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Review Article

Current Perspectives on Viable but Non-culturable Bacteria in Food Safety and Public Health

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Abstract

The viable but non-culturable (VBNC) state is defined as an adaptive mechanism for microorganisms adjusting to stressful conditions. Although VBNC bacteria are alive and metabolically active, they are unable to grow on routine culture media. Nevertheless, the potential capacity of VBNC pathogens to retain virulence activity and further resuscitate into the culturable state in favorable conditions constitutes a major hazard to food safety and public health. Food processing, transformation, and storage, as well as non-thermal techniques, can provoke pathogens toward VBNC induction. The distinct characteristic of VBNC bacteria led to the emergence of novel culture-independent techniques to prevent the misinterpretation of food safety. To deepen our knowledge of the molecular aspect of the VBNC state, several mechanism-oriented studies investigated the metabolic activity of VBNC bacteria and their correlation with different stressful conditions. This review aims to discuss the molecular mechanisms and genomic factors underlying the induction and resuscitation of the VBNC state. The study will further highlight innovative detection methods to provide a comprehensive perspective for future studies in the emerging fields of research concerning VBNC state, food safety, and public health.

Keywords: VBNC, Food safety, Public health, Waterborne pathogen, Foodborne bacteria

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Background

Escherichia coli and foodborne pathogen *Vibrio cholera* were the first bacteria demonstrated to enter the viable but non-culturable (VBNC) state, forcing the researchers to revisit some fundamental notions regarding pathogen survival and viable counting methods (1). Stressful conditions lead to a biologically inactive form of life termed the VBNC state that is undetectable by typical bacteriological media culture. However, VBNC bacteria preserve their membrane integrity, low levels of gene expression, and traceable metabolic functionality (2). The presence of detectable metabolic activity distinguishes the VBNC state from dormancy, which is a convertible characteristic of a metabolic shutdown. This state will further allow bacteria to utilize nutrients and maintain their genome and virulence activity. Thus, bacterial capacity for colonization and growth can be recovered by the resuscitation process under certain favorable conditions (3). The regain of virulence capacity following the VBNC state might be independent of the resuscitation ability of bacteria and relies on both factors stimulating the VBNC state or the resuscitation process. Furthermore, the results of recovery methods are strain-specific and not all VBNC strains can undergo resuscitation (4).

Foodborne disease, including diarrhea, nausea, vomiting, and kidney and liver dysfunction, has raised a substantial concern in every region globally. Traditional culture-based strategies for quantifying microorganisms and determining the viability of bacterial cells through colony formation have faced challenges owing to the presence of dormancy and VBNC state (5). The potential capacity of VBNC bacteria to cause infection emphasizes the great significance of the VBNC state in food safety and public health (6). The frequent exposure of food to a variety of environmental conditions may provide adequate stress for VBNC induction. Moreover, the previous detection of VBNC bacteria during the food process and the fact that VBNC pathogens can maintain toxin production

ability and recover within the human body, have attracted widespread attention to VBNC in the food industry (7,8). Several microbial pathogens can enter the VBNC state in a diversity of food products, including vegetables, fruit juice (9), chicken (10), and seafood (11). Further, not only VBNC bacteria have been detected in drinking water, but also it is suggested that conventional disinfection methods in water treatment systems can stimulate VBNC induction (12). It might be more practical to grasp an ecological perspective regarding the VBNC state and consider it as a typical defensive strategy for non-spore-forming bacteria.

This review focuses on discussing the characteristics and persistence of VBNC bacteria and their importance in food safety and public health. Accordingly, the most recent studies are considered to further highlight recent advances in detecting the techniques of VBNC cells to provide a perspective for future investigations in this dynamic field of research.

