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Liu, Z., Sun, P., He, X. et al. (7 more authors) (2025) Synergistic inactivation effect of ultrasound and nano-emulsified basil essential oil on the metabolic responses of Salmonella on sprouts. International Journal of Food Microbiology, 431. 111082. ISSN 0168-1605

https://doi.org/10.1016/j.ijfoodmicro.2025.111082

Synergistic inactivation effect of ultrasound and nano-emulsified basil essential oil on the metabolic responses of *Salmonella* on sprouts

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Abstract

This study investigated the effectiveness and mechanisms of ultrasound, nano-emulsified basil essential oil, and their combination (NBEO_US) in inactivating *Salmonella enterica* cells laid over pea sprouts. The results demonstrated that the combined treatment was more effective than individual treatments in inactivating the target. This led to a decrease of 4.5 to 5.1 log CFU/g. Transmission electron microscopy showed that NBEO_US exacerbates the disruption of the bacteria morphology. Additionally, the leakage of cell constituents (proteins and nucleotide) demonstrated that NBEO_US disrupted the structural integrity of *S.* enterica cells. In addition, the metabolomics analysis using ^{1}H NMR showed that NBEO_US had a detrimental effect on energy and amino acid metabolism in bacterial cells, specifically affecting glycolysis and amino acid production. Also, NBEO_US affected the Embden-Meyerhof-Parnas pathway in *S. enterica* cells by decreasing the activity of hexokinase, phosphofructokinase, and pyruvate kinase. Finally, the application of NBEO_US resulted in a substantial ($P < 0.05$) increase in the hardness of the treated pea sprouts while simultaneously decreasing their lightness. The present investigation illustrates the synergistic antibacterial mechanism of ultrasound and nano-emulsified basil essential oil against *S. enterica* strains using sprouts as a food model. Understanding the microbiological changes in metabolic pathways resulting from this combined treatment might enable the optimization of sanitization strategies by specifically targeting vulnerable metabolic pathways. This, in turn, promotes the safer production of fresh produce.

Keywords: Foodborne pathogen; antibacterial mechanism; NMR, metabolomics, fresh produce

1. Introduction

Salmonella is among the most significant and prevalent foodborne pathogens globally (Bajpai et al., 2012). It incites disease in humans and food-producing animals through an array of virulence factors controlled by over 200 pathogenic genes (Marcus et al., 2000; Sheng et al., 2020). According to the 2021 data from the European Centre for Disease Prevention and Control (ECDC), salmonellosis is the second most common bacterial foodborne infection in Europe, with an estimated 60, 050 cases occurring annually (Naushad et al., 2023). In recent years, the rise in consumption of fresh agricultural products has resulted in increased outbreaks associated with contaminated fresh agricultural products. Among resolved foodborne outbreaks, pea sprouts have been identified as a high-risk vector for transmitting *Salmonella*, which has been implicated as a primary source in 64 investigated outbreaks (Bhandare et al., 2024; Chahar et al., 2023). Based on the high prevalence and severity of *Salmonella* contamination in sprout products, the sprout industry urgently requires effective disinfection measures targeting *Salmonella* to ensure safety.

Synthetic food preservatives have been applied as antiseptics for sprouts due to their strong antioxidant and inactivation effect on microorganisms (Li et al., 2022). However, residual amounts of synthetic chemical compounds and aqueous solutions in the sprouts are potential risks to food safety. The naturally extracted plant-derived essential oils (EOs) are generally recognized as safe (GRAS) and provide antibacterial activity through secondary metabolites of terpenoids and phenolic compounds (FDA, 2013). As a broad-spectrum antimicrobial agent, basil essential oil (BEO) can effectively inhibit *Salmonella* spp., *Listeria* spp., and other foodborne pathogens (Meenu et al., 2023).

However, EOs can be limited by the higher costs compared with synthetic compounds and the need for high concentrations to achieve antimicrobial effects. Consequently, the primary focus regarding EOs revolves around advancing technologies to enhance their antibacterial efficacy (Cho et al., 2020). In emerging food technology, the advent of nano-sized EO emulsions, characterized by their precise minuteness at the nanoscale, signifies a transformative breakthrough. The diminutive particle size enhanced stability and solubility, ensuring optimal dispersal in food media. Recent studies found that nano-emulsified *Thymus daenensis* essential oil exhibited superior disinfection capabilities to traditional EOs (Moghimi et al., 2016).

