raw Milk, Domiati Cheese and Kareish Cheese. Middle East J. Appl. Sci., 5(1): 171–176.

Idriss SHE, Foltys V, Tancin V, Kirchnerova K, Tancinova D, Zjecau K (2014). Mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Nitra, Slovakia, Slovak J. Anim. Sci., 47 (1): 33-38.

ISO 6888-1 (2021). International Organization for Standardization, 2021. Microbiology of Food and Animal Feeding Stuffs-Horizontal Method for the Enumeration of Coagulase-positive *Staphylococci* (*Staphylococcus aureus* and Other Species)- Part 1: Technique Using Baird-Parker Agar Medium.

Katayama Y, Ito T, Hiramatsu K (2000). A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. Antimicrob Agents Chemother. 2000 Jun;44(6):1549–55. https://doi.org/10.1128/AAC.44.6.1549-1555.2000

Kell D, Heyden E, Pretorius E (2020). The biology of lactoferrin an iron-binding protein that can help defend against viruses and bacteria. Front. Immunol., 11, 1221. https://doi.org/10.3389/fimmu.2020.01221.

Khan A, Hussain R, Javed MT, Mahmood F (2013). Molecular analysis of virulent genes (coa and spa) of Staphylococcus aureus involved in natural cases of bovine mastitis. Pak. J. Agric. Sci., 50: 739-743.

Kimang'a AN (2012). A situational analysis of antimicrobial drug resistance in Africa: are we losing the battle? Ethiop. J. Health Sci. 22:135–43.

Kreausukon K, Fetsch A, Kraushaar B, Alt K, Müller K, Krömker V, Zessin KH, Käsbohrer A, Tenhagen BA (2012). Prevalence, antimicrobial resistance, and molecular characterization of methicillin-resistant Staphylococcus aureus from bulk tank milk of dairy herds. J. Dairy Sci., 95(8): 4382–4388. https://doi.org/10.3168/jds.2011-5198

Kroemer S, Galland D, Guérin-Faublée V, Giboin H, Woehrlé-Fontaine F (2012). Survey of marbofloxacin susceptibility of bacteria isolated from cattle with respiratory disease and mastitis in Europe. 2012. Vet. Rec., 170, 53. https://doi.org/10.1136/vr.100246.

Kumar S, Manoharan M, Ilanchezian S, et al. (2012). Plasmid analysis and prevalence of Multidrug resistant Staphylococcus aureus reservoirs in Chennai city, India. Internet J. Microbiol. 2012;7–1. 40. Subramani S, Vignesh S. MAR index study and MDR character analysis of a few golden Staph isolates. Asian J. Pharm. Life Sci. 2012;2(2).

Kutila T, Pyörälä S, Saloniemi H, Kaartinen L (2003). Antibacterial effect of bovine lactoferrin against udder pathogens. Acta vet. Scand. 2003. 44: 35-42. https://doi.org/10.1186/1751-0147-44-35

Lang J, Yang N, Deng J, Liu K, Yang P, et al. (2011). Inhibition of SARS Pseudovirus Cell Entry by Lactoferrin Binding to Heparan Sulfate Proteoglycans. PLoS ONE. 6(8): e23710. https://doi.org/10.1371/journal.pone.0023710.

Latorre D, Puddu P, Valenti P, Gessani S (2010). Reciprocal interactions between lactoferrin and bacterial endotoxins and their role in the regulation of the immune response. Toxins., 2: 54– 68. https://doi.org/10.3390/toxins2010054

León-Buitimea A, Garza-Cárdenas CR, Garza-Cervantes JA, Lerma-Escalera JA , Morones-Ramírez JR (2020). The Demand for New Antibiotics: Antimicrobial Peptides, Nanoparticles, and Combinatorial Therapies as Future Strategies in Antibacterial Agent Design. Front. Microbiol. 11:1669. https://doi.org/10.3389/fmicb.2020.01669.

Leitch E, Willcox M (1999). Elucidation of the antistaphylococcal action of lactoferrin and lysozyme. J. Med. Microbiol., 48: 867– 871. https://doi.org/10.1099/00222615-48-9-867

Liu H, Dong L, Zhao Y, Meng L, Wang J, Wang C, Zheng N (2022). Antimicrobial Susceptibility, and Molecular Characterization of Staphylococcus aureus Isolated From Different Raw Milk Samples in China. Front. Microbiol. 13:840670. https://doi.org/10.3389/fmicb.2022.840670

Magiorakos AP, Srinivasan A, Care RB, Carmeli, Y, Falagas ME, Giske CG (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant Bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 2012 Mar;18(3): 268–81. https://doi.org/10.1111/j.1469-0691.2011.03570.x.

