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Cronobacter sakazakii local isolates response to acid stress and their resuscitability

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Abstract

Cronobacter spp. has been reported to cause meningitis, necrotizing enterocolitis, and septicemia in a group of infants through the consumption of powder infant formula. These bacteria are reported to withstand various stress conditions such as heating, drying, low water activity, low pH, etc. A local isolate of *Cronobacter sakazakii* YRt2a was reportedly survived and entered Viable But Non-Culturable (VBNC) conditions during desiccation stress. This study aims to study the behavior of local isolates of *Cronobacter* spp. in response to acid stress and its resuscitability. *C. sakazakii* E2 and YRt2a were grown in TSB at pH 3.0±0.2 or 3.5±0.2. The number of culturable cells and viable cells were enumerated by the Total Plate Count and Direct Viable Count methods, respectively. Resuscitation was done by growing the stress or VBNC cells in TSB with or without sodium pyruvate, catalase, Tween 20, or *Cronobacter* autoinducer. The results showed that *C. sakazakii* E2 and YRt2a entered VBNC state after 60 mins of exposure to pH 3.0±0.2, while remained culturable after 120 minutes exposure to pH 3.5±0.2. TSB with or without sodium pyruvate, catalase, Tween 20, or *Cronobacter* autoinducer could resuscitate the stress or VBNC cells of *C. sakazakii*. Stress or VBNC state experienced by *C. sakazakii* in response to acid tends to be transient and can be resuscitated. *C. sakazakii* experiencing stress or VBNC may pose a risk for food safety.

1. Introduction

Cronobacter spp. is able to cause meningitis, necrotizing enteritis, and septicemia in certain of infants through the consumption of powder infant formula. These pathogenic bacteria are reported to be resistant to various stresses such as heating (Arroyo *et al*., 2009; Dancer *et al*., 2009), drying (Breeuwer *et al*., 2003; Dancer *et al*., 2009), low water activity (Dancer *et al*., 2009) and low pH (Dancer *et al*., 2009). These bacteria grow rapidly at pH 5.0, the range of gastric pH for newborns and infants (Álvarez-Ordóñez *et al*., 2014) and have a minimum pH for growth at pH 3.9 (Dancer *et al*., 2009). These bacteria were also reported to experience slow growth at pH 3.5, rapidly lose viability at pH 3.0 (Edelson-Mammel *et al*., 2006), and do not survive at pH 2.5 (Fakruddin *et al*., 2014).

Foodborne pathogenic bacteria respond to stress inside and outside various hosts (Rychlik and Barrow, 2005; Begley and Hill 2015). *Cronobacter sakazakii* responded to stress during contact with the surface of food equipment made of copper alloys (Elguindi *et al*., 2012) or during biofilm formation on stainless steel surfaces (Sinaga *et al*., 2016). Food processing such as drying different atmospheric relative humidities (Lang *et al*., 2017), oven drying (Musa *et al*., 2017), freeze-drying and spray-drying (Wan-Ling *et al*., 2010) can cause bacteria to experience stress. Similarly, exposure to Clidox-S and Quatricide disinfectants (Li *et al*., 2013), desiccation (Elguindi *et al*., 2012; Jameelah *et al*., 2018) and acidification (Edelson-Mammel *et al*., 2006) also cause stress. Exposure of foodborne pathogenic bacteria to stress conditions causes low transcription activity, increased ribosomal RNA degradation, and non-coding RNA regulation (Deng *et al*., 2012), reduced cytoplasmic membranes fluidity (Yang *et al*. 2014), slow enzymatic reactions and metabolic rate decline (Maserati *et al*., 2017).

Yousef and Courtney (2003) describe the stresses faced by foodborne microorganisms vary during the food production process. Microorganisms can experience three stages of stress. First, mild stress that could reduce

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or restrain the growth rate and maintain culturability. Second, moderate stress, which is sublethal stress that reduces or restrains the growth rate and results in the loss of ability to be cultured. Third, extreme or severe stress which is generally lethal and results in the death of most populations. When cells are under stress, cells respond in various ways such as synthesizing protective protein, temporary increase in lethal factor resistance, cell transformation into latent conditions or Viable But Non-Culturable/VBNC, avoidance of host defense mechanisms, and adaptive mutations (Yousef and Courtney, 2003).

