https://doi.org/10.17113/ftb.61.02.23.7595 preliminary communication

# Thymol-Loaded Polymeric Nanoparticles Improve the Postharvest Microbiological Safety of Blueberries

Running head: Thymol-loaded Polymeric Nanoparticles Improve the Quality of Blueberries

Syarifah Ab Rashid[1,2], Woei-Yenn Tong[3*], Chean-Ring Leong[1], Wen-Nee Tan[4], Chee-Keong Lee[5,6], Mohd Razealy Anuar[3], Siew-Hway Teo[3], Siti Khalida Abdull Lazit[3], Jun-Wei Lim[7,8] and Nur Amiera Syuhada Rozman[1]

[1]Universiti Kuala Lumpur, Branch Campus Malaysian Institute of Chemical and Bioengineering Technology, Lot 1988 Kawasan Perindustrian Bandar Vendor, Taboh Naning, 78000 Alor Gajah, Melaka, Malaysia
[2]School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[3]Universiti Kuala Lumpur, Institute of Medical Science Technology, A1-1, Jalan TKS 1, Taman Kajang Sentral, 43000 Kajang, Selangor, Malaysia
[4]Chemistry Section, School of Distance Education, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[5]Bioprocess Technology Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Minden, Penang, Malaysia
[6]Renewable Biomass Transformation Cluster, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Minden, Penang, Malaysia
[7]HICoE-Centre for Biofuel and Biochemical Research, Institute of Self Sustainable Building, Department of Fundamental and Applied Sciences, Universiti Teknologi PETRONAS, Seri Iskandar, 32610 Perak Darul Ridzuan, Malaysia
[8]Department of Biotechnology, Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, Chennai 602105, India

Received: 7 December 2021
Accepted: 23 February 2023



## SUMMARY

*Research background*. The presence of *Yersinia enterocolitica* on raw food products raised the concern of yersiniosis as most of the berries were consumed raw. This event is a challenging issue for food safety division since it could raise foodborne diseases among humans. Thus, it is crucial to implement an effective sanitation before the packaging process.

*Corresponding author:
Phone: +6065512000
Fax: +6065512001
E-mail: wytong@unikl.edu.my

*Experimental approach.* This study was aimed to synthesize and characterize thymol-loaded polyvinyl alcohol (Thy/PVA) nanoparticles sanitizer for postharvest treatment of blueberries. Thy/PVA nanoparticles were characterized by spectroscopic and microscopic approaches, prior to the analyses of antimicrobial properties.

*Results and conclusions.* In average, the diameter size of the nanoparticles was 84.7 nm, with a surface charge of -11.73 mV. Based on Fourier transform infrared (FTIR) observation, the Thy/PVA nanoparticles have notably shifted on the frequency at 3275.70, 2869.66, 1651.02 and 1090.52 $cm^{-1}$. A rapid burst was observed at the first h of release study, and 74.9 % of thymol was released from PVA nanoparticles. The largest inhibition zone was displayed by methicillin-resistant *Staphylococcus aureus* (MRSA), followed by *Y. enterocolitica* and *Salmonella typhi*. However, amongst these bacteria, the inhibition and killing of *Y. enterocolitica* required a lower concentration of Thy/PVA nanoparticles. The treatment has successfully reduced the bacterial load of *Y. enterocolitica* on blueberries by 100 %.

*Novelty and scientific contribution*. Thymol is a plant-based chemical with no reported adverse effects to humans. In this study, the use of nanotechnology has improved the stability and physicochemical properties of thymol by using PVA as encapsulant. This nanoparticles-based sanitizer could potentially promote the postharvest microbiological safety of raw berries, which may become an alternative practice of food safety.

**Keywords**: antimicrobial activity; blueberries; nanoparticles; postharvest; thymol

## INTRODUCTION

Fruits and vegetables are the most common sustenance vehicles implicated in outbreaks *(1)*. In 2010, the US Department of Agriculture revealed that about 18.9 billion pounds of fresh fruits and vegetables are wasted annually due to spoilage (*1*). This occasion accounts for 19.6 % of all edible food lost in the US (*2*). In terms of food safety, fresh fruits and vegetables are considered a high risk for microbial contamination. Microbial spoilage of these sources is usually due to the raw materials and postharvest processing equipment contact (*1*). Besides human contact with fruits and vegetables during picking, microbial contamination also caused by unsafe water used for washing the fruits and vegetables during pre-harvesting and post-harvesting processes.

*Yersinia enterocolitica* is a coccobacillus-shaped Gram-negative bacterium. It is a psychrophilic bacterium that can grow and survive at low temperature (4 ºC). It is frequently isolated from rodents, domestic animals, and water contaminated by these animals (*3*). Fruits and vegetables

can be contaminated by faeces of these domestic animals or the person handling the products (*4*). Besides, the imported fruits and berries were in conjunction with the increased threat of yersiniosis as most of the berries were consumed raw (*5*). Due to this risk factor, in the USA alone, the pathogen causes 640 hospital admission cases, 117,000 illnesses and 35 deaths (*6*). Several outbreaks were associated with frozen berries since *Y. enterocolitica* grew well at refrigeration temperature (*7*). Thus, sanitizers are used to prevent microbial growth on berries.

Chemical-based sanitizers are commonly used as a post-harvest treatment for fruits and vegetables to restrain the growth of spoilage bacteria. The common chemical-based sanitizers are sodium hypochlorite, iodine, hydrogen peroxide and quaternary ammonium compounds (*8*). However, these chemicals could cause skin irritation, mucous membrane damage, carcinogenic and mutagenic effects (*9*). They also affect ecological system once released into air, water and soil. More importantly, chemical-based sanitizers also cause food deterioration such as loss of nutritional quality, colour and flavour (*8*). Due to the consumers' demand for safe and good quality food, chemical-based sanitizers are often substituted with natural alternatives (*9*). However, nonchemical-based sanitizers are less effective than chemically synthesized compounds due to the poor stability (*10*). The natural compounds also interact negatively with food components, which affect the food quality (*8*).

Thymol (5-methyl-2-isopropylphenol, $C_{10}H_{14}O$) is a bioactive compound present in thyme oil (*Thymus vulgaris*). Moreover, it holds a Generally Recognized as Safe (GRAS) food ingredient status (*11*). This compound is a colourless crystalline substance that provides strong flavour, pleasant odour and strong antiseptic property. However, owing to its poor stability and high volatility, thymol application is restricted in food systems. Furthermore, the compound has low water solubility at neutral pH (*12*). Its pungent taste and smell also interfere with the protein and fat present in food, which causes poor palatability (*11*). All these shortcomings limit the usage of thymol as an antimicrobial agent in the food system.

Nanotechnology can be applied to improve the stability and physicochemical properties of thymol. Nanoparticles are particulate substances or solid particles within a 2-100 nm size range (*13*). The nanoscale size influences the physicochemical properties of natural compounds. The nanoparticles usually exhibited better biological activities (*14*). Nanotechnology demonstrates novel structures of a material. It can be applied to improve the physical, chemical and biological properties of materials (*15*). Thus, in this study, nanotechnology was applied to synthesize and characterize thymol with polyvinyl alcohol (PVA) as an encapsulant material. The antimicrobial efficiency of synthesized nanoparticles was evaluated on food spoilage microorganisms. More importantly, we

also determine the efficiency of thymol nanoparticles as fruit sanitizer to inhibit the growth of *Y. enterocolitica* on frozen berries.

