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ANTIBACTERIAL ACTIVITY OF CHITOSAN AND ITS OLIGOMERS ON SOME PATHOGENIC BACTERIA ISOLATED FROM SOME MILK PRODUCTS

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ABSTRACT

Chitosan and its oligomers N-acetyl chitooligosaccharides and chito-oligosaccharides prepared by deacetylation and chemical hydrolysis, respectively and have a broad antimicrobial spectrum to Gram-positive (*Staph.aureus* and *List. monocytogenes*) bacteria and Gram-negative (*Sal.typhimurium*). *Staph.aureus, List. monocytogenes* and *Sal. typhimurium* were isolated in lower percentages (23.3, 0 and 6.67%, respectively) from talaga cheese, followed by zabadi-baladi and kariesh cheese. This study focused on antimicrobial activity of chitosan and its oligomers by inoculation it and the isolated bacteria in yoghurt. 1% of chitosan completely inhibited the *Staph.aureus, List. monocytogenes* at 5th and 3rd day, respectively and Chitosan exhibited a bacteriostatic effect on *Sal. typhimurium*. while 0.1% of N-acetyl chitooligosaccharides and chito-oligosaccharides had reduced the count of *List. monocytogenes* and bactericidal effect on *Staph.aureus* and *Sal. typhimurium*. The antimicrobial properties of chitosan and its oligomers also, the perception of palatability to consumers toward it were discussed.

Key words: Chitosan, Zabadi baladi, Cheese, Antibacterial, Oligomers.

INTRODUCTION

Chitosan is nontoxic, a natural polymer, found in the exoskeletons of crustaceans (Rinaudo, 2006). It's found commercially in the waste products of the marine food processing industry (Limam *et al*., 2011). Recent studies have focused on conversion of chitosan to oligosaccharides (termed chitooligosaccharides, COS)—because the latter are not only readily soluble in water due to their shorter chain lengths, and free amino groups in D-glucosamine units, but also easily absorbed through the intestine, quickly getting into the blood flow (Kim and Rajapakse, 2005).

Chitosan and its oligomers are known for its various biological properties such as antioxidant, anti-inflammatory, cholesterol lowering, immunity enhancing, antitumor, neuroprotective, antimicrobial and antifungal which makes chitosan and its oligomers very useful polysaccharide for human health (Varun *et al*., 2017). Mechanism of inhibition of microbial cells by chitosan and its oligomers via its polycationic nature which electrostatically binds with the microbial surface and interferes with metabolism of bacteria or by blocking the transcription of RNA from DNA by adsorption on DNA after penetration to the cell (Liu *et al*., 2001). Some authors have stated that chitosan generally showed stronger effects for Gram-positive bacteria (e.g. *Listeria monocytogenes, Bacillus megaterium, B. cereus, Staphylococcus aureus, Lactobacillus plantarum, L. brevis, L. bulgaris, etc.*) than for Gram-negative bacteria (e.g. *E. coli, Pseudomonas fluorescens, Salmonella typhimurium, Vibrio parahaemolyticus*, etc.) (Goy *et al*., 2009). Conversely, it has been demonstrated that hydrophilicity in Gram-negative bacteria is significantly higher than in Gram-positive bacteria, making them most sensitive to chitosan (No *et al*., 2002).

Dairy products are most important products may be subjected to contamination by some pathogenic microorganisms through the unhygienic manipulation, transmission or during storage of these products. So, the chitosan is becoming more widely used in the food industry, particularly in the production of dairy products (Evdokimov *et al*., 2015). No *et al*. (2002) reported that the addition of chitosan to acidic foods enhances its effectiveness as natural preservative. Finally, Lee *et al.* (2004) concluded that a packaging material coated with a combination of nisin and chitosan improves significantly the microbial stability of milk.

This study aimed to elucidating the antimicrobial properties of chitosan and it's oligomers on different pathogenic microorganisms isolated from dairy product.

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MATERIALS AND METHODS

I- Collection of samples:

A total of 90 random samples of zabadi-baladi, Kareish cheese and Talaga cheese (30 of each) where collected from different markets and dairy shops in Assiut City, Egypt. The samples were collected in package as marketed to the consumer and sent to the laboratory in an insulated box with a minimum of delay to be examined.

II- Isolation of some pathogenic bacteria:

A- Isolation and identification of *staph. aureus* according to AOAC (2001) on paired barker agar, morphological examination and confirmatory tests done according to Bennett and Lancette (2001).

