



Formation and Control of the Viable but Non-culturable State of Foodborne Pathogen *Escherichia coli* O157:H7

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As a common foodborne pathogen, *Escherichia coli* O157:H7 produces toxins causing serious diseases. However, traditional methods failed in detecting *E. coli* O157:H7 cells in the viable but non-culturable (VBNC) state, which poses a threat to food safety. This study aimed at investigating the formation, control, and detection of the VBNC state of *E. coli* O157:H7. Three factors including medium, salt, and acid concentrations were selected as a single variation. Orthogonal experiments were designed with three factors and four levels, and 16 experimental schemes were used. The formation of the VBNC state was examined by agar plate counting and LIVE/DEAD[®] BacLight[™] bacterial viability kit with fluorescence microscopy. According to the effects of environmental conditions on the formation of the VBNC state of *E. coli* O157:H7, the inhibition on VBNC state formation was investigated. In addition, *E. coli* in the VBNC state in food samples (crystal cake) was detected by propidium monoazide–polymerase chain reaction (PMA-PCR) assays. Acetic acid concentration showed the most impact on VBNC formation of *E. coli* O157:H7, followed by medium and salt concentration. The addition of 1.0% acetic acid could directly kill *E. coli* O157:H7 and eliminate its VBNC formation. In crystal cake, 25, 50, or 100% medium with 1.0% acetic acid could inhibit VBNC state formation and kill *E. coli* O157:H7 within 3 days. The VBNC cell number was reduced by adding 1.0% acetic acid. PMA-PCR assay could be used to detect *E. coli* VBNC cells in crystal cake with detection limit at 10⁴ CFU/ml. The understanding on the inducing and inhibitory conditions for the VBNC state of *E. coli* O157:H7 in a typical food system, as well as the development of an efficient VBNC cell detection method might aid in the control of VBNC *E. coli* O157:H7 cells in the food industry.

Keywords: *E. coli* O157:H7, VBNC, food system, control, detection

HIGHLIGHTS

- The induction of the VBNC state of *Escherichia coli* by nutritional conditions, acetic acid concentration, and salt concentration was investigated.
- In crystal cake, the VBNC state of *E. coli* could be inhibited by adding 1.0% acetic acid in volume fraction at 4 or -20°C .
- Propidium monoazide–polymerase chain reaction (PMA-PCR) assays could be applied in detection of the VBNC state of *E. coli*.

INTRODUCTION

Escherichia coli is one of the major bacterial contaminants associated with foodborne infections worldwide (Zhao et al., 2010b; Sayad et al., 2016; Bao et al., 2017a,b; Xie et al., 2017a; Liu et al., 2018a,b; Renuka et al., 2018; Xu et al., 2020). Since food safety accident caused by *E. coli* was first reported in the United States in 1982, various reports have been posted in China, the United Kingdom, Japan, Australia, and other countries. In recent years, *E. coli* has gradually developed into a health and safety issue of worldwide concern (Xu et al., 2007, 2008a,b, 2011b, 2016a,b; You et al., 2012; Lin et al., 2016; Miao et al., 2016, 2017a,b, 2018; Zhao et al., 2018a,b). The high incidence rate of *E. coli* in food products underlies the urgent need of appropriate strategies for early detection (Zhao et al., 2010b,c, 2011, 2014 2018; Wang et al., 2011; Xu et al., 2011c, 2012b; Lin et al., 2017; Miao et al., 2017c; Liu et al., 2019; Miao et al., 2019). *E. coli* O157:H7, capable of producing Shiga toxin or Shiga-like toxin, can cause hemorrhagic colitis and hemolytic uremic syndrome in human beings (Neil et al., 2012). Shiga toxin causes various diseases including bloody diarrhea and severe hemolytic uremic syndrome, leading to kidney failure. *E. coli* O157:H7 is a typical foodborne pathogen, colonizing in food systems and drinking water (Ercumen et al., 2017; Bourelly et al., 2018; Zeinhom et al., 2018).