## MATERIALS AND METHODS

### *Synthesis of thymol nanoparticles*

Thymol nanoparticles were synthesized by using PVA as encapsulant (*16*). Firstly, 0.3 g of thymol (Solarbio, Beijing, China) was mixed in 5 mL of 25 % ethanol (Thermo Fisher, Massachusetts, USA). Next, 50 mL of 2 % Pluronic F127 (Sigma-Aldrich, Missouri, USA) was mixed with thymol solution by using silent crusher (Heidolph, Germany). Then, 50 mL of 2 % PVA solution (Sigma-Aldrich, Missouri, USA) was added and mixed at 10,000 rpm for 5 minutes until a clear solution was observed. Then, the solution was kept in a freezer (-80 °C), prior to freeze-drying process (Labconco Freeze Dry System, Missouri, USA). The freeze-dried nanoparticles were kept at desiccator prior use, and these particles are called “Thy/PVA nanoparticles” in this study. A control was provided by replacing the thymol solution with ethanol. In this study, these particles are called "blank nanoparticles". For antimicrobial assays, the nanoparticle was dissolved in 20 % Tween 20 to desired concentration and flowed through a filter (0.22 μm pore size, Millipore Sigma, Massachusetts, USA) prior to use.

### *Transmission electron microscope (TEM)*

The size and shape of the developed nanoparticles were determined *via* transmission electron microscopy (Philips CM12, Eindhoven, Netherlands). To fix the sample for microscopic observation, a droplet of Thy/PVA nanoparticle solution was dropped on a carbon-coated copper grid. It was followed by a drop of uranyl acetate stain. The sample was left to dry at an ambient temperature, prior to microscopic observation under TEM.

### *Surface charge*

To determine the surface charge of thymol nanoparticle, dynamic light scattering (DLS) measurement was executed in clear disposable zeta cell with zeta analyser (Malvern Zetasizer Nano-ZS90) (Malvern Instruments Ltd., Malvern, UK). The test temperature was set at 25 ˚C.

### *Encapsulation efficiency*

The encapsulation efficiency of nanoparticles is defined by the amount of the bioactive ingredient (thymol) encapsulated into the nanoparticles (*17*). Thy/PVA nanoparticles were first dissolved in ethanol at ratio of 1:10 (*W/V*). The sample was subjected to ultrasonic bath for 20 minutes to release the thymol into solvent phase. The amount of thymol released was determined by gas chromatography system (Auto System XL Model, Perkin Elmer, Massachusetts, USA) equipped with fused silica capillary column (30 m × 0.32 mm I.D., 0.25 µm film thickness). Hydrogen gas was applied as carrier gas with a flowrate of 1 mL/min. A total of 0.2 µL of sample was subjected to the chromatographic system. The injection and detector temperature were set up at 250 °C. The column temperature was set at 100°C for 1 minute and programmed to increased 15 °C/min to 240 °C for 1 minute. Thymol standards were prepared at the concentration range of 62.50 to 1000 µg/mL, to construct a calibration curve. The amount of thymol present in the sample was estimated according to the calibration curve. The encapsulation efficiency was calculated according to Eq.1.

$$\mathrm{EE}\ (\%) = \frac{\text{Amount of thymol entrapped}, w}{\text{Initial amount of thymol added}, w} \times\ 100\ \% \qquad /1/$$

*Fourier transform infrared (FTIR) analysis*

FTIR was performed to study the chemical interactions between thymol and its encapsulant. The FTIR spectra of Thy/PVA nanoparticle, PVA and unencapsulated thymol were determined using Thermo Scientific Nicolet iS10 FT-IR Spectrometer (Massachusetts, USA). The examination of the FTIR spectra was implemented in the infrared region over a wavenumber of 4000-600 $cm^{-1}$ at room temperature (25 °C).

*Thymol release property*

Firstly, 100 mg of Thy/PVA nanoparticle was placed in phosphate buffer solution (10 mL, pH=7.4). The solution was placed in an incubator shaker and agitated at a speed of 120 rpm, 37 °C. Then, 500 µL of the test sample was withdrawn at fixed time points, specifically at 1, 2, 4, 8, 24, 48, and 96 h. The amount of thymol released in the test medium was analysed with gas chromatography as per protocol described for encapsulation efficiency study. The experiment was implemented three replicates in separate occasions. The graph of amount of thymol released against time was plotted to study the drug release behaviour of the nanoparticle system.

*Test bacteria*

The antimicrobial efficiency of Thy/PVA nanoparticles was tested on foodborne microorganisms. The test microorganisms include both Gram-positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* [MRSA]) and Gram-negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Yersinia enterocolitica*). These bacteria were previously isolated from contaminated food samples (*17-18*). The inoculum size of bacterial suspensions was adjusted to $1 \times 10^8$ CFU/mL by comparing the turbidity with 0.5 Mc Farland standard, prior to the experiment.

*Kirby Bauer analysis*

Kirby Bauer analysis was done to screen the antimicrobial activity of the nanoparticles (*18*). A total of 3 test substances were used, including Thy/PVA nanoparticle (10 mg/mL), positive control (Chloramphenicol 100 µg/mL) and negative control (blank nanoparticles dissolved in 20 % Tween 20). At first, the bacterial suspension was swabbed on the surface of Mueller Hinton agar (Merck, USA) using a sterile cotton swab. Then, 20 µL of test substance was pipetted on the sterile paper discs (6 mm diameter) and placed on the surface of agar. The plates were kept at 37 °C for 24 h. Lastly, the diameters of inhibition zones derived from the paper discs were recorded. The test was done in triplicate.

*Broth microdilution assay*

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of Thy/PVA nanoparticle were tested on test bacteria that showed significant susceptibility on Kirby Bauer analysis (*18*). The assay was performed in a flat bottom 96 well cell culture plate (Biologix Research Co, Kansas, USA). The inoculum was prepared by adding 1 mL of the bacterial suspension into 9 mL sterile double strength Mueller Hinton broth (Merck, New Jersey, USA). Then, 100 µL of Thy/PVA nanoparticles at different concentrations (1.25 to 20.00 mg/mL) were added to obtain 200 µL as the final volume in each well. The final concentrations of Thy/PVA nanoparticles were set from 0.63 to 10.00 mg/mL. For growth control, the inoculated broth was added with 20 % Tween 20 solution. For sterility control, sterile broth was added with Thy/PVA nanoparticles at various concentrations (0.63 to 10.00 mg/mL). Then, the plate was kept at 37 °C for 24 h. After that, 20 µL of 0.2 mg/mL *p*-iodonitrotetrazolium violet salt was pipetted into each well. The plate was then stored at 37 °C for 1 h in dark condition. The colour changes from yellow to pink indicates the presence of bacterial growth. The MIC was documented as the lowest concentration of Thy/PVA nanoparticle which retards the bacterial growth. To determine the MBC, one loopful of sample from each well was

suitably streaked on the Mueller Hinton agar plate. The plates were kept at 37 °C for 24 h and the viability of the test bacteria was monitored. MBC was fixed as the lowest concentration of Thy/PVA nanoparticle needed to kill the test bacterium. The assay was triplicated.