B- Isolation and identification of *listeria monocytogenes*: They isolated on Oxford agar plates after enriched in Listeria enrichment broth and identification was performed according to Hitchins *et al*. (2016).

C- Isolation and identification of *Salmonella typhimurium* according to Wallace *et al*. (2016): They pre-enriched on buffered peptone water then enriched on Rappaport-Vassiliadis (RV) broth and isolated on XLD agar. They identified according to FDA (2016).

III- Serological identification of Salmonellae*:*

This part has been done in Serology Department in Animal Health Research Institute, El-Giza, Egypt. Serological identification of Salmonellae was carried out according to Kauffman – White scheme (Kauffman, 1974 and Popoff *et al.,* 2004) for the determination of Somatic (O) and flagellar (H) antigens using Salmonella antiserum (DENKA SEIKEN Co., Japan).

IV- Preparation of N-actyl chito-oligosaccharides and chito-oligosaccharides:-

N-actyl chito-oligosaccharides (NAc-COS) were prepared by chemical hydrolysis of chitosan as follow: 1g of chitin is hydrolyzed by 50 ml 7N HCl at 70 ºC during 3h. Chito-oligosaccharides (COS) were prepared by hydrolyze of 1g of chitosan in 50 ml 6N HCl at 56 ºC during 3h. (Chang *et al*., 2000 and Benhabiles *et al.,* 2012).

V- Bacterial suspension inoculation:

The isolated strains were inoculated into Muller Hinton broth and incubated to the growth phase at 37ºC. The growth density was adjusted to match a MacFarland 0.5 standard (10^5 CFU/ml). (Ruparelia *et al.,* 2008).

VI- Antimicrobial properties of chitosan and its oligomers (Benhabiles *et al*., 2012):

Raw milk was boiled for 10 min. then suddenly cold and inoculated with 2% yoghurt culture at 45ºC. One ml of each strain suspension (which prepared as before) mixed with 100 ml of prepared milk and divided into suitable sterile jars, chitosan, NAc-COS and COS were add at concentrations 1, 0.1 and 0.1% for each respectively, and another positive control jars without chitosan and oligomers, incubated at 40ºC until curdling. Control jar (free from strains suspension, chitosan and oligomers as a negative control) was also stored. The jars were stored at refrigerator temperature (5±2 ºC). The inoculated jars were examined bacteriologically for the count of *S. aureus* using Baird-Parker media, *Listeria monocytogenes* using Oxford agar plates and *Sal. typhimurium* using XLD agar (37ºC for 24-48h) after curdling (at time zero) and, every 2 days until the end of the experiment.

VII- Sensory evaluation:

Control yoghurt jars (free from the previous microorganisms but inoculated with chitosan and oligomers at concentrations of 1, 0.1 and 0.1%, respectively) were prepared as previously mentioned and each was subjected to the previous treatments. Thirty consumers were selected in teams of different ages, sex (15 females and 15 males), and education to taste the trials. The perception of consumers toward samples with various conc. of chitosan was studied with respect to two different attributes (flavor and palatability) (Fernandes *et al.,* 2008). The level of agreement was scored as strongly agree (SA), agree (A), disagree (D), and strongly disagree (SD) according to Nelson and Torut (1981).

RESULTS

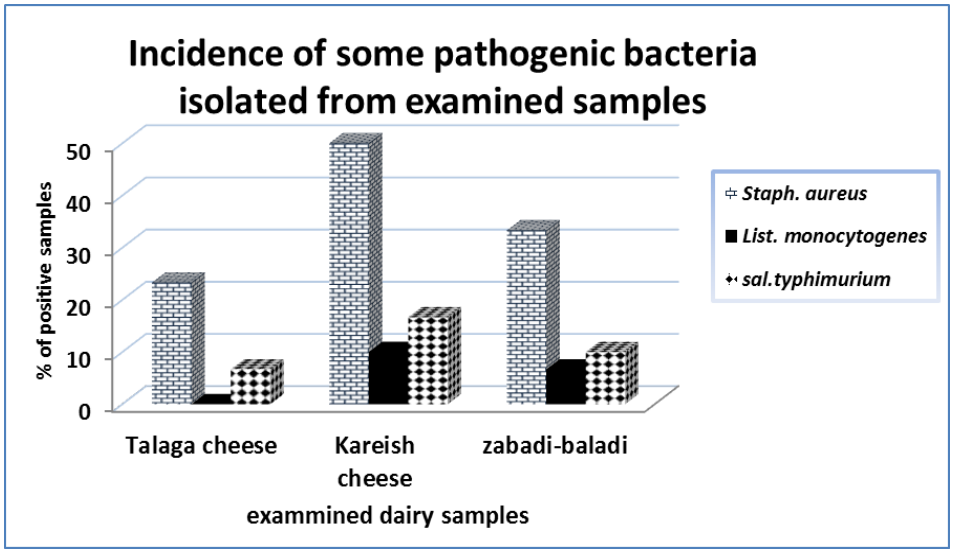

Fig. 1: Incidence of some pathogenic bacteria isolated from some diary samples.

