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Stress response of *Salmonella* Newport with various sequence types toward plasma-activated water: Viable but nonculturable state formation and outer membrane vesicle production

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ABSTRACT

This study aims to investigate the response of *Salmonella* Newport to plasma-activated water (PAW), a novel disinfectant that attracts attention due to its broad-spectrum antimicrobial efficacy and eco-friendliness. In this work, we demonstrated that *S*. Newport of different sequence types (STs) could be induced into the viable but nonculturable (VBNC) state by PAW treatment. Notably, a remarkable 99.96% of *S*. Newport ST45 strain entered the VBNC state after a 12-min PAW treatment, which was the fastest observed among the five *S*. Newport STs (ST31, ST45, ST46, ST166, ST2364). Secretion of outer membrane vesicles was observed in ST45, suggesting a potential strategy against PAW treatment. Genes related to oxidative stress (*sodA*, *katE*, *trxA*), outer membrane proteins (*ompA*, *ompC*, *ompD*, *ompF*) and virulence (*pagC*, *sipC*, *sopE2*) were upregulated in the PAW-treated *S*. Newport, especially in ST45. A reduction of 38–65% in intracellular ATP level after PAW treatment was observed, indicating a contributor to the formation of the VBNC state. In addition, a rapid method for detecting the proportion of VBNC cells in food products based on *pagC* was established. This study contributes to understanding the formation mechanism of the VBNC state in *S*. Newport under PAW stress and offers insights for controlling microbial risks in the food industry.

1. Introduction

Nontyphoidal *Salmonella* (NTS) is a major foodborne pathogen causing self-limiting enteric infection and severe invasive diseases (Marchello et al., 2022). It is estimated that NTS is responsible for 93 million enteric infections and 155,000 deaths annually, with more than 80% of the cases being foodborne (Majowicz et al., 2010). *Salmonella enterica* serotype Newport (*S*. Newport) is a serotype of NTS that is of significant concern to the global public health community. *S*. Newport is commonly found in a variety of foods, including eggs, meat, fruit, vegetables and grains (Jackson et al., 2013). In recent years, the prevalence of *S*. Newport has risen in many countries. According to the survey results, the proportion of *S*. Newport ranked fifth among *Salmonella* detected in the European Union, second in the United States and the top ten in China (Cheng et al., 2019; EFSA, ECDPC, 2023; Wu et al., 2018). Multilocus sequence typing (MLST) based on nucleic acid sequence has been used to type *S*. Newport, offering a detailed classification compared with serotyping (Tang et al., 2019).

Current research efforts are mainly focused on elucidating differences in prevalence, host, antibiotic resistance, and genetic traceability of different sequence types (STs) (Elbediwi et al., 2020; Harbottle et al., 2006; Hudson et al., 2023; Pan et al., 2018). However, limited attention has been paid to variations in stress tolerance among different STs in response to food processing-associated stressors.

Plasma-activated water (PAW) is a new disinfectant prepared by the cold plasma treatment of water (Zhou et al., 2020). PAW contains abundant reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydroxyl radicals, ozone, hydrogen peroxide (H_2O_2),

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nitrates and nitrites, which contribute to the microbial inactivation through inducing oxidative stress (Thirumdas et al., 2018; Zhou et al., 2020). Because of its low cost, environmentally friendly nature, and minimal influence on food quality, PAW is receiving more attention from the food industry (Esua et al., 2021). Several studies have demonstrated the disinfection effect of PAW on *Salmonella* in food products, such as eggs, tofu, tomatoes, blueberry, fresh-cut lettuce, and baby spinach (Frías et al., 2020; J. Wang et al., 2023; Q. Wang et al., 2023). However, there is limited research on the response of *Salmonella* to PAW. Ritter et al. (2018) treated *Salmonella* Enteritidis with cold plasma, observing upregulation of proteins associated with carbohydrate catabolism, nucleotide metabolism, RNA transcription and redox processes, which might contribute to *Salmonella* cells' tolerance to PAW.

Entering the viable but nonculturable (VBNC) state is a survival strategy for bacteria under adverse stresses (İzgördü et al., 2022). Bacteria in the VBNC state is unable to grow on routine culture media, potentially leading to false negatives and the underestimation of contamination levels (Salive et al., 2020). VBNC bacteria can recover culturability when conditions are suitable, posing a potential threat to food safety (İzgördü et al., 2022). Notably, a few studies have reported that PAW had the potential to induce *Staphylococcus aureus* and *Escherichia coli* into the VBNC state, suggesting more examination on PAW sterilization (Hu et al., 2024; Z. Xu et al., 2018). However, a notable gap in the literature regarding a systematic investigation into PAW-induced VBNC is lacking and the underlying mechanisms remain unclear.

In our study, we focused on five common STs of *S*. Newport (ST31, ST45, ST46, ST166, ST2364) in China (Paudyal et al., 2020) and subjected them to PAW treatment. Our study aimed to estimate the formation of the VBNC state by determining both culturable and viable cell counts. We further investigated the response of *S*. Newport to PAW stress at the cellular and molecular levels, including outer membrane vesicles (OMVs) secretion, gene expression and ATP level. Furthermore, a method of detecting VBNC *S*. Newport based on the *pagC* gene was established. Through this work, we aim to enhance understanding of the mechanism underlying the VBNC state formation and provide the basis for the effective control of *S*. Newport contamination.

2. Materials and methods

2.1. Preparation of S. Newport samples

The *S*. Newport strains used in this research were isolated from food and environment (Table 1). All *S*. Newport strains were kept in sterile glycerol at −80°C. The frozen stock of strains was streaked onto Xylose Lysine Deoxycholate agar (XLD agar; Qingdao Hope Bio-Technology, Qingdao, China) and then incubated at 37°C for 24 h. A single colony was picked and incubated into 10 mL Nutrient Broth (NB; Qingdao Hope Bio-Technology, Qingdao, China) at 37°C for 24 h. *S*. Newport cells were harvested by centrifugation (6000 rpm, 4°C, 10 min), washed, and resuspended in 0.85% sterile saline solution.

Table 1
Strains used in this research.