Stress conditions in bacteria can induce cells to enter the VBNC state, *i.e.* bacteria can no longer be cultured on conventional culture media but still have viability (Ramamurthy *et al*., 2014). Foodborne pathogens in VBNC state can pose a risk if they continue to be undetected by the standard laboratory tests applied (Highmore *et al*., 2018). Some bacteria experiencing VBNC can be resuscitated inappropriate conditions so that they restore the cells initial state and can be metabolically active again (Oliver, 2005; Oliver, 2010; Pinto *et al*., 2011; Pinto *et al*., 2015). Resuscitation can be done with media such as the autoinducer supernatant (Pinto *et al*., 2011), a medium containing catalase (Na *et al*., 2006; Zeng *et al*., 2013; Imamura *et al*., 2015), Tween 20 (Zeng *et al*., 2013), pyruvate and Luria-Bertani medium (Na *et al*., 2006).

Research on *Cronobacter* spp. response to acid stress and whether they experience VBNC are limited. This study aims to observe the response of *Cronobacter* spp. to acid exposure, their ability to enter the VBNC state and to be resuscitated. Knowledge gained from research on the response to acid stress could explain why the bacteria could survive stomach acid. Study in the resuscitation of VBNC *Cronobacter* spp. can be used for the development of their detection methods.

2. Material and methods

The bacteria used in this study were *C. sakazakii* E2 isolated from follow-up formula (Estuningsih *et al*. 2006) and *C. sakazakii* YRt2a (JF800182) from powder infant formula (Dewanti-Hariyadi *et al*., 2010).

Materials used include Tryptic Soy Agar (TSA) (Oxoid Ltd., UK), Tryptic Soy Broth (TSB) (Oxoid Ltd., UK), Brain Heart Infusion Broth (BHIB) (Merck, Darmstadt, Germany), Brilliance Enterobacter *Sakazakii* Agar (BESA) (Oxoid Ltd., UK), Buffered Peptone Water (BPW) (Oxoid Ltd., UK), Phosphate Buffered Saline (PBS) 10X [700 mL distilled water; 80 g sodium chloride (NaCl); 2 g of potassium chloride (KCl); 14.4 g sodium phosphate (Na2HPO4); 2.4 g monopotassium phosphate (KH2PO4)], sodium pyruvate (0.1% v/v) (100mM; Sigma-Aldrich, USA), catalase from mice liver (1% w/v, 3500 units/mgProt.) (1200 units/mgProt; Sigma-Aldrich, USA), Tween 20 (3% v/v) (Merck, Darmstadt, Germany), *Cronobacter* autoinducer (50% dilution in TSB; 1:1), yeast Extract (YE) 0.025% w/v (Oxoid Ltd., UK), Nalidixic Acid (NA) 0.002% w/v (Sigma-Aldrich, USA), Acridine Orange (AO) 0.0026% w/v (Sigma-Aldrich, USA), Gram's staining, hydrochloric acid (HCL) 10 N, ethanol [70%] and distilled water.

The instruments used include glass or stainless steel microbiology laboratories equipment and general analysis for microbiological laboratories equipment, analytical balance, centrifugation tubes, centrifugation (Hermle Labortechnik, Wehningen, Germany), filter (pore filter size 0.22 µm), pH meter (Eutech pH 700, Eutech Instruments Pte Ltd., Singapore), sterile stainless steel chips 1x1cm (SSC) affixed to the microscope slide, light microscopes (Olympus, Olympus Corporation, USA), and fluorescent microscopes (Olympus, Olympus Corporation, USA).

2.1 Acid stress induction and evaluation of cell culture and viability

A total of 0.1 mL of 18-hour culture of *C. sakazakii* was inoculated into 10 mL TSB media added with 10 N HCl such that it had a pH of 3.0±0.2 or pH 3.5±0.2 (Edelson-Mammel *et al*. 2006) and the initial number of the bacteria was ca. $\pm 10^6$ CFU/mL. Similarity, the same amount of was inoculated into TSB with pH 7.0±0.2 as a control. After incubation at room temperature for 0, 5, 15, 30, 45, 60, 75, 90, 105, and 120 mins samples were taken and the number of culturable cells was enumerated by the total plate count (TPC) method (BAM 2001). The number of colonies was transformed into log values (CFU/mL).

