



SURFACE DECONTAMINATION OF *SALMONELLA ENTERICA* SEROVAR *TYPHIMURIUM* ON SHELL EGGS BY VAPORIZED ETHYL PYRUVATE AND PLANT HYDROSOLS

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ABSTRACT

In this study, the decontamination effects of plants hydrosols, and vaporized ethyl pyruvate were investigated against *Salmonella enterica* serovar *Typhimurium* on shell eggs. For hydrosol treatments, the first inoculum level was 6.8 log cfu/g. Inoculated eggs treated with thyme, sage, basil, and their mixed hydrosols and sterilized DI water as a control for 10, 30, 60, and 90 s. Mixed and thyme hydrosols provided the highest log reduction (3 and 3.4 log) in 90 s that can be considered favorable for food decontamination treatments. Inhibition level was determined in the initial population 6.2 log CFU/g of *S. typhimurium* inactivation at 100µL, 500 µL, and 1000 µL EP concentrations under ambient and refrigerator conditions for 3, 5, and 7 days. Almost complete inhibition was observed with 1000 µL of EP at 7 days of storage at 20°C. At refrigerator temperatures, the maximum log reduction was 4.2 after 7 days of storage. In conclusion, this study showed that vaporized EP applications, which are accepted as GRAS, have high antimicrobial activity against *S. typhimurium* in shell eggs. Hydrosol treatments had lower inhibition levels of *S. typhimurium* compare to EP treatment. Hydrosol treatments had lower inhibition levels of *S. typhimurium* compared to EP treatment.

1.Introduction

Eggs and egg products are often consumed worldwide as a balanced and nutritional protein resource (Alkaya, Erdogdu, Halkman, & Ekiz, 2016; Georgescu, Apostol, & Gherendi, 2017). According to American Egg Board (AEB) per capita egg consumption was 276 per person in 2017. Also, worldwide egg production has been rising to approximately 80 million metric tons of eggs by 2017, up from 37.4 million metric tons in 1990 (Statista, 2017). Centers for Disease Control and Prevention (CDC) reports that Salmonellosis causes about 1.2 million infections, 23,000 hospitalizations, and 450 deaths every year in the United States (CDC,

2019a) and costs the United States \$2.8 billion annually (Kim, Moreira, & Castell-Perez, 2011). *Salmonella* poses a great risk in the shell, and when the shell is broken, contamination occurs inside the egg. The product can cause infection when consumed raw or insufficiently cooked. According to the Public Health Agency of Canada (2003), the human oral infectious dose for *Salmonella* species has been determined as 102 – 103 CFU (Humphrey, Whitehead, Gawler, Henley, & Rowe, 1991). Salmonellosis is a zoonotic disease or infection that can be spread between animals and humans directly or indirectly. Eggs and egg products are one of the most common cause of salmonellosis outbreaks

(EFSA, 2009). For humans, salmonellosis is usually linked to infected food consumption (Gabriela Isabel Favier, Estrada, Otero, & Escudero, 2013). The most recent shell egg linked salmonellosis outbreak (*Salmonella enteridis*) has been reported by CDC was on September 8, 2018 in the U.S. which caused 12 hospitalizations (CDC, 2019b). The presence of *Salmonella* on eggshells not only major concern because of contamination risks but also it can penetrate into the egg from pores if egg cuticle has damaged. The cuticle can be damaged at the especially post washing process with usage of chemicals. Therefore, *Salmonella* needs to be inactivated or removed from the egg surface without damaging the cuticle (Keklik, Demirci, Patterson, & Puri, 2010).