Previous studies have shown that ultrasound (US) combined with essential oil nano-emulsion can improve antibacterial application. Ultrasound is a widely recognized non-thermal technique applied to inactivate microorganisms (Carstens et al., 2019). Sonoporation involves the application of Ultrasound waves, which exert mechanical forces on the cell membrane, leading to alterations in its permeability and enhancing the efflux of intracellular amino acids and peptides (Erriu et al., 2014; Wang et al., 2021). Ultrasound waves above 20 kHz penetrate substances into cells, creating many gas bubbles in a liquid phase (Lauteri et al., 2023). When the bubbles cannot be retained in the solution, they condense and create strong shock waves, shear force, and mechanical force, disrupting the bacteria's membrane (Firouz et al., 2023). However, singly utilizing the Ultrasound as an activation method might reveal limited effectiveness; combining the Ultrasound with other sterilizing technologies has been proven to be a successful bactericidal strategy (Millan-Sango et al., 2016). Recent studies have shown that Ultrasound, combined with different treatments for bacteria inactivation, can achieve a better antibacterial effect (Yang et al., 2023; Yoon et al.,

2021). However, research on Ultrasound and BEO nano-emulsions and their combined antibacterial effect against *Salmonella* inoculated onto sprouts has not been reported.

This study investigated the antibacterial effects of nano-emulsified basil essential oils (NBEO), Ultrasound, and their combination treatment (NBEO_US) on the *S. enterica* strains inoculated onto pea sprouts. The impact of three treatment groups on the physicochemical properties of the pea sprouts was also investigated. Specifically, the study differentiated the variances among different treatment methods and elucidated the antibacterial mechanisms at each type of metabolic level.

2. Materials and methods

2.1 Bacterial preparation

Salmonella enterica subsp. *enterica serovar* Enterica (ATCC 13076), *Salmonella enterica* subsp. *enterica serovar* Typhimurium (ATCC 14028), and *Salmonella enterica* subsp. *enterica serovar* Newport (ATCC 6962) were obtained from the Department of Food Science and Technology, National University of Singapore. Each *S. enterica* strain was cultured in 30 mL Tryptic Soy Broth (TSB, Oxoid, Basingstoke, UK) and incubated overnight at 37 °C. Cell pellets were collected by centrifuged at 8,000 × *g* for 10 min at 4 ℃ and washed twice with 0.1% peptone water (Sigma-Aldrich, St. Louis, MO, USA). Ultimately, the pellets were resuspended in 200 μL of 0.1% peptone water for subsequent inoculation of pea sprouts. Also, the bacteria were resuspended for further analysis.

2.2 BEO composition analysis via GC-MS

Basil (*Ocimum basilicum*) essential oil (BEO) was purchased from NOW Foods

(Bloomingdale, Illinois). The compositions of BEO were measured using gas chromatograph 7890A equipped with a 7200C mass spectrometer (Agilent Scientific Instruments, California, USA) (Moghimi et al., 2016; Zhang et al., 2023). BEO was filtered and diluted in n-hexane at a ratio of 1: 200. The conditions of GC were as follows: HP-5MS column (30 m × 0.25 mm, 0.25 μm); carrier gas: Helium (99.999% purity); flow rate: 1mL/min; split ratio 1:20; inlet temperature: 250 °C; temperature program: 65 °C isothermal for 3 min, 5 °C/min ramp to 280 °C and isothermal for 30 min. A 70-eV potential was set for the electron ionization mode to ionize particles with high-energy electrons. Using the peak area shown in GC results, the relative contents of the detected components were determined.

2.3 Preparation and characterization of nano-emulsified basil essential oil

For nano-emulsion preparation according to a previous study with some modifications (Hai et al., 2022). An aqueous mixture of BEO (5% w/v), Tween 80 (0.3% w/v), and corn oil (0.5% w/v) was homogenized at 8,000 rpm for 10 min at 4 ℃ to produce a coarse nano-emulsion. The coarse nano-emulsion was treated with an ultrasound crusher (Scientz-II D; Ningbo Scientz, Zhejiang, China) at high power (20 kHz, 500 W) for 9 min with on and off pulse mode every 2 s. The characteristics of the oil droplet in NBEO were measured using a ζ-potential cum Nano Particlesizer (Brookhaven Instruments, New York, USA). The droplet size, ζ potential, and polydispersity index (PDI) of NBEO were monitored for 90 d at 25 °C (Salvia-Trujillo et al., 2013).

