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ORIGINAL ARTICLE

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The effect of emulsifier type and oil inclusion on stress-related gene expression of *Salmonella typhimurium* in oil-in-water emulsion

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Abstract

Salmonella has been associated with numerous outbreaks from contaminated food products, including emulsions. Emulsions are influenced by emulsifier type and oil presence, which can have varying degrees of stress or protection on bacteria. Although our previous research has shown that emulsifier solutions, rather than emulsions, provide a protective effect on *Salmonella typhimurium* after thermal treatment, the underlying mechanism remains unclear. This study selected *S. typhimurium* as the model microorganism and utilized the same emulsifiers (Tween 20, Tween 80, Triton X-100) to create emulsifier solutions and emulsions with the same oil fraction (60% (v/v)) to examine their effect on the expression of nine selected genes (rpoE, rpoH, otsB, proV, fadA, fabA, dnaK, ibpA, ompC) associated with stress response. Specifically, the study observed variations in gene expression under normal and thermal stress at 55°C. After 20-h incubation, Triton X-100 emulsion caused an upregulation of stress-related genes, rpoE, otsB, and fabA, suggesting stressful environment. After thermal treatment, *S. typhimurium* in Triton X-100 solution showed a longer 5-log reduction time with increased proV and decreased fabA and ompC expression, suggesting enhanced thermal protection compared to its emulsion. Conversely, Tween 80 solution increased fabA and ompC expression, indicating greater membrane fluidity and passive diffusion, potentially reducing thermal resistance. However, according to the upregulation of ibpA, this effect was likely mitigated by the overproduction of heat shock proteins. Notably, Triton X-100 environments exhibited the most significant gene expression changes after heat treatment, whereas Tween 80 without oil was the most inhospitable for bacterial survival. These findings inform bacterial responses under various conditions, aiding food safety strategies.

KEYWORDS
emulsifier type, emulsion, gene expression, oil, thermal treatment

1 | INTRODUCTION

Salmonella is a Gram-negative, rod-shaped bacterium that can cause a range of illnesses in humans, including diarrhea, fever, and abdominal cramps (Coburn et al., 2007). It is commonly transmitted through the consumption of contaminated food products, including meat, poultry, eggs, and dairy products (O'Bryan et al., 2022; Sher et al., 2021). One transmission way of *Salmonella* is through the consumption of emulsions, which are mixtures of two immiscible liquids that are stabilized by the addition of emulsifiers (Akbari & Nour, 2018; Prachaiyo & McLandsborough, 2003; Warren, 1998). Additionally, emulsifiers are commonly used in food processing to improve the texture, stability, and sensory qualities of products (Marhamati et al., 2021). By reducing the surface tension between the two liquids, emulsifiers are able to mix and form a stable emulsion (Cambiella et al., 2006). However, emulsifiers may also affect the survival and virulence of pathogens like *Salmonella* (Zhang et al., 2015). For example, some emulsifiers can disrupt the cell membranes of bacteria, making them more susceptible to environmental stresses and less able to cause infection (Nielsen et al., 2016). The impact of oil on bacteria has been documented in previous research (Senhaji & Loncin, 1977). Studies indicate that oil may offer protective benefits for certain types of bacteria, shielding them from potential harm caused by environmental stressors like high temperatures (Ren, 2021; Yang, 2020). However, the degree of protection provided by oil can vary based on the specific type of bacteria and environmental factors present. It is worth noting that although oil may provide a level of protection, it does not confer complete immunity from harmful agents or environmental conditions. On the other hand, the stress-related RNA expression of *Salmonella* is an important indicator of the pathogen's ability to survive and thrive in different environments (Roncarati & Scarlato, 2017). RNA expression refers to the process by which genes are transcribed into RNA molecules, which then serve as templates for the synthesis of proteins (Cooper et al., 2009). Specifically, the expression of certain genes, such as *rpoE* and *ompC*, can be used to monitor the survival and virulence of *Salmonella* in different environments (Du et al., 2011; Li et al., 2021).

The type of emulsifier and the presence of oil in emulsions are important factors that can significantly influence the stress-related RNA expression of *Salmonella*. Considering the need to control the proliferation of this pathogen in food, particularly in oil-in-water emulsion products like tahini with 59% oil content, it is vital to study how different emulsifiers and the presence of oil influence the stress-induced RNA expression of *Salmonella* in such emulsions (Szpinak et al., 2022). Our previous research has shown that a high oil fraction in emulsion system can extend the lag phase of *Salmonella typhimurium* growth; however, emulsifier solutions alone (Tween 80 and Triton X-100), rather than emulsions, have been found to be protective against thermal inactivation (Tsai & Tikekar, 2023). To build on this knowledge, this study aims to investigate the effects of different emulsion conditions on stress-related gene expression in *Salmonella*. Specifically, we will analyze stress-related gene expression (*rpoE, rpoH, otsB, proV, fadA, fabA, dnaK, ibpA, ompC*) after 20 h of incubation at 37°C and after thermal treatment at 55°C. By characterizing how *Salmonella* responds to various environmental stresses Tryptic soy broth (TSB, Tween 20 solution and its emulsion, Tween 80 solution and its emulsion, Triton X-100 solution and its emulsion), effective strategies to mitigate its growth and spread in food products can be developed.

2 | MATERIALS AND METHODS

2.1 | Materials

Salmonella enterica serotype Typhimurium (CVM98) was obtained from the stock collection of the Department of Nutrition and Food Science at the University of Maryland, College Park. *S. enterica* serotype Typhimurium with green fluorescent protein gene (GFP) labeled was purchased from ATCC (14028GFP). Tryptic soy agar (TSA) and TSB were purchased from BD Biosciences (236920, 211825). Phosphate buffer saline tablets were obtained from Fisher Scientific (BP2944-100), whereas buffered peptone water was sourced from Thermo Scientific Remel Agar (R452672). Emulsifiers Tween 20, Tween 80, and Triton X-100 were procured from Fisher Scientific (BP337-500, BP338-500), and Acros Organics (21568-2500), respectively. Vegetable oil (Crisco) was purchased from a local grocery store and stored in the dark at room temperature. For RNA extraction, RNAprotect Bacteria Reagent was obtained from Qiagen (76506), whereas RNA isolation and purification were performed using the RNeasy Mini Kit (74104; Qiagen). The concentration of extracted RNA was determined using the Qubit RNA BR Assay Kit (Q10210; Molecular Probes, Invitrogen), and RNase-free water from Fisher Scientific (7732185) was used to prevent potential RNase contamination. For the real-time quantitative PCR (qPCR) experiment, primers (standard desalted) were purchased from IDTDNA (Integrated DNA Technologies) (Table 1). PowerTrack SYBR Green Master Mix was procured from Invitrogen (A46109).

