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Studying the effect of chitosan on *Bacillus cereus* producing cereulide toxin in milk and some dairy desserts.

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Abstract

A total of 150 samples of raw milk and two types of refrigerated processed dairy desserts dishes (cooked rice with milk and Mehalabia dishes) (50 for each) were collected from dairy shops and local restaurants in Assuit City, Egypt. **ISO 7932:2004** method was used for enumeration of *Bacillus cereus* (*B. cereus*), afterwards a PCR was performed to confirm the presence of cereulide toxin (*ces*) gene. In addition, the effect of different concentrations of chitosan and chitosan nanoparticles (CNPs) (0.05, 1%) on *B. cereus* inoculated in pasteurized milk, the shelf life was evaluated by clot on boiling test and Ph value. Their pathological effect was detected by orally administration to experimental rats to investigate their adverse effect on rats liver and intestine using histopathological examination. The most prominent potent bactericidal effect was observed in CNPs 1%, *B. cereus* count reached to undetectable level at 5th day of refrigerated storage. The pasteurized milk inoculated with *B. cereus*, chitosan and CNPs showed some pathological lesions in rats treated with *B. cereus,* while the chitosan 0.5% had antibacterial activities without cytotoxic effect on rat tissues, alternatively to CNPs 1%, which exist edematous tips of intestinal villi and mild kupffer cells activation in liver. Therefore, our results contribute data that are primary to indicate the risk of food poisoning due to *B. cereus* and trials to control that in food by careful using of nanotechnology. However, the additional researches are needed to safe using of this technology even on natural nano-materials as it at nano-size gain new properties.

Keywords: *Rice, dairy desserts, chitosan nanoparticles, B. Cereus, cereulide toxin, milk.*

Introduction

Bacillus cereus is a spore forming opportunistic environmental wide spread pathogen, cause food-borne outbreaks in human (**Yu *et al.,* 2019**). **Zhao *et al.* (2020)** had isolated *B. cereus* from different dairy products, and considered it as a potential risk pathogen need to contribute an effective prevention and control program. In addition, several publications reported an increasing number of foodborne intoxication cases caused by *B. cereus*. In the European Union about 500 –700 annually reported confirmed cases of foodborne outbreaks linked to *B. cereus* toxins (**Messelhäußer and Ehling-Schulz, 2018**). *Bacillus cereus* strains have shown to produce seven different toxins (**Papan *et al.,* 2019**); the most dangerous one is cereulide toxin, which pre-formed in food. This toxin is a small cyclic dodecadepsipeptide encoded by the *ces* gene. The cereulide is heat and pH stable, highly resistant to protease activity and it remains active through the gastro-intestinal passage but, due to the lack of a suitable assay, it considered the least well known (**Ceuppens *et al.,* 2012 and Zhang *et al.,* 2015**). The toxin

produced in food during vegetative growth, and after the toxin produced, no treatment can destroy this stable molecule, including proteolysis and extreme pH **(Chica *et al.,* 2019**).

Generally, food matrices rich in carbohydrates, such as pasta and rice, as well as milk and dairy products have the highest risk of causing cereulide intoxications (**Papan *et al.,* 2019**). Outbreaks of *B. cereus* foodborne illness associated with consumption of cooked rice have been reported worldwide (**Kumari and Sarkar, 2014; Yang *et al.,* 2017 ; Alvarenga *et al.,* 2018).**

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. The antimicrobial activity of chitosan depends on the degree of deacetylation and a molecular weight (**Tantala *et al.,* 2012**). Chitosan is safe, non-toxic and can interact with polyanions to form complexes and gels (**Sukmark *et al.,* 2011**). It has many antibacterial mechanisms activity, as intercellular leakage hypothesis is widely accepted (**Silalahi *et al.,* 2016**); blockage of nutrient flow by forming a polymer layer around bacterial cells (**Fernandes *et al.,* 2009**). In addition, it may affect the structure of the phospholipid bilayer in the cell membrane resulting in the release of some of the cellular components (**Yusman, 2006 and Dutta and Dutta, 2011**). Because chitosan has many advantages, properties and mostly safe, so it used in nanotechnology as chitosan nanoparticles (CNPs) depend on ionic gelation method, which describes the crosslinking reaction of chitosan with sodium tripolyphosphate (TPP) **(Nguyen *et al.,* 2017 and Sreekumar *et al*., 2018)**

Therefore, the objective of this research was to detect the incidence and count of *B. cereus* in raw milk and two dairy desserts and detect cerulide producing toxin (*ces* gene) in isolates by using PCR. Another purpose was to investigate the potential of applying chitosan and its nanoparticles as a natural food preservative to control the growth of *B. cereus* in pasteurized milk during storage. In addition, to provide a histopathological evaluation for the antimicrobial activity of chitosan and CNPs in different concentrations against *B. cereus* on intestine and liver of experimental rats.

Materials and Methods

The prevalence of emetic *B. cereus* strains in some food samples:

Collection of samples:

A total of 150 raw milk, cooked rice with milk and Mehalabia dishes samples (50 samples each) were collected from dairy shops of different localities in Assiut city, Egypt and brought to the laboratory by means of refrigerated transport within a timeframe of max 2 h. There, all samples were prepared according to **APHA (2004).**

Identification and Enumeration of *B. cereus* strains:

Enumeration of *B. cereus* is performed by using **ISO 7932:2004** method, where the samples were plated on mannitol egg yolk phenol red polymyxin (MYP; Oxoid, Hampshire, UK) medium and then incubated for 18–48 h at 30 °C. The presumptive cultures of *B. cereus* were inoculated on Kim and Goepfert medium with polymyxin B as the selective agent and permit presumptive identification by the lecithinase reaction on the egg yolk and the inability of *B. cereus* to catabolize mannitol. Motility, endospore formation, followed by species confirmation using biochemical tests.

Identification of *ces* gene of *B. cereus* by PCR:

This part was done at Biotechnology Unit, Animal Health Research Institute, Giza, Egypt. Genomic DNA was obtained using the QIAamp Bacterial DNA Kit (Qiagen, Germany, GmbH) following the manufacturer's specifications. Polymerase chain reaction (PCR) amplification was conducted to detect the cereulide synthetase gene (*ces*) with a 20 ml reaction mixture consisting of 50 ng genomic DNA, 12.5 ml PCR Premix TaqTM (Takara, Japan), and 2 mM of each primer according to **Ehling-Schulz *et al.*, 2006**. The primers used in this study are F: GGTGACACATTATCATATAAGGTG& R: TAAGCGAACCTGTCTGTAACAACA.

Primers were supplied from Metabion

(Germany).

The antibacterial activity of chitosan and CNPs to inhibit the growth of *B. cereus*:

1. Preparation of chitosan solution

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

2. Preparation of CNPs:

The Nanoparticles were prepared following the procedure described by **Calvo *et al.* (1997)** and characterized by HRTEMModel JEOLJEM-100CX II in the Electron Microscopy Unit, Assiut University, Egypt. Briefly: a 2.5 mg/mL chitosan solution were prepared by dissolving chitosan in a 0.05% (v/v) acetic acid solution and leaving it under stirring for 24 h. The pH was adjusted to 5.5 with a 0.5 M sodium hydroxide solution and diluted in deionized water to the final desired concentrations. Sodium Tripolyphosphate (TPP) was dissolved in deionized water to a final concentration of 0.25 mg/mL. Then, the TPP solution was added to the chitosan solution drop wise (0.3 mL/min) at different TPP: chitosan ratios under vigorous magnetic stirring at room temperature. The resulting suspension was then left to jellify for 30 min.

3. Bacterial suspension inoculation:

The isolated strains were inoculated into Muller Hinton broth and incubated to the growth phase at 37 ºC. The growth density was adjusted to match a MacFarland 0.5 standard (8 $\log_{10}$ CFU/ml) according to **Yu *et al.*, (2019**).

4. Antimicrobial properties of chitosan and CNPs:

Five replicates of pasteurized milk samples were used for the antibacterial activity. Pasteurized milk samples were purchased from a retail market and examined by phosphatase test to confirm the efficacy of pasteurization **(Sharma and Rajput, 2014).** Four treatments of chitosan and CNPs at two different concentrations (0.5 and 1%) were used in the experiments; two controls, inoculated (control positive) and none inoculated with *B. cereus* (control negative), were tested. All samples were stored at refrigerator temperature (4±1 ºC) and examined every 2 days until the end of the experiment. Freshness pasteurized milk in all control and treated samples were examined using Clot on Boiling test and pH value was determined by pH meter.

