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Influence of nonionic and ionic surfactants on the antifungal and mycotoxin inhibitory efficacy of cinnamon oil nanoemulsions

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Abstract

The influence of ionic surfactants (cationic surfactant lauric arginate and anionic surfactant lysolecithin) on the physical properties, antifungal and mycotoxin inhibitory efficacy of Tween 80 stabilized cinnamon oil-in-water nanoemulsions was investigated. Nanoemulsion droplets of similar particle diameter (~ 100 nm), but variable electrical characteristics, were formed by mixing 0.1 wt% ionic surfactant with 0.9 wt% Tween 80 before homogenization. The nanoemulsions were physically stable over 28 days at 23 ^{o}C. The antifungal activity (against mycelial growth and spore germination) and mycotoxin inhibitory activity of cinnamon oil nanoemulsions bearing positive, neutral, and negative charge surface was then evaluated against two chemotypes of *Fusarium graminearum*. In general, the cinnamon oil played a decisive role in the resulting antifungal and mycotoxin inhibitory activities. The surfactant charge had a limited impact on the antifungal mycotoxin inhibitory activities of cinnamon oil in the nanoemulsions. Both ionic surfactant-based cinnamon oil nanoemulsions showed greater activity in inhibiting mycelial growth and mycotoxin production of *F. graminearum* than those based on Tween 80. Treatment of mycelium with cinnamon oil nanoemulsions resulted in the loss of cytoplasm from fungal hyphae, and accounted for the antifungal action. These results have important implications for the design of essential oil based nanoemulsions as effective antifungal delivery systems in foods.

Keywords: Essential oil, antimicrobial, mycotoxin, emulsifier charge, nanoemulsion, mycelial growth.

1. Introduction

Fusarium head blight (FHB), caused by *Fusarium graminearum* and a number of other *F. spp*, is a global disease that can devastate wheat, barley, and other cereal grains. [1] In addition to the yield losses, FHB infected grains can be associated with the occurrence of mycotoxins that can inhibit protein synthesis and modulate immune responses. [2] The most common mycotoxin found in cereal grains is deoxynivalenol (DON), a type B trichothecene produced predominantly by *F. graminearum*. [3] Many of the *Fusarium* species that infect grains produce mycotoxins in the field, during storage under suboptimal conditions, and sometimes during food processing. [4] Surveillance of cereal-based retail foods and drink has demonstrated that mycotoxins can be transferred to the final products through contaminated raw materials, thus representing an threat to animal and human health. [5] As such, efficient strategies to not only inhibit the growth of *F. graminearum* growth, but also to prevent the mycotoxin accumulation are critically required for public health, as well as economic benefit.

The utilization of synthetic fungicidal preservatives to control fungal spoilage and ensure food safety has been a routine practice, worldwide, for decades. This however has become a major challenge for food industry due to the rise in consumer demand for natural or "label-friendly" food preservatives in food products. Plant essential oils (EOs) are gaining interest, owning to their properties against a wide spectrum of food-related spoilage and pathogenic microorganisms. [6,7] As potential natural inhibitors, EOs may replace the domination of synthetic fungicidal preservatives to effectively suppress fungal growth and mycotoxin production. Nevertheless, the practical application of EOs as natural preservatives in food is still limited mainly because of their poor water solubility, high volatility, and low antifungal efficacy in food systems. Encapsulation of EOs in nanoemulsion-based delivery systems has shown promising results to address these obstacles.[8]

A number of studies have shown that essential oils in nanoemulsion delivery systems exhibited enhanced solubility, physical stability, and antifungal efficacy when compared to bulk EOs. [9,10,11] For instance, a recent study reported that the mycelial growth of *Aspergillus niger* was totally inhibited by applying 0.25 μg cinnamon oil/g nanoemulsion, while only 75% inhibition of mycelial growth was observed when employing the same concentration of bulk cinnamon oil.[12] Our recent study also found that clove oil nanoemulsions improved inhibitory activities towards mycotoxin production by two *F. graminearum* isolates when compared to bulk clove oil.[13]

The performance of EOs incorporated into nanoemulsions against the growth of microorganisms largely relies on the formulation, particularly the type of surfactants. [14] In general, fungi/ bacteria carry a negative surface charge under culture conditions. One proposed antifungal mechanism of these surfactants is based on their ability to disrupt the integral fungi membrane by a combined hydrophobic and electrostatic adsorption phenomenon at the membrane/water interface, thus giving rise to fungi membrane disruption. As such, the antifungal or antimicrobial activity of encapsulated essential oils might be expected to increase when they are incorporated into cationic surfactants. On the basis of such as premise, the synergistic antimicrobial activity of cinnamon leaf oil or thyme oil nanoemulsion, when stabilized with a positive charged LAE, was demonstrated against *Listeria monocytogenes* growth. [15,16] Meanwhile, there have been some contradictory results published. For example, clove oil nanoemulsion stabilized with the negatively charged gum arabic showed prolonged antibacterial activities against Gram positive bacterial growth than that with a nonionic surfactant Tween 80. [17] Cinnamon leaf oil nanoemulsion formulated with either negatively charged Q-Naturale or a whey protein isolate had higher antifungal activity than those formulated with Tween 80. [12] More surprisingly, a handful of surfactants such as lauric arginate (LAE), chitosan, and sodium dodecyl sulfate (SDS) have shown inherent antifungal activities. [15,

[18] However, LAE and SDS showed stronger fungal inhibitory activity in solution rather than in thyme essential oil nanoemulsions, indicating the inherent antifungal activity of surfactants might be mitigated under certain nanoemulsion systems. [14] In order to achieve greater performance of EOs, it is therefore important to not only design a physically stable nanoemulsion delivery system to incorporate EOs, but moreover to understand how formulation and the charge differences of surfactants may potentially impact the antifungal efficacy and inhibition of mycotoxin production of the EO nanoemulsions.

In this study, cinnamon essential oil (CEO) was utilized as a model EO to formulate antifungal nanoemulsions. It is one of the most widely produced and consumed EOs that concomitantly demonstrates antifungal activities against *F. spp*. [19,20] The main objective of the current research was to develop physically stable CEO nanoemulsions containing droplets with different surface charges by employing cationic LAE, nonionic Tween 80, and anionic lysolecithin. The antifungal activities of CEO nanoemulsions against mycelial growth and spore germination, and the mycotoxin inhibitory activity against two chemotypes of *F. graminearum* were investigated. In addition, we aimed to determine whether using a combination of antifungal agents (CEO and LAE) within a single nanoemulsion system would have a synergistic effect on the overall antifungal efficacy of CEO.

2. Materials and methods

2.1 Materials

Cinnamon essential oil (CEO), Tween 80, carboxymethyl cellulose, glacial acetate acid, sodium acetate trihydrate, mirex used as an internal standard in mycotoxin analysis, osmium tetraoxide, and Bis(trimethylsilyl)acetamide (BSA)/trimethylchlorosilane (TMCS)/Trimethylchlorosilane (TSIM) kit were purchased from Millipore Sigma Co. (St. Louis, MO, USA). Powdered enzyme

modified lysolecithin (ALCOLEC EM:40 % phosphatidylcholine) was kindly provided by the American Lecithin Company (Oxford, CT, USA). Lauric arginate LAE 20 was donated by the A&B Ingredients Company (Fairfield, NJ, USA). Medium-chain triglyceride (MCT, NEOBEE M-20) was kindly provided by the Stepan Company (Bordentown, NJ, USA). All chemicals were used as received without further purification. Potato dextrose agar (PDA) was purchased from AMRESCO (Solon, OH, USA). Potato dextrose broth was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Mung beans and long grain white basmati rice were purchased in a local market (Fargo, ND, USA). All solutions were prepared using ultrapure distilled de-ionized water (DDW, 18.2 MΩ cm, Barnstead ultrapure water system, Thermo Fisher Scientific, USA).