TABLE 1 Selected genes and primers used in this study.

Gene	Direction	Primer sequence (5′ → 3′)	References
16s rRNA	F	CGATCCCTAGCTGGTCTGAG	Castelijn et al. (2012)
	R	GTGCAATATTCCCCACTGCT	
rpoE	F	GTCTACAACATGACAAACAAAAACAAATGC	Humphreys et al. (1999)
	R	CCTTTTCCACTATCCCGCTATCGTCAACGC	
rpoH	F	GTTTCCTTCGCCGTACACTG	Kang et al. (2018)
	R	CCACCATTTCAACCTCATCC	
otsB	F	ACCTTGATGGCACATTGGCAGA	Li et al. (2012)
	R	ACGCCCTGAAATCAATGCCA	
proV	F	CCACAATGGTACGCCTTCTCA	Chen and Jiang (2017)
	R	GCATGAGCGCAAATGACTGGA	
fadA	F	ATCTCTCCGCCCACTTAATGCGTA	Fong and Wang (2016)
	R	AGCCTTGCTCCAGCGTTTGTTGTA	
fabA	F	ACTCCCTGCGCCGAACATGC	Chen et al. (2014)
	R	CACTTCGCCCACGCCCAGAG	
dnaK	F	CGATTATGGATGGAACGCAGG	Fong and Wang (2016)
	R	GGCTGACCAACCAGAGTT	
ibpA	F	GCCGCCAACCGTTTCA	Carroll et al. (2016)
	R	TGCTTACCGTGAGCGTTCCT	
ompC	F	ACGCTGCTGCATAAAGTTGTCA	Kollanoor Johny et al. (2017)
	R	CCGATGTTCTGCCGGAGTT	

Abbreviations: F, forward; R, reverse.

2.2 | Methods

2.2.1 | Emulsion preparation

Different volumes of TSB (10 and 4 mL) were thoroughly mixed with 2% (v/v) of Tween 20, Tween 80, or Triton X-100. Specific amounts of vegetable oil (0 and 6 mL, respectively) were added to each emulsifier-containing TSB solution to obtain a total volume of 10 mL. This resulted in 0% and 60% (v/v) oil-in-water emulsions. The solutions were then homogenized using an ultrasonic processor (FB505; Fisher Scientific, 200 W, 3 min) to create stable emulsions.

2.2.2 | Bacteria growth

S. typhimurium was retrieved from a frozen culture and streaked onto TSA plates, which were incubated overnight (~20 h) at 37°C and could be stored at 4°C for up to a month. Prior to conducting experiments, a single colony of *S. typhimurium* was inoculated into a 15 mL sterile tube filled with TSB and was incubated overnight (~20 h) at 37°C until the early stationary phase was reached, yielding approximately 9 log CFU/mL. With appropriate dilution to achieve ~5 log CFU/mL, bacteria were inoculated into TSB (as a control), emulsifier solutions, and emulsions. These inoculated solutions were incubated at 37°C for 20 h. Subsequently, pellets were collected by centrifugation at 5000 *g* for 10 min.

2.2.3 | Thermal treatment

S. typhimurium was cultured in TSB, emulsifier solutions, and emulsions overnight (~20 h) at 37°C. Following this, 200 μL of each sample was subjected to 45 min of heat treatment in a 55°C water bath (Model: 28L-M; Fisher Scientific). After thermal treatment, bacterial pellets were collected by centrifugation at 5000 *g* for 10 min.

2.2.4 | Confocal imaging

The GFP-labeled *S. typhimurium* was cultured in 60% emulsions overnight (~20 h) at 37°C, after which it was collected for confocal imaging. Before imaging, the bacterial samples were diluted at least 10,000. The imaging was carried out using an SP5X Laser Scanning Confocal with an inverted microscope (Leica Microsystems CMS GmbH). A 63× oil immersion objective was utilized to enhance the magnification of the images.

2.2.5 | RNA expression

RNA was extracted from bacterial samples that have been grown for 20 h and subjected to 45 min of thermal treatment at 55°C. A control group, consisting of stationary phase *S. typhimurium* cells harvested directly from TSB, was also included. To stabilize gene expression levels and minimize RNA synthesis and degradation during extraction and subsequent processing, RNAprotect Bacteria Reagent was used (Rocha et al., 2020). Specifically, the samples were combined with RNAprotect Bacteria Reagent in a 1:2 ratio (samples to reagent), vortexed, and incubated at room temperature for 5 min. The mixture was centrifuged at 5000 *g* for 10 min, and the supernatant was discarded. The resulting cell pellets were stored at −80°C for further processing for up to 1 week.

RNA isolation and purification from the stabilized samples were conducted using the RNeasy Mini Kit with an automated QIAcube instrument (Qiagen) following the manufacturer's protocol. The concentration of the extracted RNA was determined using the Qubit RNA BR Assay Kit on a Qubit 2.0 Fluorometer (Invitrogen), as per the manufacturer's instruction. The QuantiTect Reverse Transcription Kit was used to perform reverse transcription to complementary DNA (cDNA), which includes a genomic DNA (gDNA) wipeout buffer to eliminate any contaminating gDNA present in the RNA extract. The resulting cDNA was stored at −20°C for up to 1 month for future processing.

2.2.6 | Real-time quantitative PCR (qPCR)

Preparation for qPCR involved primer preparation and RNA quality control. The designed primers were dissolved in RNase-free water to create individual working stock solutions at a concentration of 8 μM. RNA quality was assessed by measuring the $OD_{260/280}$ ratio, as impurities in the RNA sample can lead to inaccurate quantification (Fleige & Pfaffl, 2006). An $OD_{260/280}$ value between 1.8 and 2.0 indicated high-quality RNA that was free from protein and phenol contamination.

