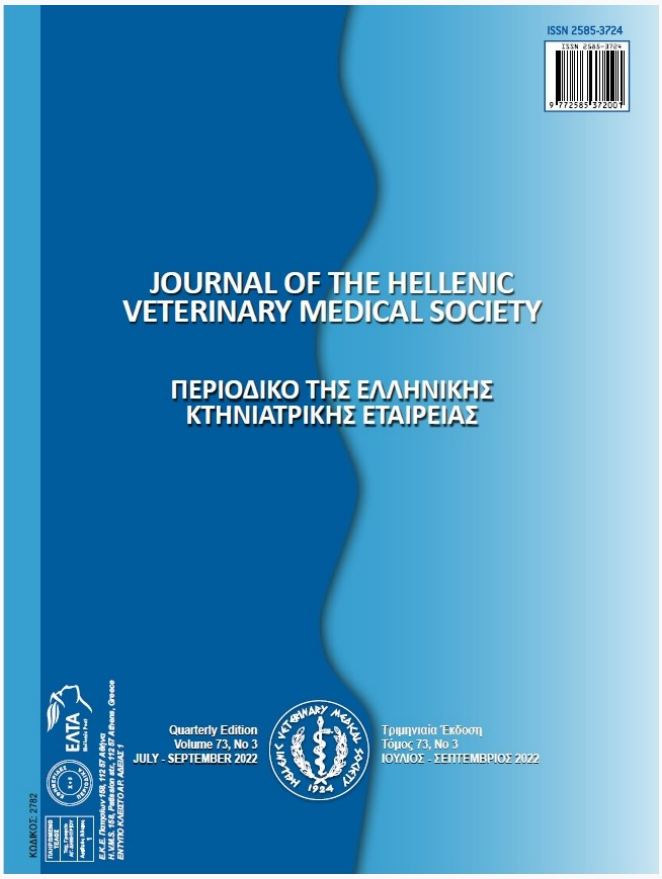
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 3 (2022)



Influence of lysozyme utilization with lactic acid bacteria in yoghurt on some foodborne pathogens

A Ismail, A Deeb, I Alhawary, W Elkassas, Y Hegazy

doi: 10.12681/jhvms.27796

To cite this article:

Ismail, A., Deeb, A., Alhawary, I., Elkassas, W., & Hegazy, Y. (2022). Influence of lysozyme utilization with lactic acid bacteria in yoghurt on some foodborne pathogens. *Journal of the Hellenic Veterinary Medical Society*, *73*(3), 4535–4544. https://doi.org/10.12681/jhvms.27796

J HELLENIC VET MED SOC 2022, 73 (3): 4535-4544
ΠΕΚΕ 2022, 73 (3): 4535-4544

Research article
Ερευνητικό άρθρο

Influence of lysozyme utilization with lactic acid bacteria in yoghurt on some foodborne pathogens

A.M. Ismail [1], A.M.M. Deeb [2], I.I. Alhawary [2,3], W.M. Elkassas [1], Y.M. Hegazy [4*]

[1]*Department of Food Hygiene, Animal Health Research Institute, Kafrelshiekh Lab, Agriculture Research Center (ARC), Egypt*

[2] *Food control department, Faculty of Veterinary Medicine, Kafrelsheikh Univeristy, Egypt*

[3] *Fish Processing and Biotechnology, Dean of faculty of Aquatic and Fisheries Science, Kafrelsheikh Univeristy, Egypt*

[4] *Department of Animal medicine (Infectious Diseases), Faculty of Veterinary Medicine, University of Kafrelsheikh, 5-Elgeish street, Kafrelsheikh, Egypt*

ABSTRACT: Despite the existence of many different food preservation methods, foodborne pathogens are still the most common related problems to dairy products. Developing alternative natural methods to control such problems is necessary. This research was planned to assess the bacteriological quality of yoghurt samples commercially available in Kafr El-Sheikh city, Egypt. Also to study the impact of natural antibacterial agents on the inhibition of selected foodborne pathogens artificially inoculated in yoghurt samples. The obtained results revealed poor bacteriological quality of the examined samples, as total staphylococci and coliforms were detected in 80% and 90% yoghurt samples, with mean values of $2.4 \times 10^3 \pm 0.13 \times 10^3$ CFU/g and $3.6 \times 10^5 \pm 2.4 \times 10^5$ MPN/g, respectively. *Staphylococcusaureus* was noticed in 4% of examined samples, polymerase chain reaction (PCR) confirmed the presence of virulence gene (clumping factor A- clfA) in one isolate. *Escherichiacoli* was detected in 14% of yoghurt samples, then serologically identified as O146:H5 (2), O111:H2 (2), O125:H11 (1), O26:H10 (1), and O158:H7 (1). Artificially inoculated yoghurt with *S. aureus* (7.39 log10 CFU/g) and *E. coli* (8.3 log10 CFU/g), were used to investigate the ability of hen egg-white lysozyme either with LAB classic or with ABT-5 (*L. acidophilus LA5+ S. thermophiles + Bifidobacterium Bb12*) starter cultures to inhibit the growth of these pathogens. During refrigerated storage, all yoghurt batches showed a reduction in pH value. It was observed that both pathogens couldn't be detected in the yoghurt batch containing LAB classic starter + heated lysozyme. Furthermore, *S. aureus* was completely inhibited in that containing ABT-5 cultures alone, while *E. coli* decreased by 6.23log. Conversely, there was not complete inhibition in other batches containing (LAB classic starter, LAB classic starter+ native lysozyme, ABT-5 culture+ native lysozyme and ABT-5 culture+ heated lysozyme) as the reduction rate during all storage periods for *S. aureus* count was 1.66, 2.63, 4.47,and 4.19 log10 CFU/g, While for *E. coli* count was 3.3, 3.81, 5.53, and 4.89 log10 CFU/g, respectively. This study highlighted the importance of adding natural antibacterial agents like lysozyme with LAB culture to yoghurt as a bio-control strategy to overcome foodborne pathogens that cause a public health hazard.

***Keywords*:** Hen Egg White Lysozyme; ABT-5;*S. aureus*; clfA;*E. coli*

Corresponding Author:
Yamen M. Hegazy, Fac. Vet. Med. Kafrelsheikh University Egypt
E-mail address: yamen12@yahoo.com

Date of initial submission: 11-08-2021
Date of acceptance: 11-01-2022

INTRODUCTION

Yoghurt is considered the most generally consumed fermented dairy product all over the world due to its high nutritive value and therapeutic properties (He et al., 2005).Although fermented dairy products are commonly considered to be safe, due to their acidic nature, inadequate hygienic conditions during processing, manipulation, and distribution decrease the quality of these products and increase the microbiological load which constitutes a public health hazard (Karagozlu et al., 2007).Foodborne pathogens like *S. aureus* and *E. coli* are the most commonly predominant human pathogenic bacteria associated with such low-quality fermented milk(Cancino-Padilla et al., 2017).

