International Journal of Food Microbiology 291 (2019) 104–110

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Synergistic antimicrobial activities of essential oil vapours against *Penicillium corylophilum* on a laboratory medium and beef jerky

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ARTICLE INFO

Keywords:
Essential oil vapours
Penicillium corylophilum
Beef jerky
Intermediate-moisture food
Synergistic antifungal effect
Xerophilic mold

ABSTRACT

This study was done to determine the antifungal activities of essential oil (EO) vapours of 97 plants against *Penicillium corylophilum* and to test combinations of EO vapours for synergistic antifungal effects. Among 97 commercially available EOs extracted from plant parts, garlic, cinnamon bark, may chang (mountain pepper), citronella, thyme thymol, oregano, spearmint, and thyme linalool EO vapours exhibited relatively strong antifungal activities. The minimal inhibitory concentrations of these EO vapours were 0.0390–0.6250 μL/mL. A combination of cinnamon bark, citronella, and may chang EO vapours, as well as a combination of cinnamon bark and citronella EO vapours, showed synergistic inhibitory activities to *P. corylophilum* on a laboratory medium. A combination of cinnamon bark, citronella, and may chang EO vapours had synergistic activity in inhibiting growth of *P. corylophilium* on beef jerky. Observations reported here provide basic information valuable when developing strategies to inhibit the growth of *P. corylophilum* and possibly other moderately xerophilic molds on intermediate-moisture foods.

1. Introduction

Penicillium corylophilum is a moderately xerophilic ascomycetous mold (Bok et al., 2009; Nagdeve, 2015). Xerophilic molds, including *P. corylophilum*, are known to cause a surface spoilage on intermediate-moisture foods (a_w 0.60–0.85) such as dried meats (Pitt and Hocking, 2009a; Vermeulen et al., 2012). Ajiboye et al. (2011) investigated microorganisms associated with dried meat in Nigeria and reported that *Penicillium* spp. have been isolated. Coetzee et al. (2005) demonstrated that ca. 24.44% of biltong (dried and cured meat products similar to beef jerky) in southern African countries were contaminated with *Penicillium* spp. Lee et al. (2004) prepared beef jerky and they reported that the most of the fungal organisms detected in jerky were *Penicillium* spp. In addition, *P. corylophilum* is known to produce citrinin, a toxin with strong nephrotoxicity, as a secondary product of metabolism (dos Santos et al., 2012). Citrinin has been designated as a Group 3 carcinogen by the International Agency for Cancer Research (Flajs and Peraica, 2009). Conidia (conidospores) of *Penicillium* species are commonly found in the environment and can cause airborne contamination of intermediate-moisture foods (Garrett et al., 1998; Pitt and Hocking, 2009a). To prevent spoilage and minimize public health concerns, it is necessary to develop strategies to inhibit the growth of *P. corylophilum* on these foods.

Traditional methods to prevent the growth of molds on foods often involve the use of synthetic chemical agents. Recently, as consumers' demands for organic dried foods have increased, essential oils (EOs), some of which have natural antimicrobial activity, have attracted attention as antifungal agents. EOs are volatile oily liquids extracted from plant materials (Burt, 2004; Singh et al., 2006). The biggest limitation for the application of EOs to food is that they generally have strong flavors and aromas. Because treatment of foods with high concentrations of EOs may adversely affect the flavor (Nazer et al., 2005), it will be necessary to develop preservation technologies that minimize the amount of residual EO after treatment. One approach to minimize the amount of EO remaining in foods after treatment is the use of vapour-phase EO (EO vapour). In contrast to liquid-phase EO, EO vapour is not directly added to foods, and therefore may have a relatively minor effect on sensorial properties (Seo et al., 2015; Tyagi and Malik, 2011). In addition, some EO vapours inhibit microbial growth more effectively at relatively low concentrations than do liquid EO (Fisher and Phillips, 2006; Goñi et al., 2009; Tyagi and Malik, 2011).

To minimize the amount of EO vapour needed to effectively treat foods, the minimal inhibitory concentration (MIC) against the target microorganism must first be determined. The MIC has been generally defined as the lowest concentration of an antimicrobial agent needed to

