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Citric acid can force *Staphylococcus aureus* into viable but nonculturable state and its characteristics

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ABSTRACT

Pathogens in viable but nonculturable (VBNC) state can escape traditional detection methods based on culturable ability, thus bringing risks to food safety and human health. Considering *Staphylococcus aureus* as a kind of primary foodborne pathogen, this study attempted to investigate whether citric acid, a food additive commonly used, can force *S. aureus* into VBNC state along with low temperature. Treated with citric acid solution (pH 4.0) at 4 °C, *S. aureus* was confirmed to enter into VBNC state after induction for 18 days. Meanwhile, resuscitation was achieved in culture medium rather than in nutrition-free saline solution. In VBNC cells, ATP concentration still maintained at a high level, as about two-thirds of exponential-phase cells. For survival, intracellular structure of VBNC cells changed remarkably, including irregular cell shape, denser cytoplasm, space between cell wall and cell membrane, and decreased density of nuclear region. Notably, resistance of VBNC cells to simulated gastric fluid improved when compared with exponential-phase cells. What are noted above suggests that VBNC state adopted by *S. aureus* might be a survival strategy to the adverse environment (acidity stress and low temperature). In conclusion, our study sounds an alarm for the safety of citric acid-containing foods.

1. Introduction

Staphylococcus aureus, one of zoonotic pathogens, can cause a variety of infections, from skin and soft tissue to pleuropulmonary infections (Tong et al., 2015), or even death. In addition, it is known that *S. aureus* can secrete different kinds of staphylococcal enterotoxins (SEs) accounting for bacterial alimentary intoxication (Bachert et al., 2012). Generally, it is easy to be contaminated for meat, dairies, eggs, fish and its products (Hennekinne et al., 2012). A great number of countries have experienced the epidemic of *S. aureus*. According to the US Center for Disease Control, alimentary toxicosis caused by *S. aureus* ranked in the second place, and such poisoning incidents in China are not uncommon as well (Zhao et al., 2016a).

In 1982, the concept of "viable but nonculturable" (VBNC) state was firstly described by Xu et al. (1982), and then it was regarded as a physiological self-protection strategy of non-spore-forming bacteria (Oliver, 2010; Orruño et al., 2017). Many kinds of pathogenic bacteria can enter into VBNC state while coping with some adverse conditions, including lower or higher temperature, acidity stress, nutrient starvation, higher pressure, and so on (Zhao et al., 2017). Although VBNC cells still survive and keep active in biological synthesis and metabolism, they cannot form colonies on standard media and be detected through traditional enumeration methods. Moreover, once meeting with the appropriate condition, VBNC cells begin to resuscitate and grow again (Pinto et al., 2015). Therefore, VBNC pathogens pose risks to food safety and human health, and kinds of induction conditions, resuscitation ability and methods, and the biological characteristics of VBNC cells should be figured out for the purpose of evaluating and downgrading the risks.

Up to now, there have emerged some detection methods for VBNC cells, for instance, nucleic acid dye assay, respiration detection, molecular biological assay (RT-PCR and PMA-qPCR), immunological assay and flow cytometry (Zhao et al., 2017). For VBNC cells, the physiological changes do not only take place in morphology, but also in metabolism and resistance to some unfavorable conditions comparing to normal cells. Xu et al. (1982) demonstrated that *Vibrio cholerae* cells became smaller after transferring into VBNC state, and then a great deal of studies emerged referring to the changes of cell morphology in broader varieties of VBNC bacteria. In physiological metabolism, the main characteristics are reduction in material transport capacity, respiratory rate and synthesis of macromolecular substances (Chen et al., 2018; Morishige et al., 2015). Nevertheless, in VBNC cells, there are still ATP concentration at relatively high level (Lindbäck et al., 2010), so is the expression of some genes (Yaron and Matthews, 2002),

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illustrating VBNC cells are still metabolically active and viable. It has not been clarified about resistance so far, but several researchers concluded that VBNC cells did improve their resistance to unfriendly circumstances. Wong and Wang (2004) found that *Vibrio parahaemolyticus* increased resistance to heat (42 °C and 47 °C), low osmotic pressure (0% NaCl) and acid (pH 4) after entering a VBNC state. It was found that VBNC *Escherichia coli* became more resistant to nine typical antibiotics than normal cells (Lin et al., 2017).