The qPCR experiment was conducted using a C1000 Touch Thermal Cycler coupled with a CFX96 Optical Reaction Module (Bio-Rad), with modifications to the manufacturer's recommended protocol based on previous research (Nolan et al., 2006). In brief, each 10 μL reaction mixture contained 5 μL of Master Mix, 0.5 μL of cDNA samples (~150 ng), 7.15 μL of both forward and reverse primers (400 nM), 0.25 μL of yellow sample buffer, and 3.75 μL of molecular-grade water. The PCR reaction consisted of 40 cycles following an initial enzyme activation step (95°C for 2 min), with 5 s of denaturation (95°C) and 30 s of annealing (60°C). A subsequent dissociation step was performed by increasing the incubation temperature from 65 to 90°C with a 0.1°C/s ramp. The resulting melt curve was analyzed to detect potential contamination or primer binding. The relative expression of each target gene in the samples was calculated using the $2^{-\Delta\Delta CT}$ method, as described by Livak and Schmittgen (2001) in the following equation:

$$\text{Fold change} = 2^{-\Delta\Delta C_T} \ldots\ldots \quad (1)$$

The purpose of Equation (1) is to compare the gene expression levels of the target gene with those of a reference gene. This equation can be expanded as shown in the following equation (Schmittgen & Livak, 2008):

$$\Delta\Delta C_T = [(C_T \text{ of target gene} - C_T \text{ of referencegene})_{\text{sample}} - (C_T \text{ of target gene} - C_T \, of \, \text{referencegene})_{\text{control}}] \ldots\ldots \quad (2)$$

In this case, 16S ribosomal RNA (16s rRNA) was used as the reference gene and stationary phase *S. typhimurium* cultured in TSB as untreated control sample.

2.2.7 | Statistical analysis

cDNA for qPCR analysis was generated from three independently treated samples, each with two on-plate replicates. Statistical significance was determined using two-way analysis of variance analysis in GraphPad Prism 6 software, applying Tukey's honestly significant difference post hoc test at a significance level of $\alpha = 0.05$. In addition, multidimensional scaling (MDS) analysis and hierarchical cluster analysis (HCA) were conducted in JMP Pro 15.2.0. It is worth noting that the threshold for a significant difference in this study was set at one $\log_2$ fold change in gene expression.

3 | RESULTS AND DISCUSSION

3.1 | Confocal imaging

To determine whether bacteria had entered oil droplets, confocal microscopy images were obtained after all samples were cultured at 37°C for 20 h. Fluorescence-labeled *S. typhimurium* was used to visualize the bacteria, whereas small pores in the image were indicative of oil droplets. As shown in Figure 1, the length of the labeled bacteria was approximately 3 μm, whereas the diameters of the small pores were less than 1 μm. Therefore, it is unlikely that the observed protective effect on bacteria in emulsion samples after thermal treatment resulted from bacterial movement to oil droplets due to the larger size of the bacteria. Other mechanisms, such as emulsifier stress, oil stress, and high temperature, may have contributed to the observed protective effect following thermal treatment, as well as the extended lag phase observed when *S. typhimurium* was inoculated in emulsions with a high oil fraction (Tsai & Tikekar, 2023).

The potential mechanism of the protective effect of emulsifiers after thermal treatment observed in a previous study remains to be fully elucidated (Tsai & Tikekar, 2023). Although bacterial movement to oil droplets is unlikely to be the cause of the effect, other mechanisms may be at play. One possible explanation is the emulsifier stress induced by thermal treatment, which can cause changes in the surface properties of the emulsion droplets, such as surface charge, hydrophobicity, or released compounds, and lead to the formation of a protective barrier around bacterial cells (Ellouze et al., 2021; Mohammad & Taha Daod, 2021). For instance, our previous study found that emulsifiers with positive charge, such as whey protein isolate with positive charge, had a higher 5D value compared to negatively charged whey protein isolate. This result implies that the interaction between the positively charged emulsifier and cell membrane may be stronger, potentially providing greater protection against thermal treatment (Tsai & Tikekar, 2023). Another possible explanation is the stress caused by the high temperature itself, which can cause changes in bacterial membrane permeability and fluidity, and disrupt cellular functions, ultimately leading to the effect on bacterial inactivation (Cebrián et al., 2019; Guillén et al., 2021). Thus, the protective effect in this study could be attributed to various stress response mechanisms, including emulsifier stress and high temperature stress.

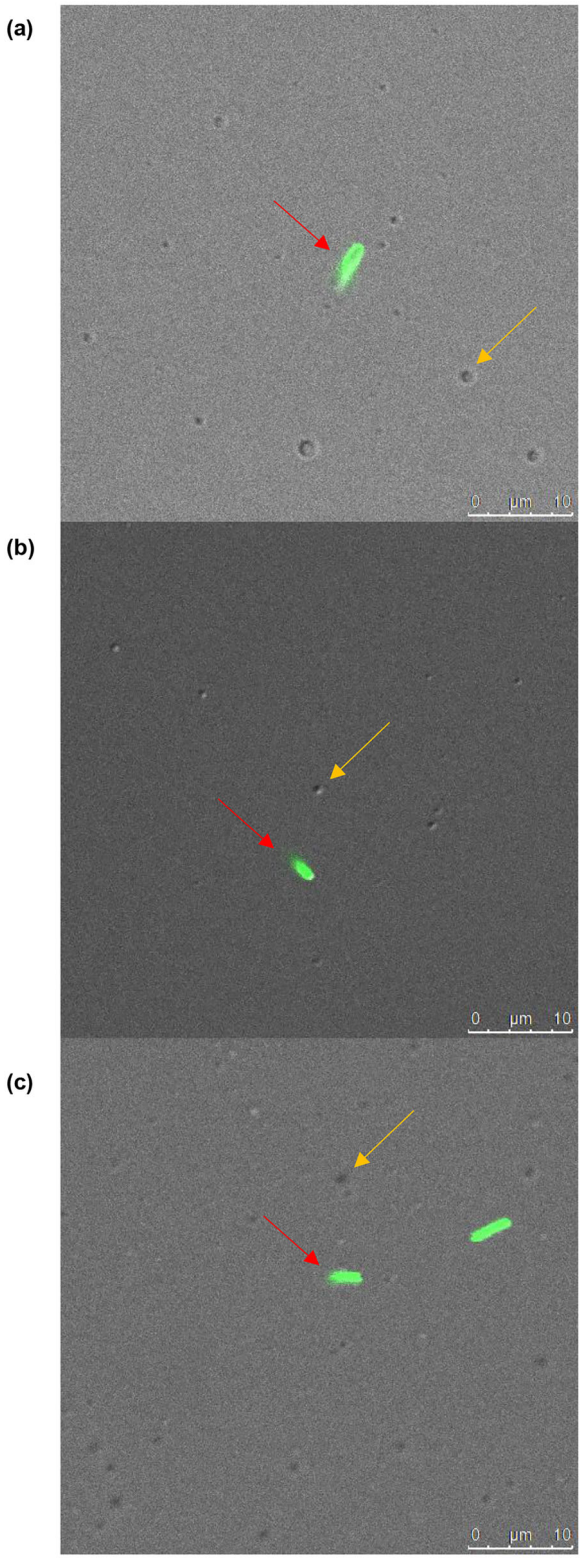

FIGURE 1 Fluorescent images of green fluorescent protein (GFP)-labeled *Salmonella typhimurium* inoculated in three different emulsions: (a) Tween 20 stabilized 60% emulsion, (b) Tween 80 stabilized 60% emulsion, and (c) Triton X-100 stabilized 60% emulsion, taken in the merge of bright field and GFP modes. In the images, oil droplets are indicated by yellow arrows, whereas GFP-labeled *S. typhimurium* are indicated by red arrows.