Mashael A, Ashwag S (2021). The prevalence of Staphylococcus aureus and methicillin resistant Staphylococcus aureus in milk and dairy products in Riyadh, Saudi Arabia. Saudi J. Biolog. Sci. 28 (2021) 7098–7104. https://doi.org/10.1016/j.sjbs.2021.08.004

McClure JA, Conly JM, Lau V, Elsayed S, Louie T, Hutchins W, Zhang K (2006). Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-susceptible from -resistant staphylococci. J. Clin. Microbiol. 44: 1141-114. https://doi.org/10.1128/JCM.44.3.1141-1144.2006

Murdock CA, Matthews KR (2002). Antibacterial activity of pepsin-digested lactoferrin on foodborne pathogens in buffered broth systems and ultra-high temperature milk with EDTA. J. Appl. Microbiol. 93: 850-856. https://doi.org/10.1046/j.1365-2672.2002.01762.x

Naas T, Fortineau N, Spicq C, Robert J, Jarlier V, Nordmann P (2005). Three-year survey of community-acquired methicillin-resistant Staphylococcus aureus producing Panton-Valentine leukocidin in a French university hospital. J. Hosp. Infect. 61: 321–329. https://doi.org/10.1016/j.jhin.2005.01.027

Ombarak RA, Marwa A, Saad A, Elbagory M (2019). Bio preservative Effect of Lactoferrin against Foodborne Pathogens Inoculated in Egyptian Soft Cheese "Karish Cheese"Alexandria. J. Vet. Sci. www.alexjvs.com. AJVS. Vol. 63 (2): 97-103 Oct. 2019 https://doi.org/10.5455/ajvs.76015

Patel R, UHL JR, Kohner P, Hopkins MK, Cockerill FR (1997). Multiplex PCR Detection of *vanA*, *vanB*, *vanC-1*, and *vanC-2/3* Genes in Enterococci. J. Clin. Microbiol., P. 703–707. https://doi.org/10.1128/jcm.35.3.703-707.1997

Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S, Hartigan PJ (2011). Veterinary Microbiology and Microbial Disease. 2nd ed., Wiley-Blackwell, J Wileyand Sons Ltd Publication, UK.

Quintieri L, Caputo L, Monaci L, Cavalluzzi MM, Denora N (2020). Lactoferrin-Derived Peptides as a Control Strategy against Skinborne Staphylococcal Biofilms. *Biomed.* 8(9):323. https://doi.org/10.3390/biomedicines8090323.

Rall VL, Vieira FP, Rall R, Vieitis RL, Fernandes A J, Candeias JM, et al. (2008). PCR detection of staphylococcal enterotoxin genes in Staphylococcus aureus strains isolated from raw and pasteurized milk. Vet. Microbiol. 132, 408–413. https://doi.org/10.1016/j.vetmic.2008.05.011

Reznikov EA, Sarah S, Comstock JL, Hoeflinger Mei, W, Michael J, Miller, S, Donovan M (2018). Dietary Bovine Lactoferrin Reduces *Staphylococcus aureus* in the Tissues and Modulates the Immune Response in Piglets Systemically Infected with *S. aureus* . *Curr. Develop. Nutrit.*, Volume 2, Issue 4, April 2018, nzy001, https://doi.org/10.1093/cdn/nzy001.

Roseanu A, Florian P, Condei M, Cristea D, Damian M (2010). Antibacterial activity of lactoferrin and lactoferricin against oral *Streptococci*. Romanian Biotechnolog. Lett., 15: 5788– 5792.

Ruparelia JP, Chatterjee AK, Duttagupta SP , Mukherji S (2008). Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater. 4, 707–716. https://doi.org/10.1016/j.actbio.2007.11.006

Sahebnasagh R, Horieh S, Parviz O (2014). The Prevalence of Resistance to Methicillin in Staphylococcus aureus Strains Isolated from Patients by PCR Method for Detection of mecA and nuc Genes. Iranian J. Publ. Health, 43 (1): 84-92.

