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USING LACTOFERRIN AS A TRAIL TO CONTROL E COLI AND STAPH. AUREUS ISOLATED FROM SOME TYPES OF CHEESE

AZHAR MOHAMMED HASSAN [1]; JAKLEEN HALIM TAWFIK BEBAWY [2]; MARY RAFAT HAFAZ [3] AND WALAA S. HASAN [4]

[1] Prof. of Microbiology in Agriculture Research Institute-Animal Health Research Institute
[2, 3] Researcher in Agriculture Research Institute-Animal Health Research Institute
[4] Colleague of Milk Hygiene Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt.

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ABSTRACT

In this study, the effect of lactoferrin as an antibacterial activity on the phagocytic activity of *E. coli* as Gram-negative bacteria and Staph. aureus as Gram-positive bacteria was investigated. The microbiological status of different types of cheese (Kareish, Domiati and Tallaga cheese) was also evaluated. A total of sixty cheese samples (20 samples each) were collected randomly from different markets and restaurants. Pathogenic bacteria including *E. coli* and *Staph aureus* were isolated and considered as indicators of the microbiological quality. The effect of lactoferrin on *E. coli* and *Staph. aureus* was evaluated to improve the quality of cheese. Lactoferrin antibacterial activity was tested using different concentrations of lactoferrin (zero, 0.5, 1.0, 5.0, 10, and 20 mg/ml) on the survivability of *E. coli* and *Staph. aureus* in different varieties of cheese. Lactoferrin showed various inhibition activity on *E. coli* viability than *Staph. aureus*, and significantly influenced the count of *E. coli* in Kareish and Domiati cheese while, and 20% of lactoferrin can inhibit the viability of *Staph. aureus* in Kareish cheese. Furthermore, their viability in Tallaga cheese was not significantly affected by lactoferrin even by using high concentration. So, lactoferrin could become a promising method to decrease the viability of *E. coli* and *Staph. aureus* in cheese.

Keywords: lactoferrin, *E. coli*, *Staph. aureus*, antimicrobial activity.

INTRODUCTION

Nowadays, lactoferrin has a wide range of physiological functions such as antiviral, antimicrobial, and anticancer activities (Farid *et al.,* 2019). Lactoferrin is an iron-binding glycoprotein that plays a number of critical protective activities in the mammalian body. And it works as an antibiotic substitute. It functions as a natural antibacterial for biopreservation, increasing the shelf life of dairy products, maintaining safety, and enhancing health by combating life-threatening disorders in newborns also help in the treatment of hepatitis, respiratory infections, and foodborne diseases (Diarra *et al.,* 2002).

Lactoferrins are thought to play an essential role in the immune system initiation because they have been found in fluids of

Corresponding author: Azhar Mohammed Hassan
E-mail address: Azharhassan769@gmail.com; aaa_mmm_1993@yaoo.com
Present address: Prof. of Microbiology in Agriculture Research Institute-Animal Health Research Institute

the body that interact with the environment and because of their broad activity against different organisms (Gruden and Poklar 2021). Furthermore, lactoferrin's bactericidal activity is caused by the contact between the positive-charged areas and anionic molecules found on the surface of some microbes, which produces an increase in membrane permeability, resulting in bacterial harm (Haversen *et al.*, 2010).

González-Chávez *et al.* (2009) demonstrated that lactoferrin inhibits growth and impairs the function of pathogens by two mechanisms; the first one is the absorption of the iron from the infection sites which is the food source of the microorganisms which creates a bacteriostatic effect. The second one is the direct interaction of lactoferrin with the infection agent as it has high levels of amylase, DNase, RNase and ATPase activity.

Cheese is considered an essential daily food for many people around the world. It has a nutritional value that provides an essential source of digestible protein and minerals, including calcium and phosphorus (Hammam *et al.*, 2020). Kareish cheese is a famous soft cheese commonly consumed in Egypt. It contains a high level of protein, amino acids, calcium, phosphorus and vitamins. Domiati cheese is one of the pickled cheese produced and consumed on a wide scale due to its pleasant taste and smooth body and texture as well as it was rich in basic supplemental elements. Tallaga cheese is characterized by its values, with a nearly neutral salty taste and is suitable for hypertensive people, all ages especially old people (Abd El-Tawab *et al.*, 2020).

