Preparation of chitosan matrices with ferulic acid: physicochemical characterization and relationship on the growth of Aspergillus parasiticus

O. Cota-Arriola**, M. Plascencia-Jatomea, J. Lizardi-Mendoza, R. M. Robles-Sánchez, J. M. Ezquerra-Brauer, J. Ruiz-García, J. R. Vega-Acosta and M. O. Cortez-Rocha

*Departamento de Investigación y Posgrado en Alimentos, Universidad de Sonora, Hermosillo, México; **Centro de Investigación en Alimentación y Desarrollo en Alimentos, CIAD, A.C., Hermosillo, México; †Laboratorio de Coloides e Interfases, Instituto de Física, Universidad Autónoma de San Luis Potosí, San Luis Potosí, México

ABSTRACT
Antioxidant, anticarcinogenic, and antimicrobial properties have been reported for ferulic acid (FA), therefore, its application interests both food and agriculture research. FA was immobilized in different chitosan (CS) matrices, physicochemically characterized and the effect on Aspergillus parasiticus ecological parameters evaluated. Nanoparticles (Nparticles), microparticles (Mparticles) and microcapsules (Mcapsules) of 35–40 nm, 30–40 µm, and 20 µm, respectively were obtained; FA incorporation in matrices affected their morphology, physicochemical properties, and their fungistatic effect. The effect of the particles was dependent on the matrix exposed. Nparticles and Mparticles showed high FA immobilization efficiency as well as a good fungistatic effect against A. parasiticus: Radial growth at 168 h was 28.46 ± 1.01 and 28.84 ± 1.36 and the inhibition of spore germination at 30 h was 57.44 ± 0.22 and 55.74 ± 2.19, for Nparticles and Mparticles, respectively compared with control cultures. Abnormalities in mycelium, hyphae, and spores morphology were observed, as well as low sporulation due particle interaction with the fungus.

Preparación de matrices de quitosano con ácido ferúlico: Caracterización físicoquímica y su relación en el crecimiento de Aspergillus parasiticus

RESUMEN
El ácido ferúlico (AF) presenta propiedades antioxidantes, anticancerígenas y antimicrobianas, siendo de interés para su aplicación en alimentos y agricultura. El AF fue inmovilizado en diferentes matrices de quitosano, se caracterizó físicoquimicamente y se evaluó su efecto sobre Aspergillus parasiticus. Se obtuvieron nanopartículas, micropartículas y microcápsulas de 35-40 nm, 30-40 µm y 20 µm, respectivamente. La incorporación de AF en matrices afectó su morfología, propiedades físicoquímicas y su efecto fungistático, dependiendo de la matriz expuesta. Las nano y micropartículas mostraron gran eficiencia de inmovilización de AF y efecto fungistático sobre el hongo: inhibición de crecimiento radial a 168 h de 28,46±1,01 y 28,84±1,36 y de germinación de esporas en 30 h de 57,44±0,22 y 55,74±2,19, respectivamente. Asimismo se observaron anomalidades en micelio y morfología de hifas y esporas, así como baja esporulación, debido a la interacción entre las partículas con el hongo. Las microcápsulas no ejercieron efecto antifúngico, sin embargo, tanto las nano- como las micro-partículas son buenos candidatos para el control de hongos.

Introduction
Chitosan (CS) is a β-1, 4-linked polymer of glucosamine (2-amino-2-deoxy-β-D-glucose). It is formed by the deacetylation of chitin (poly-N-acetylglucosamine). CS contains amino groups (–NH₂) under neutral and alkaline conditions, but they are protonated (–NH₃⁺) under acidic conditions (<pKa 6.5) ([Ilina, Varlamov, Ermakov, Orlov, & Skryabin, 2008]), which confers a wide range of properties, including biodegradability, biocompatibility and nontoxicity (López-León, Carvalho, Seijo, Ortega-Vinuesa, & Bastos-González, 2005). CS also exhibits antimicrobial properties (Rabea, Badawy, Stevens, Smagge, & Steurbaut, 2003).

The study of CS has been increased in the last few years due to its ability to form Mparticles and nanoparticles (Nparticles), which has been used mainly for biomedical and pharmaceutical applications. The pharmaceutical applicability of CS is originated by its capacity to penetrate biological barriers, to protect macromolecules such as peptides, proteins, oligonucleotides, and genes from degradation in biological media and to deliver drugs or macromolecules to a target site with subsequent controlled release (López-León et al., 2005) such as antibacterial compounds, which are able to control/minimize bacterial infections (Sanpo, Ang, Cheang, & Khor, 2009). However, the studies on the antifungal activity of CS matrices in areas such as food and agriculture are still limited.

Methods for fungi control are mainly based on chemical strategies, mainly through the use of pesticides. Their excessive as many undesirable consequences such as environment
pollution, the increase in the frequencies of resistant or tolerant pathogen populations, and the presence of chemical residues in food commodities which could represent a health risk (Reverberi et al., 2005). For these reasons, natural antimicrobials are being evaluated as viable alternatives; however, most of these natural compounds have limitations for their application in agricultural and food areas, so it is necessary to seek other approaches as well as their effective use.

Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid) has low toxicity and possesses many physiological functions, including antioxidant, anti-inflammatory, anticancer (Ou & Kwok, 2004), and antimicrobial activity (Rabea et al., 2003). Recently, it has been widely used in food, pharmaceutical, and cosmetic industries; nonetheless, its use is limited by its tendency to be rapidly oxidized in the environment, therefore, viable alternatives must be sought. It has been reported that different structures from CS-based films such as Mcapsules and Nparticles, are excellent vehicles for the incorporation, protection and controlled release of a variety of compounds such as antioxidants, enzymes, vitamins, minerals, and other nutrients (Nair, Reddy, Kumar, & Kumar, 2009), nevertheless, their properties (immobilization efficiency, controlled release, and biological activity) will depend on the matrix characteristics. The antifungal effect of CS matrices in toxigenic fungus species such as Aspergillus parasiticus has not been well studied; furthermore, the incorporation of FA in these matrices has been barely reported. Moreover, A. parasiticus is a highly toxigenic fungal species for both human and livestock, commonly found commodities like cereals. A. parasiticus produces different aflatoxins B1, B2, G1, and G2. Aflatoxin B1 (AFB1), among the four major types of aflatoxins, is the most toxic and the most potent carcinogen in humans and animals. AFB1 exhibits a high socioeconomic importance not only due to food spoilage and mycotoxin contamination, but also due to its until now ineffective pre- and post-harvest control. Only for the United States corn industry, Mitchell, Bowers, Hurburgh, and Wu (2016) estimate that aflatoxin contamination could cause losses ranging from US$5.2 million to US$1.68 billion annually. For these reasons, the aim of this study was to obtain and characterize physicochemically CS-tripolyphosphate (TPP) loaded FA matrices (microcapsules (Mcapsules), microparticles (Mparticles) and Nparticles), to determine the most efficient to immobilize FA and finally, to evaluate the most effective form to control the growth of A. parasiticus.