As a result of the health problems resulting from using artificial preservatives in dairy products, there is an urgent need for using naturally produced antimicrobial preservatives as alternatives for chemical ones without any adverse effects on public health to extend the product's shelf life and control food pathogens in refrigerated foods. Natural antimicrobial agents could be obtained from natural sources like animals, plants, and microbial sources (Pisoschi et al., 2018).

Hen egg-white lysozyme (LZ) is the most important natural antibacterial enzyme of animal origin that consists of 129 amino acids with a molecular weight of 14.3 kDa and has 4 disulfide bonds responsible for its high thermal stability(Carrillo et al., 2014).It is classified as Generally Recognized As Safe (GRAS) by the U.S. Food and Drug Administration (FDA). It could be very appropriate for milk products bio-preservation because of its absorption by casein micelles without loss in its enzymatic activity after absorption (L. de Roos et al., 1998), plus its stability in a wide range of temperature and pH (Abdou et al*.,* 2013).

LZ breakdown the bacterial cell wall of susceptible Gram-positive bacteria by catalyzing the hydrolysis of b (1-4) glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine found in peptidoglycan (the major component of the bacterial cell wall) (Arabski et al., 2015). However, its limited antibacterial effect against Gram-negative bacteria due to the presence of an additional barrier consist of protein, phospholipids, and lipopolysaccharide (LPS) surrounding the peptidoglycan layer (Mann, 2017) can be improved by modifying its hydrophobicity and cationic charge. Thermal modification caused by increasing temperatures results in a significant reduction in the enzymatic activity, but greatly improved its bactericidal action against Gram-negative bacteria (Ibrahim et al., 1996).

Likewise, Lactic Acid Bacteria (LAB) are effective natural antimicrobial agents of microbial origin that are identified as protective cultures. They have antagonistic action against some foodborne pathogenic microorganisms without adverse effect on the sensory quality of the product (Katikou et al., 2005). ABT-5 starter cultures are probiotic mixed cultures of LAB that consisting of both thermophilic (mainly *Streptococcus thermophilus*) and mesophilic strains (such as *Bifidobacterium* and *Lactobacillus acidophilus*) have been commonly used in commercial fermented dairy products today (Buriti et al., 2007).

Probiotics improve human health state, in addition to their antagonistic effect against foodborne pathogens (Yesillik et al., 2011). Its antimicrobial properties are due to the production of inhibitory substances such as bacteriocins, organic acids (lactic acid and acetic acid),H_2O_2, and hydroxylated fatty acids(Russo et al., 2017).

The current study was planned to evaluate yoghurt commercially available in Kafr El-Sheikh city bacteriologically as well as investigate the antibacterial activity of native and modified LZ,for the first time up to our knowledge,either with LAB classic starters or in combination with a probiotic culture against*S. aureus* and *E. coli* in manufactured yoghurt.

MATERIALS AND METHODS

Samples collection and preparation

Fifty yoghurt samples were randomly collected from different supermarkets in Kafr El-Sheikh city, Egypt. All samples were transported quickly to the laboratory in an insulated ice-box for bacteriological examination. Serial dilutions (10- fold) were prepared from these samples using 0.1 % sterile peptone water (Oxoid) and suitable dilutions were used for staphylococcal and coliforms counts (ISO 6887-2: 2003).

Bacteriological examination

Total staphylococci count andisolation of *S. aureus* (ISO 6888-1: 2003)

The staphylococcal count was carried on Baird Parker agar medium plates (Oxoid) that incubated at 37°C for 48 hrs. Circular, smooth or rough appearance, convex, gray-black colonies were enumerated and the total staphylococci count /g was calculated.

Then, typical colonies of *S. aureus* (dark convex colonies with a thin white margin that bounded by a clear area spreading into the opaque medium) were picked and cultured on a nutrient semisolid medium for biochemical identification using the following tests: catalase, hemolysis, mannitol, oxidase and coagulase test according to(Quinn et al., 2002).

PCR was used for the detection of virulence adhesion gene (clumping factor A- clfA)in *S. aureus* at Animal Health Research Institute, Dokki, Egypt.The extraction of bacterial DNA from *S. aureus* isolates was carried out according to QIAamp DNA mini kit instructions using specific primers (Table 1). PCR Master Mix (total volume 25µl) according to Emerald Amp GT PCR mastermix (Takara) Code No. RR310A kit was used. This mix included 12.5 µl Emerald Amp GT PCR mastermix (2x premix), 4.5µl PCR grade water, 6 µl Template DNA, 1 µl from each forward and reverse primer (20 pmol). The cycling conditions were: primary denaturation at 94˚C for 5 min, followed by secondary denaturation (35 cycles) at 94˚C for 30 sec, annealing at 55˚C for 40 sec, extension at 72˚C for 45 sec, then final extension at 72˚C for 10 min. About 20 µl of each PCR product was used in gel electrophoresis according to (Sambrook et al., 1989) with modification. A 100 bp DNA ladder was used as a marker for PCR product. This gel contained 1.5% agarose gel and 0.5µg/ml ethedium bromide (for staining). The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Total coliform count and isolation of *E.coli*

Coliforms count was carried out using the Most Probable Number (MPN/g) (ISO 4831: 2006). A loopful from positive MacConkey's broth tubes (yellow color with gas production) was streaked onto Eosin Methylene Blue agar plates (EMB) (Oxoid) then incubated at 37 ºC for 24 hrs (APHA, 2004). The purified colonies were cultured on a nutrient semisolid medium for biochemical identification using the following tests: motility, indole production, methyl red, Voges - Proskauer, citrate utilization, hydrogen-sulphide production, urease, nitrate reduction, oxidation-fermentation test and fermentation of sugars according to (MacFaddin, 2000).

The suspected *E. coli* strains were serologically confirmed at Animal Health Research Institute, Dokki, Egypt, by using rapid diagnostic *E. coli* antisera sets (Denka Seiken Co., Japan) according to (Kok et al., 1996).