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https://doi.org/10.1016/j.ijfoodmicro.2018.11.023
Received 20 July 2018; Received in revised form 12 November 2018; Accepted 18 November 2018
Available online 20 November 2018
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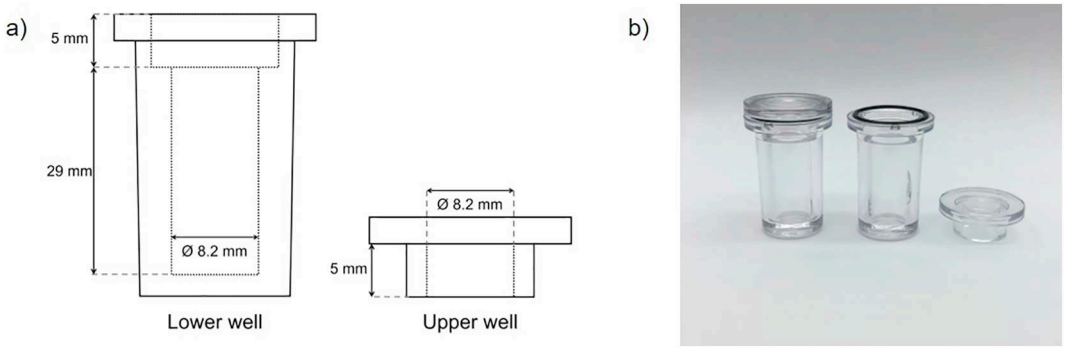

Fig. 1. a) Schematic diagram of the experimental apparatus (vial) used to determine minimal inhibitory concentrations (MICs) and synergistic antifungal effects of essential oils.
b) Photograph of the experimental apparatus (vial).

inhibit growth of a microorganism after overnight incubation (Andrews, 2001). The definition of MIC and associated experimental protocols have focused largely on measurement of the MIC of liquid and solid chemicals against bacteria. An optimal method to determine the MIC of EO vapour against molds has not been extensively studied. One method used to minimize the MIC of EO vapours in foods is to apply a combination of vapours that exert synergistic antifungal effects. To determine a synergistic antimicrobial activity of a combination of antimicrobial agents, an experimental protocol called the checkerboard assay has been described (Moody, 1992). The checkerboard assay has typically been used to determine the synergistic effects between liquid antimicrobial agents. We are not aware of reports using the checkerboard assay to measure synergistic antifungal activities of EO vapours.

The purpose of this study was to measure the MICs of EO vapours against a xerophilic mold, *P. corylophilum*, to determine if combinations of EO vapours exhibit synergistic antifungal activity against *P. corylophilum* on a laboratory medium, and to confirm that combinations of EO vapours exert synergistic antifungal activity in beef jerky, an intermediate-moisture food.

2. Materials and methods

2.1. Strains of P. corylophilum used

Two strains of *P. corylophilum* were used: strain KACC 40688 (unknown origin) and strain KCTC 16681 (isolated from a contaminated pie). Each cryopreserved strain was streaked on dichloran 18% glycerol agar (DG18 agar; Oxoid, Hampshire, England) and incubated at 25 °C for 120 h. To prepare conidial suspensions, 10 mL of sterile 0.1% peptone water containing 30% glycerol (PW30G) were dispensed onto each plate, and colonies were detached using a sterile plastic spreader (90050; SPL Life Sciences, Gyeonggi, Republic of Korea). The mixture of colonies and 0.1% PW30G was vigorously vortexed and filtered through sterile gauze. The number of conidia in each suspension was determined using a sterile disposable hemocytometer (DHC-N01; INCYTO, Cheonan, Republic of Korea). Suspensions of each strain were diluted in 0.1% PW30G to give a concentration of ca. 10^5 to 10^6 conidia/mL. A mixture of the two strains prepared by combining 5 mL of each diluted conidial suspension was used as an inoculum.

2.2. Essential oils

Ninety-seven commercially available liquid EOs were purchased from Neumond-Düfte der Natur GmbH (Raisting, Germany) and EuroAroma (Gewerbegebiet, Germany). The selected EOs used are listed in Supplementary Table 1. The inhibitory activities of these EO vapours against *P. corylophilum* were determined. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Darmstadt, Germany) was used as a diluent for EOs.

2.3. Determination of antifungal effects of EO vapours against P. corylophilum on a laboratory medium

2.3.1. Experimental apparatus

To determine if the 97 EO vapours exhibit antifungal activity against *P. corylophilum*, an experimental apparatus developed by Lee et al. (2018) was used. Fig. 1 shows a schematic diagram and photo of the experimental vial. The polycarbonate vial consisted of an upper well (ca. 0.28 mL inner volume; 8.2 mm diameter × 5.0 mm high) containing DG18 agar inoculated with *P. corylophilum* and a lower well (ca. 1.0 mL; 8.2 mm diameter × 20 mm high) containing EO vapour generated by spontaneous vaporization of liquid EO. The EO vapour in the headspace of lower well was contacting with the *P. corylophilum* on the agar in the upper well.

