Video Article

The Effect of the Application of Thyme Essential Oil on Microbial Load During Meat Drying

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Abstract

Meat is a high protein meal that is used in the preparation of jerky, a popular food snack, where preservation and safety are important. To assure food safety and to extend the shelf life of meat and meat products, the use of either synthetic or natural preservatives have been applied to control and eliminate foodborne bacteria. A growing interest in the application of natural food additives for meat has increased. Microorganisms, such as *Escherichia coli*, contaminate meat and meat products, causing foodborne illnesses. Therefore, it is necessary to improve the meat conservation process. However, the use of essential oils when the meat is being dried has not been deeply studied. In this regard, there is an opportunity to increase the value of dried meat and reduce the risk of foodborne illnesses by applying essential oils during the drying process. In this protocol, we present a novel method of applying thyme essential oil (TEO) during meat drying, specifically in vapor form directly in a drying chamber. For evaluation, we use Minimal Inhibitory Concentration (MIC) to detect the number of harmful bacteria in the treated samples compared to raw samples. The preliminary results show that this method is a viable and alternative option to synthetic preservatives and that it significantly reduces microbial load in dried meat.

Video Link

The video component of this article can be found at https://www.jove.com/video/57054/

Introduction

Drying as a traditional method to preserve foods has been used since ancient times. Nowadays, there is a growing interest in drying as an effective method for food preservation[1,2,3]. It is used to make a variety of specially processed meats. One of the most well-known is jerky.

Jerky, one of the oldest methods for meat preservation, is based on curing and drying to lower water activity and therefore to extend its shelf life[4]. Nowadays, jerky as a preserved cured meat is still very popular, where food safety, flavor, and texture are essential. Jerky preparation can be used for almost any type of meat, including beef, pork, poultry, or game[5], and it requires chopping the meat in lean strips and drying it. Usually, marinating the meat in a curing solution or smoking are used along with drying to give the jerky its characteristic flavor[6].

Despite the vast interest of drying to truly preserve food, the risk of foodborne outbreaks by *E. coli* from poorly dried meat is critical and needs to be controlled. There are some studies reporting foodborne gastroenteritis outbreaks particularly with *E. coli* O157:H7, attributed to inadequate heat processing during home-drying. Similar cases have occurred even in commercially prepared jerky[7,8,9]. Levine *et al.*[10] proposed that foodborne microorganisms can survive moderate drying conditions (approximately 60 °C) used by commercial jerky producers. *E. coli* O157:H7 outbreaks of foodborne diseases in the middle of the 1990s were attributed to ground dried meat products[6,11]. Interestingly, in all the previous cases, the main risk is caused by bacterial pathogens recognized as viable but non-culturable (VBNC). Under various stresses such as temperature changes or starvation, the *E. coli* cells could enter a particular state known as the VBNC state[12,13]. The VBNC cells may then be resuscitated back to culturable cells by exposure to suitable conditions and then present a threat to human health due to foodborne contamination[14,15]. This means that if the meat is consumed immediately after drying the product it is safe. However, in the case of inadequate storage, such as increased humidity, there is a high risk of reactivation of pathogens and microbial growth.

Besides drying and marinade methods, there is a high demand from consumers to use natural products as an alternative to additives to improve food quality[16,17]. There has been a particular interest in the application of natural food additives for meat instead of classical synthetic preservatives[18,19,20,21]. Even though there is a lack of sufficient experimental evidence in the use of essential oils when drying the meat, early research in this field already demonstrates positive results[22,23].

Since the Middle Ages, people have recognized Essential Oil Compounds (EOCs) for their antimicrobial, insecticidal, and antiparasitic chracteristics[24,25,26]. Today, EOCs are part of one of the most important group of bioactive natural compounds. Among the different EOCs, thymol is one of the most well-known. It is composed of more than 85% of TEO[23]. This phenol prevents microbial and chemical deterioration

when added to food. Additionally, its antibacterial properties might be improved in combination with other natural preservatives[2,27,28,29,30]. Nowadays, thyme (*Thymus vulgaris*), a herb that belongs to *Lamiaceae* family, has been recognized as a flavoring agent as well as a very effective meat preservative[31]. A study by García-Díez *et al.*[30] on meat products found that TEO displayed a wider inhibition pattern against foodborne pathogens when compared to other essential oils. Therefore, there is an opportunity to increase the value of dried meat and reduce the risk of foodborne illnesses by applying essential oils during the drying process.

