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Inhibitory activity of chitosan nanoparticles and *Spirulina platensis* extract against *Candida albicans* in thermally treated milk

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ABSTRACT

Milk is a suitable medium for the growth of many microbes, in particular, fungi and yeasts that change the physical and chemical properties of milk and in the final dairy products. In the current study, the target was to apply natural components to control growth of *Candida albicans* in pasteurized milk. Chitosan (CS), chitosan nanoparticles (CSN), and *Spirulina platensis* (SP) and its extract (SPE) were used. The MIC (12.5 to 100 mg/ml) of the natural components against *Candida albicans* as a model for fungi family was determined their effect in broth and in pasteurized milk. CS and CSN were the most effective natural component inhibit the growth of *C. albicans* strain with concentration 25 mg/ml followed by SPE with inhibitory activity against *C. albicans* at concentration 100 mg/ml while, *Spirulina platensis* (SP) showed the least inhibitory activity with the same concentration. By application in pasteurized milk CSN showed the best candidacidal effect with inhibitory 1.2 $\log_{10}$cfu/ml after 15 days at refrigerated storage as adding CSN extended shelf life of pasteurized milk. In conclusion, we recommend adding chitosan nanoparticles in milk at concentration 25mg/ml to limit the fungal growth

Introduction

Food spoilage became an important issue for humans with regards to food safety and security. Nowadays, up to one third of all food is spoiled or squandered before consumption, which represents about 1.3 billion tons per year (FAO, 2011).

Yeasts and molds can grow in a large variety of food including raw materials such as milk, as well as processed products leads to severe economic losses for food manufacturers due to fungal deterioration (Filtenborg *et al*., 1996). Moreover, these fungi are acidotolerant, xerotolerant, and/or psychrotolerant, and to some extent can tolerate chemical preservatives, besides, their presence in dairy products may result in several types of food spoilage, e.g., visible growth of the fungus at the product surface, and the production of metabolites causing off- flavors, as well as visible changes in color and/or texture (Ledenbach & Marshall, 2010). In addition to organoleptic properties' deterioration, spoilage molds such as Penicillium and Aspergillus spp. can also produce mycotoxins (Hymery *et al*., 2014). The risk of mold and yeast become more obvious in raw milk products as kareish cheese (Kure *et al*., 2004). So, it is urgent to produce raw milk free from fungi or their mycotoxin and food poisoning agents for production final dairy products.

Control of fungal spoilage is a major concern for food industrials and scientists that are looking for efficient solutions to prevent and/or limit fungal growth or development in dairy products. Different traditional methods use chemical preservatives which are considered as food additives. Chitosan is a linear polymer of beta-(1-4)-linked N-acetyl-2-amino-2-deoxy-d-glucose derivative obtained by partial deacetylation of chitin by enzymatic or chemical processes (Kaur and Dhillon, 2013). Due to its biodegradable, biocompatible and nontoxic characteristics, chitosan has recently gained more interest for the applications in food and pharmaceutics. Among other properties, Chitosan has a great potential as antifungal agent to treat diseases caused by human pathogenic fungi (Kumar, 2000; Peña *et al*., 2013).

Nanotechnology can play an essential role in addressing these issues by promoting the safety of raw milk (Bandara *et al*., 2020). Nanoparticles display unique physical and chemical features because of effects such as the quantum size effect, mini size effect, surface effect and macro-quantum tunnel effect. Formulation of chitosan into nanoparticles form was found to increase its antifungal effect significantly. Therefore, it is anticipated that chitosan nanoparticles have the potential of becoming a powerful and safe natural antifungal agent (Yien Ing *et al*., 2012).

Nano chitosan is a natural material with excellent physicochemical properties. It is environmentally friendly and bioactive. Nano-chitosan can be prepared by sodium tripolyphosphate (Janes *et al*., 2001). Chitosan-based nanoparticles (CNPs) have been used in agriculture as pesticides, herbicides, insecticides, and to obtain better quality food products with a higher yield (Kumaraswamy *et al*., 2018).

Spirulina (Arthrospira) platensis is a photosynthetic, multicellular, and filamentous blue-green microalga, which naturally grows in warm climates. Because it can be used as a food, animal feed, dietary supplements or functional foods Spirulina became the object of intensive research (Belay, 2008). Spirulina also possesses other biological functions such as antiviral, antibacterial, antifungal, anti-inflammatory and antiparasitic activities (Khan *et al*., 2005).

In sight of these facts, the current study aimed to investigate different natural compounds to control fungi in milk so, our focused aim toward using chitosan and chitosan nanoparticles against *Candida albicans*. In addition to investigate spirulina and spirulina extract effect during refrigerating storage of milk.

