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Original Article

Production of viable but nonculturable state in *Salmonella* isolates by combination of acidity, osmotic pressure, and freezing

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Summary
This study aimed to investigate the production of viable but nonculturable (VBNC) state in food-borne *Salmonella* strains by the combination of environmental conditions, including acidity, osmotic pressure, and freezing. Three cocktails of *Salmonella* serotypes (*S.* Typhi, *S.* Typhimurium, and *S.* Enteritidis) with the origin of beef and mutton were used in this study. The plate counting method and bacterial resuscitation were used to evaluate the VBNC state. The obtained results showed that the freezing induced the VBNC state in *S.* Typhi, *S.* Typhimurium, and *S.* Enteritidis. Acidic condition (pH 4.5) alone caused the death of all three *Salmonella* serotypes, but the combination of freezing, osmotic pressure, and low pH-induced VBNC state in all three serotypes. The results can be used as a base for further research in this field.
Keywords: Food-borne, Freezing, Resuscitation, *Salmonella*, VBNC.

Introduction

Foodborne pathogenic bacteria are considered as one of the most critical sources of zoonotic diseases (Zhao et al., 2014). This group of pathogens can cause the spread of disease in human and livestock communities and, in this way, will cause a lot of damage (Pajohi Alamoti et al., 2022). *Salmonella* spp. is one of the most important foodborne pathogens, which are classified as zoonotic bacteria. *Salmonella* spp. cause infections and deaths worldwide (even in developed countries) every year. The main source of human contamination by this bacterium is its transmission through food of animal origin, such as meat, poultry, and eggs (Manafi et al., 2020). *Salmonella* spp. can develop VBNC state. The capabilities of a bacterium to establish VBNC state can affect its abilities, such as biofilm formation ability (Li et al., 2020).

The VBNC state in bacteria was discovered and reported for the first time about four decades ago (Xu et al., 1982). Bacteria in the VBNC state do not grow under usual conditions and do not form any colonies in normal culture methods. If the conditions are appropriate, these bacteria are revived and continue to grow by forming colonies (Yan et al., 2021). When the bacterium is in the VBNC state, it becomes difficult to detect and isolate it. Also, the bacterium undergoes morphological and genetic changes in this state,

which can further increase the ability to infect (Xu et al., 1982). Resuscitation of bacteria in VBNC state is one of the significant risks of these pathogens. In these cases, due to the changes they have had, bacteria can show more pathogenicity than before (Wei and Zhao, 2018). The number of microorganisms that have the ability to form the VBNC state has increased. One of the most important conditions that have increased this ability in microorganisms is the conditions related to food storage and processing (Dong et al., 2019). Food-borne bacteria are exposed to the conditions of the food environment, such as osmotic pressure and temperature conditions; such conditions affect the growth and behavioral characteristics of bacteria (Highmore et al., 2018). Given to the studies conducted on the importance of pathogenicity of food-borne *Salmonella* spp. and also the increasing importance of the VBNC state in bacterial pathogenicity (Li et al., 2017), the effect of the conditions related to food storage and processing environments on the induction of VBNC state in *Salmonella* isolates of food origin was investigated in this study.

Materials and methods

Bacterial strains

Seven previously isolated *Salmonella* serotypes were used in the current study (Table 1). The bacterial strains were stored at -20 °C with 30% glycerol. Before the experiments, the bacteria were re-cultured twice in TSB media (Trypticase Soy Broth, QUELAB, Canada) and incubated at 37 °C for 24 h.

Table 1. The origins of bacterial strains used in the current study

Code	Serotype	Origin
21b	*S.* Enteritidis	Beef
32sh	*S.* Typhi	Mutton
44b	*S.* Enteritidis	Beef
43b	*S.* Enteritidis	Beef
49b	*S.* Typhi	Beef
51sh	*S.* Typhimurium	Mutton
52b	*S.* Typhimurium	Beef

Preparation of Bacterial cocktails

A previously described method was used to prepare the bacterial cocktail of each strain with some modifications (Lastra-Vargas et al., 2020). In the first step, the growth curves of all seven serotypes were plotted. Then, based on the growth curve, each strain was cultured in 10 mL of TSB, and then the cocktails were made by mixing 1 mL of the same serotype with a dilution of 1×10^8 CFU/mL.

