ANTIMICROBIAL EFFECTS OF VAPOR PHASE THYMOL, MODIFIED ATMOSPHERE AND THEIR COMBINATIONS AGAINST *SALMONELLA* SPP. ON RAW SHRIMP

by

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ABSTRACT OF THE THESIS

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Salmonella contamination on raw shrimp is a big food safety concern in the U.S.. This research evaluated the inhibition effects of vapor phase thymol, modified atmosphere (MA) and their combination against *Salmonella* spp. on raw shrimp. Growth profiles of a *Salmonella* spp. cocktail (6 strains), inoculated onto the surface of raw shrimp, treated with vapor phase thymol at three levels (0, 0.8 and 1.6 mg l^{-1}), or MA (59.5% CO_2 + 39.5% N_2 + 1% O_2), both alone and in combination, at three temperatures (8, 12 and 16 ℃), were determined. Lag time and maximum growth rate of *Salmonella* spp. under each treatment were estimated using Baranyi & Roberts models. Results indicated that both vapor phase thymol and MA treatments alone

inhibited the growth potential of *Salmonella* spp. effectively, extending the lag time by 10 - 100% and reducing the maximum growth rate by 14 - 71% compared with controlled samples at experimental temperatures (8, 12 and 16 ℃). Combination treatments of vapor phase thymol and MA exhibited greater inhibition effectiveness than each individual treatment and a synergistic antimicrobial effectiveness could be observed on the lag time extension. To the maximum, at 12 ℃, lag time of *Salmonella* spp. was extended 59.6% more by the combination treatment of 0.8 mg l^{-1} thymol + MA (36.97 hrs) than those effects combined from 0.8 mg l^{-1} thymol treatment and MA treatment alone (23.16 hrs in total). Linear regression models of lag time and maximum growth rate for *Salmonella* spp. on raw shrimp under multiple stresses were also developed and validated. The vapor phase thymol + MA combination strategy could be potentially utilized for *Salmonella* inhibition during the long distance and temperature abused raw shrimp import process.

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1 INTRODUCTION

Shrimp has become an important food component globally. In the year of 2006, its worldwide production amount had increased to 6.6 million metric tonnes and its total trade value had increased to 24 billion USD [1]. U.S. is a huge market for raw shrimp consumptions and the demand is not sufficient to be satisfied by its domestic productions. Therefore, a large amount of raw shrimp needs to be imported from foreign countries such as Thailand and Indonesia [2]. Due to the limited sanitation conditions during cultivation and handling, shrimp imported from these countries have frequently been detected to associate with microbial safety issues, among which, *Salmonella* contamination has been dominating [3].

Salmonella are gram-negative anaerobic rods and are considered as pathogenic for humans [4]. *Salmonella* are responsible for Salmonellosis, which has become a major concern to the public health and a significant cost to the modern society [5]. In order to reduce the big economic losses resulted from shrimp import rejections as well as to enhance the microbial safety level to the consumers' concerns, it’s not only necessary but also urgent to find a strategy that is effective against *Salmonella* growth on raw shrimp and could be potentially applied into the raw shrimp import industry.

2-isopropyl-5-methylphenol, also known as thymol, is a natural monoterpene phenol derivative of cymene, found in oil of thyme, with a pleasant aromatic odor and strong antiseptic properties [6]. It’s considered as GRAS (Generally Recognized as

Safe) by FDA. Vapor phase thymol has been widely applied for *Salmonella* inhibition [7-9]. However, in order to be effective, the relatively high dose needed in the treatment could result in additional aroma which can greatly affect the original sensory profiles of the products [10].

Modified atmosphere (MA) is another widely applied solution to enhance the microbial safety level of various food commodities. CO_2 is typically used in these applications. CO_2 has been generally proven as effective against gram-negative bacteria [11] (including *Salmonella*) [12, 13]. However, its effectiveness may not be sufficient enough to retard the microbial growth to the desired level [12, 13] .

The combination of vapor phase thymol and MA, could overcome the limitations of utilizing each strategy alone and exhibit an enhanced antimicrobial effectiveness. It has been proven by a few researchers on some selected food commodities [14-17]. For example, *Guillen* and others found that by using MA (CO_2) with vapor phase thymol, the quality deteriorations of table grapes (such as weight loss, color changes and texture softening) were significantly inhibited and the microbial counts (molds, yeasts and mesophilic aerobics) were drastically decreased [16].

This combinational inhibition effect was systematically researched against *Salmonella* spp. on raw shrimp in this study. Growth profiles of *Salmonella* spp. inoculated on raw shrimp, under treatments of vapor phase thymol at three

concentrations (0, 0.8 and 1.6 mg l^{-1}) and MA (60% CO_2 + 40% N_2, ± 1% each), both alone and in combination, incubated at three temperatures (8, 12 and 16 ℃), were obtained. Growth characteristics (lag time and maximum growth rate) for each treatment were obtained by using Baranyi & Roberts models through combase website. The inhibition effects of vapor phase thymol, MA and their combination were evaluated and compared. Linear regression (polynomial) models were developed and validated for the growth characteristics (lag time and maximum growth rate) of *Salmonella* spp. on raw shrimp under multiple stresses, for the purpose of safety assessment and shelf life prediction.

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2 LITERATURE REVIEW

2.1 Shrimp

2.1.1 Definition

According to the Food and Agricultural Organization (FAO) glossary for aquaculture, a shrimp is defined as “a decapod crustacean of the suborder Natantia, in the largest phylum in the animal kingdom -- the Arthropoda, and is characterized by jointed appendages and a periodically molted exoskeleton.” [18].

2.1.2 Nutrition Profiles

Shrimp is very helpful for human's health. It has a high content of polyunsaturated fatty acids, especially Omega-3 fatty acids [19]. According to a survey conducted by the U.S. National Health and Nutrition Authority (1999 - 2000), shrimp was the second principal source for omega-3 fatty acids among all the commonly consumed seafood [20]. Shrimp also has a high content of quality proteins and various minerals such as Mg^{2+} and Ca^{2+} [20].

2.1.3 Production

According to Table 1, in the year of 2006, an amount of approximately 6.6 million metric tonnes of shrimp was produced from both capture and aquaculture, totaling a value of more than 24 billion USD. Compared with the year of 2000, the production amount was increased by 56% and the total value was increased by 32% [1].

Table 1 World shrimp production from 2000 to 2006

Source	Value	2000	2001	2002	2003	2004	2005	2006
Capture	1000 tonnes	3087	2955	2966	3543	3527	3420	3460
	US$ million	11,175	10,411	9788	11,621	11,357	11,458	11,764
Aquaculture	1000 tonnes	1162	1347	1496	2129	2446	2716	3164
	US$ million	7310	7492	7879	8355	9536	10,501	12,486
Total	1000 tonnes	4249	4302	4462	5672	5973	6136	6624
	US$ million	18,485	17,893	17,667	19,976	20,893	21,959	24,250

2.1.4 Consumption

According to the reports of United States National Marine Fisheries Services (NMFS), the shrimp consumption per capita in the U.S. was increased from 1.0 kg in 1989 to 1.8 kg in 2005 [21]. In the year of 2001, shrimp has surpassed canned tuna becoming the most largely consumed seafood in the U.S. [22].

2.1.5 Trade

World shrimp export trade was increased remarkably over the last 20 years. According to Table 2, in the year of 1986, the trade amount was estimated to be approximately 0.93 million metric tonnes while 20 years later, in the year of 2006, it increased to approximately 3.2 million metric tonnes. The trade value followed the same trend. In the year of 1986, the total value was estimated to be approximately 4.7 billion USD while in the year of 2006 it tripled to approximately 14 billion USD. Also, the share percentage of shrimp export trade amount in total fishery product was doubled over the past 20 years from 3.18% in the year of 1986 to 6.03% in the year of 2006 [1].

Table 2 International exports of shrimp by FAO ISSCAAP

	World Export Shrimp			Share in Total Fishery Product Exported (%)		
	1986	1996	2006	1986	1996	2006
Tonnes	938,102	1,601,147	3,244,871	3.18	3.68	6.03
US$ 1000	4,740,789	9,957,324	14,138,751	20.71	18.87	16.47

2.1.6 Safety

The shrimp market in the U.S. relies on foreign imports, majorly from developing countries with poor sanitation conditions during cultivation [2]. Therefore, many kinds of pathogens have been detected from the imported shrimp, among which, *Salmonella* is dominating. According to Table 3, *Salmonella* was responsible for 35.6% seafood (including shrimp) import rejections in the U.S. in total [3]. Also in the EU, from 1999 - 2002, *Salmonella* had been the second major cause for shrimp import rejections [3].

Table 3 Seafood import refusals by US FDA from July 2001 to June 2003

Year	Month	No. of Refusal	Refusal Caused by *Salmonella*
2001	July	122	20
	August	146	40
	September	59	14
	October	136	50
	November	121	39
	December	83	18
2002	January	177	71
	February	184	35
	March	213	38
	April	126	20
	May	174	41
	June	143	41
	July	136	53
	August	121	27
	September	115	39
	October	260	108
	November	125	15
	December	153	30
2003	January	298	42
	February	194	27
	March	210	37
	April	320	119
	May	281	76
	June	202	57
Total		3977	1057

2.2 Salmonella

2.2.1 Definition and Characteristics

Salmonella are gram-negative, non-spore forming, facultatively anaerobic and mobile rod (usually 0.7–1.5 × 2–5 μm in dimensions), belonging to the family of Enterobacteriaceae with more than 2500 serovars. They are considered as potential pathogens in both animals and human [4].

2.2.2 Salmonellosis

Salmonella are responsible for Salmonellosis, which has become a major concern for the public health and a significant cost to the modern society. In the U.S.,

for instance, a 1.4 million non-typhoidal *Salmonella* infection were reported annually resulting in 168,000 visits to physicians, 15,000 hospitalizations and 580 deaths [23], for a total cost estimated to be 3 billion USD in the year of 2005 [24].

2.2.3 Regulations

Currently there is not any internationally universal agreement on the "acceptable levels" of *Salmonella* on shrimp. But there are still some policies being established in many individual countries, as listed in Table 4. Generally speaking, countries including the U.S., Australia, the EU and Hongkong agreed that for raw or cooked ready-to-eat shrimps, *Salmonella* species should not be detected (so called "zero tolerance policy") [25-28]. This policy is also supported by International Commission of Microbiological Specification for Food (ICMSF) [29].

Table 4 Microbiological criteria/guidelines for *Salmonella* on shrimps

Countries/ Food Authority	*Salmonella* Criteria/Guidelines/Specification/Maximum Limits	
	Raw Shrimp (Fresh/Frozen)	RTE Shrimp/Cooked Shrimp
Australia	nil in 25 g	nil in 25 g
EU		absent in 25 g
US	adulterant	adulterant
Hongkong		absent in 25 g
ICMSF	nil in 25 g	nil in 25 g

2.3 Solutions for *Salmonella* Inhibition

2.3.1 Vapor Phase Antimicrobials

An antimicrobial is a substance that kills or inhibits the growth of microorganisms such as bacteria, fungi, or protozoans. Among all the antimicrobials, essential oils in vapor phase have drawn more and more scientific attentions currently,

because of its natural property and great antimicrobial effectiveness for applications require indirect contact.