The number of viable cells was calculated by incubating the above samples in mixtures of YE and NA for 18 hrs under dark conditions. A total of 0.05 mL of the sample suspension was pipetted onto the SSC, AO was added and incubated at room temperature in dark conditions for 5 mins. The SSC was then rinsed with sterile PBS and air-dried. The SSC was observed under a fluorescence microscope at a wavelength of 395 nm and emissions of 509 nm. The viable cells were enumerated in ten fields as the cells that fluoresce green light and the results were expressed into Log cells/mL (modification Sinaga *et al*., 2016). The light green fluorescence cells were obtained when AO dyes bind to dsDNA (Neeraja *et al*., 2017). Stress condition is defined when there is a difference between the number of culturable cells and viable cells. Meanwhile, VBNC is defined when there

are no culturable cells on agar media but there are still viable cells as observed under the fluorescence microscope.

2.2 Resuscitation of stressed or VBNC C. sakazakii

Resuscitation of *C. sakazakii* E2 and YRt2a experiencing stress or VBNC state was carried out in TSB medium, TSB with Sodium pyruvate (0.1% v/v) (Sinaga *et al*., 2016), TSB with Catalase from mice liver (1% w/v) (Zeng *et al*., 2013 with modification) or TSB with Tween 20 (3% v/v) (Zeng *et al*., 2013 with modification) as well as in TSB containing *Cronobacter* autoinducer using most probable number (MPN) approach (Pinto *et al*., 2011 with modification).

For resuscitation in TSB containing sodium pyruvate, catalase or Tween 20, as much as 0.1 mL of stress or VBNC *C. sakazakii* was added into 10 mL of resuscitation media and incubated at 37^{o}C for 24 hrs. After incubation, 0.1 mL of the suspension was serially diluted and plated on TSA and incubated 37^{o}C for 24-48 hrs. Enumeration of bacteria after resuscitation in TSB, TSB with sodium pyruvate, TSB with Catalase, TSB with Tween 20 were conducted as above.

Resuscitation in media containing autoinducer was done by the MPN method (BAM 2010). *Cronobacter* autoinducer was made by growing *C. sakazakii* E2 or *C. sakazakii* YRt2a for 18 hrs in TSB. The culture obtained was centrifuged (1789 x *g*, 4^{o}C, 30 mins) and the autoinducer was obtained by filtering the supernatant through a 0.22 µm filter. As much as 0.5 mL suspension of stressed or VBNC cells was added into 4.5 mL of TSB containing *Cronobacter* autoinducer (1:1) and incubated at 37^{o}C for 24 hrs. A set of control was also made in TSB. Enumeration of *C. sakazakii* after resuscitation was made using MPN Table (BAM 2010).

3. Results

3.1 Acid stress induction and evaluation of culturable and viable cells

The response of *C. sakazakii* E2 to acid exposure is presented in Figures 1 and 2, while the response of *C. sakazakii* YRt2a to acid exposure is presented in Figures 3 and 4. Exposure to low pH inhibits the growth of *C. sakazakii* E2 and YRt2a as seen from the decrease in the number of culturable cells. As a control, the number of *C. sakazakii* grown at pH 7.0 increased to 7.69 log for E2 and 7.20 log for YRt2a after 2 hours of incubation.

C. sakazakii E2 (Figure 1) entered stress condition after 30 mins of exposure to pH 3.0, while, *C. sakazakii* YRt2a (Figure 3) entered stress condition after 15 mins of exposure to pH 3.0. The stress condition was shown by the difference in the number of colonies of *C. sakazakii* and the number of the viable cells. At pH 3.0, both isolates reached below the detection limit (1.4 log CFU/mL or <2.5x10^{1} CFU/mL) before 60 mins of incubation. E2 isolate can no longer be cultured (<1 CFU/mL) after 60 mins while YRt2a isolate became unculturable at 120 mins. During the incubation period, the number of viable cells for both isolates were always higher than those of culturable cells (TPC) (Figure 1 and 3). After exposure to pH 3.0 for 60 mins, *C. sakazakii* E2 entered VBNC while *C. sakazakii* YRt2a reached VBNC after in the 120 minutes exposure to acid stress condition.