E. coli and coliforms are often used as indicator microorganisms and the prevalence of *E. coli* implies a risk that other enteric pathogens may be present in the sample (Moawad *et al.*, 2021). *Staph. aureus* may be the main cause of several food intoxication outbreaks. The presence of *Staph. aureus* in cheese usually indicates contamination of milk may be from diseased udder or external surface of the dairy animals. Furthermore, contaminated, unclean hands of the workers, sneezing and coughing (Salem *et al.*, 2016).

The current study was conducted to detect the incidence of *E. coli* and *Staph. aureus* in Kareish, Domiati and Tallaga cheeses. Also, to evaluate the lactoferrin antibacterial action at various concentrations (zero, 0.5, 1, 5, 10, and 20 mg/ml) on the viability of these bacteria in various cheeses.

MATERIALS AND METHODS

1. Isolation of pathogenic organisms:

60 samples of white cheese including Kareish, Domiati and Tallaga cheese samples (20 samples for each) were collected from supermarkets and groceries. The samples were transferred to the lab in an icebox for examination.

1.1. Isolation of *E. coli*

10 g of working sample were aseptically measured and added to 90 ml of 2% sodium citrate, previously heated at 45°C and thoroughly mixed in the vortex until complete homogenization. Then, dilutions (up to 10−7), containing 2% sodium citrate, were prepared. After that, 1 ml of each dilution was inoculated on EMB and MacConkey agar. The inoculated media were incubated at 37°C/24 h. All Gram-negative, catalase-positive, and oxidase-negative isolates were picked on nutrient agar slope for further examination (Murdock *et al.*, 2007)

1.2. Isolation of *Staph. aureus*

Firstly, 25 g of sample were diversified with 225 ml sterile saline solution (0.85%) in plastic bags to prepare ten-fold serial dilutions. By means of a sterile pipette, 0.1 ml was transported from each dilution and spread using the spreader onto Baird-Parker Agar plates. The plates were then incubated

at 35 °C and examined after both 24 h± 2 h and 48 h± 2 h. After 48 hours, the characteristic morphology of typical colonies of *Staph. aureus* (black or grey, shining, convex and surrounded by a halo zone) was enumerated. Atypical colonies (which have the same characteristic morphology but the clear zone and the opalescent ring is absent) were also enumerated. The number of coagulase-positive *Staphylococci* was determined per gram of cheese. Characterized colonies (five colonies from typical and atypical colonies) were taken and tested for catalase positivity and coagulase for confirmation. Each selected colony was inoculated onto tubes containing Brain Heart Infusion broth and incubated at 35°C for 24 h±2h. 0,1 ml of each culture was aseptically added to 0,3 ml of the rabbit plasma in sterile tubes and incubated at 35°C. The tubes were observed for clotting of the plasma after 4 to 6 h of incubation and inspected after 24 h more.

2. Antibacterial activity assay:

2.1. Lactoferrin preparation:

Lactoferrin was purchased from Jarrow FORMULAS, Superior Nutrition and Formulation, Los Angeles, CA 90035-4317. The LF was dissolved in sterile distilled water and stored at − 20° C until needed.

2.2. Tested strains:

E. coli and *Staph. aureus* (two strains) were used which were previously isolated from Kareish, Domiati and Tallaga cheese samples.

2.3. Lactoferrin's antibacterial action in broth (Atef Yekta *et al.*, 2010)

One ml of the previously isolated *E. coli* strain was placed into sterile test tubes containing 1 ml of broth supplemented with various lactoferrin concentrations (zero, 0.5, 1.0, 5.0, 10, and 20 mg/ml) and incubated at 37°C for 24 hours. Viable bacteria were counted by spread plating of appropriate bacterial serial dilutions onto EMB plates.