2.3.2. Screening EO vapours for inhibition of P. corylophilum

DG18 agar was sterilized by heating at 121 °C for 15 min, then kept in a waterbath at 50 °C to maintain in a molten state. Molten DG18 agar was dispensed into the upper well of the sterilized experimental vial and solidified at room temperature (22 ± 2 °C) for 30 min. *P. corylophilum* conidial inoculum (10 μL, 10^5 to 10^6 conidia/mL), prepared as described above, was inoculated onto the DG18 agar surface and dried in a laminar flow biosafety hood at 22 ± 2 °C for 30 min. According to Seo et al. (2015), who measured the MICs of EO vapours against *Escherichia coli* O157:H7, five EO vapours (cinnamon bark, thyme thymol, oregano, peppermint, and thyme linalool) exhibited strong inhibitory activity, with MICs of 0.6250 μL/mL or less. Based on their results, a concentration of 0.6250 μL/mL was used as the reference concentration to screen for inhibitory activities of EO vapours against *P. corylophilum*. To apply EO vapour at 0.6250 μL/mL, a sterilized 8 mm-diameter paper disc (Advantec Toyo Kaisha, Tokyo, Japan) was placed in the lower well of the experimental vial. Liquid EO was diluted two-fold with DMSO three times, and 5 μL of the diluted EO was deposited on a paper disc in the lower well in each vial. When the EO is fully vaporized in the vial (headspace: 1 mL), the concentration of EO vapour in the headspace is 0.6250 μL/mL. Immediately after depositing the diluted EO on the paper disc, the lower and upper wells in the experimental vials were placed together, and the junction between the wells was sealed with Parafilm (Bemis, Neenah, WI, USA). After incubation of the sealed vials at 25 °C for 120 h, DG18 agar was visually examined for the presence of *P. corylophilum*. EO vapours (0.6250 μL/mL) that prevented colony formation were selected for further study. In all experiments, the diluted liquid EO deposited in each vial was assumed to be completely vaporized.

2.3.3. Determination of the MIC values of EO vapours against P. corylophilum

Upper wells containing DG18 agar inoculated with *P. corylophilum* were prepared using the procedure described in Section 2.3.2. EO

vapours shown to have antifungal activity against *P. corylophilum* in the screening test were serially diluted two-fold in DMSO; 5 μL of diluted EO was deposited on paper discs in the lower well to create EO vapour concentrations of 0.0195, 0.0390, 0.0781, 0.1563, and 0.3125 μL/mL. For the negative and positive controls, diluted EO was not deposited on discs. Immediately after depositing the diluted EO, the lower and upper wells were placed together and sealed with Parafilm. The experimental vials were incubated at 25 °C for 120 h and DG18 agar was visually examined for colonies of *P. corylophilum*. The lowest concentration of EO vapours preventing colony formation was considered as the MIC.

2.3.4. Determination of combinations of EO vapours with synergistic antifungal activity against P. corylophilum

Combinations of two or three EO vapours were evaluated for synergistic inhibitory activity against *P. corylophilum* using a modified checkerboard assay. Sixteen experimental vials (4 rows × 4 columns) were prepared. Molten DG18 agar was deposited in the 16 upper wells and inoculated with *P. corylophilum* using the procedure described above (Section 2.3.2). Liquid EO A and EO B were serially diluted two-fold using 1 MIC to 1/8 MIC, as was done using single EOs. Then, 2.5 μL of EO A (1, 1/2, 1/4, and 1/8 MIC) was deposited on each paper disc in four lower wells in each row of experimental vials; 2.5 μL of EO B (1, 1/2, 1/4, and 1/8 MIC) was also deposited in four lower wells in each column. This resulted in 16 combinations of EO A (1, 1/2, 1/4, and 1/8 MIC) and EO B (1, 1/2, 1/4, and 1/8 MIC) in 16 experimental vials. Immediately after depositing the diluted EO on paper discs, the lower and upper wells of the experimental vials were placed together, sealed with Parafilm, and incubated at 25 °C for 120 h. The DG18 agar was examined for colonies of *P. corylophilum*. When no colonies of *P. corylophilum* were formed, the fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) were calculated to confirm or disprove the occurrence of synergistic antifungal activities. When the FICI value was less than or equal to 0.5, the combination of EO vapours was considered to be synergistic, whereas when the FICI value was > 0.5 and < 1.0, the combination of EOs was judged to be partially synergistic. The method for calculating FICI was as follows (Moody, 1992):

$$\text{FIC} = \frac{\text{MIC of EOs in combination}}{\text{MIC of individual EO}}$$

FICI=Sum of the FICs of EOs

To measure synergistic activity of a combination of three EO vapours against *P. corylophilum*, 64 experimental vials (4 rows × 4 columns × 4 sets) were prepared. Three liquid EOs (EO A, EO B, and EO C) were serially diluted two-fold to give 1/2 MIC to 1/16 MIC. Then, 1.7 μL of EO A (1/2, 1/4, 1/8, and 1/16 MIC) was deposited on each paper disc in the 4 lower wells in each row and 1.7 μL of EO B (1/2, 1/4, 1/8, and 1/16 MIC) was deposited on paper discs in 4 lower wells in each column. In total, 4 sets of 16 vials each were prepared. Next, 1.7 μL of EO C (1/2, 1/4, 1/8, or 1/16 MIC) was deposited on paper discs in the 16 vials of each set. As a result, 64 combinations of EOs A (1/2 MIC to 1/16 MIC), B (1/2 MIC to 1/16 MIC), and C (1/2 MIC to 1/16 MIC) were tested. After incubating of the sealed vials at 25 °C for 120 h, DG18 agar was visually examined for growth of *P. corylophilum*. Synergistic antifungal effects of combinations of EOs were confirmed by calculating of the FIC and FICI according to the equation shown above.

