

## STUDYING THE EFFECT OF CHITOSAN ON *BACILLUS CEREUS* PRODUCING CERULIDE 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 Assiut City, Egypt. ISO7932 method was used for enumeration of *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* was studied 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 qualities.

**Keywords:** Rice, Nanoparticles, Histo-Pathology, Mehalabia, Rat, Liver toxicity

### INTRODUCTION

*B. 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*. For instance, European Union about 600–700 annually reported confirmed cases of foodborne outbreaks linked to *B. cereus* toxins (Messelh auser 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 performed 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; 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; 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; Sreekumar *et al.*, 2018)

Therefore, the objective of our study was to detect the incidence and count of *B. cereus* in raw milk and two dairy desserts then detect the cereulide 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.

### MATERIAL 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 (1998).

#### Identification and Enumeration of *B. cereus* strains

Enumeration of *B. cereus* is performed by using ISO7932:2004 method, where the samples were plated on mannitol egg yolk phenol red polymyxin (MEYP, Oxoid, Hampshire, UK) medium and then incubated for 24–48 h at 30 °C (Tallent *et al.*, 2012). 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 (Kim and Goepfert, 1971). Motility, endospore formation, followed by species confirmation using biochemical tests as described by Kim et al. (2011).

**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 Taq™ (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***

**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).

**Preparation of CNPs**

The Nanoparticles were prepared following the procedure described by Calvo et al. (1997) and characterized by HRTEM Model JEOLJEM-100CX II in the Electron Microscopy Unit, Assiut University, Egypt. Briefly: a 2.5 mg/mL chitosan solution was 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 jellyify for 30 min.

**Bacterial suspension inoculation**

The isolated strains were inoculated into Muller Hinton broth and incubated to the growth phase at 37 °C. The growth density was adjusted to match a MacFarland 0.5 standard (8 log<sub>10</sub> CFU/ml) according to Yu et al., (2019).

**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 °C) 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.

**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 terms 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 gweight 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<sub>10</sub> 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<sub>10</sub> CFU/ml and chitosan 0.5, 1%, respectively. Group 9 and 10 rats take milk inoculated with *B. cereus* 8 log<sub>10</sub> 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 15<sup>th</sup> 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 <0.01 was considered significant. The Graph Pad Prism software (GraphPad, Inc., San Diego, USA) (version 5) was employed for the statistical analysis.

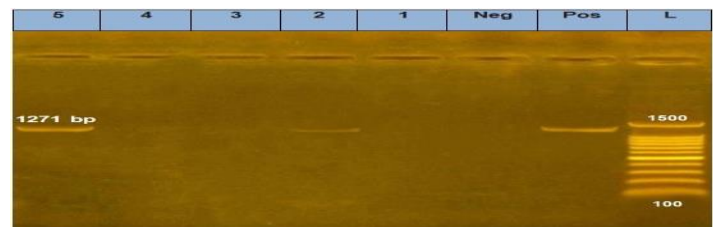
**RESULTS**

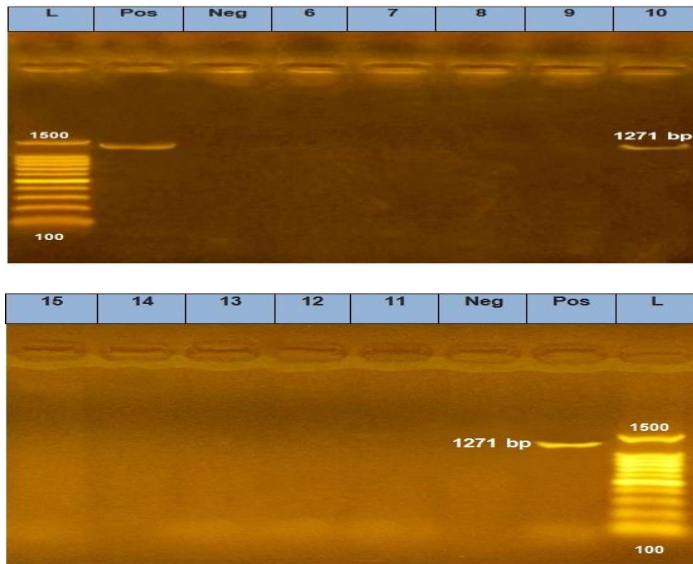
**The prevalence of emetic *B. cereus* strains in some food samples**