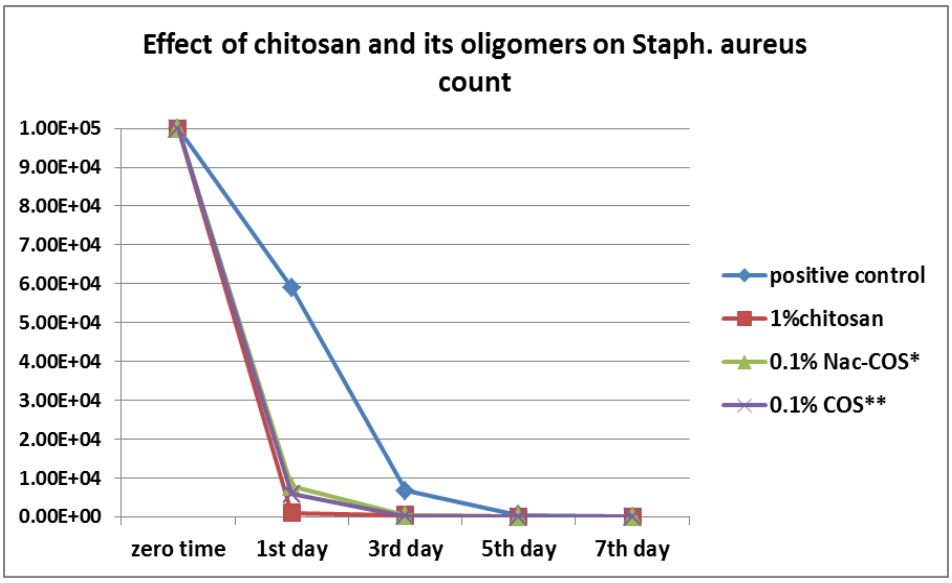

Fig. 2: Effect of chitosan and its oligomers on *Staph.aureus* inoculated in yogurt
***** N-actyl chito-oligosaccharides ****** Chito-oligosaccharides

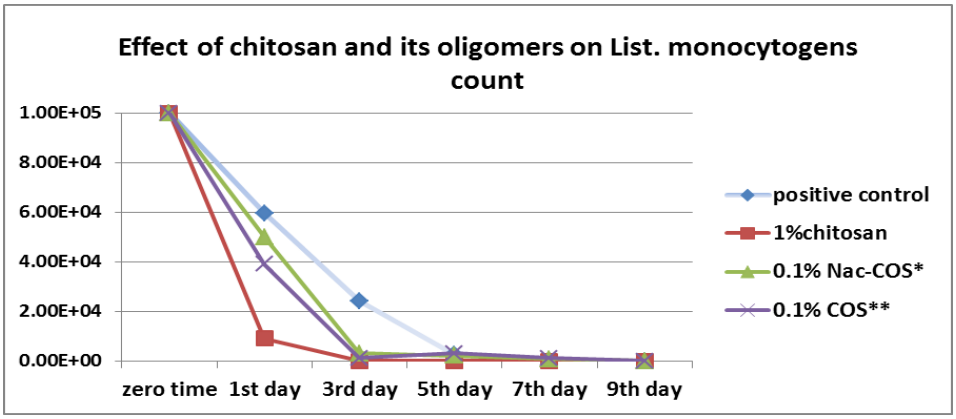

Fig. 3: Effect of chitosan and its oligomers on *List. monocytogens* inoculated in yogurt
***** N-actyl chito-oligosaccharides ****** Chito-oligosaccharides

