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Clove oil-in-water nanoemulsion: Mitigates growth of *Fusarium graminearum* and trichothecene mycotoxin production during the malting of *Fusarium* infected barley

Jing Wan[a], Zhao Jin[a], Shaobin Zhong[b], Paul Schwarz[a], Bingcan Chen[a], Jiajia Rao[a,*]

[a] Department of Plant Sciences, North Dakota State University, Fargo, ND 58108, United States
[b] Department of Plant Pathology, North Dakota State University, Fargo, ND 58108, United States

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ABSTRACT

Fusarium mycotoxin contamination in malting barley is of great concerns in malting industry. Our recent study found that clove oil nanoemulsions can act as highly efficient antifungal agents *in vitro*. Therefore, we explored the efficacy of clove oil nanoemulsions on *Fusarium* growth and mycotoxin during malting process. The impact of emulsifier types (Tween 80, BSA and quillaja saponins) on the formation of clove oil nanoemulsion, the mitigation effects on mycotoxin levels and fungal biomass, and the clove oil flavor residues on malts were measured. We observed that 1.5 mg clove oil/g nanoemulsion showed a negligible influence on germinative energy of barley, while still efficiently eliminated the DON levels and toxicogenic fungal biomass as quantified by Tri5 DNA content. Tween 80-stablized clove oil nanoemulsion displayed higher mycotoxin inhibitory activity and less flavor impact on the final malt. The results indicated the potential application of essential oil nanoemulsion during the malting process.

1. Introduction

Barley (*Hordeum vulgare* L.) ranks fourth in world cereal production and is important as a feed and food. Approximately 70% of barley is utilized for the production of malt, which is almost exclusively used by the brewing and distilling industries (Hückelhoven, Hofer, Coleman, & Heß, 2018). Fusarium head blight (FHB) is perhaps the largest food safety issue facing these industries (Schwarz, 2017). This fungal disease can be economically devastating as it causes both significant losses in grain yield and quality. Quality losses are often associated with the production of *Fusarium* mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), and zearalenone (ZEA). DON is most common, and has been detected in many of the world's barley and malt producing regions (Pestka & Smolinski, 2005). Although good agricultural practices in conjunction with the well-controlled storage conditions can control *Fusarium* mycotoxins in barley to a certain extent, the complete prevention of *Fusarium* mycotoxins in the raw materials is not always practically achievable.

Previous studies have shown that mycotoxin levels such as DON can increase during the malting of *Fusarium* contaminated grains and that DON can be transferred to beer at a very high level of recovery (Lancova et al., 2008). Malting is a controlled grain germination process which includes the steps of grain cleaning, steeping, germination and kilning. The ideal way to control mycotoxins in malt and beer is to avoid utilizing infected grains. This, however, is not always possible, particularly in years when the occurrence of FHB is widespread. In general, barley with DON levels greater than 0.5 mg/kg is not used for malting. In many cases, barley with DON $<$ 0.5 mg/kg is acceptable, as mycotoxin levels may appreciably diminish during the steeping process and remain low to below the limit of detection on the final malt. Conversely, some samples with low initial DON levels may occasionally exhibit aberrant behavior, where DON increases significantly during the malting process. A recent research study evaluated the fate of the DON during malting in 20 naturally FHB-infected barley samples from eastern China (Yu et al., 2019). Initial levels were all below 0.7 mg DON/kg, and in general levels decreased or remained constant on the malts. However, two samples exhibited the increases following malting. These aberrant samples represent a worst-case scenario in that the DON levels of malt cannot be predicted by levels in the grain. Methods to inhibit or reduce the production of mycotoxins during the malting process, therefore, may be of value, and could permit the safe utilization of a larger portion of the crop. Control measures could be particularly useful to the growing craft malting segment, as by definition craft maltsters must utilize at least 50%

* Corresponding author.
E-mail address: jiajia.rao@ndsu.edu (J. Rao).

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locally produced grain. As many of these craft operations are in regions impacted by FHB, there could be increased pressure to utilize mycotoxin contaminated grain in years where the disease is widespread. In addition to the food safety concerns, the chemical composition and resultant malting and brewing quality of the barley also can be decreased by *Fusarium* infection (Vegi, Schwarz, & Wolf-Hall, 2011).

There has been limited research on mycotoxin mitigation strategies for FHB-infected barley during the malting process. Approaches have included physical treatments, as well as, chemical and biological agents. Some treatments may either leave undesirable residues in the final malt or have a significant negative impact on the barley germination (Laitila & Hill, 2015). Hence, there is still a need for strategies that can effectively suppress the production of mycotoxins in barley during the malting process without adversely impacting the germinative energy of malt.