2.2. Nanoemulsion preparation

Cinnamon oil nanoemulsions was prepared using 95 wt% of aqueous phase and 5 wt% of oil phase. The aqueous phase consisted of 1 wt% total surfactant mixture (Tween 80, Tween 80: LAE=9:1, and Tween 80: lysolecithin = 9:1, **see Section 3.1**) that was dispersed in 94 wt% buffer solution (10 mM acetic acid buffer, pH 3.5). The oil phase was prepared by mixing cinnamon oil with MCT at a mass ratio of 2:3 prior to homogenization. The oil phase was then mixed with the aqueous phase using a high-speed hand blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min. The mixture was further homogenized using a high pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) at 15,000 psi for three passes. The nanoemulsion consisting of pure MCT in oil phase was used as a control. After homogenization, the nanoemulsions were collected and stored at 23 °C for a long term storage stability study. The following four types of nanoemulsions were prepared and used in subsequent studies on antifungal efficacy and inhibition of mycotoxin production.

(i) Cinnamon oil-in-water nanoemulsion containing nonionic surfactant (COE-Tween 80): 2 wt%

cinnamon oil, 3 wt% MCT, and 1 wt% Tween 80;

(ii) Cinnamon oil-in-water nanoemulsion containing cationic surfactant (COE-LAE): 2 wt% cinnamon oil, 3 wt% MCT, 0.9 wt% Tween 80, and 0.1 wt% LAE;

(iii) Cinnamon oil-in-water nanoemulsion containing ionic surfactant (COE-Lysolecithin): 2 wt% cinnamon oil, 3 wt% MCT, 0.9 wt% Tween 80, and 0.1 wt% lysolecithin;

(iv) MCT oil oil-in-water nanoemulsion containing cationic surfactant (MCT-LAE): 5 wt% MCT, 0.9 wt% Tween 80, and 0.1 wt% LAE.

2.3. Particle size and ζ-potential measurement

Particle size, size distribution, and zeta-potential of all nanoemulsions during storage time at 23 ℃ were measured by a dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK). The detailed procedures are described by Jing and coworkers. [13]

2.4. Antifungal activities of nanoemulsions on mycelial growth

Two chemotypes of *F. graminearum* isolates (10-124-1 and 10-125-1) were selected for evaluation of the antifungal efficacy of cinnamon oil nanoemulsions. Isolate 10-124-1 is a representative of DON and 15-acetyl-deoxynivalenol (15ADON) producers and isolate 10-125-1 is a representative of deoxynivalenol (DON) and 3-acetyl-deoxynivalenol (3ADON) producers.[21] These isolates were stored at −80 °C and refreshed on PDA plates. The PDA cultures were incubated in darkness at 23 °C for 5 days prior to use.

For the antifungal tests, 500 μL diluted nanoemulsions were introduced to the surface of PDA plates. A plate prepared by adding the same amount of buffer solution was considered as a control. An agar plug of 5 mm diameter containing fungal mycelial from 5-day-old culture on PDA was

placed at the center of plate and incubated at 23 °C for 4 days. The diameter of mycelial growth on each plate was measured and compared to the control. The mycelial growth inhibition (MGI) rate was calculated as MGI rate (%) =100 × (mycelial growth diameter of control – mycelial growth diameter of treatment) / mycelial growth diameter of control.

2.5. Scanning electron micrograph (SEM) analysis

SEM was conducted as described by A. Kheiri and their co-works with some modifications.[22] Isolate 10-124-1 was grown on PDA plates for 4 days. Then, 2 mL of each nanoemulsion was sprayed evenly on the surface of mycelium and incubated for 24 h. Mycelium treated with 2 mL of acetate buffer (10 mM pH 3.5) was considered as a control. After incubation at 23 °C for 24 h, the entire specimens of each treatment were fixed in 2.5% glutaraldehyde for 24 h and rinsed 3 times with 0.02 M phosphate buffer. Afterwards, the samples were treated with 1% osmium tetroxide for 1 h and washed using distilled water. Subsequently, the specimen was dehydrated in a graded ethanol series (30% to 100%), critical-point dried, and sputter coated with gold. The specimen was then observed with a SEM model JSM-6490LV (JEOL, Peabody, USA) at an accelerating voltage of 15 kV.

2.6. Antifungal activities of nanoemulsions on spore germination of *F. graminearum*

Spores from two *F. graminearum* isolates were incubated on carboxymethyl cellulose (CMC) medium (15.0 g CMC, 1.0 g NH_4NO_3, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g yeast extract per liter distilled water) media in the light at 23 °C for 2 weeks. This was then filtered through a double layer of Miracloth (Sigma, St. Louis, USA) to obtain the spore solution. The concentration of spore

solution was measured using a hemacytometer (Levy Ultraplane, PA, USA) and adjusted to 1.0×10^6 spores/mL. One milliliter of spore suspension was mixed with 0.8 mL nanoemulsion, and 100 μL aliquots of the mixture were spread onto the surface of PDA plates. Acetate buffer (10 mM, pH 3.5) was used as a control. Plates were incubated at 23 °C for 8 h and lactophenol cotton blue (VWR International, PA, USA) was applied to the inoculation sites on plates to stop the spore germination. The spore germination was observed using an Olympus bright field microscope (BX51 Olympus, Olympus Optical, Hamburg, Germany) and germinated spores were counted using a hemacytometer. A spore was considered germinated when the germ tube was longer than one-half of the spore diameter. The percentage of spore germination rate was calculated according to this formula: % Germination rate = the number of germinated spore / the number of total spore × 100. Three replicates were conducted for each treatment, and a minimum of 100 spores were counted in each replicate.

2.7. Influence of nanoemulsions on mycotoxin production in rice culture

Experiments measuring the inhibition of mycotoxin production were carried out in solid rice culture according to the previously reported method with some modifications.[18] Briefly, long-grain rice kernels were soaked in deionized water for 10 h in a beaker and the excess water was then decanted off. Forty grams of soaked rice kernels were added into a 250 mL Erlenmeyer flask and autoclaved at 121 °C for 1 h. After cooling to room temperature, the sterile rice culture was inoculated with 2 mL of the *F. graminearum* spore solution and 1.6 mL of the nanoemulsions. Rice cultures treated with the same amount of acetate buffer (1.6 mL) were considered as controls. The final concentration of cinnamon oil in the rice cultures was 800 μg/g rice. Samples were incubated in the dark at 23 °C for 10 days, and then stored at -80 °C for 24 h and lyophilized (Model: 10-MRSM, VirTis, Gardiner, USA) for 4 days. Mycotoxins in the rice cultures, including

DON, 15ADON, and 3ADON, were extracted and analyzed using an Agilent 6890N gas chromatograph (GC) coupled with 5973 mass spectrometer (MS) (Agilent Technologies, Santa Clara, USA) as previously described. [23]

2.8. Statistical analysis

All measurements were performed at minimum in triplicate using freshly prepared samples (i.e., new samples were prepared for each series of experiments) and were reported as mean ± standard deviation. One-way analysis of variance (ANOVA) was conducted using IBM SPSS 22 (IBM Corp., USA), and significant difference of mean value was defined at $p < 0.05$ by Tukey's test.