2.4 Inoculation on pea sprouts

Fresh pea sprouts were purchased from FairPrice, Singapore, which were dried after rinsing the surface with peptone water (Sigma Aldrich, St. Louis, Mo., U.S.A.). Each

portion of sprout samples was 10.0 ± 0.3 g. To sterilize the surface of the pea sprouts, all samples were laid flat on the surface and irradiated with UV light on both the front and back sides for 30 min (Guo et al., 2022; Hadjok et al., 2008). After that, samples (10 g) without inoculation were suspended in 90 mL of peptone water (0.1%) and plated on Xylose lysine deoxycholate (XLD) agar (Sigma-Aldrich, St Louis, MO, USA) to ensure no survival of naturally contaminating *Salmonella*. Each sample was inoculated with an individual *Salmonella* strain by aseptically depositing the concentrated cell suspension (200 µL) which was prepared in Section 2.1 at 15 random locations. Then the samples were subjected to a 3 h drying process within a laminar flow biosafety cabinet. The inoculated population of *Salmonella* was verified by suspending 10 g of inoculated samples into 90 mL of peptone water (0.1%) and plating on XLD. The initial level of *Salmonella* in all samples was ensured to be approximately 8 log CFU/g.

2.5 Sanitising treatments on pea sprouts

The washing method was based on the previous studies with appropriate modifications (Wang et al., 2022). The inoculated samples (10 g) were assigned to four treatments: Control (100 mL DI water), Ultrasound (100 mL DI water, 241 W/cm^2), NBEO (100 mL, 0.5 mg/mL NBEO), and NBEO_US (100 mL NBEO, 241 W/cm^2). Ultrasound treatment was performed with a 6 mm diameter ultrasonic probe, the probe was immersed at least 2 cm below the liquid surface, avoided contact with pea sprouts, and the power parameter was set at 241 W/cm^2 with on and off pulse mode every 5 s (Gul et al., 2018). For NBEO_US treatment, samples immersed in NBEO were subjected to sonication using previous parameters. During processing, all samples were immersed in various solutions for a duration of 10 min to guarantee the complete sanitization effectiveness of the treatments. Subsequently, the samples were gently rinsed with

phosphate-buffered saline (0.2 mol/L, pH 7.5) in order to neutralize any remaining NBEO. The verification of successful neutralization was achieved by evaluating the pH of the final wash solution. Following 20 min dehydration period in the laminar flow biosafety cabinet, each sample was transferred to a sterile stomacher bag containing 90 mL of 0.1% sterile peptone water and homogenized in stomacher (IUL Instruments, Germany) for 120 s. Then, 100 µL of the treated samples were spread on XLD plates and incubated at 37 °C for 48 h.

2.6 Determination of intracellular materials leakage

The membrane integrity was evaluated according to the concentrations of cell constituents in the supernatant after being treated with different treatments. The test was designed based on Hai et al. (2022) with modifications. Briefly, the bacteria suspension of each *S. enterica* strain was centrifuged, washed based on the method of 2.1, and resuspended in phosphate-buffered saline buffer. Where the concentration of bacteria cells was 8 log CFU/mL to obtain enough leakage contents. After four treatments: Control (DI water), Ultrasound (241 W/cm^2), NBEO (0.5 mg/mL), and NBEO_US (0.5 mg/mL NBEO, 241 W/cm^2), each group of *S. enterica* suspension was centrifuged at (8,000 × *g*, 10 min, 4 ℃) to obtain supernatant samples. The absorbance at 260 nm for the supernatants from four treatment groups was measured using an UV-Vis spectrophotometer (UV-2600, Shimadzu, Tokyo, Japan). In order to quantify protein leakage, 100 µL of supernatant was mixed with an equal amount of Bradford reagent and incubated for 5 min. Then the absorbance at 595 nm was measured using a Plate reader (Agilent Scientific Instruments, California, USA). Bovine Serum Albumin (BSA) (Sigma-Aldrich, Singapore) was employed as standard.