Osmotic pressure conditions

TSB media were firstly prepared with NaCl (4.5%), and then 1 mL of fresh bacterial suspension was inoculated into 9 mL of these media and incubated at 37 °C for 24 h. Afterward, 100 µL of the above-mentioned culture was transferred to TSB and incubated at 37 °C for 20 h (Shah and Bergholz, 2020).

Acidity conditions

At first, TSB was prepared and acidified to pH of 4.5 using pure lactic acid. Then, 1 mL of fresh bacterial suspension was inoculated into 9 mL of acidified media and incubated at 37 °C for 24 h. Finally, 100 µL of the above-mentioned culture was transferred to normal TSB and incubated at 37 °C for 20 h (Kang et al., 2018).

Freezing conditions

The prepared bacterial suspensions were placed in closed tubes and stored at -20 °C for 96 h. Then, the samples were transferred to TSB and incubated at 37 °C for 20 h (Thongbai et al., 2006).

Combinatory conditions

Bacterial cocktails were exposed to environmental conditions in three treatments (Table 2). One group was also considered as a control group. Bacterial cocktails were inoculated into TSB media with

4.5% NaCl and the pH 4.5 (lactic acid) and then stored in a freezer at -20 °C for 96 h.

Table 2. Combinations of acidity, osmotic pressure, and freezing for induction of VBNC state in *Salmonella* isolates

Treatment	Condition		
	NaCl (%)	pH (Adjusted by lactic acid)	Freezing (h)
1	4.5	7.3	96
2	0	4.5	96
3	4.5	4.5	96
Control	0	7.3	0

Plate Counting Method

The plate counting method with slight modification was used to evaluate the VBNC state induction (Zeng et al., 2013; Li et al., 2020). At first, the bacteria were subjected to acid, osmotic pressure, and freezing treatments. After being exposed to the treatments, the samples were incubated in TSB (Qeulab, Canada) media for 24 h at 37°C, and then the samples were cultured in TSA (Quelab, Canada) media and the colonies were counted. Also, in parallel, the treated samples were incubated in TSB media with 5% of Tween 20® (Polysorbate 20) at 37 °C for 24 h, and Tween 20 was used as a resuscitation agent. Then, the samples were cultured and counted again in TSA media. Differences between the numbers of recovered colonies were considered as bacteria suspected of VBNC status. Schematic of conditions for production and evaluation of VBNC state are shown in Figure 1.