3. Results and Discussion

3.1. Impact of surfactant type on cinnamon oil nanoemulsion formation

In general, the antifungal efficacy of EO nanoemulsions is strongly reliant on their formulation (e.g., oil phase composition and emulsifier) and physical characteristics of nanoemulsion droplets, such as particle size and electrical characteristics. Ostwald ripening is a common problem responsible for the instability of essential oil emulsions or nanoemulsions due to the relatively high water solubility of essential oil leading to the mass transport of dispersed phase from one droplet to another. The use of water-insoluble lipids has been widely reported as an effective solution to retard Ostwald ripening in nanoemulsions. [24] Hence, in addition to the antifungal cinnamon oil (40%), the oil phase of the nanoemulsion also consisted of 60% MCT, which we found to be an effective Ostwald ripening inhibitor that prevents droplet growth in nanoemulsions (unpublished data). Herein, we exclusively examined the influence of Tween 80, LAE, and lysolecithin, representatives of nonionic, cationic and anionic surfactant, respectively, on the formation and stability of CEO nanoemulsions. Our preliminary study along with others suggested that physically

stable CEO nanoemulsions cannot be achieved with just LAE or lysolecithin. [15,25] Therefore, we mixed the nonionic surfactant (0.9 wt% Tween 80) with either LAE or lysolecithin (0.1 wt%) before homogenization. The particle size and ζ-potentials of CEO nanoemulsion droplets stabilized with different types of surfactants was determined (**Fig. 1**).

Fig. 1 inserted

All CEO formulations had a similar mean particle diameter ranging between 91 and 95 nm, which supports the successful formation of nanoemulsions (**Fig. 1A**). The mean particle size of the 100% MCT nanoemulsion was 121 nm. This was slightly higher than that of the CEO nanoemulsions, and can likely be attributed to the increase in lipid phase viscosity and interfacial tension.

As an important physical characteristic, the electrical charge of droplets not only has a significant impact on their physical stability, but also on their antifungal efficacy. As shown in **Fig. 1B**, the initial nonionic Tween 80 (1 wt%) stabilized CEO nanoemulsion (CEO-Tween 80) was slightly negative (-0.9 ± 0.1 mV) at pH 3.5, which can be attributed to the presence of some anionic impurities in Tween 80 (such as free fatty acids) or adsorption of anionic species from the water (such as hydroxyl ions) to the droplet surfaces. [14] When 0.1 wt% anionic lysolecithin was used as a co-surfactant, the ζ-potential of CEO nanoemulsion (CEO-Lysolecithin) became much more intensely negative (-16.0 ± 0.1 mV). This suggested that the negatively charged lysolecithin molecules replaced some of the nonionic Tween 80 molecules and adsorbed to the interface of the oil-in-water nanoemulsion. Conversely, when 0.1 wt% LAE was applied to the delivery system, the droplet charges of CEO nanoemulsions (CEO-LAE and MCT-LAE) became strongly positive, with the ζ-potential of nanoemulsions being 14.6 ± 0.5 mV and 16.8 ± 0.4 mV, respectively. It indicated that positively charged LAE was able to deposit on the droplet surface of the nanoemulsions. The results suggest that CEO nanoemulsions with a range of different surface

charge characteristics can be prepared by mixing nonionic surfactants with different types of ionic surfactant solutions prior to the homogenization.

3.2. Storage stability of cinnamon oil nanoemulsions

A prerequisite for application nanoemulsions as delivery systems for antifungal agents in the food industry is that nanoemulsion-based delivery systems maintain their physical stability throughout the intended shelf-life. We therefore evaluated the long term stability of antifungal CEO nanoemulsions by monitored the change of mean particle diameter and particle size distribution of nanoemulsions for 28 days at 23 ^{o}C.

Fig. 2 inserted

As shown in **Fig.2A**, the mean particle size of CEO-LAE and CEO-Lysolecithin were highly stable against droplet growth through the whole storage time. On the other hand, the mean particle diameter of nanoemulsions stabilized with 100% Tween 80 gradually increased from 92 to 144 nm over the course of 28 days' storage. Nevertheless, there was no visible creaming or depletion flocculation in all tested nanoemulsions after 28 days storage at 23 ^{o}C (**Fig.2A** inserted picture). The results implied that the physical stability of CEO nanoemulsions was improved substantially in the presence of ionic surfactants (LAE and lysolecithin). Meanwhile, the particle size distributions of all tested CEO nanoemulsions were very similar before and after storage (**Fig. 2B**), which again highlights their good stability against droplet growth. The results also proved that the use of MCT can effectively produce a stable CEO nanoemulsion by inhibiting Ostwald ripening.

3.3. Effect of cinnamon oil nanoemulsions on mycelial growth

The mycelial growth inhibition (MGI) rate of cinnamon nanoemulsions was determined by comparison with acetate buffer (10 mM, pH 3.5) controls. We did not examine the antifungal

activity of nanoemulsions prepared with 100% cinnamon oil due to their extremely unstable physical nature. Since LAE and cinnamon oil both possess antifungal activity, one would expect nanoemulsions containing dual antifungal agents (CEO-LAE) might have different antifungal activity than those with a single agent (CEO or MCT-LAE). Therefore, the antifungal activity of a 100% MCT nanoemulsion stabilized with LAE were also examined to determine the antifungal activity of LAE itself (**Fig. 3A**).

Fig. 3 inserted

The results clearly showed that the MCT-LAE nanoemulsion exhibited minimum antifungal activity (< 16%) in terms of inhibition of mycelial growth in both *F. graminearum* isolates (**Fig. 3A**). The antifungal activity of LAE has been documented with *A. niger*, *Penicillium chrysogenum,* and *P. digitatum*, but generally under conditions of high concentration. [26–28] For instance, Xu and coworkers reported that remarkable leakage of intracellular protein from *P. digitatum* was observed after treatment with 400 μg/ml of LAE solution. [27] The different fungi used in this study likely explain the observation of low antifungal activity from LAE. Although MGI rate of all CEO nanoemulsions was remarkably higher than that of MCT-LAE, there was no appreciable difference among them with regard to surfactant type, with MGI ranging from 83 to 89%.

Interestingly, electrostatic interactions seemed to exist between the nanoemulsion droplets and the two isolates as evidenced by measuring ζ-potential of the systems at pH 3.5. The initial ζ-potential of *F. graminearum* isolate 124-1 and 125-1 was -6.44 and -2.97 mV, respectively. This signifies that two isolates have different physiological properties (**Fig. 3B**). After mixing with nanoemulsions, the ζ-potential of the two isolates varied strikingly, and was largely determined by the type of surfactant coated on the nanoemulsion droplets. In general, no significant changes of ζ-potential were observed in fungal solutions with the Tween 80 stabilized nanoemulsions. Albeit

the presence of ionic surfactants stabilized CEO nanoemulsions substantially increased the magnitude of ζ-potential in fungal solution, the type of surfactants rendered opposite impact on the sign of ζ-potential. A positive charge was observed exhibited when the fungal solutions were mixed with cationic LAE stabilized CEO emulsions, whereas a negative change occurred with the ionic counterpart. These results support the existence of electrostatic interactions (attraction and repulsion) between the charged surface of nanoemulsion droplets and the *F. graminearum* isolates. Such electrostatic interactions between charged nanoemulsions and fungi cell membrane, however, was not supported to be the results for the enhanced antifungal activity in the current study.