Material and methods

Materials

Low viscosity CS (Sigma-Aldrich) from shrimp head, with a degree of deacetylation <85%, molecular weight of 130 KDa and <1% protein and residual ash, was used. Sodium TPP and FA were also purchased from Sigma-Aldrich.

Microorganism and growth conditions

A monosporic culture of A. parasiticus (ATCC 16992) was grown and kept in potato dextrose agar (PDA) until its use. Inoculum was made by spreading spores on PDA and resuspended in Tween 80. The concentration of spores was determined by counting in a Neubauer chamber and adjusted to 1 x 10⁵ spores/mL.

Preparation of CS-TPP matrices with FA incorporated

Three types of CS-TPP matrices with FA were elaborated by the ionotropic gelation method. To elaborate Mcapsules, a 0.6% CS solution in 0.05 M acetic acid was prepared. FA (0.8 g) was dissolved in 2% TPP, (FA-TPP). CS solution was sprayed into the FA-TPP (3:4, v/v) at 50 ml/min and stirred for 15 min at 500 rpm. To elaborate Mcapsules, a 0.6% CS solution in 0.05 M acetic acid was prepared. Then, an emulsion was made by mixing CS, soy lecithin and Tween 80 (10.0:5.0:5.0, v/v, respectively). FA (0.8 g) was dissolved in 2% TPP, (FA-TPP). The emulsion was sprayed into the FA-TPP (3:4, v/v) at 50 ml/min and stirred for 15 min at 500 rpm. Finally, to elaborate Nparticles, a 0.2% CS solution in 0.05 M acetic acid was prepared. FA (0.8 g) was dissolved in 2% TPP, (FA-TPP). CS solution was sprayed into the FA-TPP (3:4, v/v) at 50 ml/min and stirred at 500 rpm for 15 min.

Characterization of CS-TPP matrices with FA immobilized

The three types of matrices were dialyzed against 0.05 M Tris buffer, pH 7.0, for 18 h in a 12 kDa membrane (Sigma-Aldrich). The pH was adjusted to 5.6 by adding 0.05 M sodium acetate buffer and lyophilized for the physicochemical characterization and bioassays on A. parasiticus. Matrices yield was obtained with the final weight after lyophilization of 1000 mL of the initial solution.

Morphology and size

The average size and morphology of the Mparticle and Mcapsule matrices with immobilized FA were examined using a scanning electron microscope (SEM), JEOL brand, model DSM-54101V, equipped with an EDS. In addition, they were analyzed by transmission electron microscope (TEM), JEOL brand, model JEM-2010F operated at 200 kV (Liu & Gao, 2009; Zhou et al., 2011).

Fourier transformed infrared spectroscopy

The main functional groups in the CS-TPP matrices with immobilized FA were determined using a Fourier transformed infrared spectrocope (FT-IR) (Perkin Elmer Spectrum GX FT-IR) (Liu & Gao, 2009; Zhou et al., 2011).

Zeta potential

The zeta potential is the electric potential in the double interfacial layer; this means, is the place where the diffuse and the Stern layers are bonded. Its value is related to the stability of the colloidal dispersions, indicating the repulsion degree among adjacent particles charged in a dispersion. Likewise, this technique is amply used for electric charge quantification in the double layer. Nparticles CS-TPP (with and without FA) were suspended and washed in two different pH buffer acetate 0.02 M (pH 5.0 or pH 5.6) using a 12 KDa membrane (Sigma-Aldrich). Material inside the membrane was diluted (1:100, v/v) using distilled water to avoid particles aggregation. 2 mL of the CS-TPP matrices with
immobilized FA suspension in a cell were tested for zeta potential using a Zetasizer Nano-25 (UK Malvern Instruments) equipment at 25°C (Liu & Gao, 2009).

**Total immobilization of FA**

The total amount of immobilized FA in the different matrices was determined as free and linked FA. Free FA is the one that is not interacting with the CS-TPP and linked FA is the one that is interacting with the matrix. Free and linked FA in each of the CS-TPP matrices was determined according to the Folin–Ciocalteu method for total phenols. To quantify spores/mL of A. parasiticus, an inoculum of $1 \times 10^5$ spores/ml of A. parasiticus was deposited in each hole and was incubated at 28°C. Radial extension growth of the colonies was measured every 12 h and compared with control media until control colony reached the plate border. Controls consisted of agar Czapek dox at pH 5.6 using acetate buffer, CS 0.5% at pH 5.6, TPP 2% at pH 5.6, and matrices without FA at pH 5.6. The rate percentage of growth inhibition (GI) and the radial expansion of the colony (cm h$^{-1}$) were determined from the slope resulting from the radius versus time graph as described by Cota-Arriola et al. (2011) using the acidified agar Czapek dox control as reference. The GI was calculated as follow:

$$GI = \left(1 - \frac{R_i}{R_c}\right) \times 100,$$

where $R_c$ is the mean value of the colony radius in the 5.6 pH agar Czapek control media, and $R_i$ is the colony radius in presence of the different treatments (matrices with and without FA, CS pH 5.6, and TPP pH 5.6).

**Spore germination**

Four coverslips were placed inside of a 8-cm glass Petri dish with 21 mL of Czapek liquid medium and 9 mL of a solution containing 15 mg of CS-TPP matrices with immobilized FA (proportion 7:3, v/v, respectively) at pH 5.6. Plates were inoculated with $1 \times 10^5$ spores/mL of A. parasiticus and manually agitated for homogenization. The plates were incubated at 28°C and every 6 h coverslips containing spores were removed and germinated and non-germinated spores were directly counted using an optical microscope (Olympus CX31, Japan) with 40X objective. A spore was considered germinated when the length of its germinal tube reached one-half of the spore diameter (Plascencia-Jatomea, Viniegra, Olayo, Castillo-Ortega, & Shirai, 2003). Controls consisted of agar Czapek dox at pH 5.6 with and without FA at pH 5.6, CS 0.5% pH 5.6, and TPP 2% pH 5.6. The number of germinated spores, the percentage of spore germination inhibition, and the germination rate (spores h$^{-1}$) were determined as described by Cota-Arriola et al. (2011). Spore germination inhibition (FI) was calculated as follow:

$$FI = \left(1 - \frac{|S_i|}{S_c}\right) \times 100,$$

where $S_c$ was the percentage of spores germinating in the control (agar Czapek dox at pH 5.6) and $S_i$ was the percentage of spores germinating in the different treatments and controls.