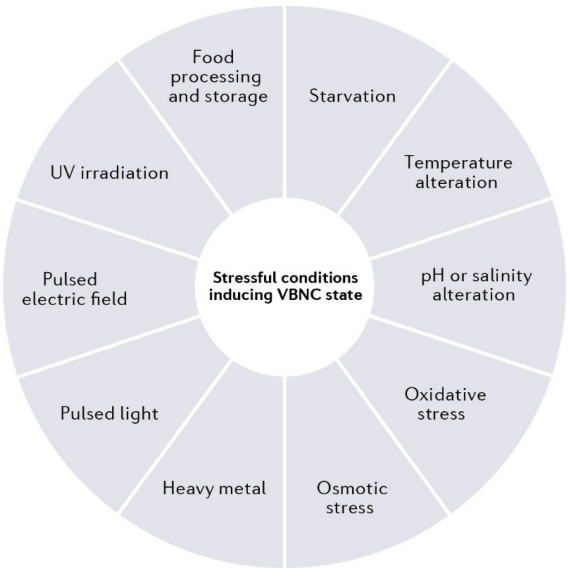

Figure 1. Main Stress Factors Stimulating Particular Bacteria Toward VBNC Induction.

VBNC State

Induction of VBNC State

The main characteristic of the VBNC state is the ability of bacterial cells to tolerate stressful environmental conditions. Although large-scale studies are yet to determine the comprehensive impacts of different conditions on VBNC induction, several physical and chemical stress factors are suggested to disturb the natural balance of bacterial growth conditions and lead the bacteria to induce the VBNC state (13). Starvation (14), extreme or low temperature (15), osmotic (16) and oxidative stress (17), UV irradiation (18), pulsed light, pulsed electric field (19), heavy metal (20), and acute alteration of pH or salinity (21), as well as food processing and preservation strategies (22), are the main uncovered factors stressing the bacteria to VBNC state as shown in Figure 1.

Although stress conditions for VBNC induction have been well studied, the molecular mechanism and genetic control underlying the VBNC state have received little attention. Due to the presence of a vast diversity of VBNC bacterial species, regulatory strategies are suggested to be strain-specific. Several studies uncovered the involvement of different mechanisms in VBNC induction, including RelA promotion and ToxR reduction in *V. cholera* (23), RpoS, MarA, YgfA, RelE (24), VapC, and HipA upregulation, and VapB downregulation in *E. coli* (25), and ClpP accumulation in *Legionella pneumophila* (26). In stress conditions, *Campylobacter jejuni* encodes high-temperature response protein B, polyphosphate kinase (PPK), and GltD and GlnA, which encode the required proteins for glutamine and glutamate production (27). The key enzymatic activity of PPK is the modulation of polyphosphate accumulation that mediates stress response, survival, colonization, and the virulence of several bacteria. Given that the PPK mutant *C. jejuni* demonstrated a reduced capacity of VBNC formation, PPK regulatory genes might be potential therapeutic targets in ceasing *C. jejuni* reinfection (28). Furthermore, a stringent response following amino acid starvation leads to RelA-dependent ppGpp synthesis, which might provoke VBNC formation. Additionally, it has been indicated that *E. coli* mutants on ppGpp production exhibited a reduction in VBNC induction (29). It is also reported that in *Salmonella enterica*, deletion in the C-terminal domain of ClpX will delay the induction of the VBNC state (30).

Characteristics of VBNC Bacteria

Despite the loss of culturability on conventional media, VBNC bacteria retain the absorptive capacity and express particular genetic materials; thereby, it is suggested that VBNC cells have several common properties with culturable cells. Nonetheless, a number of physiological conversions such as deceleration in nutrient absorption, protein synthesis, and macromolecular metabolism occur through VBNC induction (31). Collectively, major variations underlying the VBNC state include cellular morphology, metabolic activity, stress toleration, and genetic regulation.

