Alexandria Journal of Veterinary Sciences
www.alexjvs.com

AJVS. Vol. 64 (1): 88-98 Jan. 2020
DOI:

Evaluation the Efficacy of Lysozyme Hydrolysates-Loaded Chitosan Against Food Borne Pathogens in Raw Milk During Cooling Storage

Dina, A.B. Awad[1,*] and Sobhy, A. El Sohaimy [2]
[1]Department of Food Hygiene, Faculty of Veterinary Medicine, Benha University, Egypt.
[2]Food Technology ARID Land Research Institute, City of Scientific Research and Technology Application, Borg Elarb, Alexanderia.

Key words:
Chitosan, Lysozyme, Raw milk, Antibacterial activity, Food borne pathogens.

***Correspondence to:**
dina.awad@fvtm.bu.edu.eg

Article History
Received: Nov. 012019
Accepted: Dec. 30 2019

ABSTRACT

Milk preservation is closely related with its microbiological quality. The spoilage may occur at any stage from production, during processing and till consumption. Many potent antimicrobials were discovered recently but, it is important to test their efficacy against undesirable bacteria inside the food materials that help to improve food safety and validity. Our research was focused on comparative antibacterial activities between chitosan, lysozyme and their different mixtures against different undesirable bacterial strains by agar well diffusion assay. Then apply the selected antibacterial substances in raw cow milk contaminated with food borne pathogens and spoilage bacteria then monitoring the bacterial growth or inhibition. Our preliminary investigation showed that, chitosan 0.5% exhibited the largest inhibition zones diameter followed by lysozyme hydrolysates with chitosan complex against *Salmonella enteritidis* and *Bacillus subtilus* in-vitro by agar well diffusion method. During application in raw cow milk, lysozyme hydrolysates and chitosan complex exhibited powerful bactericidal effect followed by chitosan especially against *Clostridium perfringens, Staphylococcus aureus* and *Listeria monocytogenes* after 24h from cooling storage of raw cow milk. The bactericidal activity of lysozyme and lysozyme hydrolysates were greatly enhanced upon their combination with chitosan. But, the bactericidal activity of lysozyme hydrolysates with chitosan complex exhibited great killing power than the conjugation between lysozyme with chitosan complex at same concentrations. This may be attributed to chitosan oligomers and lysozyme peptides acting in a synergistic manner in penetrating and killing the undesirable bacteria. Although chitosan was effective in inhibiting the growth of spoilage microorganisms but, it is induce changes in raw milk pH. Accordingly, we suggest lysozyme hydrolysates with chitosan complex will be a promising antibacterial additive to produce a highly safe raw milk with recommendation further future studies to explore its antibacterial mechanism.

1.INTRODUCTION

Food safety and quality are the master concerns for consumers and food technologists. Although the current improvement in the preservation technology, it does not prevent the outbreaks of food borne bacterial disease, or food spoilage and food waste (Hussain, 2013). Moreover, the negative public health hazards effects of commercial chemical preservatives prompts an increasing preference of consumers for the replacement of chemical preservatives by 'natural' alternatives that are derived from biological systems (Amit *et al.*, 2017 and Roman *et al.*, 2017).

Milk is synthesized in alveoli of the mammary gland and is virtually sterile when secreted into alveoli of the udder (Tolle, 1980).

Once raw milk leaves the udder it becomes susceptible for microbial contamination from different sources such as the environmental conditions around the udder and from the surface of milk handling and storage equipments (Bramley and Mckinnon, 1990). It is well known that the microbial alternation is responsible for the great losses of food and hence, over the years, various chemical and physical processes have been developed to extend the shelf-life of foods (Dutta *et al.,* 2010). So, to meet the growing consumers demand for natural preservatives without chemical preservatives, there is an urgent need to find new antimicrobials to compact these problems so, many antimicrobial substances recently developed like chitosan and lysozyme.

Chitosan is primarily composed of a linear polysaccharide consisting of β-(1-4)-linked glucosamine and N-acetyl-D-glucosamine. It is prepared by alkaline deacetylation of chitin, which is present in the exoskeleton of marine crustaceans and insects and in the cell walls of most fungi and some algae (Ma *et al.,* 2017). It considered as a natural most promising bio-polymer for future applications which included to the GRAS (Generally Recognized as Safe) category by the FDA also characterized by its excellent biodegradability, biocompatibility, antimicrobial activity, non-toxicity, and its economic advantages (Kim *et al.,* 2007 and Ahmed *et al.,* 2014). It is exhibits bacteriostatic or bactericidal effects against a wide range of microorganisms so possess numerous tech-nological and physiological properties useful in foods (Devlieghere *et al.,* 2004).