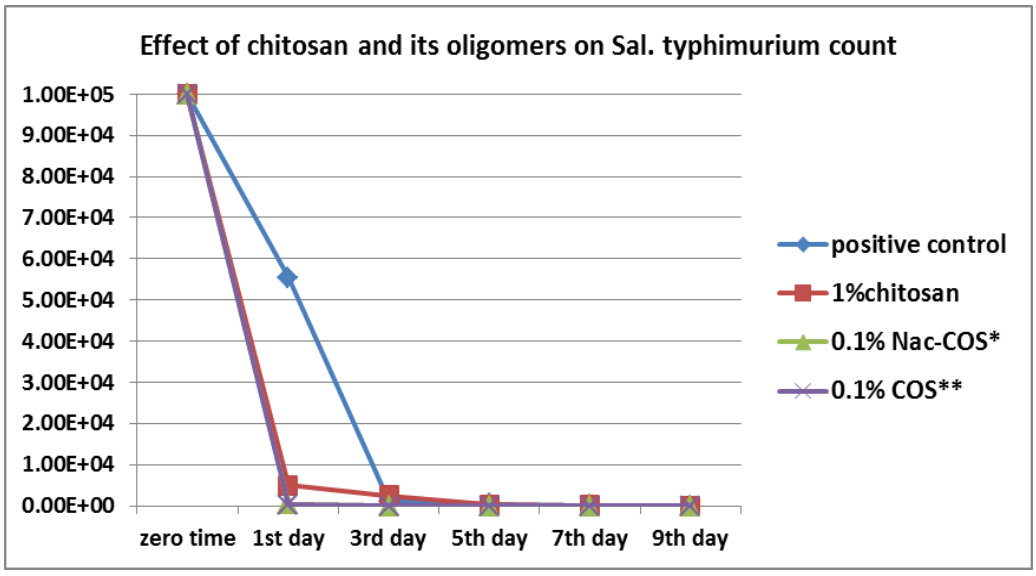

Fig. 4: Effect of chitosan and its oligomers on *Sal. typhimurium* inoculated in yogurt
***** N-actyl chito-oligosaccharides ****** Chito-oligosaccharides

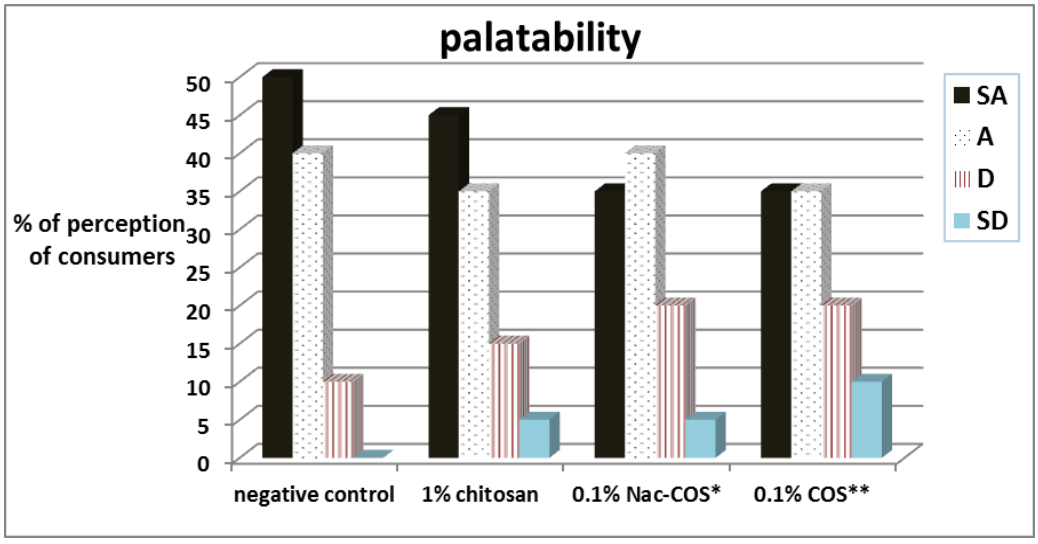

Fig. 5: Palatability of chitosan and its oligomers in yoghurt
***** N-actyl chito-oligosaccharides ****** Chito-oligosaccharides
****** strongly agree (SA), agree (A), disagree (D), and strongly disagree (SD)