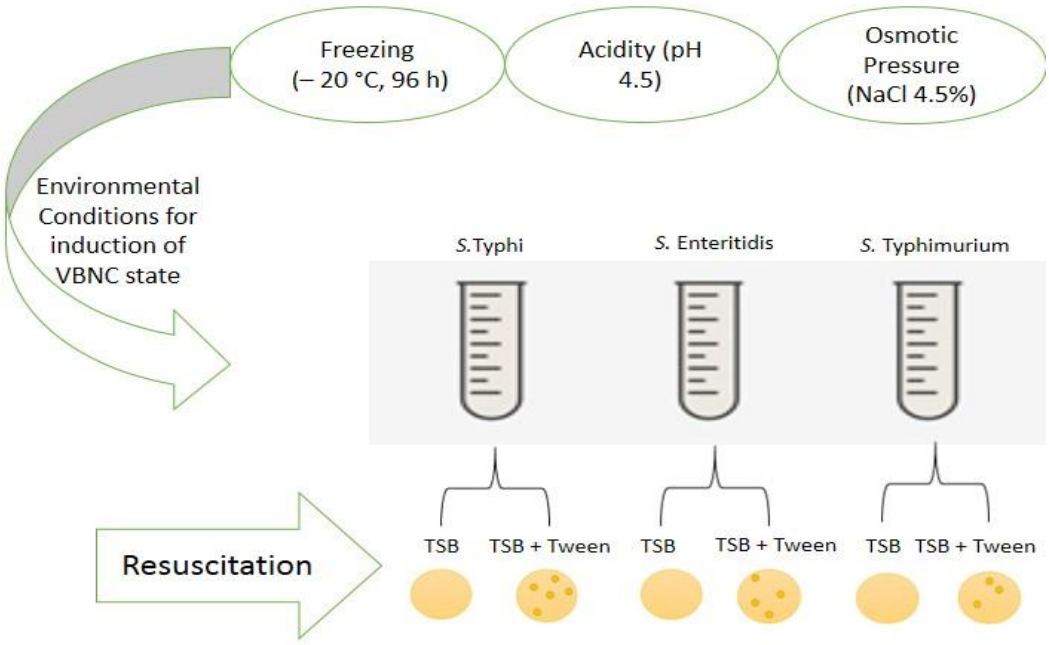

Fig. 1. Schematic of conditions for production and evaluation of VBNC state in *Salmonella* isolates

Statistical analyses

The data were statistically analyzed using SPSS version 20 software. For this purpose, the Univariate Analysis of Variance and a significance level of $P < 0.001$ were used. All experiments were performed in three replicates.

Results and discussion

According to the results, which are shown in Figure 2-4, osmotic pressure (4.5% NaCl), along with freezing (-20 C for 96 h), could not cause a significant change in the growth rate of *S.* Typhi. However, in the case of *S.* Typhimurium and *S.* Enteritidis, it could increase the resuscitated bacteria. In the second treatment, which was the combination of freezing and acidic media (pH 4.5), none of the three strains grew, indicating bacterial death.