Among all the available essential oils, vapor phase thymol has been reported by many researchers to have good antimicrobial effectiveness against *Salmonella* [7-9]. Thymol, also known as 2-isopropyl-5-methylphenol, is a natural monoterpene phenol derivative of cymene. It is found in oil of thyme, and extracted as a white crystalline substance with a pleasant aromatic odor and strong antiseptic properties [6]. The chemical structure and properties of thymol are listed in Figure 1 and Table 5 [6].

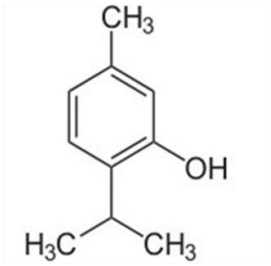


Figure 1. Structure of thymol

Table 5 Properties of thymol

Properties	Thymol
Molecular formula	$C_{10}H_{14}O$
Molar mass	150.22 g mol^{-1}
Density	0.96 g cm^{-3}
Melting point	51 ℃
Boiling point	232 ℃
Solubility in water	Insoluble

However, in order for it to be effective, the relatively high doses needed in the treatment could possibly bring in additional aroma which can affect the original sensory properties of the products [10].

2.3.2 Modified Atmosphere

Modified atmosphere (MA) is a widely applied solution to extend shelf life and improve the quality level of various food commodities. The modification of internal package atmosphere may take place at the level of total pressure or partial pressures of component gas components. The gas mixture applied in MA applications usually consists of N_2, O_2 and CO_2. Among them, CO_2 has been widely used to inhibit the growth of many gram-negative [11] bacteria including *Salmonella* [12, 13]. However its antimicrobial inhibition effectiveness may not be very strong [12, 13].

2.3.3 Combination of Vapor Phase Essential Oils and MA

The combination of vapor phase essential oils (such as thymol) and MA could overcome the limitations of utilizing each strategy alone (mentioned above) and exhibit an enhanced antimicrobial effectiveness, which has been proven by a few researchers on some selected food commodities [14-17]. For example, *Guillen* and others found that by using MA (CO_2) with vapor phase thymol, the quality deteriorations of table grapes (such as weight loss, color changes and texture softening) were significantly inhibited and the microbial counts (molds, yeasts and mesophilic aerobics) were drastically decreased [16].

3 OBJECTIVES AND TASKS

3.1 Rationale

1) Imported shrimp have frequently been detected to associate with *Salmonella* contamination in the U.S., which has placed a big safety burden to the general public.

2) Thymol is a natural antimicrobial with strong antiseptic properties. Its vapor phase has been reported to have great antimicrobial effects against *Salmonella*. However, the antimicrobial effects could be limited by the concentration capable to be added due to sensory concerns.

3) Modified atmosphere (MA) is a widely applied technology in food preservation area for microbial inhibition. Generally speaking, CO_2 used in MA can be effective against the growth of gram-negative bacteria including *Salmonella*. However, by applying MA alone, the inhibition effect is usually not strong.

4) Vapor phase thymol and MA combination has been proven to have an enhanced antimicrobial effects on some selected food commodities.

3.2 Objective

To investigate the antimicrobial effects of vapor phase thymol, MA and their combination against *Salmonella* spp. on raw shrimp as well as to develop linear regression models for the lag time and maximum growth rate of *Salmonella* spp..

3.3 Specific Tasks

1) Study Thymol Antimicrobial Ability

To preliminarily study the antimicrobial abilities of thymol against *Salmonella* through direct contact liquid phase inhibition zone test and minimum inhibition concentration (MIC) test preliminarily, then confirm the results with indirect contact vapor phase inhibition zone test and MIC test.

2) Study MA Antimicrobial Ability

To preliminarily study the antimicrobial ability of MA against *Salmonella* on agar.

3) Study Vapor Phase Thymol + MA Antimicrobial Ability

To preliminarily study the antimicrobial ability of vapor phase thymol + MA against *Salmonella* on agar.

4) Investigate Vapor Phase Thymol + MA Antimicrobial Effect on Shrimp

To investigate the antimicrobial effects of vapor phase thymol and MA, both alone and in combination against *Salmonella* spp. on raw shrimp at different temperatures (8 ℃, 12 ℃ and 16 ℃).

5) Model Development and Validation

To develop and validate linear regression (polynomial) models for the growth characteristics (lag time and maximum growth rate) of *Salmonella* spp. on raw shrimp under multiple stresses.

4 MATERIALS AND METHODS

4.1 Materials

Filter paper (cut into a circle with a diameter of 20 mm) and petri dishes were purchased from Fisher Scientific Inc. (Suwanee, GA, USA). BHI (Brain Heart Infusion) agar powder was purchased from MP Biomedicals (Solon, OH, USA). BHI broth powder was purchased from OXOID LTD (Basinstoke, Hampshire, England). XLT4 agar powder and peptone powder were purchased from BD Company (Sparks, MD, USA). Phosphate buffered saline (PBS) tablets were purchased from Sigma (St. Louis, MO, USA). 10 ml disposable glass tubes were purchased from VWR Scientific Products (West Chester, PA, USA). 500 ml Mason Jars were purchased from TMs Ball Corporation (Daleville, IN, USA). Parafilms were purchased from Pechiney Plastic Packaging (Menasha, WI, USA). 99.5+% thymol powder and 99.5+% ethanol (EtOH) were purchased from ACROS Company (Morris Plains, New Jersey, USA).

Modified atmosphere (MA) gas (60% CO_2 + 40% N_2, $\pm$0.5% each) was ordered from Airgas East Inc (Cheshire, CT, USA). A Series 5890A gas chromatography was purchased from HP Company (Palo Alto, CA, USA) and a Model 902D CO_2/O_2 detector was purchased from Quantek Instruments (Grafton, MA, USA).

Salmonella spp. (including *S*. typhimurium ATTC14028, *S*. Senftenberg ATTC8400, *S*. enteritidis ATTC13076, *S*. typhimurium FSIS 026, *S*. weltevreden FDA19143 and *S*. typhimurium ATTC29630) were obtained from

USDA-ARS-ERRC (Wydmoor, PA, USA). Fresh frozen shrimps (Size: 40/50) were purchased from Sahlman Seafoods Inc (Tempa, FL, USA). Shrimp radiator was purchased from Lockheed Georgia Company (Marietta, GA, USA).

4.2 Bacteria Species and Culture Conditions

Salmonella spp. (including *S*. typhimurium ATTC14028, *S*. Senftenberg ATTC8400, *S*. enteritidis ATTC13076, *S*. typhimurium FSIS 026, *S*. weltevreden FDA19143 and *S*. typhimurium ATTC29630) were utilized in the research.

4.2.1 Stationary Phase Bacteria Obtaining

A loopful of each strain was transferred from a -80 ℃ stock culture into a 10 ml Brain Heart Infusion (BHI) broth and incubated at 37 ℃ for 6 hrs. An equal amount of cell suspension of each strain was then separately transferred to a fresh 10 ml BHI broth and incubated at 37 ℃ for 24 hrs (at this point, the bacteria concentration in the BHI broth reached approximately 10^9 CFU ml^{-1} and was within stationary phase).

4.2.2 Exponential Phase Bacteria Obtaining

A loopful of each strain was transferred from a -80 ℃ stock culture into a 10 ml Brain Heart Infusion (BHI) broth and incubated at 37 ℃ for 6 hrs. An equal amount of cell suspension of each strain was then separately transferred to a fresh 10 ml BHI broth and incubated at 37 ℃ for 24 hrs. After that the bacteria broth was diluted by 10^5 times with new BHT broth, and then incubated at 37 ℃ for 2 hrs (at this point, the

bacteria concentration in the BHI broth reached approximately 10^6 CFU ml^{-1} and was within exponential phase).

4.3 Essential Oil Anti-*Salmonella* Abilities Test

4.3.1 Liquid Phase Direct Contact Inhibition Zone Test

This method was used for first round anti-*Salmonella* ability test of thymol. 0.1 ml 10^5 CFU ml^{-1} stationary phase (*bacteria 1*) or exponential phase (*bacteria 2*) *Salmonella* spp. was spread on to BHI agar plates. 0.1 g of 0, 2, 4 and 8 g l^{-1} thymol 95% EtOH solutions were added to filter paper discs respectively and then the paper discs were placed at the center of BHI agar plates already spread with *Salmonella*. All BHI agar plates were stored at 37 ℃ for 24 hrs and then the diameter of the inhibition zone size (mm) in each plate was measured.

4.3.2 Liquid Phase Direct Contact Minimum Inhibition Concentration (MIC) Test

This method was used for second round anti-*Salmonella* ability test of thymol. 2 ml BHI broth (containing 10^3 CFU ml^{-1} *Salmonella* spp., within stationary phase) was added into 10 ml glass tubes. Then 0.1 ml of 0, 0.05, 0.1, 0.2, 0.4 and 0.8 g l^{-1} thymol 95% EtOH solutions were added to the test tubes respectively. All the test tubes were covered carefully and stored at 37 ℃ for 24 hrs. Then the microbial count from each test tube was enumerated on XLT4 agar plates.

4.3.3 Vapor Phase Indirect Contact Inhibition Zone Test

The method was used to further investigate the anti-*Salmonella* ability of vapor phase thymol. 0.1 ml 10^5 CFU ml^{-1} *Salmonella* spp. (within stationary phase) was spread on to BHI agar plates. 0.1 g of 0, 8, 16 and 32 g l^{-1} thymol 95% EOH solutions were added to filter paper discs respectively and then the paper discs were placed at the center of the lid of the BHI agar plates already spread with *Salmonella*. All plates were sealed with parafilms tightly and stored at 37℃ for 24 hrs. Then the diameter of the inhibition zone size (mm) in each plate was measured.

4.3.4 Vapor Phase Indirect Contact MICTest

The test was used to confirm the anti-*Salmonella* effect of vapor phase thymol. 0.1 ml 10^5 CFU ml^{-1} *Salmonella* spp. (within stationary phase) was added in to 500 ml glass jars (containing 25 g of XLT4 agar in the bottom) and spread evenly on the agar. 0.4 g of 0, 8, 16, 32 and 64 g l^{-1} thymol 95% EOH solutions were added respectively to filter paper discs that were stuck onto the walls of the jars in advance. Jars were closed and stored at 37℃ for 24 hrs and then the microbial colonies formed on the agars were counted.

4.4 Modified Atmosphere (MA) Anti-*Salmonella* Effect Test

The test was used to preliminarily investigate the anti-*Salmonella* effect of MA (59.5% CO_2 + 39.5% N_2 + 1% O_2). 0.1 ml 10^4 CFU ml^{-1} *Salmonella* spp. (within stationary phase) was added in to 500 ml glass jars (containing 50 g of BHI agar in the bottom) and spread evenly on the agar. Half of the jars were flushed with 60%

CO_2 + 40% N_2 (±0.5% each) gas at a flow rate of 500 ml min^{-1} for 2 min and the other half of them were left without treatments as controls. Jars were closed and stored at 37℃ for 24 hrs and then the microbial colonies formed on the agars were counted.

4.5 Vapor Phase Thymol + MA Anti-*Salmonella* Effect Test

The test was used to preliminarily investigate the anti-*Salmonella* effect of vapor phase thymol and MA (59.5% CO_2 + 39.5% N_2 + 1% O_2) in combination against *Salmonella* spp..