Effect of natural antibacterial agents on survival of *S. aureus* and *E. coli* in manufactured yoghurt

Preparations of antibacterial agents

Starter cultures used in yoghurt manufacture

The LAB classic starter (YC-X11) containing *S. thermophilus* and *L. delbrueckii ssp. Bulgaricus* and the probiotic culture ABT-5 consisting of *L. acidophilus LA5*, *S. thermophiles*,and *Bifidobacterium Bb12*(Christian Hansen, Denmark) were used.

Lysozyme solution

Hen egg-white lysozyme (Tokyo Chemical Industry CO., LTD. 6-15-9 Toshima, KITA-KV Tokyo, Japan) stock solution of 1mg/ml in potassium phosphate buffer (10 mM, pH 6) was prepared according to (Masschalck et al., 2000), then divided into two parts, one part was kept refrigerated at 4°C and the other part was modified thermally by heating at 80°C for 20min, then insoluble aggregates were removed by centrifugation (3000×g, 15 min) (Ibrahim et al., 1996). The final concentration of native lysozyme (LZ) and heated lysozyme (HLZ) was 50µg/ml.The enzymatic activity of LZ was measured against *Micrococcus lysodekticus* by a turbidimetric method using a spectrophotometer (Touch et al., 2004).

Bacterial test strains

S. aureus and *E. coli*O_{111}:H_2 that were previously isolated in this study and subjected for confirmation were selected according to their virulence and serotype, respectively, then cultivated in sterile nutrient broth at 37°C for 24 hrs. The bacterial population was determined by the pour plate method after preparation of 10- foldserial dilutions to reach the level of 2.5×10^7 (7.39 log10 CFU/ml for *S. aureus*)and 2×10^8 (8.3 log10 CFU/ml for *E. coli*) CFU/ml.

Table 1. Oligonucleotide primers sequences (Metabion, Germany)

Gene	Sequence	Amplified product	Reference
S. aureus clfA	GCAAAATCCAGCACAACAGGAAACGA CTTGATCTCCAGCCATAATTGGTGG	638 bp	Mason et al.(2001)

Table 2. Bacterial load of the examined yoghurt samples

Tested microorganisms	No. of examined samples	Positive samples		Count /g		
		No.	%	Min.	Max.	Mean± SE
Staphylococcal count	50	40	80	1.0×10^{1}	5.6×10^{4}	$2.4\times10^{3}\pm0.13\times10^{3}$
S. aureus		2	4			
Coliforms count		45	90	7.4	1.1×10^{7}	$3.6\times10^{5}\pm2.4\times10^{5}$
E. coli		7	14			

Treatments

Yoghurt was manufactured according to (Robinson et al., 2002);raw cow's milk was heated in a water bath at 90 ºC with agitation for 10 min then cooled to 42 ºC. Then, it was divided into six batches(8 plastic cupsfor each batch)as follow:

batch 1: contained 2 % (v/v) LAB classic starter cultures (YC-X11-Yoflex®)

batch 2: contained 2 % (v/v) LAB classic starter + LZ solution

batch 3: contained 2 % (v/v) LAB classic starter + HLZ solution

batch 4: contained 2 % (v/v) ABT-5 culture

batch 5: contained 2 % (v/v) ABT-5 culture+LZ solution

batch 6: contained 2 % (v/v) ABT-5 culture + HLZ solution

Every batch was doubled, one inoculated with *S. aureus* (48 plastic cups) and the other inoculated with *E. coli*(48 plastic cups) at the rate of 7.39 and 8.3 log10 CFU/g, respectively. All batches were incubated at 45 ºC until yoghurt curd (50g yoghurt in each cup) was formed. After curdling, the batches were kept at 4 ºC for 14 days. Samples were examined immediately after fermentation (curd time) and at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 10^{th}, 12^{th}, and 14^{th} days of storage period to determine pH values using a pH meter (Jenway, UK) and evaluate the inhibitory effect of these natural antibacterial agents against both pathogens. The colony-forming units count (CFU/g) was calculated by culturing onto the specific media for each pathogen.

Statistical Analysis

Statistical analysis was performed using SPSS Version 16 software (SPSS, IL, USA). Data were analyzed using the General Linear model, Repeated Measures ANOVA to compare the significant effect of different treatment groups on pH of the yoghurt samples and also on the total *S. aureus* and *E. coli* count after 14 days of storage.Duncan's Multiple Range Test (DMRT) was used to determine significant differences between mean values. The alpha level for determination of significance was set at 0.05.

RESULTS

The results exhibited that staphylococci were detected in (40) 80% of the examined samples (Table 2) with counts ranged from 1.0×10^{1} to 5.6×10^{4}CFU/g. *S. aureus* was isolated from (2) 4 % of the examined samples.The result obtained from PCR (photo 1) recovered that the clfA virulence gene was detected in one studied strain of 638 pb.

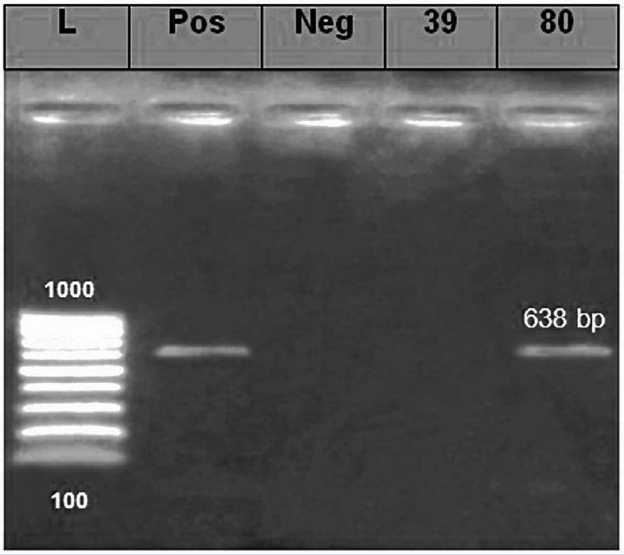
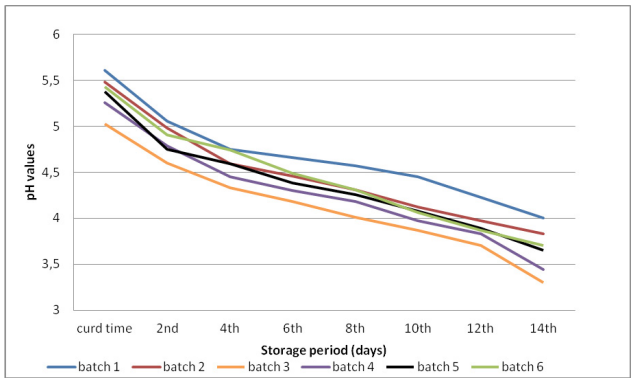