The standard detection method for foodborne pathogens is agar plate counting (Xu et al., 2008a,b, 2009; 2010; 2011a; 2012a; 2016c; 2017a; 2017b; 2018; Zhong et al., 2013; Zhang et al., 2015; Bao et al., 2017c; Xie et al., 2017b; Jia et al., 2018; Wen et al., 2020). However, this method fails in detecting viable but non-culturable (VBNC) state cells that were first reported in 1982 (Xu et al., 1982). Up to now, 85 types of microorganisms have been found to enter the VBNC state, including 18 non-pathogenic bacteria and 67 pathogenic bacteria (Ramamurthy et al., 2014). Studies have shown that *E. coli* has the ability to enter the VBNC state (Yaron and Matthews, 2002; Zhang et al., 2015; Liu et al., 2017b; Afari et al., 2019). VBNC cells differ from normal cells in both physiology and morphology. However, VBNC cells acquire high ATP concentrations (Lindback et al., 2010) and metabolic activity based on the expression of certain genes (Yaron and Matthews, 2002).

Bacteria in the VBNC state fail to form colonies in routine detection agar plates under certain environmental stresses. However, these cells have food spoilage and pathogenic capacity (Liu et al., 2017b). Factors including adverse nutrition levels,

temperatures, and osmotic pressures, which are frequently encountered during food processing and storage environments, have been reported to induce bacteria entering the VBNC state. With the failure in detection by routine method and the capability in causing food safety problems, the VBNC state of *E. coli* O157:H7 is a major concern in the food industry (Liu et al., 2017b).

Crystal cake is a traditional Chinese snack that has the characteristics of rich nutrition. Its rich nutrients and sufficient water make it a natural medium for various pathogenic bacteria and spoilage bacteria, including *E. coli*. In this study, crystal cake was applied as a representative food system to study the formation, control, and detection of the VBNC state of *E. coli* O157:H7 under food processing and storage conditions.

MATERIALS AND METHODS

Cultivation of *E. coli* O157:H7

Escherichia coli O157:H7 ATCC25922 was stored at -80°C in Luria-Bertani (LB) broth containing 20% (v/v) glycerol. It was streaked on LB agar plate and grown at 37°C for 24 h. Then, a single colony was inoculated into 2 ml of LB broth and incubated at 37°C for 12 h using a shaker incubator set at 150 r/min. The bacteria suspension was diluted 1:100 in fresh medium and cultured for 4 h to the log phase upon further experiments.

Induction of the VBNC State

According to the optimal culture conditions of *E. coli* O157:H7, and the principle of reverse adjustment, conditions that are unfavorable to bacterial growth were selected as candidate factors to induce the VBNC state. Three factors including nutritional state, salt concentration, and acidity were taken as a single variable. To investigate possible VBNC induction conditions, three factors and four levels of orthogonal experiments were designed, and 16 protocols (Table 1) were applied. Log phase cells were centrifuged for 10 min at 5000 r/min and the pellet was washed once with $1 \times$ phosphate buffer solution and then resuspended in the induced solution. The initial concentration of the bacterial solution was diluted to approximately 10^7 CFU/ml. To avoid the effects of repeated freezing and thawing, the bacterial suspension under each condition was mixed and divided into multiple 1.5-ml centrifuge tubes, followed by induction in refrigerators at 4 and -20°C , respectively.

Determination of Culturable Cell Number During Storage

The culturable cell number in each of the VBNC inducing bacterial culture was determined every 3 days by a plate counting method. Briefly, the culture of *E. coli* O157:H7 was serially diluted in 0.9% NaCl, spread on LB agar plates, and incubated at 37°C for 24 h. When the culturable number is 1 CFU/ml, the cells were considered non-culturable and possibly enter the VBNC state (Piao et al., 2019).

Determination of Viable Cell Number

To determine if the non-culturable cells are in the VBNC state after exposure to the respective treatment conditions, the

TABLE 1 | The experimental methods of orthogonal array design of VBNC induction of *E. coli* O157:H7.