Another natural commercial antimicrobial protein is hen egg white lysozyme (LZ), which consisted of a single polypeptide chain with 129 amino acid residues (Mine, 1995). It is strongly basic protein with isoelectric point (pI) of 10.7 and has 4 disulfide bridges leading to its high thermal stability (Huopalahti *et al.,* 2007). Its molecular weight 14.4 kDa and constitutes 3.5% of hen egg albumen. It is highly active against *Micrococcus* and *Bacillus* species but demonstrating lower activity against Gram-negative bacteria**.** One of the major antimicrobial mechanisms of lysozyme is the degradation the mucopolysaccharide part in the peptidoglycan structure of Gram-positive bacteria and to a lesser extent in some Gram-negative bacteria. It is called muramidase activity or lytic activity (Burley and Vadehra,1989; Bera *et al.,* 2005). Lysozyme is stable at a wide pH range and heating temperatures, therefore it has a great potential to serve as a promising natural antimicrobial food preservative (Mine, 1995; Gill and Holley, 2003). It has been classified as a food additive that may be used as a food ingredient in many foods such as hard cheeses to control spoilage bacteria, in Europe lysozyme is allowed as a food additive with E number labeled E1105 (Losso *et al.,* 2000). It can be acceptable to apply it in food processing to prolong shelf life of foods, especially when used in accordance with good manufacturing practice (WHO, 1993).

So, to control the great economic losses from the spoilage of foods each year and minimize the spread of food borne diseases, the world has been focused on preservation of the food as a protection from various micro-organisms. So this study was undertaken to evaluate the antibacterial activity of chitosan and lysozyme then establish a trial to broad the antibacterial spectrum of lysozyme by peptic hydrolysis or by loading on chitosan. After that we make highlight on their potential effectiveness application as a natural antimicrobial additive to compact food borne pathogens and spoilage bacteria in raw cow milk during cooling storage and the feasibility to be used as natural preservative of raw milk.

2. MATERIALS AND METHODS

2.1. Reagents and microorganisms

Hen egg white lysozyme. Its activity 22400 U/mg was purchased from Dalian Greensnow Egg Products Development Co., LTD., China. Pepsin crystalline (10000 U/mg obtained from porcine stomach mucus) obtained from Nacalai Tesque, INC. Kyoto, Japan. Chitosan 89.9% deacetylation with molecular weight 120KDa was purchased from Acros Organics company, Geel, Belgium. Tryptic soy broth (TSB), tryptic soy agar (TSA), Muller–Hinton Agar and Brain heart infusion broth (BHI) were obtained from Merck, Germany. Different Bacterial strains include *Salmonella entertidis, Bacillus subtilis, Clostridium perferingens*, *Staphylococcus aureus* and *Listeria monocytogenes* were obtained from Food Hygiene

Department, Animal Health Research Institute, Dokki, Giza, Egypt.

2.2 Preparation of lysozyme hydrolysates

Lyophilized lysozyme (LZ) was dissolved in milli-Q water adjusted to pH 3.0 with 1N HCl. Pepsin was added to the protein solution at enzyme-to-substrate (E/S) ratio of 1:50 (w/w). This mixture was incubated with mild stirring at 37°C for 2hr. Reactions were quashed immediately by heating at 80°C for 5min. Then placed on ice for 5min to irreversibly inactivate pepsin enzyme action. Insoluble particles were removed by centrifugation at 5000 rpm for 5min at 4ºC and the resulting supernatants lyophilized, referred to lysozyme hydrolysates (LZH) (Carrillo *et al.*, 2014).

2.3 Preparation of chitosan solution

Different concentrations of chitosan solutions were prepared by dissolving different concentrations (2.0, 1.0, 0.5, 0.25 and 0.125% w/v) chitosan in 1% aqueous acetic acid solution. To achieve complete dispersion of chitosan, the solution was stirred at room temperature for 3h. Then filtrated through a Whatman No. 3 filter paper. The resultant filtrate solution referred to chitosan solution labeled (C) (Ojagh *et al.*, 2010).

2.4 Preparation of antibacterial mixtures

Lysozyme (LZ) and lysozyme hydrolystes (LZH) were integrated and loaded into chitosan solution (C) at a ratio (1:1 w/v) gradually with mild shaking to achieve final concentration (0.5, 0.250 and 0.125%) from each one separately producing LZC and LZHC complex which referred to lysozyme chitosan and lysozyme hydrolysates chitosan complexes respectively.

2.5 In vitro evaluation the antibacterial activity

Using agar well diffusion assay according to Yang *et al*., (1992) with some modifications as described by Tahara and Kanatani (1996) as follows: the indicator pathogenic bacterial strains, one act as Gram positive bacteria *Bacillus subtilis*, and the other Gram negative bacteria *Salmonella enteritidis* were activated. Then their concentration was adjusted according to measuring the absorbance at 675nm to 1×10^6 cfu/ml. 0.1ml from each strain was inoculated into sterilized Petri dishes and poured on Muller Hinton agar then leaving the plates for solidification, wells were made on the solidified agar with sterile cork borer (10 mm in diameter) then inoculated 100µl from (LZ, LZH, LZC, LZHC, and C) with concentrations 0.5% of each. The plates were kept at room temperature for 1h to allow proper diffusion. The plates were incubated at 37ºC/24h and then examined for clear circular inhibition zone around the wells.