**Antifungal activity of CS-TPP matrices with FA immobilized on A. parasiticus**

**Radial growth**

In order to measure radial growth, the deposition technique was used. When Petri dishes containing 7 mL of agar Czapek dox (solid medium) were at 50°C, 3 mL of a solution containing 15 mg of the CS-TPP matrices with immobilized FA (7:3, v/v) at pH 5.6 was added, mixed, and allowed to cool. A hole of 0.5 cm in diameter was made in the center of Petri dishes. An inoculum of $1 \times 10^5$ spores/ml of A. parasiticus was deposited in each hole and was incubated at 28°C. Radial extension growth of the colonies was measured every 12 h and compared with control media until control colony reached the plate border. Controls consisted of agar Czapek dox at pH 5.6 using acetate buffer, CS 0.5% at pH 5.6, TPP 2% at pH 5.6, and matrices without FA at pH 5.6. The rate percentage of growth inhibition (GI) and the radial expansion of the colony (cm h$^{-1}$) were determined from the slope resulting from the radius versus time graph as described by Cota-Arriola et al. (2011) using the acidified agar Czapek dox control as reference. The GI was calculated as follow:

$$GI = \left(1 - \frac{R_i}{R_c}\right) \times 100,$$

where $R_c$ is the mean value of the colony radius in the 5.6 pH agar Czapek control media, and $R_i$ is the colony radius in presence of the different treatments (matrices with and without FA, CS pH 5.6, and TPP pH 5.6).

**Spore germination**

Four coverslips were placed inside of a 8-cm glass Petri dish with 21 mL of Czapek liquid medium and 9 mL of a solution containing 15 mg of CS-TPP matrices with immobilized FA (proportion 7:3, v/v, respectively) at pH 5.6. Plates were inoculated with $1 \times 10^5$ spores/mL of A. parasiticus and manually agitated for homogenization. The plates were incubated at 28°C and every 6 h coverslips containing spores were removed and germinated and non-germinated spores were directly counted using an optical microscope (Olympus CX31, Japan) with 40X objective. A spore was considered germinated when the length of its germinal tube reached one-half of the spore diameter (Plascencia-Jatomea, Viniegra, Olayo, Castillo-Ortega, & Shirai, 2003). Controls consisted of agar Czapek dox at pH 5.6 with and without FA at pH 5.6, CS 0.5% pH 5.6, and TPP 2% pH 5.6. The number of germinated spores, the percentage of spore germination inhibition, and the germination rate (spores h$^{-1}$) were determined as described by Cota-Arriola et al. (2011). Spore germination inhibition (FI) was calculated as follow:

$$FI = \left(1 - \frac{|S_i|}{S_c}\right) \times 100,$$

where $S_c$ was the percentage of spores germinating in the control (agar Czapek dox at pH 5.6) and $S_i$ was the percentage of spores germinating in the different treatments and controls.

**Table 1. Immobilization and immobilization efficiency (EE) of FA in CS-TPP matrices.**

| Treatment | Immobilization ferulic acid (mgGAE/100 mg) | Zeta potential (Mv) |
|-----------|-------------------------------------------|---------------------|
|           | Immobilization ferulic acid (mgGAE/100 mg) | pH 5.0 pH 5.6 |
| Nparticle | Free FA                                   | Linked FA          |
|           | 16.80 ± 0.69$^a$                        | 3.16 ± 0.29$^a$  |
| Nparticle | Linked FA                                 | Free FA            |
|           | 18.44 ± 0.56$^b$                        | 6.33 ± 1.16$^b$  |
| Nparticle | Mparticle                                | Free FA            |
|           | −55.46 ± 3.79$^b$                       | 7.93 ± 0.29$^b$  |
| Nparticle | FA                                       | Linked FA          |
|           | 25.00 ± 0.70$^c$                        | 19.03 ± 0.71$^c$  |
| Mparticle | FA                                       | Mparticle/FA       |
|           | 21.66 ± 0.94$^d$                        | 19.14 ± 0.36$^d$  |

Data followed by their standard deviation are means from five experiments. Treatments mean values followed by different superscripts are significantly different ($P \leq 0.05$).

Datos seguidos de su desviación estándar, de medias de cinco experimentos. Valores de tratamientos con letras distintas son significativamente diferentes ($P \leq 0.05$).

**Spore germination**

Four coverslips were placed inside of a 8-cm glass Petri dish with 21 mL of Czapek liquid medium and 9 mL of a solution containing 15 mg of CS-TPP matrices with immobilized FA (proportion 7:3, v/v, respectively) at pH 5.6. Plates were inoculated with $1 \times 10^5$ spores/mL of A. parasiticus and manually agitated for homogenization. The plates were incubated at 28°C and every 6 h coverslips containing spores were removed and germinated and non-germinated spores were directly counted using an optical microscope (Olympus CX31, Japan) with 40X objective. A spore was considered germinated when the length of its germinal tube reached one-half of the spore diameter (Plascencia-Jatomea, Viniegra, Olayo, Castillo-Ortega, & Shirai, 2003). Controls consisted of agar Czapek dox at pH 5.6 with and without FA at pH 5.6, CS 0.5% pH 5.6, and TPP 2% pH 5.6. The number of germinated spores, the percentage of spore germination inhibition, and the germination rate (spores h$^{-1}$) were determined as described by Cota-Arriola et al. (2011). Spore germination inhibition (FI) was calculated as follow:

$$FI = \left(1 - \frac{|S_i|}{S_c}\right) \times 100,$$

where $S_c$ was the percentage of spores germinating in the control (agar Czapek dox at pH 5.6) and $S_i$ was the percentage of spores germinating in the different treatments and controls.
**Morphometric parameters of hyphae and spores**

Samples from the spore germination test were taken at 12 and 18 h. The hyphae and spore average diameter were measured with an image analyzer Pro Plus Version 6.3 software (2008 Media Cybernetics Inc., USA) using an optical microscope with a 40X objective (Olympus CX31, Japan) connected to an Infinity 1 camera (Media Cybernetics, USA).

**Statistical analysis**

Statistics on a completely randomized design were determined using a one-way analysis of variance (ANOVA). JMP software version 5.0 (SAS Institute Inc., USA) with a significance level of $P = 0.05$, was used. Means between homogeneous groups were separated using Tukey’s multiple comparison test (Tukey’s post hoc test) with a confidence interval of 95%.