In this protocol, we present a novel method of applying TEO during meat drying, specifically using it in vapor form directly in a drying chamber. For evaluation, we use the MIC to determine the absence of pathogenic bacteria in treated samples compared with raw ones. The preliminary results show that this method is a highly effective alternative to synthetic preservatives and that it significantly reduces microbial load in dried meat.

Protocol

1. Meat Preparation

1. Obtain a short loin of beef (fresh beef from *biceps femoris*) from a local butchery and transfer it to the lab.
   NOTE: It is recommended to transport the loin of beef at ambient temperature (20 - 25 °C), for a period not longer than 20 min in a hermetic sealed bag.
2. To sterilize the outer surface of the beef muscle, in a laminar safety cabinet, wash the muscle by spraying with 70% (v/v) ethanol for 10 s using a squeeze bottle of 500 mL. Apply 0.025 g of ethanol per 1 cm^2 of muscle surface.
3. Remove the outer surface of the meat aseptically with a knife to avoid ethanol remaining in the muscle interior. Remove approximately 3 mm of the muscle interior to keep the muscle's surface uniformity.
4. Package the muscle in a plastic sealed bag and transfer it to a freezer.
5. Store the muscle at -18 °C for 1 day. Then, thaw the frozen muscle at 4 °C for 6 h.
   NOTE: For thawing, it is recommended to move the muscle from the freezer to the fridge.
6. In a laminar safety cabinet, slice each muscle into 0.5 cm thick slices with a meat cutter. Then, with a knife cut it into small 5 × 2.5 cm^2 rectangular samples.
7. Package the rectangular meat samples in plastic bags and store them in the freezer at -18 °C for later use.

2. Preparation of Standardized Inoculum and Inoculation Procedure in a Laminar Safety Cabinet

1. Prepare standardized inoculum (1.5 × 10^8 CFU/mL) of *E. coli* ATCC 25922 to inoculate the meat samples.
   1. For the preparation of the stock inoculum, first dispense the lyophilized bacterial culture (delivered by the supplier) into a 15-mL sterilized tube pre-filled with 10 mL of sterilized Buffered Mueller Hinton Broth (BMHB). Cultivate this suspension for 24 h at 37 °C.
      1. Prepare the bacterial stock solution as follows: Take approximately 0.1 - 0.2 mL of bacterial suspension and dilute into a 20-mL vial closed with a rubber stopper with an aluminum cap pre-filled with 15 mL of sterilized BMHB. Cultivate this suspension for 24 h at 37 °C.
      2. Store inside the refrigerator at 4 °C for the preparation of the standardized inoculum.
   2. From the stock solution (see step 2.1.1) of *E coli* take approximately 0.1 - 0.2 mL of bacterial suspension and dilute in a 15-mL plastic sterilized tube pre-filled with 10 mL of sterilized Buffered Mueller Hinton Broth (BMHB). Incubate the tube at 37 °C for 24 h.
   3. For the preparation of the standardized inoculum (1.5 × 10^8 CFU/mL), add small amounts of this suspension in a 15-mL sterilized tube pre-filled with 10 mL of sterilized BMHB.
   4. Thoroughly vortex the mixture and measure the optical density (OD) at 600 nm by a densitometer[32].
   5. Repeat steps 2.1.3 - 2.1.4 until the OD expressed as the McFarland value is increased by 0.5 compared to the value of clean BMHB.
2. For the inoculation procedure, place the rectangular meat samples in two different aluminum foils (20 cm x 30 cm), one for the control samples and the second for the inoculated meat samples.
   1. Over the second aluminum foil, inoculate the raw rectangular meat samples with 800 µL of bacteria suspension of the selected strain (this corresponds to 1.2 × 10^8 CFU per meat sample) by evenly distributing the inoculum on the surface.
      1. Pipette 400 µL on one side of the sample and gently spread using a sterile cell spreader on the surface. Let them dry for 10 min. Repeat the same procedure for the rest of the suspension on the other side of the sample.