The alteration in cellular morphology during the transition toward the VBNC state leads to cell dwarfing and rounding. Additionally, a reduction in the cell size is a possible strategy in VBNC cells to restrict the energy requirement (32). In contrast to Gram-negative bacteria, some Gram-positive bacteria such as *Enterococcus faecalis* demonstrated an expansion in the cell size (33). It is further indicated that Gram-positive bacteria are more susceptible to the induction of the VBNC state (34). The VBNC cells of *Helicobacter pylori* represented a coccoid appearance while preserving their virulence capacity with limited metabolic activity (35). Rod-shaped *Vibrio parahaemolyticus* in the exponential phase also transforms to a cocci form in the VBNC state and becomes more flexible (36). Nevertheless, similar morphological changes are reported in non-VBNC cells, indicating the unreliability of cellular appearance

to reveal the VBNC state (37). Considering the major part of the cell wall in shaping cellular morphology, the aforementioned changes might be the consequences of cell wall conversion. It is proposed that resuscitation-promoting factors (Rpfs) can recover the culturable form of bacteria by lysing cell wall peptidoglycan (38). Moreover, the cell wall of *E. faecalis* was reported to be less prone to physical interruption, and the chemical analysis of its peptidoglycan exhibited an enhancement in cross-linking and lipoteichoic acids (33). In addition to the cell wall, VBNC cells might further modify membrane composition to preserve membrane fluidity with unsaturated and short-chain fatty acids. The prevention of fatty acid production in *Vibrio vulnificus* is associated with reduced survival and VBNC induction (39).

Modification of metabolic activity in VBNC cells is responsible for alterations in the synthesis of proteins, fatty acids, and peptidoglycans, which eventually determines the composition of cell walls and membranes (32). In this state, following the reduction in the concentration of nucleic acid, a dense cytoplasm will surround the compact chromosomal DNA (40). Despite the reduction in total energy production in the VBNC state, the promotion in electron transfer and adenosine triphosphate (ATP) level can provide the limited energy required to preserve cellular homeostasis (41). Furthermore, some studies indicated an enhancement in protein production as protein aggregation is a defensive strategy that facilitates bacterial adaptation to environmental stress and antibiotic intervention through VBNC induction (42). However, several metabolic pathways maintain their activity during VBNC induction. In *Rhodococcus biphenylivorans*, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase are reported with similar activities in culturable and non-culturable forms (43). Additionally, *Bacillus stratosphericus* is demonstrated by XTT cell viability assay to preserve its respiration, which is a pivotal metabolic process for cell survival (44).

Resuscitation of VBNC Bacteria

Salmonella enteritidis was the first bacterial model used to represent resuscitation from the VBNC state, in which the bacteria recovered culturability following the addition of heart infusion broth (45). Resuscitation is a remarkable capacity of the VBNC state, giving a reversible characteristic to VBNC cells to regain normal levels of physiological and metabolic activities (46). A number of strategies are developed for detecting true resuscitation and excluding the regrowth of culturable bacteria such as the extreme dilution of VBNC cells with artificial seawater to remove nutrients and prevent the presence of culturable bacteria (47). Nonetheless, there is only a period of time following VBNC induction termed as "resuscitation window", in which the bacteria can undergo resuscitation. Thereafter, the number of resuscitation will significantly reduce and bacteria die ultimately (37). The term the resuscitation window mostly relies on bacterial species and incubation period, as well as the conditions of entering and recovering from the VBNC state (48).

Resuscitation from the VBNC state requires particular conditions, including the removal of stress conditions, addition of rich nutrients, stabilization of osmotic pressure, degradation of hydrogen peroxide, and the presence of a host (49). *Arcobacter butzleri* can enter this state in seawater at 4 °C and recover the normal state within 9 days of nutrient addition (50). Although the vital nutrients for resuscitation are poorly understood, it is suggested that a combination of methionine, glutamine, threonine, serine, and asparagine can accelerate *E. coli* recovery from the VBNC state (51). It is further indicated that owing to the reduction in superoxide dismutase functionality in the VBNC state, the addition of reactive oxygen species scavengers such as pyruvate can stimulate resuscitation (52). Moreover, biological stimuli are natural promoters for resuscitation, in which particular VBNC species including but not limited to *E. coli*, *Francisella tularensis*, and *C. jejuni* can resuscitate and grow in the presence of eukaryotic cells, mammal cells, and embryonated eggs, respectively (49). In addition to environmental inducers, the cell-free supernatant of bacteria can promote resuscitation probably by quorum sensing autoinducer AI-2 (53). A *Micrococcus luteus* cytokine Rpf is also reported to induce the resuscitation of the specific species of the *Mycobacterium* genus (54). Nevertheless, due to the presence of a vast variety of bacterial species and stimulus factors, the molecular mechanism of the recovering process is far from full elucidation.