2.7 Transmission electron microscopy (TEM) analysis of *S. enterica*

TEM observations were carried out to investigate the ultrastructure alterations of the effect of Ultrasound, NBEO, and NBEO_US on the integrity of *S. enterica* cells. Samples were prepared based on 2.6, then centrifuged for 5 min at 4 °C at 12,000 × *g*, and collected precipitation was fixed in 2.5% glutaraldehyde for 16 h at 37 °C. The samples were then exposed to the copper mesh for 5 min. The copper mesh was dyed with 4.5% (w/w) phosphotungstic acid for 5 min, dried, and analysed using TEM (JEM-3010, JEOL, Tokyo, Japan).

2.8 Extraction of metabolites in *Salmonella*

The metabolites of *S. enterica* were extracted according to a previous study (Lin et al., 2024) and divided into Control, Ultrasound, NBEO, and NBEO_US groups. Inoculated pea sprouts (200 g) were used in each group for metabolic analysis. Following the treatment of the pea sprouts, the solutions were promptly collected and centrifuged at 8,000 × *g* for 1 min at 4 °C to remove pea sprout debris. *S. enterica* cells pellets were harvested from the suspensions by centrifugation at 12,000 × *g* for 15 min (4 °C). The pellets were mixed with 1 mL of ice-cold methanol-d4 (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and immediately frozen the samples using liquid nitrogen (three cycles of freeze-thawing) to breakdown the cell membrane. The metabolites in ice-cold methanol-d4 were stored overnight (−20 °C), and the supernatant obtained after centrifugation (12,000 × g, 20 min, 4 °C) was subsequently used for NMR analysis (Lin, Wang, et al., 2022). Trimethylsilyl propanoic acid was added to the supernatant as an internal reference at a final concentration of 1 mM. The mixture was vortexed thoroughly, and 600 µL was subsequently transferred to an NMR tube for further analysis. Metabolite's extraction of different groups was prepared in

triplicate.

2.9 Analysis of metabolites using ^{1}H NMR spectroscopy

The samples were analyzed using a Bruker DRX-500 NMR spectrometer (Bruker, Rheinstetten, Germany) equipped with a Triple Inverse Gradient probe (Huang et al., 2022). The standard Bruker NOESY pulse sequence was used to obtain the ^{1}H spectrum with a width of 10 ppm. The acquisition time was set at 3.3 s, with a relaxation delay of 2 s, and the temperature was maintained at 25 °C. The obtained spectra of Control, Ultrasound, NBEO, and NBEO_US groups were processed using Mestre-nova (Mestreab SL, Santiago, Spain) to produce the organized database to analyze multiple variables. The qualitative and quantitative analyses of metabolites were performed using the Chenomx NMR suite software (Chenomx Inc., Canada).

2.10 Activity of enzymes analysis in the Embden-Meyerhof-Parnas (EMP) pathway

After treating pea sprouts, different treatment solutions were collected and centrifuged respectively based on the method of section 2.8. The *S. enterica* cells pellets' enzyme activity of the hexokinase (HK), pyruvate kinase (PK), and phosphofructokinase (PFK) were measured using the HK, PK, and PFK assay kits (Keming Biotechnology, Jiangsu, China).

2.11 Color and texture analysis of sprouts

Pea sprout leaves were evaluated for color changes using a Minolta Colorimeter CM-3500d (Konica Minolta, Tokyo, Japan). The samples' color values were independently replicated three times for the Control, Ultrasound, NBEO, and NBEO_US groups (Del-

245 Valle et al., 2005; Kasputis et al., 2024). Measuring the firmness of the upper leaves of
246 pea sprouts involves assessing the tissue's resistance when subjected to a cutting force.
247 Position the fixture with the prepared pea sprout sample under the texture analyzer's
248 probe (TA-XT2i, Stable Micro System, Surrey, UK) fitted with a knife blade (TA-42).
249 The maximum peak force was recorded as firmness (Li et al., 2018).
250
251 **2.12 Statistical analysis**
252 All measurements were conducted in triplicate independently. Experimental data were
253 analyzed statistically using two-way analysis of variance (ANOVA) by SPSS software
254 (version 26.0; IBM Corp., Armonk, NY). The significance of the difference was defined
255 at $P < 0.05$.
256
257 **3. Results and discussion**
258 **3.1 Composition of BEO**
259 Table 1 lists the primary BEO compounds that were identified by GC-MS. Estragole
260 constitutes about 97.62% of the overall component, which was discovered to be the
261 predominant component in BEO. Estragole is a common compound found in several
262 aromatic plants (Da Costa et al., 2021), which has been discovered to possess a
263 significant antibacterial effect against *Pseudomonas syringae* (Song et al., 2016). Other
264 minor compounds such as eucalyptol (1,8-cineole) and linalool are also reported to be
265 antibacterial against *Salmonella* (Prakash et al., 2019; Sun et al., 2018).
266
267 **3.2 Characteristics of basil nano-emulsions**
268 This study measured droplet size, PDI, and ζ potential to assess three important physical
269 properties of the emulsion, which can significantly affect the stability of colloidal