DISCUSSION

The aim of this study is to investigate some of dairy products for presence of some important pathogenic microorganisms that can make a risk for human and how control it by using natural materials as chitosan and its oligomers to help in controlling these pathogens from transmission specially in developing countries. So, in Fig. (1) clarified that *Staph. aureus*, *List. monocytogenes* and *Sal. typhimurium* could be isolated from Talaga cheese in percentages of 23.3, 0 and 6.67% respectively. While, from Kariesh cheese 50, 10 and 16.67% and from Yoghurt 33.3, 6.67 and 10% respectively. These results is lower than that obtained by Vitas *et al.* (2004) who isolated *List.monocytogenes* from soft cheese in 8.1%, Hussien *et al.* (2013) *Stap.aureus* in 85% and *list.monocytogenes* in 20% from Kariesh cheese. But, higher than results obtained by Bostan *et al.* (2006), Pelisser *et al.* (2009), and Ertas *et al.* (2010). Rahimi (2013) who reported *Staph.aureus* in 11.1% from soft cheese and 0% from yoghurt. Entry of foodborne pathogens via contaminated raw milk into dairy food processing plants can lead to persistence of these pathogens in biofilms, and subsequent contamination of processed milk products and exposure of consumers to pathogenic bacteria, pasteurization may not destroy all foodborne pathogens in milk, and inadequate or faulty pasteurization will not destroy all foodborne pathogens. Furthermore, pathogens such as *List. monocytogenes* can survive and thrive in post-pasteurization processing environments, thus leading

to recontamination of dairy products. These pathways pose a risk to the consumer from direct exposure to foodborne pathogens present in unpasteurized dairy products as well as dairy products that become re-contaminated after pasteurization (Oliver *et al.,* 2005).

As shown in Fig.2 1% chitosan after 1st day, 0.1% NAs-COS and 0.1% COS reduced the count of *Staph. aureus* until complete inhibition of the growth in yoghurt at 3rd day in all trails. While, in positive control *Staph.aureus* could be detected in reduced count nearly until the 5th day of experiment and that may be returned to increase the acidity of yoghurt and Staph. organisms are the most sensitive bacterial species to acidity (Bergdoll and Lee Wong, 2006). Similarly result recorded by, Fernandes *et al.* (2008) and Shanmugama *et al.* (2016) who showed the antibacterial effects of chitosan and chitooligomers against *S. aureus* and *E. coli* at 0.1% concentrations. Also, Amin (2014) studied that 1% chitosan could be complete the inhibition of *Staph.aureus* and enterotoxin type C at 6th day in yoghurt trials. Goy *et al.* (2016) reported that chitooligomers have been inhibited many bacteria. A reduction on the growth colonies, where the chitosan concentration is confirmed to play important role in the antimicrobial activity. the chitosan is consistently more active against the Gram-positive *Staph. aureus* than Gram-negative bacteria. Antimicrobial activity of chitosan and its oligomers may be due to interference in the metabolism by binding to the surface of the bacteria or by blocking of transcription of DNA and RNA by binding to the DNA after penetration into cell. Chitooligomers showed antimicrobial action against pathogenic organisms and significantly inhibited *Staph.aureus* (Varun *et al.,* 2017).

While, in Fig. 3 results clarified that 1% chitosan completely inhibited *List.monocytogenes* at 3rd day but 0.1% of NAs-COS at 5th day and COS reduce the count and could not be detected at 7th day, reduction in *List.monocytogenes* counts with time progress in positive control trials. Similar results recorded by Jovanovi´c *et al.* (2016) and Hromiš *et al.* (2017) *List.monocytogenes* complete inhibited by1% chitosan and less effected by its oligomers (NAs-COS and COS). No *et al.* (2002) studied the antibacterial activities of chitosans and chitosan oligomers with different molecular weights (Mws) were examined against Gram-negative and Gram-positive bacteria (*Listeria monocytogenes*). Chitosans showed higher antibacterial activities than chitosan oligomers and markedly inhibited growth of most bacteria tested although inhibitory effects differed with Mws of chitosan and the particular bacterium. Chitosan generally showed stronger bactericidal effects with Gram-positive bacteria than Gram-negative bacteria in the presence of 0.1% chitosan.

The mechanism of action of chitosan and its oligomers is differ from Gram positive and Gram negative bacteria so, the effect of them on Gram negative bacteria as *Sal.typhimurium* shown in Fig. 4 as results revealed that complete inhibition in growt of *Sal.typhimurium* at 3rd day for 0.1% of NAs-COS and COS but just reduction in counts of it by 1% chitosan and thus in agreement with results recorded by Kong *et al.* (2010) and Hromiš *et al.* (2017). Also, Benhabiles *et al.* (2012) showed antimicrobial effect of chitooligomers against many pathogenic organism, i.e., *Stap. aureus* and *Sal. typhimurium* at 0.1% concentration of chitooligomers. The oligosaccharides NAc-COS and COS showed lower MIC values (0.003%), compared to chitin and chitosan, for all Gram negative bacteria strains tested, indicating their stronger antimicrobial activity (Li *et al.,* 2010).

