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Utilization of lactoferrin to inhibit *E. coli* and *S. aureus* isolates from milk and kariesh cheese

S.A. Yassin[1] and H.A. Abd El-hady[2]

[1]Department of Food Hygiene, [2]Department Bacteriology, Animal Health Research Institute, Kafrelsheikh Lab, Agriculture Research Center, Kafrelsheikh, Egypt

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Correspondence:
S.A. Yassin
shereen_color@yahoo.com

Abstract

This study aims identify *E. coli* and its β-lactamase encoding genes, *S. aureus* and its enterotoxin genes isolated from milk and Kariesh cheese. Moreover, we evaluated the antibacterial effect of lactoferrin against these pathogenic bacteria. Sixty samples in total (30 each of raw milk and Kariesh cheese) were collected from various retail-markets in Kafrel-Sheikh Governorate. The percentage of *E. coli* isolates found in raw milk and Kariesh cheese reached 43.3% and 36.6%, respectively, while *S. aureus* isolates were recorded at 50% and 23.3% (from raw milk and Kariesh cheese). Twenty-four strains of *E. coli* were serogrouped, of which 3 strains out of 24 were O_{17}, O_{91} and O_{159}, 6 strains were O_{127} and 9 strains were O_{26}. PCR analysis for β-lactamase encoding genes in *E. coli* indicated that all eight isolates were 100% positive for blaTEM and blaSHV genes while 5 (62.5%) *S. aureus* isolates were positive for enterotoxin production. Five (62.5%) isolates produced Seb, 2(25%) produced Sec while the Sea gene was not detected in *S. aureus* isolates. The results indicate that lactoferrin 5% had a significant inhibitory effect on *S. aureus* and *E. coli* when they were inoculated into Kariesh cheese. The findings show that dairies didn't take enough hygiene precautions, and we advise following stringent hygiene procedures when dairy products are milked, processed and distributed. To control the growth of *E. coli* and *S. aureus* in dairy products,lactoferrin is thought to be a potential strategy.

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Introduction

Food producers and consumers worldwide, including in Egypt, are concerned about access to wholesome and safe food. Milk and its products are considered a staple food for people of all ages, as they contain numerous components that make them a highly nutritious. However, these benefits also make them an excellent environment for the growth of microbes, and the risk of contamination increases if dairy milk or milk products are improperly processed or handled (1). Foodborne illnesses are a significant global issue. Consuming contaminated dairy products, which may have a natural taste and smell, but are infected with dangerous microbes such as Salmonella, *Escherichia coli*, *S. aureus*, Campylobacter jejuni, Bacillus cereus, and Listeria monocytogenes, a major cause of outbreaks (2). The presence of *E. coli* is a valid indicator of fecal contamination which suggests the potential existence of enteropathogenic and/or toxigenic microbes that pose a threat to public health (3). The regular use of antibiotics is no longer effective against bacteria, which has become a public health and clinical concern. Both developed and developing nations are becoming increasingly concerned about the rising rates of resistance among *E. coli* strains which is a major factor in the inability to treat both human and animal infections (4). *S. aureus* is one of the primary pathogens associated with the consumption of raw milk and dairy products, and it is a significant cause of food poisoning worldwide. According to (5), *S. aureus* can contaminate milk through infected producing animals or through human sources during milking

and handling through lesions on the hands or arms produced by the bacteria or through coughing and sneezing during respiratory diseases. Adding natural antimicrobials to dairy milk and milk products after processing can help reduce the likelihood of infection with these bacterial diseases. Lactoferrin is one such protein that shows promise as a bio preservative increasing the shelf life of dairy products, maintaining safety, and enhancing health by combating life-threatening disorders in newborns, respiratory infections, hepatitis and foodborne diseases that can all be treated with it (6). Lactoferrin is a naturally occurring protein that binds to iron and is a member of the transferrin protein family. It can be found in milk, saliva, and other mammalian excretory fluids (7). Lactoferrin has gained increasing attention because it is "Generally accepted as being Safe" according to the Food and Drug Administration (FDA) (8). Numrous studies have observed antiviral, antifungal, anti-inflammatory and antibacterial activities for this protein (7, 9). Two mechanisms primarily account for lactoferrin's antimicrobial effect. The first is the absorption of iron from infection sites, which serves as the microbes' primary food source. Thus, a bacteriostatic effect is produced. The second involves lactoferrin's direct interaction with the infection-causing agent since it contains high levels of amylase, DNase, RNase, and ATPase activity. Therefore, LF can suppress the organism by hydrolyzing the nucleic acids of bacteria (10).

Therefore, the objective of this research was to detect *S. aureus* and *E. coli* in milk and Kareish cheese sold in the retail market in KafrelShiekh Governorate. Additionally, we evaluated the activity of lactoferrin to prevent the growth of these harmful microbes.

Materials and methods

Ethical approve

The experimental design was performed in accordance with the Guidelines for Animal Experimentation of the Ethics Review Committee of the Animal Health Research Institute, Giza, Egypt (Approval No 24429, Approval date 13/6/2021).

Sample collection and preparation

The sixty samples (30 raw milk and 30 Kareish cheese) were obtained from dairy shops, local markets and supermarkets widely distributed across Kafrel-Shiekh Governorate, Egypt from January to June 2022.Within one hour of purchase all samples were transported to the lab for analysis inside an icebox (2-5°C). The milk sample (10 ml), was mixed with 90 ml of sterile buffered peptone water (Oxoid, Ltd, Basingstoke, UK). For the Kareish cheese, 25 grams of cheese were dispensed into a sterile flask containing 225 ml of sterile buffered peptone water and mixed using a Lab-blender for 2-4 minutes (11).