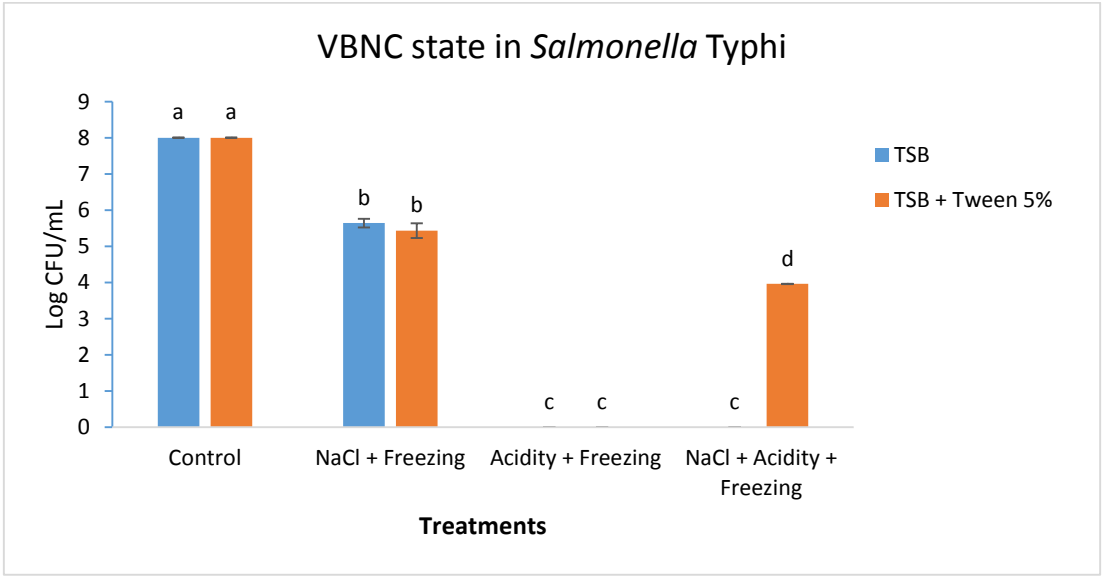

Fig. 2. Effects of treatments on VBNC state induction in *S.* Typhi

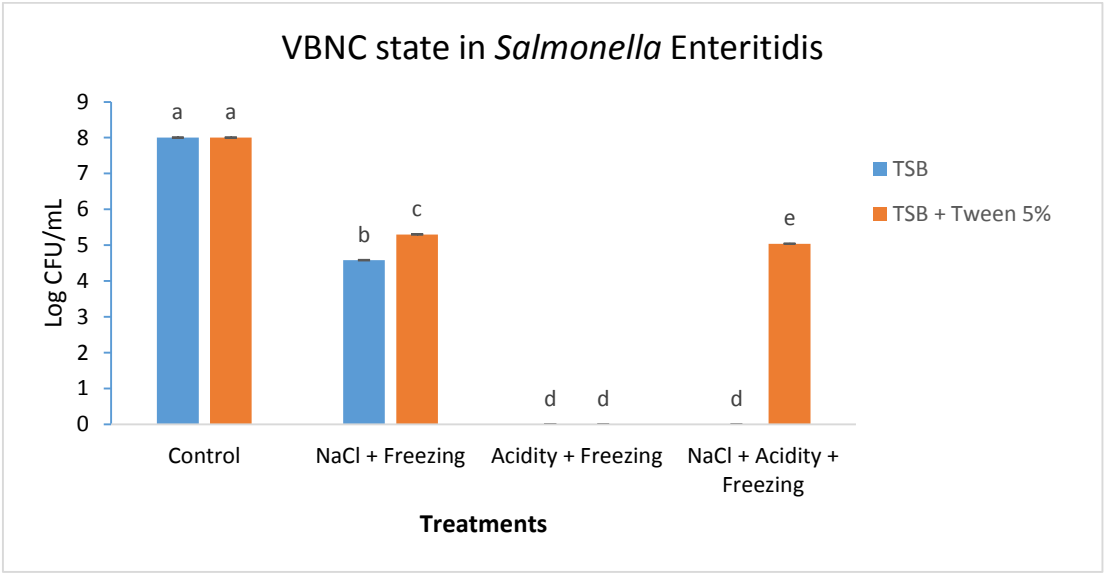

Fig. 3. Effects of treatments on VBNC state induction in *S.* Enteritidis

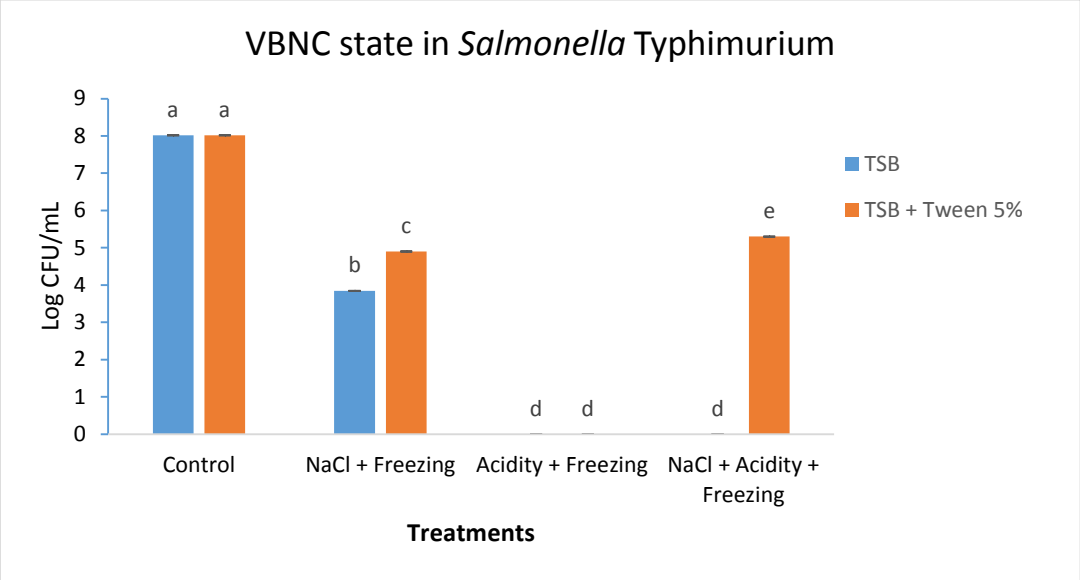

Fig. 4. Effects of treatments on VBNC state induction in *S.* Typhimurium

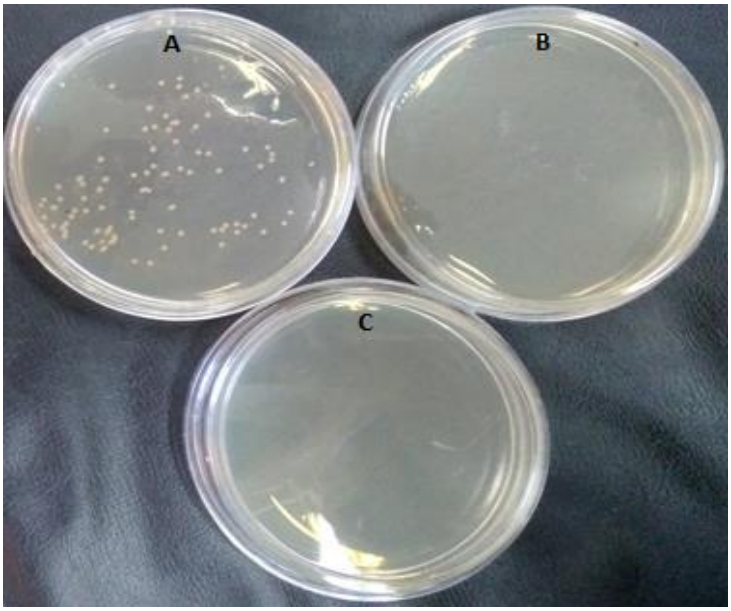

Fig. 5. Resuscitation of VBNC state in *Salmonella* spp.; the presence of resuscitation agents led to the revival of the growth of bacteria in the VBNC state (A: *S.* Typhi with 5% Tween 20, B: *S.* Typhi treated with acidic pH and freezing (with 5% Tween 20), C: *S.* Typhi (without Tween 20).

In the third treatment, which was the combination of osmotic pressure, low pH, and freezing, no growth was observed without resuscitation. After using Tween 20® and resuscitation, the growth in all three strains was observed. The growth was higher in the case of *S.* Typhimurium, pointing to the greater ability of this isolate in survival and formation of VBNC state.

The presence of tween 20 significantly increased the resuscitation of bacteria, which was in the VBNC state. Also, in all three strains, the presence of osmotic pressure caused a decrease in bacterial

growth even in the presence of resuscitation agent, which was significant in all three bacterial strains ($P < 0.001$).

Studies related to VBNC state in pathogenic bacteria are relatively novel and new cases are developing day by day (Truchado et al., 2020). The inability to induce the VBNC state in high acidity has been seen in the case of *Salmonella* spp. (Li et al., 2020). Also, the mentioned study showed that low temperatures and osmotic pressure can induce VBNC state in *Salmonella* spp., which is consistent with the results of our study. The presence of temperature factors, osmotic pressure, and acidity together cause changes in fatty acids in the bacterial cell membrane. This action, together with the changes in the membrane channels, reduces the volume of the cell, a situation in which the bacterial cell can adapt to harsh environmental and nutritional