*Bacterial growth curve*

*Y. enterocolitica* was used for this assay to investigate the effect of Thy/PVA nanoparticle concentration on bacterial growth. This bacterium was selected as it displayed the lowest MIC and MBC values. A total of 100 µL of bacterial inoculum was inoculated into 5 mL of sterile Mueller Hinton broth. To obtain a final volume of 10 mL, 4.9 mL of Thy/PVA nanoparticle at concentrations of 2.50 and 5.00 mg/mL was combined into each Erlenmeyer flask (50 mL). The study was done in triplicate. The Thy/PVA nanoparticles were tested at 1.25 mg/mL (MIC) and 2.5 mg/mL (MBC). Blank nanoparticles in Tween 20 solution were used as a control. All the flasks were incubated at 37 °C and agitated at 120 rpm in an incubator shaker. At every 6 h, 500 µL of culture broth was withdrawn aseptically for duration of 48 h. The growth of *Y. enterocolitica* was evaluated spectrophotometrically using a microplate reader (Thermo Scientific Varioskan LUX, Massachusetts, USA) at 600 nm. Sterile medium with Thy/PVA nanoparticle was used as a control. The growth curves were plotted as absorbance (*A*) at 600 nm versus incubation time (*t*).

*Antimicrobial efficacy on berries: Food model*

The antimicrobial efficacy of Thy/PVA nanoparticle solution was evaluated on blueberries (*19*). Blueberries (Berries Paradise, Mexico) used were purchased from local supermarket in April 2019. Blueberries utilised for this study were 11 days prior to the expiration date printed on the packaging. The blueberries were stored at 4 °C prior use and washed thoroughly with tap water. Then, *Y. enterocolitica* was inoculated by immersing the blueberries in 10 mL of freshly prepared bacterial inoculum for 60 minutes at 25 °C. The thymol nanoparticle solution was prepared at the concentration of 5 mg/mL (MBC for *Y. enterocolitica*). The blueberries were immersed in 10 mL of thymol nanoparticles solution for 20 minutes. The blueberries were finally rinsed with sterile distilled water. Tween 20 solution (20 %; *V/V*) with blank nanoparticles was set as a control.

*Antimicrobial efficacy on berries: Bacterial load*

Initially, 100 g of blueberries were placed on a sterile petri dish for a duration of 5 days. The petri dishes were sealed with parafilm tape to prevent the contamination from other sources. The petri dishes were kept at 15 °C. The study was done in triplicate. The blueberries were sampled on daily basis. The morphology of the blueberries was observed. To determine the bacterial load of *Y. enterocolitica*, 25 g of the sample was added with 250 mL of sterile peptone water (Oxoid, Basingstoke, UK). The sample was homogenized using a stomacher (Seward 80, West Sussex, UK). Then, 1 mL of crushed sample was serially diluted with sterile peptone water until the colony counts fall within the appropriate range, which is 30–300 colonies per plate. Next, 100 µl of the diluents were spread on Mac Conkey agar (Oxoid, Basingstoke, UK) plates by using a spreader. The plates were placed in an incubator for 48 h and temperature was set at 37 °C. The number of colonies present were observed under colony counter. The experiment was triplicated. The result was presented as logarithm of the number of the viable cells (CFU/mL) versus incubation time (*t*). Then, student t-test was performed using Microsoft Excel to determine the statistical difference between the 2 test groups.

*Statistical analysis*

All the experiments were performed in triplicate, and results were presented as average ± standard deviation. Student t-test was performed to analyse the statistical significance of different test groups in microbiological load study on food models. Statistical experiments and analyses were carried out using the software STATISTICA 7.1 (StatSoft, Tulsa, OK, USA) (*20*).

## RESULTS AND DISCUSSION

Nanoparticle-based drug delivery systems are widely used, especially in preventing postharvest microbial growth on food. This system promises excellent bioavailability, good encapsulation efficiency, controlled chemical release and low toxicity level. The chemical compatibility between the test drug and polymeric encapsulant is a key to a successful nanoparticle delivery system (*21*). PVA was selected in this study because it is an FDA-approved polymer that can be used in contact with food (*22*).

In this study, the size and morphology of Thy/PVA nanoparticles were characterized *via* TEM. The average diameter of thymol nanoparticles was 84.7±11.2 nm. Besides, thymol nanoparticles have spherical shapes and smooth surfaces. The result was in line with Zhang and co-researchers (*17*),

which reported thymol-loaded zein nanoparticles with spherical shape and smooth surface. No sign of agglomeration of nanoparticles was observed based on the electron microscopy analysis. Thymol is a hydrophobic compound while PVA is a water-soluble polymer that contains a vinyl group (*23*). Pluronic F-127 was combined in the nanoparticle formulation to avoid the agglomeration of nanoparticles by maintaining their surface energy. The higher the amount of Pluronic F127, the smaller the particle size of nanoparticles (*24*). Thus, the particles formed by using a high amount of Pluronic F127 were micelles, because it prevents the coalescence between the nanoparticles, thus improving the stability of nanoparticles (*16*). In this study, the amount of Pluronic F127 is sufficient to decrease the size of nanoparticles to a size range that less than 100 nm.

The surface charge of the nanoparticles is important in determining the efficiency and sustainability of the drug delivery process. With DLS, the surface charge of thymol nanoparticles was measured. The result showed that the synthesized nanoparticles possessed a zeta potential of -11.73 mV, with a conductivity of 6.655 mS/cm. Zeta potential is the electrokinetic potential retained by a molecule at the shear plane of a colloid particle that moves under an electric field (*15*). The stability of the nanoparticles depends on the total potential energy. Therefore, the magnitude of the zeta potential indicates the stability of a nanoparticle system. High repulsion energy represented by a particle with a large negative or positive zeta potential value (*21*). This occurrence prevents the particles from agglomeration. The high zeta potential value of the nanoparticles has justified the TEM observation.

In total, the encapsulation efficiency of Thy/PVA nanoparticles was 64.99 %. The high encapsulation efficiency showed that PVA was suitable to encapsulate thymol. The encapsulation efficiency was notably higher than the previous studies, which were reported by Li and co-researchers (*25*) and McClements (*15*). The type and amount of polymeric matrix could influence the encapsulation efficiency. Besides, the high encapsulation efficiency is also due to the optimal stirring speed during nanoparticle preparation (*24-25*). High stirring speed creates high shear stress that causes viscous droplets dispersion. In addition, when the encapsulant concentration increased, the encapsulation efficiency also increased.

The chemical interactions and functional groups of thymol, thymol nanoparticles and PVA were studied using FTIR spectroscopy (Fig. S1). In the FTIR spectrum of thymol, characteristic absorptions were observed at 3176 $cm^{-1}$ (-OH stretching), 2957 and 2926 $cm^{-1}$ (-CH stretching), 1620 $cm^{-1}$ (aromatic C=C stretching) (*26-27*). On the other hand, PVA showed absorptions at 3262 $cm^{-1}$ (-OH stretching), 2952 and 2907 $cm^{-1}$ (-CH stretching) and 1417 and 1085 $cm^{-1}$ (-C-O- stretching) (*28*). After the nanoencapsulation process, thymol nanoparticles showed absorptions at 3275 $cm^{-1}$ due to OH stretching (*29*). The absorption was shifted, which may be due to hydrogen-bonded interactions

between thymol and PVA. In addition, -CH stretching was observed at 2869 $cm^{-1}$, aromatic C=C stretching at 1651 $cm^{-1}$, -C-O stretching at 1090 $cm^{-1}$. The smaller intensity of absorption was observed in the spectrum of thymol nanoparticles, which may be attributed to a low concentration of thymol in the nanoparticles. The FTIR spectra proved that thymol was successfully encapsulated into the PVA matrix.