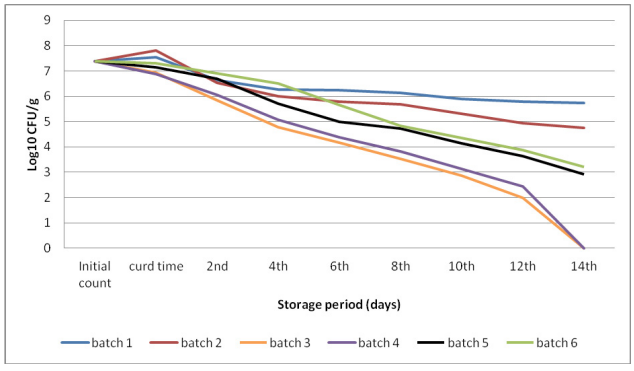
Photo 1. Agarose gel electrophoresis of cPCR amplified of *S. aureus* clfA gene. L: 100bp DNA ladder, Pos: control positive for clfA gene, Neg: control negative

Coliforms were detected in 45 (90%) of examined yoghurt samples with counts varied between 7.4 to 1.1×10^{7} MPN/g. Whereas, *E. coli* was isolated from 7 (14%) of the examined samples (Table 2). The isolated *E. coli* strains were serologically identified to serogroups as follows O_{146}:H_5 (2), O_{111}:H_2 (2), O_{125}:H_{11} (1), O_{26}:H_{10} (1), and O_{158}:H_7 (1) (Table 3).



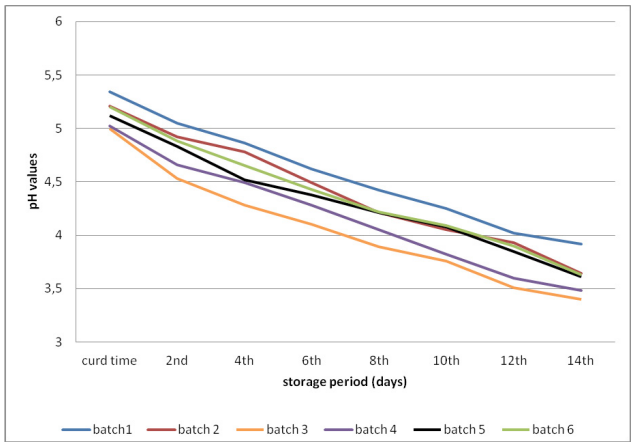
batch 1: LAB classic starter cultures batch 2: LAB classic starter + LZ
batch 3: LAB classic starter + HLZ batch 4: ABT-5 culture
batch 5: ABT-5 culture+ LZ batch 6: ABT-5 culture + HLZ

Figure 1. Changes in pH values in yoghurt batches inoculated with *S. aureus* during refrigerated storage (4°C)



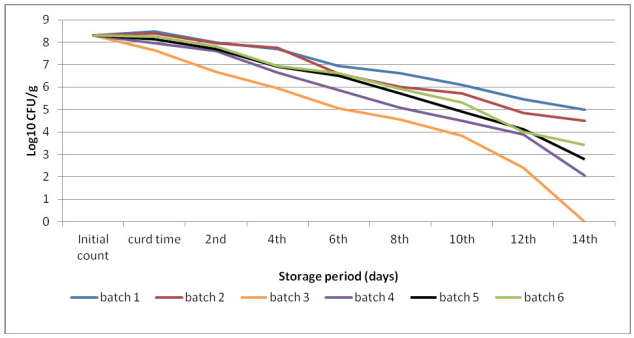
batch 1: LAB classic starter cultures batch 2: LAB classic starter + LZ
batch 3: LAB classic starter + HLZ batch 4: ABT-5 culture
batch 5: ABT-5 culture+ LZ batch 6: ABT-5 culture + HLZ

Figure 3. Behaviour of *S. aureus* in yoghurt batches during refrigerated storage (4°C)



batch 1: LAB classic starter cultures batch 2: LAB classic starter + LZ
batch 3: LAB classic starter + HLZ batch 4: ABT-5 culture
batch 5: ABT-5 culture+ LZ batch 6: ABT-5 culture + HLZ

Figure 2. Changes in pH values in yoghurt batches inoculated with *E. coli* during refrigerated storage (4°C)



batch 1: LAB classic starter cultures batch 2: LAB classic starter + LZ
batch 3: LAB classic starter + HLZ batch 4: ABT-5 culture
batch 5: ABT-5 culture+ LZ batch 6: ABT-5 culture + HLZ

Figure 4. Behavior of *E. coli* in yoghurt batches during refrigerated storage (4°C)

Table 3. Serological identification of the isolated *E. **coli*** from the examined yoghurt samples

E. coli serogroups	No.
O_{146}:H_5	2
O_{111}:H_2	2
O_{125}:H_{11}	1
O_{26}:H_{10}	1
O_{158}:H_7	1

Figures (1-2) display the pH changes in yoghurt batches inoculated by *S. aureus* and *E. coli* during refrigerated storage. The pH values gradually decreased (p<0.05) as the storage period extends from initial values at the termination of fermentation (curd time) 5.61, 5.48, 5.03, 5.26, 5.38, and 5.43 to 4.00, 3.83, 3.30, 3.44, 3.65, and 3.70 at the day 14 of storage period (4 ºC), respectively, for yoghurt batches inoculated by *S. aureus*. And from 5.34, 5.21, 5, 5.02, 5.12, and 5.2 initially to 3.92, 3.64, 3.40, 3.48, 3.61, and 3.63 at the end of the storage period for batches inoculated by *E. coli*, respectively.

Figure (3) showed viable counts of *S. aureus* in all batches decreased significantly (p< 0.05)as the storage period extended. The counts decreased from the inoculation count (7.39 log10 CFU/g) to 5.73, 4.76, 2.92, and 3.2 log10CFU/g in batches (1, 2, 5& 6),respectively.Whereas, *S.aureus* growth in yoghurt batch 3 that containing HLZwith LABclassic starter and

batch 4 with ABT-5 culture alonecouldn't be detected on the 14th day of refrigerated storage.

