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# Induction of a viable but non-culturable state in *Salmonella* Typhimurium is correlated with free radicals generated by thermosonication

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ABSTRACT

The viable but non-culturable (VBNC) state in bacteria is gaining more and more attention around the world, mainly due to it is not only an important strategy for adaption to a stressful environment but also may possibly pose a threat to food safety and public health. It was found that a small subpopulation of *Salmonella* Typhimurium in pure culture was induced into a VBNC state during thermosonication (TS) processing in our previous study, though few known about the situation in real food and how bacteria were induced into that special state. Base on the speculation that free radicals generated during TS affected induction of VBNC, the relationship between them was investigated preliminarily. It was observed that higher intensity of TS treatment, such as higher power, elevated temperature and prolonged duration resulted in more viable *S.* Typhimurium cells in carrot juice been inducted into VBNC state. The observed results showed that VBNC incidence indexes were 1000 as TS treatments at 57–62 °C and 380 W for 6 min, and 53 °C and 380 W for 8–10 min were applied, indicating a 100% VBNC state of viable cells in those cases. The ESR spectra revealed three kinds of free radicals, including carbon centered (ethanol) radicals, hydroxyl radical and hydrogen protons were generated in carrot juice during TS processing. The intensity of free radicals was tied to the TS processing parameters and also influenced the occurrence of VBNC. A nonlinear sigmoidal curve of the intensity of free radicals VS the VBNC incidence index in three stages, including a slow phase (with free radicals intensity of 0–0.10), a rapid growth phase (with free radicals intensity ranged 0.10–0.14) and a final equilibrium phase (with free radicals intensity greater than 0.14), was observed and well fitted with the Boltzmann model. Moreover, the significance of free radicals generated during TS processing for induction of VBNC state was verified and confirmed with 0–200 mM sodium pyruvate. The obtained results may contribute to understand the complicated phenomenon and guide the application of TS as a decontamination technique.

## 1. Introduction

It is generally accepted that pasteurization of fruits and vegetables products is vital to both industry and consumers, and thermal sterilization is usually employed. However, it has been found consume time, destroy active ingredients in food, and even produce unpleasant flavors that affect the overall quality of fruits and vegetables products (Mtaoua et al., 2017). Nowadays there are many scholars also vigorously studying application of non-thermal sterilization technologies in fruits and vegetables products. Thermosonication have been proven to have an efficient germicidal effect (Anaya-Esparza et al., 2017; Ferrario et al., 2015; Lee et al., 2009). For instance, Lee et al. (2009) have found that TS treatment could significantly reduce the number of viable *Escherichia coli* K12 in phosphate buffer. Ferrario et al. (2015) also adopted TS to reduce bacteria in nature squeezed apple juice, and observed 5.8-log reduction at 56 °C and 600 W and consequently deemed its availability in sterilization. In our previous study, a up to 7.67-log reduction of *Salmonella* Typhimurium in beef peptone yeast broth subjected to TS (at 53 °C, 380 W, 30 min) was detected using plate counting assay, though 2.84-log viable counts was observed using RT-qPCR assay (Liao et al., 2018). The results suggest that about 99.999% *S.* Typhimurium cells were sterilized by TS in this case, while a pretty small subpopulation of them were induced into a viable but non-culturable (VBNC) state.

VBNC bacteria are under a kind of dormant state in unfavorable terrible environments, and it is generally believed that VBNC state is a unique physiological state that is widely present in non-spore bacteria (Pinto et al., 2015). It cannot be detected by conventional methods such

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as cultivation, and may lead to underestimating in actual tests. With the rapid development of biotechnology, the existence of VBNC state in non-spore bacteria has been confirmed. The VBNC state bacteria are highly resistant to and can survive for a long time under external environmental pressure. Besides that, it is important to emphasize that they may be resuscitated when external pressure is removed or an appropriate environment been given (Amel et al., 2008). It has been reported that factors, such as improper temperature, UV irradiation (Zhang et al., 2015b), hydrogen peroxide (Morishige et al., 2013), oligotrophic environment (Jiang and Chai, 1996), high permeability (Gauthier et al., 1987), heavy metals, chlorine disinfectants (Chowdhury et al., 1997), high pressure $CO_2$ treatment (Zhao et al., 2016) and so on, may induce bacteria into VBNC state. Some of them were disinfection or inactivation technologies, even including non-thermal inactivation technology. It was believed that researching on VBNC state may be conducive to understand those technologies and followed by more efficient usage of them.

To date, few known about how bacteria were induced into VBNC state by TS. Generally, the bactericidal effect of TS is mainly derived from two aspects, including ultrasound and heat. The bactericidal action of ultrasound is mainly derived from its mechanical function and chemical action (Manas and Pagan, 2005). The mechanical effect is mainly derived from cavitation effect and powerful impact force during ultrasonic processing. The chemical action is mainly derived from production of free radicals, thus destroying the internal structure and composition of bacteria (Pingret et al., 2013). Free radicals are an atom or a group that has no electron. It formed when the covalent bond is split apart under the external conditions such as light and heat (Ferkous et al., 2017). Formation of free radicals was proposed to be involved in cellular inactivation in bacteria exposed to severe stress, e.g. by acid under aerobic conditions (Mols et al., 2010). It has reported that UV irradiation and hydrogen peroxide produce free radicals during processing, and both of them are confirmed to be bactericidal and to result in the induction of VBNC state in a subpopulation spontaneously (Li et al., 2014). A number of literatures have reported that TS processing can also produce free radicals (Butz and Tauscher, 2002; Merouani et al., 2015). Nevertheless, what was the significance of free radicals to induction of VBNC state, especially during TS processing? In our previous study, the VBNC incidence index decreased with addition of sodium pyruvate during TS processing, and it was speculated that free radicals produced during TS processing affected induction of the VBNC state in *S.* Typhimurium, as sodium pyruvate acted as a free radical scavenging agent (Liao et al., 2018). A further study of characterization and quantification of the free radicals generated during TS processing and how they interaction with VBNC state should be conducted.