Protocol number	LB medium concentration (%)	NaCl concentration (m/v) (%)	Acetic acid concentration (v/v) (%)
1	0	0.9	0
2	25	0.9	0.3
3	50	0.9	0.7
4	100	0.9	1
5	25	5	0
6	0	5	0.3
7	100	5	0.7
8	50	5	1
9	50	10	0
10	100	10	0.3
11	0	10	0.7
12	25	10	1
13	100	15	0
14	50	15	0.3
15	25	15	0.7
16	0	15	1

TABLE 2 | Inhibition assay of acidity on the formation of VBNC state of *E. coli* O157:H7.

Protocol number	LB medium concentration (%)	NaCl concentration (m/v) (%)	Acetic acid concentration (v/v) (%)
1	100	10	0.7
2			1
3	100	15	0.7
4			1
5	50	15	0.7
6			1

LIVE/DEAD® BacLight™ bacterial viability kit (Thermo Fisher Scientific, China) was used. Five hundred microliters of the non-culturable bacterial cell sample was centrifuged at 5000 r/min for 15 min, washed with saline twice, and resuspended in saline. Subsequently, 1.5 µl of SYTO 9 dye and 1.5 µl of propidium iodide were added to the sample, followed by mixing and 30 min incubation in the dark. After incubation, 5-µl cells were captured between a slide and a coverslip and used for fluorescence microscopy. The appearance of green cells indicates the existence of VBNC cells.

Inhibition of VBNC State by Acidity and Nutritional Conditions

According to the VBNC state induction process, appropriate conditions were selected to inhibit the formation of the VBNC state. The configured medium was resuspended, and the initial concentration of *E. coli* O157:H7 was adjusted to 10⁷ CFU/ml. The bacterial solution with a volume of 30 ml was stored at 4 and -20°C, respectively. The number of cultivable bacteria was measured by plate counting every 3 days (Tables 2, 3).

TABLE 3 | Inhibition assay of nutritional status on the formation of VBNC state of *E. coli* O157:H7.

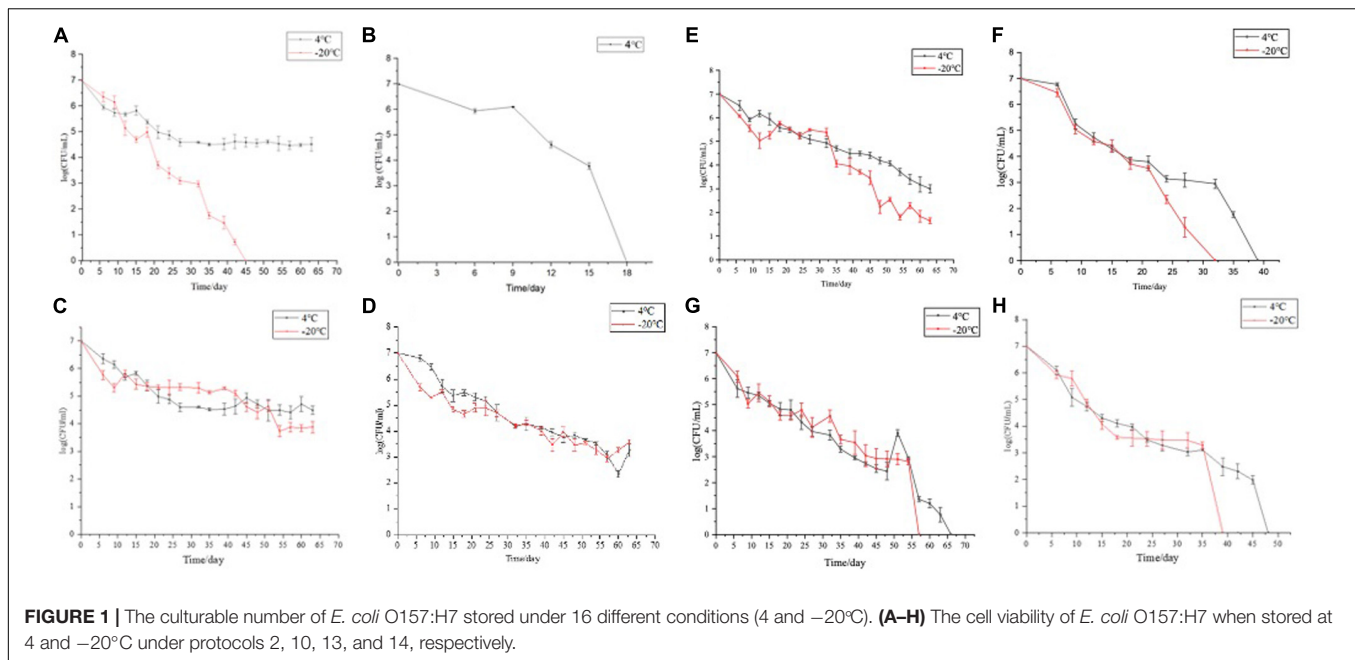
Protocol number	LB medium concentration (%)	NaCl concentration (m/v) (%)	Acetic acid concentration (v/v) (%)
1	0	10	0.3
2	25		
3	0	15	0
4	25		
5	0	15	0.3
6	25		

