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THE EFFECTIVENESS OF CHITOSAN ON THE TOXIGENIC STAPH. AUREUS IN YOGHURT

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ABESTRACT

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Chitosan and its derivatives, which are known to possess multiple functional properties, have attracted considerable interest due to their biological activities and potential applications in the pharmaceutical, food, agricultural and environmental industries. Many researchers have focused on chitosan as a potential source of bioactive material in the past few decades. This study focuses on the antibacterial and antitoxin activity of chitosan against *S. aureus* and it's enterotoxin type C. Chitosan inhibit growth of *S. aureus* which inoculated at 10^5 in yoghurt at 5^{th} day in all trails at different concentrations (conc.) (0.5, 1 and 2%). Enterotoxin type C inhibited at 10^{th} day at 0.5% conc. in yoghurt. While, at 1% and 2% conc. of chitosan complete inhibition of toxin occurred at 6^{th} day. Positive control trial, the toxin still detected until the end of the experiment. Chitosan haven't flavor, but have 55% of consumers were strongly agree to addition of 0.5% chitosan, 40% of consumers to 1% chitosan and 20% for 2% chitosan. The conc. of chitosan at 1and 2% have the same antibacterial and antitoxin effect, so this study advised to use chitosan at 1% conc. to reduce the public health hazards of *S. aureus* and it's enterotoxin.

Keywords: *Chitosan, Staphylo coccus aureus, Toxin type C, Yoghurt.*

INTRODUCTION

Chitosan is a natural nontoxic biopolymer produced by the deacetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Currently, chitosan has received extensive attention for its variable applications in the biomedical, food, and chemical industries (Arai *et al.,* 1968 and Kumar *et al.,* 2004).

Recent research has focused on the possibility of developing chitosan as a natural disinfectant (Harish Prashanth *et al.,* 2005). It has also been used in the removal of waterborne pathogens in waste water and as a food preservative by applying a coat on the exterior of vegetable and fruit products **(**Cummings *et al.,* 2009).

Yoghurt is one of the best known food products that may contain probiotics and is currently increasing supplementation with prebiotics, a type of fiber that stimulates the growth of specific bacteria in the gut (Laparra *et al.,* 2008). Synbiotic is a new concept to describe this kind of product and is popular among dairy manufactures in Europe (Yeung *et al.,* 2005). Because chitosan is an animal-origin carbohydrate polymer, which is included in the definition, it is being used as a new source of dietary fiber helping to meet consumer requirements all over the world that are increasingly interested in products high in dietary fiber (Harish Prashanth and Tharanathan, 2007 and Elleuch *et al.,* 2011).

On the other hand, *S. aureus* is found as a commensal organism on the squamous epithelium of the anterior nares up to 20% of the population at any one time, however, it has been estimated that *S. aureus* can transiently colonize up to 60% of the human population (Foster, 2004). The pathogenicity of *S. aureus* is determined by the production of toxins, these toxins can be harmful to the host and cause skin diseases and other complications, such as endocarditis, meningitis as well as toxic shock syndrome [TSS] (Mims *et al.,* 2004). Likewise, *S. aureus* can cause a wide range of infections ranging from minor skin abscesses to more serious invasive diseases. *S. aureus* can produce an enterotoxin that is the causative agent of *S. aureus* gastroenteritis. This gastroenteritis is self-limiting, characterized by vomiting and diarrhea one to six hours after ingestion of the toxin with recovery in eight to 24 hours. Symptoms include nausea, vomiting, diarrhea, and major abdominal pain (Becker *et al.,* 2003). *S. aureus* commonly causes boils, carbuncles, furuncles and impetigo, but after gaining access to the blood, may also be a major cause of endocarditis, osteomyelitis, pneumonia, TSS and septicemia (Lowy, 1998). Chitosan possesses some ideal properties of

polymeric carriers for nanoparticles such as biocompatible, biodegradable, nontoxic, and inexpensive. These properties render chitosan a very attractive material as a drug delivery carrier. In the last two decades, chitosan nanoparticles have been extensively developed and explored for pharmaceutical applications (Roberts, 1992). Furthermore, Chitosan has the ability to inhibit entertoxin production by *S. aureus* (Patrick, 2007) so, in this study the effect of chitosan on *S. aureus* count and enterotoxin type C inoculated in yoghurt was examined.

MATERIALS and METHODS

1- Bacterial suspension inoculation:
Reference strain of *S. aureus* was used, which previously have demonstrated their ability for enterotoxin type C production (reference strain from Animal Health Research Institute, Giza with code NCTC 7447 / ATCC 6538). *S. aureus* was grown in staph selective broth for 24-48h at 37 ^{o}C. Then the suspension was adjusted to the turbidity of a 0.5 McFarland standard by adding sterile saline to achieve a strain concentration of approximately $1x10^{5}$ colony forming units /ml (Gentilini *et al.,* 2000).