The need to guarantee the microbiological safety of eggs without affecting their sensory and nourishing quality has prompted enhancement in the traditional procedures and the improvement of novel non-thermal methods (Lasagabaster, Arboleya, & De Marañon, 2011). Some non-thermal techniques that have been used for eggshell decontamination are pulsed UV light (Keklik et al., 2010; Lasagabaster et al., 2011). UV radiation (Chavez, Knape, Coufal, & Carey, 2002; Gabriela I Favier, Escudero, & de GUZMaÁN, 2001) herbal antimicrobial products ionized water (Davies & Breslin, 2003), atmospheric pressure plasma (Stolz et al., 2015), pulsed electric fields (Sampedro, Rodrigo, Martínez, Barbosa-Cánovas, & Rodrigo, 2006) etc. Each one of them has its own limitations and advantages. Hydrosols as the byproducts of hydro or steam distillation of natural plants may also be used as a natural decontamination method. The advantages of hydrosol use are cost effectiveness and not being harmful to health. Previous studies show that different plant hydrosols were effective on variety of products as a sanitizing agent (Tornuk et al., 2011). Many plant hydrosols that have antimicrobial properties have been used for this purpose. Törnük and Dertli (2015) (Tornuk & Dertli, 2015) have studied on the effectiveness of sage,

rosemary, oregano, and thyme hydrosols on parsley samples; Tornuk et al.(2011) (Tornuk et al., 2011) have evaluated black cumin, bay leaf, rosemary, and sage hydrosols on carrots and apples (Tornuk et al., 2011). Both studies demonstrated that hydrosols could be an effective and natural alternative method to improve food safety.

Ethyl pyruvate (EP) is a stable lipophilic therapeutic agent that is also known as an antioxidant. EP and its use in food are classified as generally recognized as safe (GRAS) by US Food and Drug Administration (Tornuk & Durak, 2015). It has been used as a decontamination method for several products especially on fresh fruits and vegetables recently. Törnük and Durak (2014) (Tornuk & Durak, 2015) evaluated the effectiveness of vaporized EP on fresh parsley on specific bacteria and ensured full inhibition of growth. Strawberries and cherries were also evaluated by (Bozkurt et al., 2016) and found that vaporized EP and cold storage together had great potential to delay fungal deterioration and preserve the quality of strawberries and cherries. However, the effect of EP and hydrosols have not been investigated on shell eggs. Hence the study presented here aimed to reduce the *Salmonella typhimurium* load of shell eggs via some plant hydrosol applications and vaporized EP. As hydrosol types, thyme, basil, and sage hydrosols were preferred for their high antimicrobial capacity.

2. MATERIALS AND METHODS

2.1. Bacterial culture and inoculum preparations

As a target pathogen, *S. enterica* ser. Typhimurium ATCC 140288 was provided from Acibadem Food Control Lab, İstanbul, Turkey. Stock cultures were preserved at -80 °C in a 20% glycerol solution. The working culture was grown on nutrient agar (NA; Merck, Darmstadt, Germany) plates at 25 °C for a day, then stored at refrigerator at 4 °C. A colony of *S. typhimurium* was aseptically transferred from the NA into 10 ml of nutrient broth (NB; Merck,

Darmstadt, Germany) and then incubated at 37°C for 24 h.

2.2. Preparation of plant hydrosols

In order to prepare plant hydrosols, dried leaves of thyme (*T. vulgaris* L.), sage (*S. officinalis*) and basil (*O. basilicum*) were provided from a spice warehouse in Istanbul, Turkey. Hydrosols were obtained as the following method of (Sağdıç, 2003). 100 g of each ground plant material was placed into a flask (2 L) with 1 L of distilled water for 2 h with a Clevenger apparatus (Ildam, Turkey). After hydrodistillation, essential oil was separated and obtained hydrosols were kept in sterile bottles at 4 °C until use.

2.3. Inoculation of egg samples

Grade A shell eggs had been purchased from a nearby Istanbul, Turkey auction. First, surface of the eggs were washed with tap water in order to get rid of dirt. Then, the eggs were soaked in 70% (v/v) of ethanol for disinfection. Before the inoculation, disinfected eggs were dried on sterile filter paper for 30 minutes in laminar flow. 0.1 ml of *S. enterica* ser. Typhimurium ATCC 140288 was inoculated onto each egg. The eggs were left for drying approximately 50 minutes at room temperature. Inoculated eggs were used for hydrosol and ethyl pyruvate applications.