Elimination of the VBNC State in Crystal Cake

According to the National Food Safety standards (GB4789.3-2016) in China, 25 g of crystal cake was added to 225 ml of saline, and the concentration of this medium was determined to be 100, 25, and 50% of the food sample culture medium and is configured by dilution and used after autoclaving. Subsequently, *E. coli* O157:H7 was cultured to the logarithmic phase and centrifuged at 4°C, the supernatant was discarded, and the suspension was resuspended three times with physiological saline. The bacterial cells were resuspended using the sterilized food sample medium, and the concentrations of *E. coli* O157:H7 were adjusted to 10⁷ CFU/ml, respectively, and a sterile acetic acid solution was added to fix the volume fraction of acetic acid to 1.0%. The induction solution was stored at 4°C, and the culturable number and activity were detected by a plate method combined with a LIVE/DEAD® BacLight™ bacterial viability kit.

PMA-PCR Assays

Twenty-five grams of crystal cake was mixed with 225 ml of physiological saline and sterilized, and *E. coli* in the VBNC state was added. Bacterial suspensions with initial concentrations of 10⁶, 10⁵, 10⁴, 10³, 10², and 10 CFU/ml were obtained. Five hundred microliters of the bacteria suspension was added to a 1.5-ml centrifuge tube, and the PMA working solution was added to a final concentration of 5 µg/ml (to distinguish between the VBNC state and dead cells). After mixing, the samples were kept at room temperature for 10 min in the dark. Subsequently, the centrifuge tube was placed on ice, and 15 cm away from a 650 W halogen lamp for 5 min to complete the binding of PMA and DNA. The PMA-DNA was centrifuged at 10,000 r/min for 5 min, and the supernatant was discarded. DNA was then extracted using a bacterial group DNA extraction kit (Dongsheng Biotech, Guangzhou). The extracted DNA was detected by PCR. PCR assay was performed in a 25-µl volume and with 0.6 µM primers (*rfbE*-F: TTGGCATCGTGTGGACAGGGTAGGACCGCAGAGGAAAG A; *rfbE*-R: TGGGACAGGTGTGCTACGGTTTCCACGCC AACCAAGATC). The thermal profile for PCR mixtures was 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52°C for 60 s, and 72°C for 90 s and a final extension cycle at 72°C for 7 min. The amplified products (5 µl/well) were analyzed by gel electrophoresis in 2% agarose gels and stained with ethidium



bromide for 10 min. A negative control was included using sterile water instead of culture or DNA template.