5. Sensory evaluation:-

Control Pasteurized milk jars (free from the previous prepared *B. cereus* suspension but inoculated only with chitosan and chitosan nanoparticles at concentrations of 0.5 and 1%, respectively) were prepared as previously mentioned and each was subjected to the previous treatments. Thirty consumers were selected in tearms of different ages, sex (15 females and 15 males) and qualification to the trials. The perception of consumers toward samples with various conc. of chitosan was studied with respect to different attributes (flavor, color and consistency), in addition to their resultant expressed in overall acceptability (OAA). The level of agreement was scored as strongly agreed (SA), agreed (A), disagreed (D), and strongly disagreed (SD) according to (**Nelson and Torut, 1981**)

Animals and experimental design:

The experimental procedure was approved by the Ethics Committee of the animals to the Faculty of Veterinary Medicine, Assiut University. Fifty female, albino rats aged two months and 120± 20 g weight were purchased from laboratory animal house, Faculty of Medicine, Assiut University for experimental studies. Rats were kept under laboratory conditions of 65% humidity, 24-26°C and daily light/dark cycle, fed on balanced standard pellets and water was available. Animals allowed 7 days for acclimatization before starting the experiment.

Antimicrobial properties of chitosan and CNPs on experimental rats animals:

The rats were divided into 10 experimental groups (5 animals each) as following: Group 1

(control negative) rats take milk without any treatment. Group 2 (control positive) rats take milk inoculated with *B. cereus* 8 $\log_{10}$ CFU/ml. Group 3 and 4 rats take milk with chitosan 0.5 and 1%, respectively. Groups 5 and 6 rats take milk with CNPs 0.5 and 1%, respectively. Group 7 and 8 rats take milk inoculated with *B. cereus* 8 $\log_{10}$ CFU/ml CFU/ml and chitosan 0.5, 1%, respectively. Group 9 and 10 rats take milk inoculated with *B. cereus* 8 $\log_{10}$ CFU/ml and CNPs 0.5, 1%, respectively. Each rat from tested groups was daily inoculated with 2ml volume of treated milk by oral gavage for 15 days. At the end of experiment all rats groups were individually weighed, euthanatized and sacrificed for tissue specimens' collection.

Pathological studies:
At the end of experiment, on 15th day, all rats were sacrificed, examined for postmortem findings. Tissue specimens from intestine and liver were collected, fixed in 10% neutral buffer formalin, dehydrated, cleared, and embedded in paraffin blocks. Paraffin sections of 4µm thickness were prepared and stained by Hematoxylin & Eosin **(Suvarna *et al.*, 2013)**, and examined microscopically for detection of histopathological alterations.

Statistical analysis:
The effect of different concentrations of the chitosan and its nanoparticles on *B. cereus* count in pasteurized milk was analyzed using one-way analysis of variance and repeated measures ANOVA and the differences among group means were analyzed using the Tukey's multiple comparisons test. A P-value of <0.05 was considered significant. The Graph Pad Prism software (GraphPad, Inc., San Diego, USA) (version 5) was employed for the statistical analysis.

Results
Of 150 examined samples, the incidence of *B. cereus* was 40, 52 and 62 % with mean count 3.9±0.53, 4±0.72 and 4.7±0.38 log CFU/ ml or g of raw milk, cooked rice with milk and mehalabia samples respectively (Table 1). The maximum count of *B. cereus* in this study was reported in mehalabia samples (log 5.7 CFU / ml) (Table 1). The toxic genes profiles indicated that *ces*, the specific gene for emetic *B. cereus* strain, was detected (Photo 1, 2 and 3).

Table (1). Incidence and count of *B. cereus* in the examined samples.

Samples	Positive samples		*B. cereus* count ($\log_{10}$ CFU/ ml)		
	No./50	%	Min.	Max.	Mean ±StD
Raw milk	20	40	2.0	5.6	3.9±0.53
Cooked rice with milk	26	52	2.0	5	4±0.72
Mehalabia	31	62	2.0	5.7	4.7±0.38

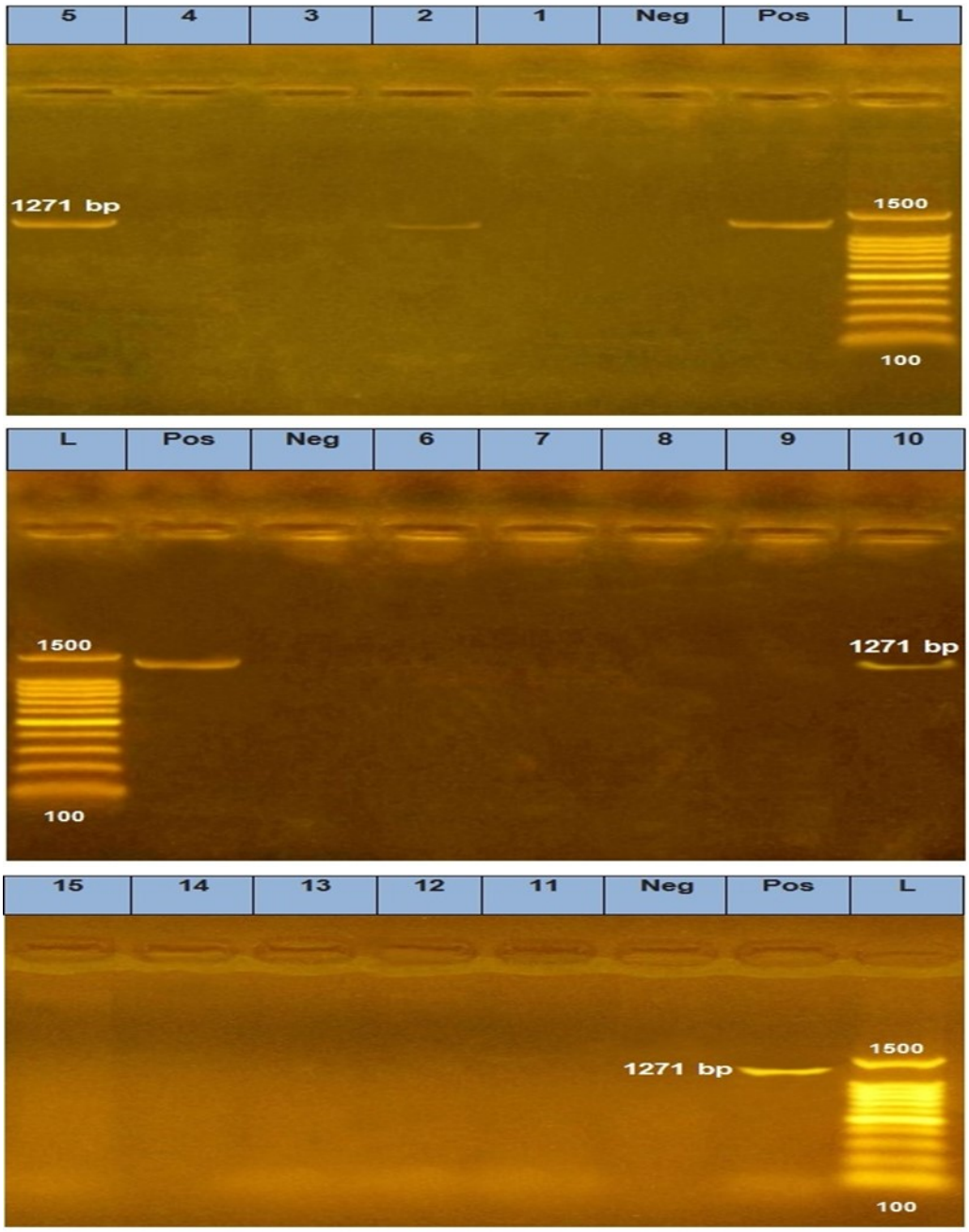

Photo (1, 2 and 3): Gel electrophoresis of the PCR amplification products obtained with toxic gene of cereulide (*ces*). Emetic *B. cereus* strains (lanes 2, 5 and 10) and non-emetic *B. cereus* strains (lanes 1, 3, 4, 6-9 and 11-15). L: Molecular marker; Lane pos: Positive control; Lane Neg: Negative control.