Sequence types	Labels	Sources
31	ST31-1	Mussel
	ST31-2	Chicken heart
	ST31-3	Pork
45	ST45-1	Beef
	ST45-2	Razor clam
	ST45-3	Feces of patients
46	ST46-1	Squid
	ST46-2	Undulating Venu
	ST46-3	Chicken breast
166	ST166-1	Duck claw
	ST166-2	Feces of patients
	ST166-3	Feces of patients
2364	ST2364-1	Feces of patients
	ST2364-2	River snail
	ST2364-3	River

2.2. Preparation of PAW

A 250 mL beaker containing 50 mL of ultrapure water was placed in an ice bath and positioned under the nozzle of the cold plasma device (Shenzhen Tonson Tech Automation Equipment, Shenzhen, China). The distance between the nozzle and the liquid level was maintained at 8 cm. The plasma input power was set to 650 W and a 30-s treatment was conducted to obtain PAW. The pH value and oxidation-reduction potential (ORP) of PAW were measured by the FE28 pH meter (Mettler Toledo, Shanghai, China) and the PHB-1 ORP meter (Sanxin, Shanghai, China) respectively. The concentrations of H_2O_2 were measured by a hydrogen peroxide assay kit (Beyotime, Shanghai, China). Ozone levels were estimated by the Spectroquant ozone test kit (Merck, Darmstadt, Germany). The determination of nitrite concentration was conducted by N-(1-naphthy1)-ethylenediamine hydrochloride method, measuring absorbance at 538 nm by UV 2600 UV–Vis Spectrophotometer (Shimadzu, Kioto, Japan). The determination of nitrate levels followed the method described by Shen et al. (2016), measuring absorbance at 220 nm. The physicochemical properties of PAW used in this study are listed in Table 2.

2.3. PAW treatment of S. Newport

The treatment of bacteria with PAW followed the method described by Bai et al. (2020) with slight modifications. Specifically, *S*. Newport suspension of 1 mL containing approximately 8 $\log_{10}$ CFU/mL was mixed with 9 mL of PAW and subsequently shaken. Following treatment for 3, 6, 9, and 12 min, samples were collected and combined with a neutralizing solution containing 0.5% $Na_2S_2O_3$ and 0.85% NaCl at a ratio of 1:9 to neutralize PAW. For the untreated group, PAW was mixed with the neutralizing solution at a ratio of 1:9 first and then mixed with the bacterial suspension at a ratio of 1:9.

2.4. Quantification of culturable and viable S. Newport cells

The quantification of culturable *S*. Newport cells was conducted by the plate count method. After appropriate 10-fold serial dilutions, the *S*. Newport suspension was plated on the Plate Counting Agar (PCA; Qingdao Hope Bio-Technology, Qingdao, China) medium, and subsequently incubated at 37 °C overnight. Colonies within the range of 30–300 were counted and recorded from the plate. The linear model was used to fit the inactivation of *S*. Newport culturable cells, as shown in Eq. (1) (Karaman and Erkmen, 2001):

$$\log_{10}(N_t / N_0) = -kt \quad (1)$$

where t is the treatment time, N_t is the concentration of *S*. Newport cells at time t (CFU/mL), N_0 is the initial concentration of *S*. Newport cells, and k is the inactivation rate (min^{-1}).

The quantification of viable cells was conducted by propidium monoazide-quantitative polymerase chain reaction (PMA-qPCR)

Table 2
Physicochemical properties of plasma-activated water (PAW) treated by 30-s plasma activation.

Properties	pH	Oxidation-reduction potential	Hydrogen peroxide	Ozone	Nitrite	Nitrate
Values	3.22 ± 0.03	531 ± 3 mV	0.19 ± 0.01 mmol/L	0.72 ± 0.02 mg/L	42.11 ± 0.58 mg/L	13.87 ± 1.53 mg/L

(Golpayegani et al., 2019; Liu et al., 2018; Xiao et al., 2013). The standard curve used in this work has been reported in our previous work (Zhang et al., 2023).

Referring to Liao et al. (2020), the total DNA of samples was extracted using TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China). The extracted DNA was stored at −20°C. qPCR was performed on a Real-Time PCR System (QuantStudio 3, Thermo Fisher Scientific, Waltham, MA, USA). Gene *invA* (forward primer: 5′-ATTCT GGTACTAATGGTGATGA-3′, reversed primer: 5′-GCCAGGCTATCG CCAATAAC-3′) was chosen for the quantification of *S*. Newport (Bell et al., 2015; Zhang et al., 2020). Each qPCR reaction contained 25 μL Platinum™ SYBR™ Green qPCR SuperMix-UDG (ThermoFisher Scientific Co., Ltd., USA), DNase/RNase free water (22 μL), 1 μL reverse primers (10 μM),1 μL forward primers (10 μM) and 1 μL the extracted DNA. The cycling procedure of qPCR was as follows: hold stage (1 cycle), 50°C for 2 min and 95°C for 2 min; PCR stage (40 cycles), 95°C for 15 s and 60°C for 30 s.

The number of viable cells was determined by PMA-qPCR and the culturable cells were quantified with the plate count method (Liao et al., 2017). The proportion of VBNC cells in the bacteria suspension (P_{VBNC}, %) was calculated using Eq. (2):

$$P_{VBNC} = (N_1 - N_2)/N_1 \quad (2)$$

where N_1 is the number of viable cells (CFU/mL) and N_2 is the number of culturable cells (CFU/mL).

2.5. Estimation of outer membrane vesicles production

Dead *S*. Newport cells were prepared by heating at 95°C for 10 min (Zhang et al., 2023) and PAW-treated cells were prepared through 12-min treatment. Transmission electron microscopy (TEM) observation method was referred to Elhenawy et al. (2016) with slight modifications. A copper mesh was placed in the bacterial suspension with a concentration of about 8 $\log_{10}$ CFU/mL for 10 min to adsorb cells and was aspirated then. The copper mesh was stained in 0.25% hydrogen peroxide acetate for 1 min and aspirated. The mesh was observed by transmission electron microscopy (JEM-1010, JEOL, Tokyo, Japan) after drying.

2.6. Total RNA extraction and gene expression analysis of S. Newport

About 10^8 cells of *S*. Newport were prepared for RNA extraction using TRIzol™ reagent (Thermo Fisher Scientific, Waltham, USA). The concentration, purity, and integrity of RNA were confirmed by NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The removal of residual DNA and the reverse transcription of RNA was conducted by a PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio Inc., Shiga, Japan) according to the instructions. cDNA was stored at −80°C for qPCR. qPCR assay was the same as in section 2.3 and the primers used were listed in Table 3. The relative expression level of genes was analyzed by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Determination of relative protein expression

S. Newport samples weighing about 1 g each were collected for parallel reaction monitoring (PRM) mass spectrometry analysis. Proteins of *S*. Newport were extracted referring to the method described by Leary et al. (2013) and cleaved into peptides using trypsin. Spectral libraries were constructed and proteins were analyzed by EASY-nLC 1200 UHPLC system coupled with Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, USA). The peptides were re-dissolved in 0.1% formic acid in water. Peptide sample of 2 μL was loaded onto an analytical column (25 cm × 75 μm, 1.9 μm resin) and separated with 120 min-gradient starting at 4% buffer B (80% acetonitrile with 0.1% formic acid) followed by a stepwise increase to 30% in 108 min, 90% in 2 min and stayed for 10 min. The column flow rate was maintained at 600 nL/min with the temperature of 55°C. The electrospray voltage was set to 2 kV. For data acquisition, PRM settings were as follows: Full MS scans in the mass range from m/z 350 to 1,200 were acquired with a resolution of 120,000, automatic gain control (AGC) target of 2e5 and a maximum injection time of 50 ms. MS2 spectra were acquired with a resolution of 30,000, AGC target of 2e5 and a maximum injection time of 70 ms. The melist exported from SpectroDive was imported into the mass list table in the PRM mode. Raw files of the targeting runs were analyzed in SpectroDive 10.4 with the default settings.