TABLE 2 Function of target genes in this study.

Genes	Function	Reference
16s rRNA	Reference gene	Castelijn et al. (2012)
rpoE	Regulation of stress response, including heat stress	Humphreys et al. (1999)
rpoH	Regulation of heat shock response	Kang et al. (2018)
otsB	Synthesis of sugar trehalose, which served as a protectant against a variety of stress	Li et al. (2012)
proV	Help bacteria survive from osmotic stress	Chen and Jiang (2017)
fadA	Fatty acid metabolism, specifically in beta-oxidation pathway that breaks down fatty acids for energy production	Fong and Wang (2016)
fabA	Fatty acid biosynthesis that catalyzes the formation of unsaturated fatty acids	Chen et al. (2014)
dnaK	Fold and stabilize other proteins and protect them from stress-induced denaturation	Fong and Wang (2016)
ibpA	Small heat shock protein to protect proteins from aggregation and denaturation during heat stress	Carroll et al. (2016)
ompC	Channel formation on the outer membrane of Gram-negative bacteria, allowing the diffusion of small molecules and wastes	Kollanoor Johny et al. (2017)

3.2 | RNA expression

3.2.1 | Function of selected stress-related genes

Table 2 summarizes the target genes and their functions. The expression levels of *rpoE*, *rpoH*, and *ibpA* are involved in the activation of specific heat shock proteins after thermal treatment. In various stress environments, including heat, oxidation, UV-light, and desiccation, the alternative σ factor, σE, is regulated by the transcriptional regulator *rpoE*, which controls its expression levels (Amar et al., 2018; Guillén et al., 2021). Activated *rpoE* under stress conditions leads to the upregulation of outer membrane proteins and the β-barrel assembly machinery complex, which works to alleviate stress on the outer membrane (Palmer & Slauch, 2020). Studies have found that the *rpoE* mutant *Salmonella* exhibited reduced sensitivity to heat shock temperature, deficiencies in intracellular survivability, and non-virulence in mouse infection models (Dawoud et al., 2017). The *rpoH* gene plays a critical role in the induction of heat shock proteins through σ factor 32 (Ban et al., 2015; Roncarati & Scarlato, 2017). Exposure to thermal treatment has been observed to result in an increase in *rpoH* expression (Dawoud et al., 2017; Yura & Nakahigashi, 1999). Moreover, *rpoH* has been reported to act as an RNA thermometer at high temperatures (Narberhaus et al., 2006). However, in a subset of Gram-negative bacteria, such as *Pseudomonas*, the *dnaK* chaperone system has been reported to negatively regulate *rpoH* expression (Kobayashi et al., 2011). *IbpA* is a crucial member of the σ factor E and H regulon that plays a vital role in coping with thermal treatment (Roncarati & Scarlato, 2017). This gene encodes the small heat shock protein, IbpA, which has been shown to possess RNA thermometer properties as well and exhibits higher expression levels under stress (Canals et al., 2019; Waldminghaus et al., 2009). The significance of *ibpA* in response to thermal stress has been demonstrated by the growth defect observed in *ibpA* mutants at 40°C (Krajewski et al., 2013). The upregulation of *ibpA* also confers the benefit of preventing endogenous protein aggregation induced by heat shock, thereby enhancing survivability under thermal treatment (Kuczynska-Wisnik et al., 2002).

The *dnaK* gene encodes for the chaperone protein Hsp70, which plays a crucial role in protecting other proteins from heat stress (Fong & Wang, 2016; Gruzdev et al., 2012). Mild thermal treatment has been shown to upregulate the expression of *dnaK* in *S. typhimurium* (Berk et al., 2005; Gruzdev et al., 2012), leading to increased production of Hsp70 and enhanced heat resistance in bacteria (Fong & Wang, 2016). The DnaK protein, which is also controlled by the *dnaK* gene, is involved in assisting in the folding of newly synthesized proteins, preventing the aggregation of misfolded or denatured proteins, and facilitating the disaggregation of protein aggregates (Agashe et al., 2004). It also plays a crucial role in protecting cells from thermal and oxidative stress, by stabilizing proteins and preventing their denaturation or aggregation under stress conditions (Katikaridis et al., 2021). Moreover, the DnaK/DnaJ chaperone system has been identified as a regulator of *rpoH* expression. It has been reported that the upregulation of σ factor 32, induced by *rpoH* and other heat shock protein-related genes, can result in the downregulation of *dnaK* expression (Da Silva et al., 2003).

OtsB and *proV* are related to stress detection through the synthesis of trehalose and the alteration of osmotic

pressure. The present study examined the expression of *otsB* as an indicator of metabolic response. OtsB is an essential enzyme in the trehalose biosynthesis pathway, and it encodes for trehalose-6-phosphate (T6P), a metabolite derived from glucose (Balaji et al., 2005; Fong & Wang, 2016; Li et al., 2012). Trehalose plays a vital role in maintaining the appropriate osmotic gradient potential in cells (Finn et al., 2013). Previous studies have shown that the upregulation of *otsB* occurs following thermal treatment (Abdelhamid & Yousef, 2020). Specifically, the upregulation of *otsB* implied an increased demand for metabolites to be transferred to trehalose-6-phosphate (T6PP) to maintain osmotic potential. Additionally, the expression of *proV* is a reliable indicator of proline accumulation and changes in osmotic pressure within the cell (Abdelhamid & Yousef, 2022; Gruzdev et al., 2012). Proline is one of the most important solutes that helps bacteria maintain normal osmotic pressure (Takasu & Nagata, 2015). Thus, upregulation of *proV* indicates increased adaptation and survival of bacteria in stressful environments (Chen & Jiang, 2017). In contrast, previous research has shown that reduced expression of *proV* results in lower importation of osmoprotectant compounds such as proline, leading to increased susceptibility to stressful environment in *Salmonella* (Abdelhamid & Yousef, 2022).