The microbiological target of protonated chitosan's action would be the cytoplasmic membrane of sensitive cells. Cellular damage can lead to the disruption of the cellular integrity of the membrane. The outer membrane of Gram-negative bacteria could act as a barrier and could be responsible for preventing chitosan from reaching the cytoplasmic membrane. Although the cytoplasmic membrane should be sensitive to chitosan, the outer membrane protects the cells (Coma *et al.,* 2003). Also, Concerning the bacteria surface polarity, the outer membrane of Gram-negative bacteria consists essentially of lipopolysaccharides containing phosphate and pyrophosphate groups which render to the surface a density of negative charges superior to that observed for Gram-positive ones (membrane composed by peptidoglycan associated to polysaccharides and teichoic acids). This supports the evidence that the leakage of intracellular material observed by chitosan in Gram-negative is superior to that reported in Gram-positive bacteria (Chung and Chen, 2008).

The chitosan and its oligomers use in the dairy industry is very promising because it allows to profitably process milk protein and carbohydrate raw materials, excluding significant energy costs. The processed products have curative properties, which make them attractive for a consumer and, as a consequence, competitive at food market (Evdokimov *et al.,* 2015). So, the perception of consumers toward samples subjected to various chitosan and oligomers conc. with respect to two different attributes (flavor and palatability) were regarded in Fig.5, 45% of consumers were strongly agree to addition of 1% chitosan while, only 35% of consumers for 0.1% for NAc-COS and COS. These results were in agreement with Amin (2014) but Duggan and Waghorne (2001) recorded that addition of chitosan to yoghurt shown dynamic rheological more better differences than those prepared without chitosan. Chitosan and its

oligomers have little flavor effect when added to yoghurt at different concentrations.

In conclusion this study revealed the presence of antimicrobial activity of chitosan and its oligomers against most important food born pathogen. Chito-oligomers would have advantages as new antimicrobial agents due to their higher activity and since they are also more readily soluble in water than the native polysaccharides (chitosan). N-acetyl chitooligosaccharides (NAc-COS) and chito-oligosaccharides (COS) both have similar antibacterial activity. Further work is needed to better understand the mode of action of chitosan and its oligomers as antimicrobial agents. Also further study in dairy food plane to be applied in production, food packaging and field.

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نشاط مادة الشيتوزان ومشتقاتها المضاد للبكتيريا على بعض البكتيريا الممرضة المعزولة من بعض منتجات الألبان

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ان الشيتوزان ومشتقاته لهم خصائص وظيفية عدة جذبت الكثير من العلماء لدراستها ودراسة تركيبها وتأثيرها على البكتيريا وخاصة البكتيريا الضارة سواء الموجبة منها والسالبة الجرام. ولذلك تركز هذه الدراسة على اهمية نشاط مادة الشيتوزان ومشتقاته كمضاد للبكتيريا الممرضة حيث تم عزل بكتيريا الاستافيلوكوكس اوريس والليستيريا مونوسيتوجين والسالمونيلا تيفيميوريم من الجبن الثلاجة حيث كانت النسب كالاتي ٢٣,٣, ٠ و٦,٦٧٪ على التوالي ونسب اعلى فى الزبادي البلدي وتليها فى الجبن القريش. وقد ثبتت التجربة قدرة الشيتوزان في القضاء على الاستافيلوكوكس اوريس والليستيريا مونوسيتوجين المحقون في الزبادى عند اليوم الخامس والثالث على الترتيب كما استطاع الشيوزان تقليل عدد السالمونيلا تيفيميوريم والقضاء عليها مع نهاية التجربة. بينما مشتقات الشيتوزان (الاوليجوميرز oligomers) استطاعت القضاء على الاستافيلوكوكس اوريس والسالمونيلا تيفيميوريم عند تركيز ٠,١٪ وتقليل عدد الليستيريا مونوسيتوجين حتى نهاية التجربة. كما تم دراسة ومناقشة مدى قابلية الشيتوزان (١٪) ومشتقاته (٠,١٪) لدى المستهلك. وتنصح الدراسة عمل المزيد من الدراسات عن الشيتوزان ومشتقاته واذا امكن الدمج بينهما ودراسة تأثيرهما على البكتيريا الضارة الاخرى والمفسدة للمنتج وعلى اهميتهما في مجال صناعة الغذاء ومدى امكانية تطبيق ذلك في السوق المحلى لإنتاج منتج صحى سليم وله قابليه لدى المستهلك.