2.3. Lactoferrin's antibacterial action in different types of cheese (Murdock and Matthews, 2002):

Different types of cheese including Kareish, Domiati and Tallaga cheese were divided into 2 groups for each strain (*E. coli* and *Staph. aureus* strains). Each group was subdivided according to the concentrations of lactoferrin into 0.5, 1.0, 5.0, 10 and 20 mg/ml and the last group which is free from lactoferrin kept as control. Samples were preserved in a refrigerator at 4±2°C for periodical counting on the 1st, 5th and 10th day. Tenfold serial dilution was done then, 0.1 ml was spread plated onto EMB agar for *E. coli* count and Baired-parker agar for *Staph. aureus* count. All plates were incubated at 37°C for 24 h and colonies were enumerated (GSO, 2005 and EOS, 2008).

Data analysis

The effect of the addition of lactoferrin on the viability of *E. coli* and *Staph. aureus* in some types of cheese throughout the shelf life was evaluated by Repeated measure ANOVA using the Prism 5 Statistical software program. A P-value of <0.05 was considered the cutoff level for statistical significance.

RESULTS

Table 1: Prevalence of *E. coli* and *Staph. aureus* in different types of cheese samples.

Types of Cheese	*E. coli*			*Staph. aureus*		
	No. of samples	Positive samples	%	No. of samples	Positive samples	%
Kareish	20	5	25	20	3	15
Domiati	20	4	20	20	2	10
Tallaga	20	2	10	20	2	10
Total	60	11	18.3	60	7	11.7

Table 2: Initial count of *E. coli* and *Staph. aureus* in Kareish, Domiati and Tallaga cheese before adding lactoferrin.

Cheese samples	*E. coli* CFU/ml	*Staph. aureus* CFU/ml
Kareish Cheese	7.9×10^{6}	3.9×10^{4}
Domiati Cheese	4.6×10^{7}	2.5×10^{5}
Tallaga Cheese	3.6×10^{6}	5.5×10^{4}

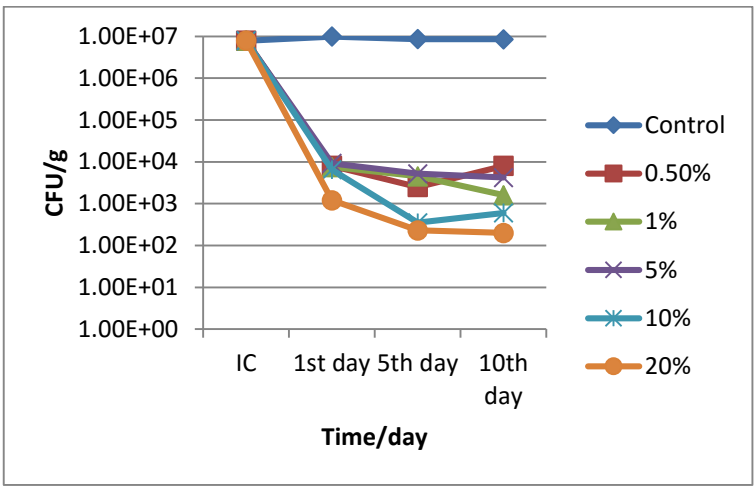

Fig.1: Effect of different concentrations of lactoferrin on *E. coli* count in Kareish cheese samples

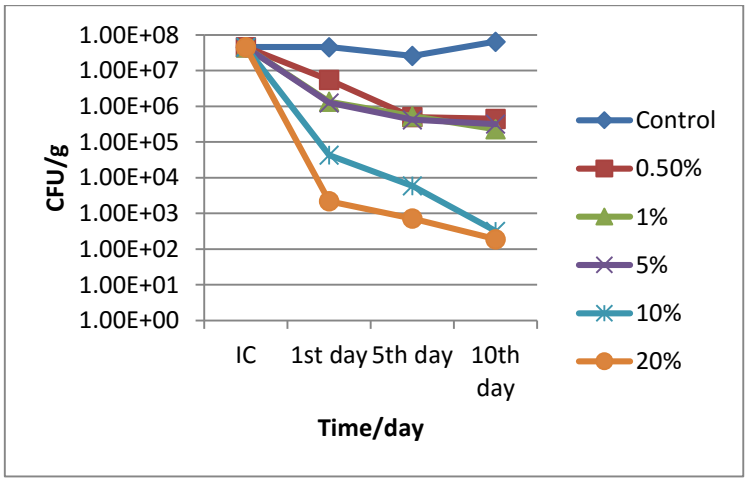

Fig.2: Effect of different concentrations of lactoferrin on *E. coli* count in Domiati cheese samples