RESULTS AND DISCUSSION

Formation of the VBNC State

To induce the formation of the VBNC state, 16 induction solutions were applied (Table 1). In induction solution 1, when stored at 4°C , the culturable cell number of *E. coli* O157:H7 was reduced to 5×10^4 CFU/ml in 66 days. When stored at -20°C , the culturable cell number of *E. coli* O157:H7 dropped to 0 in 3 days (Figure 1A). In induction solution 2, the number of culturable cells reduced to 0 after 18 days of storage at 4°C , and the number of culturable cells reduced to 0 within 3 days of storage at -20°C (Figure 1B). In induction solution 10, the number of culturable cells reduced to 0 after storing at 4 and -20°C for 39 and 33 days, respectively (Figure 1F). In induction solution 13, the culturable cell number reduced to 0 in 48 and 39 days of storage at 4 and -20°C , respectively (Figure 1G). In induction solution 14, the culturable cell number reduced to 0 in 57 and 66 days when stored at 4 and -20°C , respectively (Figure 1H). In induction solution 5, 7, and 9, although with a downtrend within 66 days, the number of culturable cells did not decrease to 0 (Figures 1C–E). In addition, in induction solutions 3, 4, 6, 8, 11, 12, 15, and 16, the number of culturable cells of *E. coli* O157:H7 reduced to 0 in 3 days.

For the induction solutions that were capable of inducing the culturable cell number of *E. coli* O157:H7 to 0 (Table 4), the existence of viable cells was determined. When the *E. coli* O157:H7 culture in induction solution 2 was stored at 4°C for 18 days, the cells were all dead, indicating that *E. coli* O157:H7 did not enter the VBNC state (Figure 2). The fluorescence results of *E. coli* O157:H7 in induction solutions 1, 10, 13, and 14 showed

that both dead and viable cells were detected, indicating that *E. coli* O157:H7 entered the VBNC state under these conditions.

Factors Affecting the Survival of *E. coli* O157:H7 Cells

In the 16 induction solutions, factors including salt, acetic acid, and medium concentrations were applied and analyzed on the effect on the survival of *E. coli* O157:H7 cells. First, the effect of salt and acid concentrations on the survival of *E. coli* O157:H7 cells was investigated. In induction solutions 3, 8, 9, and 14, the medium concentration is 50%. In induction solutions 3 (acid: 0.7%, salt: 0.9%) and 8 (acid: 1%, salt: 5%), the culturable cell number of *E. coli* O157:H7 reduced to 0 in 3 days. However, in induction solutions 9 (acid: 0, salt: 10%) and 14 (acid: 0.3%, salt: 15%), *E. coli* O157:H7 cells survived for more than 57 days. Especially in induction solution 9, the *E. coli* O157:H7 cells

TABLE 4 | The time of culturable number of *E. coli* O157:H7 decreased to 0 stored at different methods.

Protocol number	4°C	-20°C	Protocol number	4°C	-20°C
1	+	45 days	9	+	+
2	18 days	/	10	39 days	33 days
3	/	/	11	/	/
4	/	/	12	/	/
5	+	+	13	48 days	39 days
6	/	/	14	57 days	66 days
7	+	+	15	/	/
8	/	/	16	/	/

“+” stands for *E. coli* is culturable and “/” stands for the number of cultivable *E. coli* in 3 days has dropped to 0.

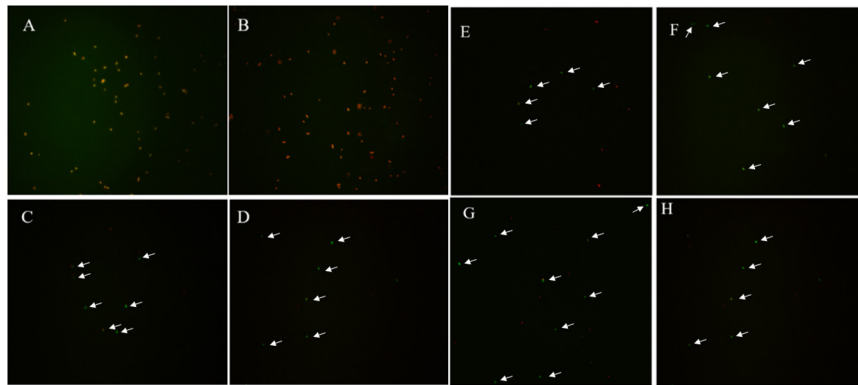


FIGURE 2 | The viability of un-culturable *E. coli* O157:H7 stored at different conditions with fluorescence. **(A–H)** *E. coli* O157:H7 inoculated in the medium configured according to the method 2, 10, 13, 14, and stored at 4 or -20°C , respectively.