Detection Methods for VBNC Bacteria

Distinct characteristics of VBNC bacteria have made the conventional microbiological detection techniques inauthentic. Hence, the unique aspect of this state, as well as the importance of VBNC bacteria in food safety and public health encouraged the scientists to the development of novel detection methods for VBNC cells. As a result, a number of innovative strategies are established to detect VBNC bacteria mainly based on their metabolic activity (Table 1).

Direct Viable Count

The direct viable count (DVC) of bacterial cells was first proposed based on the recognition of elongated cells by direct microscopy or epifluorescence microscopy (66). This method is based on the sensitivity of viable bacteria to antibiotics such as nalidixic acid, aztreonam, and ciprofloxacin, which lead VBNC cells toward elongation, while dead cells demonstrate no alteration in this regard. Consequently, nalidixic acid as a DNA replication inhibitor may not be convenient in this method (55); however, aztreonam is suitable for *Cytophaga allerginae* and *Serratia marcescens* (67), while ciprofloxacin is suitable for *Listeria monocytogenes* (68). Considering different bacterial responses to antibiotic treatments regarding their species

Table 1. Different Detection Methods for VBNC Bacteria

Method	Platform	Elements	Advantages	Limitations
Direct viable count	Based on bacterial elongation (55)	Antibiotics such as nalidixic acid, aztreonam, and ciprofloxacin (55)	Simple protocol, direct examination (55)	Low sensitivity (56)
CTC staining	Based on bacterial respiratory reaction (57)	CTC (57)	Simple protocol and typical equipment (58)	Low sensitivity and CTC toxicity (59)
LIVE/DEAD® *Bac*Light™ assay	Based on the integrity of bacterial membrane (60)	SYTO 9, PI (61)	High sensitivity, accuracy, and rapid results (62)	Different affinities of SYTO 9 with different states of cells, not specific (62)
Molecular methods	Based on the presence of bacterial mRNA (63)	RT-qPCR (64)	High sensitivity, accuracy, specific, and rapid results (63)	Selection of suitable target genes (65)

Note. CTC: 5-cyano-2,3-ditolyl-tetrazolium chloride; PI: Propidium iodide; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction; SYTO 9: A blue-excited, green-fluorescent nucleic acid stain that is cell-permeable; VBNC: Viable but non-culturable.

and nutrient uptake capacity, this method is significantly limited, thereby it is not a commonly used technique (56).

5-Cyano-2,3-ditolyl-tetrazolium Chloride (CTC) Staining Method

The electron transportation of the bacterial respiratory chain can reduce CTC to CTC-formazan, which is detectable by the epifluorescence microscopic technique (57). This method is frequently applied with DVC to give a comprehensive enumeration of viable cells as DVC demonstrates elongation and CTC staining detects cellular respiratory activity (69). Although the simple protocol and typical equipment of CTC staining resulted in a widespread application, it cannot detect extremely low levels of metabolic activity (58). Furthermore, the toxicity of CTC staining can suppress bacterial metabolic activity and thereby underestimates the authentic count of viable cells (59).

LIVE/DEAD® BacLight™ Assay

The LIVE/DEAD *Bac*Light is a commercially available kit, consisting of two DNA-binding dyes, the green fluorescent dye, the red fluorescent stain, namely, propidium iodide (PI), and SYTO 9 (61). While the SYTO 9 can penetrate intact, as well as damaged cell membranes and mark all cells, PI can only mark damaged cell membranes and therefore distinguish viable from injured and membrane-permeabilized cells (60). Under fluorescence microscopy or flow cytometry, VBNC bacteria would appear green, while the injured or dead cells would appear red. Although this method established more accuracy in counting VBNC cells than DVC and CTC staining methods, several factors such as the bleaching effect of green fluorescent staining, different affinities of viable and dead bacteria with SYTO 9, and background fluorescence should be considered when performing LIVE/DEAD assay (62).