In an effort to better understand the mechanism by which cinnamon oil nanoemulsions inhibit mycelial growth, morphological changes of *F. graminearum* isolate 10-124-1 hyphae treated with CEO nanoemulsions for 24 h were examined using SEM (**Fig. 4**). The results of SEM on the control displayed a normal morphology and linearly shaped hyphae with a smooth surface. The SEM image of cinnamon oil nanoemulsions treated *F. graminearum* revealed the alterations in the hyphae that appeared severely collapsed and flattened (arrow in **Fig. 4**) due to the lack of cytoplasm. Moreover, long-strip shaped hyphae were dissolved with the loss of linearity after treated with CEO-LAE nanoemulsion for 24 h. This observation was in agreement with the results of mycelial growth *F. graminearum* that CEO play a paramount role in inhibiting *F. graminearum* mycelial growth. A widely cited study reported that a complete inhibition of mycelial growth of toxigenic *F. moniliforme*, *A. flavus*, *A. parasiticus*, and *A. ochraceus* was accomplished after immersing the wheat grain in 500 ppm cinnamon essential oil. [29] Our findings support the proposed mechanism of action which involves cinnamon oil interfering with the enzymatic reactions of fungal cell wall synthesis, which affects morphogenesis and mycelial growth, resulting in profound morphological and ultrastructural alterations. These include disruption of the plasma membrane

and the lack of loss of cytoplasmic contents. [19,30]

Figure 4 inserted

3.4. Effect of cinnamon oil nanoemulsions on spore germination

It is well known that spore germination is the initial stage in the development of fungal mycelia. Understanding the inhibitory activity of cinnamon oil nanoemulsions on the spore germination would help corroborate their mycelial inhibition activity. The impact of cinnamon oil nanoemulsions stabilized with different types of surfactants on the spore germination inhibition (SGI) rate of *F. graminearum* was evaluated after 8 h (**Fig. 5)**.

Figure 5 inserted

Consistently, no inhibitory activity was detected with application of the MCT-LAE nanoemulsion. As discussed earlier, the antifungal efficacy of LAE is dependent on the application system, LAE state (e.g., solution *vs* nanoemulsion), and target fungi. For instance, previous studies revealed that the use of LAE in an oil-in-water emulsions or solid lipid particles reduced their antimicrobial effectiveness. [31] In contrast, all the cinnamon oil nanoemulsions exhibited 100% SGI rate across the tested two isolates. Since the spore germination was completely inhibited by applying CEO-LAE and no inhibition was observed in MCT-LAE, we concluded that inhibition of spore germination mainly stemmed from cinnamon oil. The proposed mechanisms for cinnamon oil to inhibit fungi spore germination include their ability to denature the enzymes and selective inactivation the synthesis of essential mRNA participating in the germination process. [32] From the result, it appeared that surfactant type and charge had negligible effect on the observed SGI rate. This can be explained by the fact that cinnamon oil plays the sole and independent role against spore germination.

3.5 Effect of cinnamon oil nanoemulsions on mycotoxin production

Reduced fungal growth is not always associated with the suppression of mycotoxin production since they might have involved different modes of action. In food chain, one key food safety issue that remains unresolved in the food chain, is how to avoid the consumption of foods contaminated with mycotoxin. Thus, it is crucial to evaluate the effect of CEO nanoemulsions on the inhibition of mycotoxin produced by *F. graminearum*. Spores from isolates 124-1 or 125-1 were mixed with CEO nanoemulsions prior to inoculation into rice cultures. Again, the mycotoxin inhibitory activity of MCT-LAE nanoemulsion was assessed and compared with that of the CEO-LAE nanoemulsion. The effect of nanoemulsions on the production of DON and its derivatives in the rice cultures was assessed, and the results were expressed as the mycotoxin inhibition rate (%) (**Fig. 6**).

Figure 6 inserted

In general, all nanoemulsions showed inhibition of mycotoxin production. Among the two *F. graminearum* isolates, isolate 10-124-1 was most sensitive to the action of cinnamon oil nanoemulsions. As shown in **Fig. 6**, the inhibition rate of DON produced by isolate 10-124-1 and 10-125-1 in rice culture with the treatment of CEO-LAE nanoemulsion was 98.4% and 92.9%, respectively. As aforementioned, physiological differences in the characteristics of the two *F. graminearum* isolates was witnessed in light of the significant difference in the surface charge of spore solutions (**Fig. 3B**). Generally, *F. graminearum* isolates are considered as a meta-population consisting of numerous relatively independent developing populations due to their genetic and biological differences in performance. [33] Different responses of *F. graminearum* isolates against the same antifungal agent have been previously reported. [18,34] As an example, Pagnussatt and coworkers found that a phenolic extract from *Spirulina* algae exhibited different inhibition

activities for mycelial growth and mycotoxin productions between the two *F. graminearum* isolates used. [34]

Likewise, the oil phase composition (MCT and cinnamon oil) had an appreciable influence on the mycotoxin inhibition rate. Unlike its activity in preventing mycelial growth, the MCT-LAE nanoemulsion generated certain degree of inhibition on mycotoxin production in this study. The inhibition rate of DON and 15ADON in rice culture treated with MCT-LAE was 48.3% and 36.7 %, respectively (**Fig. 6A**). However, there were minimal to no antifungal activity of MCT-LAE nanoemulsions against mycelial growth and spore germination. As mentioned above, different mechanisms of action of EOs might be involved in the inhibition of fungal growth and mycotoxin production. This was evidenced in a previous study that showed aflatoxin production to be completely inhibited by addition of 4 μL/mL essential oil from *C. virosa* var. *latisecta,* while the growth of mycelial was still observed.[35] The presence of CEO in nanoemulsion significantly promoted the inhibition of DON productions (≥ 92%) in both isolates, suggesting that cinnamon oil governs the inhibition of mycotoxin production by *F. graminearum*. The effects and the possible mode of actions of essential oils on mycotoxin production of some toxigenic fungal genera like *F. spp*. and *A. spp* have been previously reported. [33] For instance, ergosterol is specific to fungi and is the major sterol component of fungal cell membrane that be responsible for maintaining fungi cell function and integrity. Previous researchers suggested that eugenol and/or cinnamaldehyde in cinnamon oil induced inhibition of ergosterol, leading to a reduction in Ochratoxin A production. [36] Another recent study indicated that turmeric essential oil disrupted the integrity of the plasma membrane and mitochondria of *A. flavus*, which induced fungal metabolic stagnation and down-regulation of mycotoxin gene expression in the mycotoxin biosynthesis pathway. [37]