The survival pattern of *E. coli* in yoghurt batches-(Figure 4) showed that the viable counts of *E.coli* in all batches decreased significantly ($p< 0.05$) during the refrigerated storage period.The counts decreased from the initial count (8.3 log10CFU/g) to 5, 4.49, 2.07, 2.77, and3.41 log10CFU/gin batches (1, 2, 4, 5& 6), respectively.However, *E. coli* growth was not detected on day 14thof refrigerated storage only in yoghurt batch 3 (LAB classic starter + HLZ).

As regards the result tabulated in (Table4), the hydrolytic activity of LZ and HLZ was measured against *Micrococcus lysodeikticus* by spectrophotometric method. Enzymatic activity of denatured LZ decreased to 52.31% with about 47.69% loss in hydrolytic activity.

Table 4. Hydrolytic activity of lysozyme

lysozyme activity (Units / mg)		
LZ	HLZ	Reduction %
2.225	1.164	47.69

DISCUSSION

In the current study, staphylococci were detected in 80% of examined yoghurt samples found in the markets and ready to be used by consumers with a mean count of $2.4 \times 10^3 \pm 0.13 \times 10^3$ CFU/g. Yabaya and Idris (2012) found a lower staphylococcal count in collected yoghurt samples from different retail outlets within Kaduna Metropolis; 2 $x10^2$ to 7 x 10^2CFU/g. Conversely, higher staphylococcal counts were obtained by Omar et al.(2018);8.3 $x10^5$ to1.4 $x10^7$ CFU/gin collected yoghurt samples from different regions in Sharkia governorate, Egypt. The presence of staphylococci in yoghurt usually indicates improper hygienic conditions during manufacturing, handling, and storage, as contamination may be originated from using mastitic milk and food handlers with unclean hands or respiratory infection (Akram et al., 2013).

S. aureus was detected in 4 % of examined yoghurt samples.These samples were incompatible with the permissible limits reported by Egyptian standards (EOSQC, 2005), which stated that yoghurt must be free from *S. aureus*.Kandil et al. (2018), Meshref et al.(2019), Abd El-Halem et al.(2019), and Younis et al.(2020) isolated *S. aureus* from 36%, 88%, 5.7%,and 52 % of yoghurt samples collected from different governorates in Egypt, respectively.This variation in *S. aureus* incidence rates between studies may refer to the variation in the manufacturing practices and personal hygiene followed during yoghurt production. The presence of *S. aureus* in yoghurt even in low incidence must be considered a public health hazard, as the organism may lose its viability while its enterotoxins remain to elicit staphylococcal food poisoning symptoms (Erkmen, 1995).In the current study, the presence of virulence factor (clfA) in one of the two *S. aureus* isolates that considered one of the essential adhesion factors that responsible for the clumping of blood plasma protein fibrinogen allowing the organism to circulates in the blood and forms a biofilm (Vaudaux et al., 1995), also it has been identified as a virulence factor in an endocarditis case (Heilmann, 2011).

Coliforms are a group of Gram-negative bacteria of public health concern that frequently contaminate dairy products through several sources causing food illnesses (Giammanco et al., 2011).According to EOSQC (2005), coliforms count in yoghurt should be less than 10 CFU/g, in this study 86% of yoghurt samples were failed to comply with this permissible limit.Previous Egyptian studies detected coliforms at different rates as 50%, 76.67% of yoghurt samples examined by El-Leboudy et al.(2017),Fathi et al.(2019),respectively. High levels of coliforms in yoghurt may not only indicate fecal contamination but also give an indication of using low-quality raw milk, improper heat treatment, unhygienic manufacturing practices or contamination after processing,poor personnel hygiene,and using of unclean equipment and/or contaminated water (El-Kholyet al.,2014).

E. coli is used as a marker organism for fecal contamination and reflects a possible presence of other enteric pathogens and/or toxigenic organisms which constitute a public health threat (Singh and Prakash, 2008). *E. coli* was isolated from 14% of examined yoghurt samples. These samples were failed to comply with EOSQC (2005) as yoghurt must be free from *E. coli*. The contamination rate of *E. coli* in our examined samples was less than those reported by Fathi et al.(2019), Younis et al.(2020),and Hassan et al.(2021) who detect it in 36.96%, 80%, and 25%, respectively, in their examined yoghurt samples collected from different localities in Egypt, while Sayed (2012) couldn't isolate *E. coli*. The different prevalence rates between studies may indicate poor hygienic conditions during yoghurt manufacturing and handling, as its presence in yoghurt referred to post pasteurization contami-

nation either before or during the packaging process (Omola et al., 2014).

The relatively high incidence of *E. coli* in yoghurt samples reported in the present and previous studies seems to confirm the survival of *E. coli* in both low pH and temperature and, subsequently, the implication of acidic foods as yoghurt in food poisoning diseases caused by *E. coli*(Sharp et al.,1995). The isolated *E. coli* strains were serotyped to O_{146}:H_5 (2), O_{111}:H_2 (2), O_{125}:H_{11} (1), O_{26}:H_{10} (1), and O_{158}:H_7 (1), most of these serogroups are commonly involved in food poisoning sporadic and outbreak cases all over the world (Valilis et al., 2018).

The effect of natural antibacterial agents on the survival of *S. aureus* and *E. coli* in manufactured yoghurt was studied. It was observed that pH values of yoghurt batches containing LZ and HLZ with LAB classic starter culture were slightly lower than that containing LAB classic starter culture alone, this reduction may be attributed to increasing the bacterial activity of starter culture that fermented lactose leading to more lactic acid production (Hofi and El-Shibiny, 1978). Also, this confirmed that starter culture (*S. thermophilus* and *L. Bulgaricus*) don't affected by the antibacterial activity of LZ and agreed with Vinderola et al. (2002), Turchia et al.(2017), but the mechanism of this resistance needs more investigations. Moreover, probiotic yoghurt batches showed a slight decrease in pH compared to that with LAB classic starter culture alone, this drop in pH is due to probiotics not only produce lactic acid but also acetic acids resulting in low pH of yoghurt (Mobarez et al., 2008).

S. aureus was not detected in both yoghurt batches containing HLZ with LAB classic starter and ABT-5 culture alone at the end of cold storage. While the lowest reduction was belonging to batch 1 with LAB classic starter culture alone.