2.4. Antifungal effects of combined EO vapours against P. corylophilum on beef jerky

2.4.1. Construction of airtight containers

A round polystyrene dish (91.3 mm dish diameter × 38.2 mm internal height, model 310100; SPL Life Sciences, Pocheon, Republic of Korea) fitted with a lid was used as an airtight container. Fig. 2 shows a schematic diagram illustrating the structure of the container. A small Petri dish lid (58 mm diameter × 8.5 mm high, model 11060; SPL Life Sciences, Pocheon, Republic of Korea) was placed inside the airtight container and a piece of beef jerky (2 × 4 cm, 2–3 mm thick) was placed on top of the lid. The upper well of the experimental vial used to determine the MIC of *P. corylophium* on DG18 agar was placed upside-down next to the small Petri dish lid. To maintain 93% RH in the airtight container, 20 mL of saturated potassium nitrate solution (a_w 0.930 ± 0.005; Daejung, Siheung, Republic of Korea) was deposited in the airtight container and held at 25 °C for at least 12 h before the experiment was conducted.

2.4.2. Inoculation of the surface of beef jerky with P. corylophilum

Beef jerky certified as organic was used to avoid chemical preservatives. The jerky was purchased through an online market and stored at room temperature (22 ± 2 °C) until used. The jerky was cut into 2 cm × 4 cm pieces (2–3 mm thick) just before using in the experiment. Both sides of the jerky were surface-sterilized by exposing to UV radiation at room temperature for 30 min. An inoculum (100 μL, ca. 10^5 conidia/mL) of *P. corylophilum* was deposited (10 spots) on the surface of each piece of beef jerky and dried for 1 h in a laminar flow biosafety hood (22 ± 2 °C).

2.4.3. Reduction of P. corylophilum on beef jerky by treatment with single and combined EO vapours

Beef jerky inoculated with *P. corylophilum* (2.9 log conidia/cm^2) was placed on top of the lid of a small Petri dish in the treatment container and a single liquid EO was deposited in the upper well of the vial. The amount of liquid EOs deposited in the paper disc in each vial gave concentrations of vapours (0.0097, 0.0195, 0.0390, or 0.0781 μL/mL) upon vaporizing in the atmosphere within the container (ca. 218.5 mL of headspace). In experiments using combinations of EO vapours, cinnamon bark, citronella, and may chang EO were deposited on paper discs at a 1:2:1 ratio to give a total concentration of 0.0097 μL/mL

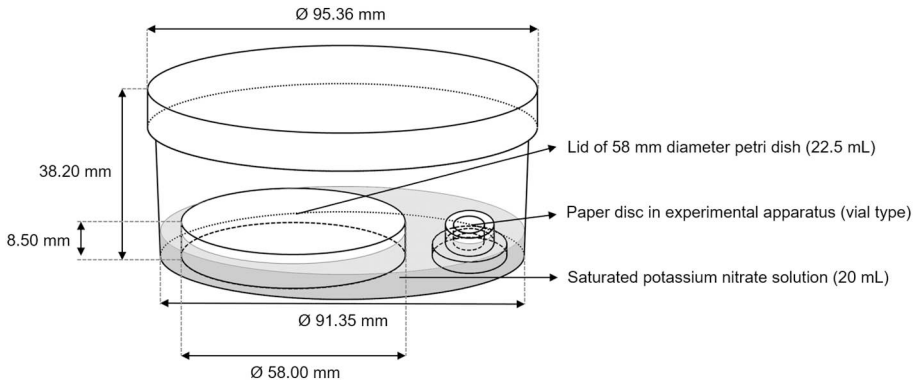

Fig. 2. Schematic diagram of the airtight container in which beef jerky was treated with vapour phase of essential oils.

Table 1
The minimal inhibitory concentrations (MICs) of essential oil vapours against a two-strain mixture of *P. corylophilum* inoculated on DG18 agar.