E. coli isolation and identification

For the prepared milk and cheese samples 1mL was mixed with 9 mL of MacConkey broth (Oxoid, Uk) and incubated for 24 hours at 37°C (12). After incubatin the MacConky broth was streaked onto MacConkey Agar (MCA) (Oxoid, UK) and Eosin Methylene Blue agar (EMB) (Oxoid, UK). The streaked plates were aerobically incubated at 37°C for 24 hours (13). Further biochemical tests were conducted to confirm the identification of the isolates (14). *E. coli* can be identified by serology as stated in (15). The isolates were identified serologically using rapid diagnostic *E. coli* antisera sets (Denka Seiken Co., Japan) to determine the Enteropathogenic types of *E. coli*.

Isolation and identification of *S. aureus*

The prepared samples were streaked onto 5% sheep blood agar (HiMedia, India) and Baird-Parked agar (Oxoid) and then incubated at 37°C for 24 hours. The presumptive colonies of *S. aureus* were selected for morphological and biochemical tests for further identification (16).

Detection of B-lactamase -encoding genes of *E. coli* and enterotoxin genes of *S. aureus*.

Extraction of DNA was carried out using the QIAamp DNA mini kit (Qiagen GmbH, Germany) with some modifications according to the manufacturer's instructions. The primers used were provided by Metabion (Germany) (Table 1). PCR amplification was performed using uniplex PCR. The PCR products were then electrophoresed on a 1% agarose gel (Applichem GmbH, Germany). After photographing the gel using a gel documentation system (Alph Innotech Biometra), the data were analyzed using computer software.

Lactoferrin

This investigation utilized bovine lactoferrin (China, Shaanxi Pioneer Biotech Co., Ltd). LF solutions concentrations of 3% and 5% were prepared in distilled water, sterilized using a 0.45 µm filter, and used immediately.

The antimicrobial activity of Bovine Lactoferrin against isolated *E. coli* and *S. aureus* by agar well diffusion test

The impact of LF on bacterial growth was investigated through the use of an agar well diffusion test to establish the appropriate concentration to be used in the making of cheese. Plates of trypticase soy agar (TSA) were prepared by adding 10 mL of semi-soft TSA (0.5%, w/v) per plate, and a concentration containing 100 µL (5×10^6 CFU/mL) of an overnight culture of each pathogen (*E. coli* and *S. aureus*) was prepared and evenly spread on the dry surface of the TSA plate using a sterile cotton swab. Using a clean cork pourer, several 6 mm wells were created in the agar plate and received a 10-µL aliquot from each concentration of bovine lactoferrin, which was left to air dry for five minutes. After

48 hours of incubation at 37°C, the plates were examined for zones of inhibition. The tests were performed in triplicate (19).

The antimicrobial activity of Bovine Lactoferrin on *E. coli* and *S. aureus* inoculation in Karish cheese

The Kariesh cheese was manufactured (20). In brief, cow's milk was pasteurized at 80°C for 15 seconds, cooled to 40°C and rennet, (3g /100 kg) from Hansen Laboratories (Copenhagen, Denmark) was added along with salt at a concentration of 1%. The milk was then inoculated with 5×10^5 CFU/g of each microbe (*S. aureus* and *E. coli*). The inoculated milk was separated into two main parts: The control part (C), which was inoculated with *S. aureus* and *E. coli* without the addition of LF, and the Treated part (T), which was inoculated with *S. aureus* and *E. coli* and treated with the proper concentration of lactoferrin that had the highest antibacterial action against *E. coli* and *S. aureus* determined by the agar well diffusion method. The impact of LF on bacterial growth was investigated through the use of an agar well diffusion test to establish the appropriate concentration to be used in the making of cheese. Plates of trypticase soy agar (TSA) were prepared by adding 10 mL of semi-soft TSA (0.5%, w/v) per plate, and a concentration containing 100 µL (5×10^6 CFU/mL) of an overnight culture of each pathogen (*E. coli* and *S. aureus*) was prepared and evenly spread on the dry surface of the TSA plate using a sterile cotton swab. Using a clean cork pourer, several 6 mm wells were created in the agar plate and received a 10-µL aliquot from each concentration of bovine lactoferrin, which was left to air dry for five minutes. After 48 hours of incubation at 37°C, the plates were examined for zones of inhibition. The tests were performed in triplicate (19).

The treated and control inoculated cheese were repackaged in polyethylene bags and kept at 5°C. To count the inoculated microbes, 25 g of cheese from both the control and treated parts were examined at zero-day, 1st, 3rd, 5th and 7th days of the storage period. Homogenization of the cheese samples weighing 25 g with 2% sodium citrate was performed, and tenfold serial dilutions were made on each day of examination for *S. aureus* on Baird-Parker media containing tellurite egg yolk and for *E. coli* on Eosin Methylene Blue agar (EMB) medium. Three replicates of the trial were conducted, and the mean of the outcomes for each treatment was recorded, as described in (21).

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions

Target	Target gene	Primers sequences	Amplified segment (bp)	Reference
E. coli	*blaTEM*	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	(17)
	blaSHV	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	
S. aureus	*Sea*	GGTTATCAATGTGCGGGTGG CGGCACTTTTTTCTCTTCGG	102	(18)
	Seb	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164	
	Sec	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451	

Sensory evaluation

Kareish cheese was manufactured (20). In brief, cow's milk was pasteurized of at 80 ºC for 15 seconds, then cooled to 40 ºC and rennet, 3g /100 kg (Hansen Laboratories, Copenhagen, Denmark) was added. Cow's milk was divided into three groups: group one (negative control) with no lactoferrin, and groups two and three were inoculated with lactoferrin concentrations of 3% and 5% respectively for sensory evaluation. The treated milk was incubated overnight at 30ºC to get coagulation of the cheese and 1% salt was applied between each layer of cheese. Treated and control cheeses were kept at a cold temperature of 4±2ºC.The sensory properties of the cheese samples were assessed at 0-day, 5th, 10th, 15th, and 20th day of storage until the onset of spoilage symptoms according to the International Dairy Federation recommendation (22). The sensory properties of the Kariesh cheese samples were assessed by the staff at the Food Hygiene Laboratory, Department of Animal Health Research Institute. Each sample was graded by panels using a weighted scale of 100 points, with 20 points provided for color and appearance, 35 points for texture and body and 45 points provided for flavor.