2- Detection of enterotoxin type C concentration in the inoculated broth by ELISA (Ewald, 1988):
Accurately, RIDASCREEN set C (Art No.: R4101, R-Biopharm AG, Darmstadt, Germany) is an enzyme immunoassay for the determination of *S.aureus* enterotoxin C by using its definite kits.

According to the test kit manual, a loopful of the culture was mixed in sterile buffer saline and then shaken for 15 minutes. After centrifugation for 10 minutes at 3500 r.p.m, sterile filtration of the supernatant was applied. An aliquot (100 µl per kit well) of this solution was used in the test. Further, the last well was represented as positive control. They were mixed gently and incubated for one hour at room temperature (20-25°C) in the dark.

The liquid was dumped out of the wells into a sink to remove all of the remaining liquid from the wells. Therefore, the wells were then filled with 250 µl of washing buffer and the liquid was poured out again. The washing step was repeated 3 more times to remove the unbound conjugate.

Subsequently, 100 µl of enzyme conjugate were added to each well and incubated for one hour at room temperature in the dark after mixing gently. The liquid was dumped out of the wells into a sink and the wells were each filled with 250 µl of the washing buffer. The liquid was poured out again and the wells were emptied to remove all of the remaining liquid. The washing step was repeated 3 more times again.

Afterwards, 50 µl of substrate and 250 µl of chromogen solutions were added to each well. The solutions were mixed gently and incubated for 30 minutes at room temperature in the dark. Finally, 100 µl of the stop solution (1M H_2 SO_4) were added to each well with gentler mixing.

By using ELISA, the absorbance was measured at 450 nm in an ELISA plate reader (ELX800, BioTek Instruments, Bad Friedrichshall, Germany).

3- The effectiveness of chitosan on the toxigenic *S. aureus* in yoghurt:
Pasteurized milk was purchased from a retail market and heated to 45^{o}C and inoculated with 2% yoghurt culture. One ml of each strain suspension (which prepared as before) mixed with 100 ml of prepared milk and divided into 4 suitable sterile jars, chitosan was added to 3 parts at concentrations of 0.5, 1 and 2%, 1 part without chitosan as a positive control, incubated at 40 ^{o}C until curdling. Control jar (free from strain suspension and chitosan as a negative control) was also incubated. The jars were stored at refrigerator temperature (5±2 ^{o}C). The inoculated jars were examined bacteriologically for the count of *S. aureus* using Baird-Parker media (37^{o}C for 24-48h) (I.C.M.S.F., 1978) and for presence of enterotoxin type C by ELISA (Ewald, 1988) after curdling (at time zero) and, every 2 days until the end of the storage period (14 days) and detected by the pH was measured in the control jars by using pH meter.

4- Sensory evaluation:
Control yoghurt jars (free from *S. aureus* but inoculated with chitosan at concentrations of 0, 0.5, 1 and 2%) were prepared as previously mentioned and each was subjected to the previous treatments. Thirty consumers were selected in teams of different ages, sex (20 females and 10 males), and education to taste the trials. The perception of consumers toward samples with various conc. of fennel honey was studied with respect to two different attributes (flavor and palatability). 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

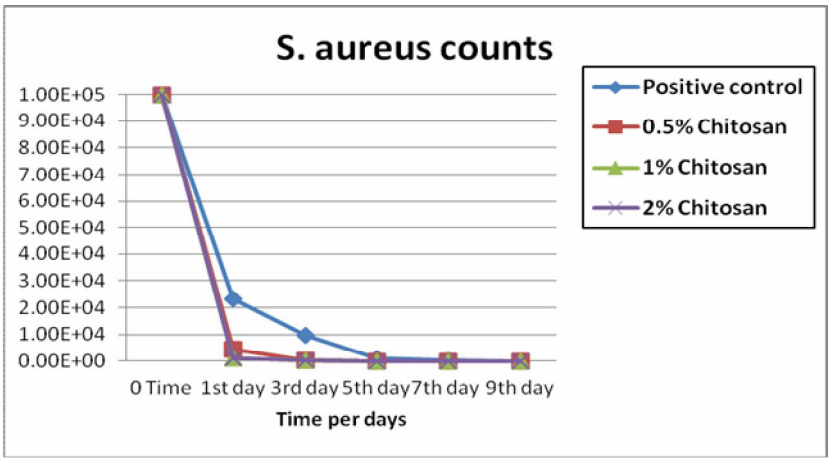

Fig.1: Effect of chitosan on *S. aureus* counts inoculated in yoghurt.

Table1: Effect of chitosan on enterotoxin type C produced by *S. aureus* inoculated in yoghurt.