Materials and methods

Materials

Low molecular weight (LMW, MW = 70 kDa) chitosan ($C_8H_{15}NO_6$) powder was purchased from Sigma-Aldrich (Germany). Dried *Spirulina platensis* powder was obtained from Animal Health Research Institute, Dokki, Egypt. Glacial acetic acid, phosphate buffered saline tablets (PBS)

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pH 7.4, tripolyphosphate (TPP), glycerol was supplied by Sigma Chemicals Co. (USA). Sabouraud Dextrose broth and Dichloran Glycerol Medium Base obtained from Himedia, India. Reference strains of *Candida albicans* (ATCC10231) strain was purchased from MIRCEN, Ain shams University, Egypt. All chemicals were of analytical grade and used as received.

Preparation of Chitosan Solution

Chitosan solution (CS) was prepared by dissolving 0.06 g of LMW chitosan in 3 mL of 1% v/v acetic acid solution. The solution remained under magnetic stirring for 24 h. Then, the solution was filtered by filter paper for subsequent use. The pH of the solution was later adjusted to 5.6 by adding sodium hydroxide solution 40 g/l to ensure acidic condition would not interfere with the antifungal determination (Ziani *et al*., 2009).

Preparation and characterization of chitosan nanoparticles solution

Chitosan nanoparticles (CSN) were prepared according to the procedure based on the ionotropic gelation via the interaction of low molecular weight chitosan powder with tripolyphosphate (TPP) (Calvo *et al*., 1997). Briefly, chitosan powder and TPP were dissolved in purified water to obtain solutions. The nanoparticles were spontaneously formed upon incorporation of 1.2 ml of TPP solution in 3 ml of the chitosan powder solution, with an infusion pump dripping under mild magnetic stirring at room temperature. Glycerol was used for centrifugation to enhance the dissolving ability of centrifuged nanoparticles. The morphological examination of nanoparticles was conducted by transmission electron microscopy (TEM) (JEOL JEM-2100 high resolution transmission electron microscope) as Quasi-spherical shape, 20.0±5.0 nm semi aggregated particulates, with Off-White color appearance according to Fig. 1.

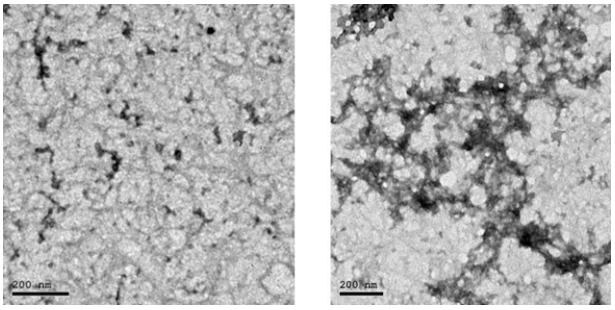

Fig. 1. Illustrate the transmission electron microscopy (TEM) of chitosan nanoparticles produced.

Preparation of Spirulina platensis extracts

Ethanolic extract was prepared by stirring 100 g of freeze-dried *Spirulina platensis* (SP) with 300 mL of ethanol for 24 h at 40°C in the dark. After filtration, the obtained extract was concentrated in a rotary evaporator, and then dried in a lyophilizer. The resulting extract of *Spirulina platensis* referred to (SPE) was kept at 4°C until use (Hlima *et al*., 2019).

Anticandidal assay of chitosan & Spirulina platensis in broth

The minimum candidacidal concentrations of different treatments including chitosan (CS), chitosan nanoparticles (CSN), *Spirulina platensis* (SP) and *Spirulina platensis* extract (SPE) against *C. albicans* have been evaluated by using a micro titer plate (96 wells), serial dilutions of different treatments. All of them were dissolved in Sabouraud Dextrose broth and transferred into the plate wells to obtain final concentrations in the range of 12.5–100 mg/ml including the inoculums of yeasts. Yeast inoculums at conc. 10^5 cfu/ml (100 µl) were added to each well and the plates were then incubated at 30ºC for 48h. 100 µl from each serial dilution was spread on Sabouraud Dextrose agar plates and incubated at 30ºC for 48 h. for detection of candidacidal effect of such growth. The count for three replicates was determined in mean $\log_{10}$ cfu/ml (Tayel *et al*., 2010).