2.6 Evaluation the antimicrobial activities in raw milk (in-vivo)

Raw cow milk obtained from faculty of veterinary medicine farm, Benha University, Egypt. Inoculated with different pathogenic bacterial strains around 10^6 cfu/ml. Then the different selected antibacterial agents (LZ, LZH, LZC, LZHC, and C) were inoculated to achieve final concentration (0.5, 0.250 and 0.125%) in presence of positive control which contain pathogenic bacteria only. All the treated groups stored in refrigerated temperature for 24h. Before and after cooling storage one ml of each treated group diluted in physiological saline for serial dilution for counting on TSA and incubated overnight at 37°C then cfu/ml was recorded. The experiment was repeated three time and the mean data is expressed as log cfu/ml (Prudencio *et al.*, 2014).

2.7 Statistical analysis

Statistical comparisons were made by using the mean data of three trials. The mean counts were expressed as log cfu/ml.

3. RESULTS

Microbial growth in food is a major cause of food spoilage and milk is perishable food easily susceptible for bacterial contamination. An attempt had been done to improve the safety and to delay spoilage by using chitosan and lysozyme and/or their different conjugations. Based on measuring the diameter of zone of inhibition (mm) in agar well diffusion assay, different concentrations (2.0, 1.0, 0.5,0.250 and 0.125%) of chitosan were prepared. Firstly, the antibacterial activity of chitosan had been tested against *Salmonella enteriditis* as example for Gram negative bacteria which not all antibacterial substances able to inhibit its growth in food. It was found that the highest concentration of chitosan 2% had displayed the lowest antibacterial activity with mean inhibition zone diameter reach to 10mm while, the chitosan concentration 0.5% had been displayed the highest antibacterial activity against *Salmonella enteriditis* with mean inhibition zone diameter reach to 22mm as shown

in fig.(2) So, we depend on chitosan concentration 0.5% as the lowest effective concentration to complete our current study.

The antibacterial activity of the selected concentration of chitosan and native lysozyme 0.5% were tested against two indicator pathogenic bacteria namely *Salmonella enteriditis* as example for Gram negative bacteria, and *Bacillus subtilis* as example for Gram positive bacteria. Results showed that chitosan had a higher in-vitro antibacterial activity than lysozyme at the same concentration with mean inhibition zone diameter 22mm and 24mm when testing against *Salmonella enteriditis* and *Bacillus subtilis*, respectively while, lysozyme gave mean inhibition zone diameter 10mm and 14mm when tested against the same bacteria, respectively, as in fig.(3,4). But it was observed, that lysozyme under peptic hydrolysis (LZH) produce a higher inhibition zone diameter than native lysozyme with average 12mm and 18mm when testing against *Salmonella enteriditis* and *Bacillus subtilis*, respectively.

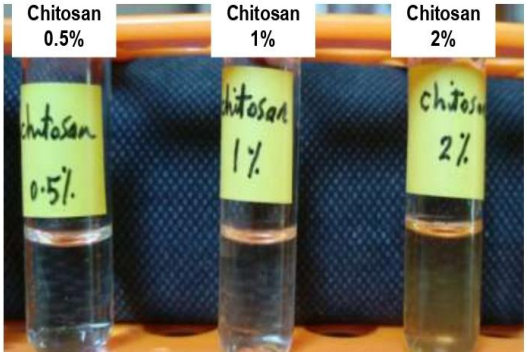

Fig. (1): Solubility of different concentrations of chitosan using 1% acetic acid showing the difference in color and viscosity.

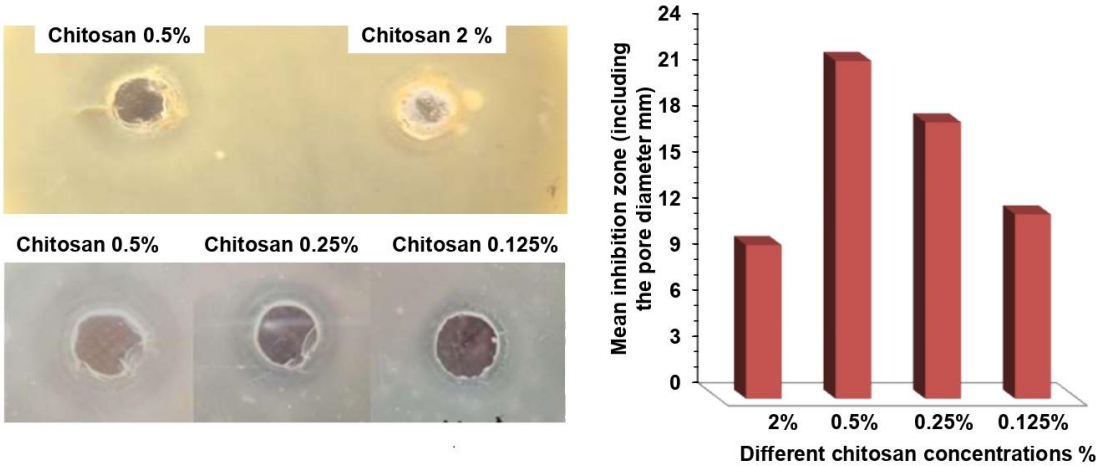

Fig. (2): Agar well diffusion assay of different chitosan concentrations against *Salmonella enteritidis*.