Fatty acid metabolism genes, *fadA* and *fabA*, provide evidence for membrane alternation. *FadA* plays a crucial role in the tricarboxylic acid cycle by breaking down long-chain fatty acids into acetyl-CoA to generate ATP (Li et al., 2012). Previous studies have suggested the expression of *fadA* is downregulated in *Salmonella* under thermal treatment due to the degradation of fatty acids on the cell membrane (Fong & Wang, 2016). However, in some cases of desiccation and starvation, *fadA* has been observed to be upregulated (Deng et al., 2012). This upregulation may be attributed to the increased demand for ATP to synthesize stress response proteins (Seeras, 2017). In addition, *fabA* gene encodes for 3-hydroxydecanoyl acyl carrier protein dehydratase, which is responsible for the biosynthesis of unsaturated fatty acids (Magnuson et al., 1993). Upregulation of *fabA* was observed under stressful environment, indicating increased membrane fluidity and lower phospholipid phase transition temperature of *S. typhimurium* (Wang et al., 2015; Yun et al., 2016). In contrast, the downregulation of *fabA* was detected after pulsed electric field treatment, which suggests a decrease in the biosynthesis of unsaturated fatty acids and an increase in heat resistance (Yun et al., 2016). The heat sensitivity of bacteria under thermal treatment can be determined by their membrane fluidity, where a more rigid membrane displays greater heat resistance (Álvarez-Ordóñez et al., 2008). Therefore, the regulation of *fabA* expression can impact the membrane fluidity of *S. typhimurium*, which in turn affects the heat sensitivity of the bacteria.

Lastly, *ompC* is another gene involved in detecting stress and the pathogenicity of *Salmonella* through the alteration of waste passive diffusion from channels on the membrane. *OmpC* encodes the outer membrane porin, OmpC, as reported by Arockiasamy et al. (2004). During *S. typhimurium* infection, OmpC is the primary antigen detected by human antibodies, making the expression of *ompC* an indicator of the bacteria's pathogenicity throughout the infection period (Muthukkaruppan et al., 1992). In response to severe environmental stress, bacteria may increase their pathogenicity for survival (Flint et al., 2016). On the other hand, higher *ompC* expression is also associated with increased passive diffusion of small molecules and wastes through the channel, which can be influenced by the presence of stress in the environment (Sugawara & Nikaido, 1994). As a result, measuring the expression of *ompC* may serve as a useful marker for assessing the level of stress experienced by the bacteria.

3.2.2 | Expression level of stress-related genes in *S. typhimurium* after 20-h incubation in TSB, emulsifier solutions, and emulsions

The control group for this study consisted of *S. typhimurium* cultured in TSB at 37°C for 20 h. When *S. typhimurium* was introduced into a solution containing Tween 20, a significant downregulation of *proV* gene was observed ($p < 0.05$). Conversely, when the bacteria were exposed to a 60% emulsion stabilized by Tween 20, there was a downregulation of *rpoE*, *rpoH*, *ibpA*, and *proV* genes compared to the control group cultured in TSB ($p < 0.05$). When the bacteria were subjected to Tween 80 solution, there was a downregulation of *proV* and *fadA* genes ($p < 0.05$). Yet, when exposed to 60% emulsion stabilized by Tween 80, only *proV* gene was downregulated compared to the control group ($p < 0.05$). Interestingly, when Triton X-100 was used as the culturing medium, there was no difference observed between the control and treatment groups ($p > 0.05$). However, when the bacteria were inoculated into Triton X-100 stabilized 60% emulsion, there was an upregulation of *rpoE*, *otsB*, and *fabA* genes compared to the control group (TSB; $p < 0.05$) (Table 3).

Based on the aforementioned observations, it is reasonable to hypothesize that the incorporation of emulsifiers in the tested environments may have led to a reduction in the importation of osmoprotectant compounds, such as proline. This hypothesis is supported by the downregulation of *proV*, a gene involved in proline transport (Chen & Jiang, 2017), across all tested environments except for

TABLE 3 Log_2 fold change in the relative expression of genes in *Salmonella typhimurium* in Tryptic soy broth (TSB), Tween 20 solution (T20), Tween 80 solution (T80), Triton X-100 solution (X100), Tween 20 stabilized 60% emulsion (T20E), Tween 80 stabilized 60% emulsion (T80E), and Triton X-100 stabilized 60% emulsion (X100E) after 20 h incubation at 37°C.

	rpoE	*rpoH*	*ibpA*	*dnaK*	*otsB*	*proV*	*fadA*	*fabA*	*ompC*
TSB	0.00 ± 0.34^{b}	0.00 ± 0.20^{a}	0.00 ± 0.27^{ab}	0.00 ± 0.39^{ab}	0.00 ± 0.44^{b}	0.00 ± 0.29^{a}	0.00 ± 0.34^{abc}	0.00 ± 0.88^{b}	0.00 ± 0.23^{ab}
T20	0.51 ± 0.57^{ab}	−0.19 ± 0.61^{a}	0.16 ± 0.09^{a}	−0.10 ± 0.54^{ab}	0.49 ± 0.54^{ab}	−1.95 ± 0.25^{b}	−1.09 ± 0.39^{c}	0.51 ± 0.66^{ab}	0.03 ± 0.27^{ab}
T80	−0.25 ± 0.14^{b}	−0.09 ± 0.15^{a}	−0.65 ± 0.67^{abc}	−0.62 ± 0.40^{b}	0.58 ± 0.31^{ab}	−2.06 ± 1.09^{b}	−2.92 ± 0.39^{d}	−0.38 ± 0.47^{b}	−1.29 ± 0.07^{b}
X100	0.29 ± 0.97^{ab}	−0.11 ± 0.36^{a}	0.17 ± 0.23^{a}	0.66 ± 0.44^{ab}	−0.01 ± 0.24^{b}	−1.56 ± 0.62^{ab}	0.60 ± 0.49^{ab}	0.96 ± 0.66^{ab}	0.57 ± 0.01^{a}
T20E	−2.10 ± 0.53^{c}	−2.01 ± 0.18^{b}	−2.06 ± 0.78^{c}	0.08 ± 0.41^{ab}	0.03 ± 0.53^{b}	−2.96 ± 0.25^{b}	−0.47 ± 0.17^{abc}	−0.04 ± 0.57^{b}	−0.25 ± 0.21^{ab}
T80E	−0.67 ± 0.42^{bc}	−0.78 ± 0.54^{ab}	−1.52 ± 0.20^{bc}	1.12 ± 0.30^{a}	0.50 ± 0.26^{ab}	−2.52 ± 0.05^{b}	−0.94 ± 0.20^{bc}	0.52 ± 0.56^{ab}	−0.13 ± 0.63^{ab}
X100E	1.80 ± 0.47^{a}	−0.03 ± 0.09^{a}	0.71 ± 1.24^{a}	1.10 ± 0.19^{a}	1.74 ± 0.33^{a}	−1.42 ± 0.82^{ab}	0.98 ± 0.25^{a}	1.78 ± 0.09^{a}	0.82 ± 0.30^{a}

Note: Lowercase letter labels (a, b, c, etc.) were used to indicate significant differences among groups. The determination of significant difference was based on a two-way analysis of variance (ANOVA). Groups with the same letter were not significantly different ($p > 0.05$), whereas those with different letters were significantly different ($p > 0.05$).