Anticandidal activity of chitosan and Spirulina platensis in milk

Skimmed cow's milk had been subjected to laboratory pasteurization then inoculated with *C. albicans* at dose of 10^5 cfu/ml, and divided into five groups as the following:

Group 1: Control positive (*C. albicans*).
Group 2: Treated with 100 mg/ml *Spirulina platensis*.
Group 3: Treated with 100 mg/ml *Spirulina platensis* extract.
Group 4: Treated with 25 mg/ml chitosan.
Group 5: Treated with 25 mg/ml chitosan nanoparticles.
Group 6: (control negative): Pasteurized skim milk free from *C. albicans*.

All groups stored at refrigerator at 5°C for 15 days. Then examined for counting *C. albicans* using Dichloran glycerol media at time intervals of 0, 5, 10 and 15 days. The microbial counts for the pasteurized milk were done visually in triplicates, and the average of each treatment expressed as $\log_{10}$ cfu/ml.

Statistical analysis

All the experiments were conducted in triplicate and the results were expressed using one-way ANOVA analysis. Differences among means were tested for significance ($P<0.05$) as described by (Hill & Lewicki, 2006). Statistical analysis of the data was carried out employing analysis of variance (ANOVA).

Results

Candidacidal activity of chitosan and chitosan nanoparticles in broth

All chitosan treatments could effectively inhibit the growth of *C. albicans* in a concentration-dependent manner. The anticandidal potency varied among chitosan and chitosan nanoparticles as illustrated in Fig. 2. Chitosan solution (CS) with MIC of 12.5 mg/mL was found to have less antifungal activity against *C. albicans* compared with chitosan nanoparticles (CSN). *Candida albicans* growth rate was 6.88 and 6.18 $\log_{10}$cfu/ml for CS and CSN, respectively at concentration of 12.5 mg/ml where the control was 8.56 $\log_{10}$cfu/ml. Although both CS and CSN at concentration 100, 50 and 25 mg can completely inhibit *Candida albicans* growth, chitosan Nanoparticles were the most effective agent to inhibit the growth of *C. albicans* strain.

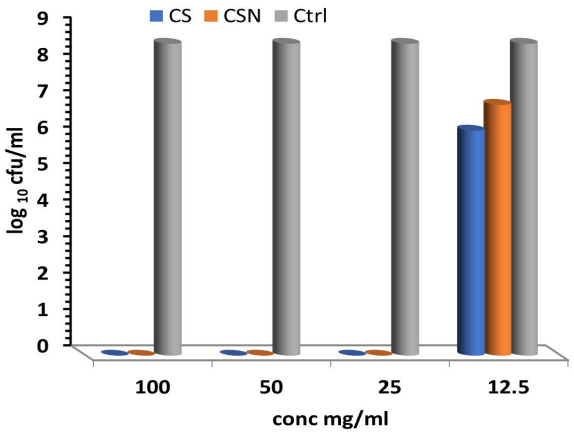

Fig. 2. Candidacidal effect of chitosan and chitosan nanoparticles in different concentrations (100, 50, 25, 12.5 mg/ml).

Candidacidal activity of Spirulina platensis, Spirulina platensis extract in broth

Spirulina platensis (SP) and its extract (SPE) followed CS and CSN had

inhibitory activity against *C. albicans*. Generally, the anticandidal activity decreased with decreasing the concentration used. At concentration of 100 mg/ml, SP could decrease *Candida albicans* growth rate to 4.9 log$_{10}$cfu/ml whereas SPE could inhibit 3.22 log$_{10}$cfu/ml. At the same time, *Spirulina platensis* (SP) and its extract showed the least inhibitory activity against *C. albicans* at concentration 12.5 mg/ml with inhibitory activity 5.78 and 5.4 log$_{10}$cfu/ml respectively, where the control was reach to 8.56 log$_{10}$cfu/ml as in Fig. 3.

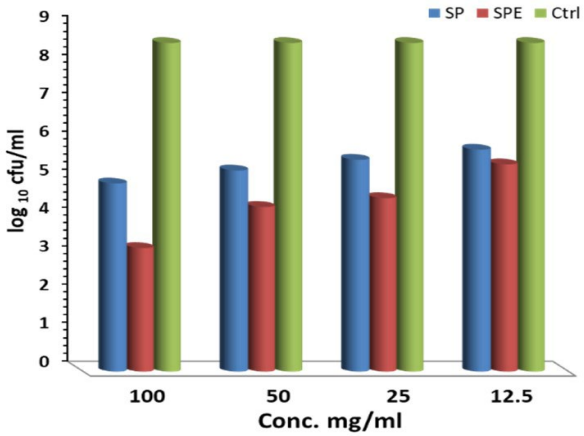

Fig. 3. Candidacidal effect of *Spirulina platensis* and *Spirulina platensis* extract in different conc (100, 50, 25, 12.5 mg/ml).