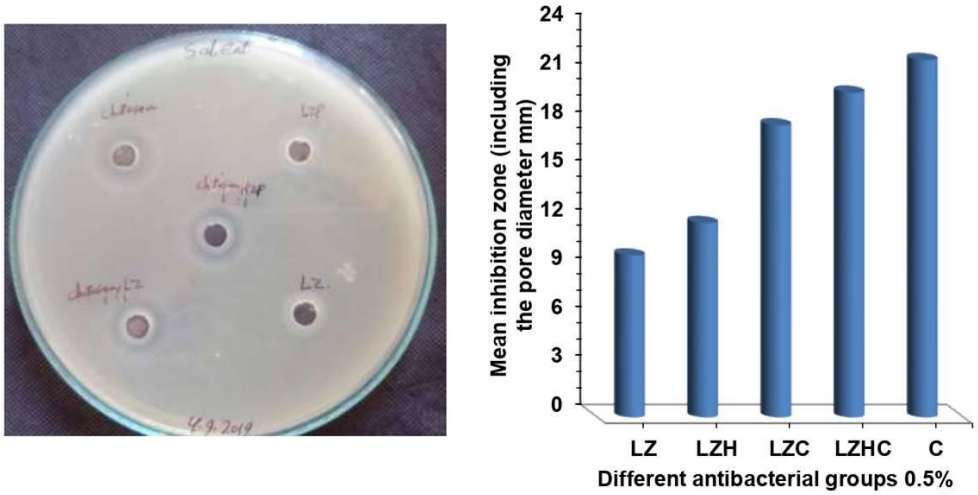

Fig. (3): Agar well diffusion assay of different antibacterial groups 0.5% against *Salmonella enteritidis* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex, (C) referred to chitosan. The test repeated three times and the mean data expressed in mm.

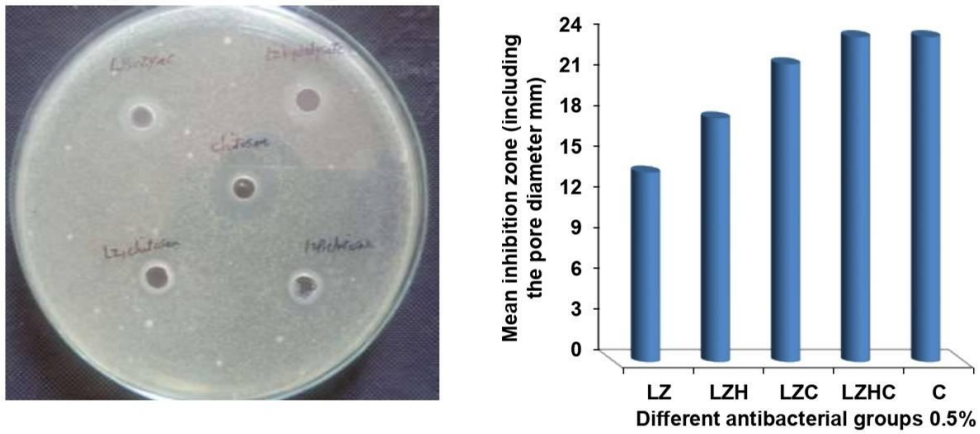

Fig. (4): Agar well diffusion assay of different antibacterial groups 0.5% against *Bacillus subtilus* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex and (C) referred to chitosan. The test repeated three times and the mean data expressed in mm.

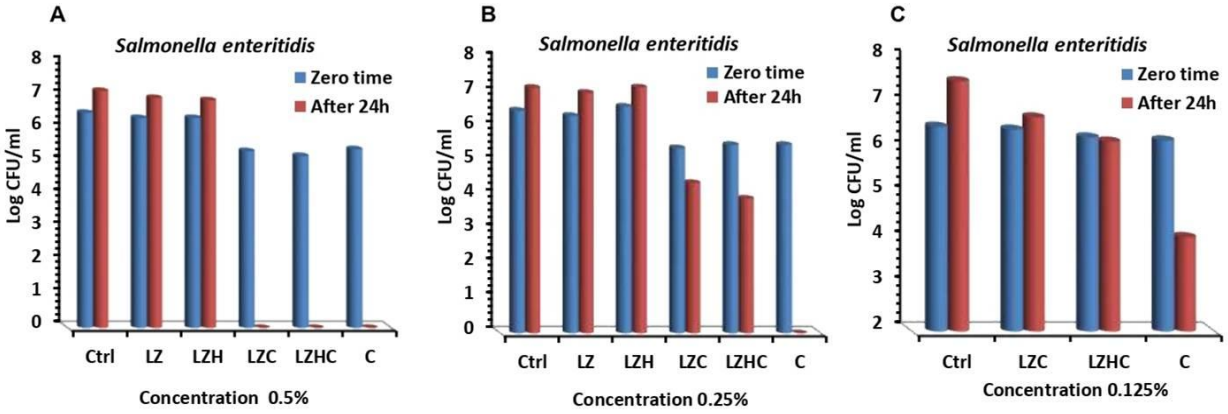

Fig. (5): The bactericidal activity of different concentrations of selected antibacterial substances in raw cow milk stored at cooling temperature for 24h, contaminated with 10^6 CFU/ml *Salmonella enteritidis* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex, (C) referred to chitosan and (Ctrl) referred to control group contain the bacterial strain without antibacterial substance. The count expressed as the mean log cfu/ml.