Currently, most studies on VBNC state referring to gram-negative bacteria, but there are few studies on the gram-positive one, particularly on *S. aureus*. Clements and Foster (1998) discovered that it was effective to induce a starvation-survival state of *S. aureus* under nutrient limiting conditions, and these cells resumed rapid growth once the nutrition upshifted. Masmoudi et al. (2010) found that *S. aureus* could survive for a long time in natural seawater at 22 °C, while it turned to VBNC state by changing the temperature to 4 °C. Besides, antibiotic pressure was proved to have the ability to induce VBNC state of *S. aureus* when growing in biofilms (Pasquaroli et al., 2013).

Citric acid exists in variety of fruits and vegetables, especially in citrus fruits and is responsible for their tart taste. The concentration of citric acid in citrus fruits ranges from 0.005 mol/L for oranges and grapefruits to 0.30 mol/L in lemons and limes (Apelblat, 2014). As a type of food acids, it is wide utilization in food and beverage, especially in soft drinks and candies (Apelblat, 2014). It is worth mentioning that citric acid can inhibit microbial growth and reproduction by lowering the environmental pH. Whether this kind of unfavorable condition caused by citric acid would stimulate microbial cells to enter into VBNC state? But there is no answer from current studies. Considering food safety, the VBNC state formation under citric acid treatment needs to be researched urgently.

The aim of this research was to investigate the effect of citric acid on VBNC state formation in *S. aureus*, and the resuscitation ability and biological characteristics of VBNC cells were also studied. This research provides data support and theoretical guidance for the study of VBNC pathogens in foods containing citric acid.

2. Materials and methods

2.1. Bacterial strain and culture conditions

The strain used in this study was *S. aureus* NCTC 8325-4, which was kindly donated by Professor Baolin Sun working in the University of Science and Technology of China. Before each experiment, the strain was activated as follows. The stocked strain at −80 °C was streaked on tryptic soy agar (TSA) plate and incubated at 37 °C for a whole day. After that, a single colony was inoculated into tryptic soy broth (TSB), and was incubated at 37 °C and 200 rpm for 12 h.

2.2. Induction of VBNC state of S. aureus by citric acid treatment

The above culture was inoculated into TSB at a dilution of 1:100, and the new mixture was incubated at 37 °C and 200 rpm until it reached to the exponential phase (OD_{600} = 2.8) according to the growth curve (data not shown). Then 10 mL of the exponential phase culture was centrifuged at 8000*g* and 4 °C for 15 min, and the cells were washed twice with sterile 0.85% (*w/v*) NaCl solution under the same condition (Zhao et al., 2013). After that, the washed culture was suspended in 1 L of a citric acid-Na_2HPO_4 buffer solution (pH 4.0) to a concentration of approximately 10^8 CFU/mL, and the solution obtained was named as “induction solution”. The concentration of citric acid in the induction solution is 0.1312 mol/L. Subsequently, the induction solution was placed at 4 °C to induce VBNC state of *S. aureus*. The number of culturable cells in induction solution had been determined every day. In contrast, the control group was conducted in the same procedure, except for substituting the citric acid-Na_2HPO_4 buffer solution (pH 4.0) with a 0.85% NaCl solution (pH 7.0) to confirm the role of citric acid during induction. Two independent experiments were performed.

2.3. Determination of the culturable and VBNC state of S. aureus

In order to determine the number of culturable cells in induction solution, plate counting method was used. The sample was ten-fold diluted with a 0.85% NaCl solution, and pour-plated on TSA. Each dilution sample was measured in triplicate. After incubation at 37 °C for 24 h or 48 h, the number of colonies was counted. When it was < 1 CFU/mL, ten-fold concentration measure was adopted according to the method reported by Zhao et al. (2013). Until the number decreased under detection limit level, i.e. the number of culturable cells below 0.1 CFU/mL, it implied that all survived cells may have entered into a nonculturable state already (Baffone et al., 2003).

In addition, the *S. aureus* cells in the nonculturable state were stained with a Live/Dead *Bac*Light bacterial viability kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions, which was detected by a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, USA) for the percentage of viable cells.