Regarding the effect of different concentrations of chitosan and CNPs on *B. cereus* count inoculated in pasteurized milk. Firstly, detect the size and shape of nanoparticles using TEM as shown in photo (4). Also, detection of freshness by Clot on boiling test which revealed positive results by the 13th day of the experiment in positive control sample and in pasteurized milk inoculated by 0.5% chitosan samples so, these samples were not tested (Table 2). In this study, the mean pH value of pasteurized milk control sample ranged from 6.8±0.04 to 3.4±0.2 by the end of the experiment. While by adding chitosan to pasteurized milk, it ranged from 6.8±0.05 to 4.2±0.09 and 5.8±0.04 in concentrations 0.5 and 1%, respectively. Nearly similar values obtained in pasteurized milk sample with CNPs. It reached 5.7±0.07 and 5.9±0.08 by the day 15th of the experiment for concentration 0.5 and 1% of CNPs, respectively (Table 2). It was found that *B. cereus* mean count decreased from 8 $\log_{10}$ CFU/ml at zero time to 3.8 $\log_{10}$ CFU/ml in positive control sample after 11th days of storage at 4±1 ºC (Table 3). On the other hand, both concentrations of chitosan 0.5 and 1% had displayed antibacterial activity against B. cereus 1.10 and 1.2 log CFU/ml by the 9th day of the experiment. CNPs still had good bactericidal activity at concentration 0.5% after 7 days from cooling storage comparing with control group at which the bacterial growth mean count reached 6.5 $\log_{10}$ CFU/ml as shown in Table (3). The most prominent potent bactericidal effect was observed in CNPs 1%, *B cereus* count reached to undetectable level after 5 days of refrigerated storage.

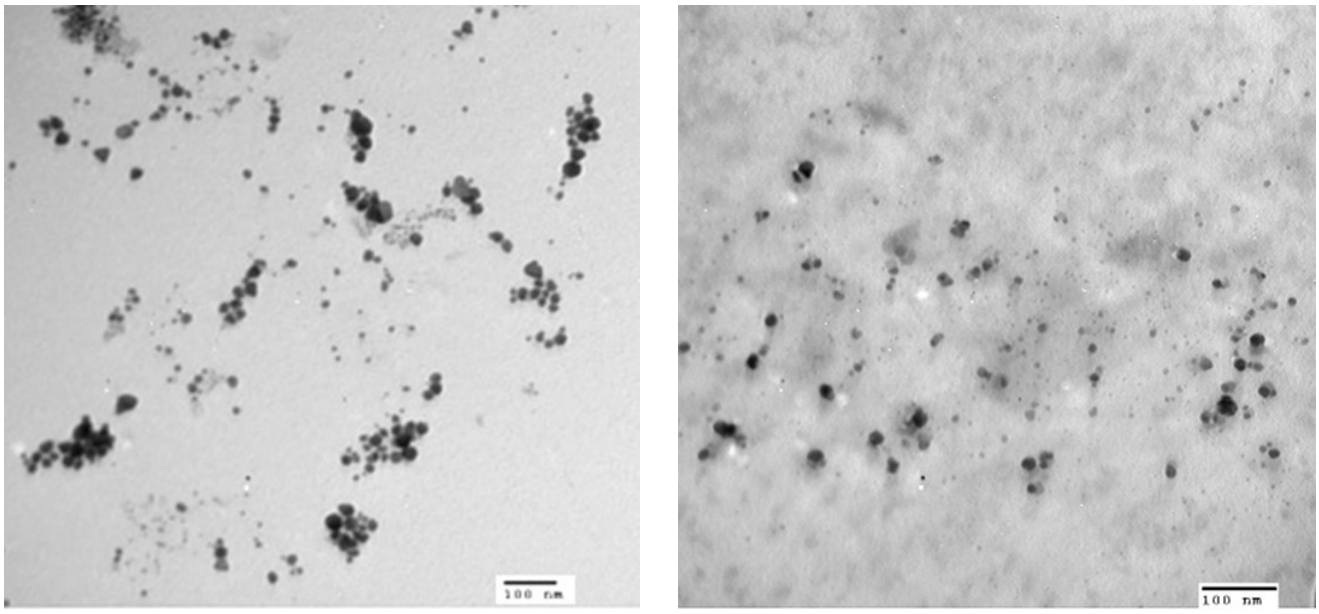

Photo (4): TEM for chitosan nano-particles with average size 27.6 nm.

Table (2). Mean pH and detection of freshness of pasteurized milk treated with chitosan and CNPs

Storage period by days	pH					Clot On Boiling				
	Control	Chitosan		CNPs		Control	Chitosan		CNPs	
		0.5%	1%	0.5%	1%		0.5 %	1 %	0.5 %	1 %
0	6.8±0.04	6.8±0.05	6.8±0.05	6.7±0.04	6.8±0.05	-ve	-ve	-ve	-ve	-ve
1st	6.5±0.06	6.5±0.03	6.6±0.06	6.5±0.05	6.6±0.04	-ve	-ve	-ve	-ve	-ve
3rd	6.3±0.05	6.3±0.06	6.5±0.05	6.5±0.07	6.5±0.06	-ve	-ve	-ve	-ve	-ve
5th	6.1±0.07	6.3±0.08	6.3±0.07	6.4±0.08	6.5±0.06	-ve	-ve	-ve	-ve	-ve
7th	5.8±0.07	6.1±0.07	6.3±0.05	6.4±0.05	6.4±0.05	-ve	-ve	-ve	-ve	-ve
9th	5.3±0.03	5.9±0.05	6.2±0.08	6.3±0.07	6.3±0.04	-ve	-ve	-ve	-ve	-ve
11th	5.1±0.05	5.4±0.09	6.0±0.05 *	6.2±0.06	6.2±0.05*	-ve	-ve	-ve	-ve	-ve
13th	4.2±0.04	4.5±0.09	5.8±0.08*	6.1±0.09	6.1±0.05*	+ve	+ve	-ve	-ve	-ve
15th	3.4±0.2	4.2±0.09	5.8±0.04*	5.7±0.07	5.9±0.08*	+ve	+ve	-ve	-ve	-ve

Mean ± SE of five replications
*Significant difference (P value <0.05)

Table (3). Effect of incorporation of different concentrations of chitosan and CNPs on *B. cereus* count (log_{10} CFU/ ml) inoculated in pasteurized milk stored at 4±1 ºC.

Storage period by days	-ve control	+Ve control	Chitosan		CNPs	
			0.5%	1%	0.5%	1%
0	-ve	8	8	8	8	8
1st	-ve	8.3	7.8	6.11	7.9	6.7
3rd	-ve	8.6	6.8	3	5.3	3.7*
5th	-ve	7.9	3	2.8	2.4*	ND
7th	-ve	6.5	1.10*	1.2*	ND	ND
9th	-ve	6.1	ND	ND	ND	ND
11th	-ve	5.8	ND	ND	ND	ND
13th	-ve	NT	NT	ND	ND	ND
15th	-ve	NT	NT	ND	ND	ND

ND: Not Detected (below than one log_{10} CFU/ml)
NT: Not Tested (due to deterioration of samples which detected by pH and clot on boiling test as shown in table 2)
Mean of five replications
*Significance difference (P value <0.05).