survived for more than 66 days. In induction solutions 4, 10, and 13, the medium concentration is 100%. In induction solution 4 (acid: 1%, salt: 0.9%), *E. coli* O157:H7 all died in 3 days and did not enter the VBNC state. In induction solutions 10 (acid: 0.3%, salt: 10%) and 13 (acid: 0, salt: 15%), *E. coli* O157:H7 cells were able to survive for 33–48 days. These results suggest that the effect of acetic acid concentration on the survival of *E. coli* O157:H7 is stronger than that of salt concentration.

Second, the effect of acid and medium conditions on the survival of *E. coli* O157:H7 cells under a certain salt concentration were investigated. In induction solutions 1, 2, 3, and 4, the salt concentration is 0.9%. In induction solutions 1 (medium: 0, acid: 0) and 2 (medium: 25%, acid: 0.3%), *E. coli* O157:H7 cells survived for more than 66 and 18 days at 4°C , respectively. However, in induction solutions 3 (medium: 50%, acid: 0.7%) and 4 (medium: 100%, acid: 1%), the *E. coli* O157:H7 cells all died in 3 days. It showed the effect of acid on the survival of *E. coli* O157:H7 is stronger than that of nutrients. In addition, when the acid concentration is 1% in induction solutions 4, 8, 12, and 16, *E. coli* O157:H7 cells died in 3 days regardless of the medium and salt concentrations. Especially in induction solution 4 (medium: 100%, acid: 0.9%) with the premium centration of medium and acid, *E. coli* O157:H7 cells still died in 3 days, indicating that acidity has the most significant effect on the survival of *E. coli* O157:H7 cells.

Third, under the same acetic acid concentration, the effects of salt and medium concentrations on the survival of *E. coli* O157:H7 were investigated. In induction solutions 6, 10, and 14, the acetic acid concentration is 0.3%. In induction solution 6 (medium: 0, salt: 5%), *E. coli* O157:H7 cells died within 3 days. However, in induction solutions 10 (medium: 100%, salt: 10%) and 14 (medium: 50%, salt: 15%), although the salt concentration increased, *E. coli* O157:H7 cells were able to survive from 33 to 66 days due to the high medium concentration. It indicated that the increase in the medium concentration eliminated the adverse effect of the increase in salt concentration. Among the factors on the survival of *E. coli* O157:H7, the effect of the medium concentration might be greater than that of the salt concentration.

When the environment changes, porin is crucial for the survival of *E. coli*. The major external protein omp (ompF, ompC) of *E. coli* is regulated by *envZ* (osmotic pressure sensor protein) and *ompR*. Studies have showed that wild-type, *ompF*, and *ompC* mutant strain of *E. coli* can enter the VBNC state under environmental pressure (pH, osmotic pressure, and starvation stress conditions), but in the *envZ* mutant strain, they cannot enter the VBNC state. This shows that the *envZ* mutant strains cannot sense changes in the external environment. When the strains are exposed to adverse conditions, they cannot enter the VBNC state (Pienaar et al., 2016). The expression of (p) ppGpp (guanosine pentaphosphate or guanosine tetraphosphate) synthetic genes *relA* and *spoT* are identified to upregulate in the VBNC state of *E. coli* O157:H7 (Magnusson et al., 2005; Mishra et al., 2012). Compared with normal strains, mutants that failed to synthesize (p) ppGpp lost culturability more quickly, and their ability to enter the VBNC state was significantly reduced. When (p) ppGpp was overexpressed, the number of VBNC cells was significantly increased (Boaretti et al., 2003). When bacteria suffered from amino acid starvation, the expression of *relA* or *SpoT* increased. (p) ppGpp synthesis increased and degradation decreased. In addition, exopolyphosphatase (PPX) was degraded by (p) ppGpp of polyphosphates (Ayrapetyan et al., 2018).