2.4. Application of ethyl pyruvate (EP)

Inoculated egg samples were placed in previously autoclaved egg boxes. EP was applied to eggs using by Durak (2012) (Durak, Churey, Gates, Sacks, & Worobo, 2012) method with some modifications. EP was pipetted into the boxes that have *Salmonella* inoculated eggs at the amount of 250 ul, 500 ul and 1000 ul. Then, the boxes were closed and wrapped out with stretch film. EP-treated and control samples stored at room temperature (~20 °C) and at refrigerator (4°C) for up to 7 days.

2.5. Application of Hydrosol

Inoculated eggs were soaked into thyme, sage, basil and the mix of these three hydrosols. Eggs were put into sterilized beakers containing 200 mL of each sanitizing hydrosol (thyme, sage, basil and mixed) for 0, 10, 30, 60, 90 seconds. Hydrosols were renewed for each trials. *Salmonella* load of hydrosol treated and control samples enumerated. The analysis conducted duplicate.

2.6. Enumeration of *Salmonella* and determination of growth inhibition rate (GIR)

EP and hydrosol treated eggs were separated from their shells by using spoon and tweezers aseptically and put into stomacher bags. Shells weighed approximately 10 g and incorporated with 90 mL sterile 0.1% pepton water. The sample was homogenized by stomacher for 60 s then serially diluted with 0.1 percent peptone water followed by spread plating. The plates were incubated for 24 h at 37°C then plates were enumerated and the *salmonella* population was expressed as log CFU/g. logarithmic values. In addition to bacterial count, growth inhibition rates (GIRs) of *S. enterica* ser. Typhimurium was determined caused by hydrosol treatment calculated using the following equation (Eq. 1) as applied by Sagdic (2003)(Sagdic, 2003):

$$\%GIR = (PC - PT) / PC \times 100$$

where PC and PT are control populations and the samples being treated at a given time,, respectively.

2.7. Statistical analysis

Each treatment was conducted two times. Windows-based S.A.S. version 8.2 statistical analysis software (SAS Institute Inc., Cary, NC, 1989-2019) was used for analysis. For the statistical analysis of the microbial reductions, two way analysis of variance (ANOVA) was employed. The significance of the differences in mean values was determined using Tukey's multiple range method.

3. Results and discussion

3.1. Application of hydrosol

In this study, antimicrobial activity of thyme, sage, basil and the mix of these three hydrosols and different concentration of EP were evaluated in the inhibition of *S. typhimurium* inoculated shell eggs. The initial population of *Salmonella* on shell eggs confirmed as 6.8 log CFU/g by plating on Nutrient agar for hydrosol study. Inoculated eggs treated with plant hydrosols and sterilized DI water as a control for 10, 30, 60 and 90 s. Water treatment (control) was found to be ineffective ($P > 0.05$) at 10 and 30 s treatments,

however it was effective after 60 s compared to untreated eggs. This reduction was statistically lower than all the hydrosol treatments ($p < 0.05$). There was significant ($P < 0.05$) reduction on *Salmonella* population even in 10 s treatments for all type of hydrosols (Table 1). 1.5 to 2 log reductions were observed in 30 s and all hydrosol types showed significant ($P < 0.05$) difference from the control. Mixed and thyme hydrosols provided the highest log reduction (3 and 3.4 log) in 90 s that can be considered favorable for food decontamination treatments (Table 1).

Table 1. Antibacterial activity of the plant hydrosols on the *S. typhimurium** inoculated shell eggs.

Hydrosol type	Treatment Time (s)			
	10	30	60	90
Control	6.3±0.07 ^{Aa}	5.9±0.03 ^{Aa}	5.5±0.1 ^{Ba}	5.4±0.2 ^{Ba}
Thyme	6.1±0.04 ^{Ab}	5.4±0.01 ^{Bc}	4.3±0.05 ^{Cd}	3.9±0.04 ^{Dd}
Sage	5.9±0.03 ^{Abc}	5.6±0.08 ^{Bbc}	4.5±0.01 ^{Cc}	4.3±0.05 ^{Cc}
Basil	6.1±0.04 ^{Ab}	5.8±0.05 ^{Bab}	5.0±0.08 ^{Cb}	4.8±0.05 ^{Cb}
Mixed	5.8±0.03 ^{Ac}	4.8±0.01 ^{Bd}	3.7±0.01 ^{Ce}	3.4±0.01 ^{De}

*Initial inoculation levels of *Salmonella* was 6.2 log cfu/g.