systems. The nano-emulsion exhibited an average droplet size ranging from 198.14 – 228.35 nm, as reported in Table 2, indicating a well-defined particle size distribution. Moreover, the PDI value indicates the width of the droplet size distribution (Liu et al., 2023). Additionally, the PDI value is a measure of the breadth of the droplet size distribution, ranging from 0 to 1. The closer the PDI value is to 1, the broader the droplet size distribution (Liu et al., 2023). From Table 2, the PDI values of BEO under a range of 0.149 to 0.252 were all below 0.30, indicating excellent emulsion stability (Pérez-Córdoba et al., 2018). Additionally, the ζ potential of -24.779 – 33.906 mV suggested low stability of the nano-emulsion. All these characteristics remained relatively unchanged during the 90-day storage period, confirming the outstanding physical stability of these nano-emulsions during storage at 25°C for 90 days.

3.3 Antibacterial effect of different treatments against *S. enterica*

The antibacterial effects on three strains of *S. enterica* were assessed by analyzing the survival of populations on XLD plates following different treatment conditions. Ultrasound, NBEO, and NBEO_US treatments effectively reduced the population of *S. enterica* on pea sprouts. Three treatments were reduced by approximately 0.71 – 5.35 log CFU/g on three *S. enterica* strains (Table 3). The Ultrasound treatment exhibited a relatively mild bacteriostatic effect (0.71 – 1.18 log CFU/g), while the NBEO treatment showed a slight increase compared to the Ultrasound treated group (1.04 – 1.35 log CFU/g). However, when compared with the Ultrasound and NBEO treatment alone, the reduction of *S. enterica* was significantly higher ($P < 0.05$) in the NBEO_US treatment (4.35 – 5.35 log CFU/g). The results indicated that *S.* Newport (ATCC 6962) was the most vulnerable to NBEO_US after comparing with other strains, reduced by 4.91 – 5.35 log CFU/g. Conversely, the *S.* Typhimurium (ATCC 14028) experienced the least

decrease. Overall, the combined use of ultrasound and NBEO should better antibacterial effects at lower concentrations and shorter treatment times compared to treatment alone. Additionally, in order to elucidate the fundamental reasons for the significant differences in various treatments, it would be beneficial to investigate the metabolic responses and bactericidal mechanisms of *S. enterica* during different treatment approaches.

3.4 Changes in intracellular materials leakage of *S. enterica*

Ultrasound and NBEO treatments can damage the cell membrane of *S. enterica*, causing cytoplasm constituent leakage. This phenomenon can be interpreted by the occurrence of protein and nucleic acid leakage (Hollander & Yaron, 2021). As shown in Fig. 1A, the Control group detected a relatively low protein concentration (0.29 – 2.86 μg/mL). The concentration showed a significant increase ($P < 0.05$) after three strains of *S. enterica* were treated with Ultrasound (6.96 – 9.23 μg/mL), NBEO (16.56 – 18.83 μg/mL) and reached peak concentration when combing these two methods (22.27 – 23.70 μg/mL). Combine treatment demonstrated excellent antibacterial effectiveness against three strains of *S. enterica*. The leakage of nucleic acid was indicated by the absorbance under 260 nm (Moghimi et al., 2016). As shown in Fig. 1B, the results and increasing trends are similar to protein leakage. Although low absorbance was detected in the Control group, the absorbance significantly increased after being treated with Ultrasound (0.297 – 0.367 of OD_{260}), NBEO (1.029 – 1.194 of OD_{260}), and NBEO_US (1.313 – 1.661 of OD_{260}) treatments ($P < 0.05$). Intracellular content leakage results provide strong evidence suggesting that NBEO_US can disrupt the cell membrane of *S. enterica* and combining these two treatments could be more effective. The results also indicate that NBEO is more advantageous than Ultrasound in disrupting cell

membranes under current treatment conditions. Overall, NBEO_US may target the cell membrane and induce the loss of intracellular macromolecular contents by disrupting the membrane integrity, which results in cell death.