Effects of Acidity and Nutrition on Formation of the VBNC State

Among the factors affecting the survival of *E. coli* O157:H7, acetic acid concentration plays a major role, followed by medium and salt concentrations. Considering the actual situation in the food system and in the process of food processing, exploring the concentration of acetic acid and culture medium to control the normal state of *E. coli* O157:H7 and VBNC is meaningful.

When the acetic acid concentration is 1%, the *E. coli* O157:H7 cells died within 3 days in induction solutions 3 (medium: 100%, salt: 10%), 4 (medium: 100%, salt: 15%), and 6 (medium: 50%, salt: 15%). When the acetic acid concentration is 0.7%, *E. coli* O157:H7 could survive in induction solutions 1 (medium: 100%, salt: 10%) and 3 (medium: 100%, salt: 15%). In induction solution

TABLE 5 | Inhibition of acidity on the formation of VBNC state of *E. coli* O157:H7.

Protocol number	Culturable		Viable	
	4°C	-20°C	4°C	-20°C
1	+	+	ND	ND
2	/	/	-	-
3	+	+	ND	ND
4	/	/	-	-
5	/	/	-	-
6	/	/	-	-

“+” stands for *E. coli* is culturable, “/” stands for *E. coli* is not culturable, “-” stands for *E. coli* is not active, and “ND” stands for *E. coli* activity not detected.

TABLE 6 | Inhibition of nutritional status on the formation of VBNC state of *E. coli* O157:H7.

Protocol number	Culturable		Viable	
	4°C	-20°C	4°C	-20°C
1	/	/	-	-
2	+	+	ND	ND
3	+	+	ND	ND
4	+	+	ND	ND
5	/	/	-	-
6	+	+	ND	ND

“+” stands for *E. coli* is culturable, “/” stands for *E. coli* is not culturable, “-” stands for *E. coli* is not active, and “ND” stands for *E. coli* activity not detected.

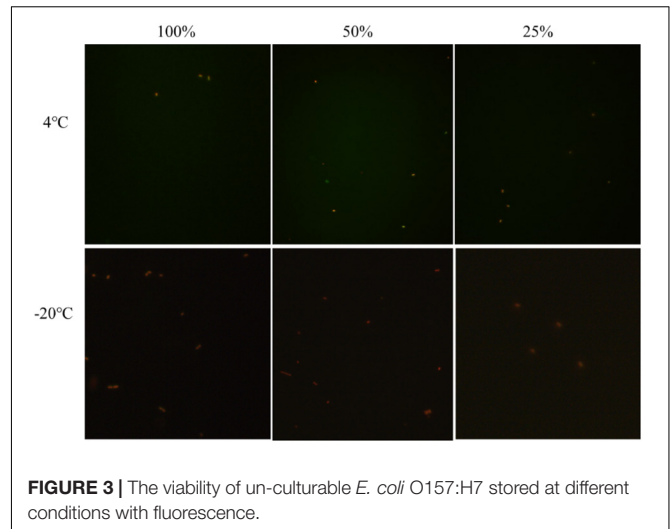
5 (medium: 50%, salt: 15%), *E. coli* O157:H7 cells died within 3 days. When the medium concentration is higher than 50%, treatment with 1.0% acetic acid directly killed *E. coli* O157:H7 without entering the VBNC state (Table 5).

When the medium concentration is 25%, *E. coli* O157:H7 cells survived more than 3 days in induction solutions 2 (salt: 10%, acid: 0.3%), 4 (salt: 15%, acid: 0), and 6 (salt: 15%, acid: 0.3). When the medium concentration is 0, in induction solutions 1 (salt: 10%, acid: 0.3%) and 5 (salt: 15%, acid: 0.3%), the number of culturable and viable cells was both 0 in 3 days. In induction solution 3 (salt: 15%, acid: 0), *E. coli* O157:H7 cells were able to survive for more than 3 days. Under weak acid and high salt conditions, the formation of the VBNC state of *E. coli* O157:H7 was controlled by changing the nutritional condition (Table 6).