A–D Different superscript uppercase letters show significant ($P < 0.05$) differences between treatment time within the same hydrosol type.

a–e Different superscript lowercase letters show significant ($P < 0.05$) differences between hydrosol time within the same treatment time.

Tornuk et al. (2011) (Tornuk et al., 2011) studied thyme, sage, black cumin, rosemary and bay leaf hydrosol inhibitory effects against *Salmonella typhimurium* and *Escherichia coli* O157:H7 inoculated on apples and carrots. They found that sterile tap water was ineffective in reducing *S. typhimurium* and *E. coli* O157:H7, where we found that washing was effective for eggs after 60s. The reason for this may be the surface structure of egg shells. They also demonstrated 1.1 – 1.5 log reduction for thyme hydrosol on *Salmonella* inoculated apple

and carrot respectively. The maximum log reductions for sage hydrosol were 0.7 and 0.9 log. Our reduction rates are slightly higher but similarly, we observed a higher decontamination effect in thyme than sage hydrosol. Similar to our findings, Öztürk et al. (2016) (Ozturk, Tornuk, Caliskan-Aydogan, Durak, & Sagdic, 2016) achieved 3 log reduction by thyme hydrosol application for 60 mins on iceberg lettuce samples. Growth inhibition rate (GIR) of *Salmonella* obtained by hydrosols is shown in Fig 1.

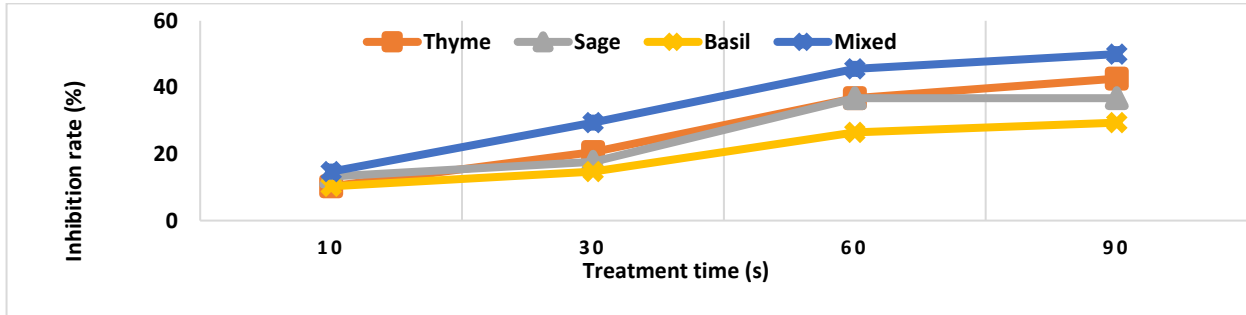


Figure 1. Growth inhibition levels of *S. Typhimurium* on shell egg samples by treatments of several plant hydrosols

This figure clearly shows the effect of treatment time and hydrosol types on *Salmonella* inhibition efficiency. Mixed hydrosol reached the highest inhibition rate with 50% at 90 s that is followed by thyme hydrosol by 42%. The lowest inhibition rate belongs to basil hydrosol with 29%. Sage hydrosol inhibited same rate of *Salmonella* at 60 and 90 s treatments which was around 37%.

3.2. Application of EP

Table 2 shows the effect of different concentrations (100µL, 500 µL and 1000 µL) of EP on *Salmonella* inactivation for 3, 5 and 7 days at ambient and refrigerator conditions. The initial population of *Salmonella* on shell eggs was 6.2 log CFU/g. Control and EP-treated eggs were monitored over 7 days. Almost the complete inhibition was observed with 1000 µL of EP at 7 days of storage at ambient temperature (Table 2).