Table 3
Primers used in this research.

Gene	Sequence (5′-3′)
16S rRNA	F: GCGGCAGGCCTAACACAT
	R: GCAAGAGGCCCGAACGTC
pagC	F: GAAAACGGGATTTGCCTGGG
	R: AGAAACGGTATCCAACCCCG
trxA	F: GGCAAATTGACCGTTGCC
	R: CTTTGGTTGCCGCCACTT
ompC	F: AGTAAGTCGCGCCGACAT
	R: TTTGGTCTGCGTCCGTCT
ompA	F: ATACTCGATACCGCCCGC
	R: GCGTGCAGACACCAAGTC
ompD	F: TTCGACTTCGGTCTGCGT
	R: AACCAGATCCTGGTCGCC
ompF	F: ACCTACGCCATCGCCATT
	R: TGCTGGCGGTTTGTTGAC
sopE2	F: TGTTGTGGCGTTGGCATC
	R: CTCATTGCTTCCGCGAGT
sipC	F: AGCGCGAATATTGCCTGC
	R: GCCCGTGAAAGTTCACGT
sodA	F: TCGAGCGTGACTTCGGTT
	R: CTTTCAGCACCAGCCACG
katE	F: TCAGCCGTTACTTCACCCAT
	R: CGATGTTAAAGGCCGAGTGG

2.8. Determination of the intracellular ATP concentration

The intracellular ATP concentration of *S*. Newport before and after PAW treatment was determined by a commercial ATP assay kit (Beyotime Biotechnology, Beijing, China) according to the instructions.

2.9. Establishment of the detection of VBNC proportion based on pagC

The flow diagram illustrating the detection process is shown in Fig. 1. A mixture of three strains of ST45, totaling 1 mL, was prepared at the ratio of 1:1:1, resulting in a final concentration of 8 $\log_{10}$ CFU/mL. The bacteria suspension was treated with PAW to achieve a proportion of VBNC cells reaching 99.9%, which was approximated to be regarded as 100%. VBNC cells were mixed with untreated cells to obtain bacterial suspension with VBNC cell proportions of 5%, 25%, 50%, 75%, and 100%. The extraction of total RNA and RT-qPCR assay was conducted following the method in section 2.6. The relative expression level of the *pagC* was determined by using 16S rRNA as the reference gene. The correlation curve was plotted to verify the relationship between the relative expression of *pagC* and the proportion of VBNC cells.

The chicken, eggs, and cherry tomatoes used in this work were purchased from the local market. Chicken samples were prepared into cubes and inoculated with *S*. Newport of approximately 10^8 CFU/each.

The treatment and inoculation methods of eggshells were adapted from Georgescu et al. (2017) with slight modifications. Eggshells were immersed in 75% ethanol to kill background microorganisms and inoculated with *S*. Newport of approximately 10^8 CFU/fragment.

Cherry tomatoes were treated according to the method of He et al. (2021) with slight modifications as follows: several cherry tomatoes were wiped with 75% ethanol to sterilization. *S*. Newport suspension was added to each tomato, reaching 10^8 CFU/each.

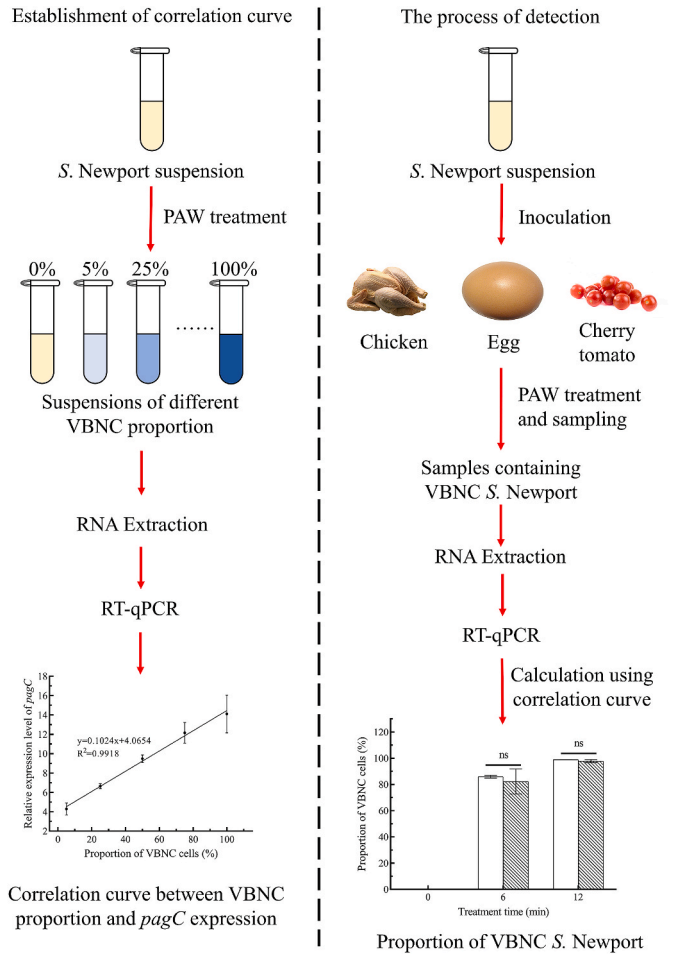

Fig. .1. The flow diagram of the detection of VBNC *S.* Newport proportion based on *pagC*.

The inoculated areas of samples were mixed with PAW for treatment and then vortexed to obtain bacteria suspension, which was detected by PMA-qPCR and the method based on gene *pagC*, respectively.