On the other side, lysozyme after its conjugations with chitosan producing lysozyme chitosan complex (LZC) acquired more potent antibacterial activity than native lysozyme alone (LZ) with mean inhibition zone diameter 18mm and 22mm against *Salmonella enteriditis* and *Bacillus subtilis*, respectively, as presented in fig. (3,4). In addition, the activity of LZH after its combination with chitosan (LZHC) become more potent than LZH alone with inhibition zone diameter 20mm and 24mm when testing against *Salmonella enteriditis* and *Bacillus subtilis*, respectively, as shown in fig.(3,4).

By application the different antibacterial substances, the focus of our current study, in raw cow milk contaminated with five different food borne pathogens and spoilage bacteria, then stored it in refrigerated temperature for 24h to evaluate the in vivo antibacterial activity.

In raw milk contaminated with *Salmonella enteriditis* around 10^6 cfu/ml, we found that LZ and LZH at concentration 0.5% didn't show any bactericidal effect against *Salmonella enteriditis* but, after their conjugations with chitosan 0.5% (LZC and LZHC complex) become acquired potent bactericidal activity as in fig. (5). But, the efficacy of the bactericidal activity was dose dependent manner as their bactericidal activity reduced at concentration 0.25% as shown in fig.(5B,C). In the same time, LZ and its hydrolysates or their antibacterial conjugates lost their bactericidal activities, chitosan still had good bactericidal activity 4.09 log CFU/ml at lower concentration 0.125% comparing with control group at which the bacterial growth mean count reached 7.5 log CFU/ml after 24h from cooling storage as shown in fig.(5C).

Similar results were observed in raw milk contaminated with *Bacillus subtilus* around 10^6 cfu/ml, we found that LZ and LZH at any concentration used didn't show any bactericidal effect against *Bacillus subtilis* but, their conjugations with chitosan (LZC and LZHC at concentration 0.5% and 0.25%) acquired potent bactericidal activity as in fig. (6A,B). We observed LZHC complex and chitosan 0.125% producing the higher killing power on *Bacillus subtilis* with mean bacterial growth count were 5.14and 5.24 log CFU/ml respectively, after 24h from refrigerated storage as shown in fig.(6C).

When raw cow milk contaminated with *Clostridium perfringens*around 10^6 cfu/ml, we found that LZ at concentrations 0.5 and 0.25%

exhibited slight bactericidal effect against *Clostridium perfringens*as shown in fig. (7A,B) with mean bacterial growth count was 5.7 and 5.8 log CFU/ml respectively, comparing with control group with mean bacterial growth value 7.3 log CFU/ml after 24h from refrigerated storage. Our results reveals that, LZH acquired better bactericidal effect than native LZ at concentrations 0.5 and 0.25% as observed in fig. (7A,B) with mean bacterial growth count was 4.5 and 4.7 log CFU/ml respectively, comparing with control group with mean value 7.3 log CFU/ml after 24h from refrigerated storage. LZHC 0.5% produce the best bactericidal effect with mean bacterial growth count 3.2 log CFU/ml comparing with LZC complex and chitosan of the same concentration 0.5% with mean bacterial growth count 3.2 and 4.5 log CFU/ml respectively, against *Clostridium perfringens* after 24h from refrigerated storage as shown in fig. (7A).

In case of raw milk contaminated with *Staphylococcus aureus* and *Listeria monocytogenes*, LZ and LZH behaved in the same manner as pervious, exhibited very slight bactericidal effect comparing with control group after 24h from refrigerated storage, more clear in fig.(8,9,A). The most prominent potent bactericidal effect was observed in LZHC 0.125% complex with mean bacterial growth count reached to 2.8 log CFU /ml comparing with control group with mean bacterial growth count 6.3 and 6.5 log CFU /ml in *Staphylococcus aureus* and *Listeria monocytogenes* respectively, after 24h from refrigerated storage as shown in fig.(8,9C). The effectiveness of chitosan 0.125% was lost on *Staphylococcus aureus* while, the higher concentration of chitosan 0.5% exhibit moderate bactericidal effect with mean bacterial growth count 4.7 log CFU/ml after 24h from refrigerated storage. It was observed that, chitosan in raw milk contaminated with *Listeria monocytogenes* exhibited potent bactericidal activity even with low concentration 0.125% with mean bacterial growth count 3.5 log CFU /ml after 24h from refrigerated storage as shown in fig.(9C). But, LZHC complex exhibited the most powerful bactericidal activity at the same concentration with mean bacterial growth count 2.8 log CFU /ml after 24h from refrigerated storage as shown in fig.(9C).