Table 2. Antibacterial activity of the EP on the *S. typhimurium** inoculated shell eggs at 20°C and 4°C

20 °C Growth inhibition level (log cfu/g)				
Storage Time (Days)				
EP Concentrations (µL)	1	3	5	7
Control	0.4±0.08 ^{ABb}	0.3±0.01 ^{Bd}	0.6±0.04 ^{Ad}	0.3±0.01 ^{Bd}
250µl	0.3±0.01 ^{Db}	1.0±0.11 ^{Cc}	1.3±0.09 ^{Bc}	1.8±0.11 ^{Ac}
500µl	0.6±0.02 ^{Db}	1.4±0.01 ^{Cb}	2.3±0.04 ^{Bb}	2.8±0.04 ^{Ab}
1000µl	2.6±0.15 ^{Da}	3.0±0.11 ^{Ca}	3.5±0.06 ^{Ba}	5.4±0.01 ^{Aa}
4 °C Growth inhibition level (log cfu/g)				
Storage Time (Days)				
EP Concentrations (µL)	1	3	5	7
Control	0.2±0.01 ^{Bd}	0.4±0.03 ^{Ad}	0.5±0.01 ^{Ad}	0.4±0.03 ^{Ad}
250µl	0.3±0.01 ^{Cb}	0.8±0.01 ^{BCc}	0.9±0.12 ^{Bc}	1.3±0.05 ^{Ac}
500µl	0.5±0.01 ^{Dc}	1±0.01 ^{Cb}	1.7±0.02 ^{Bb}	2.3±0.01 ^{Ab}
1000µl	2.2±0.03 ^{Da}	2.6±0.03 ^{Ca}	3±0.42 ^{Ba}	4.2±0.2 ^{Aa}

*Initial inoculation levels of *Salmonella* was 6.2 log cfu/g.

A-D Different superscript uppercase letters show significant ($P < 0.05$) differences between treatment time within the same EP concentration.

a-d Different superscript lowercase letters show significant ($P < 0.05$) differences between EP concentration within the same treatment time.

One day storage of eggs with 250 ve 500 µL EP had no statistical difference ($P>0.05$) from the control sample at 20 °C. On the other hand, 1000 µL of EP treatment was effective even at 1 day storage. Tornuk and Durak (2015) evaluated EP efficiency on high and low inoculation levels of *E.coli* O157:H7 and *S.aureus* inoculated parsleys. Similar to our findings, 1000 µL EP treatments completely inhibited the *E.coli* O157:H7 population and enabled 100% growth inhibition levels at room temperature. At refrigerator temperatures, the maximum log reduction was 4.2 after 7 days of storage. There was a significant ($P<0.05$) effect of EP

concentration for all levels. After 5 days of storage, 3.5 and 2.3 log cfu/g reduction was observed at 1000 and 500 µL EP concentrations, respectively. Durak et al. (2012), assessed the effectiveness of EP on *E. coli* O157:H7 inoculated green onions and spinach at 4 and 10 °C. They observed full inhibition at 7th days for 4 °C and for 5th days for 10 °C at 420 g/L concentration of EP. Similar to our study, they also observed faster decontamination at higher temperatures as expected. GIR of *Salmonella* obtained by EP treatments are shown in Fig 2 and Fig 3.