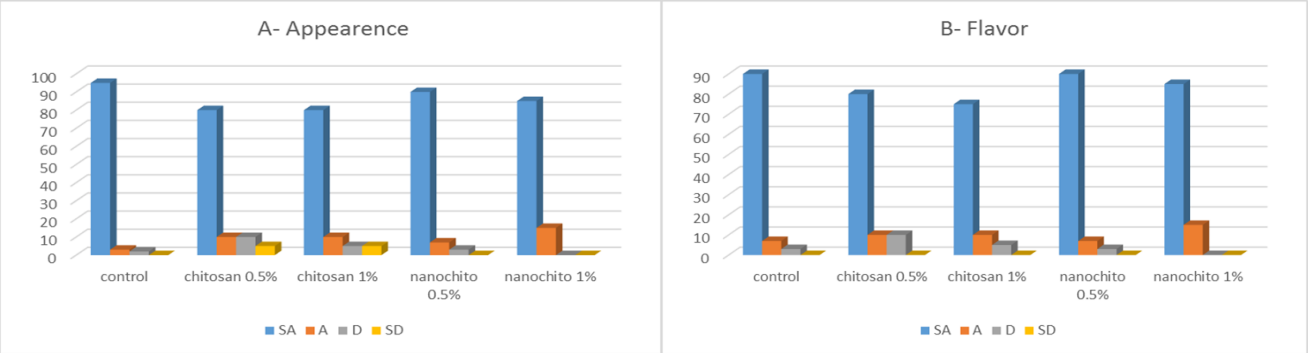

Figure (1). Level of agreement of consumer acceptability to appearance (A), and flavor (B) of Pasteurized milk inoculated with different concentrations of chitosan and CNPs.

Body weight: No significant changes were observed in the values of body weight in different experimental groups along the period of the experiment.

Pathological findings:

Postmortem examination: No characteristic gross of pathological lesions in rats of different groups except group (2) which administrated milk with *B. cereus* (8 $\log_{10}$ CFU/ml), most rats showed mild congested and hyperemic intestine and enlarged pale liver.

Light microscopic examination:

The intestine in Group 1 (control negative) and Groups 3, 4 and 5 showed normal structure of intestinal villi with intact lamina epithelialis (Fig. 2 A, B and C). While, The liver of same group's demonstrated normal hepatic parenchyma (Fig. 3 A, B). In Group (6), rats take milk with 1% CNPs revealed mild edema appeared at the tip of some villi of the intestine (Fig. 2D and E) and mild proliferation of kupffer cells in sinusoids of liver (Fig. 3 C). Control positive milk with *B. cereus* only (Group 2) showed swollen villi, goblet cells hyperplasia, some villi appeared blunted associated with congestion of lamina propria and edema in submucosa (Fig. 2 F, G and H). While, the liver of the same group showed characteristic vacuolar hepatocytes degeneration, perivascular mononuclear cells infiltration and kupffer cells proliferation in perivascular and hepatocyte plates (Fig. 3 D and E). Group (7) which rats take milk with *B. cereus* and 0.5% chitosan, the intestine showed mild hyperplasia of goblet cells and slight aggregation of mononuclear inflammatory cells in villus core (Fig. 2 I and J) and the liver showed mild vacuolar degeneration of hepatocytes (Fig. 3 F). In Group (8), the intestine appeared almost normal mucosa except for slight increase in goblet cells population (Fig. 2 K and L) and liver showed almost normal hepatocytes (Fig. 3 G). On the other hand, Group (9) which rats take milk with *B. cereus* and 0.5% CNPs, the intestine showed increase population of goblet cells and some villi showed slight edematous tips with mild vacuolar degenerative changes in overlying epithelium (Fig. 2 M and N). In addition, the liver showed mild necrobiotic changes of hepatocytes (Fig. 3 H). In group (10) which rats take milk with *B. cereus* and 1% CNPs, intestinal villi showed nearly normal mucosa except for appearance of edema at the tips of some villi (Fig. 2 O and P), liver showed mild sinusoidal dilatation and some engorged with RBCs accompanied by kupffer cells activation (Fig. 3 I).

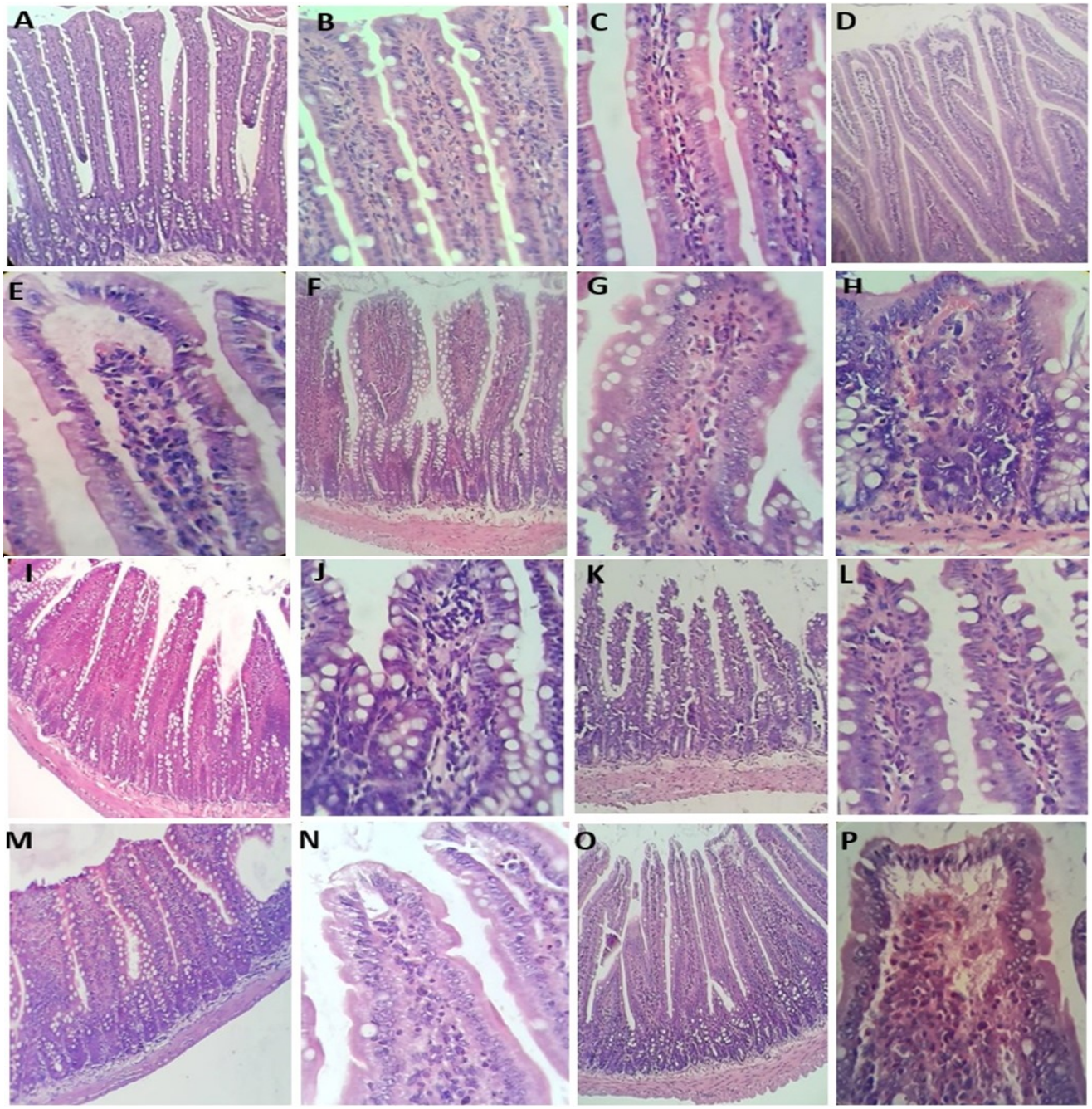

Figure (2). Photomicrograph of rat intestine in different groups, panels show (A&B) normal mucosa of intestinal villi and the higher magnification (x400) from group (1, 3 and 4). (C) Almost normal intestinal villi from group (5). (D&E) mild edema appeared at the tips of villi and the higher magnification (x400) from group (6). (F, G&H) swollen villi, goblet cells hyperplasia and edema in submucosa, and the high power from group (2). (I&J) mild goblet cells hyperplasia and the high power view slight aggregate of mononuclear cells in villus core from group (7). (K& L) almost normal mucosa except for slight increase in goblet cells and the higher magnification from group (8). (M&N) increase population of goblet cells and the high power view mild edema at the tip of villi from group (9). (O&P) nearly normal mucosa except for appearance of edema at the tips of villi and the higher power from group (10). (H&E stain low power x100, high power x400).