**Results and discussion**

**Acquisition and morphology of CS-TPP matrices with immobilized FA**

Poncelet (2006) defines the Nparticle as a particle with size of $<1.0$ µm and the Mparticle and Mcapsule as those particles with sizes of $<1.0$ mm. According to his classification, the obtained matrices were Mcapsules, Mparticles and Nparticles of CS-TPP with immobilized FA (Figure 1). After lyophilization, Mparticles and Nparticles with immobilized FA (Mparticles/FA and Nparticles/FA); they showed irregular morphology and a white color, with sizes of 30–40 µm and 35–40 nm (Figure 1), respectively, and a yield of 28.3 and 24.5 g/L, respectively. Mcapsules with FA (Mcapsules/FA) presented spherical morphology and a uniform diameter of 20 µm, (Figure 1) with a yield of 37.9 g/L. They exhibit a yellow color due to the presence of soy lecithin. The irregular shape of the Mparticles and Nparticles may be due to competition between the carboxyl group of the FA and the phosphate group of TPP to interact with the CS amino group. The spherical shape of the Mcapsules could have been due to less interaction among the components in the presence of soy lecithin, which forms a layer between the CS-TPP matrix and the FA.

**Infrared spectroscopy of CS-TPP matrices with immobilized FA**

Infrared spectra from the main components of the matrices with and without FA are presented in Figure 2. The characteristic peaks of CS, TPP, and FA were observed as well as their interactions. Concerning Mparticles and Nparticles without FA (Mparticles and Nparticles), the presence of the characteristic band of the primary amine of CS to 1644–1646 cm$^{-1}$ was observed (Woranuch & Yoksan, 2013). Also, the band of TPP (P=O) was present, 1103 and 889–750 cm$^{-1}$, corresponding to the stretching vibrations of the P=O group, and a band at 540 cm$^{-1}$ corresponding to the deformation vibration of P=O, which indicated the presence of CS-TPP in both matrices (Ibezim et al., 2011). Similarly, in the spectrum of the Mcapsule without FA (Mcapsule), the characteristic bands of CS and TPP mentioned above were observed, and another band around 1739 cm$^{-1}$ was observed, which corresponded to the C=O group of lecithin (Wang, Luo, & Xiao, 2014). In the spectra of Mparticle/FA, Nparticle/FA, and Mcapsule/FA, the appearance of a new peak at 1596–1529 cm$^{-1}$ was detected. It corresponded to the characteristic band of the C=C from the FA aromatic ring, which indicated its immobilization in the matrices (Sun, Wang, Kadouh, & Zhou, 2014). Also, a frequency shift and a decreased intensity of the peaks corresponding to CS and TPP were observed, compared with the spectra of the matrices without FA, indicating an ionic interaction between the CS-TPP and a possible ionic interaction between the carboxyl group of the FA with the CS amino groups.

**Total immobilization and immobilization efficiency (EE) of FA in matrices of CS-TPP**

Table 1 shows data for the total immobilization of FA and the efficiency of immobilization (EE) by the matrices. The immobilization efficiencies of Nparticles and Mparticles were statistically similar. Mcapsules had the lowest immobilization efficiency but were the most efficient to encapsulate free FA because of its larger size, however, they had less linked FA ($P \leq 0.05$). This may have been due to the presence of lecithin and Tween that can form an interlayer between CS-TPP and the FA, affecting the ionic interaction between the COO$^-$ and the NH$_3^+$ groups of FA and CS, respectively.

---

**Image References**

*Figure 1.* Micrographs of CS-TPP matrices with immobilized FA. Transmission electron microscope (a) and scanning electron microscope (b–c). (a) Nparticle/FA, (b) Mparticle/FA, and (c) Mcapsule/FA.

*Figura 1.* Micrografías de matrices de CS-TPP con FA inmobilizado. Microscopía electrónica de transmisión (a) y Microscopía electrónica de barrido (b–c). (a) Npartícula/AF, (b) Mpartícula/AF, (c) Mcápsula/AF.
The immobilization efficiencies of Nparticles and Mparticles were statistically similar, but Mparticles showed higher total FA encapsulation. In addition, Nparticles showed high amount of linked FA, which may have been due to the compact nature and small size of the structure. This brought the carboxyl group of FA and the amino group of CS close enough to favor an ionic interaction. Furthermore, in the Mparticles, significant amounts of linked FA were detected, which may have been due to the concentration of CS used, making the amino groups more available to interact with the FA carboxyl group. Several studies have reported variable values of immobilization efficiency, due to many intervening factors such as concentration, polymer properties, crosslinking agent, degree of crosslinking, size and morphology of the matrix, and others (Agnihotri, Mallikarjuna, & Aminabhavi, 2004).

Zeta potential of CS-TPP matrices with immobilized FA

The zeta potential or surface charge is an essential parameter in the characteristics of a particle, mainly through its influence on stability (Du, Niu, Xu, Xu, & Fan, 2009), and determines the antimicrobial potential on fungi and bacteria (Du, Xu, Xu, & Fan, 2008). Table 1 shows the zeta potential of the elaborated matrices at two pH levels, 5.0 and 5.6. The Nparticle and Mparticle at pH 5.6 had low surface charges (+), which may indicate that not all of the amino groups from CS were loaded and could be due to the excess of TPP added for the crosslinking (Liu & Gao, 2009). However, the immobilization of FA in the Nparticles and Mparticles at pH 5.6 increased their surface charge (+), which may be due to the competition between the FA and TPP for linkage to the amino group of CS or to structural rearrangement of the particle by the immobilization of FA, exhibiting more free amino groups on the surface (Liu & Gao, 2009). In addition, the Nparticles and Mparticles with or without immobilized FA at pH 5.0 had increased surface charges (+) compared with those at pH 5.6. This may have been due to a high protonation of the amino groups, or possibly to a rearrangement of the particles by lowering of the pH, which is in agreement with Liu and Gao (2009). The Mcapsules with or without immobilized FA showed a negative surface charge; this may have been caused by the addition of soy lecithin, which increased the presence of phosphate groups on the surface of the Mcapsule, affecting its electrostatic properties. Zeta potential is a crucial parameter for stability of aqueous nanosuspensions. For a physically stable nanosuspension solely stabilized by electrostatic repulsion, a zeta potential of ±30 mV is required as a minimum (Müller, Jacobs, & Kayser, 2001). All these data suggested that CS Nparticles and CS Nparticles loaded metal ions prepared here were stable. Several studies have reported that the antimicrobial activity of CS under acidic conditions is due to the protonation of –NH₂ on the C-2 position of the D-glucosamine repeat unit (Ali, Joshi, & Rajendran, 2010). Positively charged CS binds to the bacterial cell wall surface which is negatively charged and disrupt the normal functions of the membrane affecting the nutrient transport into the cell or by promoting the leakage of intracellular components. Du et al. (2009) found that antibacterial activity of CS Nparticles loaded metal ions was directly proportional to potential zeta.

Figure 2. FT-IR spectra of CS-TPP matrices with immobilized FA. Blue, red, green, and black lines correspond to the peaks of the main functional groups of CS, TPP, FA, and lecithin, respectively.