Statistical analysis

IBM SPSS software program version 20.0 (Armonk, NY: IBM Corp) was used to analyze the data. Quantitative data were presented in terms of percentage and numbers. The Kolmogorov-Smirnov test was used to verify the normality of the data distribution. Descriptive statistics such as range (minimum and maximum), mean, standard deviation, and median were calculated for the quantitative data. The significance level was set at 5% for all analyses.

Results

The prevalence of *E. coli* and *S. aureus* in raw milk and Kariesh cheese is presented in table 2. Out of a total of 60 samples (30 for raw milk and 30 for Kariesh cheese). 13 samples (43.3%) from raw milk and 11 samples (36.6%) from Kariesh cheese tested positive resulting in a total of 24 positive samples (40%). In contrast, 15 samples (50%) from raw milk and 7 samples (23.3%) from Kariesh cheese tested positive for *S. aureus*, resulting in a total of 22 positive samples (36.6%).

Table 2: Prevalence of *E. coli* and *S. aureus* in raw milk and kariesh cheese samples.

Product	*E. coli*			*S. aureus*		
	No. of samples	Positive samples	%	No. of samples	Positive samples	%
Raw milk	30	13	43.3	30	15	50
Kariesh cheese	30	11	36.6	30	7	23.3
Total	60	24	40	60	22	36.6

The results presented in table 3, clearly show the serological identification of *E. coli* strains as follows: O17:H18 (23.1%), O91:H21 (23.1%), O127:H6 (23.1%), and O26: H11 (30.8%) for raw milk samples. The serological identification of isolated *E. coli* in Kariesh cheese was O159 (27.3%), O26:H11 (45.4%), and O127:H6 (27.3%).

Table 3: Serological identification of *E. coli* isolated from raw milk and Kariesh cheese

Type of product	Serogroup	Strain characterization	No. of +ve	%
Raw milk (n=13)	O17: H18	EPEC	3	23.1
	O91: H21	EHEC	3	23.1
	O127: H6	ETEC	3	23.1
	O26: H11	EHEC	4	30.8
Kareish cheese (n=11)	O159	EIEC	3	27.3
	O26: H11	EHEC	5	45.4
	O127: H6	ETEC	3	27.3

The results presented in table 4 and figures 1 and 2 indicate that eight *E. coli* strains (four from raw milk samples and four from Kariesh cheese) were analyzed to detect beta-lactamase resistance genes. All examined *E. coli* strains tested positive for the blaTEM gene 100% and the blaSHV gene 100%.

Table 4: PCR results for detection of Beta-lactamase resistance *E. coli* genes blaTEM and blaSHV

Isolated *E. coli* samples	Origin	blaTEM	blaSHV
1	Raw milk	+	+
2	Raw milk	+	+
3	Raw milk	+	+
4	Raw milk	+	+
5	Kareish cheese	+	+
6	Kareish cheese	+	+
7	Kareish cheese	+	+
8	Kareish cheese	+	+

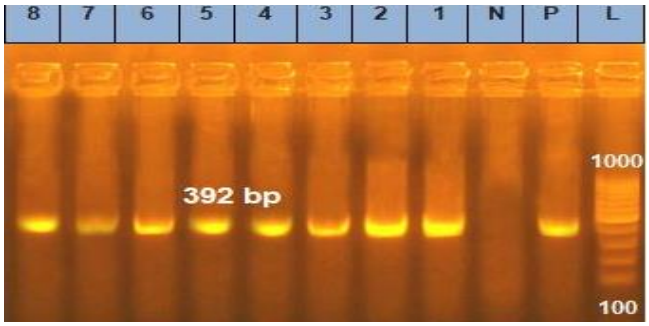

Figure 1: Agarose gel electrophoresis for the PCR results of the blaSHV (392bp) gene in *E. coli*. Lane L:100-1000bp molecular size marker. Lane pos: control positive *E. coli* blaSHV at 392 bp. Lane neg: control negative. Lane 1 to 8; positive blaSHV gene.

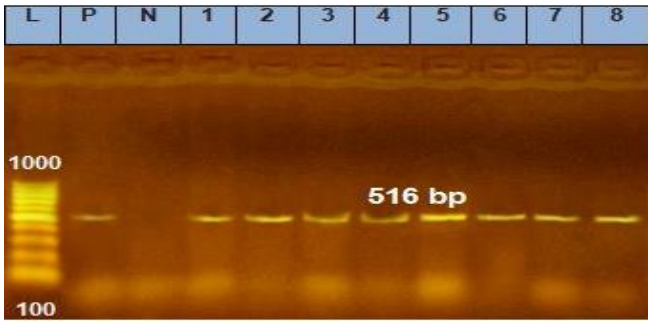

Figure 2: Agarose gel electrophoresis for the PCR results of blaTEM (516 bp) gene in *E. coli*. lane L: 100-1000bp molecular size marker. Lane pos: control positive *E. coli* blaTEM at 516 bp. Lane neg: control negative. Lane 1 to 8: positive blaTEM gene.