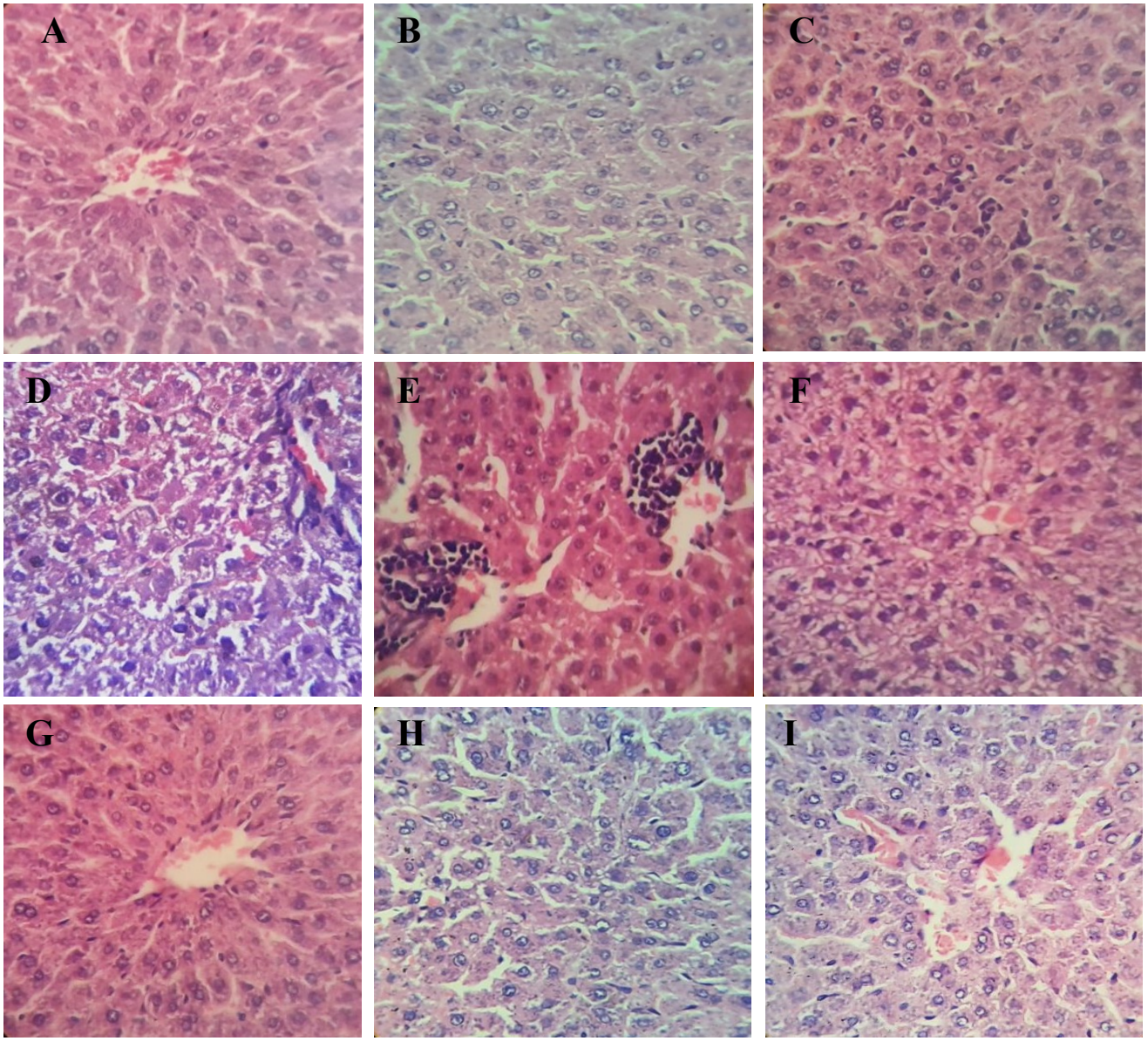

Figure (3). Photomicrograph of rat liver in different groups, panels show (A) normal hepatic parenchyma from group (1, 3, 4). (B) Almost normal hepatic tissue from group (5). (C) Mild kupffer cells activation from group (6). (D&E) hepatocellular vacuolar degeneration, clumps of lymphocytes and kupffer cells in hepatic parenchyma from group (2). (F) Mild hepatocellular vacuolar degeneration from group (7). (G) Almost normal hepatocytes from group (8). (H) Mild necrobiotc changes in hepatocytes from group (9). (I) Mild dilatation of sinusoids and activation of kupffer cells from group (10). (H&E stain x400)

Discussion

The combined abilities of *B. cereus* spores and toxins, to survive in pasteurization and of certain strains to multiply at low temperatures make this organism a unique milk-borne pathogen. Since *B. cereus* grows best in a starch-rich environment **(Samapundo *et al* 2011)**, cooked rice with milk and Mehalabia are popular dessert prepared and sold all day by dairy shops or local restaurants in Egypt and many middle east countries. They produced by adding whole rice grains or rice flour or cornstarch to milk, cooked and stored at refrigeration temperature. In the present study, high incidence of *B. cereus* (52 and 62%) was reported in cooked rice with milk and mehalabia compared to raw milk samples. These investigated dairy products are all known to be subjected to different types of heat treatment during processing according to each product. Heat treatment is not completely efficient to eliminate spore-forming bacteria in foods, among *Bacillus* spp. This high incidence may be attributed also to the improper temperature control during storage of such products. Similar incidences were reported by **Organji *et al.* (2015)** and **Mohamed *et al.* (2016)**. It was reported that *B. cereus* increase rapidly in boiled rice to 7-8 $\log_{10}$ CFU/g and produced emetic toxin at both 30-35ºC **(Agata *et al.*, 2002)**.

Several studies detected *B. cereus* emetic strains in various foods **(Mudagza and Buys, 2017; Yang *et al.*, 2017 and Frentzel *et al.*, 2018)**. Only a minority of *B. cereus* isolates may produce cereulide but the detection at least of one toxin gene could be a target marker for screening toxigenic *B. cereus* group strains in food **(Gdoura-Ben *et al.*, 2018)**.

The present article demonstrated that strains of *B. cereus* which isolated from milk and dairy desserts may contain genes encoding the toxic gene (*ces*), so the risk of food poisoning should not be neglected. For that reason, our present study was focused on the application of chitosan and CNPs in pasteurized milk contaminated with *B. cereus* as an example of spoilage and pathogenic microorganism. Significant decrease in *B. cereus* count was noticed in treated pasteurized milk with different concentrations of chitosan and the organism could not be detected by the 9th day of the experiment. While, by adding CNPs (0.5 and 1%), the count could not be detected at 7th and 5th days of storage, respectively. On the other hand no significance differences ($p > 0.05$) were observed between concentrations of chitosan and CNPs.

The effectiveness of chitosan on gram-positive or gram-negative bacteria is however, somewhat controversial. Several studies **(Dutta *et al.*, 2009 and Zaghloul and Ibrahim 2019)** detected the bactericidal effectiveness of chitosan, others stated that chitosan exhibited a bacteriostatic effect on all bacteria tested including *B. cereus* **(Benhabiles *et al.*, 2012)**. **Raafat *et al.* (2008)** declare one of the suggested hypothesis for this effect; who stated that, this difference in sensitivity is largely ascribed to the different structure of Gram positive and Gram-negative bacterial cell envelopes. As a possible mechanism of action for chitosan, antimicrobial activity is due to binding to teichoic acids present in the cell wall of Gram-positive bacteria, coupled with membrane lipids extraction, which trigger a series of event that resulted in bacterial cell death. **Tamara *et al.* (2018)** showed that *B. cereus* was generally more resistant to the CNPs and suggested that *B. cereus* had more hydrophilic and negatively charged cellular structure. Another study **(Hassan *et al.*, 2016)** investigated that the antibacterial activity of CNPs on *Bacillus* spp. was recorded in percentage 100 % inhibition at different concentrations. Furthermore, CNPs can be used as bioactive ingredients carriers and in wide approach arrays, due to their favorable biological properties such as non-toxicity, biocompatibility, biodegradability and antibacterial ability **(Zhao *et al.*, 2011)**. Increasing the degree of de-acetylation has a major effect on the antimicrobial activity of chitosan and in dependency CNPs; that due to increase the free amino groups in the produced chitosan, which leads to higher antimicrobial activity, as the -NH2, -OH groups are the main reactive site in chitosan. As, it is determinant in the charge development and solubility of chitosan **(Zaghloul and Ibrahim, 2019)**.