With regards to the impact of surfactant charge on the inhibition of mycotoxin production, the results showed that ionic surfactant based nanoemulsions exhibited a higher mycotoxin inhibition rate than nonionic based (Tween 80) one (p<0.5). The mechanisms of ionic surfactants leading to suppression of mycotoxin production are yet to be well understood. However, three possible mechanisms appear to be involved in the mycotoxin inhibition action of CEO nanoemulsions. First, the electrostatic interaction of positively charged (CEO-LAE) nanoemulsion droplets with negatively charged fungal spore cell walls, which can potentially increase the diffusion rate of the nanoemulsion droplets into the cell membrane. As a consequence, it might promote the cinnamon oil on inhibition of some key enzyme activity in fungi, thus reducing its ability to produce the toxin. The electrostatic interaction between *F. graminearum* spore and ionic surfactant stabilized nanoemulsion was shown in **Fig 3B**. For example, the initial ζ-potential of isolate 10-124-1 spore solution and CEO-LAE nanoemulsion was -6.44 and +14.6 mV, respectively. Upon mixing, the magnitude of the ζ-potential decreased to +10.30 mV compared to CEO-LAE nanoemulsion itself because of the electrostatic attraction. It was worth noting that the ζ-potential magnitude of anionic lysolecithin stabilized CEO and *F. graminearum* spore solution mixture was also decreased. One possible reason is that the electrostatic deposition of negative charged nanoemulsion droplets onto the positively charged moiety (e.g., protein) on cell wall of *F. graminearum* spore, that consists of 74.5% carbohydrate, 4.5% protein, 3.0% lipid, 4.5% ash, and 0.3% phosphorus [38]. The slightly enhanced mycotoxin inhibitory activity of ionic surfactant coated CEO may be explained by the absorbed spore layer on nanoemulsion droplet, allowing the efficient diffusion of CEO to fungal cell. Second, nanoemulsion composition may impact the efficacy of CEO to inhibit mycotoxin production. The sustained release of CEO from the nanoemulsion is anticipated due to the restrained partitioning of CEO between oil phase and aqueous phase. Meanwhile, some researcher

suggested that the complex surface composition of fungal cells might offer a large number of suitable interaction sites with the negative emulsifier stabilized EO nanoemulsion (e.g., whey protein isolate), hence driving the higher efficacy of antifungal EOs. [12]

4. Conclusions

In summary, physically stable cinnamon oil-in-water nanoemulsions can be prepared by mixing either cationic (LAE) or anionic (lysolecithin) with a nonionic surfactant (Tween 80) before homogenization. The nanoemulsion droplets displayed a wide range of electrical characteristics, from -16.0 to +16.8 mV. This study showed the decisive role of cinnamon plays in both antifungal and mycotoxin inhibitory activity. In the absence of cinnamon oil, the cationic LAE stabilized MCT nanoemulsion displayed imperceptible activity on mycelial growth and spore germination. The presence of cinnamon oil allowed nanoemulsion to inhibit mycelial growth and spore germination, as well as the mycotoxin production. The addition of 0.1 wt% ionic surfactant (LAE and lysolecithin) not only greatly enhanced the long-term physical stability, it also benefited the mycotoxin inhibitory efficacy of cinnamon oil-in-water nanoemulsion. This effect may be derived from the ionic surfactant coated cinnamon oil nanoemulsion droplets that be attracted to anionic fungi surfaces or cationic moiety of fungi cell wall, prompting the amount of cinnamon oil available to interact with the fungi cell membrane. The results of this study provide important guidelines for the design of essential oil nanoemulsion as antifungal delivery systems in the food and other industries.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Figure Captions

Figure 1. Effect of emulsifier type on mean particle size and zeta-potential of cinnamon oil nanoemulsions. Columns with different letter were significantly different between each other ($p<0.05$).

Figure 2. Effect of emulsifier types on long-term stability of cinnamon oil nanoemulsions determined by (A) mean particle size (the inserted pictures are the visual observation of nanoemulsions) and (B) particle size distribution over 28 days' storage.

Figure 3. Cell surface charge of *F. graminearum* (124-1 and 125-1) spore solution mixed with cinnamon oil nanoemulsions at pH 3.5 after storage for 12 h at room temperature (Inserted images representing the appearance of mixed fungal spore solution with different cinnamon oil nanoemulsions).

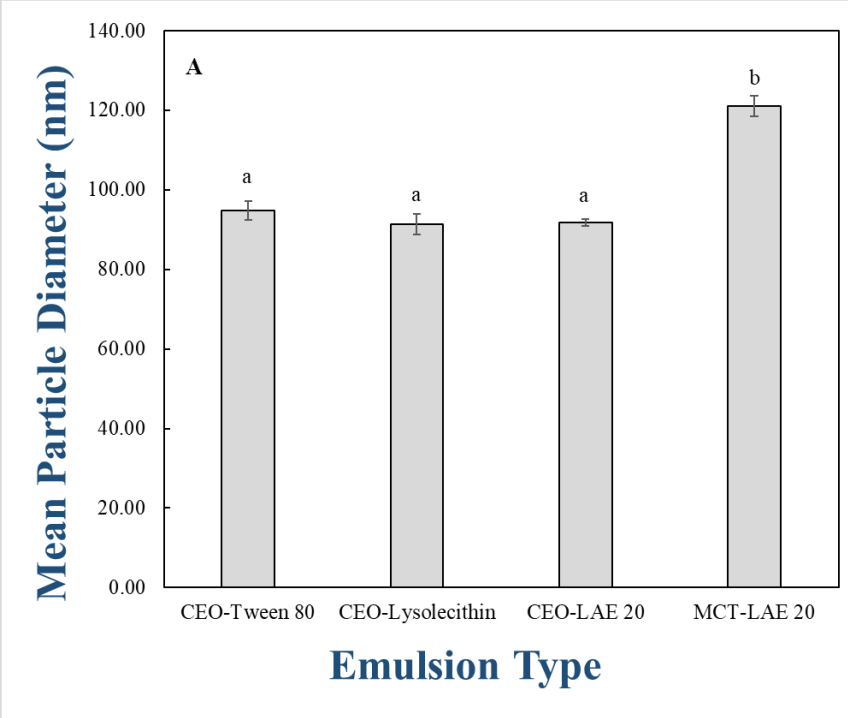
Figure 4. Effect of emulsifier type on mycelial growth of *F. graminearum* isolates. Columns with different letter were significantly different between each other ($p<0.05$).

Figure 5. Scanning electron microscopic observation of hyphae of *F. graminearum.* Hyphae samples treated by cinnamon oil nanoemulsions and control.

Figure 6. Effect of emulsifier type on spore germination rate of *F. graminearum* isolates

Figure 7. Effect of thyme oil nanoemulsions on inhibition of mycotoxins produced by *F. graminearum* isolate 10-124-1 (A) and by isolate 10-125-1 (B) in rice culture. Columns with different letter were significantly different between each other ($p<0.05$).

Figure 1


A
Mean Particle Diameter (nm)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
a
a
a
b
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
Emulsion Type