The results presented in table 5 and figures 3-5 indicate that eight *S. aureus* isolates (four from raw milk samples and four from Kareish cheese samples) were tested for the presence of enterotoxin genes. PCR analysis revealed that 62.5% (5 isolates) of the tested *S. aureus* strains were

enterotoxigenic, as they had one or two SE-genes. The Sea gene was not detected in any of the raw milk and Kariesh cheese samples. However, the Seb gene was found in 5(62.5%) of the isolated strains, and sec gene was detected in 2 (25%) of them in this study.

Table 5: PCR results for detection of enterotoxigenic *S. aureus* of Sea, Seb and Sec toxins

S. aureus sample	*Origin*	*Sea*	*Seb*	*Sec*
1	Raw milk	-	+	-
2	Raw milk	-	+	+
3	Raw milk	-	+	+
4	Raw milk	-	-	-
5	Kareish cheese	-	-	-
6	Kareish cheese	-	-	-
7	Kareish cheese	-	+	-
8	Kareish cheese	-	+	-

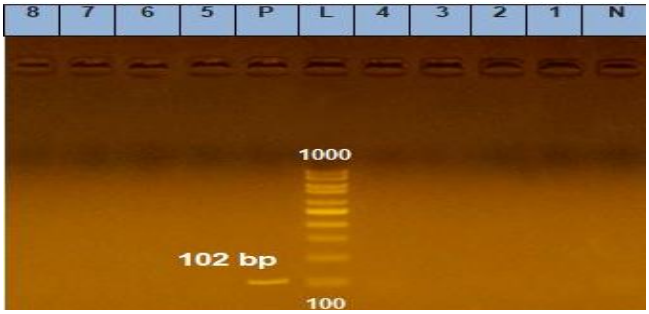

Figure 3: Agarose gel electrophoresis shows the polymerase chain reaction amplification results of the Sea enterotoxin gene for *Staphylococcus aureus*. Lane L:100-1000bp molecular size marker. Lane pos: control positive *Staphylococcus aureus* Sea enterotoxin gene at 102 bp. Lane neg: control negative. Lanes 1 to 8 are negative for the Sea enterotoxin gene.

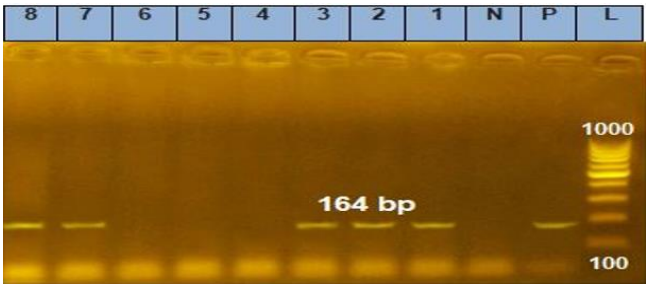

Figure 4: Agarose=gel=electrophoresis=of=PCR=products =of Seb=enterotoxin=gene for Staphylococcus aureus. Lane L:100-1000bp molecular size marker. Lane pos: control positive Staphylococcus aureus Seb enterotoxin gene at 164 bp. Lane neg: control negative. Lane 1,2,3,7 and 8: positives to Seb=enterotoxin=gene.

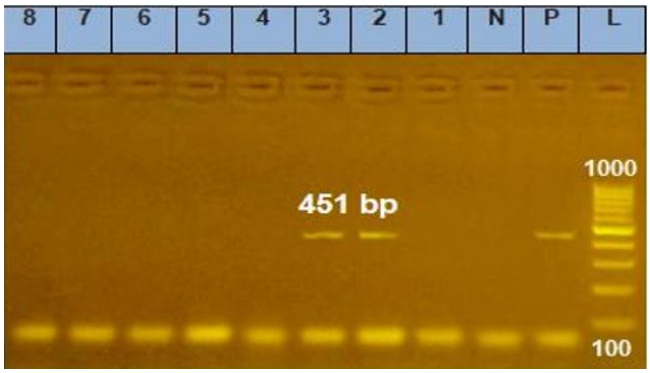

Figure 5: Agarose gel electrophoresis of PCR results of Sec enterotoxin gene for *Staphylococcus aureus*. Lane L:100-1000bp molecular size marker. Lane pos: control positive *Staphylococcus aureus* Sec enterotoxin gene at 451 bp. Lane neg: control negative. Lane 2,3: positive to Sec enterotoxin gene.

Table 6 presents the maximum observed zone of inhibition at a 5% lactoferrin concentration, which was 10.33±1.33 and 12.66±1.45 mm in diameter for *E. coli* and *S. aureus*, respectively. The zone of inhibition for *E. coli* and *S. aureus* was 8.66±0.33mm and 9.66±0.88 mm, respectively, at a 3% lactoferrin concentration.

Table 6: The antimicrobial activity of bovine lactoferrin (BL) against isolated *E. coli* and *S. aureus* by agar well diffusion test

Concentrations	Inhibition zone [Mean (mm)± S.D]	
	E. coli	*S. aureus*
3% BL	8.66±0.33	9.66±0.88
5% BL	10.33±1.33	12.66±1.45

The changes in *E. coli* counts during the storage period for treated and untreated Kareish cheese are presented in table 7, figure 6. The initial values of *E. coli* counts were 4.59 ±3.28 and 4.56±3.18 CFU/g for the control and treated Kareish cheese, respectively, and there were no significant differences ($P< 0.05$) between the two groups. However, during storage, significant differences ($P< 0.05$) were observed in the *E. coli* counts of the treated and control groups. On the 7th day *E. coli* counts in the control sample reached 6.99± 5.76 CFU/g whereas it was completely inhibited on the sixth day of storage in the treated sample. Table 7, figure 7 demonstrate the variations in *S. aureus* counts in Kareish cheese. *S. aureus* was completely inhibited on the 6th day of storage time in the treated sample, whereas in the control group, the count was 6.65±5.51 CFU/g.