conditions. Therefore, the bacteria are alive in this state, but are not able to grow in an unfavorable environment (Yoon and Lee, 2019). The presence of the resuscitation agent that causes the growth ability to return to the bacteria and is called the recovery of VBNC, was observed in the present study. The resuscitation of bacteria entering the VBNC state has been reported (Zeng et al., 2013). This study showed that cold and freezing can be effective in induction of the VBNC state, which was similar to our finding.

It was reported that the presence of cold temperature along with salt causes the VBNC state in *Vibrio parahemliticus*, and the more the NaCl concentration, the greater number of bacteria entering in VBNC state (Yoon Jae-Hyun, Lee, 2019). In the case of *Staphylococcus aureus*, the existence of the VBNC state due to freezing in the bacteria reduces the enzyme activity of the bacteria. It was also shown that resistance of the bacteria to gastrointestinal fluid was increased after their recovery (Yan et al., 2021).

Conclusion

Environmental conditions such as temperature, osmotic pressure, acidity, and nutritional deficiencies cause changes in the growth and behavior of bacteria, which can play an important role in induction of VBNC state. The results obtained from this study showed that freezing can be one of the most important factors in production of the VBNC state in food-borne *Salmonella* spp. Also, the presence of freezing along with other environmental factors of the food model (pH and osmotic pressure) play a role in creating VBNC state. The induction of VBNC state in pathogenic bacteria in food at different stages of storage and processing can increases the prevalence of these bacteria; therefore, performing further studies in this field are needed to find solutions for managing foodborne pathogens. Since the isolated serotypes could survive and create a VBNC state, they should be further studied to develop methods for controlling them in food and reducing their pathogenic capabilities.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

Ethical approval

Not applicable.