The incidence of *B. cereus* in 150 examined samples were 40, 52 and 62 % with mean count of 3.9±0.3, 4±0.7 and 4.7±0.3 log<sub>10</sub>CFU/ml 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<sub>10</sub> 5.7 CFU /ml) (Table 1). The toxic gene 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		<i>B. cereus</i> count		
	No./50 %	(log <sub>10</sub> CFU/ ml)	Min.	Max.	Mean ±StD
Raw milk	20	40	2.0	5.6	3.9±0.3
Cooked rice with milk	26	52	2.0	5	4±0.7
Cooked rice with milk	31	62	2.0	5.7	4.7±0.3



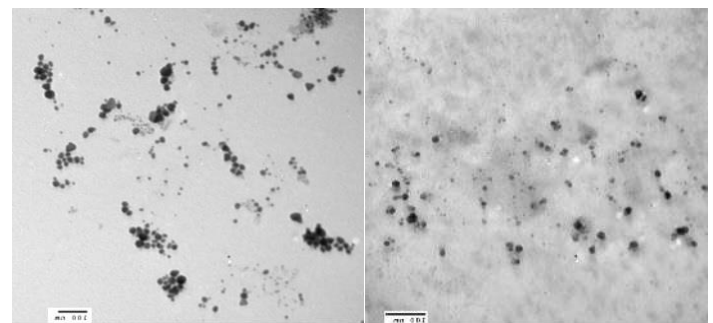


**Photo 1, 2, 3** Gel electrophoresis of the PCR amplification products obtained with toxic gene of cereulide (*ces*). Emetic *B. cereus* strains (lanes 2, 5 and 10) and non-emetic *B. cereus* strains (lanes 1, 3, 4, 6-9 and 11-15). L. Molecular marker, Lane pos. Positive control, Lane Neg. Negative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

**The antibacterial efficacy results of chitosan and CNPs to inhibit the growth of *B. cereus*.**

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 13<sup>th</sup> 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 at day 15<sup>th</sup> 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<sub>10</sub> CFU/ml at zero time to 3.8 log<sub>10</sub> CFU/ml in positive control sample after 11<sup>th</sup> days of storage at 4±1 °C (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<sub>10</sub> CFU/ml at the 7<sup>th</sup> day of the experiment). CNPs still had good bactericidal activity at concentration 0.5% after 7 days from cooling storage comparing with positive control group at which the bacterial growth mean count reached 6.5 log<sub>10</sub> CFU/ml as shown in Table (3). The most prominent potent bactericidal effect was observed in CNPs 1% in which *B. cereus* count reached to undetectable level at the 5<sup>th</sup> day 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 0.5	Chitosan 1	CNPs 0.5	CNPs 1	Control	Chitosan 0.5	Chitosan 1	CNPs 0.5	CNPs 1
0	6.8±0.04	6.8±0.05	6.8±0.05	6.7±0.05	6.8±0.04	-ve	-ve	-ve	-ve	-ve
1 <sup>st</sup>	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
3 <sup>rd</sup>	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
5 <sup>th</sup>	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
7 <sup>th</sup>	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
9 <sup>th</sup>	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
11 <sup>th</sup>	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
13 <sup>th</sup>	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
15 <sup>th</sup>	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

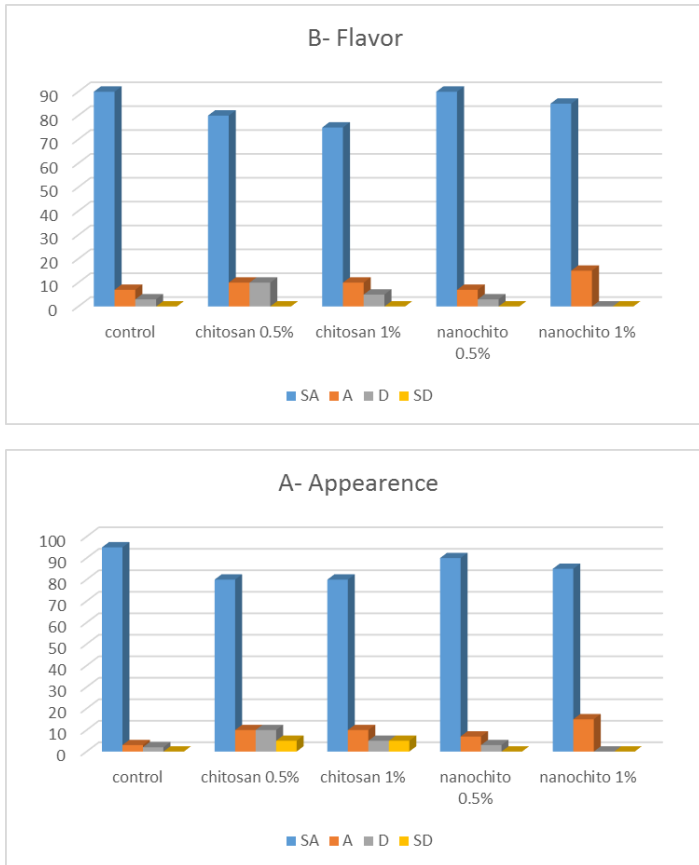
Mean ± SE of five replications  
\*Significant difference (P value <0.01)