Fig. 1 shows the thymol release pattern from PVA nanoparticles for 96 h. Overall, an initial burst release was observed at the first h of the experiment. The burst release phenomenon is important to provide sufficient thymol to the food system to inhibit bacterial growth. The rapid burst release of thymol was allied to its rapid diffusion and adsorption from the surface of the PVA nanoparticles (*30*). After that, thymol release was slow and gradual, with an average amount of 44.1 μg/mL thymol released per h. The release was in accordance with the first order of kinetic, where 74.9±5.4 % of thymol was released into the test medium. These results showed the excellent drug carrier properties of PVA. The sustainable release of thymol was due to the gradual swelling of the nanoparticles when they were exposed to the test medium. A similar trend was reported by Martin and co-researchers in 2014 (*30*). They reported that the release of thymol from nano-fibrous material showed a rapid burst release at the first 2 h, then continued by slow and gradual release until equilibrium. It is worth mentioning that PVA nanoparticles own a high surface to volume ratio and porosity, making them excellent in drug delivery. This characteristic allows the chemical to enhance its drug loading capacity and delivery (*16*). The release of thymol reached a plateau at 48 h. PVA was successfully used to improve the shelf life of the nanoparticles. The drug release pattern proved that PVA was an excellent encapsulant polymer for thymol.

Fig. 1

Kirby Bauer analysis was conducted to screen the antimicrobial spectrum of Thy/PVA nanoparticles. A total of 6 test bacteria were tested. In overall, both Thy/PVA nanoparticles showed significant antimicrobial activity on both Gram-positive and Gram-negative bacteria (Table 1). There were no halo zones displayed by solvent control and blank nanoparticles on all test bacteria. This event indicated that the inhibitory activity related to the presence of thymol itself. The largest inhibition zone was represented by MRSA (17.1±0.1 mm) and followed by *Y. enterocolitica* (15.1±0.1 mm). Both represented Gram-positive and Gram-negative bacteria, respectively.

Table 1

The quantitative analysis of antimicrobial efficiency for Thy/PVA nanoparticles was evaluated on broth microdilution assay. Generally, Thy/PVA nanoparticles exhibited significant microbicidal activity on foodborne bacteria. A wide range of MICs were observed, ranging from 1.25 to 10.00 mg/mL. The wide range of MICs signified diverse susceptibility of test bacteria to the nanoparticles.

Generally, a notable difference was monitored between the MIC and MBC of Thy/PVA nanoparticles on all test bacteria, except MRSA. The antibacterial efficiency of thymol nanoparticles on bacteria was in accordance with the concentration. A higher concentration of Thy/PVA nanoparticle was required to allow a killing effect on the test bacteria (MBC), instead of inhibiting the growth (MIC). A notably low MIC and MBC was also observed on *Y. enterocolitica*.

Growth curve study was carried out to study the killing capability of the developed nanoparticles. The investigation was performed on *Y. enterocolitica* by using its low MIC and MBC as a reference. Absorbance represents the turbidity and mirrors the growth of the bacterium in the broth medium (*18*). In general, the control growth curve showed three distinct growth phases: lag phase, exponential phase, and stationary phase (Fig. 2). Tween 20 solution which was used to dissolve the Thy/PVA nanoparticle did not demonstrate any inhibitory signs on the growth of *Y. enterocolitica*. In general, the result was in accordance with broth microdilution assay, where 99.9 % of killing efficiency was not accomplished at the concentration of MIC. Thy/PVA nanoparticle concentration was not adequate to kill the bacterial cells. The growth curve showed prolonged lag phase and the stationary phase was attained at 30 h. However, the absorbance displayed for MIC was notably lower than blank nanoparticle control. At a concentration of MBC, 99.9 % killing of bacterial cells was recorded during the study period. No significant exponential growth of *Y. enterocolitica* was monitored when exposed to thymol nanoparticle at a concentration of MBC. This event marked the efficacy of the nanoparticles in killing foodborne *Y. enterocolitica*.

Fig. 2

Similar observations were previously reported where the researchers reported significant antimicrobial activity of thymol on *S. aureus* (*31*). Thymol disrupts the outer and inner membrane of bacteria and affecting cellular activities and functions of bacterial cells (*12*). Originally, MRSA is known as a significant cause of serious healthcare-related infections. However, it has been mentioned as a source of foodborne diseases in the USA over the past three decades (*32*). *Y. enterocolitica is* a causal agent of yersiniosis, clustered as a zoonotic bacterium. It usually triggers a sporadic type of infection (*5*). It is believed that the most common transmission method for *Y. enterocolitica* is fecal-oral through contaminated food (*33*). Moreover, imported fruits, including berries, are related to the growth risk of infections by *Y. enterocolitica* (*5*). *S. typhi* is also an important zoonotic bacterium responsible for an extensive worldwide burden of gastroenteritis. This species has been involved in foodborne outbreaks in Australia, with 92 % cases to account (*34*). The small particle size of nanoparticles enhances the penetration of thymol into the bacterial cells, thus improving the antimicrobial performance (*35*).

The antimicrobial efficacy of Thy/PVA nanoparticles was finally examined in food models as postharvest treatment. *Y. enterocolitica* causes significant loss of blueberries. Blueberries are fruits with high visual appeal and nutritional values. However, they are very susceptible to microbial infection during the postharvest storage period (*36*). The treatment with thymol nanoparticles maintained the visual qualities of blueberries (Fig. S2). Apparent bacterial growth was observed on blueberries treated with blank nanoparticle control. The decay of blueberries was significantly reduced. Besides, skin colour is an important factor that affects the visual appearance of blueberries (*37*). The treatment with thymol nanoparticles also maintained the skin colour of blueberries.

The treatment of blueberries with thymol nanoparticles also significantly reduced the bacterial load of *Y. enterocolitica* (Fig. 3). The bacterial load obtained for blueberries treated with Thy/PVA nanoparticles were too few to count (TFTC) throughout the whole experimental duration. The treatment causes a 100 % reduction of bacterial load on the blueberries up to 5 days. By comparing to blank nanoparticle control, a significant difference of bacterial load was observed since day 1 ($p \leq 0.05$). The fruit coating using Thy/PVA nanoparticles has significantly prolonged the storage period of blueberries, by reducing the bacterial load that causes significant postharvest quality deterioration of the blueberries. The result was in consensus with Sun and co-researchers *(38)*. The integration of 0.5 % trans-cinnamaldehyde essential oil and chitosan coating on blueberries has provided effective protection against *Escherichia coli* and *Penicillium digitatum* at 10 °C for 7 days. Besides, it also protected the fruits from softening. The previous study by Medina and colleagues have successfully developed chitosan thymol nanoparticles protein films *(39)*. Chitosan thymol nanoparticles showed good antimicrobial activity for preservation of fresh fruits, and it also acts as water vapour barriers when the films were applied on fresh fruits. Another study by Saez-orviz and colleagues also developed thymol nanoparticles for food using polylactic acid. The gelatine film with these nanoparticles showed high transparency and excellent antimicrobial activity *(40)*. Both studies are in agreed with present study.