At pH 3.5, both *C. sakazakii* E2 and YRt2a began to experience stress after 30 mins (Figures 2 and 4). *C. sakazakii* E2 and YRt2a grown at pH 3.5 decreased more slowly than those at pH 3.0. Both isolates also remained culturable up to 2 hrs incubation, although the number of bacteria was lower than the detection limit. The viable cells as seen by the microscope also showed higher count, suggesting that the bacteria experienced stress. These results suggested that at pH 3.5, *C. sakazakii* E2 or YRt2a never entered VBNC state.

3.2 Resuscitation of stressed or VBNC C. sakazakii

C. sakazakii E2 and YRt2a either under stress or VBNC can be resuscitated in various resuscitation media. The resuscitation results of E2 and YRt2a isolates with TSB only and with TSB supplemented with sodium pyruvate, catalase and Tween 20 are presented in Table 1. TSB only can resuscitate the acid stress and VBNC cells as seen by the number of post-resuscitation cells that reach up to the 5-6 log. Cells resuscitated in TSB media supplemented with sodium pyruvate, catalase and Tween 20 had a 1-2 log higher cell number than those

Table 1. The response of stress and VBNC *Cronobacter* spp. in various resuscitation media

Isolates	Cells State	pH value	Time of exposure to acid (Minutes)	Number of bacteria post-resuscitation in the media (Log Cells/mL)			
				TSB	TSB + Sodium Pyruvate	TSB + Catalase	TSB + Tween 20
E2	VBNC	3	60	6.68	8.09*	8.02*	8.20*
YRt2a	VBNC	3	120	5.93	7.62*	8.17*	8.21*
E2	Stress	3.5	120	5.99	7.72*	8.04*	8.13*
YRt2a	Stress	3.5	120	6.06	8.09*	8.17*	8.13*

*Number of bacteria >2.5x10^{7} Cells/mL

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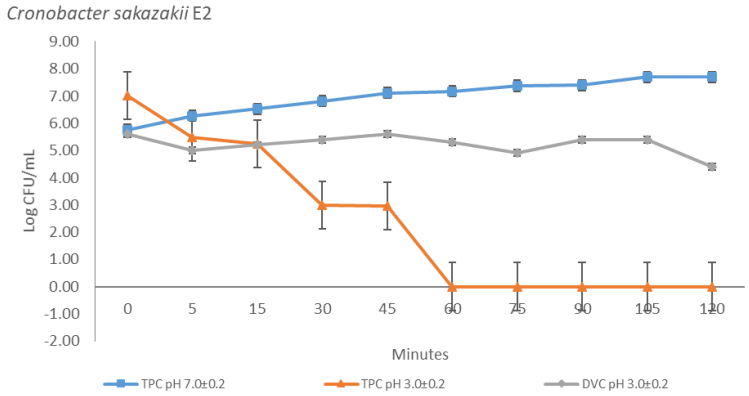

Figure 1. The fate of *C. sakazakii* E2 in response to acid exposure pH 3.0 and its control pH 7.0. (The abbreviations are expressed as TPC pH 7.0±0.2: Culturable *C. sakazakii* grown at pH 7.0; TPC pH 3.0±0.2: Culturable *C. sakazakii* exposed to pH 3.0; DVC pH 3.0±0.2: Viable *C. sakazakii* exposed to pH 3.0)