**Table 3** Effect of incorporation of different concentrations of chitosan and CNPs on *B. cereus* count (log<sub>10</sub> CFU/ ml) inoculated in pasteurized milk stored at 4±1 °C.

Storage period by days	Control		Chitosan		CNPs	
	-ve	+Ve	0.5%	1%	0.5%	1%
0	-ve	8	8	8	8	8
1 <sup>st</sup>	-ve	8.3	7.8	6.11	7.9	6.7
3 <sup>rd</sup>	-ve	8.6	6.8	3	5.3	3.7*
5 <sup>th</sup>	-ve	7.9	3	2.8	2.4*	ND
7 <sup>th</sup>	-ve	6.5	1.10*	1.2*	ND	ND
9 <sup>th</sup>	-ve	5.7	ND	ND	ND	ND
11 <sup>th</sup>	-ve	3.8	ND	ND	ND	ND
13 <sup>th</sup>	-ve	NT	NT	ND	ND	ND
15 <sup>th</sup>	-ve	NT	NT	ND	ND	ND

ND. Not Detected (below than one log<sub>10</sub> 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 <0.01).





**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.

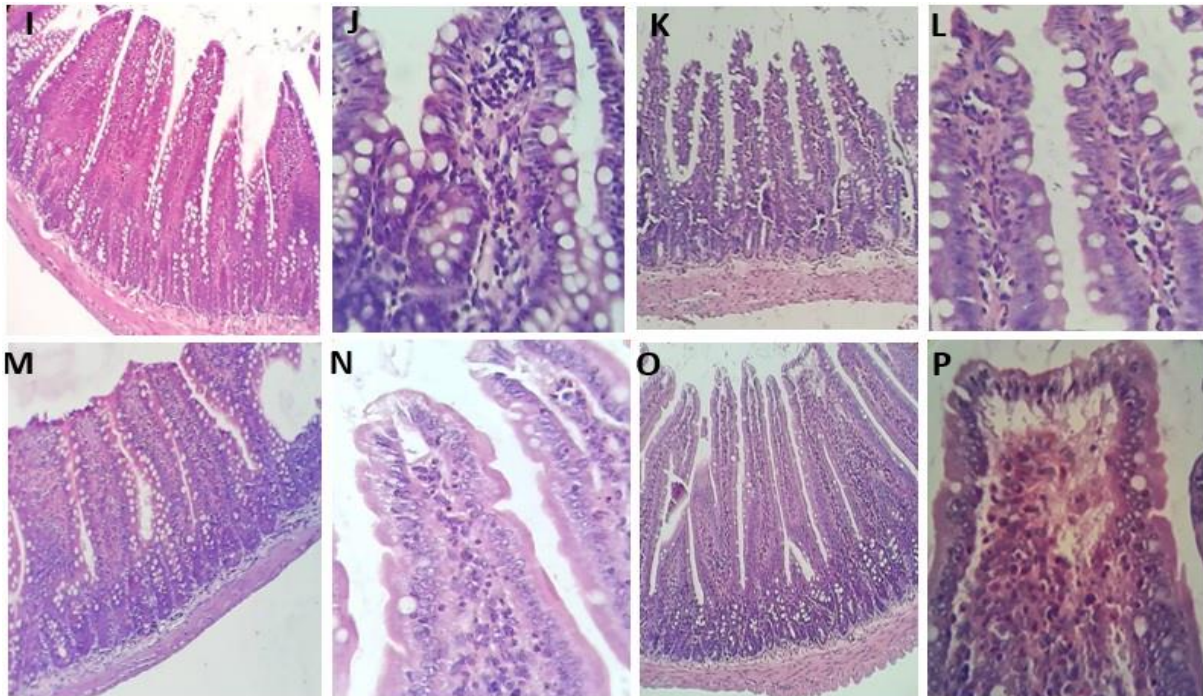
**Body weight.** No significant changes were observed in the values of body weight in different experimental groups along the period of the experiment.