Fig. 3

## CONCLUSIONS

A novel nanoparticle system was successfully developed for thymol using PVA as encapsulant. The nanoparticles exhibited a sustained release property for 48 h. Thymol nanoparticles exhibited significant inhibitory activities on both Gram-positive and negative foodborne bacteria. The nanoparticles also successfully reduced the bacterial load of *Y. enterocolitica* on blueberries. Thy/PVA nanoparticles can be potentially used as a postharvest sanitizer for fruits and vegetables, especially blueberries. The application of these sanitizers could improve the microbiological quality of fruits and

vegetables, and thus preventing food borne infections. Further investigations should be conducted to compare the efficacy of Thy/PVA nanoparticles with un-encapsulated thymol.

## ACKNOWLEDGEMENTS

The author(s) would like to thank Universiti Kuala Lumpur and Universiti Sains Malaysia.

## FUNDING

The Ministry of Higher Education Malaysia funded this work under the Fundamental Research Grant Scheme (FRGS/1/2017/STG05/UNIKL/02/5).

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

## SUPPLEMENTARY MATERIALS

Supplementary materials are available at: www.ftb.com.hr.

## AUTHORS' CONTRIBUTION

Conceptualization and writing were done by Syarifah Ab Rashid. Writing supervision and funding acquisition were done by Tong Woei Yenn. Data curation for transmission electron microscope and surface charge were handled by Leong Chean Ring. Methodology for encapsulation efficiency and FTIR were in-charged by Tan Wen Nee and Lim Jun Wei. Data curation (thymol release property, Kirby Bauer analysis) was done by Lee Chee Keong. Synthesis of thymol nanoparticles was assisted by Mohd Razealy Anuar. Statistical analysis was done by Teo Siew Hway Teo and the microbiological analyses were performed by Siti Khalida Abdull Lazit and Nur Amiera Syuhada Rozman.

## ORCID ID

A.R. Syarifah https://orcid.org/0000-0001-9189-5142
W.Y. Tong https://orcid.org/0000-0002-8697-6885
C.R. Leong https://orcid.org/0000-0002-2692-0097
W.N. Tan https://orcid.org/0000-0002-7812-3946
C.K. Lee https://orcid.org/0000-0003-2668-428X
M. A. Razealy https://orcid.org/0000-0003-2176-9612
H.T. Siew https://orcid.org/0000-0002-6862-4966

J.W. Lim https://orcid.org/0000-0003-0158-8822

## REFERENCES

*1.* Saranraj P, Stella D, Reetha D. Microbial spoilage of vegetables and its control measures: A Review. Int J Nat Prod Sci. 2012;2(2): 1-12.
https://doi.org/10.1007/978-1-4419-0826-1_6

2. Kasso M, Bekele A. Post-harvest loss and quality deterioration of horticultural crops in Dire Dawa Region, Ethiopia. J Saudi Soc Agric Sci. 2018;17(1):88-96.
https://doi.org/10.1016/j.jssas.2016.01.005

*3*. Råsbäck T, Rosendal T, Stampe M, Sannö A, Aspán A, Järnevi K, Lahti ET. Prevalence of human pathogenic *Yersinia enterocolitica* in Swedish pig farms. Acta Vet Scand. 2018;60(1):39.
https://doi.org/10.1186/s13028-018-0393-5

*4*. Ifoema MM. Incidence and public health significance of *Yersinia enterecolitica* in Enugu Urban-Enugu State, Nigeria. J Environ Sci Toxicol Food Technol. 2013;6(3):52-56.
https://doi.org/10.9790/2402-0635256

*5*. Huovinen E, Sihvonen LM, Virtanen MJ, Haukka K, Siitonen A, Kuusi M. Symptoms and sources of *Yersinia enterocolitica* infection: a case- control study. BMC Infect Dis. 2010;10(1):122.
https://doi.org/10.1186/1471-2334-10-122

*6.* Ain UQ, Ahmad S, Azam SS. Subtractive proteomics and immunoinformatics revealed novel B-cell derived T-cell epitopes against *Yersinia enterocolitica*: An etiological agent of Yersiniosis. Microb Pathog. 2018;125:336-348.
https://doi.org/10.1016/j.micpath.2018.09.042

*7*. Verbikova V, Borilova G, Babak V, Moravkova M. Prevalence, characterization and antimicrobial susceptibility of *Yersinia enterocolitica* and other *Yersinia* species found in fruits and vegetables from the European Union. Food Control. 2018;85:161-167. https://doi.org/10.1016/j.foodcont.2017.08.038

*8*. Rutala WA, Weber DJ. Disinfection, sterilization, and antisepsis: An overview. Am J Infect Control. 2019;47:3-9.
https://doi.org/10.1016/j.ajic.2015.10.038

*9*. Rutala WA, Kanamori H, Gergen MF, Sickbert-Bennett EE, Weber DJ. Susceptibility of *Candida auris* and *Candida albicans* to 21 germicides used in healthcare facilities. Infect Control Hosp Epidemiology. 2019;40(3):380-382.
https://doi.org/10.1017/ice.2019.1

*10*. Esfanjani AF, Jafari SM. Biopolymer nano-particles and natural nano-carriers for nano-encapsulation of phenolic compounds. Colloids Surf B Biointerfaces. 2016;146:532-543.

https://doi.org/10.1016/j.colsurfb.2016.06.053

*11*. Marchese A, Orhan IE, Daglia M, Barbieri R, Di Lorenzo A, Nabavi SF, Gortzi O, Izadi M, Nabavi SM. Antibacterial and antifungal activities of thymol: a brief review of the literature. Food Chem. 2016;210:402-414.
https://doi.org/10.1016/j.foodchem.2016.04.111

*12*. Meeran N, Fizur M, Javed H, Al Taee H, Azimullah S, Ojha SK. Pharmacological properties and molecular mechanisms of thymol: prospects for its therapeutic potential and pharmaceutical development. Front Pharmacol. 2017;8:380.
https://doi.org/10.3389/fphar.2017.00380

*13*. Khan I, Saeed K, Khan I. Nanoparticles: Properties, applications and toxicities. Arab J Chem. 2017;6:1-24.
https://doi.org/10.1016/j.arabjc.2017.05.011

*14*. Chen H, Zhang Y, Zhong Q. Physical and antimicrobial properties of spray-dried zein–casein nanocapsules with co-encapsulated eugenol and thymol. J Food Eng. 2015;144:93-102.
https://doi.org/10.1016/j.jfoodeng.2014.07.021

*15*. McClements DJ. The future of food colloids: Next-generation nanoparticle delivery systems. Curr Opin Colloid Interface Sci. 2017;28:7-14. https://doi.org/10.1016/j.cocis.2016.12.002

*16*. Sahadan MYB, Tong WY, Tan WN, Leong CR, Misri MNB, Chan M, Shaharuddin S. Phomopsidione nanoparticles coated contact lenses reduce microbial keratitis causing pathogens. Exp Eye Res. 2019;178:10-14.
https://doi.org/10.1016/j.exer.2018.09.011