3.5 Morphological changes of *S. enterica*

The morphological changes in *S. enterica* cells were observed by TEM. For *S.* Newport ATCC 6962, the cells of the control group (Fig. 2A1) were smooth and full, with no obvious wrinkles, complete surface, and regular morphology, and the cells were in a typical short rod shape. In the NBEO treatment group (Fig. 2A3), the cells' surface had a small wrinkling, and the cells were able to retain their fundamental form of a short rod. When the cells were treated by the Ultrasound treatment alone (Fig. 2A2), it can be observed that the cells had obvious cavitation characteristics, and some cells were broken inward (Lin, Chen, et al., 2022). For NBEO_US treatment (Fig. 2A4), most of the cells were broken, there were traces of a large number of cell contents leaking and escaping around them, and the cell morphology and structure were severely damaged (Srivastava et al., 2021). Moreover, the TEM images of *S.* Enteritidis ATCC 13076 after treatments (Fig. 2B1-B4) showed that the cells could not maintain the basic standard rod shape after Ultrasound and NBEO treatments, the cell morphology was the most severely injured after the combined treatment of NBEO_US, which is similar to the TEM images of *S.* Typhimurium ATCC 14028 after treatments (Fig. 2C1-C4). In this study, TEM analysis visually demonstrated that cells experienced varying degrees of damage following different treatments. These observations clearly confirmed that the application of ultrasound and NBEO resulted in significant physical damage to *S. enterica* cells, primarily through disruption of the cell wall and disintegration of internal cellular structures, ultimately leading to the release of cellular contents and accelerating

345 *S. enterica* cells death and disintegration.

346

347 **3.6 Discriminative metabolites change and pathways perturbation upon various**
348 **treatments**

349 To achieve a comprehensive understanding of *Salmonella*'s response to different
350 treatments, pairwise comparisons of the acquired metabolic profiles of the Control,
351 Ultrasound, NBEO, and NBEO_US groups were performed using the OPLS-DA model.
352 In this model, R^2Y represents the quality factor, and Q^2 represents the predictor factor.
353 Both factors values of all comparison groups (Ultrasound *vs.* Control: $R^2Y = 0.980$, Q^2
354 $= 0.913$; NBEO *vs.* Control: $R^2Y = 0.849$, $Q^2 = 0.671$; NBEO_US *vs.* Control: $R^2Y =$
355 0.932, $Q^2 = 0.754$) exceeded 0.5, suggesting that our models exhibited exceptional
356 interpretability and reliability (Chen et al., 2020). In addition, the OPLS-DA score plot
357 for each pair clearly showed substantial disparities (Fig. S1), suggesting that the
358 antimicrobial effects of NBEO and Ultrasound treatment were prominent at the
359 metabolic level (Zhang et al., 2021). The variable importance for projection (VIP) is
360 often used to identify possible discriminative metabolites in OPLS-DA models (Li et
361 al., 2021). In our work, metabolites with a VIP value greater than 1 were considered the
362 most important variables in the analysis, shown in Fig. 3A1-C1. Based on these
363 screened metabolites, the pathway analysis was conducted to show how three
364 treatments affected the *S. enterica* strains based on the KEGG database (Fig. 3A2-C2).

365

366 Overall, 15, 31, and 33 pathways were predicted for *S. enterica* in the Ultrasound,
367 NBEO, and NBEO_US groups, respectively. Specifically, for *S. enterica* under
368 Ultrasound treatment, arginine, proline metabolism, and tyrosine metabolism were the
369 most influential pathways (Fig. 3A2). For *S. enterica* under the NBEO treatment,

aminoacyl-tRNA biosynthesis, propanoate metabolism, and amino acid metabolism (i.e., tyrosine metabolism and proline metabolism) were significantly altered (Fig. 3B2). Lastly, for *S. enterica* under the NBEO_US treatment, the alternative pathways showed significant similarities to the NBEO groups, including aminoacyl-tRNA biosynthesis, amino acid metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, and ABC transporters metabolism. These similarities were seen to varying degrees between these two groups. (Fig. 3C2). Overall, it was discovered that most pathways impacted by NEBO_US treatment belong to the amino acid biosynthesis-related processes and the sugar metabolism pathways.