2.10. Statistical analysis

The analysis of data was carried out by IBM SPSS Software Version 25.0 (IBM Corp., Armonk, NY, USA) and significance was analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test. A value of $p < 0.05$ was considered as statistically significant. GraphPad Prism V.8.0.1 software (GraphPad Software, San Diego, CA, USA) was used for graphing. Data were expressed as mean ± standard deviations (SDs).

3. Results

3.1. The survival of S. Newport with different STs upon PAW treatment

The plate count method and PMA-qPCR were used to quantify culturable and viable *S.* Newport after PAW treatment, respectively (Fig. 2A). PMA is a dye which can enter dead cells, combining with DNA and inhibiting the amplification, while the DNA of culturable and VBNC cells is not affected by PMA (Golpayegani et al., 2019). As a result, only culturable and VBNC cells can be detected by PMA-qPCR. The number of viable cells minus culturable cells is the number of VBNC cells. PMA-qPCR is widely used to detect VBNC cells (Liao et al., 2020; Liu et al., 2018; Zhang et al., 2020). The culturable cells of five STs were reduced rapidly with the increase of treatment time. However, according to the result of PMA-qPCR, the number of viable cells remained stable over PAW treatment time. The differences between the results obtained from plate count method and PMA-qPCR indicated the entry of the VBNC state during PAW treatment. *S.* Newport strains with different STs showed varying rates of culturability decrease and entry into the VBNC state (Fig. 2B and C). ST45 displayed the highest propensity to enter the VBNC state, with 95.57% at 3 min and 99.96% at 12 min in the VBNC state.

3.2. The secretion of OMVs of S. Newport induced by PAW

The *S.* Newport ST45 strain was selected for TEM analysis due to its pronounced tendency to enter the VBNC state. The production of OMVs of *S.* Newport was assessed using TEM. Compared with untreated cells (Fig. 3A), most cells appeared to be shriveled after PAW treatment (Fig. 3C). And a transparent outer layer was observed in these cells, which was absent in cells killed by heat (Fig. 3B). Considering that 99.96% of ST45 entered the VBNC state after PAW treatment for 12 min (Fig. 2C), cells shown in Fig. 3C were predicted to be VBNC cells due to

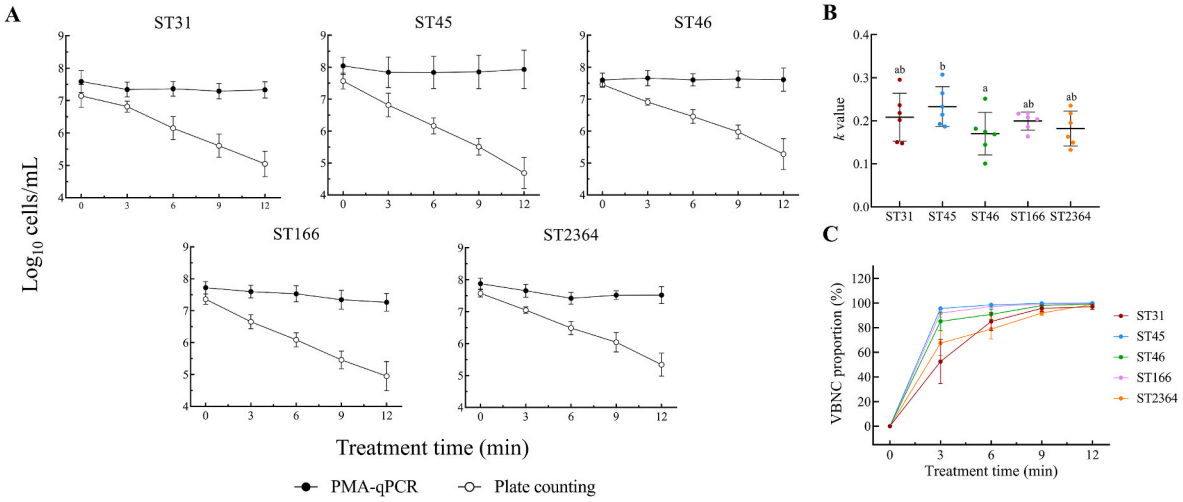

Fig. 2. (A) The total culturable (plate count) and total viable counts (PMA-qPCR) of different *S.* Newport STs treated by plasma-activated water (PAW) for various times. (B) The decline slopes (*k* values) of culturable *S.* Newport stains of different STs after PAW treatment. Different letters represent significant differences in *k* values of different STs ($p < 0.05$). (C) The changes in proportions of VBNC cells of different STs after PAW treatment.

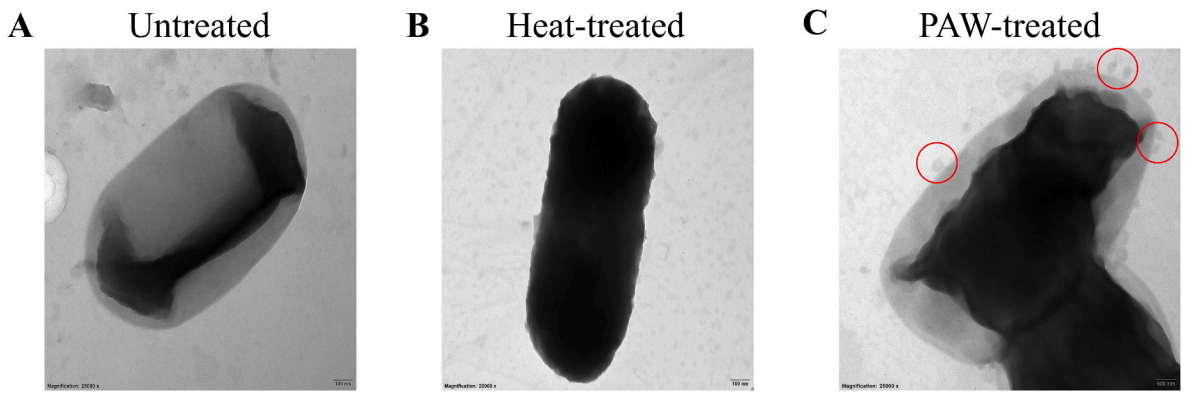

Fig. 3. TEM images of (A) untreated, (B) heat-treated and (C) PAW-treated cells of *S.* Newport strain ST45. The spheres in the red circles in Fig. 3C are considered to be outer membrane vesicles. The magnification was set as 25,000×. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

their high proportion under TEM observation. And these cells were similar to VBNC *Salmonella* observed by Salive et al. (2020), which had rough surfaces and transparent outer layers. Spherical vesicles were also observed on the surface of cells after PAW treatment (Fig. 3C), likely corresponding to be OMVs observed by Elhenawy et al. (2016).