Molecular Methods

Recent advances in molecular techniques have facilitated the detection of gene expression and metabolic activity by the reverse transcription-quantitative polymerase chain reaction (RT-qPCR), quantitative real-time PCR (qRT-PCR), and loop-mediated isothermal amplification. The incapability of the PCR to determine DNA from viable or dead cells has led to the application of DNA inhibitors such as propidium monoazide (PMA) previous to DNA extraction, which penetrates through the damaged membrane and prevents DNA amplification (70). However, a limitation of the PMA-qPCR method is the presence of dead cells with intact membranes such as particular UV-killed bacteria (71). Hence, bacterial mRNA, owing to its short half-life within the cell, is suggested as an accurate marker for determining gene expression and bacterial viability (63). Although a continuously expressed gene is targeted based on bacterial species, housekeeping and virulence genes are primary markers to be detected by the RT-qPCR and thereby determining cell viability (64). Nonetheless, the limitation of RT-qPCR is the selection of suitable target genes, which are transcribed independent of physiological alterations (65).

VBNC Pathogens

Following the discovery of VBNC cells in 1982, several researchers expressed the intention of seeking different bacterial species capable of entering the VBNC state. Further, some studies have reviewed and presented various lists of human pathogens reported to induce the VBNC state (13,37,72,73), which are further discussed as follows.

Foodborne Pathogens

The increasing population of VBNC pathogens in a vast majority of environments has a substantial impact on food safety. The frequent exposure of food to a narrow diversity of environments throughout processing, transference, and storage can provide several opportunities for VBNC induction. Freezing or refrigeration, cooking, fermentation, and additive addition (e.g., salt) are different types of stress conditions to induce the VBNC state in foodborne pathogens (74). The addition of acidic additives such as citric acid and acetic acid during the food process can lead to VBNC induction in *Staphylococcus aureus*. It is reported that the presence of sufficient nutrients in the acidic condition can stimulate *S. aureus* to induce the VBNC state, whereas the acidic condition with nutrient starvation can damage and eliminate the bacteria (75,76). Furthermore, various non-thermal techniques, including ultrasonication, cold plasma technology, irradiation, supercritical technology, pulsed electric field, high

hydrostatic pressure, pulsed ultraviolet technology, and ozone are used in the sanitization of food products without heat application (77). Several studies demonstrated the formation of the VBNC state for pathogen microorganisms by non-thermal technologies such as electrolyzed water (78), high-pressure CO_2 (41), pulsed light inactivation (79), thermosonication (18), and non-thermal plasma (80).

The potential capacity of several VBNC pathogens to preserve the expression of virulence factors and toxins has caused major concerns in food safety (81). Multiple studies indicated the development of disorders in the host following inoculation with VBNC pathogens. The injection of VBNC *V. vulnificus* into animal models can cause mortality through in vivo resuscitation (82). The isolation of VBNC *V. cholerae* from rabbit intestinal loops represented the enterotoxigenicity of this VBNC pathogen (83). Moreover, a rabbit ileal loop assay was applied to exhibit the preservation of enterotoxin production in VBNC *E. coli* H10407 (84). The detection of VBNC bacteria in mouse and human urine samples, previously considered sterile, further revealed the hazardous characteristic of VBNC pathogens in developing infectious diseases (85).