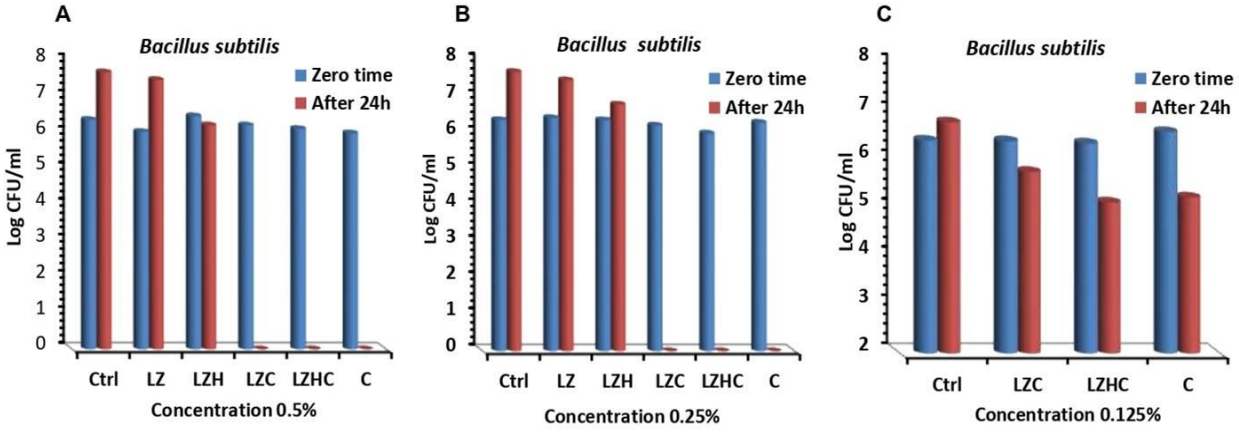

Fig. (6): The bactericidal activity of different concentrations of selected antibacterial substances in raw cow milk stored at cooling temperature for 24h, contaminated with 10^6 CFU/ml *Bacillus subtilus* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex,(C) referred to chitosan and (Ctrl) referred to control group contain the bacterial strain without antibacterial substance. The count expressed as the mean log cfu/ml.

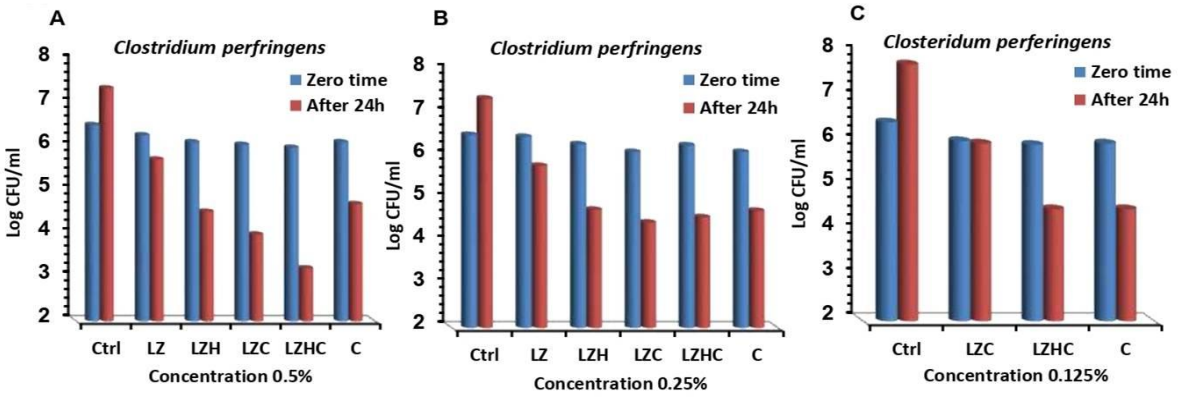

Fig. (7): The bactericidal activity of different concentrations of selected antibacterial substances in raw cow milk stored at cooling temperature for 24h, contaminated with 10^6 CFU/ml *Clostridium perferingens* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex, (C) referred to chitosan and (Ctrl) referred to control group contain the bacterial strain without antibacterial substance. The count expressed as the mean log cfu/ml.