Recently, essential oils (EOs) have received increasing attention in the food industry as they are natural antifungal agents with the board-spectrum of activities and inhibition against mycotoxin biosynthesis (Tullio et al., 2007). However, direct application of EOs as antifungal agents during food processing is impractical. This is because EOs, primarily consisting of terpenes, terpenoids and phenols, are not water soluble and have a high volatility (Rao, Chen, & McClements, 2019). Such limitations could potentially be overcome by incorporating EOs into appropriate delivery systems. EO-in-water nanoemulsions are such a delivery system, and are gaining popularity due to greater physical stability, as well as the potency to increase the antifungal activity of EOs. Our recent series of *in vitro* studies found that the antifungal and DON inhibitory activities of the five selected EO (thyme, lemongrass, cinnamon, peppermint, and clove) nanoemulsions were considerably improved over that of bulk EOs (Wan, Zhong, Schwarz, Chen, & Rao, 2019b). Thyme and clove oil nanoemulsions had the greatest antifungal and mycotoxin inhibitory activities among the five selected EOs. Still, our findings have not shown if they share the same capacity in the actual malting process. In terms of nanoemulsion formulation, a number of food-grade emulsifiers are available for producing nanoemulsions including natural (e.g., proteins and polysaccharides) and synthetic emulsifiers (e.g., Tween 80). The influence of emulsifier type on its antifungal and mycotoxin inhibitory activities during the malting process has not been addressed.

The current study was set forth to evaluate the efficacies of clove oil nanoemulsions stabilized with three different emulsifiers, i.e., bovine serum albumin (BSA), quillaja saponins (Q-Naturale), and Tween 80, on the prevention *Fusarium* growth and mycotoxin production during the micro-malting process, as well as maintaining the germinative energy of barley seeds. After applying clove oil nanoemulsions at the first steeping stage of barley malting, their antifungal and mycotoxin inhibitory activities at each stage of the micro-malting process were evaluated through the quantification of Tri5 DNA and DON, respectively. Moreover, clove oil volatile residues in the final malt products were also quantified to investigate the impact of clove oil on the malt flavor. The findings could rationalize the application of EO nanoemulsion in the malting industry to produce safe and high-quality malt.

2. Materials and methods

2.1. Materials and chemicals

Fusarium-infected malting barley (cultivar: Stellar-ND) was obtained from North Dakota State University trials conducted at Langdon ND in 2018. Inoculation using a grain spawn method was previously described (Urrea, Horsley, Steffenson, & Schwarz, 2002). The harvested barley had a DON concentration of 4.7 mg/kg. Clove oil (purity ≤ 100%), bovine serum albumin (BSA), polyoxyethylene (20) sorbitan monooleate (Tween 80), eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide were purchased from Millipore Sigma Co. (St Louis, MO, USA). Corn oil was obtained from a local supermarket (Fargo, ND, USA). Quillaja saponin (Q-Naturale) was kindly provided by Ingredion Inc. (Westchester, IL, USA). The DNeasy Plant Mini Kit was purchased from Qiagen Inc. (Valencia, CA, USA). SsoAdvanced™ Universal SYBR® Green Supermix was purchased from Bio-Rad (Bio-Rad laboratories, Hercules, CA, USA).

2.2. Preparation of clove oil nanoemulsions

The coarse clove oil-in-water nanoemulsions were produced by mixing 1.5 wt% of clove oil, 3.5 wt% of corn oil, 1.0 wt% emulsifier (Tween 80, BSA, and quillaja saponin), and 94.0 wt% of phosphate buffer (pH 7.0, 10 mM) using a high-speed hand blender (M133/128-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min. The coarse nanoemulsions were then passed through a high-pressure homogenizer (LM 20-20 Microfluidizer Processor, Westwood, MA) at 10,000 psi with two cycles for further reduction in particle size. The finished nanoemulsions were collected and stored in test tubes sealed with screw caps at a room temperature (21 ± 3 °C) for further analysis. The final clove oil concentration in nanoemulsions was 15 mg/g nanoemulsions.

2.3. Characterization of clove oil nanoemulsions

2.3.1. Mean particle size, size distribution and ζ-potential

The mean particle size, size distribution, and ζ-potential of three types of clove oil nanoemulsions were determined using dynamic light scattering instrument (Zetasizer Nano ZEN 3600, Malvern Instruments, Malvern, UK). Nanoemulsions were diluted 20× and 100× times using pH 7 phosphate buffer for mean particle size and ζ-potential measurements, respectively, to avoid multiple scattering effects.

2.3.2. Long-term stability

Long-term stability of three types of clove oil nanoemulsions were determined by measuring the mean particle size and particle size distribution during a 28-day storage.

2.4. Germinative energy of FHB-infected barley

Germinative energy (GE) was measured according to method Barley-3C of the American Society of Brewing Chemists (ASBC) (ASBC, 1999) with slight modifications. Briefly, 100 barley kernels were placed onto two filter papers in the bottom of a petri dish. Four milliliters of phosphate buffer (10 mM, pH 7.0) (as control) or 5×, and 10× diluted clove oil nanoemulsions were added to each dish to achieve the final clove oil concentrations in nanoemulsion as 1.5 mg/g and 3 mg/g nanoemulsions. Each dish was covered and stored at 21 °C with more than 90% relative humidity. Sprouted kernels were counted and removed daily for 4 days. The overall percentage of sprouted kernels were determined after 4 days of germination.