2.4. Resuscitation of VBNC state of S. aureus

Referring to Whitesides and Oliver (1997), the experiments were carried out as following with slight modification. VBNC cells were firstly centrifuged at 8000*g* for 15 min at room temperature and washed with 0.85% NaCl solution (pH 7.0), and then dissolved in TSB (pH 7.0) and 0.85% NaCl solution (pH 7.0) respectively. Next, the two groups (TSB group and NaCl group) were ten-fold diluted with its own solution (TSB or 0.85% NaCl solution). Thereafter, all of them were incubated at 37 °C, and the number of culturable cells was determined after 24 h. Each experiment was performed in triplicate.

2.5. Assay of intracellular ATP concentration in VBNC S. aureus cells

The exponential phase cells (uninduced cells) and VBNC cells were separately transferred to a non-phosphorus test tube, and the cells were pre-treated with an ultrasonic cell breaker (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, Zhejiang, China) to disrupt cell wall for followed ATP and total protein assay. And then the ATP concentration of the samples was determined by the method of colorimetry with ATP Assay Kit and the Total Protein Assay Kit (BCA method), which were both produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Each experiment was performed in duplicate, and each sample was measured in triplicate.

2.6. Microscopic observation of VBNC S. aureus cells

For analysis of intracellular changes between uninduced and VBNC *S. aureus* cells, the transmission electron microscopy (TEM) assay was employed. The method of sample preparation for TEM observation was referred to the report of Zhao et al. (2013). Finally, the sample was examined on a HITACHI H-7650 TEM (Hitachi, Ltd., Japan).

2.7. Resistance of VBNC S. aureus cells to heat and simulated gastric fluid

In order to explore the resistance to heat and simulated gastric fluid, both uninduced and VBNC cells were treated as following, and all the experiments were performed in triplicate.

Untreatment group: One milliliter of bacterial solution was centrifuged at 12,000*g* for 2 min at 25 °C. After the supernatant was discarded, the cells were resuspended with 1 mL of 0.85% NaCl solution filtered through a 0.22 μm filter.

Heat treatment group: Five milliliters of bacterial solution was added to the test tubes preheated to 72 °C or 63 °C, and maintained at 72 °C for 15 s or at 63 °C for 30 min. Afterwards, 1 mL of the treated

samples were immediately added to pre-cooled centrifuge tubes and centrifuged for 2 min (12,000*g*, 25 °C). Then the precipitates were washed once with 1 mL of filtered 0.85% NaCl solution, and finally the cells were resuspended with 1 mL of filtered 0.85% NaCl solution.

Simulated gastric fluid treatment group: Simulated gastric fluid is consisted of hydrochloric acid and pepsin. The pH value of simulated gastric fluid is 1.31 and pepsin content is 1% (*w/v*). One milliliter of bacterial solution was centrifuged for 2 min (12,000*g*, 25 °C), and then the cells was resuspended with 1 mL of simulated gastric fluid and incubated at 37 °C for 2 h. At the end of the treatment, with centrifugation under the same condition, the cells were washed once and finally resuspended with 1 mL of filtered 0.85% NaCl solution.

In order to obtain the percentage of the viable cells treated with heat and simulated gastric fluid, all the cells of the three groups above were stained with the Live/Dead *Bac*Light bacterial viability kit and then examined by the FACSCalibur flow cytometry. The survival rate of the cells with each treatment equals to the ratio of the percentage of the viable cells with and without treatment. Meanwhile, untreated and treated VBNC *S. aureus* cells above were resuscitated to examine their resuscitation ability.

2.8. Statistical analysis

Student's test was performed by using IBM SPSS Statistics 22. The significance level was 0.05.