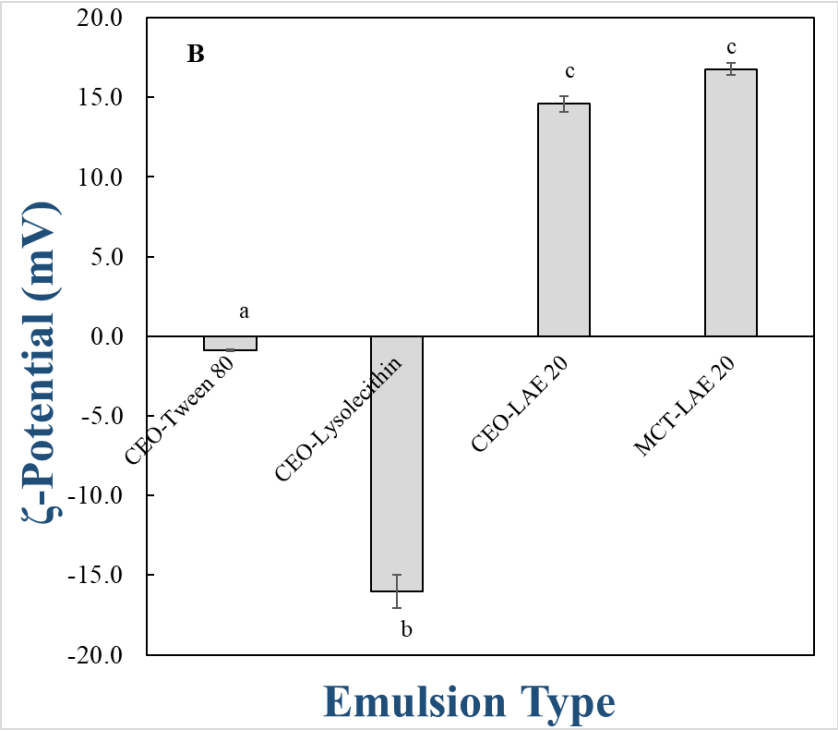
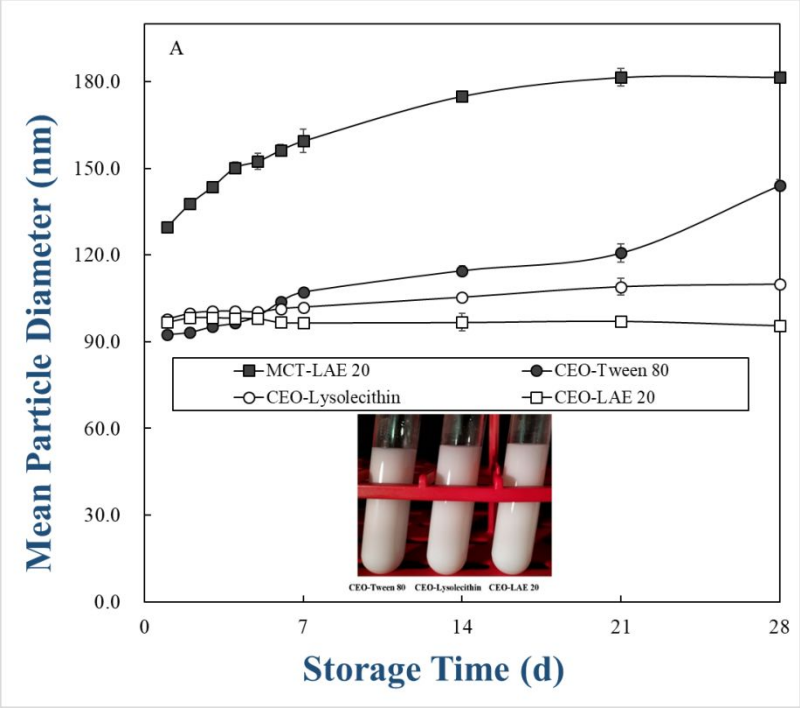

B
ζ-Potential (mV)
20.0
15.0
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
a
b
c
c
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
Emulsion Type

Figure 2


A
Mean Particle Diameter (nm)
180.0
150.0
120.0
90.0
60.0
30.0
0.0
0
7
14
21
28
Storage Time (d)
MCT-LAE 20
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
CEO-Tween 80 CEO-Lysolecithin CEO-LAE 20

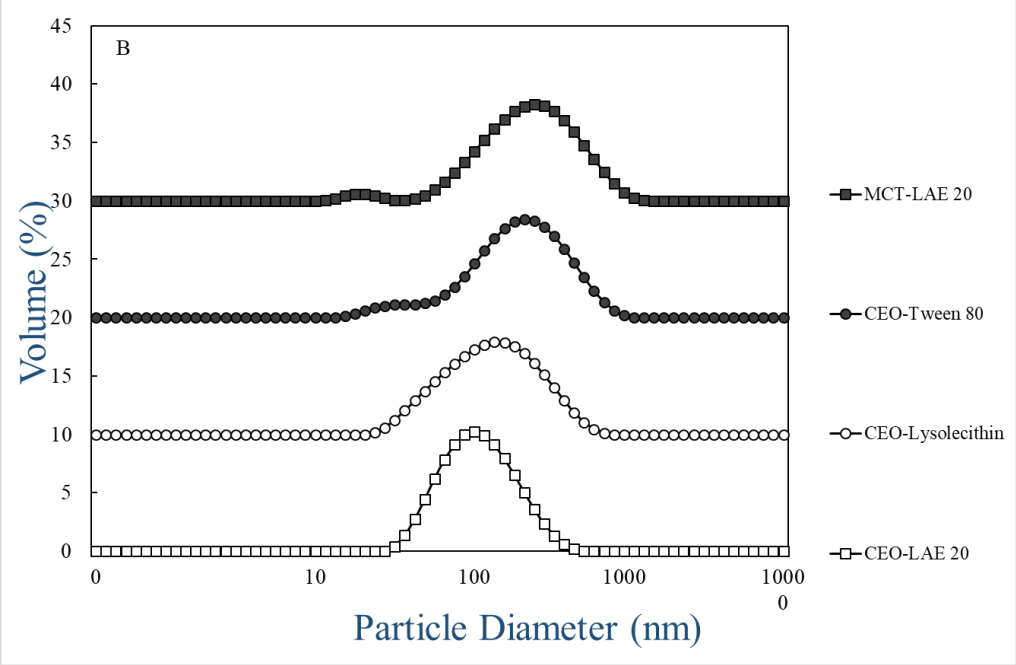

B
Volume (%)
45
40
35
30
25
20
15
10
5
0
0
10
100
1000
10000
Particle Diameter (nm)
MCT-LAE 20
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20