3. Drying and TEO Application

1. Transfer both aluminum foils containing the rectangular meat samples from the laminar safety cabinet to the dryer: cover each with aluminum foil, and then place the samples inside the dryer.
2. Carry out the drying in a standard laboratory dryer.
   NOTE: First, preheat the oven to 55 °C. This procedure can last for 20 min.
   1. Dry the control samples for 6 h at 55 °C, with drying air relative humidity values ranging from 30 - 45%.
      NOTE: Drying air relative humidity values vary in time depending on the rate of evaporation of liquid from the meat.

3. Calculate the volume of TEO applied, and express the essential oil concentration as a volume of TEO per dryer volume (mL/L air). For example, the dose of 1.5 mL of TEO in 53 L (volume of the dryer) results in a concentration of 0.028 mL/L air. To determine the MIC of TEO for *E. coli*, use doses of 1.5 mL (0.028 mL/L air), 1 mL (0.019 mL/L air), and 0.75 mL (0.014 mL/L air).
4. Before drying, for the application of the TEO vapors (with thymol as the main compound 79%), soak a filter paper (12 cm x 20 cm) with a 1.5 mL dose of TEO and place into the dryer in front of the fan.
5. Dry the TEO treated meat samples using the same procedure as for the control samples (steps 3.1 and 3.2).
   NOTE: After the drying process ends and samples are removed, switch on the oven for 3 h at 80 °C and set the air valve indication to 100% air vent in order to clean the essential oil residues from the oven.

4. Microbial Analysis

1. Before the meat inoculation with bacteria, examine the meat samples for any adulteration. The appearance of slime and the detection of any strong and pungent smells are indicative of meat spoilage. If the texture feels slimy, the bacteria may have started to multiply on the surface of the meat.
2. To assess the inoculation efficiency, test the raw inoculated samples for the presence of *E. coli* ATCC 25922 and compare them with non-inoculated control samples before the drying procedure. For this purpose:
   1. Wash each meat sample (2 control samples and 2 inoculated samples). Suspend each meat sample in a sterilized flask with buffered peptone water (8.5 g of NaCl, 1 g of peptone, 5 tablets of phosphate-buffered saline, and 1 g of Polysorbate 80 in 1 L of water) in a ratio of 1:10 (w/v) with a pH range from 7 - 7.3. Shake using a shaker at 140 rpm for 10 min at room temperature.
      NOTE: Wash immediately after the inoculation procedure.
   2. Evaluate the number of bacteria by an adjusted 6 × 6 drop plate procedure summarized by Chen, Nace, and Irwin[33] on Plate Count Agar (PCA) and MacConkey Agar (MCA).
      NOTE: The 6 × 6 drop method uses the broth micro dilution method to prepare 10-fold serial dilutions of the investigated sample with a multichannel pipette, which is less labor intensive and more economical compared to the conventional method[33,34].
   3. Cultivate the 10-fold serial sample dilutions by the 6 × 6 drop plate procedure for the evaluation of *E. coli*.
      NOTE: Particularly for the 6 × 6 drop method, for cultivation use six 5 µL-drops, from six selected dilutions of the investigated sample with a multichannel pipette. On appropriately dried Petri dishes, the drops absorb quickly into the agar and the planting by this method is very convenient and manageable[34].
   4. Incubate the Petri dishes at 37 °C for 24 h. After the cultivation period, evaluate the number of colonies of *E. coli* on the Petri dishes (CFU g^{-1} of dried meat) as described in section 5.