Essential oil			Minimal inhibitory concentration, MIC (μL/mL)
Scientific name	Common name	Plant part	
Allium sativum	Garlic	Root	0.0390
Cinnamomum zeylanicum	Cinnamon bark	Bark	0.1563
Litsea cubeba	May chang	Fruit	0.1563
Cymbopogon nardus	Citronella	Grass	0.3125
Thymus zygis CT thymol	Thyme thymol	Leaf	0.3125
Origanum vulgare	Oregano	Leaf	0.6250
Mentha spicata	Spearmint	Flowering herb	0.6250
Thymus zygis CT linalool	Thyme linalool	Flower	0.6250

(0.0024, 0.0048, and 0.0024 μL/mL, respectively), 0.0195 μL/mL (0.0048, 0.0097, and 0.0048 μL/mL, respectively), 0.0390 μL/mL (0.0097, 0.0195, and 0.0097 μL/mL, respectively), and 0.0781 μL/mL (0.0195, 0.0390, and 0.0195 μL/mL, respectively) in the atmosphere inside the container. The total volumes of EOs (single or combined) deposited on paper discs were 2.12, 4.26, 8.52, and 17.06 μL, respectively. After the liquid EO was deposited, the lid of the container was applied and sealed with Parafilm. After incubation at 25 °C for 120 h, the beef jerky was transferred to a sterile Whirl-Pak bag (catalog no. B01196WA; Nasco, Fort Atkinson, WI, USA) using sterilized forceps in a laminar flow biosafety hood (22 ± 2 °C). Sterile 0.1% PW30G (10 mL) was added to the Whirl-Pak bag containing the beef jerky and the mixture was pummeled for 1 min in a stomacher (Interscience BagMixer® 400 W; Interscience, Saint Nom, France). The mixture of jerky and 0.1% PW30G was serially diluted using 0.1% PW30G and spread-plated onto DG18 agar. The number of presumptive colonies of *P. corylophilum* was counted after incubating the DG18 agar plates at 25 °C for at least 48 h. The theoretical detection limit for spread plating was 0.1 log CFU/cm^2.

2.5. Statistical analysis

All experiments were repeated at least three times. Data were analyzed using the general linear model with SAS software (ver. 9.4; SAS Institute, Cary, NC, USA). Differences in populations of *P. corylophilum* on the surface of the beef jerky after treatment with various concentrations of a single EO or combinations of EOs were compared at the 95% significance level using Fisher's least significant difference (LSD) test ($P \leq 0.05$).

3. Results

3.1. Determination of antifungal effects of EO vapours against P. corylophilum on a laboratory medium

3.1.1. Screening EO vapours for inhibition of P. corylophilum

Ninety-seven EO vapours at a concentration of 0.6250 μL/mL were examined for their inhibitory effects against *P. corylophilum*. Eight EO vapours (garlic, cinnamon bark, may chang, citronella, thyme thymol, oregano, spearmint, and thyme linalool) were found to inhibit colony formation of *P. corylophilum* on DG18 agar. These vapours had relatively strong antifungal activity and were chosen for the measurement of MICs against *P. corylophilum*.

3.1.2. Determination of the MIC values of EO vapours against P. corylophilum

Table 1 shows the MIC values of eight EO vapours screened for inhibitory activity against *P. corylophilum* on DG18 agar at 25 °C. Garlic EO vapour (0.0390 μL/mL) showed the lowest MIC, followed by cinnamon bark (0.1563 μL/mL), may chang (0.1563 μL/mL), citronella (0.3125 μL/mL), thyme thymol (0. 3125 μL/mL), oregano (0.6250 μL/mL), spearmint (0.6250 μL/mL), and thyme linalool (0.6250 μL/mL) EO vapours.

3.1.3. Combinations of EO vapours with synergistic inhibitory activity against P. corylophilum

Table 2 presents results of pairs of eight EO vapours causing at least partial synergism in inhibiting growth of *P. corylophilum* on DG18 agar. Cinnamon bark EO vapour in combination with citronella EO vapour had the highest synergistic antifungal activity (FICI = 0.5000) when

Table 2
Pairs of essential oil vapours tested for inhibitory activity against a two-strain mixture of *P. corylophilum*, as determined using the experimental apparatus[a].

EO A	EO B	Concentration of EO (μL/mL)			FIC		FICI	Observation
		EO A	EO B	total EO	EO A	EO B		
Cinnamon bark	Citronella	0.0390	0.0781	0.1171	0.2500	0.2500	0.5000	Synergism
Garlic	Citronella	0.0195	0.0390	0.0585	0.5000	0.1250	0.6250	Partial synergism
Garlic	Thyme linalool	0.0195	0.0781	0.0976	0.5000	0.1250	0.6250	Partial synergism
May chang	Cinnamon bark	0.0195	0.0781	0.0976	0.1250	0.5000	0.6250	Partial synergism
May chang	Citronella	0.0195	0.1563	0.1758	0.1250	0.5000	0.6250	Partial synergism
Citronella	Oregano	0.1563	0.0781	0.2344	0.5000	0.1250	0.6250	Partial synergism
Cinnamon bark	Oregano	0.0195	0.3125	0.3320	0.1250	0.5000	0.6250	Partial synergism
Garlic	Thyme thymol	0.0195	0.0390	0.0585	0.5000	0.2500	0.7500	Partial synergism
Garlic	May chang	0.0195	0.0390	0.0585	0.5000	0.2500	0.7500	Partial synergism
Garlic	Oregano	0.0195	0.1563	0.1758	0.5000	0.2500	0.7500	Partial synergism
May chang	Oregano	0.0781	0.1563	0.2344	0.5000	0.2500	0.7500	Partial synergism
Garlic	Spearmint	0.0195	0.1563	0.1758	0.5000	0.2500	0.7500	Partial synergism
Citronella	Thyme thymol	0.1563	0.0390	0.1953	0.5000	0.2500	0.7500	Partial synergism
Citronella	Spearmint	0.1563	0.1563	0.3125	0.5000	0.2500	0.7500	Partial synergism
Citronella	Thyme linalool	0.1563	0.1563	0.3125	0.5000	0.2500	0.7500	Partial synergism
Spearmint	Thyme linalool	0.1563	0.3125	0.4688	0.2500	0.5000	0.7500	Partial synergism