0.1 ml 10^4 CFU ml^{-1} bacteria (within stationary phase) was added in to 500 ml glass jars (containing 40 g of BHI agar in the bottom) and spread evenly on the agar. For some parts of the jars, 0.2 g of 32 g l^{-1} thymol 95% EtOH solution was added into filter paper discs that were stuck onto the wall of these jars in advance. For some parts of the jars, 60% CO_2 + 40% N_2 gas (±0.5% each) gas was flushed at a flow rate of 500 ml min^{-1} for 2 minutes. For other parts of the jars, the same thymol treatments and MA treatments were applied in combination. All jars were closed and stored at 37℃ for 24 hrs and then the microbial colonies formed on the agars were counted.

4.6 Bacteria Inoculation on Shrimp

Raw shrimp was cleaned, de-headed, peeled and pre-irradiated using 137-Cs gamma radiation source at a dose rate of 10 kGy min^{-1} for 133.84 min. Then the irradiated shrimp was cut into 5 g pieces and put inside 500 ml glass jars. 0.05 ml 10^5

CFU ml^{-1} *Salmonella* spp. (within stationary phase) were inoculated onto the surface of shrimp samples.

4.7 Treatments on Shrimp

4.7.1 Vapor Thymol Treatment

After bacteria inoculation, 0.05 ml of 0, 8, 16, 32, 64, 128 and 256 g l^{-1} thymol 95% EtOH solutions were added respectively into filter paper discs that were stuck onto the wall of the glass jars in advance. All jars were closed and stored at 8, 12 or 16 ℃.

4.7.2 MA Treatment

After bacteria inoculation, the jars were flushed with 60% CO_2 + 40% N_2 gas (±0.5% each) at a flow rate of 500 ml min^{-1} for 2 min. Then all jars were closed and stored at 8, 12 or 16 ℃.

4.7.3 Vapor Phase Thymol + MA Treatment

After bacteria inoculation, jars were flushed with 60% CO_2 + 40% N_2 gas (± 0.5% each) at a flow rate of 500 ml min^{-1} for 2 min. Then 0.05 g of 0, 16 and 8 g l^{-1} thymol 95% EtOH solutions were added respectively into the filter papers that were stuck onto the walls of the jars in advance through the two connectors that were inlaid into the lid. All jars were closed and stored at 8, 12 or 16 ℃.

4.8 Vapor Phase Thymol Concentration Determination

A HP Series 5890A Gas Chromatography was used to determine the vapor phase thymol concentrations inside the glass jars under different thymol EtOH solution treatments. The results from GC matched with the theoretic values calculated from the equation of $\left(\frac{\text{Weight of Liquid thymol solution (g)} \times \text{Purity of thymol (\%)}}{\text{Volume of the jar (l)}}\right)$ and therefore suggested complete evaporations of thymol from its EtOH solutions into the headspace. Detailed settings for the GC were as follows: a thermal conductivity detector and a CTR I column were used; Oven temperature, injection temperature and detector temperature were set at 50, 90 and 110 ℃ respectively; Hydrogen was used as the carrier gas and its flow rate was set at 60 ml min^{-1}.

4.9 $CO_2/O_2/N_2$ Concentration Determination

For MA treated samples, a Model 902D CO_2/O_2 detector was used to detect the initial CO_2 and O_2 concentration inside the glass jars right after MA gas flushing (which was 59.5% and 1% respectively). The percentage of N_2 was obtained by subtracting the percentage of CO_2 and O_2 from 100% (which was 39.5%). During later storage, the detector was used to monitor the inner O_2 gas concentration changes.

4.10 Bacteria Enumeration on Shrimp

At proper time intervals during storage, jars were opened and shrimp samples (duplicates) were transferred into sterile stomacher bags, and 5 ml (1:1 to shrimp weight) of sterile 0.05% peptone water were added [30]. After being homogenized for 1 min at 300 rpm in the stomacher, 0.05 - 0.1 ml of the homogenized solution were

enumerated on *Salmonella*-selective XLT4 agar plates and then incubated at 37 ℃ for 24 - 48 hrs. Proper dilutions were performed, if needed, to attain reliable plate counts.

4.11 Model Development

The growth data were analyzed using the DMFit software available on the Combase website (http:www.combase.cc). Baranyi & Roberts model [31] was applied to fit the growth data to obtain lag time and maximum growth rate of *Salmonella* spp. for each treatment. Linear regression (or polynomial) models were developed and validated for lag time and maximum growth rate based on the experimental design. The SAS, v9.1 was used for data analysis.

5 RESULTS AND DISCUSSIONS

5.1 Growth Profiles of *Salmonella* spp.

5.1.1 Stationary Phase Bacteria Obtaining

In BHI Broth

Table 6 Growth profile of *Salmonella* spp. at 37 ℃ in BHI broth

Time (hr)	Duplicate 1	Duplicate 2	Dilution	Average Counts	STDV	Concentration (CFU ml^{-1})
0	105	110	10	107.5	$3.54*10^{2}$	$1.08*10^{4}$
2	168	174	10^{2}	171	$4.24*10^{3}$	$1.71*10^{5}$
4	35	40	10^{4}	37.5	$3.54*10^{5}$	$3.75*10^{6}$
6	28	29	10^{6}	28.5	$7.07*10^{6}$	$2.85*10^{8}$
8	55	66	10^{6}	60.5	$7.78*10^{7}$	$6.05*10^{8}$
10	80	53	10^{6}	66.5	$1.91*10^{8}$	$6.65*10^{8}$
12	161	139	10^{6}	150	$1.56*10^{8}$	$1.50*10^{9}$
20	61	45	10^{7}	53	$1.13*10^{9}$	$5.30*10^{9}$
22	88	93	10^{6}	90.5	$3.54*10^{7}$	$9.05*10^{8}$
24	27	42	10^{7}	34.5	$1.06*10^{9}$	$3.45*10^{9}$
26	124	138	10^{6}	131	$9.90*10^{7}$	$1.31*10^{9}$
29	334	327	10^{6}	330.5	$4.95*10^{7}$	$3.31*10^{9}$
44	299	199	10^{6}	249	$7.07*10^{8}$	$2.49*10^{9}$
56	275	292	10^{6}	283.5	$1.20*10^{8}$	$2.84*10^{9}$

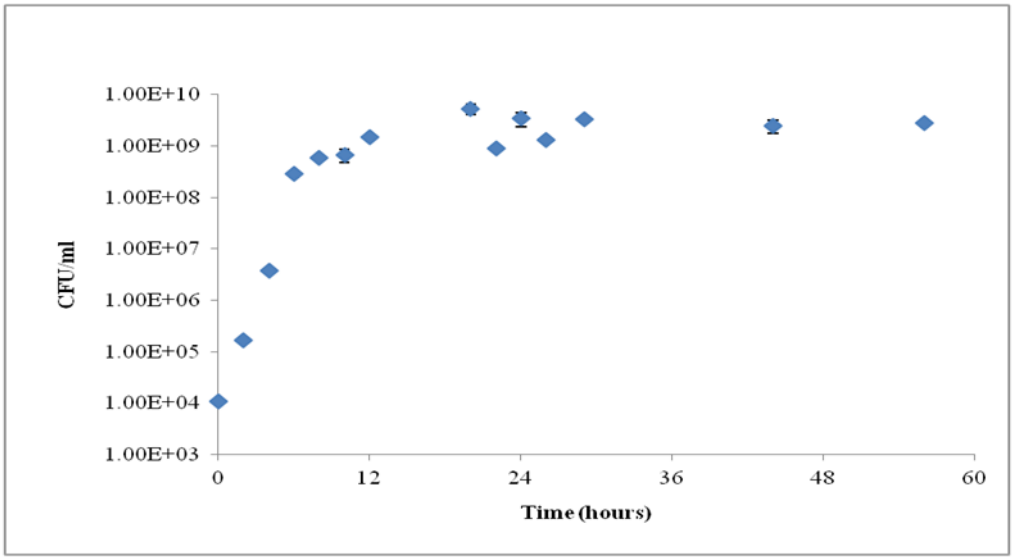


Figure 2. Growth curve of *Salmonella* spp. at 37 ℃ in BHI broth

From Table 6 and Figure 2, it could be seen clearly that with a starting bacteria concentration of around 10^4 CFU ml^{-1} in BHI broth, *Salmonella* spp. gradually propagated and finally reached a constant concentration of around 10^9 CFU ml^{-1} after 12-hr incubation at 37 ℃ (within stationary phase).

In 50% BHI and 50% PBS Solution

Table 7 Growth profile of *Salmonella* spp. at 37 ℃ in 50% BHI and 50% PBS solution

Time (hr)	Dilution	Average Counts	Concentration (CFU ml^{-1})
0	10^3	48	$4.8*10^4$
2	10^5	84	$8.4*10^6$
4	10^6	68	$6.8*10^7$
6	10^6	33	$3.3*10^7$
8	10^5	21	$2.1*10^6$
10	10^5	370	$7.7*10^7$
12	10^5	31	$3.1*10^6$

Table 7 showed that *Salmonella* spp. could grow up to a concentration of around 10^7 - 10^8 CFU ml^{-1} in 50% BHI and 50% PBS (Phosphate Buffered Saline) solution within initial 4 - 6 hrs' incubation at 37 ℃ with a starting concentration of around 10^4 CFU ml^{-1}. However, after 6 hrs, its growth suddenly stopped and the bacteria concentration was fluctuating between 10^6 – 10^8 CFU ml^{-1}. The accurate concentration was difficult to predict.

In PBS Solution

Table 8 Growth profile of *Salmonella* spp. at 37 ℃ in PBS solution

Time (hr)	Dilution	Average Counts	Concentration (CFU ml^{-1})
0	10^3	35	$3.5*10^4$
4	10^3	24	$2.4*10^4$
12	10^2	17	$1.7*10^3$
24	10	27	$2.7*10^2$

As could be seen from Table 8, with a starting bacteria concentration of around 10^4 CFU ml^{-1}, the concentration of *Salmonella* spp. in PBS solution kept decreasing (indicating within death phase). After 24-hr incubation at 37 ℃, the bacteria concentration within the solution decreased to around 10^2 CFU ml^{-1}.

From above three experiments, we could make a conclusion that BHI broth was the most reliable culture medium to get a consistent bacterial concentration of *Salmonella* within stationary phase. Generally speaking, after 24-hr incubation at 37 ℃ with a starting concentration of 10^4 CFU ml^{-1}, the concentration of *Salmonella* in BHI broth would reach around 10^9 CFU ml^{-1} and was within the stationary phase.

5.1.2 Exponential Phase Bacteria Obtaining

Table 9 Growth profile of *Salmonella* spp. at 37 ℃ in BHI broth within first 6 hrs

Trial	Time (hr)	Dilution	Average Counts	Concentration (CFU ml^{-1})
1	0h	10^2	400	$4.0*10^4$
	2h	10^4	250	$2.5*10^6$
	4h	10^6	72	$7.2*10^7$
	6h	10^7	106	$1.1*10^9$
2	0h	10^2	200	$2.0*10^4$
	2h	10^4	13	$1.3*10^6$
	4h	10^5	100	$1.0*10^8$
3	0h	10^2	289	$2.9*10^4$
	2h	10^2	105	$1.1*10^6$
4	0h	10^2	321	$3.2*10^4$
	2h	10^2	119	$1.2*10^6$

From Table 9, we could see that after 2-hr incubation at 37 ℃ in BHI broth with a starting concentration of around 10^4 CFU ml^{-1}, *Salmonella* spp. reached a consistent concentration of around 10^6 CFU ml^{-1} and at this point the bacteria were within exponential phase.