Treatment / Storage time	-ve Control	Control +ve	Chitosan concentrations		
			0.5%	1%	2%
Zero time	------	60	60	60	60
2 days	------	60	55	40	40
4 days	------	60	30	10	5
6 days	------	60	20	------	------
8 days	------	60	5	------	------
10 days	------	60	------	------	------

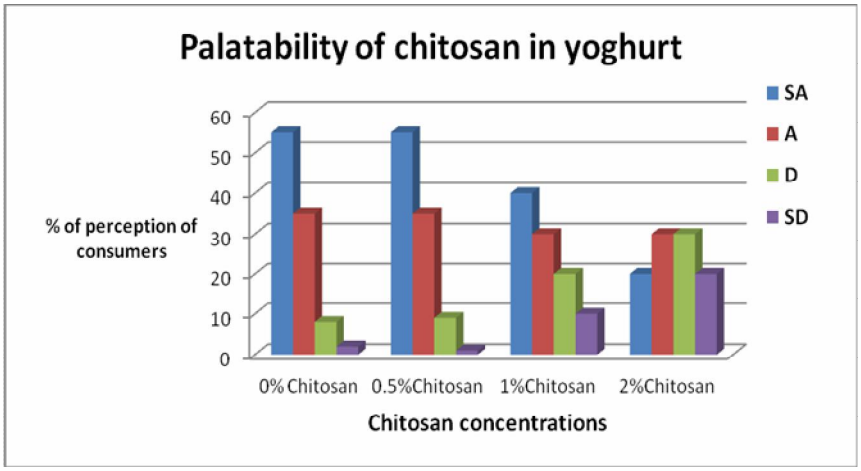

Fig.2: Palatability of chitosan in yoghurt.

***** strongly agree (SA), agree (A), disagree (D), and strongly disagree (SD)

DISCUSSION

S. aureus can multiply rapidly in food held at room temperature and the toxin can be produced by the microorganism growing in the food. This toxin is called an enterotoxin because it causes gastroenteritis or inflammation of the lining of the intestinal tract. Thorough cooking destroys the *S. aureus* bacteria, but the toxin is very resistant to heat, refrigeration, and freezing. The toxin is produced when the Staphylococcus aureus populations exceed 10^6 CFU/ gram of food. Less than 1.0 microgram of the toxin in food will produce staphylococcal intoxication symptoms.

Chitosan is the most important chitin derivative in terms of application (Salmabi and Seema, 2013).

Chitosan has attracted considerable interest because of its unique combination of properties, such as biocompatibility, biodegradability, metal complication and antibacterial activity. Chitosan has a variety of current and potential applications in various fields, for example, biotechnology (Mao *et al.,* 2001), pharmaceutics (Illum, 1998), wastewater treatment, cosmetics (Majet and Kumar, 2000), and food science (Chien *et al.,* 2007). The antibacterial activity of chitosan has been widely explored (Hong *et al.,* 2002; Tsai *et al.,* 2004 and Liu *et al.,* 2006). As shown in Fig.1 chitosan markedly inhibited the growth of *S. aureus* in yoghurt at 5th day in all trails. The activity increased with increasing concentration of chitosan. While, in positive control S. aureusstill in high concentration until 3rd day and inhibited at 7th day 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). Valero *et al.* (2009) recorded that for a cocktail of five enterotoxigenic S. aureusisolates growth has been recorded at pH 4.5. Chitosan showed significant antibacterial activity against *S. aureus* and that returned to Chitosan shows great potential in developing into a biocompatible antibiotic. Chitosan with a high molecular weight (500KD-1000KD) and maximum degree of de-acetylation is expected to show enhanced antimicrobial activity even at lower concentrations (Rhodes and Roller, 2000 and Raafat, 2008), theorized that a mild degradation of chitosan enhances its antimicrobial action, where as highly degraded chitosan displayed no antimicrobial action (Salmabi and Seema, 2013). Monarul *et al.* (2011) reported that many hypotheses have been proposed to elucidate the mechanism of antibacterial activity of chitosan. Chitosan contains three types of reactive functional groups, an amino/acetamido group as well as, both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. This electrostatic interaction results in twofold interference:i) by promoting changes in the properties of membrane wall permeability, thus incite internal osmotic imbalances and consequently restrain the growth of microorganisms (Shahidi *et al.,* 1999) and ii) by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase) (Papineau *et al.,* 1991 and Chen *et al.,* 1998).

In this study the same results are in agree with Chung and Chen, (2008); Másson *et al.* (2008) and Raafat *et al.* (2008). likewise, Patrick (2007) reported that chitosan at 3.5 and 7.0 mg/ml (final concentrations) was bactericidal for *S. aureus*.