A schematic model illustrating the antimicrobial mechanisms of the Ultrasound and NBEO was proposed based on the metabolic analysis results (Fig. 4). On the one hand, the antibacterial function of ultrasound is primarily attributed to the cavitation effect, which is induced by shock waves. This process can disrupt the structure of *Salmonella* by rupturing cavitation bubbles (Zupanc et al., 2019). Furthermore, the accumulation of free radicals produced by water hydrolysis during the implosion process can oxidize functional molecules containing reactive residues (Zhao et al., 2022). Unsurprisingly, when subjected to Ultrasound treatment, the breakdown of amino acids surpassed their synthesis. This may be related to the fact that amino acid anabolism is particularly susceptible to adverse stressors, such as mechanical force, heat, or drying (Lin et al., 2024). Moreover, among the three strains, the depletion of some essential amino acids is significantly substantial (Fig. S2), possibly due to the formation of Universal Stress Proteins (USP). These proteins can aid bacterial cells in overcoming external pressures and adapting to environmental conditions, including hypoxia and oxidative stress (Miliute et al., 2015).

On the other hand, the lipophilic properties of EO facilitate its diffusion and interaction with components within the cell (Kim et al., 2016). Increased membrane permeability may result in the efflux of cytoplasmic contents, such as K^+, which could subsequently impact ATP production (Giorgi et al., 2018). Consequently, this leads to a growth inhibition pattern and further disruption of intracellular metabolic flux (Mackenzie et al., 2020). Furthermore, significantly higher contents of lactate, allantoin, catechol, arginine, caprate, alanine, acetamide, isovalerate, guanidinoacetate, alloisoleucine, and anthranilate, etc., were recorded upon EO treatment. This could be due to the need to eliminate denatured and aggregated proteins, which inevitably occur after being treated with NBEO or NBEO_US, to maintain cellular homeostasis (Nikoo et al., 2016). Hence, unnecessary proteins are degraded by proteases into reusable amino acids (Ciechanover, 2005). Additionally, amino acids such as proline, acting as protective elements of the cell structure, are upregulated in their biosynthesis (Vettore et al., 2021).

3.7 Changes in the key enzyme of the EMP pathway

Analysis of the NMR results revealed that the NEBO_US treatment significantly affected pathways associated with glycolysis. Key enzymes in the EMP pathway were subsequently investigated. As shown in Fig. 5, the EMP pathway is a significant respiratory and metabolic pathway in most bacteria. HK, PFK, and PK are three important enzymes influencing the EMP pathway (He et al., 2021; Yang et al., 2019). Fig. 5 showed that the Control group's enzyme activities remained stable or increased, and all three enzyme activities showed significant reduction ($P < 0.05$) following different treatments with the highest pronounced reduction recognized in the combined treatment group. These results suggested that the inhibition ability of NBEO and

Ultrasound against *S. enterica* respiratory metabolism can be perceived by the change of these enzymes. For the HK enzyme, after 6 h, the Control group increased from 2.209 to 2.609 pmol/min/10^4 cells. At the same time, there was a significant decrease for all other treatments (Fig. 5A). In addition, PFK exhibited resistance to NBEO and NBEO_US, where the control treatment increased by 12.286 pmol/min/10^4 cells after 6 h of incubation. In contrast, the treatments with NBEO and NBEO_US decreased by 10.078 and 15.272 pmol/min/10^4 cells, respectively (Fig. 5B). At the same time, PK enzyme showed significant resistance to the treatment groups, after 6 h treated, the Control, Ultrasound, NBEO, NBEO_US groups reduced by 0.197, 0.906, 1.149, and 1.371 pmol/min/10^4 cells, respectively (Fig. 5C). In summary, there is a drastic decline in the trends of the three enzymes, HK, PFK, and PK, which consistent with those in Fig. 6 for *S.* Typhimurium and *S.* Newport. This suggests that by disrupting the EMP pathway, NBEO_US may effectively suppress *S. enterica*'s respiratory metabolism.