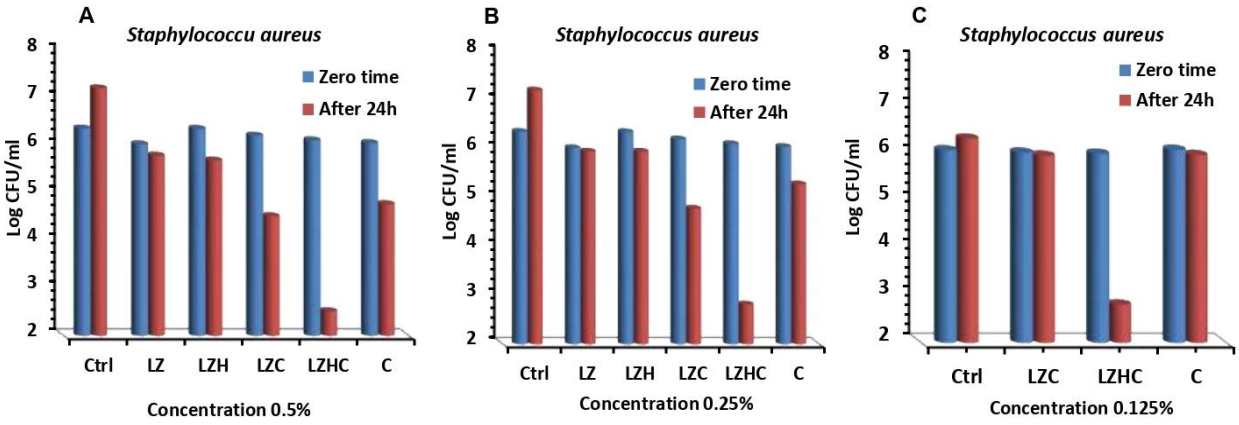

Fig. (8): The bactericidal activity of different concentrations of selected antibacterial substances in raw cow milk stored at cooling temperature for 24h, contaminated with 10^6 CFU/ml *Staphylococcus aureus* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex, (C) referred to chitosan and (Ctrl) referred to control group contain the bacterial strain without antibacterial substance. The count expressed as the mean log cfu/ml.

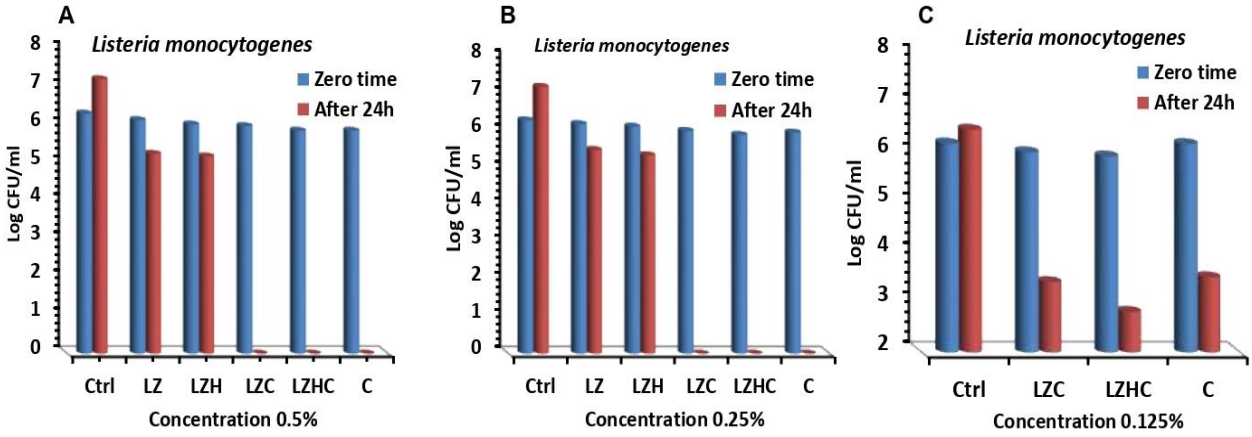

Fig. (9): The bactericidal activity of different concentrations of selected antibacterial substances in raw cow milk stored at cooling temperature for 24h, contaminated with 10^6 CFU/ml *Listeria monocytogenes* where (LZ) referred to native lysozyme, (LZH) referred to lysozyme hydrolysates produced by pepsin enzyme, (LZC) lysozyme with chitosan complex, (LZHC) referred to lysozyme hydrolysates with chitosan complex,(C) referred to chitosan and (Ctrl) referred to control group contain the bacterial strain without antibacterial substance. The count expressed as the mean log cfu/ml.