2.5. Micro-malting process

The micro-malting process was performed according to the procedure described by Karababa et al with a few modifications (Karababa, Schwarz, & Horsley, 1993). The conditions and schedule for micro-malting process were optimized in our preliminary experiments (Table 1). For the control group, FHB-infected barley (20 g, dry basis) was steeped to 45% of moisture by placing100 ml of ultrapure water in a 500 ml beaker. For the treatment group, the mixture of 10 ml clove oil nanoemulsion with 90 ml of ultrapure water was transferred to the FHB-infected barley (20 g, dry basis) in a 500 ml beaker. All samples were placed in a temperature-controlled incubator at 16 °C. The steeping schedule was set as: 6 h of steeping followed by 2 h of air-rest, then 6 h of steeping followed by 2 h of air-rest, finally 8 h of steeping. At each steeping stage, wastewater was decanted and replaced by fresh ultrapure water. After steeping, barley samples followed a germination for 4 days at 16 °C with ~95% relative humidity during the germination

Table 1
Schedule of the micro-malting process.

Micro-malting schedule		Duration (h)
Steeping	The first steeping step	6
	Air rest	2
	The second steeping step	6
	Air rest	2
	The third steeping step	9
Germination	Day 1	24
	Day 2	24
	Day 3	24
	Day 4	24
Kilning	50 °C	8
	60 °C	2
	85 °C	2

stage. Kilning was conducted by increasing temperatures in a stepwise manner as follows: the temperature was held at 50 °C for 8 h, and then 60 °C for 2 h, finalized at 85 °C for 2 h. Rootlets were removed from the kilned barley prior to analysis.

2.6. Quantification of Fusarium Tri5 DNA

The fungal DNA from barley and malts were extracted using a DNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA, USA). The Tri5 PCR primers TMT_fw (5′-GATTGAGCAGTACAACTTTGG-3′) and TMT_rev (5′-ACCATCCAGTTCTCCATCTG-3′) were employed to amplify the Fusarium Tri5 specific amplicon in the SYBR Green based quantitative real-time polymerase chain reaction (qPCR) performed by a CFX96 Real-time System thermocycler (Bio-Rad, Hercules, CA, USA) according to previously published method with some modifications (Jin et al., 2018). In brief, the total reaction volume was 10.0 μl, including 5.0 μl of SsoAdvanced TM Universal SYBR® Green Supermix, 2.0 μl of water, 0.50 μl of TMT_fw (10 pmol) and 0.50 μl of TMT_rev (10 pmol), and 2.0 μl of DNA template. The concentrations of Tri5 DNA in the original barley and the malt samples were quantified according to the standard curve. A standard curve was prepared by simultaneously performing a serial dilution of purified *F. graminearum* Tri5 amplicon generated with *F. graminearum* DNA template in barley and malts. The *F. graminearum* DNA template was extracted from mycelial cultures of *F. graminearium* grown on potato dextrose agar. The serially diluted concentration of Tri5 DNA consisted of nine levels: 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg and 100 ag. Results was expressed in mg Tri5 DNA/kg barley.

2.7. Mycotoxin quantification

Mycotoxins including DON, the 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON), nivalenol (NIV), diacetoxyscirpenol (DAS), HT-2 toxin, T-2 toxin, and zearalenone (ZEA) at each step of the micro-malting process were quantified using GC–MS as previously described (Wan, Zhong, Schwarz, Chen, & Rao, 2018). Results were expressed in mg mycotoxin/kg malt. The limits of quantitation (LOQ) and detection (LOD) of mycotoxins were 0.20 and 0.10 mg/kg, respectively.

2.8. Quantification of clove oil residues in malt

Eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide, which account for 85.5% of the chemical components, were quantified as a measure of the major volatiles in clove oil (Wan et al., 2019b). Standard curves were prepared by diluting the five chemical standards in barley flour. Each standard (60 μl) was spiked in barley flour (1 g, dry basis) in 20-ml GC vials capped by an aluminum cap with PTFE/silicone septa. The vials were sonicated in an ultrasonic bath for 60 min, while agitating at 250 rpm. After sonication, vials were equilibrated at room temperature for 24 h prior to analysis. The concentration of clove oil residues in malts were analyzed using an Agilent 7890B/5977A headspace-solid-phase microextraction-gas chromatography–mass spectrometry (HS-SPME-GC–MS) system following methods previously reported with some modifications (Xu, Jin, Lan, Rao, & Chen, 2019). Briefly, the vials were incubated for 15 min at 80 °C in the autosampler heating block under agitation at 250 rpm (CTC Analytics, Zwingen, Switzerland). Then, the SPME fiber needle (50/30 μm DVB/CAR/PDMS, Supelco Inc) was inserted into the vial for 63.5 min at 80 °C to adsorb volatiles. It was then transferred to the injector port (250 °C) for 5 min desorption. The concentrations of clove oil residues in malt were quantified according to the standard curves. Results are expressed in mg clove oil residues/kg malt.

2.9. Statistical analysis

All measurements were conducted three times using freshly prepared samples and expressed as mean ± standard deviation (SD). Statistically significant differences between mean values were determined by one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) test at a 95% confidence level.