[a] EO, essential oil; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

Table 3
Combinations of three essential oil vapours tested for inhibitory activity against a two-strain mixture of *P. corylophilum*, as determined using the experimental apparatus[a].

Concentration of EO (μL/mL)				FIC			FICI	Observation
Cinnamon bark	Citronella	May chang	Total EO	Cinnamon bark	Citronella	May chang		
0.0195	0.0390	0.0195	0.0781	0.1250	0.1250	0.1250	0.3750	Synergism
0.0195	0.0390	0.0390	0.0975	0.1250	0.1250	0.2500	0.5000	Synergism
0.0195	0.0390	0.0781	0.1366	0.1250	0.1250	0.5000	0.7500	Partial synergism

[a] EO, essential oil; FIC, fractional inhibitory concentration; FICI, fractional inhibitory concentration index.

combined at levels of 1/4 MIC (0.0390 μL/mL) and 1/4 MIC (0.0781 μL/mL), respectively, were applied.

Combinations of three EO vapours against *P. corylophilum* on DG18 agar had synergistic antifungal activity (Table 3). Cinnamon bark and citronella EO vapours (FICI = 0.5000) were selected for testing because, in combination, they clearly had a synergistic antifungal effect (Table 2). May chang EO vapour was selected for evaluation because it had partial synergistic antifungal activity (FICI = 0.6250) in combination with cinnamon bark and citronella EO vapours. A combination of cinnamon bark, citronella, and may chang EO vapours showed synergistic activity (FICI = 0.3750) at levels of 1/8 MIC (0.0195 μL/mL), 1/8 MIC (0.0390 μL/mL), and 1/4 MIC (0.0781 μL/mL), respectively.

3.2. Antifungal effects of combinations of EO vapours against P. corylophilum on beef jerky

Table 4 shows changes in populations of *P. corylophilum* on beef jerky treated with various concentrations (0, 0.0097, 0.0195, 0.0390, and 0.0781 μL/mL) of single EO vapours (cinnamon bark, citronella, or may chang) and a combination of these EO vapours (1:2:1 concentration ratio) at 25 °C and 93% RH for 120 h. The initial population of *P. corylophilum* inoculated on the surface of beef jerky (2.9 log CFU/cm^2) increased to 5.2 log CFU/cm^2 when the jerky was held at 25 °C and 93% RH for 120 h in an atmosphere without EO vapour. When beef jerky was exposed to single EO vapour, the minimum concentrations of cinnamon bark, citronella, and may chang vapours preventing an increase the population of *P. corylophilum* were 0.0390 μL/mL, > 0.0781 μL/mL, and 0.0781 μL/mL, respectively. A combination of cinnamon bark, citronella, and may chang EO vapours at 0.0195 μL/mL (0.0049, 0.0097, and 0.0049 μL/mL, respectively) inhibited the growth of *P. corylophilum*. At a concentration of 0.0195 μL/mL, the population of *P. corylophilum* on beef jerky treated with a combination of the three EO vapours was significantly lower than the population on jerky exposed to a single EO vapour ($P \leq 0.05$).

4. Discussion

Traditionally, agar dilution, solid medium diffusion, broth dilution, and broth microdilution methods have been used to measure MICs of antimicrobial agents against bacteria (Hammer et al., 1999; Souza et al., 2007; Witebsky et al., 1979), but these methods have been limited to measuring the MICs of antimicrobial agents in liquid phase. In contrast, protocols for the measuring MICs of antimicrobial agents in vapour-phase against molds on solid media have been received relatively meager research attention. To measure the MIC of EO vapours against *P. corylophilum*, we used an experimental apparatus and protocol introduced by Seo et al. (2015) and further developed by Lee et al. (2018). The MIC of EO vapour against *P. corylophilum* used in this study is defined as the lowest concentration of EO vapour that inhibited visible growth on a solid laboratory medium after inoculation and incubation at 25 °C for 5 days.