Waterborne Pathogens

Obligate and opportunistic pathogens are the two major groups of waterborne pathogens that can cause diseases in the host regardless of the health condition and in a subgroup of sensitive individuals, respectively. These microorganisms can grow as primary colonizers by attaching to solid surfaces and forming multiple- or single-strain biofilms. Similarly, they might multiply as secondary colonizers by integrating with the preformed biofilm (86). Microorganisms within mature biofilms tend to morphological heterogeneity by inducing the VBNC state and persisted formation in stressful conditions. Consequently, the flow and pressure in the drinking water distribution system can detach the biofilm and facilitate the transmission of waterborne VBNC pathogens (87). The major risk for developing waterborne diseases is related to the consumption of fecal contaminated water such as in the case of discharging wastewater in fresh water and coastal seawater (88). *S. enterica*, *Shigella* spp., *V. cholerae*, *E. coli*, *Yersinia enterocolitica*, *Campylobacter* spp., and *H. pylori* are among major water-related pathogens (86). Primary disorders following infection with waterborne pathogens are cholera, bacillary dysentery, gastroenteritis, typhoid fever, salmonellosis, and acute diarrhea (88).

A major threat to human health is the development of gastrointestinal diseases such as peptic ulcers and gastric cancer following the *H. pylori* infection. This pathogen can survive unfavorable conditions in drinking water through biofilm formation and VBNC induction (89). Although VBNC induction leads to a reduction in metabolic activity, the coccoid form of *H. pylori* can colonize and stimulate inflammation within the host gastric mucosa by expressing virulence factors (90). As a result, researchers have declared that the acquisition and transmission of *H. pylori* are closely related to the quality of drinking water (91).

Persistence of VBNC State

Virulence of VBNC Pathogens

Despite the contrary reports regarding the virulence capacity of VBNC pathogens, it is suggested that microorganisms can preserve their virulence through the VBNC state and cause infection following resuscitation. *Vibrio harveyi* is reported to cease the expression of the hemolysin gene in the VBNC state, while the resuscitated bacteria are lethal and their injection into zebrafish is reported to cause death within a week (92). As regards *V. vulnificus*, it is demonstrated that its virulence capacity is reduced to some extent by VBNC induction. However, non-culturable *V. vulnificus* can resuscitate within the mouse and thereby lead to the fatal infection of the host (82).

Several studies further investigated the correlation between the stress condition and regulation of virulence genes as some virulence genes are required for the tolerance and survival of bacteria within the host. The *L. monocytogenes* LO28 mutation in the induction of the acid tolerance response represented diminished virulence in a murine model (93). The acid adaptation of *L. monocytogenes* strains by short-term exposure of bacteria to low pH may increase the expression of virulence genes such as *inlA*, *opuC*, and *sigB*; consequently, it may promote the survival and invasiveness of these microbes (94). Additionally, the combination of different stress conditions can influence the growth and virulence of the pathogens; therefore, extreme alterations in temperature and pH of the environment might increase pathogen invasiveness and lead to outbreaks (95).

Biofilm Formation

The capacity of biofilm formation is a common attribute of several microorganisms, suggesting a dominant stage of bacterial colonization. The developmental process of biofilm formation is triggered by the aggregation and/or surface attachment of planktonic bacteria and is fulfilled by the dispersion of microorganisms from the biofilm structure (96). Due to the complex architecture of the biofilm, interior areas within biofilms are hypoxic, nutrient-limited, and acidic owing to the accumulation of waste products (97). Considering that these stress conditions can independently induce the VBNC state, pathogens deep within the biofilm structure tend to VBNC induction. In addition, the culturability of bacteria in multispecies biofilms is influenced by the metabolic activity of other strains (98). It is noteworthy that the active starvation response of biofilm bacteria is reported to establish antibiotic tolerance (99); thereby, biofilm bacteria are highly resistant to antibiotic interventions and more difficult to eradicate compared to planktonic cells (100).

Approximately 95% of the waterborne pathogens in drinking water distribution systems are located at biofilm structures, whereas only 5% are detectable through

sampling the water phase (101). Drinking water is substantially associated with the contamination of food and the development of multiple diseases, thus several studies are conducted on VBNC bacteria within biofilms. Foodborne pathogen *C. jejuni* induces the VBNC state inside biofilms to persist for 4 months and resist biocide (102). Furthermore, the reduced inflammatory activity of VBNC cells and the lower activation of macrophages can facilitate the immune evasion of biofilm bacteria (103). In addition to stress tolerance, biofilm formation is a concentration mechanism for *H. pylori* bacteria through which a small fraction of the biofilm can escape conventional microbiological sampling while containing sufficient pathogens to infect the host (104).