**Pathological findings**

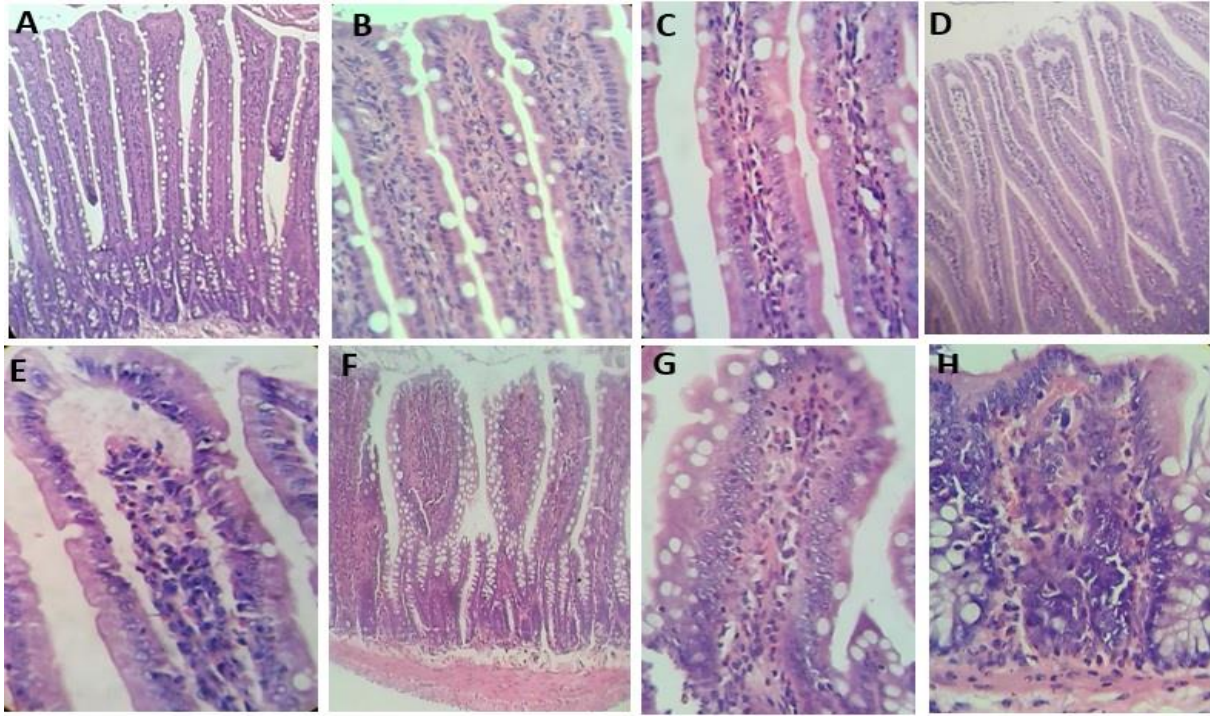
**Postmortem examination:** No characteristic gross of pathological lesions in rats of different groups except group (2) which administrated milk with *B. cereus* ( $8 \log_{10}$  CFU/ml), most rats showed mild congested and hyperemic intestine and enlarged pale liver.