5.2 Essential Oil Anti-*Salmonella* Ability Test

5.2.1 Liquid Phase Direct Contact Inhibition Zone Test

Table 10 Liquid phase direct contact inhibition zone test of thymol against *Salmonella* spp.

Thymol Concentration ($g\ l^{-1}$)	*Bacteria 1* Inhibition Zone Average (mm)	*Bacteria 2* Inhibition Zone Average (mm)
Control	0	0
EtOH	0	0
2	10	10
4	18	20
8	29	31

From Table 10, it could be seen that EtOH treatment didn't result in any visible inhibition zone. It suggested that the EtOH didn't have any anti-*Salmonella* inhibition effectiveness, which was consistent with the experiments done by *Guarda* and others [32]. For thymol, it preliminarily exhibited a good antimicrobial inhibition effectiveness against *Salmonella* spp. through its diffusion on the agar. and the effectiveness was concentration dependent.

Theoretically speaking, exponential phase bacteria usually have better activity than those in stationary phase, since the bacteria within exponential phase are propagating at the maximum speed [33]. According to "worst case scenario" principle, exponential phase bacteria should be utilized in the research. However through the experiments, for *bacteria 1* (within stationary phase) and *bacteria 2* (within exponential phase), according to statistic analysis results (results not shown), no

significant difference (P > 0.05, ANOVA, SAS v9.1) was observed. Therefore, stationary phase *Salmonella* were utilized in further research since it's easier to obtain.

5.2.2 Liquid Phase Direct Contact Minimum Inhibition Concentration (MIC) Test

Table 11 Liquid phase direct contact MIC test of thymol against *Salmonella* spp.

	Concentration ($g\ l^{-1}$)	Average Counts	Bacteria Concentration ($CFU\ ml^{-1}$)
Control	-------	630	$6.3*10^3$
EtOH	-------	609	$6.1*10^3$
Thymol	0.8	0	0
	0.4	0	0
	0.2	51	$5.1*10^2$
	0.1	128	$1.3*10^3$
	0.05	280	$2.8*10^3$

From Table 11, it confirmed that compared with controls, liquid phase thymol had effective inhibition against *Salmonella* spp. in culture broth. As the thymol concentration increased, the bacteria concentration inside the broth decreased. When 0.4 $g\ l^{-1}$ thymol was added, the bacteria concentration dropped to 0 $CFU\ ml^{-1}$and therefore could be considered as its MIC.

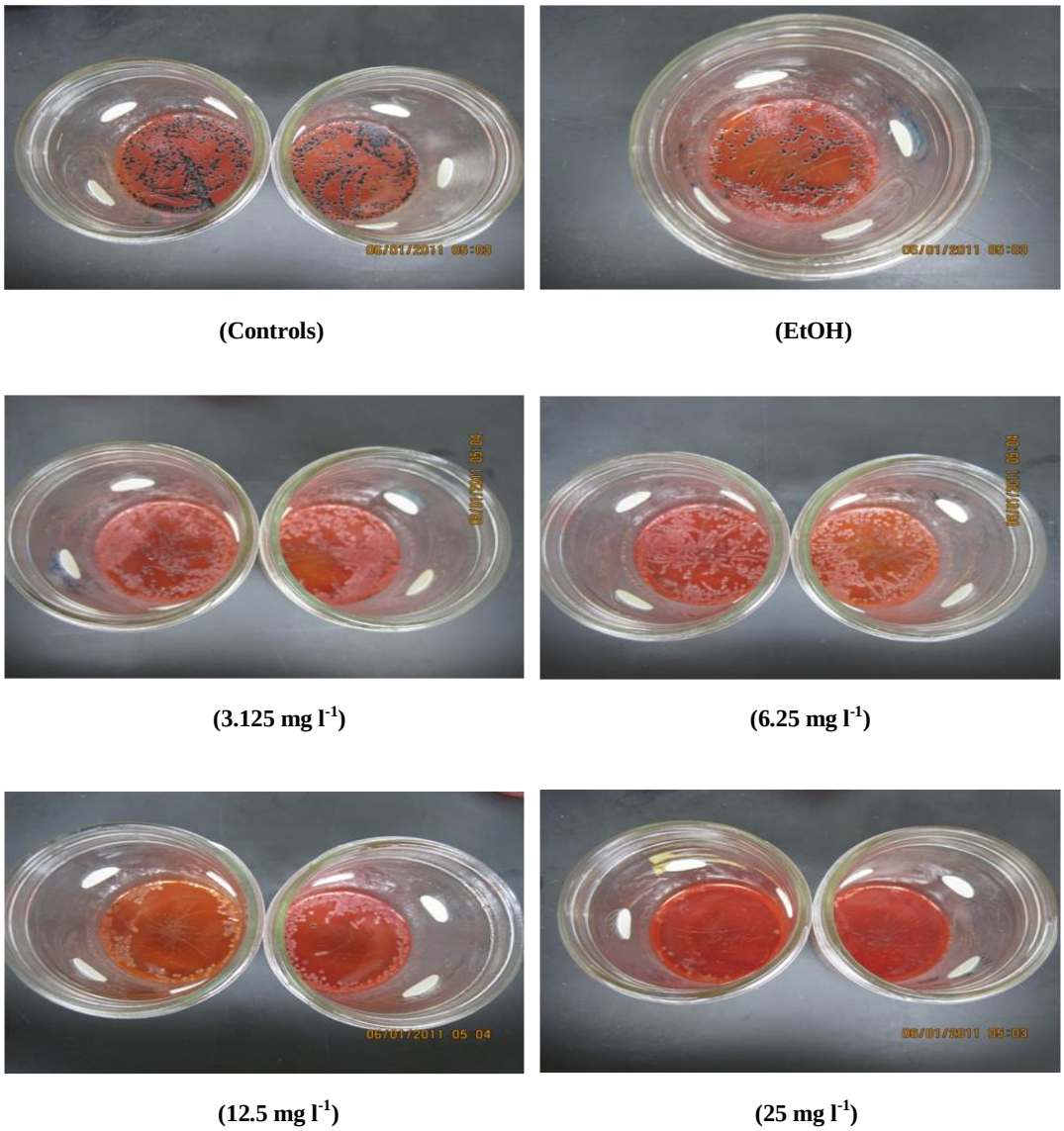
5.2.3 Vapor Phase Indirect Contact Inhibition Zone Test

Table 12 Vapor phase indirect contact inhibition zone test of thymol against *Salmonella* spp.

	Concentration ($g\ l^{-1}$)	Inhibition Zone Size (Average, mm)
Control	--------	0
EtOH	--------	0
Thymol	8	15
	16	20
	32	30

From Table 12, it could be seen that vapor phase thymol demonstrated great antimicrobial effectiveness against *Salmonella* spp. and as its concentration increased, the size of the inhibition zone increased as well. EtOH vapor didn't exhibit any inhibition effect.

5.2.4 Vapor Phase Indirect Contact MICTest



(Controls) **(EtOH)**

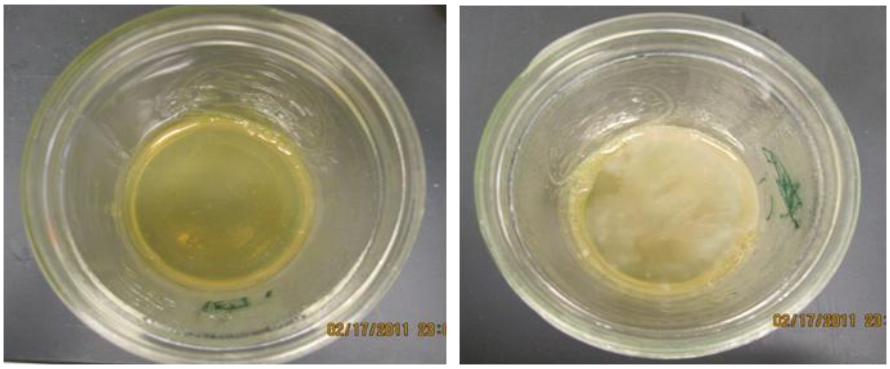
(3.125 mg l^{-1}) **(6.25 mg l^{-1})**

(12.5 mg l^{-1}) **(25 mg l^{-1})**

Figure 3. Vapor phase indirect contact MIC test of thymol against *Salmonella* spp.

Figure 3 confirmed the great antimicrobial effectiveness of vapor phase thymol against *Salmonella* spp.. 25 mg l^{-1} vapor thymol had almost complete inhibition effectiveness and could be considered as MIC in this case.

5.3 Modified Atmosphere (MA) Anti-*Salmonella* Effect Test



(With MA treatment) (Control)

Figure 4. MA effect on *Salmonella* spp.

According to Figure 4, for the jars without MA treatment, it showed that *Salmonella* spp. grew well on the surface of BHI agar, with thick colonies being formed. For the jars with MA treatment, it showed that the growth of *Salmonella* spp. was inhibited compared with controlled samples. This finding illustrated the inhibition effectiveness of CO_2 against *Salmonella* spp., which was consistent with the research work done by *Gill*, *Murphy* and others [12, 13].

5.4 Vapor Phase Thymol + MA Anti-*Salmonella* Effect Test

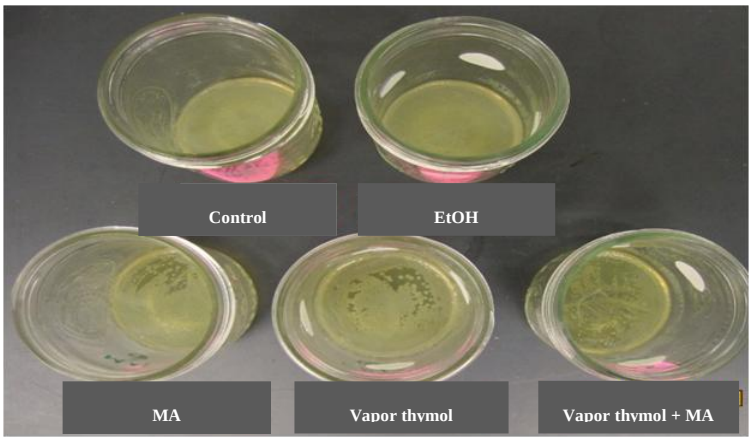


Figure 5. Vapor phase thymol, MA, vapor thymol + MA effects on *Salmonella* spp.

According to Figure 5, compared with each individual treatment, an enhanced inhibition effectiveness was clearly observed when vapor phase thymol (12.8 mg l^{-1}) + MA was used in combination [14-17]. This finding was the foundation for our future research on raw shrimp.