Antifungal activity of chitosan, chitosan nanoparticles, Spirulina platensis, Spirulina platensis extract in thermally treated milk

Data in Figure 4, showed that monitoring of *Candida albicans* growth in pasteurized milk in presence of chitosan and chitosan nanoparticles at concentration 25 mg/ml for 15 days under refrigerator storage. Data represented decreasing in *Candida albicans* count more pronounced when pasteurized milk inoculated with CSN than CS. The *Candida albicans* growth showed 5.215, 2.451, 1.796 and 1.102 log$_{10}$cfu/ml at 0, 5th,10th and 15th day of refrigerating storage, respectively when pasteurized milk inoculated with CSN of conc 25 mg/ml. On the other hand, chitosan with the same concentration 25 mg/ml could decrease the *Candida albicans* growth count 4.916, 4.501, 3.771 and 3.440 log$_{10}$cfu/ml at 0, 5 ,10 and 15 days of cooling storage, respectively.

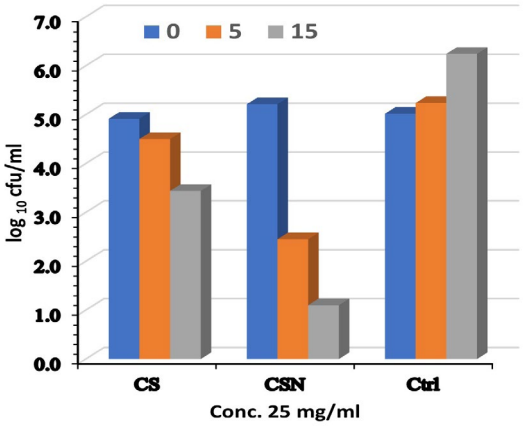

Fig. 4. Candidacidal effect of chitosan (CS) and chitosan nanoparticles (CSN) at conc. (25 mg/ml) in pasteurized milk during cooling storage period for 15 days.

Additionally, data presented in Figure 5, showed that inoculated Pasteurized milk samples treated with *Spirulina platensis* extract at conc 100 mg/ml showed decrease in *Candida albicans* growth count 5.009, 3.990, 3.212 and 2.120 log$_{10}$cfu/ml at 0, 5, 10 and 15 days at refrigerator storage, respectively.

The lowest *Candida albicans* growth rate count was showed when inoculated pasteurized milk treated with *Spirulina platensis* at rate 5.042, 4.885, 4.220 and 3.780 log$_{10}$cfu/ml at 0, 5, 10 and 15 days after refrigerator storage respectively.

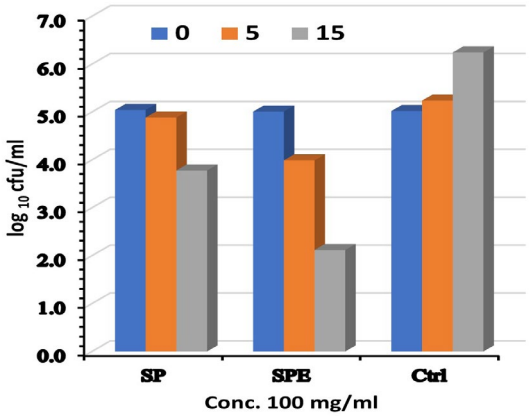

Fig. 5. Candidacidal effect of *Spirulina platensis* (SP) and *Spirulina platensis* extract (SPE) at concentration (100 mg/ml) in pasteurized milk during cooling storage period for 15 days.

Discussion

The current result revealed that nano-chitosan (CSN) had the best candidacidal effect at conc. 25 mg/ml followed by chitosan (CS) when applied in broth and when applied in pasteurized milk inoculated with specific dose 25 mg/ml of *Candida albicans* (Figs. 2 and 4). The primary target of chitosan in the first antimicrobial mechanism is the plasma membrane of fungus. Chitosan's positive charge makes it possible for it to bind with the negatively charged fungal membrane phospholipids components, this will make the membrane more permeable and cause cellular contents to flow out, which ultimately results in cell death (Liu *et al*., 2004; García-Rincón *et al*., 2010). The second method involves the chelating agent function of chitosan, which binds to trace elements and renders them inaccessible for normal fungal development (Roller & Covill, 1999). Thirdly, it was suggested that chitosan may pierce the cell walls of fungus and bind to their DNA. The production of necessary proteins and enzymes will be hampered because of this inhibiting mRNA synthesis (Kong *et al*., 2010).