*17.* Zhang Y, Niu Y, Luo Y, Ge M, Yang T, Yu LL, Wang Q. Fabrication, characterization and antimicrobial activities of thymol-loaded zein nanoparticles stabilized by sodium caseinate–chitosan hydrochloride double layers. Food Chem. 2014;142:269-275.
https://doi.org/10.1016/j.foodchem.2013.07.058

*18*. Leong CR, Azizi K, Afif M, Taher MA, Wahidin S, Lee KC, Tong WY. Anthocyanins from *Clitoria ternatea* attenuate food-borne *Penicillium expansum* and its potential application as food biopreservative. Nat Prod Sci. 2017;23(2):125-131.
https://doi.org/10.20307/nps.2017.23.2.125

*19*. Gazula H, Quansah J, Allen R, Scherm H, Li C, Takeda F, Chen J. Microbial loads on selected fresh blueberry packing lines. Food Control. 2019;100:315-320.
https://doi.org/10.1016/j.foodcont.2019.01.032

20. Sáyago-Ayerdi SG, Zamora-Gasga VM, Venema K. Prebiotic effect of predigested mango peel on gut microbiota assessed in a dynamic *in vitro* model of the human colon (TIM-2). Food Res. Int. 2019;118:89-95.
https://doi.org/10.1016/j.foodres.2017.12.024

*21*. He X, Hwang HM. Nanotechnology in food science: functionality, applicability, and safety assessment. J Food Drug Anal. 2016;24(4):671-681.
https://doi.org/10.1016/j.jfda.2016.06.001

*22*. Cao Y, Liu F, Chen Y, Yu T, Lou D, Guo Y, Ran H. Drug release from core-shell PVA/silk fibroin nanoparticles fabricated by one-step electrospraying. Sci Rep. 2017;7(1):11913.
https://doi.org/10.1038/s41598-017-12351-1

*23*. Zhu Z, Min T, Zhang X, Wen Y. Microencapsulation of thymol in poly (lactide-co-glycolide)(PLGA): Physical and antibacterial properties. Materials (Basel). 2019;12(7):1133.
https://doi.org/10.3390/ma12071133

*24*. Lee WH, Bebawy M, Loo CY, Luk F, Mason RS, Rohanizadeh R. Fabrication of curcumin micellar nanoparticles with enhanced anti-cancer activity. J Biomed Nanotech. 2015;11(6):1093-1105.
https://doi.org/10.1166/jbn.2015.2041

*25.* Li KK, Yin SW, Yin YC, Tang CH, Yang XQ, Wen SH. Preparation of water-soluble antimicrobial zein nanoparticles by a modified antisolvent approach and their characterization. J Food Eng. 2013;119(2):343-352.
https://doi.org/10.1016/j.jfoodeng.2013.05.038

*26*. Trivedi M, Patil S, Mishra RK, Jana S. Structural and physical properties of biofield treated thymol and menthol. J Mol Pharm Org Process Res. 2015; 3(2):1-10.
https://doi.org/10.4172/2329-9053.1000127

*27*. Zamani Z, Alipour D, Moghimi HR, Mortazavi SAR, Saffary M. Development and evaluation of thymol microparticles using cellulose derivatives as controlled release dosage form. Iran J Pharm Sci. 2015;14(4):1031-1040
https://doi.org/10.22037/ijpr.2015.1754

*28*. Saha N, Ali SA, Kar S, Saha P, Banthia AK. Aluminosilicate-filled composites of PVA–PVP—An improved biodegradable polymeric material. J Appl Polym Sci. 2006;102(5):4963-4970.
https://doi.org/10.1002/app.24671

*29*. Turki A, El Oudiani A, Msahli S, Sakli F. Infrared spectra for alfa fibers treated with thymol. J Glycobiol. 2018;7(130*)*:1-8.
https://doi.org/10.4172/2168-958x.1000130

*30*. Martins IM, Barreiro MF, Coelho M, Rodrigues AE. Microencapsulation of essential oils with biodegradable polymeric carriers for cosmetic applications. Chem Eng J. 2014;245:191-200. https://doi.org/10.1016/j.cej.2014.02.024

*31*. Abdelhamid SM, El-Hosseiny LS. Combined efficacy of thymol and silver nanoparticles against *Staphylococcus aureus*. Afr J Microbiol Res. 2017;11(11):450-457. https://doi.org/10.5897/ajmr2016-8387

*32*. Jones TF, Kellum ME, Porter SS, Bell M, Schaffner W. An outbreak of community-acquired foodborne illness caused by methicillin-resistant *Staphylococcus aureus*. Emerg Infect Dis. 2002;8(1):82-84. https://doi.org/10.3201/eid0801.010174

*33*. Latiful Bari Md, Anwar Hossain M, Isshiki K, Ukuku D. Behavior of *Yersinia enterocolitica* in foods. J Pathog. 2011;2011:420732. https://doi.org/10.4061/2011/420732

*34*. Zhang S, Li S, Gu W, den Bakker H, Boxrud D, Taylor A, Brown E. Zoonotic source attribution of *Salmonella enterica* serotype Typhimurium using genomic surveillance data, United States. Emerg Infect Dis. 2019;25(1):82. https://doi.org/10.3201/eid2501.180835

*35*. Nayak D, Ashe S, Rauta PR, Kumari M, Nayak B. Bark extract mediated green synthesis of silver nanoparticles: evaluation of antimicrobial activity and antiproliferative response against osteosarcoma. Mater Sci Eng C. 2016;58:44-52. https://doi.org/10.1016/j.msec.2015.08.022

*36*. Zheng X, Meng F, Huang D, Chen X, Li G, Li Y, Lin M. Research advances on technologies for postharvest storage and preservation of blueberry. J Food Safety Qual. 2016;7(9):3560-3565. http://doi.org/10.3233/JBR-190421

*37*. Zorenc Z, Veberic R, Stampar F, Koron D, Mikulic-Petkovsek M. Changes in berry quality of northern highbush blueberry (*Vaccinium corymbosum* L.) during the harvest season. Turk J Agric For. 2016;40(6):855-864. https://doi.org/10.3906/tar-1607-57

*38.* Sun X, Narciso J, Wang Z, Ference C, Bai J, Zhou K. Effects of chitosan-essential oil coatings on safety and quality of fresh blueberries. J Food Sci. 2014; 79:955–960. https://doi.org/10.1111/1750-3841.12447

*39.* Medina E, Caro N, Abugoch L, Gamboa A, Díaz-Dosque M, Tapia C. Chitosan thymol nanoparticles improve the antimicrobial effect and the water vapour barrier of chitosan-quinoa protein films. *J Food Eng*. 2019; 240:191-198.

https://doi.org/10.1016/j.jfoodeng.2018.07.023

*40.* Sáez-Orviz S, Marcet I, Weng S, Rendueles, M, Díaz M. PLA nanoparticles loaded with thymol to improve its incorporation into gelatine films. J Food Eng. 2020; 269: 109751. https://doi.org/10.1016/j.jfoodeng.2019.109751