Triton X-100 included environment. The extent of decrease in proline transportation, however, may have varied among different environments, as suggested by differences in the fold change of *proV* expression levels. Furthermore, the downregulation of *fadA*, which is associated with the degradation of fatty acids in cell membrane (Fong & Wang, 2016), was observed specifically in Tween 80 solutions. This effect may be attributed to the structural characteristics of Tween 80, including higher molecular weight and longer fatty acid chains (Bąk & Podgórska, 2016). In contrast, Triton X-100 stabilized 60% emulsion showed upregulation of *otsB* and *fabA* genes, indicating an increase in membrane fluidity and a higher rate of metabolite transportation across the membrane (Balaji et al., 2005). Consequently, Triton X-100 emulsion can be considered a stressful condition for bacterial survival.

3.2.3 | Expression level of stress-related genes in *S. typhimurium* after 45-min thermal treatment in TSB, emulsifier solutions, and emulsions

Comparing the gene expression levels before and after subjecting samples to a 45-min thermal treatment at 55°C, significant downregulation was observed in TSB for *dnaK*, *otsB*, *proV*, *fadA*, *fabA*, and *ompC* genes ($p < 0.05$). In the case of Tween 20 solution, an upregulation of *rpoH*, *ibpA*, and *proV* was noted ($p < 0.05$). Similarly, in the presence of 60% emulsion stabilized by Tween 20, an upregulation of *rpoE*, *rpoH*, and *ibpA*, coupled with a downregulation of *otsB*, *fadA*, and *ompC* were observed ($p < 0.05$). Regarding the Tween 80 environments, an upregulation of *ibpA* was observed in the solution ($p < 0.05$). In the emulsion, there was also an upregulation of *ibpA*, whereas a downregulation of *otsB*, *fadA*, *fabA*, and *ompC* was detected ($p < 0.05$). In the case of Triton X-100 samples, upregulation was observed in both solution and emulsion for *rpoE*, *rpoH*, *ibpA*, *dnaK*, *otsB*, and *proV* genes ($p < 0.05$). Additionally, *fabA* was specifically upregulated in emulsion ($p < 0.05$) (Table 4).Based on the gene expression data following thermal treatment, it can be inferred that in TSB, the induction of heat shock proteins may not have occurred, as evidenced by the expression levels of corresponding genes, such as *rpoE*, *rpoH*, and *ibpA*. The downregulation of *otsB* and *proV* genes, which are responsible for metabolite transportation and proline uptake, respectively, indicates an increased susceptibility to heat treatment. Moreover, the degradation of fatty acids and impaired biosynthesis of unsaturated fatty acids likely resulted in membrane damage. These findings provide insights into the behavior of *S. typhimurium* under typical condition.

TABLE 4 Comparative statistical analysis of pre- and post-thermal treatment effects on *Salmonella typhimurium* populations in various solutions and emulsions.

	rpoE	*rpoH*	*ibpA*	*dnaK*	*otsB*	*proV*	*fadA*	*fabA*	*ompC*
TSB	-	-	-	* (D)	** (D)	* (D)	**** (D)	* (D)	* (D)
T20	-	* (U)	**** (U)	-	-	**** (U)	-	-	-
T80	-	-	**** (U)	-	-	-	-	-	-
X100	**** (U)	**** (U)	**** (U)	**** (U)	**** (U)	**** (U)	-	-	-
T20E	-	**** (U)	**** (U)	-	* (D)	-	**** (D)	-	**** (D)
T80E	-	-	**** (U)	-	**** (D)	-	**** (D)	**** (D)	**** (D)
X100E	*** (U)	**** (U)	**** (U)	**** (U)	** (U)	**** (U)	-	** (U)	-

Note: The table details the changes in *S.* typhimurium populations when inoculated in Tryptic soy broth (TSB), Tween 20 (T20), Tween 80 (T80), and Triton X-100 (X100) solutions, as well as in 60% emulsions of Tween 20 (T20E), Tween 80 (T80E), and Triton X-100 (X100E). The populations were assessed before and after a 45-min thermal treatment at 55°C in a water bath. Significant differences in bacterial populations post-thermal treatment compared to pre-treatment levels are indicated as follows: '*' denotes $p < 0.05$, '**' denotes $p < 0.01$, and '***' denotes $p < 0.001$, with "U" indicating an increase and "D" representing a decrease, relative to the pre-thermal treatment populations.

In contrast, in emulsifier-containing environments, the upregulation of *rpoH* and *ibpA* genes suggests the induction of σ factor 32 and the synthesis of related heat shock proteins, including the small heat shock protein IbpA (Waldminghaus et al., 2009). Notably, the downregulation of *otsB* and *fadA* genes was observed in samples incubated in the emulsion stabilized with Tween 20 and Tween 80, but no similar reduction was observed in Triton X-100 environments. This result implies the degradation of fatty acids in the cell membrane and mild membrane disruption, which consequently led to the reduced expression of *otsB* and increased osmotic stress as well.

After administering a thermal treatment for 45 min at 55°C, we evaluated the gene expression levels of each sample against those of the TSB (Table 5). We noted an upregulation of *rpoE*, *ibpA*, *dnaK*, *proV*, and *fadA* genes in the Tween 20 solution ($p < 0.05$). Conversely, in the Tween 20 emulsion, only *dnaK* gene exhibited upregulation after thermal treatment compared to TSB ($p < 0.05$). In the case of Tween 80 solution, solely the *ibpA* gene was found to be upregulated ($p < 0.05$), whereas in Tween 80 emulsion, *ompC* gene showed a significant downregulation ($p < 0.05$). Across the Triton X-100 samples, all genes recorded an upregulation relative to the TSB following the thermal treatment ($p < 0.05$).