Figura 2. Espectro de FT-IR de matrices de CS-TPP con FA inmobilizado. Linea azul, roja, verde y negro corresponden a los picos de los principales grupos funcionales de CS, TPP, FA y lecita, respectivamente.
Antifungal activity of CS-TPP matrices with immobilized FA on A. parasiticus

Radial growth
In fact, the study of Mparticles and Nparticles of CS with antimicrobial potential is having a strong impact, since they have greater effect than CS in solution. For this reason, alternatives were needed to increase their antimicrobial activity by incorporating natural compounds. Table 2 shows results for the GI of A. parasiticus in the presence of matrices with immobilized FA at 98 and 168 h. It was observed that Mparticles/FA and Nparticles/FA showed greater inhibition and lower growth rates compared with matrices without FA and the controls (Buffer acetate, TPP and CS). We also found that A. parasiticus was not inhibited by the presence of Mcapsules or Mcapsules/FA; however, they promoted faster growth of the fungus. The Nparticles without FA showed greater fungistic activity compared with control cultures, and this effect could be attributed to the amino groups of CS in the particles, which can interact with fungal membrane components (phospholipids), increasing cell permeability (Palma-Guerrero et al., 2010). However, the phosphate ions present in the particles may contribute to this effect, as they indisposed divalent metals that are important for fungus development, such as Ca²⁺ and Mg²⁺, or bound to positively charged components of the membrane such as certain proteins (Palmeira-de-Oliveira et al., 2011). In addition, the fungistic effect of Nparticles may be related to their small size, having larger contact surface with the cell membrane (Ali et al., 2010). Furthermore, it was found that A. parasiticus growth and spore germination was greater in the presence of Mcapsules and Mcapsules/FA, compared with those in buffer acetate control. This effect can be attributed to the presence of lecinthin in the capsules, since it is reported that lecinthin accelerates the growth of fungus, such as in Anthracophyllum discolor, which uses it as a carbon source (Bustamante, Rubilar, & Diez, 2014). The addition of FA in Nparticles and Mparticles potentiated its inhibitory effect, compared with the matrices without FA. This effect may be due to an increase in the zeta potential (ζ) of such matrices for the presence of immobilized FA (Table 2), indicating that more amino groups are loaded, resulting in an increased interaction with the phospholipids of the cell membrane, thereby affecting cell permeability and the development of the fungus (Ali et al., 2010; Saharan et al., 2013). Furthermore, the increase in the fungistatic potential of the Mparticles/FA and Nparticles/FA, can also be attributed to the presence and release of FA (Free and linked FA in CS-TPP matrices). It has been reported that phenolic compounds exhibit antimicrobial effects against bacteria and fungi, due to their ability to interact via hydrogen bonds with proteins and amino acids from the cell wall and cell membrane, and even from cytoplasm, damaging cell permeability (Yi et al., 2014; Nakayama et al., 2013; Bossi et al., 2007; Cousinet-Mossion et al., 2010). It was also mentioned that these phenolic compounds decrease or inhibit the activity of essential enzymes for fungal growth (Bossi et al., 2007; Kandil, Li, Vasanthan, & Bressler, 2012; Yi et al., 2014). Also, phenolic compounds exhibit antimicrobial activity in their action against free radicals, generating a high amount of hydrogen peroxide, causing irreversible damage to microbial cell (Yi et al., 2014).

The effects on the morphology of A. parasiticus in the presence of CS-TPP matrices with immobilized FA are shown in Figure 3. It was observed that morphological changes in A. parasiticus were more evident in the presence of the Mparticles and Nparticles of CS-TPP with immobilized FA (Nparticles/FA and Mparticles/FA). Colonies presented white color and cottony mycelia growth, low sporulation and hypha apical growth (Figure 3), compared with those in the controls and matrices without FA, in which the mycelium was yellowish-green with high sporulation and longitudinal growth of the hyphae. Mycelium morphological changes observed in presence of Nparticles/FA and Mparticles/FA may be due mainly to the presence of CS and FA. This effect also can be attributed to the ability of FA to interact with amino acids and enzymes that are important for the development and sporulation of A. parasiticus (Holmes, Boston, & Payne, 2008; Kandil et al., 2012; Nakayama et al., 2013; Yi et al., 2014). In addition, a greater effect on the mycelium due to interactions between the Nparticle or Mparticle with the membrane could be caused by the increase in zeta potential (ζ) elicited for the addition of FA (Du et al., 2009). It was also observed that the presence of Nparticles/FA resulted in most significant, exhibiting a completely white and cottony mycelium with little sporulation, which could be due to the small size of the particles, causing greater interaction with the fungus membrane and thereby, greater effect on its morphology and growth. It was also noted that there were no obvious changes in the mycelium and sporulation of the fungus by the Mcapsules, and this was attributed to the presence of lecinthin. However, in the Mcapsules/FA, few white and cottony mycelia were observed, probably due to the presence of the FA in the matrix.

Spore germination
Spore germination is the first stage of fungal growth and is indicative of adaptation to the medium, because at this stage, the fungus produces the necessary compounds for its development (Cota-Arriola et al., 2011). It has been reported that calcium (Ca²⁺) and some enzymes, such as chitin synthase among others, play a fundamental role in the germination, mainly in the development of hyphae.

Table 2. Growth inhibition and radial extension rate of A. parasiticus in presence of CS-TPP matrices with FA in Czapek media incubated at 28°C.

| Treatment          | Growth inhibition (%) | Radial extension (cm h⁻¹) |
|--------------------|-----------------------|--------------------------|
|                    | 98 h                  | 168 h                    |
| Acetate buffer     | 0.0                   | 0.0                      |
| CS₅                | 19.24 ± 2.06d        | 15.32 ± 2.64d           |
| TPP                | 0.44 ± 0.63a         | −3.07 ± 1.08b           |
| Nparticle          | 24.22 ± 1.25c        | 16.06 ± 1.84d           |
| Mparticle          | 10.39 ± 2.12c        | 7.35 ± 1.76c            |
| Mcapsule           | −10.62 ± 0.69a       | −12.28 ± 3.92e          |
| Mcapsule/FA        | 28.64 ± 1.22a        | 28.46 ± 1.01e           |
| Mparticle/FA       | 32.52 ± 1.14a        | 28.84 ± 1.36e           |
| Mcapsule/FA        | −8.85 ± 1.30a        | −1.84 ± 1.85b           |