4. DISCUSSION

Food safety is a known great problem worldwide. Nowadays, consumers are concerned with the illness caused by some pathogenic and spoilage microorganisms in food and also for the safety of foods containing synthetic preservatives. Thus, it shows a growing interest about the replacement of synthetic preservatives with natural, effective and nontoxic antimicrobial compounds. Recently great concern on using natural antimicrobial compounds, such as chitosan and lysozyme. Our current study was focused on their application in raw cow milk contaminated with spoilage and pathogenic microorganisms. Then monitoring the effect of the selected antibacterial substances on the bacterial growth in raw milk after cooling storage. According to fig.(1) chitosan not dissolved in water but it need low acidic media for dissolving so, the widespread use of chitosan in food industry has been limited due to its insolubility in water. For food applications, chitosan is either dissolved in acetic acid to a concentration of 1–2%, or applied as a chitosan-based packaging film (Zhao *et al.*, 2018). Although the higher chitosan concentration 2% used, it produced the least inhibition zone diameter in agar well diffusion assay. This may be proposed due to the higher viscosity of chitosan at this concentration (2%) which made it so difficult to diffuse within the Muller Hinton agar or nutrient agar as shown in fig.(2). So it is not enough to depend on agar well diffusion assay in case of determination the antibacterial activity of chitosan due to the big barrier of its diffusion in agar. Agar well diffusion assay could be used as a rapid, inexpensive and in-vitro screening method to assess and differentiate between the selected antibacterial substances but not in case of chitosan due to its higher viscosity at higher concentration. The results revealed that lysozyme more active against the Gram positive bacteria *Bacillus subtilus* than Gram negative bacteria *Salmonella enteritidis*. This may be explained by that Gram-negative bacteria are less susceptible, because its outer membrane mainly consisting of lipopolysaccharide (LPS) which act as a barrier and prevents the access of lysozyme molecules to the site of action on the peptidoglycan in cell walls (Nakamura *et al.*, 1997). While this outer membrane is absent in Gram positive bacteria so lysozyme easily attack them. The antibacterial spectrum of lysozyme was enhanced using pepsin enzyme producing LZH as in fig. (3,4). This was explained by antimicrobial peptides produced from enzymatic hydrolysis. Our result agreed with (Mine *et al.*, 2004) who found internal antimicrobial peptides after enzymatic digestion within lysozyme sequence that are effective against Gram-negative bacteria, without lytic activity.

Loading of LZ and LZH on chitosan as a mixture of antibacterial complex potentially enhanced their antibacterial activity against most tested pathogenic and spoilage bacteria used in this study. This may be proposed due to chitosan is one of the few cationic polysaccharides able to attach with bacterial cell membrane (Fajardo *et al.*, 2010). Also it has a very powerful effectiveness antimicrobial properties against bacteria, moulds and yeasts (Rabea, *et al.*, 2003) this powerful activity may proposed to potentiate and enhance the LZ and LZH antibacterial activity. In addition to, lysozyme can make degradation for chitosan converting it from

polymer form to oligomers form which suggested to be acquired potent antibacterial activity (Nordtveit*et al.*, 1994, 1996). All of these suggested reasons make LZC and LZHC complexes had potent bactericidal activity. But the mixture between LZH and chitosan (LZHC) exhibited great killing power than the conjugation between native LZ and chitosan (LZC). This may be attributed to the chitosan oligomers and lysozyme peptide act in synergistic manner in penetrating and killing undesirable bacteria.

At application native LZ and LZH in raw cow milk contaminated with different food borne pathogens and spoilage bacteria used in this study, they did not show potent bactericidal activity, this may be proposed due to the low concentration (0.5%) which we have been chosen in this study. Moreover, we found LZHC complex performed more potent bactericidal than LZC complex and chitosan against Gram positive food poisoning bacteria *Staphylococcus aureus* (fig.8C) andpsychrotrophic bacteria *Listeria monocytogenes* (fig.9C). Also LZHC complex exhibited bactericidal effect against anaerobic and aerobic spore former bacteria (*Clostridium perfringens* and*Bacillus subtilis*) respectively, (fig. 6B,7A) and can kill Gram negative bacteria *Salmonella enteritidis* (fig.5B). According to the bactericidal efficacy of LZHC complex in killing most forms spoilage and pathogenic bacteria, this nominated LZHC complex to be a promising antibacterial agent could be applied in milk as a method of biopreservation. Besides that, our results reveals native LZ can affect the growth of *Clostridium perfringens* that's why it can be applied to control anaerobic spore former bacteria and late blowing in hard cheese (D'incecco *et al.*, 2016) but, LZHC complex exhibited strong bactericidal effect more than native LZ so, LZHC will be consider a promising antibacterial additive to prevent late blowing in hard cheese.

Although chitosan produce potent bactericidal effect against most bacterial forms but, It is important to examine the constituents of the food matrix and the next processing steps before apply chitosan as a natural preservative, as its application in raw milk make change in its pH (data not shown). As a conclusion, we suggest that the novel LZHC complex will be a promising antibacterial additive to produce highly safe raw milk without heat treatment also chitosan may be applied in fermented dairy products to produce highly safe and good quality product.

Conclusion

Milk spoilage may occur at any stage from milk production till its consumption. There are many synthetic and natural antimicrobials were discovered recently like chitosan and lysozyme but, it is important to test their efficacy against undesirable bacteria inside the food to improve food safety and validity. So our target was focused on testing the antibacterial activities of chitosan, lysozyme and or their mixtures against different undesirable bacterial strains in raw milk. The results revealed the bactericidal activity of native LZ and LZH could be broaden upon their conjugation with chitosan. But LZHC complex exhibited a great killing power than the LZC complex. This may be attributed to the synergistic effect of chitosan oligomers and lyszyme peptide. Although chitosan was effective in inhibiting the growth of spoilage microorganisms but, It is induce changes in the food matrix due to its low solubility in water. Accordingly, we nominate the novel LZHC complex to be a promising additive in dairy sector. Also we recommend chitosan to be applied in fermented dairy products especially kareish cheese (the most popular soft cheese in Egypt) to produce highly safe and good quality product.

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