3. Results and discussion

3.1. Induction of the VBNC state

The changes in the culturable cell numbers of *S. aureus* suspended in citric acid solution (pH 4.0) were shown in Fig. 1. Two distinct phases were presented in the curve, showing rapid decrease in earlier stage and slow decline in the latter. As shown in Fig. 1, the number of culturable cells rapidly decreased from 1.30×10^8 CFU/mL at the initial day to approximately 530 CFU/mL at the fifth day. From then on, it took thirteen days for the culturable cell numbers declined to undetectable levels (< 0.1 CFU/mL), and *S. aureus* cells finally entered into

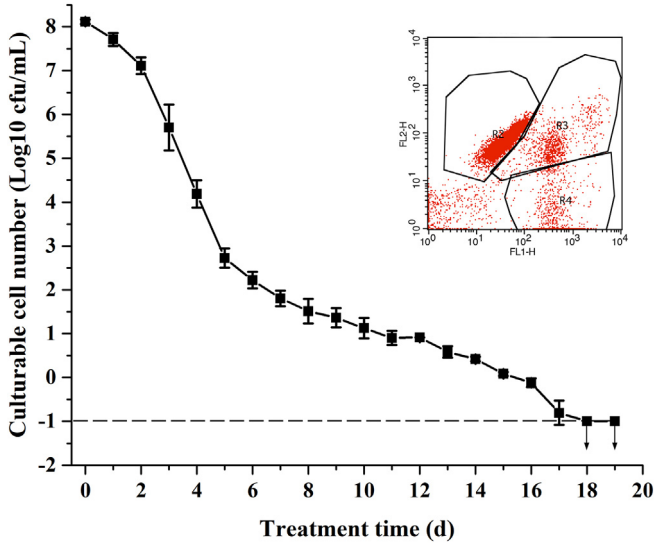

Fig. 1. Survival of *S. aureus* NCTC 8325-4 in citric acid solution (pH 4.0) at 4 °C and flow cytometric analysis of nonculturable cells on the 20th day.
FL1-H, channel for green fluorescence (viable cells); FL2-H, channel for red fluorescence (dead cells). The subpopulations were classified based on their differential staining characteristics: R2: Dead cells (74.20%) that owned debris or lysed cell structures; R3: Damaged cells (11.81%) that owned damaged membranes; R4: Viable cells (9.72%) that owned intact membranes.

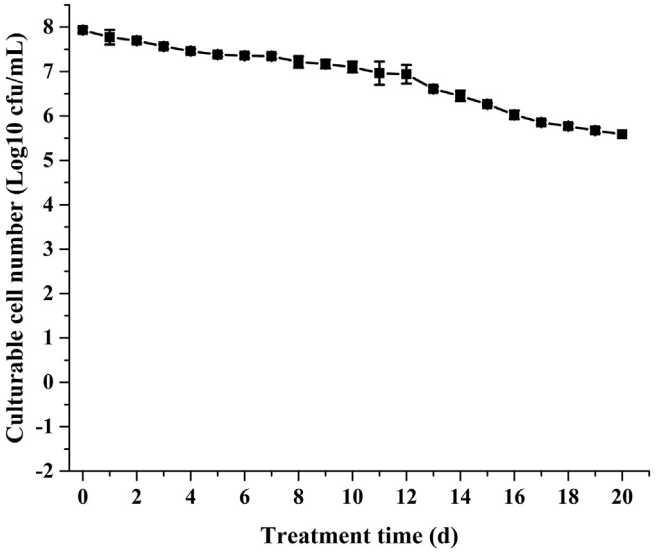

Fig. 2. Survival of *S. aureus* NCTC 8325-4 in 0.85% NaCl solution (pH 7.0) at 4 °C for 20 days.

nonculturable state on the 18th day. Declining types of culturable cell numbers in different VBNC induction researches were variant, such as one stage type (Asakura et al., 2007), two-stage type (Pasquaroli et al., 2014) and three-stage type (Zhao et al., 2013). Although with different declining types, a slow rate of decrease in the last stage was found in some studies as our finding. This phenomenon might be caused by a small number of stress-tolerant cells, which was formed during induction process. However, when incubated in 0.85% NaCl solution (pH 7.0) at 4 °C, there were still 5.86×10^5 CFU/mL of culturable *S. aureus* cells on the 18th day (Fig. 2). According to changes of the number of culturable cells in citric acid and 0.85% NaCl solution under 4 °C, the significant effect of citric acid on VBNC state formation in *S. aureus* cells was unveiled. Certainly, it cannot rule out the role of low temperature in the VBNC state induction. Moreover, colonies on TSA plate gradually became smaller since the 8th day. Unless incubated for 48 h, colonies were invisible to naked eyes. It indicated that reproduction ability of culturable cells became weaker along with the longer time incubated in citric acid solution, which might be a physiological basis for the nonculturable state formation in the cells.