3. After the drying, take two inoculated dried samples and compare them with two dried non-inoculated control samples for viable *E. coli*, respectively. To determine the presence or absence of *E. coli* of these four samples, carry out the pre-enrichment process of each meat sample as follows:
   1. Suspend each meat sample in a sterilized flask with buffered peptone water (see step 4.2.1) and shake using a shaker at 140 rpm for 10 min at room temperature. Then incubate each flask at 37 °C during 6 h for pre-enrichment.
   2. For the evaluation and cultivation of the bacteria, follow the same procedure as described in steps 4.2.2 - 4.2.4.

5. Review Results

1. After the incubation is complete, remove the Petri dishes from the incubator and review the results as follows:
   1. To evaluate the total number of colonies, examine the plates for the presence of mesophilic aerobic bacteria on PCA (white spots) and typical *E. coli* colonies (red to dark pink) on MCA. If the pathogen is absent, both agars present no growth.
   2. Count the colonies and determine the amount of *E. coli* (CFU/g^{-1} of dried meat) present.
      NOTE: Count the number of colonies (*N*) at two consecutive dilutions containing 30 or less colonies per drop (**Figure 1**). The number *N* of CFU/g^{-1} of dried meat is determined as follows[35]
      $$N = {}^{C}/_{v}\,(n_1 + 0.1n_2)d$$
      where, *C* is the sum of colonies on all drops counted, *v* is the volume of sample dilution used per drop (here, 0.05 mL), n_1 is the number of drops used at the first dilution, n_2 is the number of drops used at the second dilution, and *d* represents the dilution from which the first counts were captured.

2. To analyze the microbiological data, convert the number of colonies to log CFU g^{-1} and subject them to analysis of variance (ANOVA) for the main effects of treatment[36].
   1. Perform the Tukey Honest Significant Difference test (Tukey HSD) for multiple mean comparisons[36] and determine the significant differences between treatments.

Representative Results

We had first previously developed this method by using oregano essential oil (OEO) to enhance food safety and increase the value of dried meat. In general, the preceding experiments showed that *E. coli* goes into the VBNC state during drying as a survival strategy. This is demonstrated by the fact that there were no culturable bacteria after the drying finished[22]. Therefore, the pre-enrichment process for 6 h was necessary to allow the counting of the strain. In shorter periods, the numbers of growing cells were still very low. Consequently, the results are presented after the pre-enrichment process and excluding raw inoculated samples that indicate the control of inoculation efficiency (see **Table 1**). Overall, it was not necessary to test the TEO dose of 3 mL (0.057 mL/L air) since in our previous study[22] the *E. coli* was not found after the OEO treatments and it was evaluated for flavor as too intense by consumers. Therefore, lower concentrations of TEO were tested to define the MIC against *E. coli*.

Table 1 presents the behavior of *E. coli* in beef samples dried at 55 °C for 6 h and subjected to the pre-enrichment process for PCA and MCA. PCA shows the growth of mesophilic aerobic bacteria such as *Pseudomonas spp.* and *E. coli*. MCA identifies the presence of *E. coli*. Inoculated raw samples after inoculation (the control for inoculation efficiency) reached on average a population of 5.31 log CFU g^{-1} of bacteria, for PCA and MCA, which means there was no contamination on the meat samples at the beginning of the procedure. After drying, significant differences *(p* <0.05) were observed between non-treated samples (NoEO) and 0.75 mL, 1 mL, and 1.5 mL TEO-treated samples for both agars, respectively. This result revealed a successful performance of the TEO treatment, reducing the *E. coli* counts while increasing the essential oil dose. As well, the counts in both agars are very similar, which suggest that after pre-enrichment, the samples present *E. coli* and non-contamination with other bacteria. Significantly, *E. coli* was eliminated under the TEO treatment with the 1.5 mL dose. As a result, the TEO concentration of 0.028 mL/L of air was revealed as the appropriate MIC against *E. coli* due to a considerable decrease in VBNC *E. coli* (p <0.05) after 6 h of drying at 55 °C. The statistical differences were observed when performing multiple mean comparisons between the dose of TEO and sample type for PCA and MCA (see **Table 1**; Tukey HSD, p <0.05).