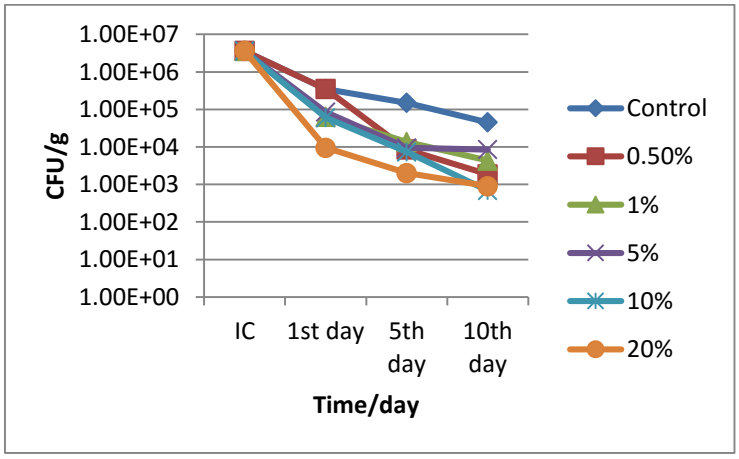

Fig.3: Effect of different concentrations of lactoferrin on *E. coli* count in Tallaga cheese samples

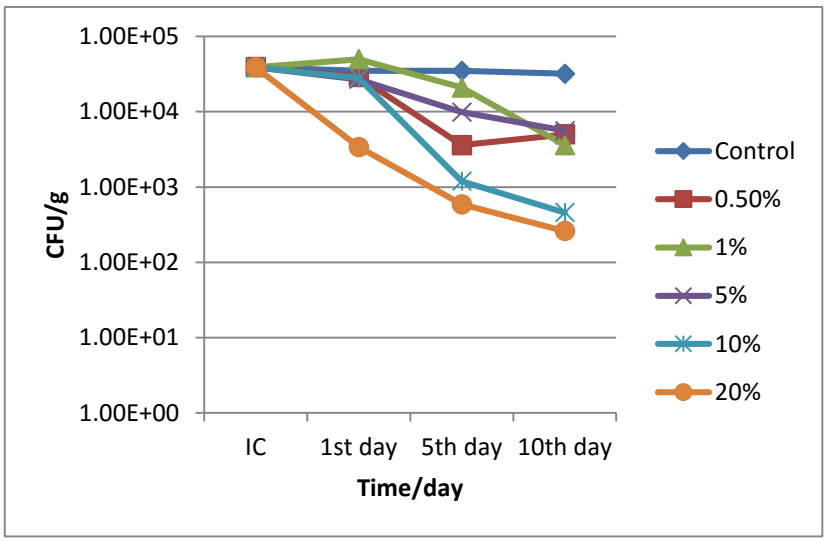

Fig.4: Effect of different concentrations of lactoferrin on *Staph. aureus* count in Kareish cheese samples