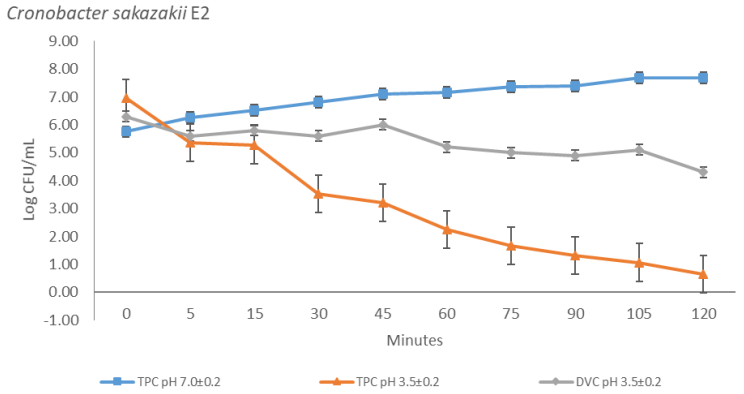

Figure 2. The fate of *C. sakazakii* E2 in response to acid exposure pH 3.5 and its control pH 7.0. (The abbreviations are expressed as TPC pH 7.0±0.2: Culturable *C. sakazakii* grown at pH 7.0; TPC pH 3.5±0.2: Culturable *C. sakazakii* exposed to pH 3.5; DVC pH 3.5±0.2: Viable *C. sakazakii* exposed to pH 3.5)

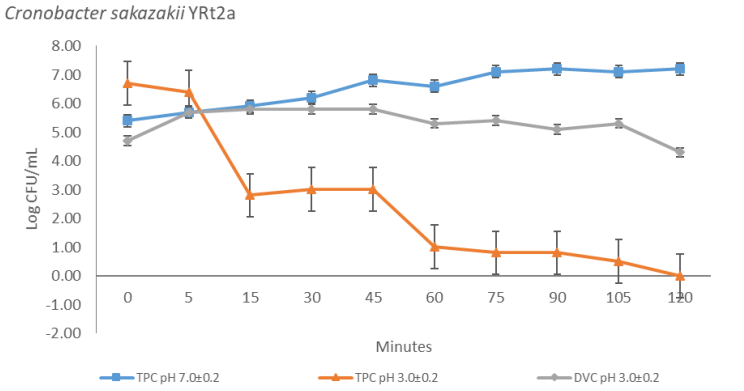

Figure 3. The fate of *C. sakazakii* YRt2a in response to acid exposure pH 3.0 and its control pH 7.0. (The abbreviations are expressed as TPC pH 7.0±0.2: Culturable *C. sakazakii* grown at pH 7.0; TPC pH 3.0±0.2: Culturable *C. sakazakii* exposed to pH 3.0; DVC pH 3.0±0.2: Viable *C. sakazakii* exposed to pH 3.0)

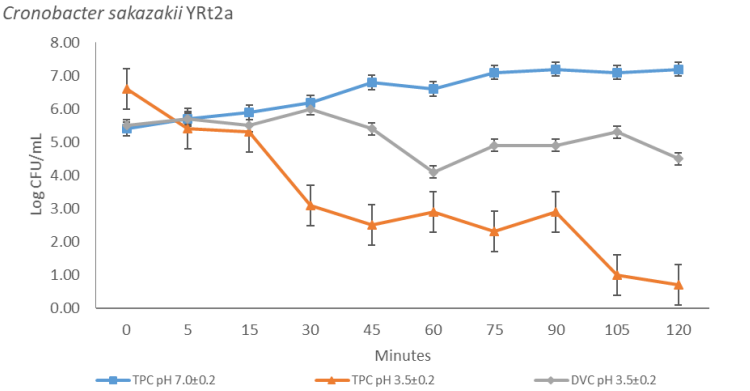

Figure 4. The fate of *C. sakazakii* YRt2a in response to acid exposure pH 3.5 and its control pH 7.0. (The abbreviations are expressed as TPC pH 7.0±0.2: Culturable *C. sakazakii* grown at pH 7.0; TPC pH 3.5±0.2: Culturable *C. sakazakii* exposed to pH 3.5; DVC pH 3.5±0.2: Viable *C. sakazakii* exposed to pH 3.5)

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Table 2. Response of stress and VBNC *Cronobacter* spp. in media containing autoinducer

Isolate	Cells State	pH value	Time of exposure to acid (Minutes)	Number of bacteria post-resuscitation in the Log MPN/mL	
				TSB	TSB + *Cronobacter* autoinducer
E2	VBNC	3	60	4.38	5.04**
YRt2a	VBNC	3	120	3.29	5.04**
E2	Stress	3.5	120	3.34	5.04**
YRt2a	Stress	3.5	120	3.41	5.04**

**Number of bacteria >1.1 x 10^5 MPN/mL

resuscitated in TSB alone. Resuscitation using TSB media supplemented with *Cronobacter* autoinducer (Table 2) also resulted in 1 log higher number than that of the control.