3. Results and discussions

3.1. Physical properties of clove oil nanoemulsions

The impact of emulsifier type on the particle size and ζ-potential of clove oil nanoemulsions produced under fixed homogenization conditions (10,000 psi for 2 cycles) was investigated (Fig. 1). The mean particle diameters of freshly prepared clove oil nanoemulsions stabilized by Tween 80, BSA, and quillaja saponins were 130.95 ± 5.40 nm, 152.78 ± 4.36 nm, and 179.82 ± 4.36 nm, respectively (Fig. 1A). The particle distribution of all samples exhibited a monomodal pattern (Fig. 1B). These results indicated that all the emulsifiers were able to fabricate ultrafine and nanoscale particles, but that Tween 80 was slightly more effective at producing smaller particles under the same homogenization conditions. Different efficacies emulsifiers on nanoemulsion formation has been reported in other studies. For example, Yang et al. observed that Tween 80 was more efficient than quillaja saponins at producing smaller droplets of middle chain triacylglycerol (MCT) oil-in-water nanoemulsions (Yang, Leser, Sher, & McClements, 2013). We also found that a smaller particle size of thyme oil nanoemulsions were formed with BSA rather than quillaja saponins (Wan, Zhong, Schwarz, Chen, & Rao, 2019a). The properties of emulsifiers are the major factors contributing to such differences, including (i) the absorption rate of emulsifier on the oil droplet surface to prevent droplet re-coalescence during homogenization; (ii) the ability of emulsifier to lower the interfacial tension; and (iii) the capability of emulsifier to generate a strong electrostatic and/or steric repulsive interaction between oil droplets (Ribes et al., 2017).

The ζ-potential of droplets is closely related to the physical stability and functionality of nanoemulsions (Ozturk & McClements, 2016). For instance, a charged nanoemulsion might interact with the charged fungal cell surfaces, thus influencing the antifungal activity of the whole nanoemulsion system (Ribes et al., 2017). Therefore, we measured the ζ-potential of clove oil nanoemulsions stabilized by three emulsifiers (Tween 80, BSA, and quillaja saponins). As shown in Fig. 1C, the ζ-potential values of clove oil nanoemulsions stabilized by Tween 80, BSA and quillaja saponins at pH 7.0 were −5.31 ± 0.16 mV, −30.65 ± 2.09 mV, and −49.64 ± 2.45 mV, respectively. In agreement with many other studies, we noticed that Tween 80 stabilized clove oil nanoemulsion possessed a slight negative charge (~−5 mV), presumably owing to the impurities of free fatty acids or polar constituents in Tween 80 (Zhu et al., 2019). Both BSA and quillaja saponins stabilized clove oil nanoemulsions exhibited a larger magnitude of negative charge compared to Tween 80. This is expected