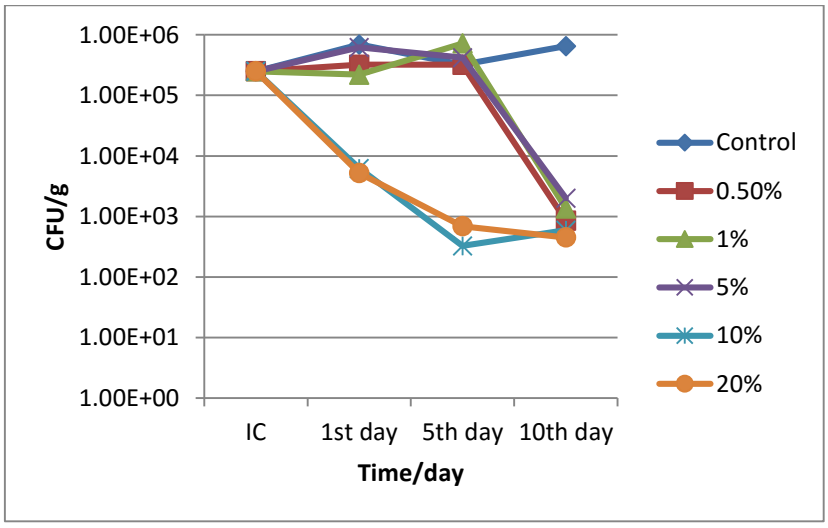

Fig.5: Effect of different concentrations of lactoferrin on *Staph. aureus* count in Domiati cheese samples

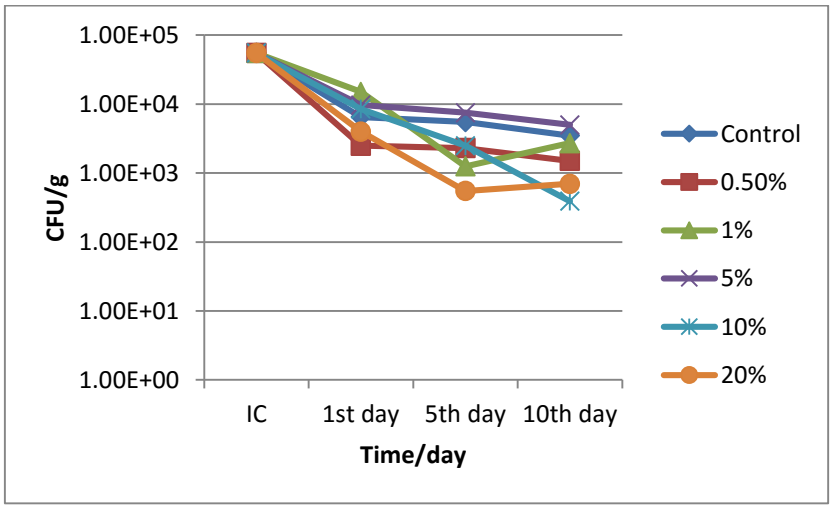

Fig.6: Effect of different concentrations of lactoferrin on *Staph. aureus* count in Tallaga cheese samples.

DISSCUSION

The microbial quality of cheese may be affected by the quality and heat treatment of milk used in the manufacture and ripening in a brine solution, as well as, transportation and storage conditions (Taha *et al.,* 2019).

Bacteriological examination revealed that *E. coli* was isolated at percentages of 25, 20 and 10% out of 20 samples of Kareish, Domiati and Tallaga cheese samples respectively.

E. coli was isolated in another study, from seven samples constituting about (29%) of the total samples as indicated by Raghda *et al.* (2021). However, Kandil *et al.* (2018). Abd El-*Tawab et al.* (2020) revealed that *E. coli* could be isolated from examined Kareish cheese samples with an incidence rate of 54%. Nearly, a similar finding was reported by El-Bagory *et al.* (2015) and higher incidence was shown by Ibrahim *et al.* (2019), while a lower incidence was detected by Awad (2016).

It is evident that *E. coli* could be isolated from examined Domiati cheese samples with an incidence of 20%. Higher findings were isolated by Ibrahim *et al.* (2019) and Raghda *et al.* (2021) while a lower incidence was shown by El-bajory *et al.* (2015). As illustrated in Table (1), *E. coli* could be isolated from Tallaga cheese samples with an incidence rate of 10%. A similar finding was obtained by Taha *et al.* (2019), while a higher result was investigated by Raghda *et al.* (2021).

The higher contamination rate of *E. coli* in Kareish cheese than in other types of cheese may be due to the differences in cheese making process and the characteristics of the final product between cheeses (Kandil *et al.,* 2018).