4. Discussion

4.1 Acid stress induction and evaluation of cell culture and viability

The results suggested that *C. sakazakii* E2 and YRt2a exposed to acid for up to 120 mins experienced stress and capable of entering the VBNC state. Both isolates showed sensitivity to acid exposure and rapidly decreased in the number of culturable cells. Wesche *et al*. (2009) stated that acid stress can occur in low pH conditions when H^+ ions cross bacterial membrane cells and acidify the cytoplasm. Loss of cation from a key metabolic could occur due to exposure to a low pH environment, e.g. the loss of Magnesium which is an integral part of ribosome integrity. Shifts in pH can also interfere with the proton motive force and change the protein profile of the outer membrane in Gram-negative cells. This can cause cells to fail to use proton pumps to remove H^+ ions from the cells (Pienaar *et al*., 2019). Acidification also disrupts key biochemical processes (Chung *et al*., 2006). After exposure to acidic conditions, there is an increase in the expression of defense enzymes to help maintain homeostatic pH in cells (Flint *et al*., 2016).

Oliver (2010) suggested that entering VBNC is a strategy of bacterial survival when environmental conditions are not suitable for normal growth. Although VBNC cells cannot be cultured, they can maintain metabolic activity and express toxic proteins (Ding *et al*., 2017), energy activity and respiratory activity (Pienaar *et al.,* 2019), membrane integrity (Lahtinen *et al*., 2008), and main regulatory gene from the general stress response (Kusumoto *et al*., 2012). Pinto *et al.* (2013) explained that cell populations that can be cultured when induced into VBNC conditions will experience a reduction in the number of cells that can be cultured to below the detection limit and other cell populations *i.e.* injury cells, VBNC cells, and dead cells will increase. Wei and Zhao (2018) revealed that the ability and speed of induction of the VBNC state were mainly related to incubation conditions and cells and species of cells.

The VBNC cells of E2 and YRt2a isolates still showed metabolic activity because the number of viable cells was always higher than the culturable cells. The DVC method is a fluorescent staining test to determine cell viability and membrane integrity (Schottroff *et al*., 2018). The fluorescence dye used in this study was acridine orange, a fluorochrome and cationic dye (Neeraja *et al*., 2017). Acridine orange emits green fluorescence light when bound to dsDNA (at 520 nm) and reddish fluorescence light when bound to ssDNA or RNA (at 650 nm) (Neeraja *et al*., 2017). This allows differentiation between viable cells and dead cells. Cells that grow actively or reproduce will glow green. In the meantime, slow or non-reproductive cells will appear orange (Fakruddin *et al*., 2013).

In this study, acid exposure was carried out in TSB media adjusted by HCl. Marieb and Hoehn (2013) explained that HCl as a strong inorganic acid will completely dissociate into protons and chloride ions. Beales (2004) explains that increasing acidity conditions will have an impact on the disruption of cellular component synthesis and induce cell death as a result of damage to the outer membrane, disruption of pH homeostasis, DNA and enzymes.

The results showed that a decrease in the number of *C. sakazakii* E2 and *C. sakazakii* YRt2a due to acid was faster when compared to those reported by several other researchers. Edelson-Mammel *et al*. (2006) showed that *Enterobacter sakazakii* (4.01C, 607, ATCC 29544, ATCC 51329, NQ1-Environ, NQ2-Environ, Environ NQ3, LCDC648, LCDC648, LCDC 674, CDC A3 (10), SK90, EWFAKRC11NNV1493) experienced a decrease in cells after 5 hrs of exposure to pH 3.0 and 3.5. Fu *et al*. (2011) also found that *Cronobacter* NC041, NC1006, and NC830 could not survive at pH 3.0 and 3.5 (pH value <4.0) to 24 hrs of incubation. Additionally, Hsiao *et al*. (2010) reported that *C. sakazakii* BCRC 13988 after 3 hrs of exposure to gastric acid simulations (pH 3.0) was more rapidly decreasing than that exposed to pH 3.5. Our results suggested both isolates, *C. sakazakii* E2, and *C. sakazakii* YRt2a possibly are more

sensitive to acid than the above-reported isolates. The sensitivity of *C. sakazakii* to acid is reported to be varied and strain- as well as acid-dependent (Alvarez-Ordóñez *et al*., 2012; Zhu *et al*., 2013).