CNPs have been very effective in increasing the shelf life of different meats and their products **(Quesada *et al.*, 2016)**. In the present study, the shelf life of pasteurized milk with

chitosan and CNPs was extended compared to control samples. Clot on boiling test was used to detect freshness of pasteurized milk. Positive control and pasteurized milk with 0.5% chitosan samples soured by the 13th day of the experiment so, these samples were not tested. On the other hand, the shelf life of pasteurized milk extended more than 15 days by adding 1% chitosan, 0.5, and 1% CNPs as shown in table (2). Generally, treated milk either by chitosan or CNPs were considered the most preferable and strongly accepted as nearly no difference from control as shown in Figure (1). That may be the chitosan in nature white color and have no special taste or flavor so, haven't effect on flavor or appearance. These results in agremeent with **Shawkat et al. (2019)** who documented that the addition of CNP had no effects on the physicochemical properties of pasturized milk.

Although chitosan produce potent bactericidal effect against most bacterial forms, neverthless it is important to examine the constituents of the food matrix **(Awad and El Sohaimy, 2020)**. The change in pH value by adding chitosan and CNPs should be taken into consideration before apply chitosan as a natural preservative. Chitosan was reported to be positively charged and have high antimicrobial activity, mainly at pH values below its pKa of 6.5 **(Chang *et al*., 2015)**. The molecular weight and viscosity development of chitosan in aqueous solution also play a significant role in the biochemical and pharmacological application of chitosan. Other major parameters are crystallinity, ash content, moisture content, heavy metal content and so on **(Rinaudo 2006)**. On the other hand, CNPs are natural materials with excellent physicochemical, antimicrobial and biological properties, which make them a superior environmentally friendly material and they possess bioactivity that does not harm humans **(Malmiri *et al.*, 2012 and Elsherif and Ali, 2019).**

Regarding to histopathological findings, The hyperplasia of villous goblet cells associated with congested lamina propria in-group (2) were considered to be a result of *B. cereus* enterotoxins which responsible for diarrheal symptoms **(Granum and Lund, 1997)**. Furthermore, the vacuolar degeneration of hepatocytes, perivascular and around hepatocyte plates aggregation of lymphocyte and kupffer cells observed in liver of *B. cereus* exposed rat (group 2) could be due to the effect of *B. cereus*, cereulide, toxin on hepatocytes mitochondria. **Yokoyama *et al.* (1999)** have also previously reported vacuoles in the cytoplasm of hepatocytes in precentral area of hepatic lobule associated with proliferation of kupffer cells in sinusoids of mice treated with cereulide. Moreover, attributed the hepatocytes vacuolar degeneration to disruption caused by cereulide toxin on mitochondrial membrane potential and de-energize mitochondria leading to impair mitochondrial fatty acid metabolism and accumulation of vacuoles in the cytoplasm **(Mahler *et al.*, 1997 and Inai *et al.*, 1997)**.

With regarding to the effect of chitosan and CNPs on *B. cereus* in groups (7, 8, 9 and 10) at both concentrations 0.5% and 1% we found that both of CNPs and large molecule chitosan had antibacterial effect on *B. cereus* and markedly reduced its enterotoxins degenerative effects on intestine and liver. However, it was observed that the large molecule chitosan especially higher concentration 1% had much better antibacterial effect without cytotoxic effects on rat's intestinal villi in-compared to the same concentration 1% of CNPs, which exerted degenerative action on intestinal epithelium similar to group (6). In spite of antibacterial effect of 1% CNPs on *B. cereus* and its toxin, but it has mild side effect on biological tissue. While, this cytotoxic effect was less prominent with lesser concentration (0.5%) of CNPs. Our findings are inconsistent with those previously reported by **Tokura *et al*., (1996)** who mentioned that large chitosan molecules revealed greater antibacterial activates than smaller chitosan molecules. Additionally, the report of **(Islam *et al*., 2011)** indicated that a concentration of 1.2 mg/ml of chitosan molecules had a good inhibitory activity against *Staph aureus*. On the other hand, some studies demonstrated that chitosan polymerization exhibited low activity against *E. coli* **(Zheng and Zhu, 2003).** In a study of **Ardila *et al*. (2017a)** indicated that the antimicrobial properties of chitosan needs at least partial solubilisation additionally requires

a direct contact between chitosan and the microbial cell surface for antibacterial activity of chitosan. Suggested that one part of antimicrobial effect of chitosan is exerted by the direct contact protonated chitosan powder with negatively charged bacterial cell wall; and the other, may exert by deposition of solubilized chitosan on bacterial surface affecting the cell permeability resulted in leakage of intracellular constituents **(Kong *et al*., 2008 and Arkoun *et al*., 2017)**. However, in another study of **Ardila *et al*., (2017b) and Elsherif *et al*., (2020)** investigated that the antimicrobial activity of CNPs is independent of the size and form of bacterial cells.

The edematous tips of intestinal villi, which observed in rats administrated milk with 1% CNPs of group (6) and confirmed by its appearance again in group (10) which take milk inoculated with *B. cereus* 8 $\log_{10}$ CFU/ml and CNPs 1% in spite of disappearance of cereulide toxic effect on liver hepatocytes of group (10). This probably returned to the cytotoxic activity of higher concentration of 1% chitosan nanoparticles on intestinal villous epithelium. Previous studies have evaluated the cytotoxicity of chitosan nanoparticles **(Huang *et al*., 2004; Qi *et al*., 2005; Kamjumphol *et al*., 2017 and Hassanen *et al*., 2019),** which may be more critical than natural large chitosan molecules, because the nanoparticles could penetrate the cells through pervasion, modified the mechanism of cellular uptake and alter the function of DNA and mRNA. **Qi *et al.* (2005)** reported a similar explanation; as they found that cytotoxic activity of CNPs against normal human hepatic cells. In addition, these results in agreement with **Loh *et al.* (2010)** who reported the cytotoxicity of 1% CNPs on human liver cells through uptake of it into the cell nucleus was observed by confocal microscopic analysis after 4 h exposure with 1% w/v of chitosan nanoparticles. Electron micrographs further suggest necrotic or autophagic cell death, possibly caused by cell membrane damage and resultant enzyme leakage and normal cells at 0.5 % concentration of CNPs. While, the liver of the same group (6) showed mild kupffer cells proliferation in sinusoids, this could be attributed to the immune stimulatory effect of CNPs **(Yeh *et al*., 2017)**. Where, it has been found to have a variable of biological properties including antimicrobial effects, activation of macrophages and accelerate wound healing through enhanced inflammatory cells infiltration in the area of injury **(Rinaudo, 2006 and Prabaharan, 2008)**

Conclusion and Recommendations

This study demonstrated that the toxin gene *ces* is present in some strains of *B. cereus* isolated from some of raw milk and dairy desserts samples. Therefore, effective prevention and control measures of emetic *B. cereus* in food is demanded. Both chitosan and CNPs showed antibacterial activity in treated pasteurized milk against *B. cereus*. The shelf life of pasteurized milk with chitosan and CNPs solution was extended in compared to control samples. Furthermore, CNPs will be used increasingly in the fields of food storage, preservation and microbial inhibition with excellent physicochemical, antimicrobial and biological properties, which make them a superior environmentally friendly material and they possess bioactivity that does not harm humans. The results of experimental study revealed that chitosan 1% and CNPs 0.5% can be applied safely against *B. cereus* in dairy products without harmful effects on biological tissues.

References

Agata, N.; Ohta, M. and Yokoyama, K. (2002). Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. Int. J. Food Microbiol. 73: 23-27.

Alvarenga, V. ; Brancini, G.; Silva, E.; Da Pia, A.; Campagnollo, F.; Braga, G.; Hubinger, M. and Sant'Ana, A. (2018). Survival variability of 12 strains of *Bacillus cereus* yielded to spray drying of whole milk. Int. J. Food Microbiol. 286:80–89.

APHA "American Public Health Association" (2004). Standard methods for examination of Dairy Products, 17th edn., Washington D.C 20001. USA.