Figure 3

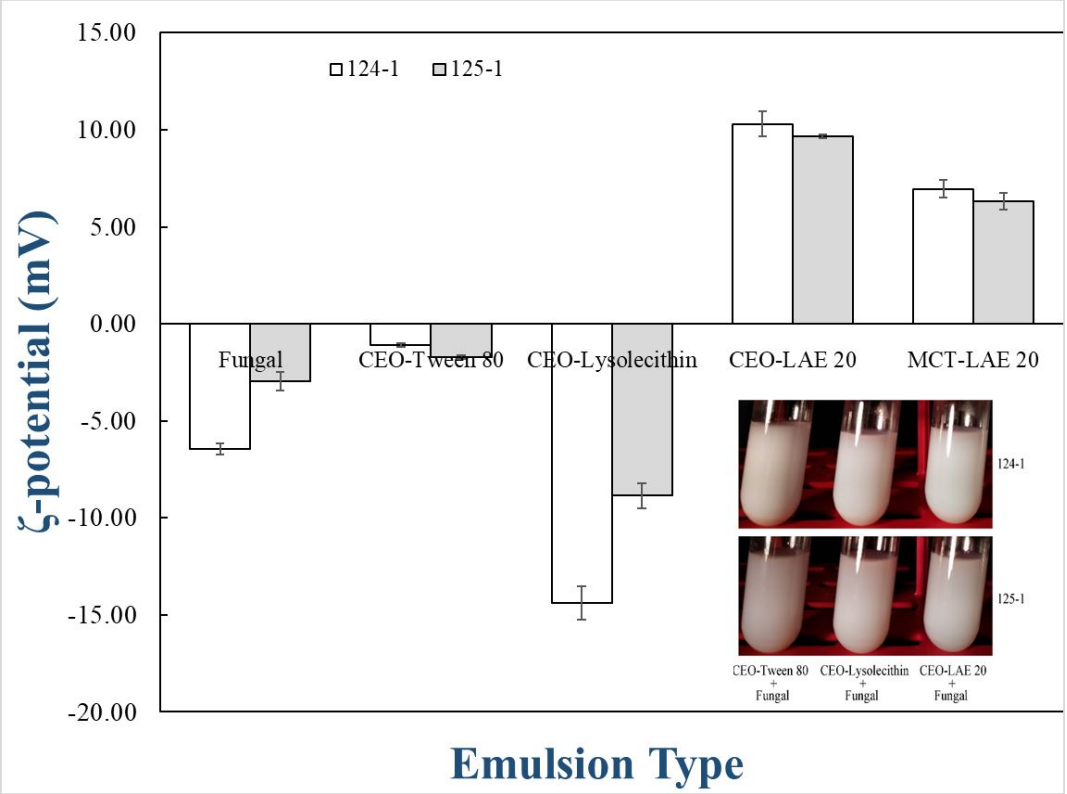

15.00
10.00
5.00
0.00
-5.00
-10.00
-15.00
-20.00
124-1
125-1
ζ-potential (mV)
Fungal
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
124-1
125-1
CEO-Tween 80 + Fungal
CEO-Lysolecithin + Fungal
CEO-LAE 20 + Fungal
Emulsion Type

Figure 4

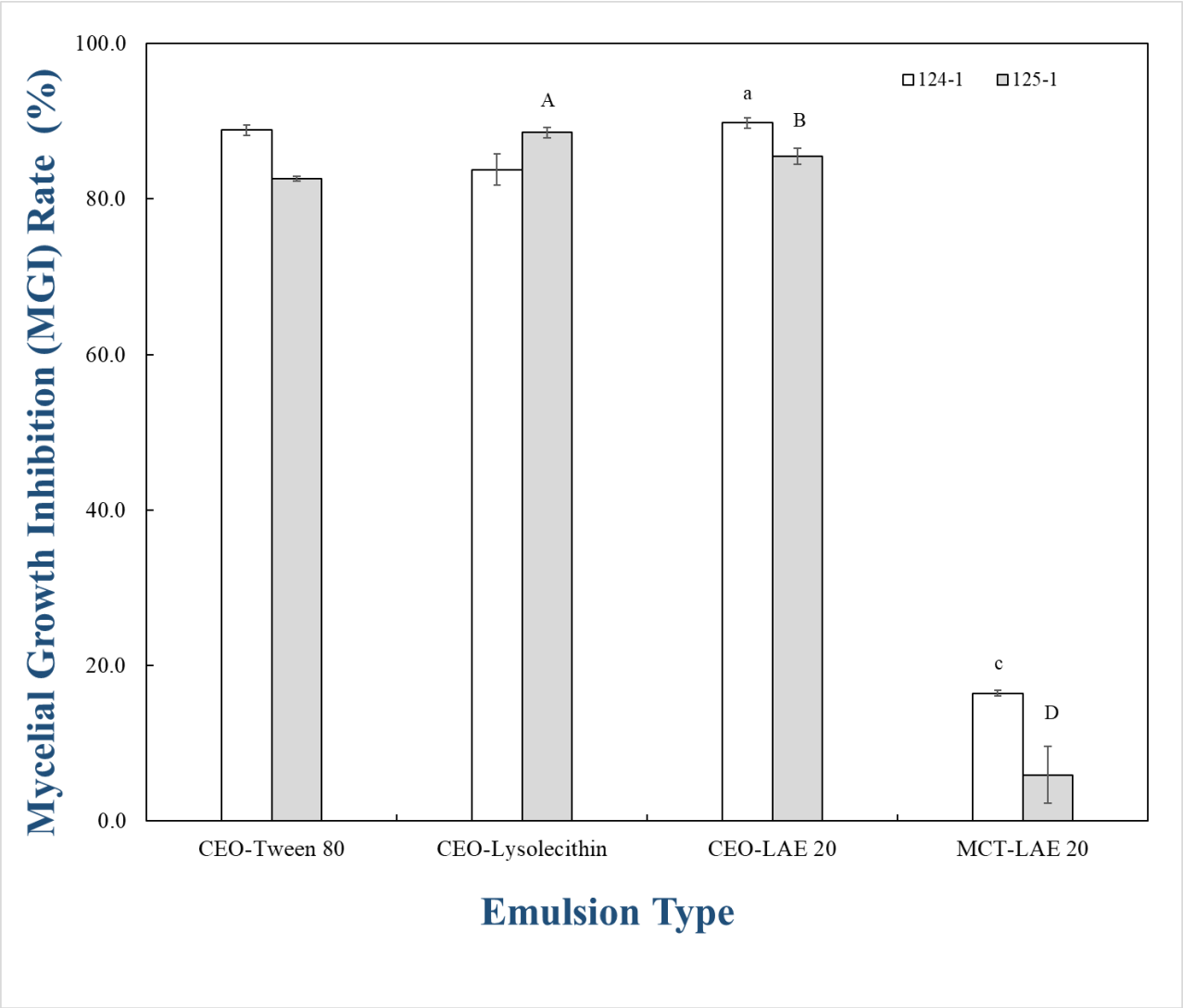

Mycelial Growth Inhibition (MGI) Rate (%)
100.0
80.0
60.0
40.0
20.0
0.0
124-1
125-1
A
a
B
c
D
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
Emulsion Type

Figure 5


15kV X10,000 1μm
15kV X10,000 1μm
CEO-Tween 80
CEO-Lysolecithin
15kV X10,000 1μm
15kV X10,000 1μm
CEO-LAE 20
Acetate Buffer