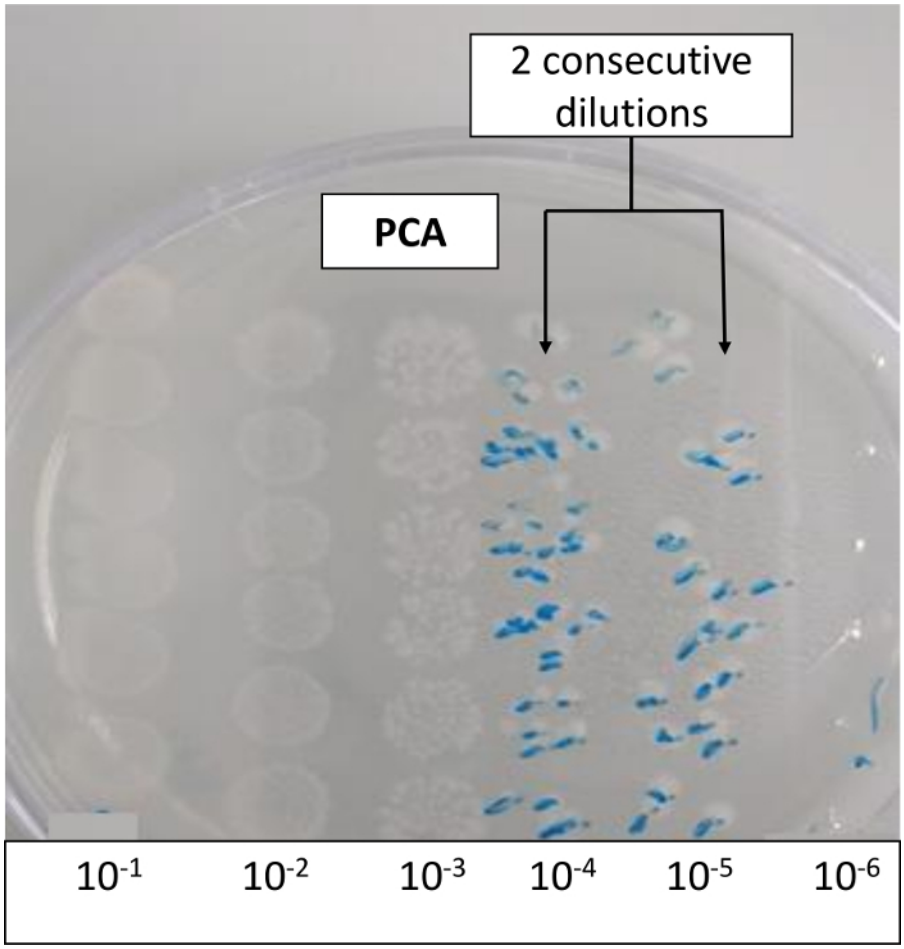

Figure 1: Demonstration of counting the number of colonies (N) at two consecutive dilutions containing 30 or less colonies per drop. This example results after the incubation of the PCA Petri dishes at 37 °C for 24 h. By utilizing the 6 × 6 drop method for cultivation, six 5 µL-drops were planted, from six selected dilutions of the investigated sample with a multichannel pipette. On appropriately dried Petri dishes, in this case PCA, the grown colonies (white spots) are enumerated from two consecutive dilutions (10^{-4} and 10^{-5}), which contain 30 or less colonies per drop. Please click here to view a larger version of this figure.