Ardila, N.; Daigle, F.; Heuzey, M.C. and Ajji, A. (2017a). Effect of chitosan physical form on its antibacterial activity against

pathogenic bacteria. J. Food Sci. 82(3): 679-686. https://doi.org/10.1111/1750-3841. 13635

Ardila, N.; Daigle, F.; Heuzey, M.C. and Ajji, A. (2017b). Antibacterial activity of neat chitosan powder and flakes. Molecules. 22(1): 100; doi:10.3390 /molecules 22010100

Arkoun, M.; Daigle, F.; Heuzey, M.C. and Ajji, A. (2017). Antibacterial electrospun chitosan-based nanofibers: A bacterial membrane perforator. Food Sci. Nutr. 5(4): 865–874.

Awad, D. and El Sohaimy, S. (2020). Evaluation the efficacy of lysozyme hydrolysates-loaded chitosan against food borne pathogens in raw milk during cooling storage. A.J.V.S. 64 (1): 86-96.

Benhabiles, M.S.; Salah, R.; Lounici, H.; Drouiche, N.; Goosen, M.F.A. and Mameri, N. (2012). Antibacterial activity of chitin, chitosan and its oligomers prepared from shrimp shell waste. Food Hydrocolloids. 29: 48–56.

Calvo, P.; Remunan-Lopez, R.; Vila-Jato, C. and Alonso, M. (1997). Chitosan and chitosan/ethylene oxide–propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm. Res. 14: 1431–1436.

Ceuppens, S.; Rajkovic, A.; Hamelink, S.; Van, T.; Boon, N. and Uyttendaele, M. (2012). Enterotoxin production by *Bacillus cereus* under gastrointestinal conditions and their immunological detection by commercially available kits. Foodborne Pathol. Dis. 9:1130–6.

Chang, S.; Lin, H.; Wu, G. and Tsai, G. (2015). pH Effects on solubility, zeta potential and correlation between antibacterial activity and molecular weight of chitosan. Carbohydr. Polym. 134: 74–81.

Chica, J.; Correa, M.; Aceves-Diez, A.; Rasschaert, G.; Heyndrickx, M. and Castan˜eda-Sandoval, L.M. (2019). Genomic and toxigenic heterogeneity of *Bacillus cereus* sensu lato isolated from ready-to-eat foods and powdered milk in day care centers in Colombia. Foodborne Patho. Dis. Volume XX, Number XX, 2019. DOI: 10.1089/fpd.2019.2709

Dutta, J. and Dutta, P.K. (2011). Antimicrobial activity of chitin, chitosan and their oligosaccharides. In: Kim SK, editor. Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological Activities and Aplications. New York: CRC Press; p. 195-209.

Dutta, P.K.; Tripath, S.; Mehrotra, G.K. and Dutta, J. (2009). Perspectives for chitosan based antimicrobial films in food applications. Food Chem. 114: 1173-1182.

Ehling-Schulz, M.; Guinebretiere, M.; Monthán, A.; Berge, O.; Fricker, M. and Svensson, B. (2006). Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol. Lett. 260: 232–240.

Elsherif, W.M. and Ali, D.N. (2019). Antibacterial effect of silver nanoparticles on antibiotic resistant *E. coli* o157:H7 isolated from some dairy products. Bul. J. of Vet. Med., 2019. DOI: 10.15547/bjvm.2019-0027

Fernandes, J.C.; Eaton, P.; Gomes, A.M.; Pintado M.E. and Malcata, F.X. (2009). Study of the antibacterial effects of chitosans on *Bacillus cereus* (and its spores) by atomic force microscopy imaging and nanoindentation. Ultramicroscopy 109: 854–860.

Frentzel, H.; Kraushaar, B.; Krause, G., Bodi, D.; Wichmann- Schauer, H.; Appel, B. and Mader, A. (2018). Phylogenetic and toxinogenic characteristics of *Bacillus cereus* group members isolated from spices and herbs. Food Control. 83: 90–98.

Gdoura-Ben, A.; Siala, M.; Zayani, M.; Grosset, N.; Smaoui, S.; Messadi-Akrout, F.; Baron, F.; Jan, S.; Gautier, M. and Gdoura, R. (2018). Isolation, identification, prevalence, and genetic diversity of *Bacillus cereus* group bacteria from different foodstuffs in Tunisia. Front Microbiol.

9:447.

Granum, P.E. and Lund, T. (1997). *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Lett. 157: 223–228.

Hassan, M.I.; Taher, F.A.; Mohamed, A.F. and Kamel, M.R. (2016). Chitosan nanoparticles prepared from *Lucilia Cuprina Maggots* as antibacterial agent. J. Egypt. Soc. Parasitol. 46(3): 519 – 526.

Hassanen, E.I.; Khalaf, A.A.; Tohamy, A.F.; Mohammed, E.R. and Farroh, K.Y. (2019). Toxicopathological and immunological studies on different concentrations of chitosan-coated silver nanoparticles in rats. International J. Nanomedicine. 14: 4723–4739

Huang, M.; Khor, E. and Lim, L.Y. (2004). Uptake and cytotoxicity of chitosan molecules and nanoparticles: Effects of molecular weight and degree of deacetylation. Pharm. Res. 21: 344–353.

Inai, Y.; Yabuki, M.; Kanno, T.; Akiyama, J.; Yasuda, T. and Utsumi, K. (1997). Valinomycin induces apoptosis of ascites hepatoma cells (AH-130) in relation to mitochondrial membrane potential. Cell Struct. Func. 22: 555-563.

Islam, M.; Masum, S.M.; Mahbub, K.R. and Haque, M.Z. (2011). Antibacterial Activity of crab-chitosan against *Staphylococcus aureus* and *Escherichia coli*. J Adv. Sci. Res. 2(4): 63–66.

ISO 7932: (2004). Microbiology of Food chain —Horizontal Method for the Enumeration of Presumptive *Bacillus cereus*—Colony-Count Technique at 30 degrees C; ISO: Geneva, Switzerland, 2004.

Kamjumphol, W.; Chareonsudjai, P. and Chareonsudjai, S. (2017). Antibacterial activity of chitosan against *Burkholderia pseudomallei*. WWW. Microbiology Open. 2018; 7:e00534. https://doi.org/10.1002/mbo3.534

Kong, M.; Chen, X.G.; Liu, C.S.; Liu, C.G.; Meng, X.H. and Yu L.J. (2008). Antibacterial mechanism of chitosan microspheres in a solid dispersing system against *E. coli*. Colloids Surf. B. 65: 197–202.

Kumari, S. and Sarkar, P.K. (2014). Prevalence and characterization of *Bacillus cereus* group from various marketed dairy products in India. Dairy Sci Technol. 94:483 –497.

Loh, J.W.; Yeoh, G.; Saunders, M. and Limab, L.Y. (2010). Uptake and cytotoxicity of chitosan nanoparticles in human liver cells. Toxicology and Applied Pharmacology. 249(2):148-157. https://doi.org/10.1016/j.taap.2010.08. 029

Mahler, H.; Pasi, A.; Kramer, J.M., Schulte, P.; Eng, G. and Scoging, A.C. (1997). Fulminant liver failure in association with the emetic toxin of *Bacillus cereus.* New Engl. J. Med. 336: 1142-1148.

Malmiri, H.J.; Jahanian, M.A.G. and Berenjian, A. (2012). Applications of chitosan nanoparticles as novel support in enzyme immobilization. American J. Biochem. Biotechnol. 8 (4): 203-219

Messelhäußer, U. and Ehling-Schulz, M. (2018). *Bacillus cereus*-a multifaceted opportunistic pathogen. Curr. Clin. Microbiol. Rpt. 5: 120–125. doi: 10.1007/s40588-018-0095-9

Mohamed, A.S.; Mohamed, E.A. and Salah, F.A. (2016). Occurrence of *Bacillus cereus* in raw milk and some dairy products in Egypt. Japanese J. Vet. Res. 64 (Supplement 2): S95-102.

Mudagza, D.T. and Buys, E.M. (2017). Diversity of *Bacillus cereus* strains in extended shelf life. Int Dairy J. 73:144–150.

Nelson, J.A. and Torut, G.M. (1981). Judging Dairy Products, 4th edition revised. Westport, CT: The AVC, 1981.