**Light microscopic examination.**

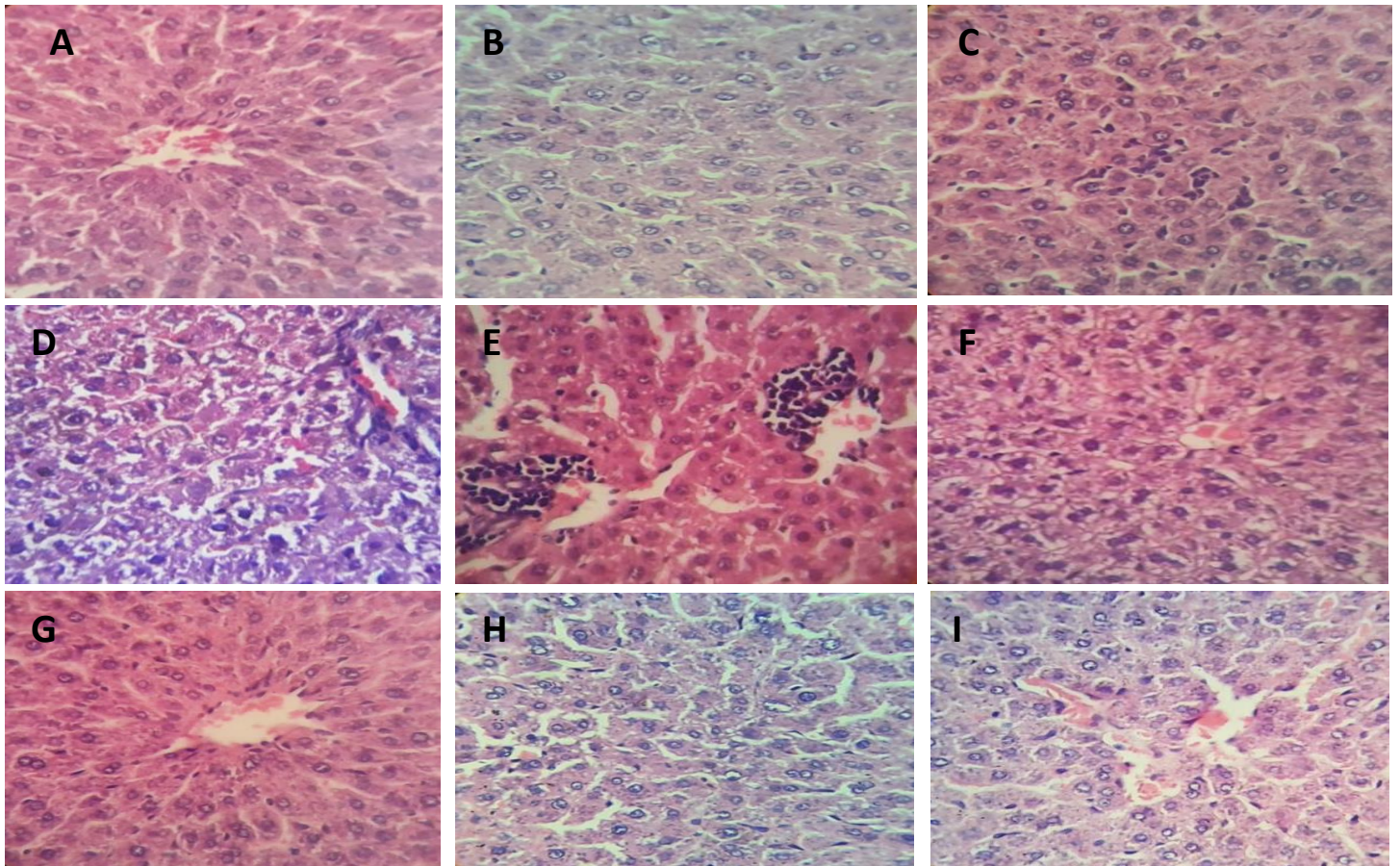
The intestine in Group 1 (control negative) and Groups 3, 4 and 5 showed normal structure of intestinal villi with intact lamina epithelialis (Fig 2 A, B and C). While, The liver of same group's demonstrated normal hepatic parenchyma (Fig 3 A, B). In Group (6), rats take milk with 1% CNPs revealed mild 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 & 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 & 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 & 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 & 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 & 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 necrobiotic 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<sub>10</sub> CFU/g and produced emetic toxin at both 30-35°C (Agata et al., 2002). Several studies detected *B. cereus* emetic strains in various foods (Mudagza and Buys, 2017, Yang et al., 2017; 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 9<sup>th</sup> day of the experiment. While, by adding CNPs (0.5 and 1%), the count could not be detected at 7<sup>th</sup> and 5<sup>th</sup> 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; Zaghoul & 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 -NH<sub>2</sub>, -OH groups are the main reactive site in chitosan. As, it is determinant in the charge development and solubility of chitosan (Zaghoul 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 13<sup>th</sup> 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.

Although chitosan produce potent bactericidal effect against most bacterial forms, nevertheless 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; 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; 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 activities 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; 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<sub>10</sub> 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; 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; Prabakaran, 2008)

## CONCLUSION

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.

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