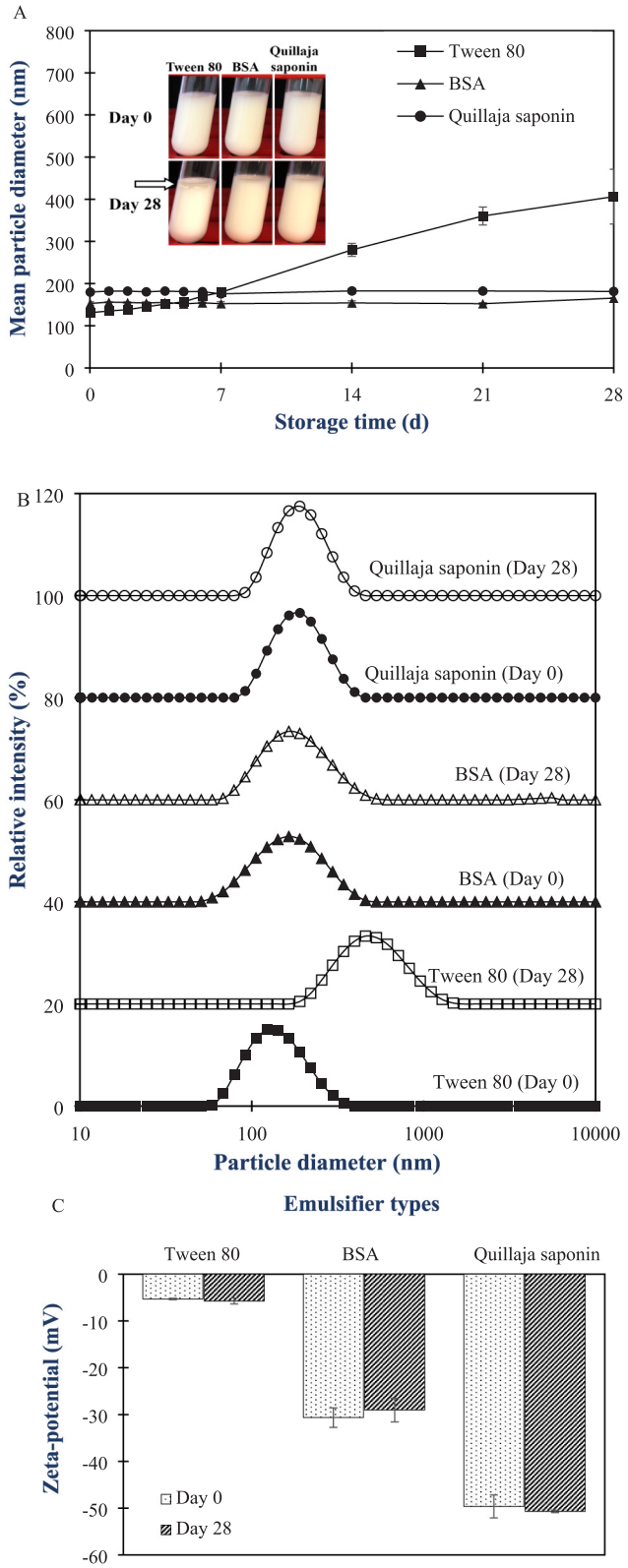

Fig. 1. Impact of emulsifier type on (A) the mean particle diameter (nm), (B) the particle size distribution (relative intensity, %), and (C) the zeta-potential (mV) of clove oil nanoemulsions stabilized by Tween 80, BSA, and quillaja saponin.

as the pH (7.0) of the nanoemulsions was above the isoelectric points of BSA (4.7) and the saponin constituents in quillaja saponins (3.25) (Tabibiazar et al., 2015).

Long-term stability of clove oil nanoemulsions is essential for determining their applications in food systems. Thus, the stability of the three types of clove oil nanoemulsions was evaluated in terms of their mean particle diameters and particle size distribution after storage at 23 °C for 28 days. As shown in Fig. 1A, the mean particle diameter of clove oil nanoemulsions stabilized by BSA or quillaja saponins were highly stable and remained constant over 28 days of storage. The size distribution pattern also remained unchanged after 28 days of storage, which further proved their long-term stability. This can be explained by the strong electrostatic repulsions generated from the high ζ-potential values of nanoemulsion droplets surrounded by BSA and quillaja saponins (Herrera-Rodríguez, López-Rivera, García-Márquez, Estarrón-Espinosa, & Espinosa-Andrews, 2019). In general, steric repulsion and electrostatic repulsion are the two predominant forces controlling the stability of nanoemulsions (Wu et al., 2019). It is commonly believed that highly charged nanoemulsion droplets with the magnitude of ζ-potential greater than 30 mV could provide a strong electrostatic repulsion between droplet, thus ensuring the long-term stability of nanoemulsions (Gurpreet & Singh, 2018). By contrast, the mean particle size diameter of the clove oil nanoemulsion containing Tween 80 increased appreciably after 7 days of storage as evidenced an increase from 130.95 to 406.33 nm diameter, concomitant with a visible oiling off in the test tube. Additionally, its particle size distribution shifted towards larger sizes. Such a high long-term instability may be caused by Oswald ripening, a common physical phenomenon occurring in EO emulsions or nanoemulsions, through the mass transport of the relatively high water solubility EO from one droplet to another (Djerdjev & Beattie, 2008). As the concentration of Tween 80 (1.0 wt%) employed in the current study was higher than its critical micelle concentration, the additional Tween 80 molecules may spontaneously self-assemble into micelles. Tween 80 micelles were capable of solubilizing and transporting clove oil molecules lingering in the aqueous phase into their hydrophobic interiors (Rao & McClements, 2012). Similar results were also found in sodium dodecyl sulfate (SDS)-stabilized octane and decane oil-in-water emulsions system, where the Ostwald ripening rate increased steeply in the presence of SDS micelles (Djerdjev & Beattie, 2008).

3.2. Influence of clove oil nanoemulsions on germinative energy (GE) of FHB-infected barley

The malt quality is essential for brewing and is reliant on the quality of the barley employed. High quality barley seeds should be able to germinate in a uniform, rapid and vigorous manner during the malting process. The germinative energy (GE) test is an indicator of the malting potential of barley seeds (Frančáková, Líšková, Bojnanská, & Mareček, 2012). It has been reported that essential oils as plant-based secondary metabolites display various levels of allelopathic effects against seed germination (Shokouhian, Habibi, & Agahi, 2016). Thus, it was important to evaluate the impact of clove oil concentration and emulsifier type on germination of barley seeds. As shown in Fig. 2, the average GE of the untreated FHB-infected barley was only about 89.5%. This is below the value of > 95% that is a criterion for many maltsters, but it should be pointed out that the GE of heavily infected barley can be damaged by invasion of mycelium and mycotoxin accumulation (Wolf-Hall, 2007).