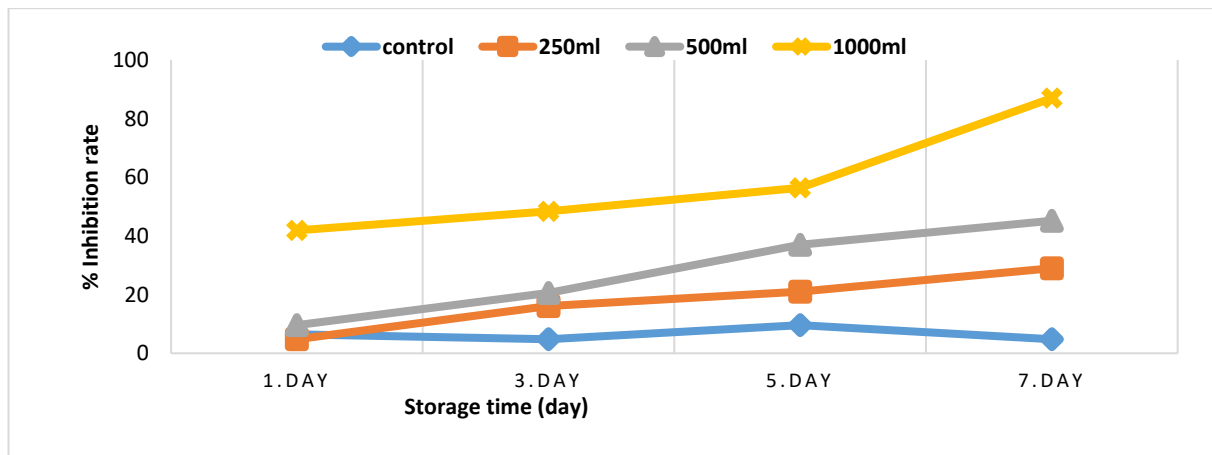


Figure 2. Growth inhibition levels of S.Typhimirum on shell eggs samples by treatments of EP at ambient temperature

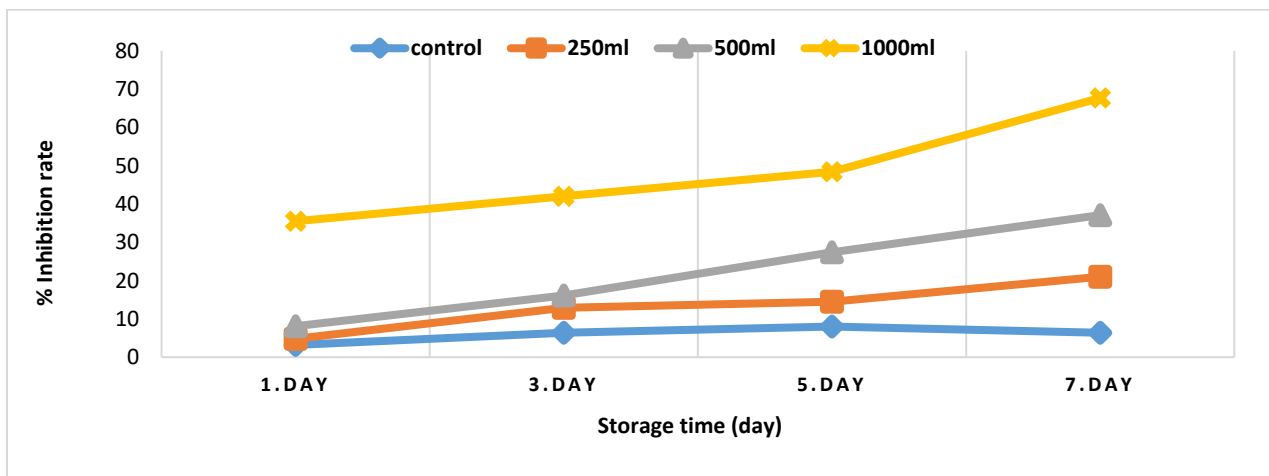


Figure 3. Growth inhibition levels of S.Typhimirum on shell eggs samples by treatments of EP at refrigerator temperature.

The concentration dependence of the inhibition levels can be seen from these figures. While the inhibition rates were under 50% for 100, 250 and 500 µL of EP concentrations; the inhibition rate at 1000 µL of EP was distinctly higher from the others ($P < 0.05$), for both temperatures.

4. Conclusions

The results obtained in this study demonstrated that vaporized EP treatments had high antimicrobial activity against *S. typhimurium* on shell eggs, and EP is already recognized as GRAS for human consumption. Plant hydrosol treatment is another natural method that promises surface decontamination of shell eggs. Compare to EP, hydrosol treatments had lower inhibition rates of Salmonella. However, especially mixed and thyme hydrosols reached 50% of inhibition rate. For further studies, different types of plant hydrosols could be used to decontaminate egg shells. Also, since hydrosol treatment maintains limited decontamination levels compared to others, it can be used in combination with another decontamination method.

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