Figure 6

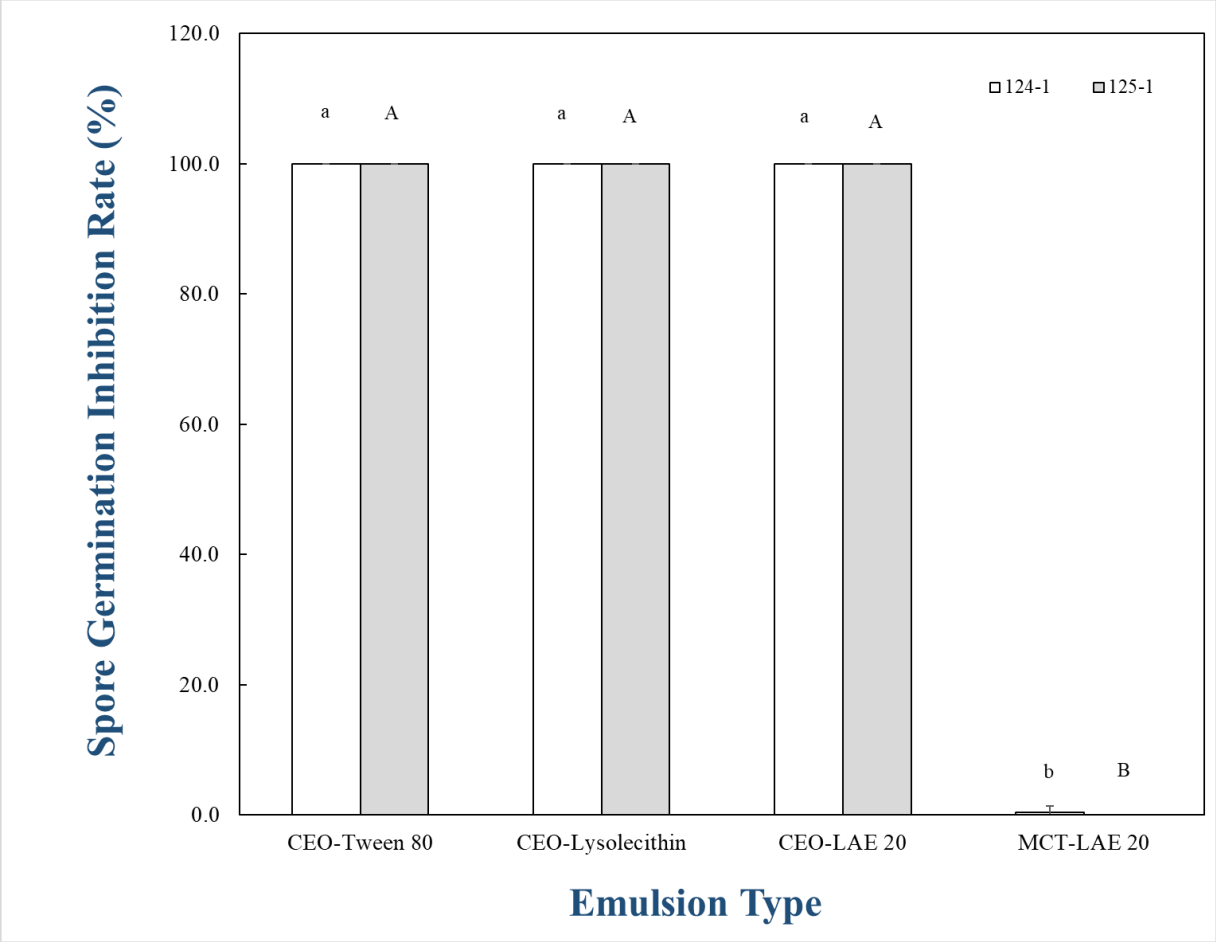
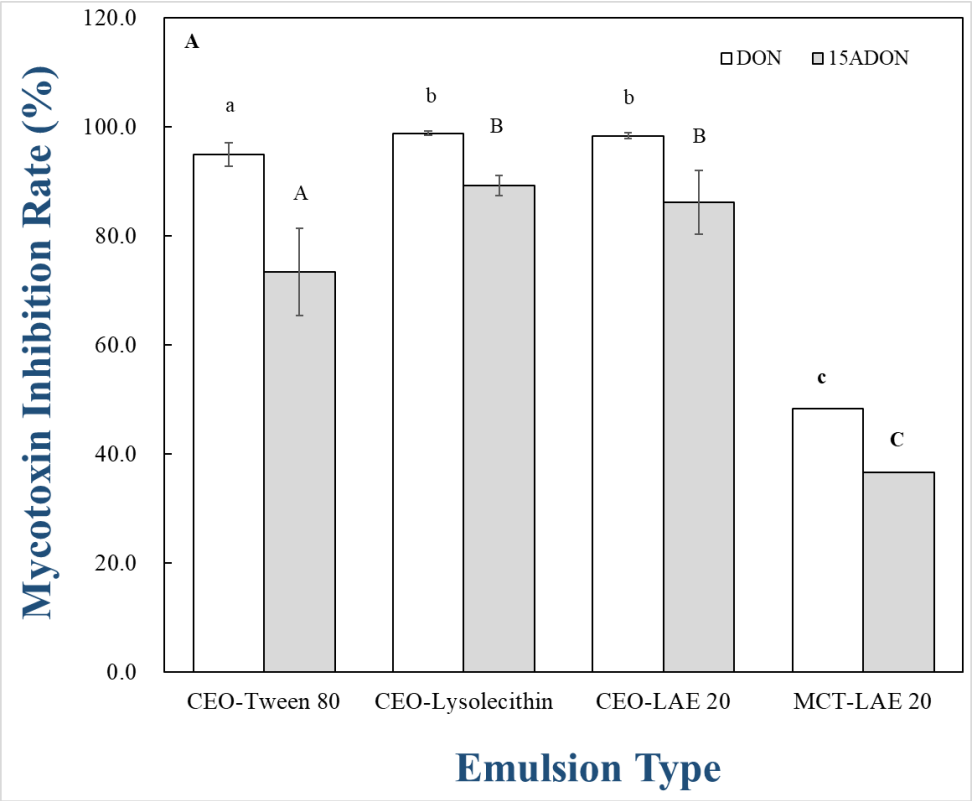
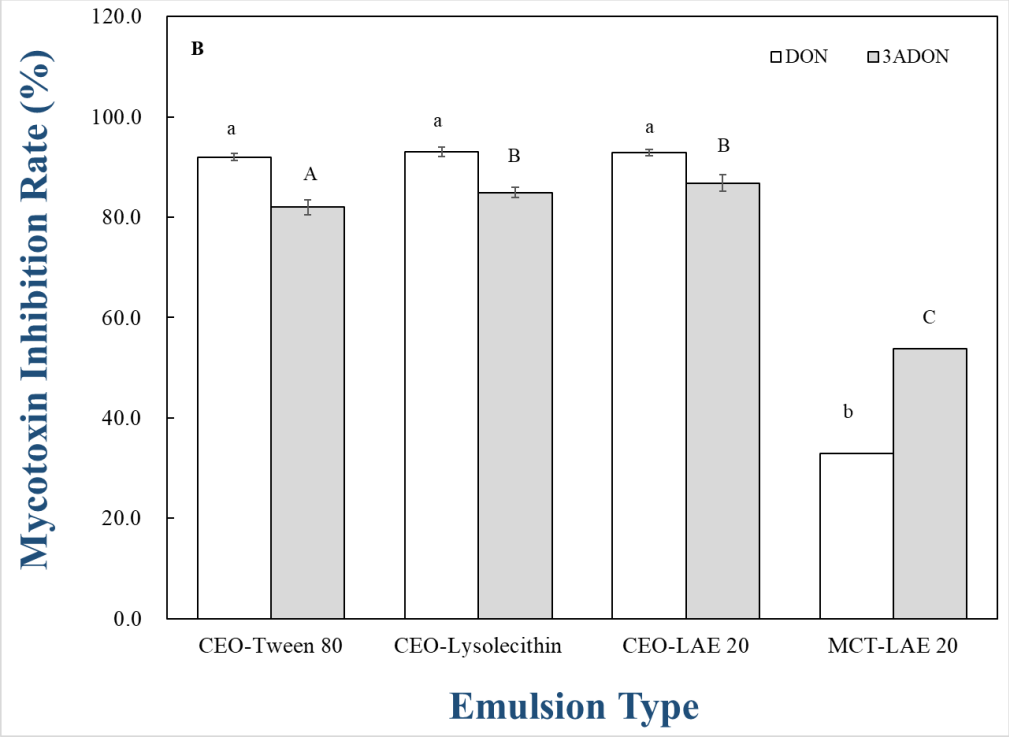

Spore Germination Inhibition Rate (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
124-1
125-1
a
A
a
A
a
A
b
B
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
Emulsion Type

Figure 7


A
DON
15ADON
Mycotoxin Inhibition Rate (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
a
A
b
B
b
B
c
C
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
Emulsion Type


B
DON
3ADON
Mycotoxin Inhibition Rate (%)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
a
A
a
B
a
B
b
C
CEO-Tween 80
CEO-Lysolecithin
CEO-LAE 20
MCT-LAE 20
Emulsion Type

Graphic Abstract