Meanwhile, the percentage of viable cells in the nonculturable cells was measured. As shown in Fig. 1, the percentage of viable cells was accounted for approximately 9.72% of the total population. The proportion of viable cells to the total cells ranges from 0.1% to 15% in the most of studies on VBNC state, thus citric acid solution combined with low temperature is an eligible choice for obtaining more VBNC cells.

Taken all together, the results above demonstrated that *S. aureus* could be induced into VBNC state by citric acid solution (pH 4.0) at 4 °C.

Incubated in seawater at low temperature, it took > 210 days for *S. aureus* 8325-4 entering into the VBNC state (Masmoudi et al., 2010); in contrast, only one month was needed for VBNC state entry in *E. coli* under the same condition (Li et al., 2016). However, so long inducing time would limit subsequent studies on VBNC *S. aureus*. In our study, *S. aureus* 8325-4 could enter into VBNC state on the 18th day when incubating in citric acid solution (pH 4.0) at 4 °C. And it demonstrated for the first time that *S. aureus* could enter into VBNC state by citric acid treatment, which indicate a potential health risk exist in citric acid-containing foods. Cunningham et al. (2009) found that another gram-positive bacterium, *Listeria monocytogenes*, could enter into VBNC state in brain heart infusion (BHI) broth (pH 4.0, adjusted by HCl) at 37 °C with or without potassium sorbate, while the cells were still culturable at the same condition when incubated at 4 °C. This study indicated that

higher temperature combined with low pH were effective in VBNC state formation in *L. monocytogenes*. The difference between the two VBNC induction conditions (low pH combined with low temperature versus low pH combined with high temperature) might be caused by the different growth characteristics of these two bacteria. *L. monocytogenes* is capable of growing at refrigeration temperatures, but *S. aureus* cannot. Therefore, in the study of VBNC state induction, conditions unfavorable to microbial growth should be considered.

It is worth noting that the experimental condition in this study is a simulated condition for citric acid-containing liquid foods stored at 4 °C. In fact, the contamination concentration of *S. aureus* in actual foods is usually lower than that in our study. Several studies have shown that the lower inoculum concentration was, the shorter time entry of VBNC spent (Dinu and Bach, 2011; Nicolo et al., 2011). Therefore, *S. aureus* would enter into VBNC state for < 18 days in actual citric acid-containing foods stored at 4 °C. Nicolo et al. (2011) found that *E. coli* could enter into VBNC state after incubated in grapefruit juice (pH value was approximately 3) for 24 h at 4 °C, and *Salmonella enterica* serovar Typhimurium entered into VBNC state either under the same condition after 48 h. Citric acid exists in grapes and grape juice (Kurt et al., 2017), which may play a role in forcing *E. coli* and *S.* Typhimurium into VBNC state in the grape juice. Based on our study and the data reported by Nicolo et al. (2011), we supposed that *S. aureus* in some soft drinks containing citric acid with low pH would enter into VBNC state in a short period when stored at low temperature. Thus, soft drinks containing citric acid, especially self-made juice without sterilization, should be alerted to the potential risks to consumers caused by VBNC *S. aureus*.

3.2. Resuscitation of VBNC S. aureus

On the 20th day of induction, the resuscitation experiment was carried out. As shown in Fig. S1, the VBNC cells with the dilution of 10^0, 10^{-1}, 10^{-2} and 10^{-3} successfully resuscitated in TSB (pH 7.0) at 37 °C after incubating for 24 h, and the culturable cell counts of these resuscitation samples all exceeded 10^8 CFU/mL. However, the VBNC cells with 10^0 to 10^{-6} dilution did not achieve resuscitation when suspending in 0.85% NaCl solution (pH 7.0) at 37 °C (data not shown).