Staph. aureus recorded lower incidence (15, 10 and 10%) from Kareish, Domiati and Tallaga cheese, respectively as shown in Table (1). Nearly similar prevalence (12.6 %) of Kareish cheese yielded *Staph. aureus* was detected by Salem *et al.* (2016). Whereas, Moawad and Khalil (2021) found the incidence rate of *Staph. aureus* in Kareish cheese and Talaga samples was 18 and 6%, respectively. Growth of *Staph. aureus* was inhibited by many factors including the addition of a starter culture, low temperature (10°C), high salt concentration (15%) and pH (~5.2). Furthermore, these factors may have prevented enterotoxin production in white-brined cheese (Al-Nabulsi *et al.,* 2020).

Lactoferrin has been shown to inhibit the growth of a number of pathogenic bacteria including *E. coli* in both in vitro and in vivo studies (Yen *et al.,* 2011). Additionally, Shashikumar and *Puranik* (2011) demonstrated that cheese treated with lactoferrin up to 20 mg/ml showed an increase in the shelf life of cheese, the cheese has a much higher hardness, resiliency, springiness and chewiness.

Table (2) showed that the initial count of *E. coli* was 7.9×10^6, 4.6×10^7 and 3.6×10^6 and and *Staph aureus* was 3.9×10^4, 2.5×10^5 and 5.5×10^4 in Kareish, Domiati and Tallaga cheeses, respectively.

All treatment with lactoferrin in Kareish cheese samples caused a significant decrease in $P < 0.05$ of *E. coli* count. The count decreased from 7.9×10^6 at zero time to 8×10^3, 1.6×10^3, 4.2×10^3, 6×10^2 and 2×10^2 after 10 days at the concentration of lactoferrin 0.5, 1, 5, 10 and 20 mg/ml, respectively, while it increased from 7×10^6 at zero time reaching 9.9×10^6 by the 1st day and decreased to 8.5×10^6 at the end of the experiment as illustrated in Figure (1).

In another study, (Atef Yekta *et al.,* 2010) discovered that using 0.5 to 10 mg/ml and 0.1 to 10 mg/ml of human or bovine lactoferrin, respectively, *E. coli O157:H7* growth was considerably reduced in broth from three to six hours after incubation. Furthermore, Taha *et al.* (2019) found that

all concentrations of lactoferrin caused a significant decrease in $P < 0.05$ on the viability of *E. coli O1* isolated from Kareish cheese after 72 h in broth at the concentration of 10 and 20 mg/ml lactoferrin.

Regarding Domiati cheese sample, a significant decrease in $P < 0.05$ of *E. coli* count was detected in all treatments with lactoferrin. The *E. coli* count decreased from 4.6×10^7 to 3.2×10^2 and 1.9×10^2 at the concentration of 10 and 20 mg/ml lactoferrin, respectively (Fig. 2). on the other hand, the inhibition limit of lactoferrin on *E. coli* count was not significant in all treatments of Tallaga cheese. The count of *E. coli* decreased from 3.6×10^6 to 4.5×10^4 by the 10^{th} day in the sample control. Whereas, the count decreased to 9×10^2 and 6.9×10^2 at the concentration of 10 and 20 mg/ml lactoferrin by the 10^{th} day (Fig. 3).

The antimicrobial activity ascribed to lactoferrin against *E. coli* showed vary between studies. This variation depends on lactoferrin purity, temperature, iron saturation level, presence of different chelating compounds, pH, water activity, food components (lipid, protein and carbohydrate) and cations (Mg2+ and Ca2+) (Rybarczyk *et al.*, 2017).

Regarding *Staph. aureus* count, high lactoferrin concentration (20 mg/ml) significantly affect the count at $P < 0.05$ compared to the control sample in Kareish cheese only (Fig. 4). The count decreased from 3.9×10^4 at zero time to 2.6×10^2 after 10 days at the concentration of lactoferrin 20 mg/ml. While no significant decrease was noticed in *Staph. aureus* count in all treatments of Domiati and Tallaga cheese with lactoferrin (Fig 5 and 6).

It was observed that the bacterial growth decreased dramatically in *E. coli* count more than in the *Staph. aureus* infection compared to the control. The variation in results between these types of cheeses may be due to the difference in acidity, salt concentrate, and the method of manufacture.