C. sakazakii E2 entered VBNC state at 60 mins while YRt2a isolate entered at 120 mins of exposure to pH 3.0. The two isolates did not enter the VBNC state during exposure to pH 3.5 for 120 mins. There have been no previous reports suggesting *Cronobacter* spp. enter VBNC because of acid stress. However, some have reported that *Cronobacter* spp. enter VBNC state because of other stress. Jameelah *et al*. (2018) reported that *C. sakazakii* YRt2a entered the VBNC state after 24 days of exposure to desiccation conditions. Sinaga *et al*. (2016) also reported that during the formation of biofilms YRt2a isolate entered VBNC state on the 63rd day. Moreover, Fakruddin *et al*. (2017) suggested that nutritional deficiencies and low temperatures experience can induce *C. sakazakii* to enter the VBNC state after 40 days of incubation.

The results also showed that local isolates of *C. sakazakii* E2 entered the VBNC faster than *C. sakazakii* YRt2a. This difference in response is thought to be due to the differences in the characteristic of the two isolates. E2 isolate was obtained from a follow-up formula (Estuningsih *et al*., 2006) while YRt2a isolate was isolated from powder infant formula (Dewanti-Hariyadi *et al*., 2010). Previous research stated that E2 isolate was more toxic (Nurjanah *et al*., 2013) and able to survive longer during oven drying (Musa *et al*., 2017) as compared to other local isolates of *C. sakazakii*. Pinto *et al*. (2011) stated that the ability of bacteria to enter the VBNC depends on the strain.

4.2 Resuscitation of stressed or VBNC C. sakazakii

The results show that both stress and VBNC cells of *C. sakazakii* can be revived in the resuscitation media used. Implies that both isolates may experience mild stress or sublethal injury. Li *et al*. (2014) in his review stated that resuscitation can be triggered by various stimulants e.g. increased temperature, nutrient concentration and the presence of local *C. sakazakii* E2 dan YRt2a host cells. Resuscitation process can be mediated by physical stimulants and various types of chemical stimulants. Another trigger for resuscitation is the presence of specific components acting as signals, e.g. amino acids, trigger-resuscitation factors (Rpf) and autoinducers. Pinto *et al*. (2011) stated that factors influencing the success of resuscitation are the strains, conditions that induce VBNC, age of VBNC cells, conditions of resuscitation media, and conditions that induce resuscitation. Based on that idea, the resuscitation process that occurs in stress cells and VBNC of *C. sakazakii* E2 and *C. sakazakii* YRt2a may be triggered by the existing stimulants in resuscitation media.

Our result implied that E2 and YRt2a isolate experience stress due to acidification in the cytoplasm because the cells gain culturability when they are removed into a neutral and rich environment such as TSB. TSB medium may act not only as a physical stimulant but also as a source of nutrients and energy to sustain homeostasis pH in the stressed or VBNC cells. Dancer *et al*. (2009) revealed that TSB has amino acids (glutamate, lysine, decarboxylase) that could increase cytoplasmic pH. The results on both isolates are in-accord with Pienaar *et al*. (2019) who revealed that neutral media can be used for bacterial proliferation and recovery. In addition, Joshi *et al*. (2014) reported that nutrient-rich media such as TSB can recover *C. sakazakii* 29004 and 29544.