The size of natural or synthetic polymer nanoparticles ranges from 10 to 1000 nm (Kreuter, 2001). Due to phenomena including the quantum size effect, micro size effect, surface effect, and macro-quantum tunnel effect, nanoparticles exhibit distinctive physical and chemical properties (Wen *et al*., 2009). Our results cleared that chitosan nanoparticles have the best result against *Candida albicans*. This agreed with (Sayed-ElAhl *et al*., 2019) who apply chitosan nanoparticles to improve colours, taste and odour of kareish cheese, which improved more than 20 days and more palatable excellent characters were noticed with increased customer acceptance. As the concentration of CSNPs grew up to 0.5%, the prolonged preservation and acceptability increased more than 20 days, and it was demonstrated that treated cheese with CSNPs had a better acceptability score evaluation, particularly if coating took place after the manufacturing of the cheese which cause prolonged safe preservation of cheese.

According to Ma and Lim, (2003) as most chitosan molecules are found extracellular, cellular absorption of chitosan nanoparticles into cells is higher than that of chitosan molecules. As a result, it was hypothesized that chitosan nanoparticles would migrate into fungal cells and obstruct the creation of both DNA and RNA. This may account for why chitosan nanoparticles have stronger antifungal activity than the free polymer or solution form of the substance. Therefore, it was anticipated that the hypothesized inhibitory mechanism of chitosan nanoparticles against *C. albicans* would involve diffusion of nanoparticles into the fungal cells, followed by inhibition of DNA or RNA synthesis, ultimately leading to a direct cell death. The strongest anticandidal activity was produced by the

smallest LMW chitosan nanoparticles. According to Tayel *et al.* (2010). LMW chitosan is superior to other varieties in its ability to kill *C. albicans*. Compared to other species of fungi, *C. albicans* was more likely to be suppressed by chitosan nanoparticles. This may be caused by the constituents of cell walls including anionic charged sialic acid (Balicka-Ramisz *et al*., 2005).

Additionally, small particle size will lead to improved uptake of nanoparticles into microbial cell because the size of particles has a significant impact in determining the antibacterial activity of nanoparticles when they enter the cell walls of microbes through carrier proteins or ion channels (Sharma *et al*., 2010). Nanoparticle surface charges were high, ranging from +50 to +54 mV. By adding a positive charge to increase the interaction between nanoparticles and negatively charged microbial cell surface, particle surface charge contributes to the inhibitory effect of chitosan nanoparticles (Wiarachai *et al*., 2012).

This ultimately causes release of intracellular substances by changing the permeability of fungal cell membranes. This is consistent with a prior study that was published and demonstrated that positively charged chitosan particles will suppress microbial growth (Vallapa *et al*., 2011).

In the same context, *Spirulina platensis* extract showed a better candidacidal effect than algae itself when tested in milk at conc 100 mg/ml (Fig. 3). This may be attributed to several microalgal extracts and extracellular products exhibit antimicrobial action. Bacillus subtilis and *Candida albicans* were the most sensitive species, and the antibacterial and antifungal properties of the algal extracts were demonstrated in both culture filtrate and entire cultures (cells and exo-metabolites). Cyclic peptides, alkaloids, and lipopolysaccharides could all contribute to the explanation for why microalgae have antimicrobial properties (Katircioğlu *et al*., 2006). This action might be brought on by the toxins released by its cells, like how certain blue-green algae create toxins with potential medicinal uses. An antimicrobial activity against numerous pathogens was found in the ethanol extract. This might be because there are bioactive metabolites present that are soluble in ethanol but not diethyl ether (Tüney Kızılkaya *et al*., 2006).

Different molecules from a wide variety of chemical classes may be responsible for *S. platensis* antimicrobial properties (Ozdemir *et al*., 2004). The presence of -linolenic acid (Demule *et al*., 1996), active fatty acids (Xue *et al*., 2002), and the synergistic interaction of lauric and palmitoleric acids may all contribute to the antimicrobial activity of *Spirulina platensis* extracts (Mendiola *et al*., 2007).

Conclusion

Milk spoilage and deterioration by mould and yeast is a problem facing many factories, and scientific research. Previous survey of milk samples showed the principal fungal pathogen implicated was the yeast genus (*Candida albicans*). In this study, we highlight controlling *Candida albicans* in milk using natural methods, in addition to addressing the use of modern methods such as the use of nanoparticles. The MIC (12.5 to 100 mg/ml) of the natural components against *Candida albicans* as a model for fungi family was determined their effect in broth and in pasteurized milk. Chitosan and chitosan nanoparticles besides *Spirulina platensis* extract consider potent anticandidal agents in milk. Our findings reveal the possibility of the application of chitosan nanoparticles and *Spirulina platensis* extract as a natural method for controlling fungal growth.

Conflict of interest

The authors declare that they have no conflict of interest

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