In induction solutions 2, 3, 4, and 6, the culturable cell number of *E. coli* O157:H7 did not decrease to 0 in 3 days. However, the culturable cell numbers of *E. coli* O157:H7 reduced to 0 after storing at 4 and -20°C for 3 days in induction solutions 1 and 5, and the cells were not viable. Thus, changing the acid concentration with cold storage might be efficient to control the formation of the VBNC state of *E. coli* O157:H7.

Elimination of the VBNC State in Crystal Cake

In the crystal cake food system with 100, 50, and 25% nutrient concentrations and 1.0% acetic acid, the culturable cell numbers of *E. coli* O157:H7 were 0 after storing at 4 and -20°C for 3 days. The results of fluorescence microscopy showed that all the *E. coli*

**FIGURE 3** | The viability of un-culturable *E. coli* O157:H7 stored at different conditions with fluorescence.

O157:H7 cells died, suggesting *E. coli* O157:H7 was not capable of entering into the VBNC state under these conditions (Figure 3). In the food system, without affecting the flavor and quality of the food, the normal and the VBNC state of *E. coli* O157:H7 was controlled and eliminated by adding 1% acetic acid.

The foodborne microorganisms in the VBNC state have food spoilage ability. Compared with normal state, VBNC state cells have reduced metabolic activity and increased resistance of the cell wall to external environmental stress, including high concentrations of antibiotics, heavy metal ions, high temperature, high salt, and higher acidity (Ayrapetyan and Oliver, 2016; Shekar et al., 2017; Schottroff et al., 2018). Due to the presence of resistance, *E. coli* O157:H7 in the VBNC state is more difficult to be completely eliminated. Under favorable conditions, *E. coli* O157:H7 in the VBNC state might be able to overcome this inactive state and become active again, thus being able to reproduce in the food system. During the processing of crystal cake, the surface of the processing equipment and the inside of the pipeline should be cleaned in time to ensure no residual nutrients, thereby inhibiting the survival of *E. coli* O157:H7. In addition, combined with a certain concentration of acetic acid treatment equipment surface (1.0% acetic acid), it ensures the elimination of *E. coli* O157:H7 and its VBNC state and avoid potential food safety risks.

Detection of the VBNC State of *E. coli* in Crystal Cake

The risk of food safety issues can be partially reduced by the efficient detection of the VBNC state of *E. coli* in food system. Propidium monoazide (PMA) is a photoreactive DNA-binding dye. Dead microorganisms lose their capability to keep their membranes intact, which leaves the “naked” DNA in the cytosol ready to react with PMA. DNA of living organisms are not exposed to the PMA, as they have a complete cell membrane. DNA extraction and PCR amplification of PMA-treated samples can effectively detect bacteria in the VBNC state. The detection limit was determined by detecting different concentrations of *E. coli* O157:H7 in the VBNC state. In this study, the detection

limit of the VBNC state *E. coli* O157 in the crystal cake food system using PMA–polymerase chain reaction (PMA-PCR) was 10^4 CFU/ml, which can effectively avoid the potential threat brought by VBNC state bacteria.

CONCLUSION

The effect of external environmental conditions on the formation of the VBNC state of *E. coli* O157:H7 was as follows: acidity > nutritional state > salt concentration. When the nutrient concentration is higher than 50%, *E. coli* O157:H7 was killed by adding 1.0% acetic acid. In the crystal cake system, when the nutrient concentration is 25, 50, and 100%, *E. coli* O157:H7 cells died in 3 days by adding 1.0% acetic acid with no VBNC cells identified. In the food system, the VBNC state formation of *E. coli* O157:H7 was inhibited by adding 1.0% acetic acid. In addition, PMA-PCR assays can be utilized in the detection of the VBNC state of *E. coli* in the food system.

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DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

JL and KW conceived the study and participated in its design and coordination. YML and T-YH performed the experimental work. CY and LC analyzed the data. YL and JL prepared and revised this manuscript. All authors reviewed and approved the final manuscript.

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