5.5 Growth Profiles of *Salmonella* spp. in BHI Broth

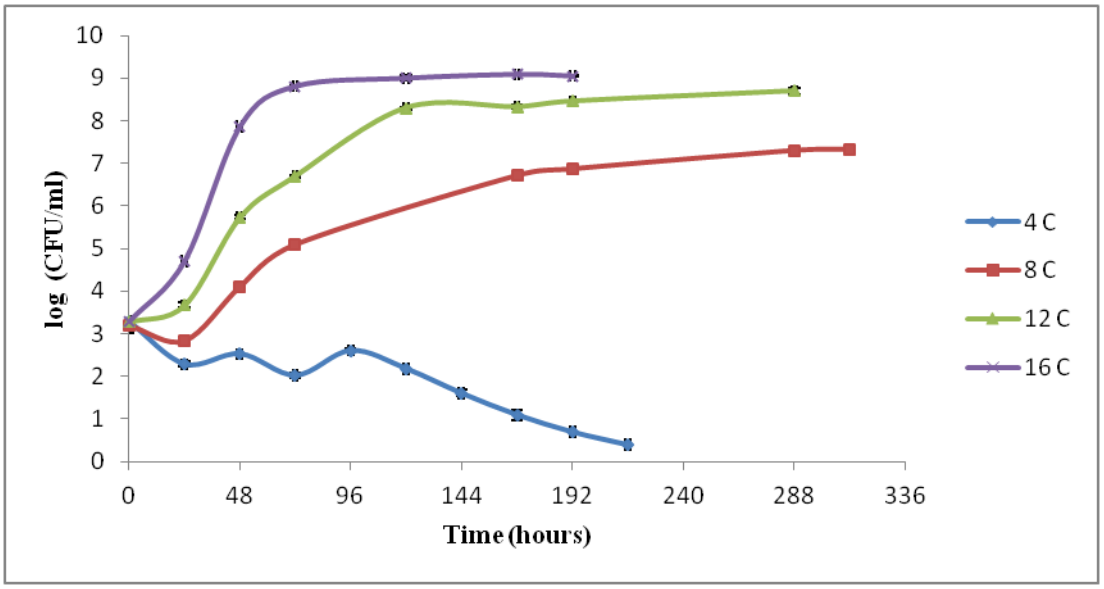
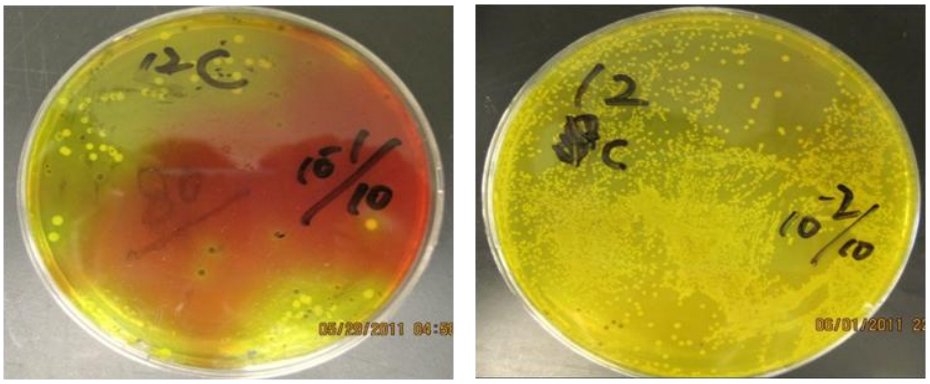


Figure 6. Growth profiles of *Salmonella* spp. in BHI broth at 4, 8, 12 and 16 ℃

It could be seen from Figure 6 that: at 4 ℃, *Salmonella* spp. could not survive. It gradually died out within 216 hrs. Therefore, this temperature was excluded in future experiments. At 8, 12 and 16 ℃, as the temperature increased, the growth of *Salmonella* spp. was promoted. At 8 ℃, it took the bacteria more than 300 hrs to reach a maximum concentration of 10^7 CFU ml^{-1}. At 12 ℃, it took the bacteria around 120 hrs to reach a maximum concentration of 10^8 CFU ml^{-1} and then gradually increased to 10^9 CFU ml^{-1}. However at 16 ℃, it only took the bacteria 72 hrs to reach a maximum concentration of 10^9 CFU ml^{-1}.

5.6 Growth Profiles of *Salmonella* spp. on Non-irradiated Shrimp



(72 hrs) (96 hrs)

Figure 7. Growth profiles of *Salmonella* spp. on non-irradiated shrimp at 12 ℃

According to Figure 7, after 72-hr incubation on non-irradiated shrimp at 12 ℃, the background bacteria had already overgrown *Salmonella* spp. (black colonies represented *Salmonella* spp. and white colonies represented background bacteria). After 96-hr incubation on non-irradiated shrimps at 12 ℃, those background bacteria had dominated on the shrimp. Other than 12 ℃. The results from 8 ℃ and 16 ℃ were similar (figures didn't show).

These background bacteria had competed the nutrients with *Salmonella* spp. and greatly influenced its growth profile, making a reliable plate count difficult. In order to enhance the accuracy of the experiment data, all samples were irradiated. After irradiation, plate enumerating results from non-*Salmonella* selective agar (BHI agar) and *Salmonella*-selective agar (XLT4 agar) showed no significant difference ($P > 0.05$, ANOVA, SAS v9.1) which indicated that background bacteria had been eliminated.

5.7 Growth Profiles of *Salmonella* spp. on Irradiated Shrimp

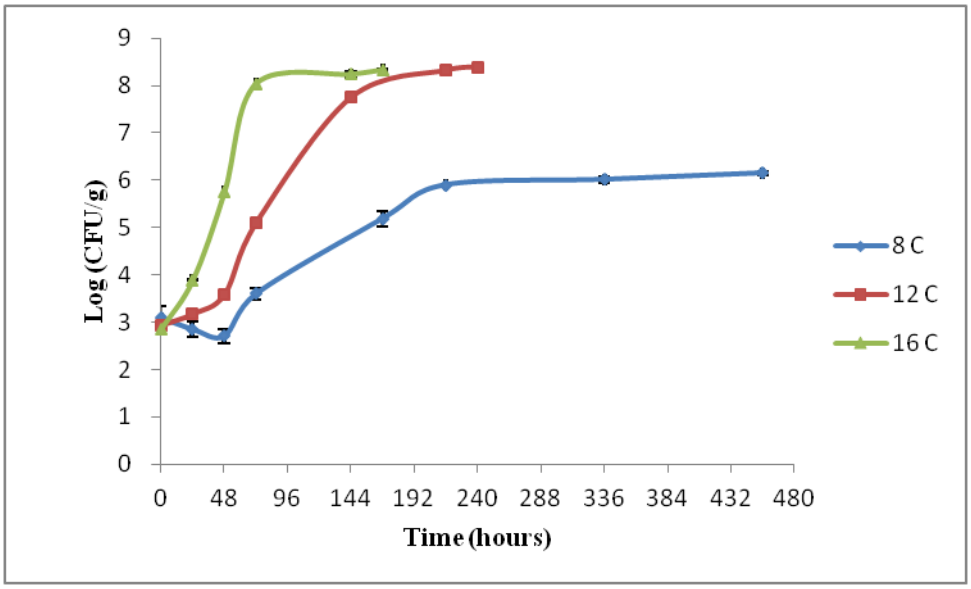


Figure 8. Growth profiles of *Salmonella* spp. on irradiated shrimp at 8, 12 and 16 ℃

Figure 8 showed that on irradiated shrimp, *Salmonella* spp. grew more slowly than in BHI broth (Figure 6). As it could be seen, at 8 ℃, it took the bacteria 168 hrs to reach 10^5 CFU g^{-1} on shrimp while in BHI broth it grew up to 10^7 CFU ml^{-1} within the same time; at 12 ℃, it took the bacteria 216 hrs to reach 10^8 CFU g^{-1} on shrimp while in BHI broth it grew up to 10^8 CFU ml^{-1} within only 120 hrs; at 16 ℃, it took the bacteria 72 hrs to grow to 10^8 CFU g^{-1} on shrimp while in BHI broth it grew up to 10^9 CFU ml^{-1} within the same time.

The results indicated that using BHI broth as a culture medium could not accurately simulate the real growth profiles of *Salmonella* spp. on shrimp. The results were not surprising and instead understandable since BHI broth is a widely accepted perfect medium for *Salmonella* growth [34, 35]. When under real situations such as on

shrimp, the bacteria growth should be slower. These finding further suggested that culture broth may not be used as a food simulant to replace real foods where foodborne pathogen is a food safety concern.

Table 13 Lag time and maximum growth rate of *Salmonella* spp. on irradiated raw shrimp at 8, 12 and 16 ℃

	Lag Time (hrs)			Maximum Growth Rate (log CFU/g*hr)		
	Duplicate 1	Duplicate 2	Average	Duplicate 1	Duplicate 2	Average
8 ℃	51.79	56.61	54.20	0.02100	0.02090	0.02095
12 ℃	27.91	32.32	30.12	0.04600	0.04580	0.04590
16 ℃	16.17	15.92	16.05	0.09530	0.09490	0.09510

Table 13 showed that: for *Salmonella* spp. on shrimp, at 8 ℃, the average lag time was 54.2 hrs and the average maximum growth rate was 0.02095 (log CFU $g^{-1}*hr^{-1}$); at 12 ℃, the average lag time was 30.12 hrs and the average maximum growth rate was 0.04590 (log CFU $g^{-1}*hr^{-1}$); at 16 ℃, the average lag time was 16.5 hrs and the average maximum growth rate was 0.09510 (log CFU $g^{-1}*hr^{-1}$). Generally speaking, as the temperature increased, the lag time decreased while the maximum growth rate increased.

5.8 Treatments for *Salmonella* spp. on Irradiated Shrimp

5.8.1 Vapor Thymol Treatment

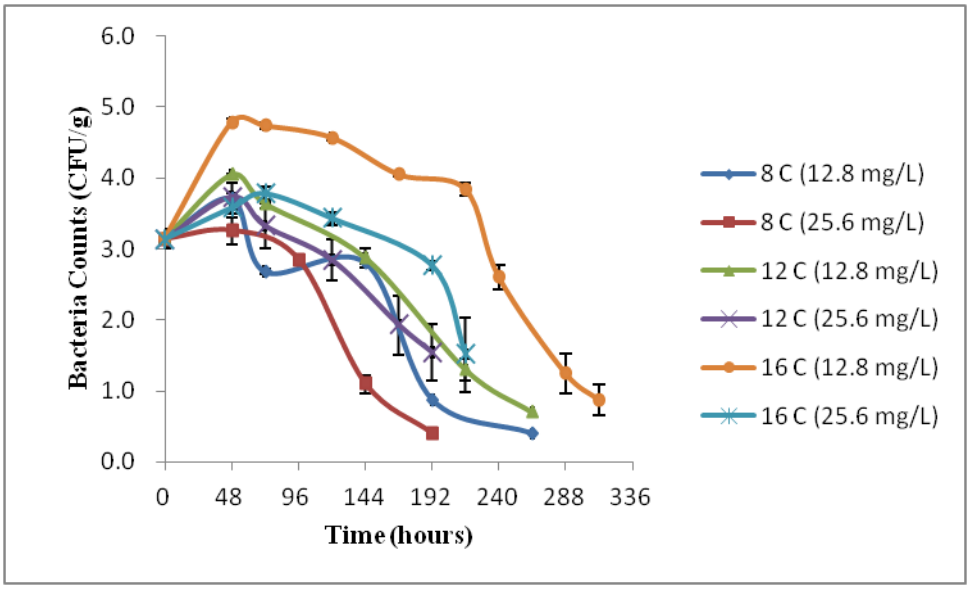


Figure 9. Growth profiles of *Salmonella* spp. on irradiated shrimp under vapor thymol treatments (12.8 and 25.6 mg l^{-1}) at 8, 12 and 16 ℃

As could be seen from Figure 13, *Salmonella spp.* on irradiated shrimp gradually died out under the treatments of 12.8 and 25.6 mg l^{-1} vapor phase thymol regardless of the temperatures. Therefore, these two vapor phase thymol concentrations were excluded in later experiments.