Several researchers have reported that some EO vapours (e.g., bay, clove, cinnamon leaf, lemongrass, mustard, and thyme) have antifungal activity against *P. corylophilum* (Guynot et al., 2003; Suhr and Nielsen, 2003, 2005); however, our study is the first to focus on determining MICs of plant EO vapours against *P. corylophilum*. Among 97 EO vapours tested for the inhibitory activity against *P. corylophilum*, garlic EO vapour showed the lowest MIC (0.0390 μL/mL), followed by cinnamon bark (0.1563 μL/mL), may chang (0.1563 μL/mL), citronella (0.3125 μL/mL), and thyme thymol (0.3125 μL/mL). Although garlic EO vapour had the lowest MIC, it did not exhibit synergistic antifungal activity in combination with other EO vapours tested. Combinations of cinnamon bark, citronella, and may chang EO vapours did show synergistic activity. The major antimicrobial components of cinnamon bark EO include cinnamaldehyde, 1,8-cineol, and eugenol (Didry et al., 1994; Hendry et al., 2009; Ranasinghe et al., 2002). It has been reported that cinnamaldehyde inhibits key enzymes related to cytokinesis and disrupts bacterial cell membranes (Hyldgaard et al., 2012). Eugenol is presumed to alter cell membrane and wall structures of yeasts, resulting in a release of cellular constituents (Bennis et al., 2004).

Table 4
Populations of a two-strain mixture of *P. corylophilum* on beef jerky treated with essential oils vapours[a].

Essential oil	Population (log CFU/cm^2)					
	Initial	Concentration of EO (μL/mL)[b]				
		0	0.0097	0.0195	0.0390	0.0781
Cinnamon bark	A 2.9 ± 0.1 c	A 5.2 ± 0.3 a	B 4.4 ± 0.3 b	A 4.5 ± 0.3 b	AB 3.2 ± 0.4 c	B 3.1 ± 0.3 c
Citronella	A 2.9 ± 0.1 e	A 5.2 ± 0.3 a	A 4.8 ± 0.2 b	B 4.0 ± 0.3 c	A 3.7 ± 0.5 cd	A 3.6 ± 0.4 d
May chang	A 2.9 ± 0.1 d	A 5.2 ± 0.3 a	A 5.2 ± 0.3 a	B 4.3 ± 0.2 b	AB 3.5 ± 0.3 c	B 2.9 ± 0.3 d
Cinnamon bark, citronella, and may chang (1:2:1)	A 2.9 ± 0.1 b	A 5.2 ± 0.3 a	A 4.9 ± 0.1 a	C 3.2 ± 0.0 b	B 3.0 ± 0.4 b	B 3.1 ± 0.3 b

[a] Beef jerky treated or not treated with EO vapour was inoculated with a two-strain mixture of *P. corylophilum* (3.1 log CFU/cm^2) and held at 93% RH and 25 °C for up to 120 h.

[b] Values in the same column that are not preceded by the same uppercase letter are significantly different ($P \leq 0.05$); values in the same row that are not followed by the same lowercase letter are significantly different ($P \leq 0.05$).

Linalool and citral are common components in citronella and may chang EOs, both having been shown to have antimicrobial activity (Gogoi et al., 1997; Nakahara et al., 2013; Onawunmi, 1989). Citronellal in citronella EO have also been reported to exhibit antimicrobial activities (Nakahara et al., 2013). Studies have shown that citral inhibits mycelial growth of *Penicillium italicum* by causing membrane damage (Tao et al., 2014). Recognizing that most studies on antimicrobial mechanisms of EOs have focused on bacteria, Hyldgaard et al. (2012) suggested that the mechanisms of the antifungal activities of EOs should be investigated.

The checkerboard assay has been used to test for synergistic effects of liquid antimicrobials. Since we wanted to test combinations of EO vapours for synergistic activity, we used a modified checkerboard assay based on the method developed by Lee et al. (2018). We believe that this is the first study designed to determine synergistic antifungal effects of gaseous antimicrobials using the checkerboard assay. Combinations of vapours showing the strongest synergistic antifungal effect against *P. corylophilum* on DG18 agar were cinnamon bark and citronella EO vapours (Table 2) and cinnamon bark, citronella, and may chang EO vapours (Table 3). When cinnamon bark and citronella EO vapours were combined, approximately 25% and 63% less were required, respectively, to cause the same inhibitory effects as cinnamon bark or citronella EO vapour alone. When cinnamon bark, citronella, and may chang EO vapours were combined, approximately 50%, 75%, and 50% less were required, respectively, to have the same inhibitory effects as cinnamon bark, citronella, or may chang EO vapour alone.