Carrot juice is a widely consumed vegetable juice mainly due to its pleasant taste, high nutritional value and several benefits to human health of carrot. However, undesirable browning reactions leading to discoloration and spoilage microbial growth in fresh carrot juice raise a request of reasonable sterilization process. A few studies have reported effective quality retention and microbial reduction of carrot juice using TS, especially with temperature higher than at 58 °C (Jabbar et al., 2015; Martínez-Flores et al., 2015). However, it was found that most of *S.* Typhimurium cells were killed by TS (53 °C, 380 W, 30 min) in commercial available carrot juice, while a pretty small subpopulation of them were induced into a VBNC state in our previous study (Liao et al., 2018). This study was to investigate the effect of TS treatment on induction of VBNC state *S.* Typhimurium in fresh carrot juice, and to analyze the relationship between induction of VBNC state and free radicals generated during TS processing more importantly, and to ultimately use TS as a disinfection technology to control undesired microbes in food in a better way.

## 2. Materials and methods

### 2.1. Preparation of carrot juice

Fresh carrot was purchased from Auchan supermarket in Wuxi. They were cleaned, peeled, cut into filaments, blanched in 0.2% citric acid (w:v = 1:1) at 90 °C for 30 min, squeezed using a household beater (HB500A, Hauswirt, Qingdao, China) to obtain carrot pulp. Obtained carrot pulp was immediately filtered with a four layers of cheesecloth to collect fresh carrot juice, which was adjusted to pH = 4.5 with 0.2% citric acid. Obtained carrot juice was tinned with glass bottles, refrigerated (4 °C), and was pasteurized (95 °C, 1 min) for later use.

### 2.2. Preparation of bacteria suspension

*S.* Typhimurium CMCC 50115 was obtained from China Microbiological Culture Collection Center (CMCC, Beijing, China) and stored at -80 °C until further multiplication culture with beef peptone yeast medium (BPY, containing peptone 10 g/L, beef extract 5 g/L, yeast power 5 g/L, glucose 5 g/L, NaCl 5 g/L, pH 7.0) followed with selectively grow on SS agar plate. A single colony was picked and transferred to BPY broth, harvest at the early stationary growth phase (at 37 °C for 10 h). Each 10 mL bacteria suspension was centrifuged at 8000 rpm for 10 min. Harvested *S.* Typhimurium cells were washed with sterilized PBS solution (pH 7.0) and resuspended with pasteurized carrot juice to obtain a working solution to a final concentration of $\sim 10^7$ CFU/mL.

### 2.3. TS processing

TS treatments were carried out by using an ultrasonic probe processor (Scientz-IID, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) with a horn micro tip diameter of 6 mm and an operating frequency ranging from 20 to 25 kHz. Each 5 mL samples were treated within a double wall cylindrical vessel (D × H = 1.6 cm × 5 cm) connected to a water bath (DC-0506, Hengping, Shanghai, China). After preheating for 1–4 min, sonication began with temperatures of samples reached at 32, 37, 42, 47, 52, 57 and 62 °C, and hold for 0–10 min. Treatments were performed in a pulsed mode (3 s on/off) with input electric powers of 10%, 20%, 30%, 40% and 60% maximum power of 950 W. After TS processing, samples were collected for immediate tests, including total counts, viability and culturability assays. Samples without TS treatment were adjusted as control (CK).

For TS-generated free radicals detection, 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO, Sigma Aldrich, St. Louis, MO, USA)was used as a spin trap, it was dissolved directly into working solution (carrot juice inoculated with *S.* Typhimurium cells) to a final concentration of 100 mM before TS treatments. All treatments were performed at least in triplicate.

### 2.4. Total counts, viability and culturability assay

For culturability assay, each 200 μL CK and TS treated sample were diluted with aseptic 0.85% NaCl solution and determined by the plate counting method.

For total counts test, each 1 mL CK and TS treated sample were collected to extract genomic DNA using a TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). Reverse transcription reactions (qPCR) adopted the QuantiFast SYBR Green PCR kit (Qiagen, Dusseldorf, Germany) and were performed in a volume of 20 μL, containing 10 μL 2× QuantiFast SYBR Green PCR Master Mix, each 0.6 μL of forward and reverse primers (10 μmol/L), 2 μL DNA template, and 6.8 μL RNase-free water.

For viability assay, each 1 mL CK and TS treated sample were collected to extract RNA using a Simply P Total RNA Extraction (Bioer Technology Co., Ltd., Hangzhou, China). Genomic DNA was removed