3.3. The stress response-related gene expression of S. Newport treated by PAW

The expression level of genes associated with oxidative stress, outer membrane proteins (OMPs) and virulence was determined to investigate *S.* Newport's response to PAW (Fig. 4).

sodA, the gene encoding manganese-dependent superoxide dismutase (Tsolis et al., 1995), exhibited approximately a 2-fold upregulation across all five *S.* Newport STs. *katE* encoding catalase HPII (Robbe-Saule et al., 2001) showed a 3-fold upregulation in ST45, significantly higher than observed in other STs ($p < 0.05$). As for *trxA* encoding thioredoxin 1 (Bjur et al., 2006), it was significantly upregulated in ST45, ST46, and ST2364, especially in ST45 with a fold change of 6.02. Additionally, the genes *ompA*, *ompC*, *ompD,* and *ompF*, responsible for encoding porins on the outer membrane (Chowdhury et al., 2022). Following PAW treatment, these genes exhibited upregulation ranging from 1.15 to 6.17-fold, except for *ompD* in ST166. Among virulence-associated genes, *pagC*, *sipC* and *sopE2* exhibited upregulation in PAW-treated *Salmonella* (Velge et al., 2012). Specifically, *pagC* was upregulated by 4.66 to 13.52-fold across the five *S.* Newport STs. Similarly, *sopE2* was upregulated in ST45, ST46, ST166, and ST2364, with fold changes ranging from 2.58 to 5.94-fold. And *sipC* was upregulated by about two folds in ST31, ST45, ST46 and ST2364.

To verify the result of gene expression, the relative expression of

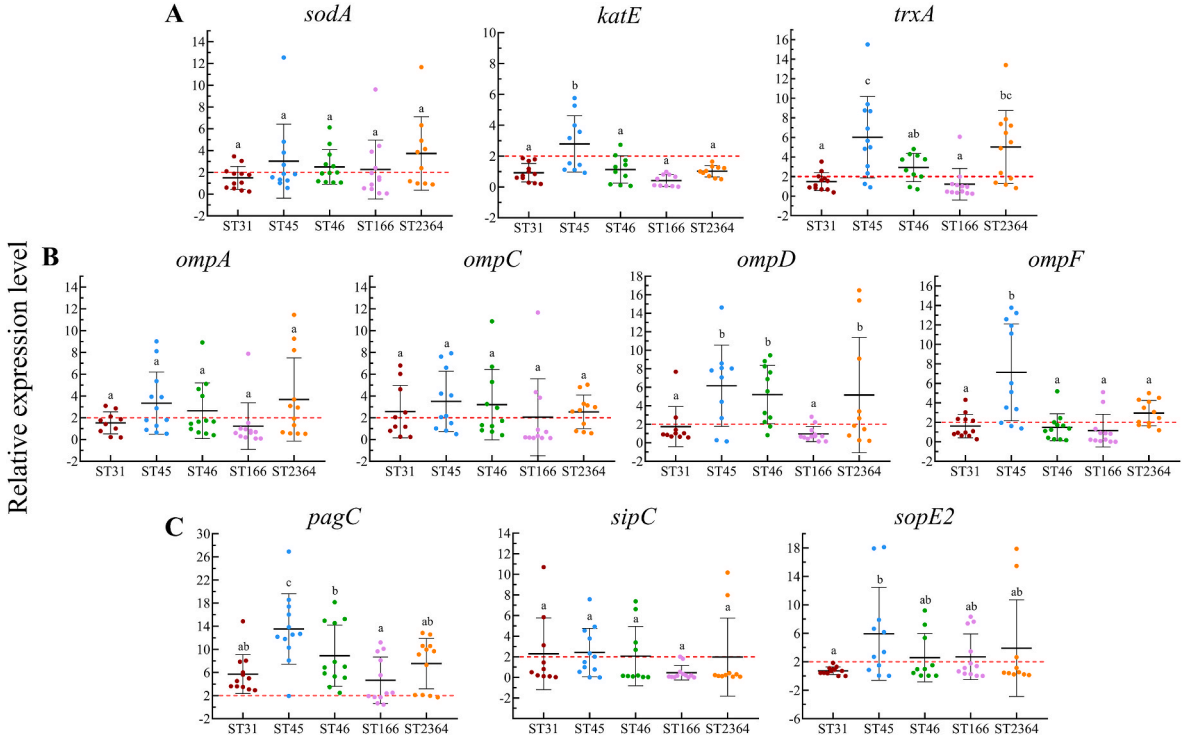

Fig. 4. Relative expression levels of genes related to (A) oxidative stress, (B) outer membrane proteins and (C) virulence in different *S.* Newport STs after PAW treatment. Different lowercases represent significant differences in the relative expression of the same gene among different STs ($p < 0.05$). The red dotted line represents the relative expression level of 2-fold. Relative expression levels more than 2-fold are considered significantly upregulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

proteins in ST45 after PAW treatment was also determined (Table 4). PagC showed significant upregulation, ranging from 6.69 to 11.15 folds, across all STs ($p < 0.05$). While other proteins were also upregulated, there was no significant difference ($p > 0.05$), except for SodA in three ST45 strains, and SopE2 and SipC in ST45-2, which was downregulated.

3.4. Changes of the intracellular ATP level in S. Newport after PAW treatment

To evaluate the energy dynamics in *S*. Newport, intracellular ATP levels were evaluated before and after PAW treatment (Fig. 5). In the untreated groups, ST45 exhibited an ATP level of 0.27 μM, significantly lower than other STs ($p < 0.05$). After PAW treatment, ATP levels decreased by 38–65% in all five *S*. Newport STs, with the lowest level recorded as 0.12 μM in ST45.

3.5. Detection of VBNC S. Newport based on pagC in food produces

To demonstrate the feasibility of gene *pagC* as a biomarker for the VBNC state, we prepared *S*. Newport suspensions containing varying proportions of VBNC cells and measured the relative expression of *pagC*. A strong correlation was shown between the relative expression of *pagC* and VBNC proportion, ranging from 5 to 100% (Fig. 6A). We subsequently applied this method to eggs, chicken, and cherry tomatoes treated by PAW, commonly contaminated with *S*. Newport. There was no significant difference between the results obtained from PMA-qPCR and the *pagC*-based method for eggs and cherry tomatoes ($p > 0.05$). However, a notable difference was observed in chicken samples ($p < 0.05$) (Fig. 6B–D).