Sasidharan S, Prema B, Yoga Latha L (2011). Antimicrobial drug resistance of *Staphylococcus aureus* in dairy products. Asian Pac J. Trop. Biomed. 2011; 1(2): 130-132. https://doi.org/10.1016/S2221-1691(11)60010-5

Schmidt T, Marleen MK, Marthie ME (2017). Molecular Characterization of Staphylococcus aureus Isolated from Bovine Mastitis and Close Human Contacts in South African Dairy Herds: Genetic Diversity and Inter-Species Host Transmission RIGINAL RESEARCH published: 06 April 2017 https://doi.org/10.3389/fmicb.2017.00511 Frontiers in Microbiology | www.frontiersin.org 1 April 2017 Volume 8

Shashikumar CSS, Puranik D (2011). Study on Use of Lactoferrin for the Biopreservation of Paneer. Trop. Agric. Res. 23 (1):70-76.

Shifeng W, Zhonga Y, Jun W, Harvey H, Yongxin Y, Rongbo F, Qijing D, Hongning J , Rongwei H (2021). Prevalence, Drug Resistance, and Virulence Genes of Potential Pathogenic Bacteria in Pasteurized Milk of Chinese Fresh Milk Bar .J. Food Prot. (2021) 84 (11): 1863–1867. https://doi.org/10.4315/JFP-21-094

Shukla SK, Stemper ME, Ramaswamy SV, Conradt JM, Reich R, Graviss EA , Reed KD (2004). Molecular characteristics of nosocomial and Native American community-associated methicillin-resistant Staphylococcus aureus clones from rural Wisconsin. J. Clin. Micobiol. 42:3752-3757. https://doi.org/10.1128/JCM.42.8.3752-3757.2004

Sinha M, Sanket K, Punit K, Sujata S, Tej PS (2013). Antimicrobial Lactoferrin Peptides: The Hidden Players in the Protective Function of a Multifunctional Protein. Int. J. Peptides. Volume 2013, Article ID 390230, 12 pages. http://dx.doi.org/10.1155/2013/390230.

Sineke N, Asante J, Amoako D, King Abia AL, Perrett K, Linda A, Bester LA, Essack SY (2021). Staphylococcus aureus in Intensive Pig Production in South Africa: Antibiotic Resistance, Virulence Determinants, and Clonality. Pathogens. 10, 317, https://doi.org/10.3390.

Stephen JC (2005). Manual of antimicrobial susceptibility testing, American Society for Microbiology, 2005. Library of Congress Cataloging-in-Publication Data, USA.

Subramani S, Vignesh S (2012). MAR Index Study and MDR Character Analysis of a few Golden Staph Isolates. Biology. Corpus ID: 86260443.

Superti F (2020). Lactoferrin from bovine milk: A protective companion for life. Nutrients., 12: 2562. https://doi.org/10.3390/nu12092562

Yan guang C, Sijin Y, Xiancai R (2020). Vancomycin resistant Staphylococcus aureus infections: A review of case updating and clinical features. J. Adv. Res. 21 (2020) 169–176. https://doi.org/10.1016/j.jare.2019.10.005

Yang F, Wang Q, Wang XR, Wang L, Li XP, Luo JY, Zhang SD, Li HS (2016). Genetic characterization of antimicrobial resistance in Staphylococcus aureus isolated from bovine mastitis cases in Northwest China. J. Integr. Agric. 2016, 15: 2842–2847. https://doi.org/10.1016/S2095-3119(16)61368-0

Zakaria AM, Zakaria H M, Abdelhiee EY, Fadl SE, Ombarak RA (2020). The Impact of Lactoferrin Fortification on the Health Benefits and Sensory Properties of Yogurt. J. Curr. Vet. Res. ISSN: 2636-4026 Journal homepage: http://www.jcvr.journals.ekb.eg. https://doi.org/10.21608/jcvr.2020.121536

Zarzosa-Moreno D, Christian AG, Luisa SR, Erick TL, Ricardo RMo, Hernández-Ramírez JO, Jesús SL, Mireya G (2020). Lactoferrin and Its Derived Peptides: An Alternative for Combating Virulence Mechanisms Developed by Pathogens. Molecules. 2020, 25, 5763. https://doi.org/10.3390/molecules25245763

Zeinhom MMA, Abed AH (2021). Prevalence, Characterization and control of Staphylococcus aureus isolated from raw milk and Egyptian soft cheese. J. Vet. Med. Res. (2021); 27 (2): 152–160. https://doi.org/10.21608/JVMR.2021.146885.

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