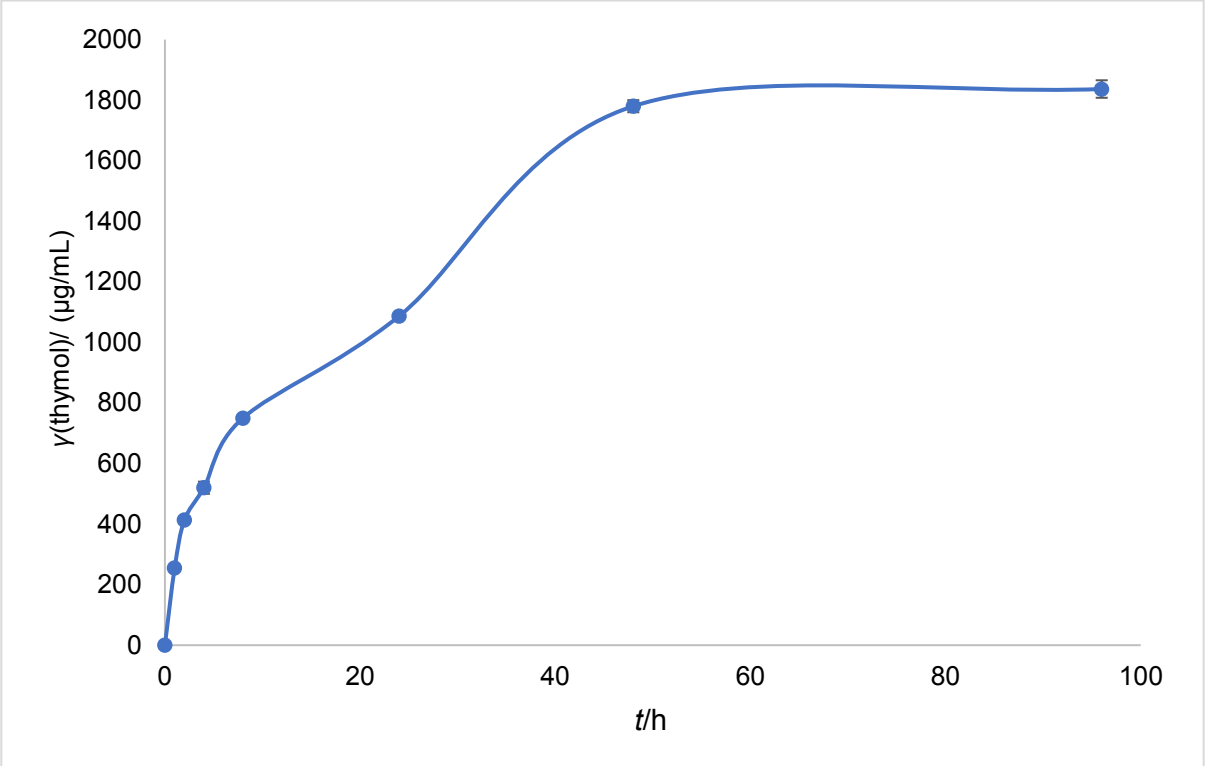


Fig. 1. The release of thymol from PVA nanoparticles reached plateau at 48 h

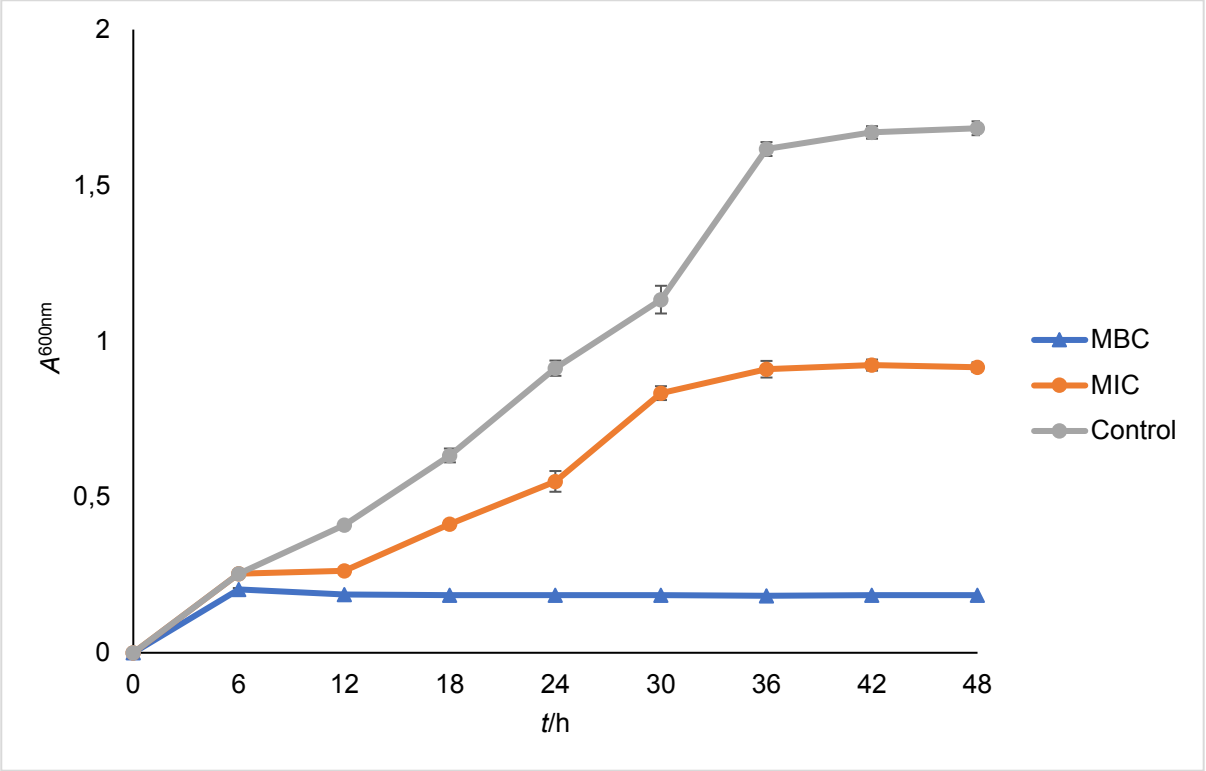


Fig. 2. The growth curve of *Y. enterocolitica* when exposed to different concentrations of thymol nanoparticles (MIC and MBC), and also control (without thymol nanoparticles). The absorbances of the culture was significantly reduced when exposed to the nanoparticles