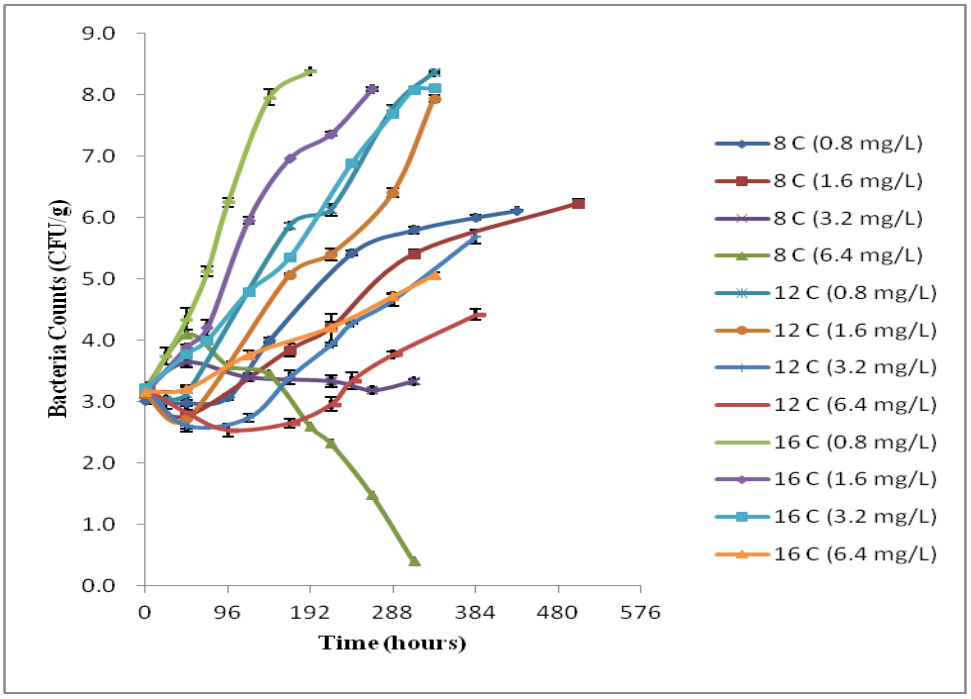


Figure 10. Growth profiles of *Salmonella* spp. on irradiated shrimp under vapor thymol treatments (0.8, 1.6, 3.2 and 6.4 mg l^{-1}) at 8, 12 and 16 ℃

As could be seen from Figure 10, under 0.8, 1.6, 3.2 and 6.4 mg l^{-1} vapor thymol treatments, the growth profiles of *Salmonella* spp. were different compared with under 12.8 and 25.6 mg l^{-1} vapor thymol treatments. Most of the bacteria could grow and the growth profiles were significantly influenced by the vapor thymol concentration treated: as the vapor thymol concentration increased, the bacteria growth was retarded. The working mechanism of thymol is not clear till now, but may result, at least partially, from a disfunction of the lipid fraction of microorganism plasma membrane, causing membrane permeability alterations [36]. Increased thymol concentration may intensify this alteration and therefore exhibited a stronger inhibition effect.

Preliminary sensory test results indicated that when the concentration of vapor phase thymol exceeded 1.6 mg l^{-1}, the sensory panels (consisted of 20 experienced graduate students from Rutgers University Food Science Department, trained in advance) started to detect the significant smell difference (unfavorable) between treated samples and control samples after 24-hr storage under all experimental temperatures ($P < 0.05$, ANOVA, SAS v9.1). Therefore, from the point of practical applications, vapor thymol concentration exceeding 1.6 mg l^{-1} was excluded in later experiments.

Table 14 Lag time and maximum growth rate of *Salmonella* spp. on irradiated shrimp under vapor thymol treatments (0.8 and 1.6 mg l^{-1}) at 8, 12 and 16 ℃

	T (℃)	Lag Time (hrs)			Maximum Growth Rate (log CFU $g^{-1}*hr^{-1}$)		
		Duplicate 1	Duplicate 2	Average	Duplicate 1	Duplicate 2	Average
0.8 (g ml^{-1})	8	90.06	93.15	91.61	0.01720	0.01730	0.01725
	12	44.59	49.65	47.12	0.02100	0.02070	0.02085
	16	20.15	22.56	21.36	0.04100	0.04000	0.04050
1.6 (g ml^{-1})	8	108.42	104.67	106.55	0.01250	0.01180	0.01215
	12	58.33	65.64	61.99	0.01690	0.01700	0.01695
	16	24.42	28.19	26.31	0.02810	0.02740	0.02775

For 0.8 and 1.6 mg l^{-1} vapor thymol treatments, the lag time and maximum growth rate of *Salmonella* spp. were listed in Table 14. Compared with controls (Table 13), vapor thymol treatments (0.8 and 1.6 mg l^{-1}) were effective on extending the lag time and reducing the maximum growth rate.

For 0.8 mg l^{-1} vapor thymol treatment, at 8 ℃, the lag time was increased by 37.41 hrs and the maximum growth rate was decreased by 0.0037(log CFU $g^{-1}*hr^{-1}$) compared with control; at 12 ℃, the lag time was increased by 17 hrs and the maximum growth rate was decreased by 0.02505 (log CFU $g^{-1}*hr^{-1}$) compared with control; at 16 ℃, the lag time was increased by 5.31 hrs and the maximum growth rate was decreased by 0.0546 (log CFU $g^{-1}*hr^{-1}$) compared with control.

For 1.6 mg l^{-1} vapor thymol treatment, at 8 ℃, the lag time was increased by 52.35 hrs and the maximum growth rate was decreased by 0.0088 (log CFU $g^{-1}*hr^{-1}$) compared with control; at 12 ℃, the lag time was increased by 31.87 hrs and the maximum growth rate was decreased by 0.02895 (log CFU $g^{-1}*hr^{-1}$) compared with control; at 16 ℃, the lag time was increased by 10.26 hrs and the maximum growth rate was decreased by 0.06785 (log CFU $g^{-1}*hr^{-1}$) compared with control.

5.8.2 MA Treatment

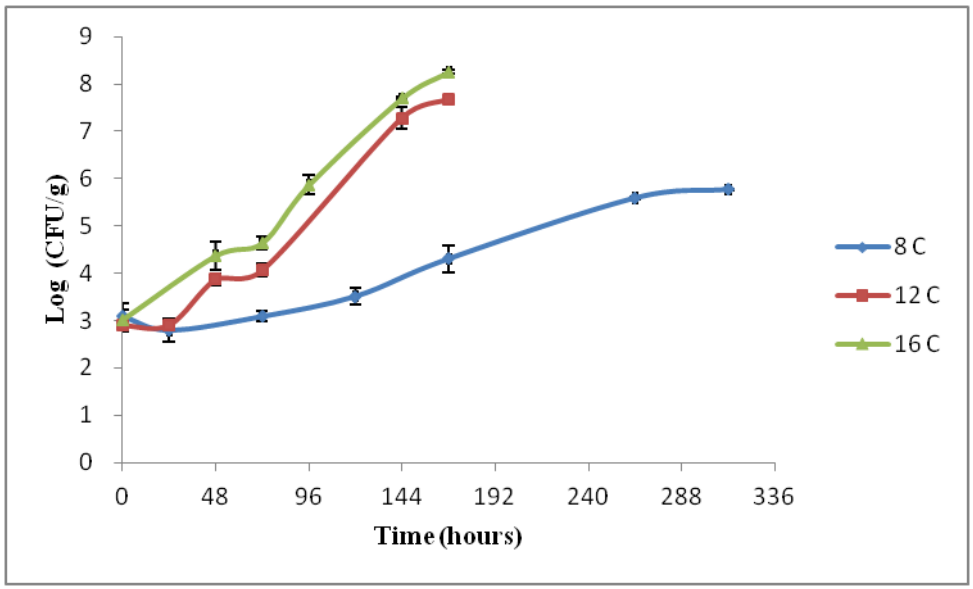


Figure 11. Growth profiles of *Salmonella* spp. on irradiated shrimp under MA treatments at 8, 12 and 16 ℃

Table 15 Lag time and maximum growth rate of *Salmonella* spp. on irradiated shrimp under MA treatments at 8, 12 and 16 ℃

	Lag Time (hrs)			Maximum Growth Rate (Log CFU $g^{-1}*hr^{-1}$)		
	Duplicate 1	Duplicate 2	Average	Duplicate 1	Duplicate 2	Average
8 ℃	90.94	87.48	89.21	0.01750	0.01680	0.01715
12 ℃	35.59	36.94	36.27	0.04000	0.03980	0.03990
16 ℃	22.79	27.90	25.35	0.03810	0.04200	0.04005

From Figure 11 and Table 15, it's clear to see that compared with controls (Table 13), under MA treatment, the lag time of *Salmonella* spp. on shrimps was increased while the maximum growth rate was decreased. At 8 ℃, the lag time was increased by 35.01 hrs and the maximum growth rate was decreased by 0.0038 (log CFU $g^{-1}*hr^{-1}$); at 12 ℃, the lag time was increased by 6.15 hrs and the maximum growth rate was decreased by 0.006 (log CFU $g^{-1}*hr^{-1}$); at 16 ℃, the lag time was

increased by 9.3 hrs and the maximum growth rate was decreased by 0.05505 (log CFU $g^{-1}*hr^{-1}$).

Mechanism of microbial inhibition by CO_2 gas was not elucidated yet, but the proposed theories were related to (a) nutrient uptake caused cell membrane function change, (b) enzyme activity inhibition, (c) dissolved CO_2 caused intracellular pH change, and (d) protein properties change [37, 38].

The CO_2 concentration determined to be 40% with 60% N_2 as the complementary gas was because according to the preliminary sensory test results of treatments using different CO_2/N_2 ratios, when CO_2 concentration was higher than 40%, the sensory panels started to notice the significant appearance difference between CO_2 treated samples and controlled samples after 24-hr storage at experimental temperatures ($P < 0.05$, ANOVA, SAS v9.1). The CO_2 treated samples had a redder color and increased extrudes on the surface compared with controlled samples. These drawbacks from CO_2 treatments have also been recorded by some other researchers [39]. After MA gas flushing, a very small proportion of O_2 (1%) was left in the headspace (detected by CO_2/O_2 detector) and was slightly increased during the storage period. This level of O_2 could be sufficient to eliminate the possibility of *Clostridium botulinum* toxin formation [40].