The bactericidal effect of yoghurt batch containing LAB classic starter with HLZ was significantly higher ($p< 0.05$)than those with LZ, this may be attributed to heat denaturation modifying native LZ (monomeric form) to oligomeric forms resulting in broadens its antibacterial activity to include some Gram-positive bacteria that resist to native LZ (Cegielska-Radziejewska et al., 2009). As, *S. aureus*carries an O-acetylation on the C-6 hydroxyl group of the N-acetylmuramyl residues of its peptidoglycan layer by the activity of integral membrane protein, OatA gene resulting in its resistance to native LZ (Bera et al., 2007).

Also, it worth to be mention that the survival of *S. aureus* were significantly lower ($p< 0.05$) in yoghurt batches with probiotic culture compared to yoghurt batch with classic starter culture alone that may be due to probiotics produce metabolites with antagonistic properties including organic acids, carbon dioxide, bacteriocins, H_2O_2, and low molecular weight antimicrobial compounds (Tejero-Sariñena et al., 2012).

Al-Delaimy and Hamamdeh (2013) and Abd El-Gawad et al. (2014) recorded that *S. aureus* was inhibited after the 10th day of cold storage in the probiotic yoghurt, while the bacteria were able to survive in traditional yoghurt sample after 15 days of storage period. While,Soliman and Ahmed (2019) stated a slight decrease in the number of *S. aureus* after 7 days of storage. Conversely, Saleem et al. (2021) reported a significant inhibitory effect of LZ on *S. aureus* when added to mozzarella cheese.

Our findings also showed that the yoghurt batch containing HLZ with LAB classic starter culture gave the best bactericidal effect toward *E. coli*as it couldn't be detected at end of the storage period.

The markedly inhibitory effect of HLZ ($p< 0.05$) compared to LZ with LAB classic starter may be attributed to thermal modification leading to the partial unfolding of native LZ extending its antibacterial activity to include Gram-negative bacteria by penetrating the lipid layer of the bacterial cell membrane forming ion pores resulting in disturbance in the electrochemical potential (Leśnierowski and Cegielska-Radziejewska, 2012).

Additionally, it was noticed that the reduction in *E. coli* count were significantly higher ($p< 0.05$) in probiotic yoghurt batches than that with LAB classic starter culture alone. This inhibitory effect could be due to probiotics produce antibacterial substances.

A previous study by Vilcacundo et al.(2018) recorded that thermal modification of LZ at 80°C for 20 min was able to decreasethe log of *E. coli*. On the other hand, Abd El-Gawad et al. (2014) revealed that 3.5×10^5CFU/g of *E. coli* were eliminated after 2 and 5 days of refrigerated storage in probiotic and traditional yoghurt samples, respectively.

The hydrolytic activity of HLZ decreased to 52.31%.This result disagreed with those of Thammasirirak et al.(2010), Vilcacundo et al.(2018) who reported that heated lysozyme at 80°C for 20min was with 74.5 and 95% lytic activity, respectively. Previ-

ous studies by Ibrahim et al.(2001), Gorbenko et al. (2007) have confirmed the fact that the antibacterial activity of thermal modified LZ is not dependent on its enzymatic activity. As, LZ has two main additional non-enzymatic bacteriolytic mechanisms of action, which are not dependent on its enzymatic function (the indirect bacteriolytic-dependent mechanisms and electrostatic communication mechanism with bacterial oligonucleotides) (Abdou et al., 2013; Arabski et al., 2015).

Combined use of HLZ with probiotics showed better antimicrobial effect against both pathogens than using LAB classic starter alone. Conversely, using probiotic culture alone had better antibacterial effect against pathogens than its using in combination with HLZ. This indicates that probiotic culture alone gave the most potent inhibitory activity on pathogens than using mixture of probiotic culture with HLZ and using LAB classic starter alone. It could be expected that LZ had an antagonistic effect either on bifidobacteria that found in ABT-culture and/or its bacteriocin. Kim et al. (2015) reported that the inhibitory activity of bacteriocins produced by probiotics could be antagonized by enzymatic degradation. Also, the susceptibility of bifidobacteria to LZ was strain-specific (Rada et al., 2010). For this reason, the resistance to LZ at 25-50 mg/l should be commended as a standard for the selection of probiotic strains suitable for use in the milk industry, as, LZ can be used as milk products preservatives at this concentration (Cardarelli et al., 2007).

CONCLUSION

Results of this study clearly indicate that the bacterial quality of locally produced yoghurt in Kafr El-Sheikh city, Egypt was inferior as compared to the Egyptian Standards. Therefore strict sanitary measures must be applied during manufacturing, storage, and distribution to produce safe and high-grade yoghurt. Moreover, our experimental results showed that the combination of HLZ with LAB classic starter exhibited a great bactericidal effect on both*S. aureus* and *E. coli* than its combination with probiotic culture.Also,using probiotic culture alone gave the most potent inhibitory activity on pathogens than using mixture of probiotic culture with HLZ and using LAB classic starter alone. So, we suggest adding these natural antimicrobials as food bio-controlling preservatives.

ACKNOWLEDGEMENTS

This study was supported by the Animal Health Research Institute, Ministry of Higher education, Egypt.

CONFLICT OF INTEREST

None declared by the authors.

REFERENCES

Abd El-Gawad IA, El-Sayed EM, El- Zeini HM, Hafez SA, Saleh FA (2014) Antibacterial activity of probiotic yoghurt and soy-yoghurt against *Escherichia coli* and *Staphylococcus aureus*. J Nutr Food Sci 4 (5): 1-6.

Abd El-Halem SG, Attia IA, El-Dera HB, El-Seedy AS (2019) Prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from raw milk and dairy products collected from Alexandria, Egypt. Alex J Food Sci & Technol 16 (2): 25-33.

Abdou AM, Kim M, Sato K (2013) Functional proteins and peptides of hen's egg origin. In: Bioactive Food Peptides in Health and Disease. Tech, Croatia-European Union: pp 115-144.

Akram N, Chaudhary AH, Ahmed S, Ghuman MA, Nawaz G, Hussain S (2013) Isolation of bacteria from mastitis affected bovine milk and their Antibiogram. Eur J Vet Med 2 (1): 38-46.

Al-Delaimy KS and Hamamdeh YM (2013) Inhibition of *Staphylococcus aureus* by lactic acid bacteria and /or *Bifidobacterium lactis* during milk fermentation and storage. J Microbiol Biotechnol Food Sci 2 (4): 2406-2419.

APHA (American public Health Association) (2004) Standard methods for the examination of Dairy products17th ed. Edited by H. Michael Wehr and Joseph H. Frank, Washington, D.C., USA.