4. Discussion

In this study, we found that PAW treatment could induce *S*. Newport into VBNC state. Five STs of *S*. Newport entered the VBNC state after PAW treatment, which was underestimated by the plate count method (Fig. 2A). Variations in response were observed among different STs, with ST45 exhibiting the highest propensity to enter the VBNC state. This raises concerns about the potential for false-negative results in the detection, which could lead to inadequate sterilization (Fig. 2B and C). ST45 is a common ST of *S*. Newport isolated from livestock and humans, known to contain drug-resistant strains (Gomes-Neto et al., 2021; Harbottle et al., 2006; Pan et al., 2019). Pan et al. (2019) analyzed the antibiotic resistance of 1,842 strains, revealing that approximately 60% of ST45 exhibited multiple antibiotic resistance, in contrast to the 1.15% and 1.66% observed in ST5 and ST118, respectively. Gomes-Neto et al. (2021) reported that ST45 exhibited higher resistance to quaternary ammonium salts compared with ST5, ST31 and ST118, primarily due to the presence of the small multidrug transporter SugE, which was unique to ST45. Further comprehensive investigation is required to reveal the underlying mechanisms driving the propensity of ST45 to develop resistance to various stresses.

Table 4
Relative expression level of proteins in ST45 *S*. Newport after PAW treatment. *$p < 0.05$ means significant differences between PAW treated group and untreated group and $p > 0.05$ means no significant difference between PAW treated group and untreated group.

Proteins	ST45-1		ST45-2		ST45-3	
	Relative Expression level	p*	Relative Expression level	p	Relative Expression level	p
SodA	0.51 ± 0.10	0.26	0.18 ± 0.06	0.06	0.25 ± 0.12	0.01
KatE	2.30 ± 1.15	0.16	1.84 ± 0.73	0.60	2.61 ± 1.05	0.42
TrxA	1.45 ± 0.56	0.61	1.78 ± 0.88	0.29	1.42 ± 0.70	0.74
OmpA	3.67 ± 0.90	0.14	2.88 ± 1.60	0.22	3.99 ± 1.80	0.36
OmpC	2.49 ± 0.57	0.08	5.42 ± 1.38	0.03	3.51 ± 1.53	0.06
OmpD	2.01 ± 0.50	0.11	4.58 ± 0.98	0.03	3.06 ± 1.12	0.08
OmpF	3.92 ± 1.01	0.01	7.63 ± 2.93	0.05	4.39 ± 1.37	0.11
PagC	9.75 ± 1.80	<0.01	6.79 ± 0.10	0.01	7.62 ± 1.31	0.05
SopE2	4.83 ± 4.77	0.27	0.97 ± 0.43	0.59	3.41 ± 2.70	0.20
SipC	4.13 ± 4.37	0.72	0.70 ± 0.33	0.39	4.36 ± 3.38	0.11

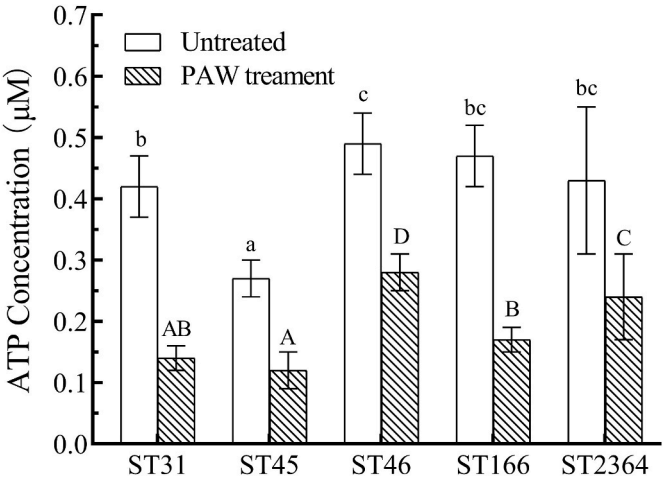

Fig. 5. Intracellular ATP level of different *S*. Newport STs before and after PAW treatment. Different lowercase letters present significant differences in different untreated groups ($p < 0.05$). Different capital letters present significant differences in PAW-treated groups ($p < 0.05$).

The observation of OMV secretion in ST45 after PAW treatment (Fig. 3C) suggested a potential strategy employed by *S*. Newport against PAW-induced stress. Bacteria membrane vesicles (MVs) are vesicles secreted by both Gram-positive and Gram-negative bacteria, and MVs generated from the outer membrane in Gram-negative bacteria are referred to as OMVs, typically comprising phospholipids, lipopolysaccharides, OMPs, and components from the periplasmic space (Toyofuku et al., 2019). OMVs have been proven to play an important role in various biological activities, including facilitation of gene transfer, secretion of virulence factors, removal of damaged proteins, supply of nutrients and ions, and sequester of antibiotics, bacteriophages, and antibodies (Furuyama and Sircili, 2021; Schwechheimer and Kuehn, 2015). OMVs have been recognized as one of the crucial mechanisms employed by bacteria in response to external stimuli, including immune attacks, heat treatment, low pH, oxidative stress, and antibiotics (Klimentova et al., 2019; Lekmeechai et al., 2018; Murray et al., 2020). It is believed that the secretion of OMVs can assist bacterial survival by removing misfolded proteins and altering the membrane structure under diverse environmental stresses (Mozaheb and Mingeot-Leclercq, 2020). In addition, OMVs have been implicated in mediating antibiotic resistance by acting as either bait or barriers for antibiotics (Liu et al., 2022). Previous studies have reported the secretion of OMVs in VBNC cells (Orta de Velasquez et al., 2017; J. Xu et al., 2018). Our results also demonstrated the production of OMVs in *S*. Newport ST45, which exhibited the highest rate of entry into the VBNC state, indicating the potential contribution of OMVs to the survival and formation of the VBNC state induced by PAW. We assume that the secretion of OMVs plays the role of removing damaged protein and easily oxidized components, thereby protecting cells from oxidative stress induced by PAW, which has been reported in previous studies (McBroom and Kuehn, 2007; Orench-Rivera and Kuehn, 2021).