5.8.3 Vapor Phase Thymol + MA Treatment

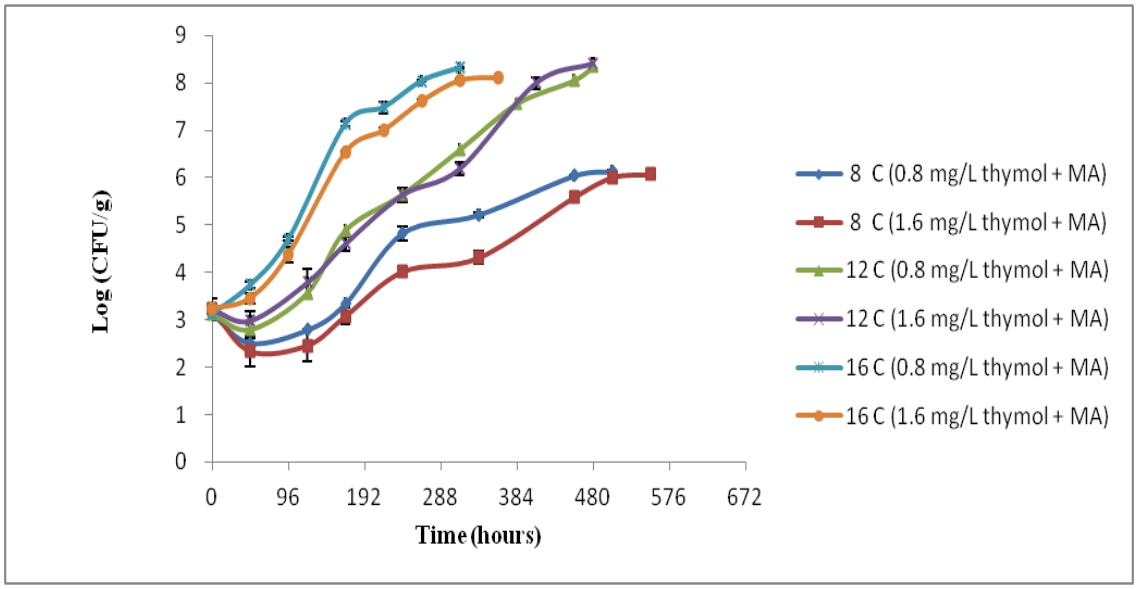


Figure 12. Growth profiles of *Salmonella* spp. on irradiated shrimp under combination treatments of vapor thymol (0.8 and 1.6 mg l^{-1}) and MA at 8, 12 and 16 ℃

Table 16 Growth characteristics of *Salmonella* spp. on irradiated shrimp under combination treatments of vapor thymol (0.8 and 1.6 mg l^{-1}) + MA at 8, 12 and 16 ℃

	T (℃)	Lag Time (hrs)			Maximum Growth Rate (log CFU g^{-1}*hr^{-1})		
		Duplicate 1	Duplicate 2	Average	Duplicate 1	Duplicate 2	Average
0.8 (g ml^{-1})	8	125.19	115.58	120.39	0.01280	0.01400	0.01340
	12	65.53	68.64	67.09	0.01560	0.01570	0.01565
	16	34.33	31.19	32.76	0.02810	0.02740	0.02775
1.6 (g ml^{-1})	8	149.2	138.68	143.94	0.00950	0.01020	0.00985
	12	77.72	78.23	77.98	0.01480	0.01520	0.01500
	16	43.50	39.68	41.59	0.02300	0.02440	0.02370

As it could be seen from Figure 12 and Table 16, under combination of vapor phase thymol (0.8 and 1.6 mg l^{-1}) + MA treatments, the lag time of *Salmonella* spp.

on shrimp was largely increased compared with vapor phase thymol (0.8 mg l^{-1} and 1.6 mg l^{-1}) and MA treatment alone while the maximum growth rate was largely decreased.

For lag time, under vapor phase thymol (0.8 mg l^{-1}) + MA combination treatment, at 8 ℃, the lag time was increased to 120.39 hrs compared with 91.61 hrs under vapor thymol treatment alone and 89.21 hrs under MA treatment alone; at 12 ℃, the lag time was increased to 67.09 hrs compared with 47.12 hrs under vapor thymol treatment alone and 36.27 hrs under MA treatment alone; at 16 ℃, the lag time was increased to 32.76 hrs compared with 21.36 hrs under vapor thymol treatment alone and 25.35 hrs under MA treatment alone.

Under vapor phase thymol (1.6 mg l^{-1}) + MA combination treatment, at 8 ℃, the lag time was increased to 143.94 hrs compared with 106.55 hrs under vapor thymol treatment alone and 89.21 hrs under MA treatment alone; at 12 ℃, the lag time was increased to 77.98 hrs compared with 61.99 hrs under vapor thymol treatment alone and 36.27 hrs under MA treatment alone; at 16 ℃, the lag time was increased to 41.59 hrs compared with 26.31 hrs under vapor thymol treatment alone and 25.35 hrs under MA treatment alone.

For maximum growth rate (MGR), under vapor phase thymol (0.8 mg l^{-1}) + MA combination treatment, at 8 ℃, the MGR was decreased to 0.01340 (log CFU g^{-1}*hr^{-1}) compared with 0.01725 (log CFU g^{-1}*hr^{-1}) under vapor thymol treatment alone and

0.01715 (log CFU $g^{-1}*hr^{-1}$) under MA treatment alone; at 12 ℃, the MGR was decreased to 0.01565 (log CFU $g^{-1}*hr^{-1}$) compared with 0.02085 (log CFU $g^{-1}*hr^{-1}$) under vapor thymol treatment alone and 0.03965 (log CFU g^{-1}*hr-1) under MA treatment alone; at 16 ℃, the MGR was decreased to 0.02700 (log CFU $g^{-1}*hr^{-1}$) compared with 0.04050 (log CFU $g^{-1}*hr^{-1}$) under vapor thymol treatment alone and 0.04005 (log CFU $g^{-1}*hr^{-1}$) under MA treatment alone.

Under vapor phase thymol (1.6 mg l^{-1}) + MA combination treatment, at 8 ℃, the MGR was decreased to 0.00985 (log CFU $g^{-1}*hr^{-1}$) compared with 0.01215 (log CFU $g^{-1}*hr^{-1}$) under vapor thymol treatment alone and 0.01715 (log CFU $g^{-1}*hr^{-1}$) under MA treatment alone; at 12 ℃, the MGR was decreased to 0.01500 (log CFU $g^{-1}*hr^{-1}$) compared with 0.01695 (log CFU $g^{-1}*hr^{-1}$) under vapor thymol treatment alone and 0.03965 (log CFU $g^{-1}*hr^{-1}$) under MA treatment alone; at 16 ℃, the MGR was decreased to 0.02350 (log CFU $g^{-1}*hr^{-1}$) compared with 0.02775 (log CFU $g^{-1}*hr^{-1}$) under vapor thymol treatment alone and 0.04005 (log CFU $g^{-1}*hr^{-1}$) under MA treatment alone.

Table 17. Lag time extended by vapor phase thymol (0.8 and 1.6 mg l^{-1}) alone, MA alone and vapor phase thymol (0.8 and 1.6 mg l^{-1}) + MA in combination

T (℃)	Thymol		MA	Thymol + MA combination	
	0.8 mg/l	1.6 mg/l		0.8 mg/l	1.6 mg/l
8	37.41	52.35	35.01	66.19	89.74
12	17.01	31.87	6.15	36.97	47.86
16	5.31	10.26	9.30	16.72	25.55

According to Table 17, we could draw a conclusion that generally speaking on extending the lag time, vapor phase thymol (0.8 and 1.6 mg l^{-1}) + MA in combination had synergistic effects. For 0.8 mg l^{-1} vapor thymol + MA combination: at 8 ℃, the synergistic effect was not obvious (*the only exception*); at 12 ℃, the combination further extended the lag time by 13.81 hrs (*59.6% more: the largest extension*); at 16 ℃, the combination further extended the lag time by 2.11 hrs. For 1.6 mg l^{-1} vapor phase thymol (1.6 mg l^{-1}) + MA combination: at 8 ℃, the lag phase was further extended by 2.42 hrs; at 12 ℃, the lag time was further extended by 9.84 hrs; at 16 ℃, the lag phase was further extended by 5.99 hrs.

This synergistic inhibition effectiveness could be explained by a so called "hurdle technology theory" that has been defined by *Leistner* [41]. According to the theory, the multiple hurdles applied in the application (in our case were vapor phase thymol and MA) could place multiple stresses to the microbe, thus disturb its homeostasis in multiple aspects which makes the repair progress more different [42].

5.9 Linear Regression Model Development

5.9.1 Based on Lag Time

With MA

Table 18 Parameters of linear regression model for lag time of *Salmonella* spp. on irradiated shrimp under vapor thymol treatment

Parameter	Estimate	Standard Error	t Value	Pr >|t|
Intercept	148.4551389	17.22344988	8.62	< .0001
Temperature	-14.6336458	2.96632489	-4.93	0.0003
Thymol Concentration	69.5390625	7.26324205	9.57	< .0001
Temperature * Thymol Concentration	-3.2878906	0.43200942	-7.61	< .0001
Temperature * Temperature	0.3918229	0.12219072	3.21	0.0075
Thymol Concentration * Thymol Concentration	-6.5013021	3.05476792	-2.13	0.0547

According to Table 18, we could develop linear regression model as below:

LAG = 148.4551 – 14.6336 T + 69.5391 THY – 3.2879 T*THY + 0.3918 T*T – 6.5013 THY*THY **(1)**

(Where LAG is the lag time in hours; T is temperature in ℃; THY is vapor thymol concentration in mg l^{-1}. The R^2 is 0.98.)

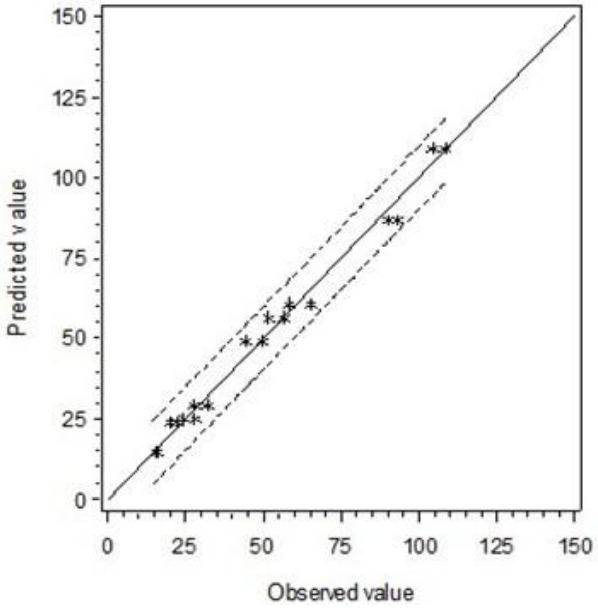


Figure 13. The predicted vs. observed values of LAG using Eq. (1)

With MA

Table 19 Parameters of linear regression model for lag time of *Salmonella* spp. on irradiated shrimp under vapor thymol + MA treatment

Parameter	Estimate	Standard Error	t Value	Pr >|t|
Intercept	274.1351389	19.74012948	13.89	< .0001
Temperature	-30.8163542	3.39976240	-9.06	< .0001
Thymol Concentration	70.4453125	8.32454239	8.46	< .0001
Temperature * Thymol Concentration	-3.0066406	0.49513437	-6.07	< .0001
Temperature * Temperature	0.9435417	0.14004515	6.74	< .0001
Thymol Concentration * Thymol Concentration	-6.8059896	3.50112868	-1.94	0.0757

According to Table 19, we could develop linear regression model as below:

LAG = 274.1351 – 30.8164 T + 70.4453 THY – 3.0066 T*THY + 0.9435 T*T – 6.8060 THY*THY **(2)**

(Where LAG is the lag time in hours; T is temperature in ℃; THY is vapor thymol concentration in mg l^{-1}. The R^2 is 0.99.)