Arabski M, Konieczna I, Tusińska E, Wąsik S, Relich I, Zając K, Kamiński ZJ, Kaca W (2015)The use of lysozyme modified with fluorescein for the detection of Gram-positive bacteria. Microbiol Res170: 242-247.

Bera A, Biswas R, Herbert S, Kulauzovic E, Weidenmaier C, Peschel A, Go¨tz F (2007)Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. J Bacteriol 189 (1): 280-283.

Buriti FCA, Cardarelli HR, Saad SMI (2007) Biopreservation by *Lactobacillus paracasei* in co-culture with *Streptococcus thermophilus* in potentially probiotic and symbiotic fresh cream-cheeses. J Food Prot 70 (1): 228-235.

Cancino-Padilla N, Fellenberg MA, Franco W, Ibáñez RA, Vargas-Bello-Pérez E (2017)Foodborne bacteria in dairy products: Detection by molecular techniques. Cienc Investig Agrar 44 (3): 215-229.

Cardarelli HRI, Saad SMI, Gibson GR, Vulevic J (2007) Functional petit-suisse cheese: measure of the prebiotic effect. Anaerobe 13 (5-6): 200-207.

Carrillo W, García-Ruiz A, Recio I, Moreno-Arribas MV (2014) Antibacterial activity of hen egg white lysozyme modified by heat and enzymatic treatments against oenological lactic acid bacteria and acetic acid bacteria. J Food Prot 77: 1732-1739.

Cegielska-Radziejewska R, Leśnierowski G, Kijowski J (2009) Antibacterial activity of hen egg white lysozyme modified by thermochemical technique. Eur Food Res Technol 228: 841-845.

El-Kholy M, El-Shinawy SH, Meshref AMS, Korny AM (2014)Screening of antagonistic activity of probiotic bacteria against some food-borne pathogens. J FBT IAU4 (2): 1-14.

El-Leboudy AA, Amer AA, El-Ansary MA, El- Asuoty MS, Ahmida MR (2017) Evaluation of some fermented milks sold in Alexandria city. Assiut Vet Med J 63 (154): 59-66.

EOSQC (Egyptian Organization for Standardization and Quality Control) (2005) "Domiati cheese 1008-3/Kareish cheese1008-4/ Ice cream 1185-1/Yoghurt 1633".

Erkmen O (1995) Behavior of *S. aureus* in Turkish feta cheese during manufacture and ripening. J Food Prot 58 (11): 1201-1205.

Fathi SS, Mohamed AS, El- Sayed MSH (2019)Coliforms contamination in raw milk and some dairy products with special reference to comparative identification of Enterobacterspp.Zagazig Vet J47 (4): 388-397.

Giammanco GM, Pepe A, Aleo A, D'Agostino V, Milone S, Mammina C (2011)Microbiological quality of Pecorino Siciliano "primosale" cheese on retail sale in the street markets of Palermo, Italy. New Microbiol 34 (2): 179-185.

Gorbenko GP, Ioffe VM, Kinnunen PKJ (2007) Binding of lysozyme to phospholipid bilayers: Evidence for protein aggregation upon membrane association. Biophys J 93 (1): 140-153.

Hassan GM, Meshref AMS, Elnewery HA, Mohamed AS (2021) Prevalence of *Escherichia coli* in milk and some dairy products in Beni-Suef governorate, Egypt. J Vet Med Res 27 (2): 161-167.

He M, Yang YX, Han H, Men JH, Bian LH, Wang GD (2005)Effects of yoghurt supplementation on the growth of preschool children in Beijing suburbs. Biomed Environ Sci18 (3): 192-197.

Heilmann C (2011) Adhesion mechanisms of staphylococci. In: Bacterial Adhesion. Springer, Netherlands: pp 105-123.

Hofi AA and El-Shibiny S (1978)The chemical composition of market yoghurt. Egypt J Dairy Sci 6 (1): 25-31.

Ibrahim HR, Higashiguchi S, Juneja LR, Kim M, Yamamoto T (1996)A structural phase of heat-denatured lysozyme with novel antimicrobial action. J Agric Food Chem44 (6): 1416-1423.

Ibrahim HR, Matsuzaki T, Aoki T (2001) Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett 506 (1): 27-32.

ISO 4831: 2006 (International Organization for Standardization (2006) Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of coliforms — Most probable number technique.

ISO 6887-2: 2003(International Organization for Standardization (2003) Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

ISO 6888-1: 2003 (International Organization for Standardization) (2003) Microbiology of food and animal feeding stuffs — Horizontal methods for the enumeration of coagulase-positive Staphylococci (*Staphylococcus aureus* and other species) Technique using Baird-Parker medium.

Kandil AA, Elhadidy M, El-Gamal A, Al-Ashmawy M A (2018)Identification of *S. aureus* and *E. coli* from dairy products intended for human consumption. Adv Anim Vet Sci6 (11): 509-513.

Karagozlu N, Karagozlu C, Ergonul B (2007) Survival characteristics of *E. coli O157:H7, S. typhimurium* and *S. aureus* during kefir fermentation. Czech J Food Sci 25 (4): 202-207.

Katikou P, Ambrosiadis I, Georgantelis D, Koidis P, Georgakis SA (2005) Effect of Lactobacillus-protective cultures with bacteriocin-like inhibitory substances'producing ability on microbiological, chemical and sensory changes during storage of refrigerated vacuum-packaged sliced beef. J Appl Microbiol99 (6): 1303-1313.

Kim DH, Chon JW, Kang IB, Kim H, Kim HS, Song KY, Seo KH (2015) Growth inhibition of *Cronobacter sakazakii* in experimentally contaminated powdered infant formula by kefir supernatant. J Food Prot78 (9): 1651-1655.

Kok TW, Worswick D, Gowans E (1996)Some serological techniques for microbial and viral infections. In: Practical Medical Microbiology. 14th ed, Edinburgh, Churchill Livingstone, UK: pp 179-204.

L. de Roos A, Walstra P, Geurts TJ (1998) The association of lysozyme with casein. Int Dairy J 8 (4): 319-324.

Leśnierowski G and Cegielska-Radziejewska R (2012) Potential possibilities of production, modification and practical application of lysozyme. Acta Sci Pol Technol Aliment 11 (3): 223-230.

MacFaddin JF (2000)Biochemical tests for identification of medical bacteria. 3rd ed, Lippincott Williams and Wilkin Washington, Washington, Philadelphia, PA 113, U.S.A.

Mann, K. (2017) Proteomics of Egg White. In: Proteomics in Food Science. Kuddus M ed, Academic Press, London, UK: pp 261-276.