Data followed by the standard deviation are means from five experiments. Treatments mean values followed by different superscripts are significantly different (P ≤ 0.05). Nparticle = nanoparticle, Mparticle = microparticle, Mcapsule = microcapsule, Nparticle/FA = nanoparticle with ferulic acid, Mparticle/FA = microparticle with ferulic acid, Mcapsule/FA = microcapsule with ferulic acid.
McIntyre, Dynesen, & Nielsen, 2001; Plascencia- Jatomea et al., 2003). Table 3 shows the percentages of inhibition of spore germination of A. parasiticus in the presence of CS-TPP matrices with immobilized FA, and in control treatments. At 12 h, it was found that the presence of TPP, Mcapsules, and Mcapsules/FA, did not inhibit the germination of spores. It was also observed that at 30 h, all treatments showed inhibitory effects compared with the control acetate buffer, with a greater effect in the presence of Mparticle/FA and Nparticle/FA. The inhibitory effect presented by CS on the germination of spores of A. parasiticus was attributed to its calcium (Ca\(^{2+}\))-chelating potential, the electrostatic interaction between protonated amino groups (NH\(_3^+\)), and the ability of the phosphate ion to electrostatically interact with major ions (Ca\(^{2+}\)) for spore germination (Palmeira-de-Oliveira et al., 2011). It was generally observed that Mparticles and Nparticles of CS-TPP potentiated the inhibitory effect of germination compared with TPP and CS solutions. This may have been due to the aforementioned mechanisms (Palmeira-de-Oliveira et al., 2011) and to the formation of the particle (smaller), presenting a greater effect on fungal germination (Ali et al., 2010; Du et al., 2008; Yien, Zin, Sarwar, & Katas, 2012). However, the incorporation of FA into Mparticles and Nparticles potentiated the inhibitory effect on spore germination, and was attributed to the increase of the zeta potential of the particles (Ali et al., 2010; Chowdappa, Gowda, Chethana, & Madhura, 2014; Saharan et al., 2013). In addition, this effect may be due to the capacity of FA (free and bound to the matrix), to inactivate enzymes that are important for fungal development, as occurred in the radial growth phase (Kandil et al., 2012; Nakayama et al., 2013; Yi et al., 2014).

Morphometric parameters and morphological changes of spores and hyphae of A. parasiticus

Changes in morphometric parameters indicated the morphological changes undergone by the fungus in the presence of adverse factors in their adaptation and development. In Table 4, the diameters of spores and hyphae of A. parasiticus in the presence of CS-TPP matrices with embedded FA and control treatments are shown. The spore diameter at 12 and 18 h increased due to the effect of CS in solution and to the CS-TPP matrices with immobilized FA (Nparticles/FA, Mparticles/FA and Mcapsule/FA), compared with other treatments. In addition, the length of hyphae was shorter, with...
significant changes in hyphae grown in the presence of CS and Nparticles/FA. Furthermore, the TPP and matrices (Nparticles, Mparticles and Mcapsules) showed no significant difference in the morphometric parameters of spores and hyphae from the control (acetate buffer), indicating that the morphology of *A. parasiticus* was normal. These results indicate that the incorporation and release of FA by different matrices caused alterations in fungal morphology, increasing the diameter of the spores. This may be because the spore, not being adapted to germinate, continues synthesizing carbohydrates, lipids, and proteins (enzymes) in order to adapt, causing greater swelling thereof (Deacon, 1993). Shorter and irregular hyphae in the presence of CS were observed, attributed to alterations in the cell wall or to changes in pressure and wall tension (Cota-Arriola et al., 2011). This can also be due to the lack of calcium (Ca$^{2+}$), which is important for the formation and elongation of the hyphae (Plascencia-Jatomea et al., 2003).

Figure 4 shows the morphology of spores and hyphae of *A. parasiticus*. It was observed that the presence of CS (Figure 4(b)) caused an increase in the diameter of spores and shorter hyphae compared with control and TPP (2.0%), in which average morphology was observed (Figure 4(a,c)). The morphology of *A. parasiticus* in the presence of Nparticles and Mparticles (Figure 4(d,e)) showed more noticeable changes, displaying shorter and irregular hyphae, relative to the control. In addition, a possible interaction between Nparticles and Mparticles with spores and hyphae was observed. However, in the presence of Mcapsules (Figure 4(f)), no obvious change was observed in hyphae or spores, nor in the interactions between the Mcapsule and fungus. This was likely because on the surface of the capsule, there are no cationic amino groups (NH$_3^+$) present to interact with the fungus membrane, being consistent with the results of Table 1 (zeta potential negative). The most obvious changes in morphology were presented in the fungus exposed to the presence of matrices with incorporated FA (Figure 4(g–i)), where deformations in spores and hyphae were observed. Morphological changes were due to the aforementioned mechanisms in the radial growth phase.

### Table 4. Morphometric parameters of spores and hyphae of *A. parasiticus* in presence of CS-TPP matrices with FA incorporated at the stage of germination of spores in Czapek liquid medium, 28°C.

| Treatment          | Spore diameter (µm) | Hyphae diameter (µm) |
|--------------------|---------------------|-----------------------|
|                    | 12 h 18 h           | 18 h                  |
| Acetate buffer     | 5.51 ± 0.89$^a$     | 5.67 ± 0.92$^a$       |
| CS                 | 6.48 ± 1.03$^b$     | 6.75 ± 1.13$^b$       |
| TPP                | 5.23 ± 0.98$^c$     | 5.72 ± 1.24$^c$       |
| Nparticle          | 5.33 ± 0.47$^d$     | 6.01 ± 1.07$^d$       |
| Mparticle          | 5.51 ± 1.02$^e$     | 6.68 ± 1.24$^e$       |
| Mcapsule           | 5.53 ± 0.75 $^f$    | 5.92 ± 1.18$^f$       |
| Nparticle/AF       | 6.52 ± 0.76$^a$     | 6.42 ± 0.78$^a$       |
| Mparticle/AF       | 6.16 ± 0.82$^a$     | 7.09 ± 1.06$^a$       |
| Mcapsule/AF        | 6.19 ± 0.75$^a$     | 7.10 ± 1.08$^a$       |

Data followed by the standard deviation are means from fifty spores measured. Treatments mean values followed by different superscripts are significantly different ($P \leq 0.05$). Nparticle = nanoparticle, Mparticle = microparticle, Mcapsule = microcapsule, Nparticle/FA = nanoparticle with ferulic acid, Mparticle/FA = microparticle with ferulic acid, Mcapsule/FA = microcapsule with ferulic acid.

**Figure 4.** Microphotographs of *A. parasiticus* spores and hyphae observed using a 40X objective after 18 h of incubation in Czapek liquid medium in presence of CS-TPP matrices with immobilized FA. (a) Control (acetate buffer), (b) Chitosan, (c) Tripolyphosphate, (d) Nparticles, (e) Mparticles, (f) Mcapsule, (g) Nparticles/FA, (h) Mparticles/FA, and (i) Mcapsule/FA.