Table 7: The antimicrobial activity of Bovine Lactoferrin on *E. coli* and *S. aureus* inoculation in Kariesh cheese during the refrigerated period

Bacteria	Groups	0 day	1st day	3rd day	5th day	7th day
E. coli	Control	4.59 ± 3.28^{A}	4.76 ± 4.07^{C}	5.55 ± 4.87^{C}	6.54 ± 5.19^{C}	6.99 ± 5.76^{C}
	T1	4.56 ± 3.18^{A}	3.79 ± 3.54^{A}	2.96 ± 1.71^{A}	1.94 ± 0.81^{A}	$<1^{A}$
S. aureus	Control	4.66 ± 3.21^{A}	4.93 ± 3.28^{C}	5.32 ± 4.05^{C}	6.15 ± 5.65^{C}	6.65 ± 5.51^{C}
	T1	4.62 ± 3.15^{A}	3.55 ± 3.04^{A}	2.41 ± 1^{A}	1.98 ± 1.09^{A}	$<1^{A}$

The different letters in the same columns, indicate a statistical difference at P<0.05.

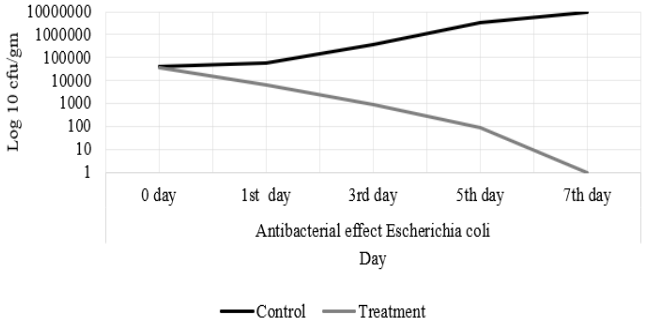

Figure 6: The antimicrobial activity of 5% bovine lactoferrin on *Escherichia coli* inoculated in manufactured kariesh cheese during a refrigerated period.

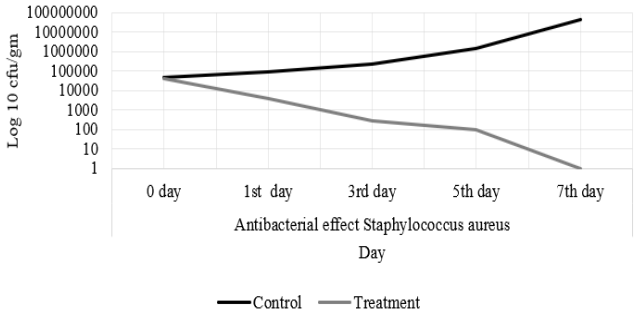

Figure 7: The antimicrobial activity of 5% bovine lactoferrin on *Staphylococcus aureus* inoculated in manufactured kariesh cheese during a refrigerated period.

Table 8 shows the changes in sensory evaluation scores (appearance, body and texture and flavor) on a 100-point scale in different experimental groups. After 20 days of storage, the quality of appearance (scored out of 20 points) was reduced, as observed by the panelists. At the end of the storage period, the highest scores were given to the 3% and 5% lactoferrin groups which were significantly better than the control cheese (P<0.05). The control cheese spoiled after 15 days of storage (Figure 8). The body and texture quality (scored out of 35 points) of the treated samples remained suitable during the 20 days of the storage period in 3% and 5% lactoferrin-treated cheese groups (P<0.05) (Figure 9). The flavor quality (scored out of 45 points) was improved in all lactoferrin-treated groups (Figure 10).

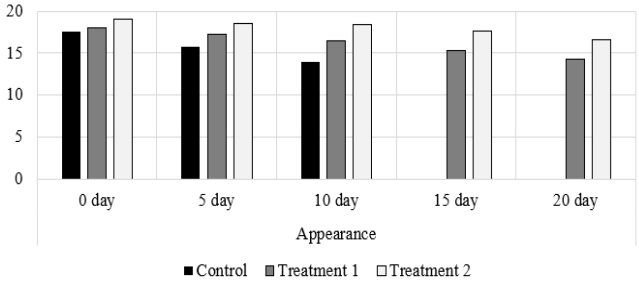

Figure 8: Appearance evaluation of manufactured Kariesh cheese during the refrigerated period (5±1ºC) (score 20 points).

Table 8: Sensory evaluation of manufactured Kareish cheese during the refrigerated period (5±1°C) (score 100 points)

Bacteria	Groups	0 day	1st day	3rd day	5th day	7th day
Appearance (20 points)	Control	17.53 ± 0.15^{A}	15.77 ± 0.25^{C}	13.93 ± 0.25^{C}	S	S
	T1	17.97 ± 0.21^{A}	17.27 ± 0.25^{A}	16.5 ± 0.2^{A}	15.3 ± 0.5^{A}	14.33 ± 0.35^{A}
	T2	19.03 ± 0.15^{B}	18.53 ± 0.47^{A}	18.37 ± 0.32^{B}	17.67 ± 0.15^{B}	16.6 ± 0.2^{B}
Body and texture (35 points)	Control	32.43 ± 0.21^{A}	29.37 ± 0.35^{C}	26.4 ± 0.1^{C}	S	S
	T1	33.7 ± 0.2^{A}	31.33 ± 0.31^{A}	28.67 ± 0.15^{A}	26.23 ± 0.21^{A}	24.17 ± 0.15^{A}
	T2	35.63 ± 0.31^{B}	33.47 ± 0.25^{B}	30.17 ± 0.21^{B}	28.37 ± 0.32^{B}	26.27 ± 0.25^{B}
Flavor (45 points)	Control	40.47 ± 0.25^{A}	36.3 ± 0.3^{C}	29.3 ± 0.2^{C}	S	S
	T1	42.4 ± 0.36^{B}	40.33 ± 0.15^{A}	38.43 ± 0.4^{A}	35.5 ± 0.36^{A}	29.17 ± 0.29^{A}
	T2	44.13 ± 0.32^{C}	42.33 ± 0.31^{B}	39.3 ± 0.2^{A}	37.47 ± 0.42^{B}	33.47 ± 0.15^{B}