Mason WJ, Blevins JS, Beenken K, Wibowo N, Ojha N, Smeltzer MS (2001)Multiplex PCR Protocol for the diagnosis ofstaphylococcal Infection. J Clin Microbiol39 (9): 3332-3338.

Masschalck B, Garc´ıa-Graells C, Haver EV, Michiels CW (2000) Inactivation of high pressure resistant *Escherichia coli* by lysozyme and nisin under high pressure.Innov Food Sci Emerg Technol 1 (1): 39-47.

Meshref AMS, Hassan GM, Riad EM, Ashour WA (2019)Studies on enterotoxigenic *Staphylococcus aureus* in milk and some dairy products. Assiut Vet Med J 65 (163): 87-97.

Mobarez AM, Hosseini Doust R, Sattari M, Mantheghi N (2008)Antimicrobial effects of bacteriocin like substance produced by *L. acidophilus* from traditional yoghurt on *P. aeruginosa* and *S. aureus*. J Biol Sci 8 (1): 221-224.

Omar SM, Abd El-Fattah HI, Abdelbasit HM, Mahgoub SA (2018)Contamination of fermented foods in Egypt with undesirable bacteria. Zagazig J Agric Res 45 (1): 317-329.

Omola EM, Kawo AH, Shamsudden U (2014)Physico-chemical, sensory and microbiological qualities of yoghurt brands sold in Kano Metropolis, Nigeria. BAJOPAS 7 (2): 26-30.

Pisoschi AM, Pop A, Georgescu C, Turcuş V, Olah NK, Mathe E (2018) An overview of natural antimicrobials role in food. Eur J Med Chem 143: 922-935.

Quinn PJ, Markey BK, Carter ME, Donnelly WJC, Leonard FC (2002) Veterinary Microbiology and Microbial Diseases. 1st published Blackwell Science, Oxford, UK.

Rada V, Splichal I, Rockova S, Grmanova M, Vlkova E (2010) Susceptibility of bifidobacteria to lysozyme as a possible selection criterion for probiotic bifidobacterial strains.Biotechnol Lett 32(3): 451-455.

Robinson RK, Tamine AY, Wszolek M (2000) Microbiology of fermented milks. In: Dairy Microbiology Handbook . 3rd ed, john Wiley and Sons, New York: pp 367-490.

Russo P, Arena MP, Fiocco D, Capozzi V, Drider D, Spano G (2017) *Lactobacillus plantarum* with broad antifungal activity: a promising approach to increase safety and shelf-life of cereal-based products. Int J Food Microbiol247: 48-54.

Saleem IM, Gulzar N, Nadeem M, Sameen A, Rafiq S, Rehman S (2021) Influence of hen egg white lysozyme N-acetylmuramide glycan hydrolase on the antimicrobial activity and textural characteristics of high-moisture mozzarella cheese. J Food Process Preserve15573.

Sambrook J, Fritscgh EF, Mentiates (1989) Molecular coloning. In: A laboratory manual. Cold spring Harbor Laboratotry press, New York.

Sayed M (2012)Laban rayeb and yoghurt: Sensory, rheological, chemical and microbiological properties. Assiut Vet Med J 58 (132): 1-26.

Sharp JCM, Reilly WJ, Coia JE, Curnow J, Synge BA (1995) *Escherichia coli O157:H7* infection in Scotland: anepidemiological overview, 1984-1994. PHLS Microbiol Digest12:134-140.

Singh P and Prakash A (2008) Isolation of *Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* from milk products sold under market conditions at Agra Region. Acta agric Slov92 (1): 83-88.

Soliman NSM and Ahmed LI (2019)Survival of *Staphylococcus aureus* in Bio-Yoghurt. Open J Appl Sci9 (7): 564-572.

Tejero- Sariñena S, Barlow J, Costabile A, Gibson GR, Rowland I (2012) In vitro evaluation of the antimicrobial activity of a range of probiotics against pathogens: Evidence for the effects of organic acids. Anaerobe 18 (5): 530-538.

Thammasirirak S, Pukcothanung Y, Preecharram S, Daduang S, Patramanon R, Fukamizo T, Araki T (2010)Antimicrobial peptides derived from goose egg white lysozyme. Comp Biochem Physiol C Toxicol Pharmacol 151 (1): 84-91.

Touch V, Hayakawa S, Saitoh K (2004)Relationships between conformational changes and antimicrobial activity of lysozyme upon reduction of its disulfides bonds. J Food Chem84 (3): 421-428.

Turchia B, Pedonesea F, Torraccaa B, Fratinia F, Mancinia S, Galieroa A, Montalbanoa B, Cerria D, Nuvolonia R (2017)*Lactobacillus plantarum* and *Streptococcus thermophilus* as starter cultures for a donkey milk fermented beverage. Int J Food Microbiol 256 (1): 54-61.

Valilis E, Ramsey A, Sidiq S, DuPont HL (2018) Non-O157 Shiga toxin-producing *Escherichia coli*-A poorly appreciated enteric pathogen: Systematic review. Int J Infect Dis 76: 82-87.

Vaudaux PE, Francois P, Proctor RA, McDevitt D, Foster TJ, Albrecht RM, Lew DP, Wabers H, Cooper SL (1995) Use of adhesion-defective mutants of *Staphylococcus aureus* to define the role of specific plasma proteins in promoting bacterial adhesion to canine arteriovenous shunts. Infect Immun 63 (2): 585-590.

Vilcacundo R, Méndez P, Reyes W, Romero H, Pinto A, Carrillo W (2018) Antibacterial activity of hen egg white lysozyme denatured by thermal and chemical treatments. Sci Pharm 86 (48): 1-17.

Vinderola CG, Costa GA, Regenhardt S, Reinheimer JA (2002) Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. Int Dairy J 12 (7): 579-589.

Yabaya A and Idris A (2012)Bacteriological quality assessment of some yoghurt brands sold in Kaduna Metropolis. JORIND10 (2):35-39.

Yesillik S, Yildirim N, Dikici A, Yildiz A, Yesillik S (2011) Antibacterial effects of some fermented commercial and homemade dairy products and 0.9% lactic acid against selected foodborne pathogens.Asian J Anim Vet Adv 6 (2): 189-195.

Younis MO, Baka ZAM, Abo-Doubara MI, El-Metwaly MAM, Mostafa AHM (2020) Utilization of Cyanobacteria extracts in improving the microbial quality of soft white cheese. J Agric Chem Biotechnol 11 (7): 223-228.