Beside physical stimulants, the presence of chemical stimulants *i.e.* sodium pyruvate, catalase and Tween 20 could play a role in the resuscitation of *C. sakazakii* stress and VBNC. Based on the results of the study, TSB supplemented with sodium pyruvate and catalase could increase the number of post-resuscitation cells E2 and YRt2a isolates. These imply that both isolates possibly accumulate hydrogen peroxide (H_2O_2) during stress. Oliver (2010) stated H_2O_2 is one factor that causes bacteria to enter VBNC. Kawasaki and Kamagata (2017) claimed that the presence of H_2O_2 binding agents e.g. catalase and sodium pyruvate is known to effectively degrade H_2O_2. Both of these compounds bind H_2O_2 in the media. Subsequently, sodium pyruvate reacts with H_2O_2 to produce acetic acid, CO_2 and H_2O_2. Meanwhile, catalase converts H_2O_2 to O_2 and H_2O through its enzymatic activity. Al-Holy *et al*. (2008) reported that the addition of sodium pyruvate (0.1%) could recover *Enterobacter sakazakii* cells (ATCC 12868, ATCC 29004, FSM 292, FSM 287) experiencing acid stress. Morishige *et al*. (2013) explained the addition of pyruvate triggers macromolecular biosynthesis activities such as DNA synthesis, protein synthesis, activating certain metabolic pathways for energy metabolism including the reduction of respiratory activity on CTC until the VBNC cell population regains the ability to be cultured. Fakruddin *et al*. (2017) have reported that VBNC *Cronobacter sakazakii* cells can be resuscitated by the addition of catalase. Catalase degrades the accumulated of H_2O_2 in the cells (both externally and internally), thus increasing the cells' ability to be cultured. This study indicates that C. *sakazakii* E2 and YRt2a could be revived by catalase, therefore very likely the bacteria produce H_2O_2 during stress. Tween 20 is another chemical stimulant that works for the resuscitation of stress and VBNC cells of E2 and YRt2a

isolates. Peres *et al*. (2011) stated that Tween 20 is a carbon source. During stress, E2 and YRt2a isolates may experience deficiencies in carbon sources, thus Tween 20 was then used by both isolates as carbon sources to increase nutrient absorption and energy metabolism. The availability of energy sources can improve the colony formation thus the ability to be cultured. Addition of Tween 20 (3%) has been reported to resuscitate VBNC cells of *Escherichia coli* O157: H7 (Afari and Hung 2018) and *Salmonella enterica* serovar Typhi (Zeng *et al*., 2013).

We also found that *Cronobacter* autoinducer can resuscitate stress and VBNC cells of *C. sakazakii*. The results suggested that there were signals in *Cronobacter* autoinducer compounds that help stimulate recovery. Research conducted by Weichart and Kell (2001) revealed that *E. coli* supernatant triggered or facilitated cell division during recovery, accelerated subsequent recovery and growth. The active components in the supernatant were dialysable, heat-stable, acid-stable, alkali-stable, proteinase-stable, small, non-protein and non-ionic organic molecules. Our result was similar to Pinto *et al*. (2011) who reported that autoinducer obtained from the culture supernatant of *E. coli* could resuscitate *E. coli.* The supernatant is a heat-stable enterobacterial autoinducer that was used to resuscitate VBNC cells of *E. coli*.

Overall, the local isolates *C. sakazakii* E2 and YRt2a experiencing stress or VBNC could be resuscitated and gain culturability. This can be seen from the formation of colonies after treatment in resuscitation media such as TSB, TSB with sodium pyruvate, TSB with catalase, TSB with tween 20 or TSB with *Cronobacter* autoinducer. The ability of both isolates to be resuscitated provides an opportunity to develop enrichment media for the detection methods of the bacteria, especially *Cronobacter* spp. that experience acid stress such as those in acid foods.

5. Conclusion

Local isolates *C. sakazakii* E2 and YRt2a experienced stress and entered VBNC state when exposed to pH 3.0. Likewise, acid-induced stress also occurred at ph 3.5 but the bacteria remained culturable. E2 isolate decreased faster during the period of acid exposure and showed more sensitivity to acid as compared to YRt2a isolate. Furthermore, Placing stressed and VBNC cells of *C. sakazakii* E2 and YRt2a in TSB at pH 7.0 was able to resuscitate the bacteria. Addition of chemical stimulants such as sodium pyruvate, catalase or tween 20 and the presence of specific components *Cronobacter* autoinduction further resuscitated and increase the culturability of *C. sakazakii* stress and VBNC. The ability of both isolates that can survive in low pH conditions and can be resuscitated increases the risk of food safety. Further research is needed to determine the effects and mechanisms of acid exposure on the expression of regulators, virulence, stress, VBNC and resuscitation genes.

Conflict of Interest

The authors declare no conflict of interest.

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