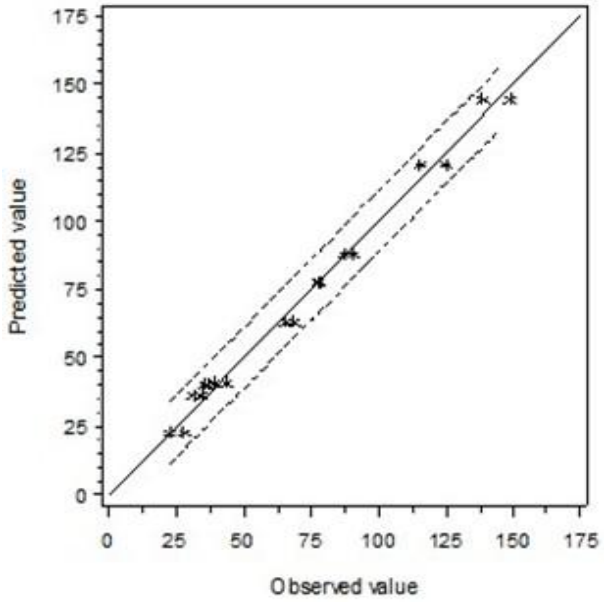


Figure 14. The predicted vs. observed values of LAG using Eq. (2)

From model (1) and (2), it could be concluded that: Lag time was majorly in a function of temperature and vapor thymol concentration. It's more sensitive to unit vapor thymol concentration change (mg l^{-1}) than unit temperature change (℃). Also, it's in a function with the secondary factors as: temperature*vapor thymol concentration, temperature*temperature and vapor thymol concentration*vapor thymol concentration. But the influences of these secondary factors are considerably smaller. The R^2 for model (1) and (2) were 0.98 and 0.99 respectively, which indicated that the linearity was good as shown in Figure 14 and 15.

5.9.2 Based on Maximum Growth Rate

Without MA

Table 20 Parameters of linear regression model for maximum growth rate of *Salmonella* spp. on irradiated shrimp under vapor thymol treatment

Parameter	Estimate	Standard Error	t Value	Pr >|t|
Intercept	-.0498513889	0.01216293	-4.10	0.0011
Temperature	0.0083677083	0.00097800	8.56	< .0001
Thymol Concentration	0.0329947917	0.01177671	2.80	0.0141
Temperature * Thymol Concentration	-.0045742187	0.00094695	-4.83	0.0003

According to Table 20, we could develop linear regression model as below:

$$MGR = -0.04985 + 0.0084\ T + 0.03300\ THY - 0.0046\ T*THY \quad \textbf{(3)}$$

[Where MGR is the maximum growth rate in log CFU g^{-1} hr^{-1}; T is temperature in ℃; THY is vapor thymol concentration in mg l^{-1}. The T*T and THY*THY terms were not significant ($P > 0.05$). The R^2 is 0.90.]

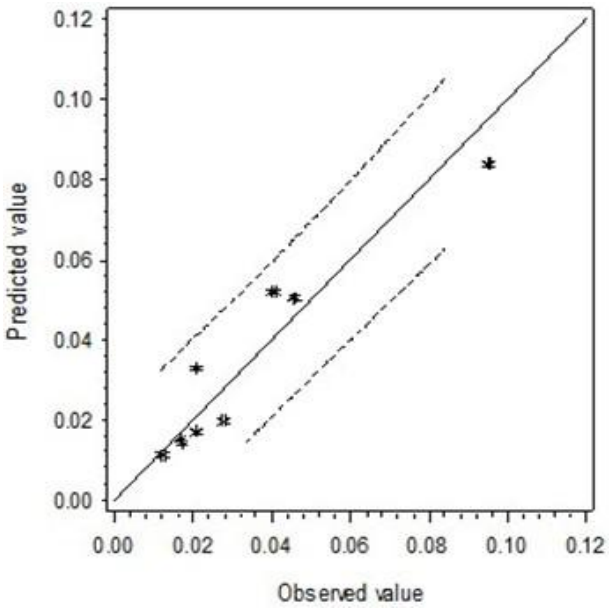


Figure 15. The predicted vs. observed values of MGR using Eq. (3)

With MA

Table 21 Parameters of linear regression model for maximum growth rate of *Salmonella* spp. on irradiated shrimp under vapor thymol + MA treatment

Parameter	Estimate	Standard Error	t Value	Pr >|t|
Intercept	0.0071083333	0.00413696	1.72	0.1078
Temperature	0.0020979167	0.00031184	6.73	< .0001
Thymol Concentration	-.0239375000	0.00562169	-4.26	0.0008
Thymol Concentration * Thymol Concentration	0.0086718750	0.00337571	2.57	0.0223

According to Table 21, we could develop linear regression model as below:

$$MGR=0.0071 + 0.0021\ T - 0.02394\ THY + 0.0087\ THY*THY \quad \textbf{(4)}$$

[Where MGR is the maximum growth rate in log CFU g^{-1} hr^{-1}; T is temperature in ℃; THY is vapor thymol concentration in mg l^{-1}. The T*T and T*THY terms were not significant ($P > 0.05$). The R^2 is 0.87.]

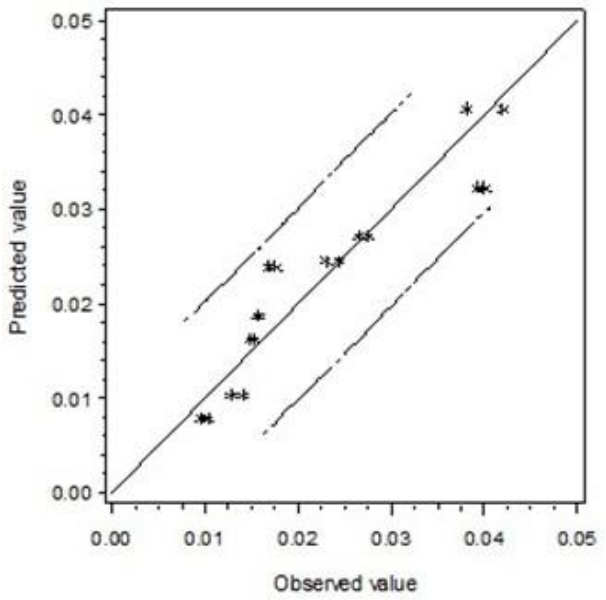


Figure 16. The predicted vs. observed values of MGR using Eq. (4)

From model (3) and (4), it could be concluded that: Maximum growth rate was in a function with vapor thymol concentration and temperature. But the unit influence of vapor thymol concentration (mg l^{-1}) was much higher than that of temperature (℃), especially when MA was applied. Also, maximum growth rate was in a function with some secondary factors: when MA was not applied, the influence from temperature*vapor thymol was significant (p=0.0003); when MA was applied, the influence from vapor thymol*vapor thymol was significant (p=0.0223). Also, the unit influences of these secondary factors were comparable with that of temperature. The R^2 for model (3) and (4) were 0.90 and 0.87 respectively, which indicated that the linearity for these two models was not as high as that for the lag time models, but still good enough for our purposes (as shown in Figure 15 and 16).

5.9.3 Model Validation

Following conditions were used to evaluate the performance of developed models: temperature (14 ℃), vapor phase thymol concentration (0.5 and 1.2 mg l^{-1}), MA (with and without). The predicted values obtained from the models and the experimental values were summarized in Table 26, in which the differences between predicted and experimental values were around 10 - 25%. For the lag time, the predicted values were under-estimated compared with experimental ones; while for the maximum growth rate, they were over-estimated. With current data, the models tended to conservatively predict the microbial growth compared with the real situations, which may become positive for the food microbial safety risk assessment.

Table 22 Predicted and experimental values of lag time (hrs) and maximum growth rate (log CFU/g/hr) at 14℃

TC (mg/l)		LAG		MGR	
		PV	EV	PV	EV
0.5	WO/MA	30.5064	34.6732	0.0521	0.0430
	W/MA	40.1064	47.6549	0.2671	0.0210
1.2	WO/MA	39.2258	44.3582	0.0301	0.0259
	W/MA	51.8543	58.0045	0.0203	0.0151

(TC: Thymol concentration; LAG: Lag time; MGR: Maximum growth rate; PV: Predicted value; EV: Experimental value; WO/MA: without MA; W/MA: with MA)

6 CONCLUSIONS

The combination of vapor phase thymol and MA showed great effectiveness in retarding the growth of *Salmonella* spp. on raw shrimp compared with controlled samples. For instance, at 8 ℃, under the combination treatment of 1.6 mg l^{-1} vapor thymol + MA, lag time of *Salmonella* spp. was extended from 54.2 hrs (without treatment) to 143.9 hrs, and maximum growth rate was reduced from 0.021 log CFU/g/hr (without treatment) to 0.0098 log CFU/g/hr. Compared with individual treatment, the combination treatment also exhibited stronger effectiveness in extending the lag time and reducing the maximum growth. A synergistic inhibition effect from the combination treatment was observed on lag time extension. To the maximum, at 12 ℃, lag time of *Salmonella* spp. was extended 59.6% more by the combination treatment of 0.8 mg l^{-1} thymol + MA (36.97 hrs) than those effects combined from 0.8 mg l^{-1} thymol treatment and MA treatment alone (23.16 hrs in total). Therefore, the combination of vapor phase thymol and MA could be potentially utilized as an effective strategy for *Salmonella* inhibition during the long distance and temperature abused raw shrimp importation process.

Linear regression models were established and validated for the growth characteristics (lag time and maximum growth rate) of *Salmonella* spp. on raw shrimp under multiple stresses. These models may be useful on shelf life prediction and safety assessment of raw shrimp.

7 FUTURE WORK

7.1 Multiple Additions of Vapor Phase Thymol

In the research, vapor phase thymol was utilized in an instant addition form. According to the study done by our research group, it was highly possible that using multiple additions could have a better inhibition effect than instant addition. Therefore, we could divide the amount of vapor phase thymol utilized in the research into several portions and add separately at different time intervals during the storage. The effectiveness could be observed and compared with the instant addition form.

7.2 Thymol Incorporating into Packaging Material

For a better inhibition effectiveness, thymol could be incorporated into packaging materials [43], then gets released from the packaging in its vapor phase. Research could be done to find an appropriate packaging material which is suitable for thymol to be incorporated effectively and released in a desired manner. Some related tests could also be done such as total extraction test and release test.

7.3 Antimicrobial Study of Vapor Thymol + MA on Spoilage Bacteria

Thymol is not only effective in pathogenic bacteria (such as *Salmonella*) inhibition which enhances the food safety, but also effective in inhibiting many spoilage bacteria such as *Pseudomonas* spp. [44], which is a major spoilage bacteria on shrimp that costs quality deterioration and shelf life expiration [45, 46]. Research could be done to investigate how vapor phase thymol would affect the growth profiles

of *Pseudomonas* spp. on shrimp. Furthermore, since MA was also reported to be effective against *Pseudomonas* spp. [47, 48], the combination inhibition effectiveness of vapor thymol + MA against *Pseudomonas* spp. could also be investigated.

7.4 Antioxidant Study of Vapor Thymol + MA on Lipid Oxidation of raw Shrimp

Besides being a good antimicrobial, thymol is a great antioxidant compound against lipid oxidation as well [49, 50]. MA technology is also believed to be effective in lipid oxidation inhibition [51, 52]. Since shrimp has a relatively high content of unsaturated fatty acid [19], the antioxidant effectiveness of vapor phase thymol + MA in combination could be investigated against lipid oxidation of shrimp through food simulant test and real food test.

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