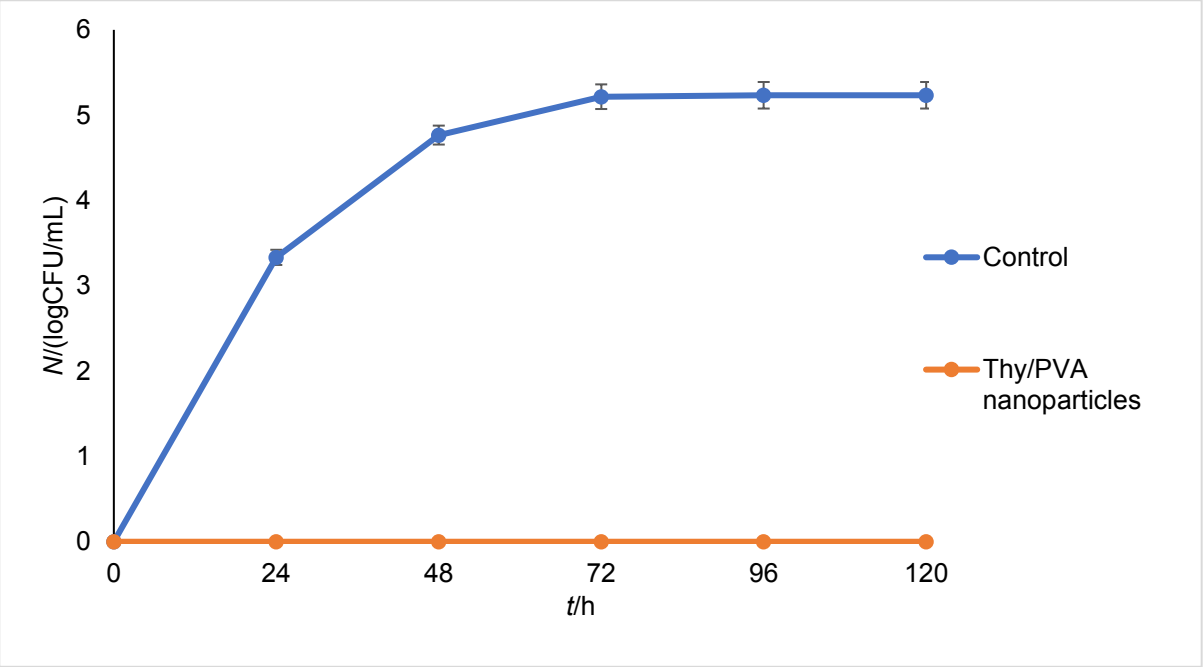


Fig. 3. The treatment of blueberries with thymol nanoparticles significantly reduced the bacterial load of *Y. enterocolitica* ($p \leq 0.05$). The bacterial loads for blueberries treated with the nanoparticles were too few to count (TFTC) throughout the whole experimental period

Table 1. The antimicrobial activity of Thy/PVA nanoparticles against foodborne bacteria, which represented by inhibition zones, MIC and MBC values

| | *d*/mm | | | Antimicrobial susceptibility | |
|---|---|---|---|---|---|
| Test Microorganisms | Thy/PVA nanoparticles | Thymol-free nanoparticles | Positive control | MIC *γ*/(mg/mL) | MBC *γ*/(mg/mL) |
| Gram-positive bacteria | | | | | |
| *S. aureus* | 9.7±0.4 | - | 20.1±0.2 | 5.00 | 10.00 |
| MRSA | 17.1±0.1 | - | 17.2±0.3 | 10.00 | 10.00 |
| *B. cereus* | 7.2±0.2 | - | 12.1±0.2 | 2.50 | 5.00 |
| Gram-negative bacteria | | | | | |
| *Y. enterocolitica* | 15.1±0.3 | - | 20.2±0.2 | 1.25 | 2.50 |
| *S. typhii* | 11.1±0.1 | - | 21.2±0.4 | 2.50 | 10.00 |
| *E. coli* | 6.4±0.2 | - | 11.2±0.4 | 2.50 | 5.00 |

(-): no inhibition zone

SUPPLEMENTARY MATERIALS

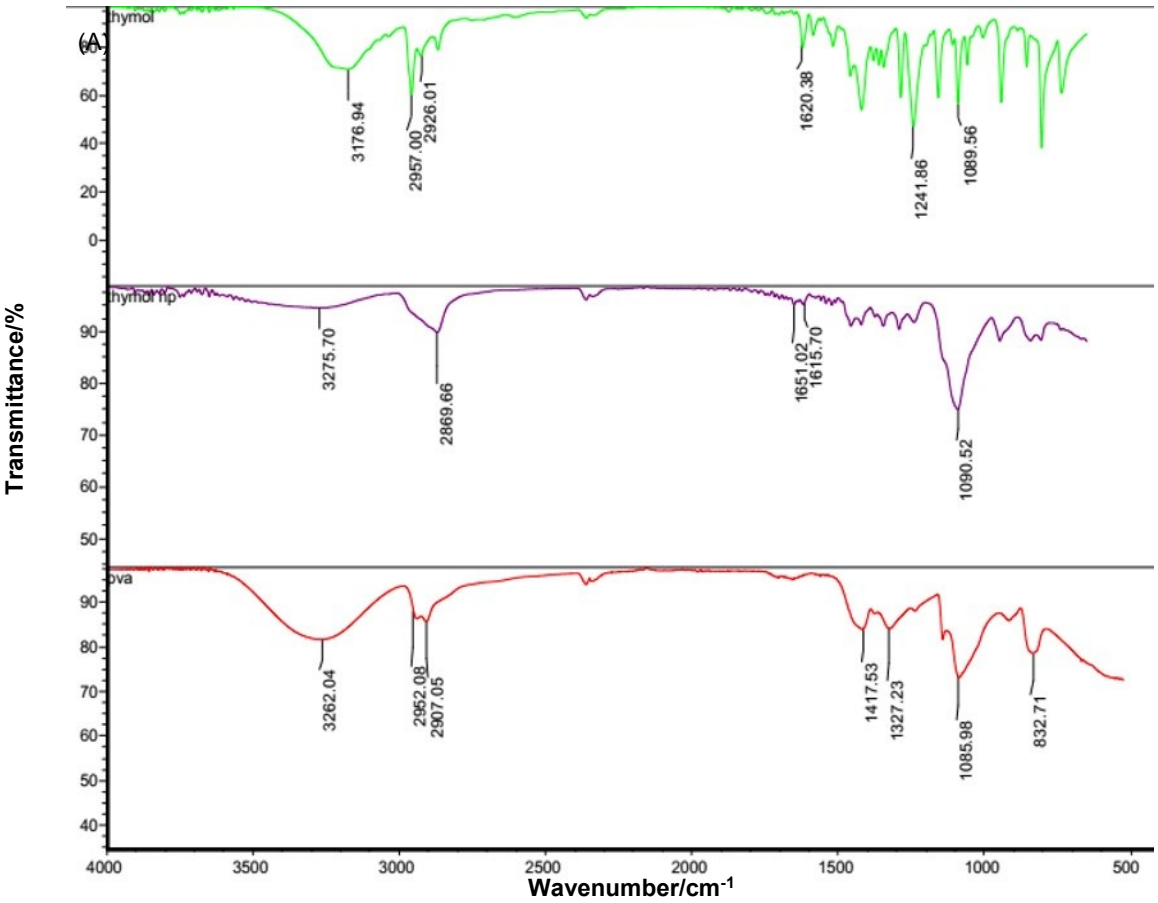


Fig. S1. FTIR spectrum for (a) thymol (b) thymol nanoparticles and (c) PVA

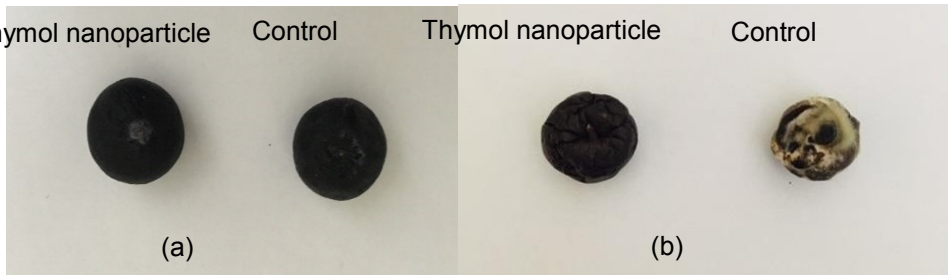


Fig. S2. The morphologies of the contaminated blueberries before (a) and after (b) treatment with thymol nanoparticles