Control: cheese without bovine lactoferrin, T1: 3% bovine lactoferrin cheese, T2: 5% bovine lactoferrin cheese, S: spoiled cheese. The different letters in the same columns, indicate a statistical difference at P<0.05.

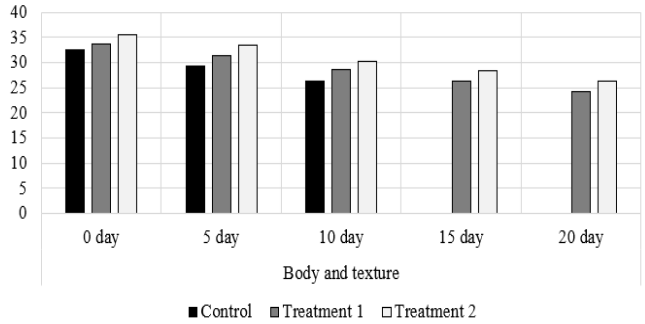

Figure 9: Body and texture evaluation of manufactured Kariesh cheese during the refrigerated period (5±1ºC ºC) (score 35 points).

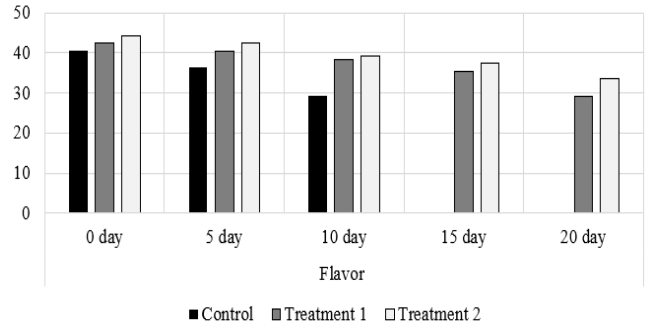

Figure 10: Flavor evaluation of manufactured Kariesh cheese during the refrigerated period (5±1ºC) (score 45 points).

Discussion

Milk and other dairy products are among the best types of food for people from birth to old age. They not only have excellent sensory qualities but also provide all the nutrients the body needs for rapid growth. Furthermore, they can help prevent or lessen the risk of many diseases caused by nutritional deficiencies (23). However, contaminated food is the primary way that harmful bacteria are transferred from animals to people, and it is the primary factor in most diseases in affluent nations, often leading to mortality and morbidity (24). The presence of *S. aureus* along with *E. coli* is a strong sign of fecal pollution and may indicate that these products were manufactured in unhygienic settings (25).

In our research, we found that the incidence of *E. coli* was 43.3% and 36.6% in examined raw milk and Kariesh cheese samples, respectively, from a total of 30 examined samples for each type. Our results for *E. coli* incidence in raw milk were similar to those reported by Ranjbar *et al.* (26), who detected *E. coli* at a rate of 42.85%.In Kariesh cheese, our results agree with El Bagoury *et al.* (27),who isolated *E. coli* at a rate of 37.1%.Higher results 76.4% were obtained by Ombarak *et al.* (28) in raw milk, and for Kariesh cheese, a higher result 74.5% was obtained by Ombarak *et al.* (28).On the other hand, lower results 16% and 34%for raw milk were detected by Zeinhom and Abdel-Latef (29) and Alsanjary and Sheet (30), and For Kariesh cheese, lower results 11.54, 16 and 10% were recorded by Chaleshtori *et al.* (31), Hussien *et al.* (32) and Alkhafaje *et al.* (33), respectively. The presence of pathogenic *E. coli* is problematic as it is the etiological agent for enteritis and several additional gastrointestinal diseases, and is recognized as a pathogen for both animals and humans. Detection of *E. coli* in milk or Kariesh cheese often indicates fecal contamination. Unhygienic food-handlers who are infected can easily contaminate milk or water that has human discharge in it. Cheese contamination may occur at various points in the production chain. Therefore, farmers must receive training in safe handling procedures and appropriate personal hygiene practices, and water used in production must be safe and essentially pathogen-free (34).

The prevalence of *S. aureus* in some tested commercial raw milk and Kariesh cheese. Among the examined products, *S. aureus* has been most frequently found in raw milk, followed by Kariesh cheese, in percentages of 50% (15/30) and 23% (7/30), respectively. This finding is in line with reports from Gajewska *et al.* (35) and Meshref *et al.* (36) who found *S. aureus* in raw milk with a prevalence of 55.6% and 52% respectively. A higher result was reported by Kou *et al.* (37), who detected *S. aureus* by 61.7%. While a lower result 19.8% and 24% were detected by Sharma *et al.* (38) and Taher *et al.* (39). For Kareish cheese, similar results were recorded by Hassan and Afify (40) and Amal and Mona (41), where *S. aureus* was isolated with a rate of24%and 26.6%, respectively. A higher result44% was obtained by Abdeen *et al.* (42), while a lower result 16.7% was obtained by Badawy *et al.* (43). Environmental pollution and cross-contamination of milk during transportation or in milk collecting centers are two possible causes of the extensive *S. aureus* presence in raw milk. Additionally, another factor contributing to the contamination of milk and dairy products is *S. aureus* excreted by sick animals (44). The principal source of *S. aureus* infection in Kariesh cheese is often the raw milk used for cheese making. Unhygienic cheese handling, massive contamination by workers who may be involved in cheese production and marketing, or both, are the main *S. aureus* carriers. Food poisoning outbreaks resulting from the consumption of fresh soft cheese containing enterotoxins have been reported (45). These results highlight the need to apply stricter hygienic practices to reduce microbial contamination, especially in traditional cheese manufacturing.