Chitosan have antitoxic effect against enterotoxin which produced by *S. aureus* and that may returned to it's a polysaccharide comprised of repeating units of glucosamine. The mechanism underlying its growth and toxin-inhibitory effects remains unknown, but may be related to its strong charge (Patrick, 2007). The polymeric size and charge suggest the agent may inhibit exotoxin production by effects on the microbial surface and thus by interfering with signal transduction. (Projan *et al.,* 1994).

In this study, chitosan inhibited enterotoxin type C at 10th day at 0.5% conc. in yoghurt. While, at 1 and 2% conc. of chitosan complete inhibition of toxin occurred at 6th day. Positive control trial, the toxin still detected until the end of experiment at the same concentration (60nm/ml) and that may returned to that the optimum pH for *S. aureus* producing enterotoxin ranged between pH 5.7 and 4.9 under aerobic conditions. It was noted that growth occurred in the absence of enterotoxin production under some conditions. No culture was able to produce enterotoxin at a pH less than 5.7 under anaerobic conditions (Paulin *et al.,* 2012). Similar results were obtained for three Staph. enterotoxin type B, and one for type C-producing strains. However a strain producing type E formed enterotoxin weakly at pH 4.8 (Genigeorgis *et al.,* 1971).

These results is in agreement with Yarwood *et al.* (2001); Novick (2003); Yarwood and Schlievert (2003); Pragman and Schlievert (2004) and Patrick (2007).

Also, Burkatovskaya *et al.* (2006) shown that chitosan, an agent that could be added to wound dressings, inhibited both bacterial growth and exotoxin production in vitro and prevented Toxic Shock Syndrome (TSS) and necrotizing fasciitis in rabbit models. It has long been known that chitosan inhibits the growth of gram positive and gram-negative bacteria, with greater antimicrobial activity against gram-positive agents (Takai *et al.,* 2002 and Rabea *et al.,* 2003). Also, Mendel and Vijay (2010) reviewed that chitosans are reported to exhibit numerous health-related beneficial effects, including strong antitoxin and antioxidative activities in

foods. A special need exists to develop a better understanding of the role of chitosans in ameliorating foodborne illness. To contribute to this effort, this overview surveys and interprets our present knowledge of the chemistry and antimicrobial activities of chitosan in solution, as powders, and in edible films and coating against foodborne pathogens, spoilage bacteria, and pathogenic viruses and fungi in several food categories. They suggested that low-molecular-weight chitosans at a pH below 6.0 presents optimal conditions for achieving desirable antimicrobial, antitoxin and antioxidative-preservative effects in liquid and solid foods.

Regarding, the perception of consumers toward samples subjected to various chitosan conc. with respect to two different attributes (flavor and palatability), 55% of consumers were strongly agree to addition of 0.5% chitosan and 40% of consumers to 1% chitosan (Fig. 2). Generally, 1% chitosan conc. considered the most preferable additives in all samples than 2% chitosan conc. Chitosan have no flavor effect when added to yoghurt at different concentrations.

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مدى فاعلية الشيتوزان على المكور العنقودى الذهبي المفرز للسموم في الزبادي

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الشيتوزان ومشتقاته ، والمعروفة بأنها تمتلك خصائص وظيفية متعددة ، وقد اجتذبت اهتماما كبيرا بسبب أنشطتها البيولوجية والتطبيقات المحتملة في الأدوية والمواد الغذائية ، والصناعات الزراعية والبيئية. وقد ركز العديد من الباحثين على الشيتوزان كمصدر محتمل من المواد النشطة بيولوجيا في العقود القليلة الماضية. وتركز هذه الدراسة على النشاط المضاد لبكتيريا المكور العنقودي الذهبي والسم المعوي نوع C المفرز منها. وقد أثبتت التجربة قدرة الشيتوزان على القضاء على بكتيريا المكور العنقودي الذهبي المحقون في الزبادي بتركيز ١٠° عند اليوم الخامس فى التركيزات المختلفة للشيتوزان (٠.٥، ١ و٢٪). وقد استطاع الشيتوزان محو السم المعوي C عند اليوم العاشر عند التركيز ٠.٥٪ بينما عند تركيز ١ و٢٪ من الشيتوزان اختفى السم المعوي C تماما عند اليوم السادس. أما العينة الضابطة المحقون بها الميكروب فقط (العينة الضابطة الايجابية) السم المعوي كان متواجد بها حتى نهاية التجربة. ومن مميزات الشيتوزان انه ليس له رائحة، وكان التركيز ١٪ أفضل تركيز له قبول لدى المستهلك. لذلك تنصح دراستنا باستخدام الشيتوزان بنسبة ١٪ للحد من المخاطر الصحية العامة من ميكروب المكور العنقودي الذهبي والسم المعوي نوع C المفرز منه.