**Figura 4.** Micrografías de esporas e hifas de *A. parasiticus* observadas a 40 X después de 18 h de incubación en medio líquido Czapek en presencia de matrices de CS-TPP con FA inmobilizado. (a) Control (Buffer de acetato), (b) Quitoxyano, (c) Tripolifosfato, (d) Npartícula, (e) Mpartícula, (f) Mcápsula, (g) Npartícula/FA, (h) Mpartícula/FA, (i) Mcápsula/FA.
and spore germination. Moreover, they were due to the increase in the zeta potential of the particle, causing greater interaction and to the presence of FA (free and bound to the matrix of CS-TPP), which can inhibit enzymes that are important for fungal development (Kandil et al., 2012; Yi et al., 2014), such as chitin synthases, responsible for catalyzing the polymerization of chitin. Furthermore, it has been reported that when the activity of chitin synthase is interrupted to form chitin in Aspergillus species, hyphae cell walls swell, appearing abnormal conidiophores (McIntyre et al., 2001).

Conclusions

The results of this study indicate that the CS-TPP matrices are effective in the immobilization, protection, and release of FA. The immobilization efficiency of FA depends on the matrix obtained and how it is in the matrix (free or linked), as we found that Mparticles had higher total immobilization of FA. Mparticles exhibited higher total immobilization of FA, and the interaction with CS matrices had effect on matrix morphology as well as physicochemical properties, depending on the matrix, increasing zeta potential (+) of Mparticles and Nparticles. These changes affected the fungistatic properties against A. parasiticus; moreover, the incorporation of FA (free and linked) into Mparticles and Nparticles significantly enhanced its fungistatic activity on growth, spore germination, and morphology of A. parasiticus; however, Mcapsules enhanced fungus growth. These results indicate that the application of CS matrices to control toxigenic fungi will depend on the composition, structure, and FA immobilization in the matrices. In addition, this study suggests that the development of CS Mparticles and Nparticles by incorporating natural antimicrobial compounds as FA is a promising technology for future application in food and agriculture areas, mainly for control of socioeconomic important toxigenic fungi.

Acknowledgments

The study was funded by the Mexican Council for Science and Technology (CONACYT) through the projects 58249 and 53493 J1 at the University of Sonora and for the scholarship to Octavio Cota-Arriola for postgraduate studies.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Universidad de Sonora/Universidad Estatal de Sonora and by CONACYT.

References

Agnihotri, S.A., Mallikarjuna, N.N., & Aminabhavi, T.M. (2004). Recent advances on chitosan based micro and nanoparticles in drug delivery. Journal of Controlled Release, 100, 5–28. doi:10.1016/j.jconrel.2004.08.010

Ali, S.W., Joshi, M., & Rajendra, S. (2010). Modulation of size, shape and surface charge of chitosan nanoparticles with reference to antimicrobial activity. Advanced Science Letters, 3, 452–460. doi:10.1166/asl.2010.1152

Bosi, A., Rinalducci, S., Zolla, L., Antonioli, P., Righetti, P.G., & Zapparoli, G. (2007). Effect of tannic acid on Lactobacillus hilgardii analysed by a proteomic approach. Journal of Applied Microbiology, 102, 787–795. doi:10.1111/j.1365-2672.2006.03118.x

Bustamante, M., Rubilar, O., & Diez, M.C. (2014). Effect of soya lecithin on solubilization and biodegradation of pentachlorophenol by Anthracophyllum discolor. American Journal of Analytical Chemistry, 5, 28–38. doi:10.4236/ajac.2014.51005

Chowdappa, P., Gowda, S., Chethana, C.S., & Madhura, S. (2014). Antifungal activity of chitosan silver nanoparticle composite against Colletotrichum gloeosporioides associated with mango anthracnose. African Journal of Microbiology Research, 8, 1803–1812. doi:10.5897/AJMIR2013.6584

Cota-Arriola, O., Cortez-Rocha, M.O., Rosas-Burgos, E.C., Burgos-Hernández, A., López-Franco, Y.L., & Plascencia-Jatomea, M. (2011). Antifungal effect of chitosan on the growth of Aspergillus parasiticus and production of aflatoxin B1. Polymer International, 60, 937–944. doi:10.1002/pi.3054

Couzinet-Mossion, A., Balayssac, S., Gilard, V., Malet-Martino, M., Potin-Gautier, M., & Behra, P. (2010). Interaction mechanisms between caffeine and polyphenols in infusions of Camellia sinensis leaves. Food Chemistry, 119, 173–181. doi:10.1016/j.foodchem.2009.06.020

Deacon, J.W. (1993). Introducción a la Micología Moderna (2nd ed.). México, D.F.: LIMUSA.

Du, W.L., Niu, S.S., Xu, Y.L., Xu, Z.R., & Fan, C.L. (2009). Antibacterial activity of chitosan triplypolyphosphate nanoparticles loaded with various metal ions. Carbohydrate Polymers, 75, 385–389. doi:10.1016/j.carbpol.2008.07.039

Du, W.L., Xu, Y.L., Xu, Z.R., & Fan, C.L. (2008). Preparation, characterization and antibacterial properties against E. coli K88 of chitosan nanoparticle loaded copper ions. Nanotechnology, 19, 085707. doi:10.1088/0957-4484/19/8/085707

Holmes, R.A., Boston, R.S., & Payne, G.A. (2008). Diverse inhibitors of aflatoxin biosynthesis. Applied Microbiology and Biotechnology, 78, 559–572. doi:10.1007/s00253-008-1362-0

Hosseini, S.F., Zandi, M., Rezaei, M., & Farahmandghavi, F. (2013). Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: Preparation, characterization and in vitro release study. Carbohydrate Polymers, 95, 50–56. doi:10.1016/j.carbpol.2013.02.031

Ibezim, E.C., Andrade, C.T., Marcia, B., Barretto, B., Odimegwu, D.C., & de Lima, F.F. (2011). Ionically cross-linked chitosan/tripolyphosphate microparticles for the controlled delivery of pyrimethamine. Ibnosina Journal of Medicine and Biomedical Sciences, 3, 77–88.