	Sample type			
	Treated samples		Untreated samples	
Dose of TEO	PE 6H_PCA	PE 6H_MCA	Raw_PCA	Raw_MCA
NoEO	3.929 (0.44)^{d}	3.833 (0.40)^{d}	5.474 (0.12)^{a}	5.516 (0.05)^{a}
0.75 mL	2.493 (0.11)^{c}	2.516 (0.22)^{c}	5.370 (0.03)^{a}	5.452 (0.24)^{a}
1 mL	1.574 (1.05)^{b}	1.579 (1.06)^{b}	5.129 (0.35)^{a}	5.123 (0.40)^{a}
1.5 mL	ND^{a}	ND^{a}	5.298 (0.09)^{a}	5.166 (0.33)^{a}

Table 1: Means (standard deviation) of behavior of *E. coli* ATCC 25922 (log CFU g^{-1}) in beef samples dried at 55 °C for 6 h in a conventional dryer subjected to pre-enrichment (PE) for 6 h and the control of inoculation efficiency (RAW) for both Plate Count Agar (PCA) and MacConkey Agar (MCA). Different letters ("a", "b", "c", "d") in the same column represent the statistical groupings of category means and indicate significant differences (p <0.05). Dose of TEO, dose of thyme essential oil; NoEO, no essential oil; ND, not detected. The p values reported are from multiple mean comparisons between the dose of TEO and sample type for PCA and MCA (Tukey HSD, p <0.05 indicates statistical significance).

Discussion

Previous research has shown that microorganisms causing foodborne diseases survive drying[10]. It is therefore necessary to apply preservatives before drying to assure food safety. In this study, we focus on using TEO. The reason is twofold: First, there is a high demand from consumers to use natural products as alternative additives to improve food quality[16]; Second, a previous study demonstrated positive results after using OEO during the meat drying process[22]. Hence, the method by the application of OEO during meat drying was extended to the use of other essential oils to control microbial load.

In a previous study, we have tested OEO to improve food safety and increase the value of dried meat. Our earlier results showed that *E. coli* was successfully inhibited by using OEO in the meat drying, since *E. coli* viable counts significantly decreased after 6 h of drying at 55 °C with 1.5 mL (0.028 mL/L air) of OEO[22]. For the present study, we implemented the method with TEO. It was demonstrated that by using this method it is possible to detect, enumerate, and reduce VBNC *E. coli* in dried meat samples. However, the use of TEO has restrictions due to organoleptic properties since it affects the taste, smell, and texture of the dried meat product. Because of this reason, it was critical to establish the MICs necessary to prevent *E. coli* growth, notably pathogenic bacteria that cause foodborne infections.

In both cases, *E. coli* was reduced under the OEO and TEO treatment with a 1.5 mL dose. As a result of both studies, the concentration of 0.028 mL/L air of OEO and TEO respectively, was indicated as the MIC against *E. coli* due to a significant decrease in the counts of VBNC *E. coli* (p <0.05) after 6 h of drying at 55 °C. The results in **Table 1** show that in samples treated with 1.5 mL of TEO, the *E. coli* was removed. In this regard, it was not necessary to test the dose of 3 mL (0.057 mL/L air) of TEO. Besides, a previous study demonstrated that the bacteria treated with the dose of 3 mL of OEO was not detected after the essential oil treatment[22]. Therefore, lower doses of TEO were used in the present protocol. This elimination of the *E. coli* is associated with the fact that TEO contains thymol, which is a very effective essential oil compound against microbial activity. Particularly, it is a predominant and the mostly recognized chemical compound against strains of *E. coli*[37,38].

This protocol has primarily been standardized to screen VBNC *E. coli* using pre-enrichment of the dried meat samples for 6 h to allow the counting of the strain (which is necessary because there were no culturable bacteria after finishing the drying). This protocol can potentially be adapted to detect other foodborne pathogens, such as, *Salmonella enteritidis* and *Listeria monocytogenes* in dried meat products, but more research in this area is needed.

Investigations dealing with foodborne pathogens are very dynamic and involve a multi-step process that might differ according to the specific situation and the local environment conditions. These investigations are important because they promote the use of natural additives in different food preservation techniques. As far as we know, these studies are the first to reveal a novel method by the application of essential oils during meat drying, specifically using them in vapor form directly in the drying chamber. The positive results show that this method is a remarkably effective choice to synthetic additives and that it significantly reduces microbial growth in dried meat. For future research, dose optimization of the application in combination with other essential oils and/or other preservation methods is recommended in order to evaluate the antimicrobial effect of those synergies.

Disclosures

The authors have nothing to disclose.

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