In our study, a dilution method was applied in the resuscitation assay, which was referred to the method adopted by Whitesides and Oliver (1997) with minor modification. This method can effectively distinguish the real resuscitation of VBNC cells from the regrowth of undetectable culturable cells. The results showed that the VBNC cells diluted 1000 times (the number of culturable cells < 0.0001 CFU/mL) achieved resuscitation in TSB, which indicated that a real resuscitation occurred. Meanwhile, such a success also confirmed that nonculturable cells induced by citric acid were indeed VBNC. Additionally, the NaCl solution (pH 7.0) combined with 37 °C could not resuscitate the VBNC cells. Nothing could be more powerful to prove that only adjusting acidity back to neutral and temperature upshift without nutrient addition was far from adequacy for resuscitation. Up to now, VBNC *S. aureus* were successfully resuscitated by some conditions, such as temperature upshift without nutrient addition (Masmoudi et al., 2010), rich medium supplemented with sodium pyruvate (or culture filtrate of *S. aureus*) as well as temperature upshift (Pasquaroli et al., 2013). The discrepancy among these resuscitation conditions might be caused by the difference in VBNC induction methods. To illustrate effects of resuscitation conditions on the resuscitation ability of VBNC *S. aureus* induced by citric acid, further resuscitation experiments using kinds of resuscitation conditions are carried out in our laboratory.

3.3. ATP concentration in VBNC S. aureus cells

The intracellular ATP concentration of the uninduced cells and the VBNC cells induced on the 20th day was both determined. The ATP concentration of the uninduced cells was 58.30 ± 2.10 mmol/gprot, while the ATP concentration of the VBNC cells (36.74 ± 2.29 mmol/gprot) was about two-thirds of the counterpart's. Such a high ATP concentration also indicated that the *S. aureus* cells treated by citric acid would rather enter into VBNC state than go to death. Lindbäck et al. (2010) also found high ATP concentration in VBNC L. *monocytogenes*. However, to our knowledge, there are very limited researches on direct measurement of ATP concentration in VBNC bacteria. Through two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis, Lai et al. (2009) found that ATP synthase was up-regulated in VBNC *Vibrio parahaemolyticus* cells. Using RNA-seq technique, genes related to ATP accumulation were up-regulated in VBNC *Rhodococcus* sp. TG3 (Su et al., 2016). The corresponding author in this study also discovered that some genes and proteins relevant to ATP production were up-regulated in VBNC *E. coli* cells by RNA-Seq transcriptomics and iTRAQ proteomic methods (Zhao et al., 2016b). Up-regulation of genes or proteins related to ATP accumulation would compensate ATP consumption in VBNC bacteria, which is a strong proof for a high ATP concentration maintenance in VBNC cells. Unfortunately, there is no omics study or other study on the expression of ATP-related genes or proteins in VBNC *S. aureus* cells. Maintaining such a high level of ATP might be a survival mechanism of VBNC *S. aureus* to cope with the adverse environment.

3.4. Morphological analysis using TEM

The uninduced cells and the VBNC cells induced on the 20th day were both observed by TEM. As shown in Fig. 3.A, many of the uninduced cells were in a division phase, and both cytoplasm and nucleic material were evenly separated by newly-formed cell walls. In addition, ribosomes were well-distributed throughout the cytoplasm matrix, and nucleoid located in the cell center owned a compact and condensed structure. Meanwhile, cell walls were intact and smooth with the cell membranes tightly fitted. With the advent of VBNC state, considerable morphological changes in the cells were observed by TEM. Compared with the uninduced ones, the electron density of the cytoplasm was increased in the VBNC cells, and a space between the cell membrane and the cell wall was observed in some of the VBNC cells (Fig. 3.B–D). The space between the cell wall and the cytoplasmic membrane might be caused by the condensed cytoplasm in the VBNC cells. These gaps led to a certain degree of distortion of the cell wall and caused irregular shape of the VBNC cells (Fig. 3.B–D). A study by Chaiyanan et al. (2001) showed similar phenomena. Chaiyanan et al. (2001) found that the cytoplasm in two species of VBNC *V. cholerae* cells both condensed, and pointed out that a dehydration of the cells might be a reason. Ribosomes are one of the most abundant macromolecules in microbial cells. Acting as macromolecular crowders, ribosomes control the rheological properties of the cytoplasm (Delarue et al., 2018). Increase of ribosome concentration would lead to cytoplasmic crowding (Delarue et al., 2018), while excessive crowding can dramatically decrease molecular motion (Miermont et al., 2013), resulting in decrease of the activity of cellular metabolism. In our study, condensation of the cytoplasm would increase ribosome concentration, thus lower the metabolic activity in the VBNC cells. This condensation in cytosol would probably be a survival strategy for VBNC cells to cope with adverse conditions. On the other hand, the nuclear region in the VBNC cells appeared decreased electron density when compared with the uninduced cells, showing a bright hollow (Fig. 3.B) or a radially distributed light-colored structure (Fig. 3.C and D). Other microorganisms capable of entering into VBNC state exhibit the similar phenomenon (Chen et al., 2009; Zhao et al., 2013). The decreased density of the nucleoid in the VBNC cells might be caused by the unwrapping of the supercoiled structure of the nucleic acid material. This change would reduce the activity of gene replication and expression, resulting in the declined cell division ability. Interestingly, VBNC cells in the division phase were observed by TEM (Fig. 3.D). But we know that VBNC cells cannot form colonies on media, i.e. they lose the division ability. It is worthwhile