PAW, rich in ROS and RNS, is widely recognized as the primary reactive agent responsible for bacterial inactivation (Cheng et al., 2020; Thirumdas et al., 2018). Therefore, in this study, we measured the expression levels of genes associated with oxidative stress. Three typical genes, *sodA*, *katE* and *trxA*, were found to be upregulated in five *S*. Newport STs after PAW treatment (Fig. 4A). SodA is an important superoxide dismutase disproportionating two molecules of superoxide to O_2 and H_2O_2, which are subsequently eliminated by catalases

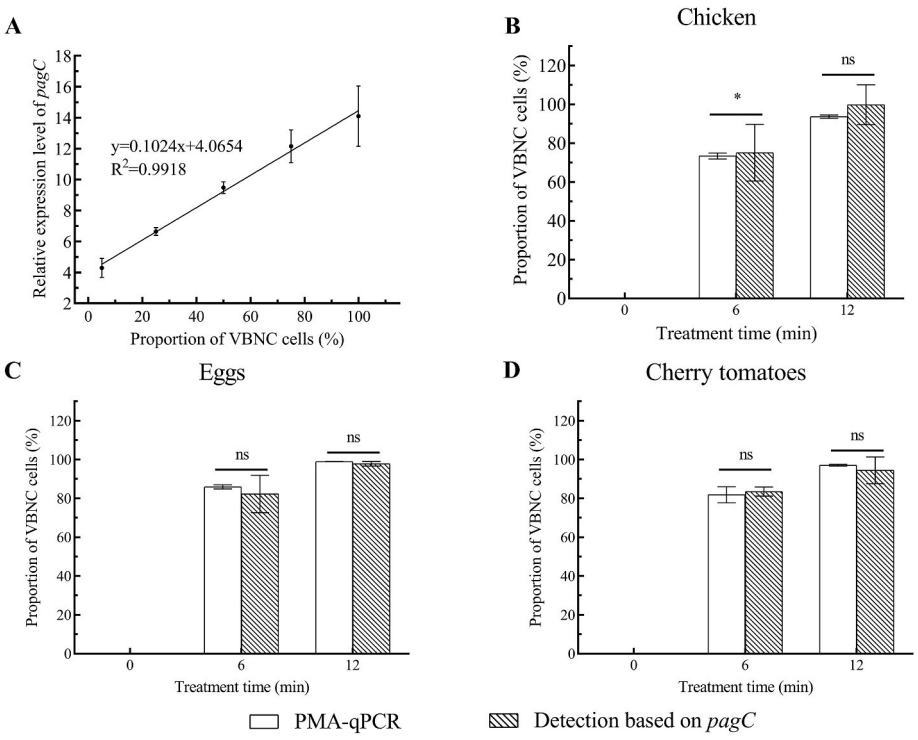

Fig. 6. (A) Correlation curve between the relative expression level of *pagC* and proportion of VBNC cells and the results of the method based on *pagC* in (B) chicken, (C) eggs and (D) cherry tomatoes. * represents the significant difference in results between PMA-qPCR and detection based on *pagC* ($p < 0.05$). ns represents no difference in results between PMA-qPCR and detection based on *pagC* ($p > 0.05$).

(Diaz-Ochoa et al., 2016). KatE is one of the major catalases in *Salmonella*, decomposing hydrogen peroxide into H_2O and O_2 (Hahn et al., 2021). The gene *trxA* encodes thioredoxin-1 which participates in reducing cytoplasmic proteins by acting as the hydrogen donor and can eliminate ROS as the reductant (Zeller and Klug, 2006). A 3-fold upregualtion of *katE* gene was observed in ST45, while it was up to 1.17-fold in other STs. Considering that ST45 exhibited the fastest entry into the VBNC state, KatE may play an important role in the resistance of *S.* Newport to PAW.

ompA, *ompC*, *ompD* and *ompF* are genes encoding porins on the outer membrane, which are related to the passive transport of hydrophilic compounds (Choi and Lee, 2019). In our study, these four genes were upregulated in most *S.* Newport strains when exposed to PAW-induced stress (Fig. 4B). OmpA is an OMP with a smaller pore compared with OmpC, OmpD and OmpF, which is believed to reduce the influx of detrimental factors (Chowdhury et al., 2022a). van der Heijden et al. (2016) found that *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) can reduce the diffusion of H_2O_2 by regulating the opening of OmpA and the closing of OmpC, which has a larger pore size. Chowdhury et al. (2022b) reported that the absence of OmpA led to nitrosative stress in *S.* Typhimurium cells. In addition, the deletion of *ompA* gene in *S.* Typhimurium resulted in the downregulation of *katG*, *katE*, *sodA*, *sodB* and genes encoding *Salmonella* pathogenicity island 2 (SPI-2) effectors, which are related to oxidative and nitrosative stress response (Chowdhury et al., 2022a,b). OmpC has a larger pore than OmpA, which can be closed during oxidative stress through its interaction with TrxA and the heat shock protein Hslt, thereby reducing the influx of H_2O_2 (van der Heijden et al., 2016). We suggested that the upregulation of *ompA* and *ompC* might be conducive to controlling the influx of ROS under PAW stress.

OmpD and OmpF are also important outer membrane proteins in *Salmonella*. In contrast to our findings, OmpD is considered to be detrimental to the survival of *Salmonella* under oxidative stress by promoting the intake of ROS, reported by Calderón et al. (2011) and Aguayo et al. (2015). Darcan (2012) reported that the expression of *ompF* was downregulated in *E. coli* under photooxidative stress. The upregulation of *ompD* and *ompF* observed in our study may be attributed to different strains and conditions inducing oxidative stress. Another possible interpretation is that the upregulation of *ompD* and *ompF* is the compensation response to OMPs loss caused by the secretion of OMVs because OMPs are common components of OMVs (Choi et al., 2011; Kothary et al., 2017; Thoma et al., 2018).

The upregulation of genes associated with virulence, particularly *pagC*, after PAW treatment suggests that *S.* Newport may retain its pathogenic potential even in the VBNC state induced by PAW treatment. This finding aligns with previous studies indicating that *Salmonella* in the VBNC state can maintain its pathogenicity (Highmore et al., 2018; Zeng et al., 2013). Three genes related to virulence were upregulated after PAW treatment, especially *pagC* (Fig. 4C). PagC is a *Salmonella*-specific protein necessary for infection and secretion of OMVs (Kitagawa et al., 2010; Lawley et al., 2006). Besides regulating virulence, *pagC* is also related to environmental stress response, reported by Li et al. (2019). And data from the COLOMBO database align with our findings, showing upregulation of *pagC* in response to oxidative stress in *Salmonella* (Moretto et al., 2015). SipC and SopE2 are virulence proteins that contribute to the invasion of *Salmonella*, produced by the *Salmonella* pathogenicity island 1 (SPI-1) type III protein secretion system (Zhou and Galán, 2001). Kim et al. (2010) observed the upregulation of SipC after H_2O_2 exposure, suggesting that SPI-1 effectors might support the survival under oxidative stress generated by macrophages during infection. Based on our findings, *S.* Newport could express virulence genes although in VBNC state. In addition, the upregulation of *pagC* and *sopE2* in *S.* Newport ST45 strains was significantly higher than other four STs, suggesting strain-specific responses. However, some researchers have also pointed out that the expression of virulence genes and proteins does not necessarily determine the pathogenicity of VBNC cells. As demonstrated by studies on other bacterial species like *Listeria monocytogenes*, the expression of virulence genes alone may not necessarily translate to pathogenicity in vivo (Cappelier et al., 2005; Lindbäck et al., 2010). Therefore, the virulence of VBNC *S.* Newport needs to be further verified.