The GE values of barley seeds decreased rapidly with an increase in clove oil concentration in the nanoemulsions. As one can see from Fig. 2, the GE of FHB-infected barley seeds was reduced to below 50% as the clove oil concentration raised from 1.5 to 3.0 mg/g. Nevertheless, there was no statistically significant difference between the GE values of the control (89.5%) and the clove oil treatments at 1.5 mg/g nanoemulsion. Nevertheless, the values were slightly lower indicating the importance of clove oil concentration in terms of maintaining the GE of barley seeds. While there was no statistically significant difference among the three emulsifiers used the nanoemulsions, barley seeds

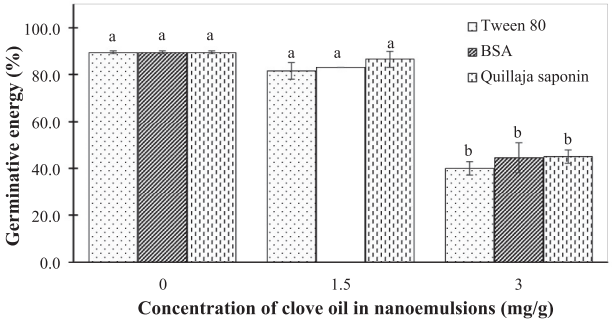

Fig. 2. Impact of emulsifier type and clove oil nanoemulsion concentrations in germinative energy of FHB-infected barley. Columns that do not share a letter are significantly different ($p < 0.05$).

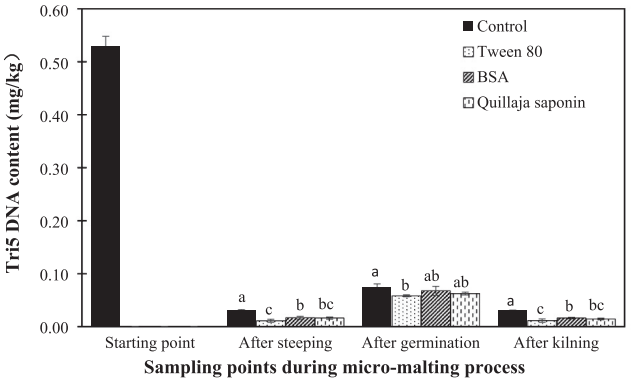

Fig. 3. Impact of emulsifier type on fungal growth during the micro-malting process of FHB-infected barley as quantified by Tri5 DNA content. Differences were compared at each micro-malting operation unit among three types clove oil nanoemulsions. Columns that do not share a letter are significantly different ($p < 0.05$).

treated with quillaja saponins-stabilized clove oil nanoemulsion trended to a slightly higher GE. For instance, with the addition of 1.5 mg/g nanoemulsion, the GE of barley seeds was 86.5%, 83.0% and 81.5% for quillaja saponins, BSA and Tween 80, respectively. On the basis of these results, the concentration of clove oil in nanoemulsion at 1.5 mg/g was chosen for the following micro-malting process. Other than EO concentration and emulsifier type, the EO type has a profound impact on GE of barley seeds. On another parallel study using thyme oil nanoemulsion prepared under same condition, we found the GE of the same barley seeds was completely suppressed after the treatment. This denoted that not all EO nanoemulsions are applicable in the malting process.

3.3. Influence of clove oil nanoemulsions on Fusarium Tri5 DNA levels during malting

Fusarium Tri genes are involved in the biosynthesis of trichothecene (Tri) mycotoxins from farnesyl pyrophosphate (FPP) (Sakuda et al., 2016). The Tri5 gene is responsible for encoding trichodiene synthase that catalyzes the first cyclization step of the trichothecene biosynthesis (Morcia et al., 2013). In recent years, the sequence of the Tri5 gene has been widely adopted as a target for the quantifying the biomass of trichothecene-producing *Fusarium* spp. in cereal grains (Burlakoti et al., 2007). The quantity of Tri5 DNA has been shown to exhibit a strong positive correlation with the development of DON in barley, wheat, and rye malts (Jin et al., 2018; Leišová et al., 2006). In this section, Tri5 DNA content in the samples in the absence/presence of clove oil nanoemulsions was quantified during each step of micro-malting including steeping, germination and killing (Fig. 3).

However, preliminary research was first conducted to determine, the optimum time point to apply clove oil nanoemulsion during the malting process. Initially, clove oil nanoemulsions were applied to barley seeds in the first steep, the second steep, or was sprayed onto barley seeds at the start of the germination phase. We found that DON levels in the final malt decreased dramatically when clove oil nanoemulsions were added in the first stage of steeping process, when compared to other treatments (Supplementary Figure). Thus, introduction of clove oil nanoemulsions to barley seeds during the first stage of steeping was selected for subsequent experiments to evaluate the role of clove oil nanoemulsions on the development of both Tri5 DNA and mycotoxins during the malting process.

The concentration of Tri5 DNA fluctuated between each step of the micro-malting process in the sense that the contents of Tri5 DNA decreased noticeably after steeping, increased during germination, and then decreased after kilning. This pattern was observed in all treatment groups including the controls. For instance, Tri5 DNA content in control barley dramatically dropped from 0.529 to 0.028 mg/kg after steeping, increased to 0.073 mg/kg after 4 days of germination, and then ended up at 0.029 mg/kg in the final kilned malt. Similar trends have been reported for the quantification of Tri5 DNA during the malting of healthy, naturally infected, and *F. graminearum* inoculated barley seeds (Vegi et al., 2011). These results again indicated that although steeping can wash off a large portion of fungi, the remnants can still grow during the germination steps and produce more Tri5 DNA.