Nguyen, T.V.; Nguyen, T.H.; Wang, S.L.; VO, P.K. and Nguyen, A.D. (2017). Preparation of chitosan nanoparticles by TPP ionic gelation combined with spray drying,

and the antibacterial activity of chitosan nanoparticles and a chitosan nanoparticle–amoxicillin complex. Res. Chem. Inter. med. 43: 3527–3537.

Ojagh, S.M.; Rezaei, M.; Razavi, S.H. and Hosseini, S.M.H. (2010). Effect of chitosan coatings enriched with cinnamon oil on the quality of refrigerated rainbow trout. Food Chem.120(1): 193–198.

Organji, S.R.; Abulreesh, H.H.; Elbanna, K.; Osman, G. and Khider, M. (2015). Occurrence and characterization of toxigenic *Bacillus cereus* in food and infant feces. Asian Pac. J. Trop. Biomed. 5: 515-520.

Papan, C.; Fo¨rster, K.; Herterich, R.; Schulze, A.; Schubert, S. and Flemmer, A. (2019). Identification and containment of a cluster of two *Bacillus cereus* infections in a neonatal intensive care unit. Can. J. Infect. Dis. Med. Microbiol. 2019:1506583.

Prabaharan, M. (2008). Review paper: chitosan derivatives as promising materials for controlled drug delivery. J. Biomater Appl. 23 (1):5-36.

Qi, L.; Xu, Z.; Jiang, X.; Li, Y. and Wang, M. (2005). Cytotoxic activities of chitosan nanoparticles and copper-loaded nanoparticles. Bioorg. Med. Chem. Lett. 2005, 15: 1397–1399.

Quesada, J.; Sendra, E.; Navarro, C. and Sayas-Barberá, E. (2016). Antimicrobial active packaging including chitosan films with Thymus vulgaris L. essential oil for ready-to-eat meat. Foods. 5: 57.

Rinaudo, M. (2006). Chitin and chitosan: properties and applications. ProgPolym Sci. 31: 603-32.

Raafat, D.; Bargen, K.; Haas, A. and Sahl, H.-G. 2008. Insights into the Mode of Action of Chitosan as an Antibacterial Compound. Appl. Environ. Microbiol. 74(12): 3764–3773. doi: 10.1128/AEM.00453-08

Samapundo, S.; Heyndrickx, M.; Xhaferi, R. and Devlieghere, F. (2011). Incidence, diversity and toxin gene characteristics of *Bacillus cereus* group strains isolated from food products marketed in Belgium. Int. J. Food Microbiol. 150:34–41.

Shawkat, S.M.; Al-Jawasim, **M. and Khaleefah, L.S. (2019).** Extending Shelf Life of Pasteurized Milk via Chitosan Nano-particles. J. Pure Appl. Microbiol. 13(4): 2471-2478

Sharma, R. and Rajput, Y.S. (2014). Rapid methods for assessing efficiency of heat treatment of milk. J. Food Sci. Technol. 51 (7): 1416–1420. doi: 10.1007/s13197-012-0635-x

Silalahi, J.; Situmorang, P.; Patilaya, P. and Ce Silalahi, Y. (2016). Antibacteria activity of chitosan and hydrolyzed coconut oil and their combination against *Bacillus cereus* and *Escherichia* coli. Asian J. Pharma. Clin. Res. 9: 69-73. doi:10.22159/ajpcr. 2016. v9i5. 11768.

Sreekumar, S.; Goycoolea, F.M.; Moerschbacher, B.M. and Rivera-Rodriguez, G.R. (2018). Parameters influencing the size of chitosan-TPP nano- and microparticles. Sci. Rep. 8: 4695.

Sukmark, T.; Rachtanapun, P. and Rachtanapun, C. (2011). Antimicrobial activity of oligomer and polymer chitosan from different sources against foodborne pathogenic bacteria. Kasetsart J. (Nat. Sci.). 45: 636- 643.

Suvarna, S.K.; Layton, C. and Bancroft, J.D. (2013). Bancroft's Theory and Practice of Histological Techniques. 7th edn. Churchill Livingstone Elsevier.

Tamara, F.R.; Chi, L.; Fwu-Long, M. and Yi-Cheng, H. (2018). Antibacterial effects of chitosan/cationic peptide nanoparticles. Nanomaterials. 8: 88. doi:10.3390/ nano8020088

Tantala, J.; Thongngam, M.; Rachtanapun, P. and Rachtanapun, C. (2012). Antimicrobial activity of chitosan and carboxymethyl chitosan from differences types and sources of chitosan. Italian J. Food Sci., XXIV: 97- 101.

Tokura, S.; Ueno, K.; Miyazaki, S. and Nishi, N. (1996). Molecular weight dependent antimicrobial activity by chitosan. In New Macromolecular Architecture and Functions. 1996; pp. 199–207. Springer Berlin Heidelberg.

Yang, Y.; Gu, H.; Yu, X.; Zhan, L.; Chen, J.; Luo, Y.; Zhang, Y.; Zhang, Y.; Lu, Y.; Jiang, J. and Mei, L. (2017). Genotypic heterogeneity of emetic toxin producing *Bacillus cereus* isolates from China. FEMS. Microbiol. Lett. 364:fnw237.

Yeh, M.Y.; Shih, Y.L.; Chung, H.Y.; Chou, J.; Lu, H.; Liu, C.H.; Huang, W.W.; Pang, S.F.; Wu, L.Y. and Chung, J.G. (2017). Chitosan promotes immune responses, ameliorating total mature white blood cell numbers, but increases glutamic oxaloacetic transaminase and glutamic pyruvic transaminase, and ameliorates lactate dehydrogenase levels in leukemia mice in vivo. Molecular Medicine Reports. 16: 2483 -2490.

Yokoyama, K.; Ito, M.; Agata, N.; Isobe, M.; Shibayama, K.; Horii, T. andOhta, M. (1999). Pathological effect of synthetic cereulide, an emetic toxin of *Bacillus cereus*, is reversible in mice /FEMS Immunol. Med. Microbiol. 24: 115-120.

Yu, P.; Yu, S., Juan, W.; Hui, G.; Ying, Z.; Xiyu, L.; Junhui, Z.; Shi, W.; Qihui, G.; Liang, X.; Haiyan, Z.; Rui, P.; Tao, L.; Zhang, J.; Qingping, W. and Yu, D. (2019). *Bacillus cereus* isolated from vegetables in china: incidence, genetic diversity, virulence genes, and antimicrobial resistance. Frontiers in Microbiol. 10 (948). doi: 10.3389/fmicb.2019.00948

Yusman, D.A. (2006). Relationship between antibacterial activity of chitosan and surface characteristics of bacterial cell wall. Thesis, Departement of Chemistry, Faculty of Mathematics and Natural Sciences. Institut Pertanian Bogor. 2006.

Zaghloul, E.H. and Ibrahim, H.A. (2019). Comparative study on antimicrobial activity of commercial and extracted chitin and chitosan from *Marsupenaeus japonicus* shells. Egyptian J. Aquatic Biol. Fisheries. 23 (2): 291 - 302.

Zhang, Z.; Feng, L.; Xu, H.; Liu, C.; Shah, N.P. and Wei, H. (2015). Detection of viable enterotoxin-producing *Bacillus cereus* and analysis of toxigenicity from ready to- eat foods and infant formula milk powder by multiplex PCR. J. Dairy Sci. 99:1047–55

Zhao, L.; Shi, L.; Zhang, Z.; Chen, J.; Shi, D.; Yang, J. and Tang, Z. (2011). Preparation and Application of Chitosan Nanoparticles and Nanofibers. Brazilian J. Chem. Eng. 28: 353 – 362.

Zhao, S.; Chen, J.; Peng, F.; Feng, H.; Yao, W.; Ali, A.; Shuzhen, L.; Haonan, J. and Yang, W. (2020). Prevalence, molecular characterization, and antibiotic susceptibility of *Bacillus cereus* isolated from dairy products in China. J. Dairy Sci. 103 (5): 3994 -4001. https://doi.org/10.3168/jds.2019-17541.

Zheng, L.Y. and Zhu, J.F. (2003). Study on antimicrobial activity of chitosan with different molecular weights. Carbohydrate Polymers. 54(4): 527–530.