References

Dong K., Pan H., Yang D., Rao L., Zhao L., Wang Y. & Liao X. Induction, detection, formation, and resuscitation of viable but non-culturable state microorganisms. *Comprehensive Reviews in Food Science and Food Safety*, 2019, 1–35.

Highmore C. J., Warner J. C., Rothwell S. D., Wilks S. A. & Keevil C. W. Viable-but-nonculturable *Listeria monocytogenes* and *Salmonella enterica* serovar *thompson* induced by chlorine stress remain infectious. *mBio*, 2018, 9, e00540-18.

Kang I., Kim D., Jeong D., Park J. & Seo K. Heat resistance of *Salmonella* Enteritidis under prolonged exposure to acid- salt combined stress and subsequent refrigeration. *International Journal of Food Microbiology*, 2018, 285, 165–172.

Lastra-vargas L., Aurelio L. M. & Palou E. Modeling *Salmonella* Typhimurium ATCC14028, ATCC cocktails survival under the effects of pH, protein, and essential oil concentration. *Journal of Food Processing and Preservation*, 2020, 44, e14718.

Li J., Feng J., Ma L., de la Fuente Núñez C., Gölz G. & Lu X. Effects of meat juice on biofilm formation of *Campylobacter* and *Salmonella*. *International Journal of Food Microbiology*, 2017, 253, 20–28.

Li Y., Huang T., Bai C., Fu J., Chen L., Liang Y., Wang K., Liu J., Gong X. & Liu J. Reduction, Prevention, and Control of *Salmonella enterica* Viable but Non-culturable Cells in Flour Food. *Frontiers in Microbiology*, 2020, 11, 1859.

Manafi L., Aliakbarlu J. & Dastmalchi Saei H. Antibiotic resistance and biofilm formation ability of Salmonella serotypes isolated from beef, mutton, and meat contact surfaces at retail. *Journal of Food Science*, 2020, 85(8), 2516–2522.

Pajohi Alamoti M., Bazargani-Gilani B., Mahmoudi R., Reale A., Pakbin B., Di Renzo T. & Kaboudari A. Essential Oils from Indigenous Iranian Plants: A Natural Weapon vs. Multidrug-Resistant *Escherichia coli*. *Microorganisms*, 2022, 10, 109.

Shah M. K. & Bergholz T. M. Variation in growth and evaluation of cross-protection in *Listeria monocytogenes* under salt and bile stress. *Journal of Applied Microbiology*, 2020, 129, 367-377.

Thongbai B., Gasaluck P. & Waites W. M. Morphological changes of temperature- and pH-stressed *Salmonella* following exposure to cetylpyridinium chloride and nisin. *LWT - Food Science and Technology*, 2006, 39, 1180–1188.

Truchado P., Gil M. I., Larrosa M. & Allende A. Detection and Quantification Methods for Viable but Non-culturable (VBNC) Cells in Process Wash Water of Fresh-Cut Produce: Industrial Validation. *Frontiers in Microbiology*, 2020, 11, 673.

Wei C. & Zhao X. Induction of viable but nonculturable *Escherichia coli* O157:H7 by low temperature and its resuscitation. *Frontiers in Microbiology*, 2018, 9, 2728.

Xu H. S., Roberts N., Singleton F. L., Attwell R. W., Grimes D. J. & Colwell R. R. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecology*, 1982, 8, 313–323.

Yan H., Li M., Meng L. & Zhao F. Formation of viable but nonculturable state of *Staphylococcus aureus* under frozen condition and its characteristics. *International Journal of Food Microbiology*, 2021, 357, 109381.

Yoon J. H. & Lee S. Y. Characteristics of viable-butnonculturable *Vibrio parahaemolyticus* induced by nutrient deficiency at cold temperature. *Critical Reviews in Food Science and Nutrition*, 2019, 60(8), 1302-1320.

Zeng B., Zhao G., Cao X., Yang Z., Wang C. & Hou L. Formation and resuscitation of viable but Nonculturable *Salmonella typhi*. *BioMed Research International*, 2013, 1–7.

Zhao X., Lin C. W., Wang J. & Oh D. H. Advances in rapid detection methods for foodborne pathogens. *Journal of Microbiology and Biotechnology*, 2014, 24, 297–312.