Upon the treatment of the infected barley with clove oil nanoemulsions, levels of Tri5 DNA were further reduced at each step of malting, when compared to the controls. This implies the antifungal activity of clove oil nanoemulsion in barley during the malting process. In particular, Tri5 DNA content in the final malt was 0.011, 0.016, and 0.014 mg/kg barley after applying clove oil nanoemulsion stabilized by Tween 80, BSA, and quillaja saponins, respectively (Fig. 3). With regard to the impact of nanoemulsion emulsifier type on Tri5 DNA content during the micro-malting process, the results clearly showed that Tween 80-stabilized clove oil nanoemulsion had the greatest inhibitory activity against fungal growth in each step of the malting process.

3.4. Influence of clove oil nanoemulsions on mycotoxins production in barley during the malting process

A primary concern from the malting industry is being able to predict and/or control the level of mycotoxins in the finished malt as they may be transferred to the beer where they can present consumer health issues. We therefore evaluated the effect of clove oil nanoemulsions on mycotoxin production in heavily infected barley at different stage of the micro-malting process.

Among all trichothecenes mycotoxins (DON, 3-ADON, 15-ADON, NIV, DAS, HT-2 toxin, T-2 toxin, and ZEA) that were assessed, only DON was detected in raw barley seeds and malt samples. The DON level in control barley samples declined sharply from 4.70 to 0.60 mg/kg after steeping, followed by a slight increase to 0.67 mg/kg at the end of germination (Fig. 4). Such dynamic changes of DON during the malting of naturally infected barley have also been observed in the previous studies (Habler et al., 2016; Schwarz, Casper, & Beattie, 2014; Vegi et al., 2011). During steeping, with periodical replacement of water, the water soluble DON located on the husk of barley seeds may be washed off, as is some fungi. However, the germination conditions allow the residual fungi to grow and produce mycotoxin, resulting in the increase of DON level. The slight reduction of DON after kilning could be explained by the removal of rootlets in the final malt. The rootlets in the germinated barley have been reported to have high levels of DON (Mastanjević et al., 2019). In addition, some of the DON present on the grain can be transformed to “masked” forms, such as deoxynivalenol-3-glucoside (Lancova et al., 2008), which was not measured in the current

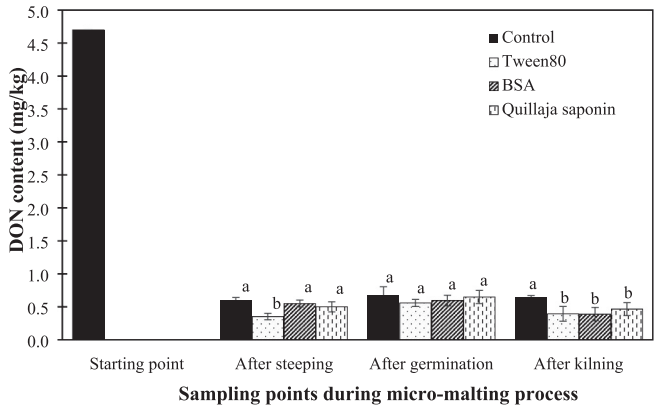

Fig. 4. Impact of emulsifier type on DON content of FHB-infected barley during the micro-malting process. Differences were compared at each micro-malting operation unit among three types clove oil nanoemulsions. Columns that do not share a letter are significantly different ($p < 0.05$).

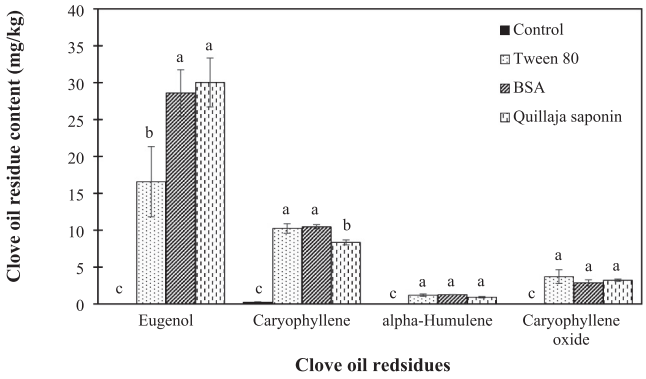

Fig. 5. Impact of emulsifier type on clove oil residues content of malts. Differences were compared among three types clove oil nanoemulsions for each clove oil residue. Columns that do not share a letter are significantly different ($p < 0.05$).

study.

A similar trend on DON development was also recorded in the treatment groups where clove oil nanoemulsion was introduced during the first stage of steeping. Moreover, all clove oil nanoemulsions showed inhibitory activities on the DON production compared with the control group as the DON in that all treated samples were less than 0.5 mg/kg after kilning. In terms of DON level in the final malt, statistically significant difference was not shown among three selected emulsifiers employed in clove oil nanoemulsion. This result suggested that the mycotoxin inhibitory activity was mainly originated from clove oil rather than the emulsifier. Interestingly, it was also noticed that Tween 80-stabilized clove oil nanoemulsion had the greatest inhibitory activity on DON production after steeping with the lowest DON of 0.35 mg/kg in barley. We postulated that the smaller particle size of clove oil nanoemulsion stabilized by Tween 80 may facilitate the higher absorption rate of nanoemulsion on barley kernel, which limited the growth of fungal and the production of DON by the increased localized concentration of clove oil.