Ilina, A.V., Varlamov, V.P., Ermakov, Y.A., Orlov, V.N., & Skryabin, K.G. (2008). Chitosan is a natural polymer for constructing nanoparticles. Doklady Chemistry, 421, 165–167. doi:10.1134/S0022396406070033

Kandil, A., Li, J., Vasanthan, T., & Bressler, D.C. (2012). Phenolic acids in some cereal grains and their inhibitory effect on starch liquefaction and saccharification. Journal of Agricultural and Food Chemistry, 60, 8444–8449. doi:10.1021/jf3000482

Liu, H., & Gao, C. (2009). Preparation and properties of ionically cross-linked chitosan nanoparticles. Polymers for Advanced Technologies, 20, 613–619. doi:10.1002/pat.1306

López-León, T., Carvalho, E.L.S., Seijo, B., Ortega-Víuesa, J.L., & Bastos-González, D. (2005). Physicochemical characterization of chitosan nanoparticles: Electrokinetic and stability behavior. Journal of Colloid and Interface Science, 283, 344–351. doi:10.1016/j.jcis.2004.08.186

McIntyre, M., Dynesen, J., & Nielsen, J. (2001). Morphological characterization of Aspergillus nidulans: Growth, septation and fragmentation. Microbiology, 147, 239–246. doi:10.1099/00221287-147-1-239

Mitchell, N.J., Bowers, E., Hurburgh, C., & Wu, F. (2016). Potential economic losses to the US corn industry from aflatoxin contamination. Food Additives & Contaminants: Part A, 33, 540–550. doi:10.1080/19440049.2016.1138545

Müller, R.H., Jacobs, C., & Kayser, O. (2001). Nanosuspensions as particulate drug formulations in therapy rationale for development and what we can expect for the future. Advanced Drug Delivery Reviews, 47, 3–19. doi:10.1016/S0169-409X(00)00118-6

Nair, R., Reddy, B.H., Kumar, C.A., & Kumar, K.J. (2009). Application of chitosan microspheres as drug carriers: A review. Journal of Pharmaceutical Sciences and Research, 1, 1–12.
Nakayama, M., Shimatani, K., Ozawa, T., Shigemune, N., Tsugukuni, T., Tomiyama, D., ... Miyamoto, T. (2013). A study of the antibacterial mechanism of catechins: Isolation and identification of Escherichia coli cell surface proteins that interact with epigallocatechin gallate. *Food Control*, 33, 433–439. doi:10.1016/j.foodcont.2013.03.016

Ou, S., & Kwok, K.C. (2004). Ferulic acid: Pharmacological functions, preparation and applications in foods. *Journal of the Science of Food and Agriculture*, 84, 1261–1269. doi:10.1002/jsfa.1873

Palma-Guerrero, J., Gómez-Vidal, S., Tikhonov, V.E., Salinas, J., Jansson, H. R., & Lopez-Llorca, L.V. (2010). Comparative analysis of extracellular proteins from Pochonia chlamydosporia grown with chitosan or chitin as main carbon and nitrogen sources. *Enzyme and Microbial Technology*, 46, 568–574. doi:10.1016/j.enzmtec.2010.02.009

Palmeira-de-Oliveira, R., Palmeira-de-Oliveira, A., Gaspar, C., Silvestre, S., Martinez-de-Oliveira, J., Amaral, M.H., & Breitenfeld, L. (2011). Sodium Tripolyphosphate: An excipient with intrinsic in vitro anti-Candida activity. *International Journal of Pharmaceutics*, 421, 130–134. doi:10.1016/j.ijpharm.2011.09.030

Plascencia-Jatomea, M., Viniegra, G., Olayo, R., Castillo-Ortega, M.M., & Olayo, R. (2013). Effect of chitosan and temperature on spore germination of Aspergillus niger. *Macromolecular Bioscience*, 3, 582–586. doi:10.1002/mabi.200350024

Poncet, D. (2006). Microencapsulation: Fundamentals, methods and applications, in surface chemistry in biomedical and environmental science. In J.P. Blitz & V.M. Gun'ko (Eds.), *Surface chemistry in biomedical and environmental sciences* (pp. 23–54). Dordrecht: Springer. doi:10.1007/1-4020-4741-X_3

Rabea, E.I., Badawy, M.E.T., Stevens, C.V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules*, 4, 1457–1465. doi:10.1021/bm034130m

Reverberi, M., Fabбри, A.A., Zjalic, S., Ricelli, A., Punelli, F., & Fanelli, C. (2005). Antioxidant enzymes stimulation in Aspergillus parasiticus by Lentinula edodes inhibits aflatoxin production. *Applied Microbiology and Biotechnology*, 69, 207–215. doi:10.1007/s00253-005-1979-1

Robles-Sánchez, R.M., Rojas-Graú, M.A., Odrizola-Serrano, L., González-Aguilar, G.A., & Martín-Belloso, O. (2009). Effect of minimal processing on bioactive compounds and antioxidant activity of fresh-cut ‘Kent’ mango (Manifera indica L.). *Postharvest Biology and Technology*, 51, 384–390. doi:10.1016/j.postharvbio.2008.09.003

Saharan, V., Mehrotra, A., Khatik, R., Rawal, P., Sharma, S.S., & Pal, A. (2013). Synthesis of chitosan based nanoparticles and their in vitro evaluation against phytopathogenic fungi. *International Journal of Biological Macromolecules*, 62, 677–683. doi:10.1016/j.ijbiomac.2013.10.012

Sanpo, N., Ang, S.M., Cheang, P., & Khor, K.A. (2009). Antibacterial property of cold sprayed chitosan-Cu/Al coating. *Journal of Thermal Spray Technology*, 18, 600–608. doi:10.1007/s11666-009-9391-5

Sun, X., Wang, Z., Kadouh, H., & Zhou, K. (2014). The antimicrobial, mechanical, physical and structural properties of chitosan-gallic acid films. *LWT-Food Science and Technology*, 57, 83–89. doi:10.1016/j.lwt.2013.11.037

Wang, X., Luo, Z., & Xiao, Z. (2014). Preparation, characterization, and thermal stability of β-cyclodextrin/soybean lecithin lecithin inclusion complex. *Carbohydrate Polymers*, 101, 1027–1032. doi:10.1016/j.carbpol.2013.10.042

Woranuch, S., & Yoksan, R. (2013). Preparation, characterization and antioxidant property of water-soluble ferulic acid grafted chitosan. *Carbohydrate Polymers*, 96, 495–502. doi:10.1016/j.carbpol.2013.04.006

Yi, S., Wang, W., Bai, F., Zhu, J., Li, J., Li, X., ... He, Y. (2014). Antimicrobial effect and membrane active mechanism of tea polyphenols against *Serratia marcescens*. *World Journal of Microbiology and Biotechnology*, 30, 451–460. doi:10.1007/s11274-013-1464-4

Yien, L., Zin, N.M., Sarwar, A., & Katas, H. (2012). Antifungal activity of chitosan nanoparticles and correlation with their physical properties. *International Journal of Biomaterials*, 20, 1–9. doi:10.1155/2012/632698

Zhou, H.Y., Zhou, D.J., Zhang, W.F., Jiang, L.J., Li, J.B., & Chen, X.G. (2011). Biocompatibility and characteristics of chitosan/cellulose acetate microspheres for drug delivery. *Frontiers of Materials Science*, 5, 367–378. doi:10.1016/s11706-011-0146-0