Synergistic antifungal effects of EO vapours against *P. corylophilum* have not been reported to date, but synergistic effects of EO vapours against other molds have been described. Edris and Farrag (2003) studied the combined effects of major constituents of peppermint and sweet basil EO vapours against phytopathogenic molds (*Sclerotinia sclerotiorum*, *Rhizopus stolonifera*, and *Mucor* sp.). They reported that, among the major constituents of peppermint EO, menthol (ca. 29.53% in the EO) showed antifungal activity but menthone (33.34% in peppermint EO) did not. However, when these components were combined in proportions present in respective EOs, the antifungal activity was enhanced. Linalool, one of the major components in sweet basil EO, showed antifungal activity, while eugenol did not, but a combination of linalool and eugenol in proportions present in sweet basil EO had increased antifungal activity. Edris and Farrag (2003) predicted that 1–8 cineol, which is a major component of sweet basil EO, along with linalool and eugenol, would also contribute to antifungal activity, and expected antifungal activity to be further increased when linalool and eugenol were combined with 1–8 cineol. In our study, we suspect that 1–8 cineol and eugenol, components of the cinnamon bark EO, had synergistic effects when together with linalool, a component of citronella EO and may chang EO. However, the mechanism of synergistic activity of cinnamon bark, citronella, and may chang EO vapours against *P. corylophilium* has not been defined.

Although *P. corylophilum* is considered to be a xerophilic mold, it is not an extreme xerophile because its growth is inhibited at a_w 0.80 (Guynot et al., 2003). *P. corylophilum* may grow and cause spoilage of foods, however, when the a_w is higher than 0.80. We conducted experiments simulating a situation where beef jerky is exposed to high RH (93%) in order to confirm the synergistic antifungal activities of combinations of EO vapours on *P. coryphilum* observed on a laboratory medium. The concentrations of cinnamon bark, citronella, and may chang EO vapours in combination (total was 0.0195 μL/mL) that inhibited the growth of *P. corylophilum* inoculated on beef jerky were lower than those resulting from a single EO vapour treatment (cinnamon bark EO vapour [0.0390 μL/mL], citronella EO vapour [> 0.0781 μL/mL], or may chang EO vapour [0.0781 μL/mL]). This indicates that the synergistic antifungal effects of combined EO vapours on *P. corylophilum* demonstrated on a laboratory culture medium were also exhibited on the surface of beef jerky. Minimum concentrations of single or combined EO vapours required to inhibit the growth of *P. corylophilum* on beef jerky were lower than the MICs of the same EOs found to inhibit growth on DG18 agar. Initially, it was expected that higher concentrations of EO vapours, compared to MICs observed on DG18 agar, would be required to inhibit the growth of *P. corylophilum* on beef jerky, because food components can reduce the bioactivity of some EOs (Gutierrez et al., 2008). Our findings suggest that intrinsic and extrinsic factors unique to beef jerky exerted a potentially adverse influence on growth of *P. corylophilum* on jerky compared to DG18 agar. For example, the a_w of beef jerky was 0.93 and the a_w of DG18 agar was 0.96. It is suspected that the a_w of DG18 agar would be more favorable than the a_w of beef jerky for growth of *P. corylophilum*. In addition, differences in nutrients in the beef jerky and DG18 agar may have affected growth of *P. corylophilum*. Beef jerky is high in proteins and lipids, and low in carbohydrates. Fungal metabolism is generally enhanced on substrates containing high levels of carbohydrates (Pitt and Hocking, 2009b). The seasoning applied to make the beef jerky used in our experiment may also have affected the growth of *P. corylophilum*. The seasoning contained garlic and ginger, both of which have been shown to have antifungal activities (Ankri and Mirelman, 1999; Shukla and Singh, 2007).

In summary, we screened 8 plant EO vapours among 97 commercially available EO vapours for inhibitory activity against *P. corylophilum*, determined the MIC values of those 8 EO vapours, evaluated combinations of EO vapours for synergistic antifungal activity on a laboratory medium, and showed that synergistic antifungal activity occurs on inoculated beef jerky treated with these vapours. It was shown that a combination of cinnamon bark (0.0049 μL/mL), citronella (0.0097 μL/mL), and may chang (0.0049 μL/mL) EO vapours had synergistic inhibitory activities to *P. corylophilum* on a beef jerky surface.

In future studies, the mechanism of synergistic activity of cinnamon bark, citronella, and may chang EO vapour against *P. corylophilium* should be precisely defined. Additional novel EO vapour combinations should be investigated. Ideally, a combination of EO vapours that controls multiple xerophilic molds capable of causing deterioration of intermediate-moisture foods should be identified. In our study, we focused on identifying combinations of EO vapours exerting synergistic antifungal activity against *P. corylophilum* on beef jerky, but we did not determine the influence EO vapours on sensorial properties of the beef jerky. Therefore, the effect of treatment of intermediate-moisture foods with combinations of EOs on sensorial properties should be evaluated. Finally, development of a technology for controlled release of EO vapours would be valuable for use in packaging systems for controlling fungal spoilage of intermediate-moisture foods.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.11.023.

Acknowledgments

This study was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea (No. 316032053WT081) and the Youlchon Foundation (Nongshim Corporation and its affiliated companies).

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