Although most *E. coli* species are not dangerous, some of them are known to generate toxins that can cause disease in people, including Enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli*, enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, and diffusely adherent *E. coli* (DAEC). The results of serological identification of examined *E. coli* strains, which is nearly similar to El-Nahas

et al. (34) that revealed O_{114}, O_{127}, O26, and O_{111} from raw milk samples and O_{127} and O_{26} from Kariesh cheese samples.

The Enterobacteriaceae family includes several antibiotic resistance determinants, making the treatment of infections more challenging. It has been found to create the majority of these ESBLs (46). *E. coli* and K. pneumoniae are the most widespread bacteria involved in the creation of extended-spectrum beta-lactamases, and environments-such as water or soil, wild-animals, pet animals, food, and humans are their reservoirs (47). Asymptomatic-colonization of antibiotic-resistant *E. coli* by intestinal flora in food animals poses a risk of human infection if consumed through the food chain (48). A similarly high prevalence of blaTEM100%was observed by Younis *et al.* (49), while a lower prevalence of blaSHV14.8%was detected by Gaffer *et al.* (50) and lower prevalence of blaTEM was detected by Mahmood and Ahmed (51).

When milk is infected with ESBL-producing bacteria and used directly-without being heated or used in cheese manufacture, the Codex Alimentarius Commission organized an international taskforce in response to this issue (52). In 2014, the Council of European Dentists (CED), the Federation of Veterinarians of Europe (FVE), and the Standard Committee of European-Doctors (CPME) released a joint press release requesting that all authorities resolve the problem of Enterobacteriaceae bacteria that produce ESBL. According to our investigation, the high incidence of ESBL producers with their resistant genes in dairy products analyzed poses a health concern to consumers.

Foodborne outbreaks of *S. aureus* intoxication have been linked in several countries to the consumption of the contaminated milk and dairy products. Consuming enough of the enterotoxins produced by Staphylococcus bacteria in food results in food poisoning (53). Heat and proteolytic enzymes do not affect Staphylococcal enterotoxins (54). When food is exposed to heat before consumption, *S. aureus* may become inactive. but the extremely stable enterotoxins may still be active (55). They pose a serious hazard to food safety as a result of their existence (56). *S. aureus*-related food poisoning causes vomiting, nausea, abdominal pain, and diarrhea. According to Balaban and Rasooly (57), the condition is self-limiting and typically resolves 24 to 48 hours after it begins.

The enterotoxin (SE) genes of Staphylococcus spp. are encoded in mobile genetic elements, such as plasmids or prophages, meaning that not all strains of this bacterium produce them, and they can be spread through *S. aureus* strains even during food preparation and processing (58). The results are nearly similar to those Sahebekhtiari *et al.* (59), who found that67% of *S. aureus* isolates harbored one or more enterotoxin genes .

In our study, the Sea gene was not detected in any raw milk and Kariesh cheese samples. This result is similar to Hegab *et al.* (60), who did not detect the Sea gene in examined cheese samples. On the other hand, the Seb gene was found in 5(62.5%) and the sec gene was detected in 2 (25%) of the isolated strains in this study. Lower results were reported by Badawy *et al.* (43), who detected Sec with an incidence of 4.5% and did not detect Seb. It was exciting to note that the majority of the *S. aureus* isolates from milk and Kareish cheese in our investigation had the Seb 62.5% gen E. These results agree with Hegab *et al.* (60), who stated that the Seb gene was the most common from the examined cheese samples.

Lactoferrin acquires its antibacterial effectiveness from its capacity to sequester iron (Fe^{+3}) away from bacteria. Iron is utilized by bacteria for the synthesis of DNA and RNA, the tricarboxylic acid cycle, the manufacture of cytochromes and toxins, as well as for energy (61). So, pathogenic bacteria may have less energy if the environment's iron levels are reduced. Lactoferrin targets the lipid A component of the LPS layer and releases it from the membrane, which reduces gram-negative bacteria's ability to survive (62). Against gram-positive bacteria, lactoferrin's antibacterial action targets the teichoic acid in the bacterial cell wall. Furthermore, data suggest that lactoferrin's effectiveness against some gram-positive bacteria may be even larger than its effectiveness against gram-negative bacteria (63).

The antibacterial activity of bovine lactoferrin at 3 and 5% concentrations against *E. coli* and *S. aureus* was evaluated in vitro utilizing the agar well diffusion method to determine the proper concentration to use for making Kariesh cheese. Table 6 shows that the maximum observed zone of inhibition at a dose of 5% lactoferrin concentration was 10.33±1.33and12.66±1.45 mm in diameter for *E. coli* and *S. aureus*, respectively. Similar outcomes were obtained by Ombarak *et al.* (22) and Karam-Allah *et al.* (64), respectively. The results also showed 8.66±0.33mm for *E. coli* and 9.66±0.88 mm for *S. aureus* at 3% concentration. The findings indicate that the tested-pathogens, *E. coli* and *S. aureus*, on agar plates responded best to 5% lactoferrin. Therefore, we used 5% bovine lactoferrin in vivo by inoculating Kariesh cheese because it demonstrated the highest antibacterial action against *E. coli* and *S. aureus*. The antimicrobial activity of bovine lactoferrin on *E. coli* and *S. aureus* inoculation in Kariesh cheese during the refrigerated period. The results demonstrate a very promising suppressive effect of 5% lactoferrin concentration as the studied pathogens were completely eradicated.