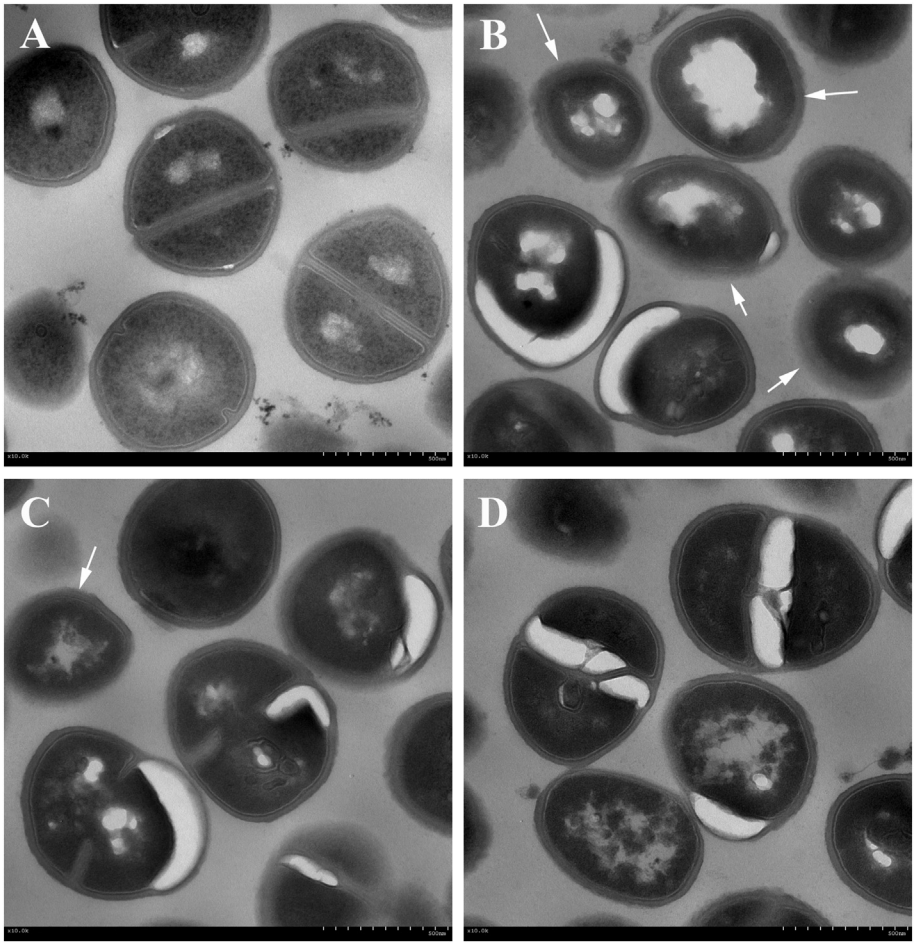

Fig. 3. Transmission electron micrographs of *S. aureus* NCTC 8325-4. A: the exponential-phase cells; B–D: VBNC cells induced with citric acid solution (pH 4.0) at 4 °C for 20 d. Cells pointed by arrows are believed to be dead.
All the images, magnification of ×10.0 k.

noted that large vacuoles between the newly-formed cell wall and cell membrane existed in the "dividing" VBNC cells (Fig. 3.D), which would impair the cell's division ability. These impaired "dividing" VBNC cells probably resuscitated firstly when encountering favorable conditions, which might be the important basis for the resuscitation of VBNC cells. In addition, some cells lost cellular structure, especially the defective cell wall morphology, or contained much less internal staining materials (Fig. 3.B and C, arrow), which are considered to be dead.