Incorporating 2% (v/v) Triton X-100 as an emulsifier resulted in an upregulation of stress-related genes, indicating the instigation of a harsh environment despite the addition of oil. Interestingly, the time required to achieve a 5-log reduction in *S. typhimurium* count, also known as the 5D value, was markedly longer in the Triton X-100 solution (795.34 ± 420.09 min) compared to the Triton X-100 stabilized emulsion (207.23 ± 60.03 min) ($p < 0.05$) (Tsai & Tikekar, 2023). This raised the question as to the cause of this significant difference, prompting us to conduct a comparative analysis of gene expression between Triton X-100 solution and emulsion following thermal treatment in this study. Our results revealed a more pronounced expression of *proV* gene and reduced expression of *fabA* and *ompC* genes in *S. typhimurium* inoculated in the solution as opposed to the emulsion ($p < 0.05$). The upregulated expression of *proV* gene suggests an accelerated exchange of metabolites and proline across the cellular membrane, potentially contributing to improved protection against thermal treatment. On the other hand, the decreased expression of *fabA* gene implies increased heat resistance via the establishment of a more rigid membrane. The finding related to *ompC* gene expression also suggests that combining oil with Triton X-100 generates a more challenging environment for bacterial growth and survival. To clarify, the enhanced thermal resistance observed in the Tween 80 solution might be due to the overproduction of heat shock proteins, which could possibly modulate membrane fluidity (De Maio & Hightower, 2021). Conversely, in the Triton X-100 environment, a more rigid bacterial membrane appears to be established, as indicated by the decreased expression of the *fabA* gene. Furthermore, a higher expression of *proV* was observed in the Triton X-100 solution compared to its emulsion counterpart, whereas no such difference was noted between the Tween 80 solution and its emulsion. This differential *proV* expression might further elucidate the distinct effects observed between the Triton X-100 and Tween 80 environments.

A comparable pattern was observed in environments incorporating Tween 80. The 5D value for *S. typhimurium* count was observed to be more extended in the solution (1914.70 ± 706.35 min) relative to the emulsion (136.45 ± 30.56 min) ($p < 0.05$) (Tsai & Tikekar, 2023). Notably, higher expression levels of *fabA* and *ompC* genes were recorded in *S. typhimurium* cultured in Tween 80 solution as opposed to those cultured in Tween 80 emulsion ($p < 0.05$). The upregulation of *fabA* and *ompC*

TABLE 5 Log_2 fold change in the relative expression of genes in *Salmonella typhimurium* in Tryptic soy broth (TSB) (TSBh), Tween 20 solution (T20h), Tween 80 solution (T80h), Triton X-100 solution (X100h), Tween 20 stabilized 60% emulsion (T20Eh), Tween 80 stabilized 60% emulsion (T80Eh), and Triton X-100 stabilized 60% emulsion (X100Eh) with 45-min thermal treatment at 55°C.

	rpoE	*rpoH*	*ibpA*	*dnaK*	*otsB*	*proV*	*fadA*	*fabA*	*ompC*
TSBh	−0.93 ± 0.54^{c}	0.18 ± 0.12^{b}	0.21 ± 1.29^{d}	−1.62 ± 0.74^{d}	−1.97 ± 0.31^{bc}	−1.72 ± 0.64^{d}	−4.57 ± 0.55^{c}	−1.80 ± 0.36^{cd}	−1.73 ± 0.43^{cd}
T20h	1.17 ± 0.24^{b}	1.63 ± 0.50^{b}	3.51 ± 0.61^{b}	0.25 ± 1.64^{bc}	−0.61 ± 1.14^{b}	0.98 ± 0.61^{c}	−2.65 ± 0.11^{b}	−0.61 ± 0.86^{bc}	−0.16 ± 0.28^{bc}
T80h	−0.30 ± 0.84^{bc}	1.21 ± 0.32^{b}	2.03 ± 0.09^{bc}	−1.27 ± 1.38^{cd}	−0.66 ± 0.46^{bc}	−1.27 ± 0.56^{d}	−4.25 ± 0.87^{bc}	−0.49 ± 0.99^{bc}	−2.07 ± 0.52^{d}
X100h	3.65 ± 0.49^{a}	5.46 ± 0.17^{a}	7.05 ± 0.12^{a}	3.09 ± 0.91^{a}	4.96 ± 0.20^{a}	5.12 ± 0.73^{a}	0.56 ± 0.51^{a}	0.98 ± 1.85^{b}	0.56 ± 1.19^{b}
T20Eh	−0.72 ± 0.92^{c}	0.51 ± 0.29^{b}	0.88 ± 0.72^{cd}	0.60 ± 0.64^{b}	−1.73 ± 0.25^{bc}	−2.25 ± 0.44^{d}	−4.43 ± 0.65^{c}	−1.53 ± 1.13^{cd}	−2.81 ± 0.19^{de}
T80Eh	−1.63 ± 0.32^{c}	0.38 ± 0.16^{b}	1.55 ± 0.38^{cd}	-0.31 ± 0.39^{bcd}	−2.27 ± 0.96^{c}	−1.51 ± 0.80^{d}	−4.83 ± 0.60^{c}	−2.59 ± 0.32^{d}	−4.20 ± 0.80^{e}
X100Eh	4.07 ± 1.04^{a}	6.21 ± 0.58^{a}	5.90 ± 0.80^{a}	3.65 ± 0.54^{a}	3.82 ± 0.05^{a}	3.01 ± 0.08^{b}	1.78 ± 0.33^{a}	3.69 ± 0.92^{a}	2.31 ± 0.39^{a}

Note: Lowercase letter labels (a, b, c, etc.) were used to indicate significant differences among groups. The determination of significant difference was based on a two-way analysis of variance (ANOVA). Groups with the same letter were not significantly different ($p > 0.05$), whereas those with different letters were significantly different ($p < 0.05$).

genes is understood to be linked to increased membrane fluidity and enhanced passive diffusion through channels. Although these changes could potentially lower heat resistance in bacteria, this effect is counterbalanced by the overproduction of heat shock proteins. Indeed, our findings reveal that in Tween 80 solution, this overproduction not only neutralizes any negative impacts but also augments the bacterium's protection against thermal treatment.