3.5. Clove oil residues in malt

Many essential oils have a strong flavor, even at trace quantities, due to the high volatile compounds they contain. This has restricted the type of food products into which they can be incorporated. Although clove oil nanoemulsions showed antifungal and mycotoxin inhibitory activities during malting, the residual flavor in the final malt may counteract their application, as malt flavor, together with hops, are the soul of the beer flavor (Chen et al., 2017). Therefore, the final malt flavor should be taken into consideration when applying clove oil nanoemulsions to mitigate mycotoxin production during malting (Broeckling et al., 2018). As previously determined in our lab, eugenol, eugenol acetate, caryophyllene, α-humulene, and caryophyllene oxide are the major chemical compositions of clove oil we used (Wan et al., 2019b). In the current study, clove oil nanoemulsion at a concentration of 75 mg/kg barley was added to barley seeds in the first stage of steeping, and the selected five chemical constitutes were quantified after kilning to evaluate the clove oil residues in final malt (Fig. 5).

In the absence of clove oil nanoemulsions, eugenol, α-humulene, and caryophyllene oxide were not detected in final malt. Interestingly, 0.24 mg/kg of caryophyllene was found in the control malt, which might be generated by malting of FHB-infected barley. After being treated with clove oil nanoemulsions (75 mg/kg barley), eugenol, caryophyllene, α-humulene, and caryophyllene oxide were detected at some extent in all final malts. Among all four detectable clove oil residues in malts, eugenol was the leading chemical constitute in malts ranging from 16.08 to 30.03 mg/kg malt, follow by caryophyllene (~10 mg/kg malt), and trace amounts of α-humulene (~1 mg/kg malt) and caryophyllene oxide (~3 mg/kg malt), which was in consistent with their percentage concentration in clove oil. Actually, both caryophyllene and α-humulene are considered as the principal volatiles of hops. Caryophyllene oxide could also be found in final beer product due to the oxidation of β-caryophyllene during the brewing process (Rettberg, Biendl, & Garbe, 2018). Surprisingly, eugenol acetate was not detected in all samples. The control and release of volatiles in food systems are collectively determined by their volatility nature, such as boiling point and molecular weight, processing operations, and food matrices. The disappearance of eugenol acetate in the final malts may be due to its less affinities with barley matrix than the other four volatiles, which was then stripped off after kilning. Among three different emulsifiers stabilized nanoemulsions, eugenol concentration (16.08 mg/kg) was significantly lower in malt treated by Tween 80-stabilized clove oil nanoemulsion than that of BSA (28.61 mg/kg) and quillaja saponin (30.03 mg/kg) stabilized ones. This result indicated that emulsifier played an essential role on the retention of volatiles. As reported widely, the antifungal activity of clove oil nanoemulsions may primarily result from the eugenol. Indeed, the barley treated with the Tween 80-stabilized clove oil nanoemulsion had the lowest Tri5 DNA content after kilning. Consequently, one would expect that the more eugenol in Tween 80 system was used for inhibition of *Fusarium* spp. growth during the micro-malting process.

4. Conclusion

In conclusion, physically stable clove oil nanoemulsions could be fabricated by mixing 5.0 wt% oil and 1.0 wt% emulsifier (BSA, quillaja saponins, and Tween 80) using high-pressure homogenization. Barley treated with a higher clove oil concentration of nanoemulsion had a lower germinative energy (GE). The negligible impact of clove oil on GE could be obtained by applying 1.5 mg clove oil/g nanoemulsion in barley. During micro-malting process, fungal biomass as reflected by Tri5 DNA and mycotoxin levels were decreased appreciable after steeping, but increased after germination, and then declined again after kilning. All clove oil nanoemulsions had the capability to inhibit fungal growth and DON production during the micro-malting process. Regarding volatiles residues of clove oil in final malt, eugenol, caryophyllene, α-humulene, and caryophyllene oxide were detected in final malts treated with clove oil nanoemulsions. Among the three emulsifiers, Tween 80-stablized clove oil nanoemulsion displayed less flavor impact on the final malt.

5. Author statement

Jing wan conducted the experiment, analyzed data and draft the manuscript. Dr. Jin Zhao provided the hands on experience on Tri 5 DNA detection. Professor Shaobin Zhong provided the fusarium contaminated barley seed from the field. Professor Paul Schwarz provided guideline and experimental design on malting processing. Professor Bingcan Chen revised the manuscript, tables and figures. Professor Jiajia Rao designed the structure of manuscript, supervised the work, revised manuscript. All authors reviewed the manuscript before submission. Professor Bingcan Chen provided the techincal support on analysis of clove oil redsidues content of malts and revised revised the manuscript, tables and figures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.126120.

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