Experimental evidence suggests that resistance to the bacteriostatic effect of lactoferrin may be attributed to the bacterial synthesis of iron chelators, which can compete with lactoferrin or transferrin for host iron. Furthermore, lactoferrin resistance has not developed with even simple systems such as the adherence fimbria of enteroaggregative *E coli* (Ochoa and Cleary, 2009). Moreover, Many Factors may hinder the viability and multiplication of bacteria in milk and cheese including the addition of salts, lowering the pH and decreasing water content and refrigeration as detected by Ombarak *et al.* (2016).

CONCLUSION

It could be concluded that lactoferrin had significantly influenced the count of *E. coli* in Kareish and Domiati cheese and the high concentration of 20% of lactoferrin can inhibit the viability of *Staph. aureus* in Kareish cheese. Furthermore, the viability of *E. coli* and *Staph. aureus* in Tallaga cheese was not significantly affected by lactoferrin even by using high concentration. So, lactoferrin could become a promising method to decrease the viability of *E. coli* and *Staph. aureus* in cheese.

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استخدام اللاكتوفيرين لمحاولة السيطرةعلى الإشيريشيا كولاى وستفيلوكوكس المعزولة من بعض أنواع الجبن

أزهار محمد حسن ، جاكلين حليم توفيق ، مارى رفعت حافظ ، ولاء شعبان على حسن

E-mail: Azharhassan769@gmail.com Assiut University web-site: www.aun.edu.eg

الغرض من هذه الدراسة معرفة تأثير اللاكتوفيرين كمضاد للبكتريا على نمو ميكروب الإشريشيا كولاى كمثال للبكتيريا السالبة لصبغة الجرام والاستاف أوريس كبكتيريا موجبة لصبغة الجرام. كما تم تقييم الحالة الميكروبيولوجية لثلاث أنواع من الجبن (قريش ودمياطى وجبنة التلاجة). تم تجميع ٦٠ عينة جبن (٢٠ عينة منها لكل من أنواع الجبن الثلاثة) بشكل عشوائي من الأسواق والسوبرماركت المختلفة بمحافظة أسيوط. تم عزل الإشريشيا كولاى والاستاف أوريس المسببة للأمراض وأعتبرت مؤشرا على الجودة الميكروبيولوجية لكل من الجبنه القريش والدمياطى وجبنة التلاجة على التوالى. أظهر الفحص البكتريولوجى أنه تم عزل بكتيريا الإشريشيا كولاى بنسبة ٢٥,٢٠ ,١٠ % أنواع الجبن الثلاثة, فى حين سجلت بكتريا الاستاف أوريس نسبة اقل ٥, ١٠ , ١٠ % من كل منها على التوالى. كما تم تقييم تأثير نشاط اللاكتوفيرين المضاد للبكتيريا باستخدام تركيزات مختلفة من اللاكتوفيرين (صفر ، ٠,٥ ، ١,٠ ، ٥,٠ ، ١٠ ، ٢٠ مجم / مل) على بقاء بكتيريا الإشريشيا كولاى والاستاف أوريس في الأنواع المستخدامة من الجبن. بناءً على النتائج التي توصلنا إليها ، أظهر اللاكتوفيرين أن إضافته بنسب مختلفة يؤثرعلى حيوية بكتيريا الإشريشيا كولاى Aوالدمياطى كما أوضحت النتائج أن التركيز المرتفع ٢٠ % مجم / مل من اللاكتوفيرين يمكن أن يؤثر بشكل معنوى على حيوية الاستاف أوريس فى جبن القريش. علاوة على ذلك لم تتأثر حيوية بكتيريا الإشريشيا كولاى والاستاف أوريس فى جبنة الثلاجة باللاكتوفيرين حتى باستخدام التركيز المرتفع (٢٠٪ مجم / مل) نسب ممكنة لتثبيط الحيوية. وخلصت الدراسة الى إمكانية استخدام اللاكتوفيرين بطريقة واعدة لتقليل نمو بكتيريا الإشريشيا كولاى والاستاف أوريس في الجبن.