3.8 Color analysis and firmness measurement

Antibacterial compounds may cause dehydration, discoloration, softening, and other visual defects in products (Lucera et al., 2012). Therefore, their potential positive or negative impacts on the physical properties of the product should also be considered. The physical properties of pea sprouts before and after the treatments were determined and compared in Table 4. The results showed that in terms of color parameters, only L^* of the Ultrasound group is significantly different from other groups. However, the difference was a small margin, and the ΔE^* indicates no significant difference between groups regarding color, which is consistent with our previous research (Chen et al., 2022). In terms of firmness, after sanitizing treatments, the firmness of the sprouts increased. The NBEO and NBEO_US groups showed the highest firmness, followed

by the Ultrasound and Control groups, which might be because NBEO reduced the firmness decrease (Zambrano-Zaragoza et al., 2014). The overall color and firmness properties of pea sprouts were consistent with the expected results. The treated samples maintained characteristics close to the untreated ones, with the effects on total color difference and firmness of the pea sprouts remaining within acceptable limits.

4. Conclusion

The bactericidal effect and potential mechanism of Ultrasound, NBEO, and NBEO_US treatments on three *S. enterica* strains inoculated onto pea sprouts were manifested in this study. The results demonstrated that NBEO_US treatment significantly inhibited the growth of three *S. enterica* strains, compromised bacterial cell membrane integrity, induced the leakage of intracellular contents, and preserved the hardness of pea sprouts. Fluctuations in marker metabolites and pathway changes reveal that glycolysis and protein synthesis are affected in *S. enterica*. Furthermore, NBEO_US could also influence the EMP pathway of *S. enterica* by reducing the activity of three key enzymes (PFK, HK, and PK). Overall, this study offers a novel, environmentally friendly, and sustainable technique to prevent pathogens and provide great potential for application in the preservation of fruits and vegetables.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National University of Singapore, Faculty of Science,

Food Science and Technology Department (E-160-00-0007-02).

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Figure Legends

Fig. 1. Protein (A) and nucleic acid (B) leakage of *S. enterica* cells under different antibacterial treatments: Control (CK), Ultrasound (US), nano-emulsified basil essential oil (NBEO), Combine (NBEO_US). (Mean ± SD, n = 3)

Fig. 2. TEM images of *S. enterica* cells under different antibacterial treatments: Control (CK), Ultrasound (US), nano-emulsified basil essential oil (NBEO), Combine (NBEO_US). The TEM images of *S.* Newport ATCC 6962 after treatments with Control (A1); Ultrasound (A2); NBEO (A3); and NBEO_US (A4). The TEM images of *S.* Enteritidis ATCC 13076 after treatments with Control (B1); Ultrasound (B2); NBEO (B3); and NBEO_US (B4). The TEM images of *S.* Typhimurium ATCC 14028 after treatments with Control (C1); Ultrasound (C2); NBEO (C3); and NBEO_US (C4). The duration of action was 10 min for all treatment groups. Magnification = 10,000 ×, bar marker = 500 nm.

Fig. 3. Major metabolites and pathway analysis of *S. enterica* cells under different antibacterial treatments: Control (CK), Ultrasound (US), nano-emulsified basil essential oil (NBEO), Combine (NBEO_US). Screened metabolites with VIP > 1 of group Control and Ultrasound (A1), Control and NBEO (B1), Control and NBEO_US (C1); Overview of the pathway analysis of group Control and Ultrasound (A2), Control and NBEO (B2), Control and NBEO_US (C2).

Fig. 4. Proposed schematic of mechanisms of action about nano-emulsified basil essential oils and ultrasound on *Salmonella.*

Fig. 5. Landscape of Embden-Meyerhof-Parnas pathway (Referenced with Lin et al. (2018)). The activities of hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) of *S.* Enteritidis under different antibacterial treatments: Control (CK), Ultrasound (US), nano-emulsified basil essential oil (NBEO), Combine (NBEO_US). (A) HK in *S.* Enteritidis; (B) PFK in *S.* Enteritidis; (C) PK in *S.* Enteritidis. The data are represented in mean ± SD of three replicates.

Fig. 6. The activities of hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK) of *S.* Typhimurium and *S.* Newport under different sanitizing treatments: Control (CK), Ultrasound (US), nano-emulsified basil essential oil (NBEO), Combine (NBEO_US). (A1) HK in *S.* Typhimurium; (A2) HK in *S.* Newport; (B1) PFK in *S.* Typhimurium; (B2) PFK in *S.* Newport; (C1) PK in *S.* Typhimurium; (C2) PK in *S.* Newport. The data are represented in mean ± SD of three replicates. Mean values labeled with different lowercase letters are significantly different from the others in same time ($P < 0.05$).