All in all, these changes of intracellular structure in *S. aureus* cells might be one of the reasons for VBNC state formation under the citric acid treatment.

3.5. Resistance to heat and simulated gastric fluid

In the heat treatment experiment, two pasteurization conditions for liquid foods sterilization were used. As shown in Table 1, after heat treatment at 72 °C for 15 s, the survival rate of the uninduced group and VBNC group was higher than 90%, while the survival rate of both groups were < 1% at 63 °C for 30 min. This indicated that the latter condition (63 °C and 30 min) could kill VBNC *S. aureus* cells in citric acid-containing foods effectively. Besides, VBNC cells exhibited no resistance changes to heat treatment when compared with the uninduced ones, as the survival rates of both groups at the same heat treatment were statistically insignificant ($P > 0.05$). The result is inconsistent with the result reported by Wong and Wang (2004), who found that VBNC *V. parahaemolyticus* cells performed higher tolerance at 42 °C and 47 °C for 60 min than the mid-exponential phase cells. Perhaps lower temperature combined with long-term treatment may be more conducive to reflect the heat resistance of VBNC cells. In addition, after heat treatment at 72 °C for 15 s, the VBNC cells with the dilution of 10^0 and 10^{-1} successfully resuscitated, but it was difficult to resuscitate when treated at 63 °C for 30 min, which also suggested that the latter pasteurization condition exhibited more effective sterilization effect on VBNC *S. aureus* cells. Considering the resistance of the VBNC cells to heat, appropriate pasteurization conditions should be adopted for sterilization of citric acid-containing foods.

Table 1
Resistance of *S. aureus* NCTC 8325-4 to heat and simulated gastric fluid.

Treatment	Survival rate(%)		*P* value
	Uninduced cells	VBNC cells	
72 °C, 15 s	94.877 ± 6.032	92.964 ± 6.767	0.733
63 °C, 30 min	0.333 ± 0.051	0.536 ± 0.291	0.300
Simulated gastric fluid, 37 °C, 2 h	0.113 ± 0.004	0.184 ± 0.005	0.000

After simulated gastric fluid challenge at 37 °C for 2 h, the survival rate of the two groups were both < 0.2%, suggesting that the gastric fluid in stomach would inactivate most of *S. aureus* cells, including the VBNC cells induced by citric acid (pH 4.0) at 4 °C. It indicated that gastric fluid could effectively protect human health. In Table 1, it turned out that the survival rate of the VBNC group was higher than that of the uninduced group ($P < 0.05$), indicating that *S. aureus* cells significantly improved their resistance to gastric fluid after entering

into the VBNC state. Wong and Wang (2004) also found that VBNC *V. parahaemolyticus* cells were more resistant to acid treatment (pH 4.0) than the mid-exponential phase cells. As the VBNC cells were induced under citric acid (pH 4.0) treatment for 20 d, it was likely to form an acid adaptive mechanism against low pH in the VBNC cells. Thus, VBNC *S. aureus* cells showed relatively higher resistance to lower pH (pH 1.31) than the uninduced ones. Nevertheless, it is worth noting that the VBNC samples used in this experiment contained about 10^7 viable cells/mL, so there was still about 2×10^4 viable cells/mL of VBNC cells left after simulated gastric fluid treatment. Furthermore, the VBNC cells with the dilution of 10^0 successfully resuscitated after simulated gastric fluid treatment. If these viable cells enter into human body and resuscitate, it will bring about risks of foodborne diseases. Therefore, food industries should have an emphasis on sterilization of citric acid-containing foods before consumption to prevent the threats caused by VBNC cells. Meanwhile, methods used to specifically detect VBNC cells should be adopted by food industries to evaluate sterilization effect, as VBNC cells cannot be detected using the conventional plate counting method.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.108254.

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