The best evaluation scores for appearance, body and texture and flavor were reported in the 5% lactoferrin-treated cheese group, followed by the 3%lactoferrin-treated cheese group. It appears that the application of lactoferrin dramatically improved the overall sensory qualities of the samples extended the shelf life of Kariesh cheese, and prevented risks to the public's health.

Our results, similar to Ombarak *et al.* (22), demonstrate that lactoferrin enhances the cheese's sensory qualities after being experimentally contaminated with microbes such as *S. aureus*, *E. coli* O_{157}:H_7, *B. cereus*, and *L. monocytogenes*.

They also show that lactoferrin prolongs the cheese's shelf life in cold storage. Hassan *et al.* (65) concluded that lactoferrin at 20%can prevent the viability of *S. aureus* in Kariesh cheese, while lactoferrin greatly affected the number of *E. coli* found in Kariesh and Domiati cheese and displayed a range of inhibitory actions on *E. coli*-viability compared to *S. aureus*. Furthermore, lactoferrin, even at high concentrations, had no obvious effects on their survivability in Tallaga cheese. Da Silva *et al.* (66) stated that inhibition of *S. aureus* growth in the cheeses occurred by lactoferrin. Al-Habty and Ali (67) revealed that lactoferrin has potent antibacterial properties against multidrug-resistant (MDR) *S. aureus*, making it suitable food preservative for yogurt with good sensorial properties.

Conclusion

This study concluded that *E. coli* and *S. aureus* were detected in raw milk and Kariesh cheese from the retail market. All *E. coli* isolates tested positive for the blaTEM gene 100% and blaSHV gene 100%. Furthermore, some isolated strains of *S. aureus* were found to harbor more than one type of enterotoxins (Seb, Sec), which pose a danger to consumers. Additionally, the data provided indicated that no hygienic practices were employed during the milking, manufacturing, and distribution of these dairies. Lactoferrin has shown promise in dairy preservation due to its potent antibacterial action and favorable sensory characteristics. The inhibitory impact of lactoferrin could act as a safety measure to reduce the spread of microbes in food and prevent the associated risks to public health.

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Conflict of interest

The authors declare that there is no conflict of interest, and all authors agree to the publication of this article.

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استخدام اللاكتوفيرين لتثبيط عزلات الإيشريكيا القولونية والمكورات العنقودية الذهبية من اللبن والجبنة القريش

شيرين عبد الفتاح يس[١] و هالة أمين عبد الهادي[٢]

[١]قسم صحة الأغذية، [٢]قسم علم البكتريا، معهد بحوث الصحة الحيوانية، فرع كفر الشيخ، مركز البحوث الزراعية، كفر الشيخ، مصر

الخلاصة

تهدف هذه الدراسة إلى تحديد الإيشريكيا القولونية وجيناتها المشفرة البتا لاكتيميز والمكورات العنقودية الذهبية وجيناتها المعوية المعزولة من الحليب والجبن القريش، بالإضافة إلى تقييم التأثير المضاد للبكتيريا للاكتوفيرين ضد هذه البكتيريا المسببة للأمراض. حيث تم جمع ستون عينة إجمالاً (٣٠ عينة من كل من اللبن الخام وجبن القريش) من مختلف أسواق البيع بالتجزئة في محافظة كفر الشيخ. بلغت نسبة عزلات الإيشريكيا القولونية في اللبن الخام والجبن القريش ٤٣,٣٪ و ٣٦,٦٪ على التوالي، بينما سجلت عزلات المكورات العنقودية الذهبية ٥٠٪ و ٢٣,٣٪ (من اللبن الخام والجبن القريش) علي التوالي. تم تصنيف ٢٤ سلالة من بكتيريا الإيشريكيا القولونية في مجموعات مصلية، منها ٣ سلالات من أصل ٢٤ سلالات كانت O_{17} و O_{91} و O_{159} و ٦ سلالات من O_{127} و ٩ سلالات من O_{26}. أشار اختبار تفاعل السلسلة المتبلمرة لجينات البتا لاكتيميز المشفرة في الإيشريكيا القولونية إلى أن جميع العزلات الثماني كانت إيجابية ١٠٠% لجينات blaTEM و blaSHV، بينما كانت ٥ عزلات (٦٢,٥٪) من والمكورات العنقودية الذهبية موجبة لإنتاج السموم المعوية. خمس عزلات (٦٢,٥٪) أنتجت جين Seb، بينما عزلاتين (٢٥%) أنتجت جين Sec بينما لم يتم الكشف عن جين Sea في عزلات المكورات العنقودية الذهبية. تشير النتائج إلى أن اللاكتوفيرين ٥٪ كان له تأثير مثبط معنوي على بكتريا الإيشريكيا القولونية والمكورات العنقودية الذهبية عندما تم تلقيحهما في الجبن القريش. تظهر النتائج أن الألبان لم تتخذ احتياطات النظافة الكافية ونصحت باتباع إجراءات النظافة الصارمة عند حلب منتجات الألبان ومعالجتها وتوزيعها. من أجل السيطرة على نمو الإيشريكيا القولونية والمكورات العنقودية الذهبية في منتجات الألبان، يُعتقد أن اللاكتوفيرين هو استراتيجية محتملة.