Expression level of proteins was determined to validate the results of gene expression level. No significant difference ($p > 0.05$) in the changes in protein expression was observed between PAW-treated and untreated groups, except for PagC (Table 4). Instability in mRNA translation in VBNC bacteria might contribute to the inconsistent results between gene and protein expression observed in this work. The reduction in the expression of proteins associated with translation such as ribosomal proteins was observed in VBNC bacteria, indicating a downregulation of translational machinery and leading to a decrease in protein synthesis (T. Xu et al., 2018; Zhao et al., 2016). This phenomenon could be attributed to various factors, including the depletion of cellular energy resources, such as ATP, which is essential for translation (Shan et al., 2017).

Our research also observed a decrease in the ATP level after PAW treatment, consistent with the findings in various VBNC bacteria (Ganesan et al., 2007; Liao et al., 2020; Pu et al., 2019). It's worth mentioning that the ATP level in ST45 was significantly lower than other strains, indicating a correlation between ATP levels and the formation of the VBNC state. Although the formation mechanism of the VBNC state remains unclear, some researchers have proposed that ATP depletion plays an important role (Li et al., 2024; Liao et al., 2020; Yang et al., 2023). Based on our results, we proposed a possible mechanism of the formation of the VBNC state following PAW treatment (Fig. 7). The PAW treatment induced oxidative stress and caused cellular damage in *S.* Newport. In response to PAW stress, the secretion of OMVs was enhanced to remove the harmful substances, and the expression of stress response-related genes was upregulated, potentially leading to a large amount of ATP consumption. To conserve energy, *S.* Newport reduced its metabolism activity and entered the VBNC state as an adaption to the PAW stress.

The combination of PMA-qPCR and the plate count method has been widely utilized for quantifying VBNC cells in previous studies (Golpayegani et al., 2019; Liu et al., 2018; Xiao et al., 2013). However, this approach typically extends more than one day to complete the protocols, and requires additional staining and photo-activation steps due to the use of PMA. Some research has attempted to detect VBNC bacteria based on mRNA, exploiting its short half-life of mRNA which enables it to serve as a reflection of cell viability (Dong et al., 2020). J. Xu et al. (2018) proposed that the protein PagC could serve as a biomarker for detecting VBNC *Salmonella*. In our study, we observed a proportional relationship between the expression of *pagC* and the ratios of VBNC cells, indicating the potential of *pagC* as a biomarker for VBNC *S.* Newport (Fig. 6A). Based on that, we proposed a detection method for quantifying the proportion of VBNC *S.* Newport cells using the *pagC* gene. Our work applied the developed method to assess contamination levels in chicken, eggs, and cherry tomatoes, which are commonly associated with *S.* Newport contamination (Fig. 6 B to C). Our method only requires RNA extraction and qPCR process, offering advantages in terms of reduced time compared with PMA-qPCR. However, further optimization is required to enhance the stability of this method, and additional methods are needed for precise quantification.

5. Conclusions

Our findings indicated that *S.* Newport could enter the VBNC state after PAW treatment. We observed variations in the response among five STs, with ST45 being the most prone to enter the VBNC state. The secretion of OMVs was observed and regarded as the strategy for *S.* Newport to survive under PAW stress. The expression of genes related to oxidative stress, outer membrane proteins and virulence was upregulated as the response to PAW. We proposed that these responses consumed much energy, leading to reduced intracellular ATP levels, ultimately causing the formation of the VBNC state in *S.* Newport. In addition, we established a method based on gene *pagC* to detect the proportion of VBNC cells contaminated in food products. The findings of this work contribute to understanding the mechanism underlying the VBNC state formation and assessing the application of PAW, thus assisting in controlling the risk of *S.* Newport contamination during food processing.

CRediT authorship contribution statement

Yuhao Sun: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Rui Gao:** Conceptualization, Investigation, Data curation, Writing – original draft. **Xinyu Liao:** Conceptualization, Methodology, Data curation, Writing – review & editing. **Mofei Shen:** Methodology, Writing – review & editing. **Xiuqin Chen:** Methodology, Writing – review & editing. **Jinsong Feng:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. **Tian Ding:** Funding acquisition, Supervision, Project Leader and overall

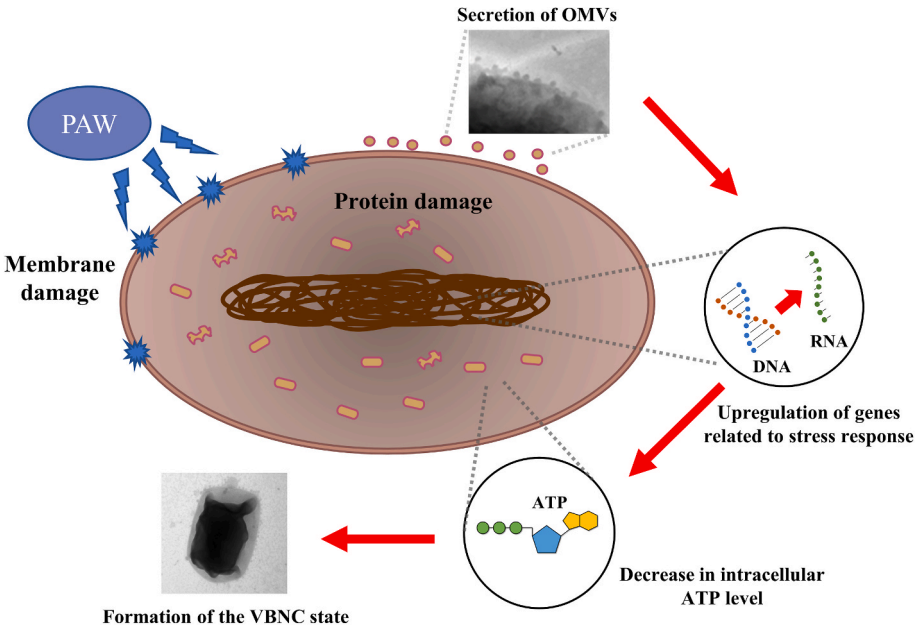

Fig. 7. Schematic diagram of the proposed formation mechanisms of VBNC *S.* Newport under PAW stress.

management of the study, Conceptualization, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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