3.2.4 | Comparison among gene expression of *S. typhimurium* inoculated in different environments

To understand the relationships between different samples, this study applied MDS and HCA (Figures 2 and 3). These analytic approaches revealed three distinct clusters: (a) samples before thermal treatment, except for Tween 80 solution; (b) Tween 80 solution before thermal treatment, TSB, Tween 20 solution, Tween 80 solution, and their corresponding emulsions after thermal treatment; and (c) Triton X-100 environments after thermal treatment.

In summary, Tween 80 solution was the only environment that caused changes in bacterial gene expression after 20 h of incubation at 37°C. Moreover, after thermal treatment, Triton X-100 environments exhibited significant differences in bacterial gene expression compared to the other samples. Specifically, Tween 80 without oil was the harshest environment for bacterial survival. This was evident after 45 min of thermal treatment at 55°C, which showed that Tween 80 without oil had a potent antibacterial effect, even before any thermal treatment was applied. Conversely, the Triton X-100 environment became the most challenging for bacteria after thermal treatment, although this was not observed before thermal treatment. Further research is needed to fully understand the mechanisms underlying these observed gene expression patterns in different environments.

4 | CONCLUSION

This research examined the gene expression and survival patterns of *S. typhimurium* under various emulsifier environments and thermal treatments, offering insights into the dynamic stress responses of bacteria. Confocal microscopy findings indicated that the protective effects observed in emulsion samples after thermal treatment were unlikely to be a consequence of bacterial migration into oil droplets. Rather, emulsifier-induced stress, oil stress, and high temperatures appeared to be the key factors contributing to this protection. We observed

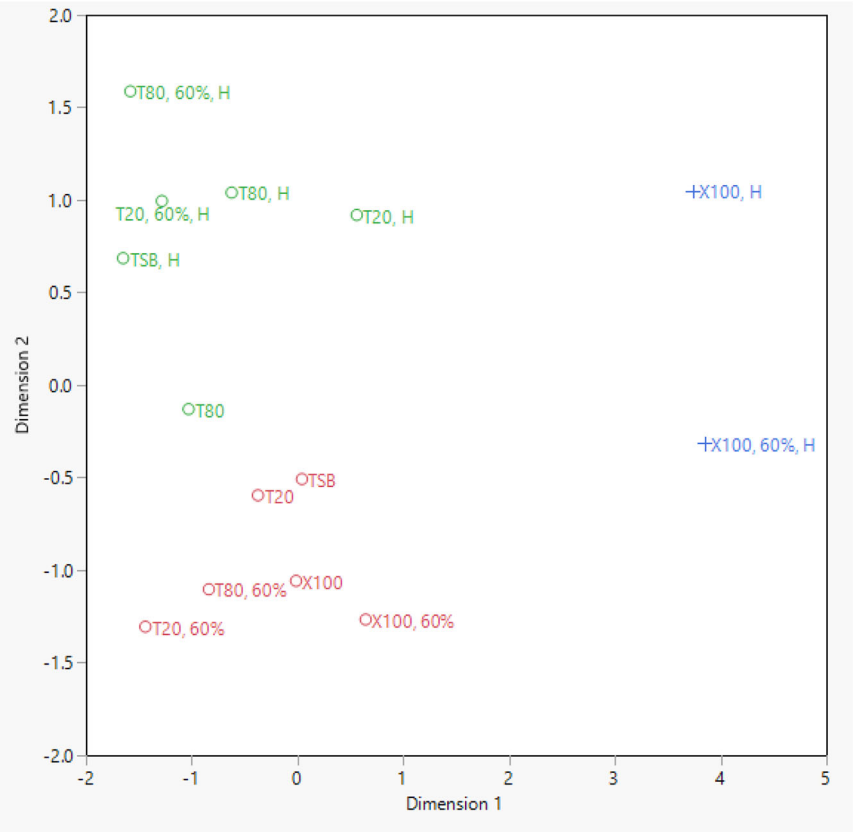

FIGURE 2 Multi-dimension scaling plot based on selected stress-related gene expression data among all samples, including Tryptic soy broth (TSB), Tween 20, Tween 80, Triton X-100 solutions and their 60% emulsions, before and after thermal treatment at 55°C.

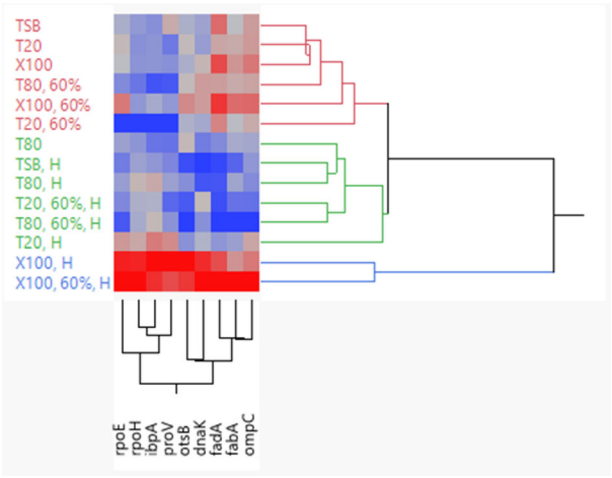

FIGURE 3 Hierarchical cluster analysis based on selected gene expression among all samples, including Tryptic soy broth (TSB), Tween 20, Tween 80, Triton X-100 solutions and their 60% emulsions, before and after thermal treatment at 55°C.

significant changes in bacterial gene expression in environments containing Tween 80 and Triton X-100. For instance, the upregulation of stress-related genes in environments with Triton X-100, especially after thermal treatment, suggested an activation of stress response mechanisms. Similarly, the overproduction of heat shock proteins in the Tween 80 solution was seen to enhance the thermal resistance of bacteria. Additionally, the study recorded substantial variations in 5D values, demonstrating the duration required for bacterial inactivation could be strongly influenced by the type of emulsifier used and the presence or absence of oil. Specifically, we found Tween 80 solution without oil to be the most unfavorable environment for bacterial survival, whereas the Triton X-100 environment was the most challenging following thermal treatment. In essence, the study elucidates the intricacies of bacterial gene expression patterns in response to various emulsifier environments and thermal stress, providing a better understanding of microbial behavior under these conditions. Further research is encouraged to fully unravel the underlying mechanisms influencing these gene expression patterns, with the aim of developing more effective microbial control strategies, particularly for food safety applications.

AUTHOR CONTRIBUTIONS

Shawn Tsai: Methodology; conceptualization; investigation; data curation; validation; formal analysis; visualization; writing—original draft. **Rohan V. Tikekar**: Conceptualization; methodology; supervision; funding acquisition; project administration; resources; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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