Antibiotic Resistance

The VBNC state by definition is the adaptation of cells to stressful conditions to be less influenced by exogenous stressors. The aforementioned biofilm formation is additional protection, in which microorganisms form an extracellular matrix to escape the host immune defense and establish antibiotic resistance. Moreover, the combination of morphological alterations with a low metabolic rate in VBNC cells will reduce the effectiveness of antibiotics targeting substances or metabolic pathways of culturable bacteria (105). Despite the morphological similarity between the VBNC and culturable forms of particular bacteria, the microorganism can withstand antimicrobial substances owing to epigenetic alterations. In VBNC *E. coli*, significantly expressed genes are reported to be involved in substance transportation such as efflux pumps to reduce the accumulation of toxic compounds and gene regulators such as transcriptional regulatory factors, stress regulators, and virulence genes (106). Further, VBNC induction can be the consequence of antibiotic treatment as various concentrations of ciprofloxacin might lead to quinolone-induced VBNC *E. coli* (107). Similarly, this might be an explanation for the frequent reinfection of *H. pylori*-infected individuals who undergo remission following an antibiotic intervention.

Food Safety and Public Health

As a worldwide challenging issue, food safety is the protection of the food supply chain from contamination by pathogens and chemical compounds (108). Various documents have confirmed the presence of VBNC pathogens in food and drinking water (109). The diversity of the surrounding environment during food processing and the detection of VBNC pathogens in food have widely challenged public health. Experimental studies have indicated the capacity of VBNC bacteria to resuscitate and lead the host toward a lethal state (82). Accordingly, solid evidence suggests that VBNC pathogens might be responsible for foodborne outbreaks. In 1998, contamination of salted salmon roe with *E. coli* O157 caused an outbreak in Japan. The underestimated number of viable pathogens due to the presence of VBNC *E. coli* was proposed as the main reason for the outbreak (110). The induction of VBNC *Salmonella oranienburg* following osmotic stress in dried processed squids led to another foodborne outbreak in Japan (111). The *E. coli* O104:H4 strain expressing genes characteristics of enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) resulted in an outbreak and several cases of bloody diarrhea and hemolytic uremic syndrome by encountering copper ions or particular tap water and thereby entering the VBNC state (112). Nevertheless, the presence of favorable conditions for bacterial resuscitation is of great significance for determining the pathogenicity of VBNC cells. VBNC *S. typhimurium* LT4 was demonstrated to recover only through oral administration, suggesting the gastrointestinal tract as a favorable environment for resuscitation (113). However, *S. typhimurium* ATCC 14028 failed to recover into a culturable cell by transmission through the digestive tract (114).

Limitations and Future Perspective

Decades of research have shed light on the characteristics and potential capacity of VBNC cells to provide novel strategies for eliminating VBNC pathogens. Despite advances in the biotechnology and promotion of detection techniques, limitations in the study design prevent us to deepen our knowledge regarding the induction and resuscitation of the VBNC state. Heterogeneous cultivation conditions encompassing culturable, non-culturable, injured, and dead bacteria can interfere with study analyses concerning morphological properties and metabolic activities associated with the induction and recovery of VBNC bacteria. Complete isolation of VBNC cells from heterogeneous systems and application of microfluidic technology and time-lapse microscopy may allow us to establish a more accurate platform for studying VBNC bacteria at the single-cell level. Furthermore, the development of sensitive and easily operated detection methods for overcoming the shortcoming of current methods should be a priority in the modern field of food sciences. Finally, genomic and proteomic studies need to address common and strain-specific mechanisms by which pathogens can transform from a culturable into a VBNC state and vice versa.

Conclusions

The VBNC state has been indicated as a pivotal characteristic of bacteria to survive stressful conditions and further develop infectious diseases in the host. The complexity of the VBNC concept as a corresponding mechanism of adaptation has drawn substantial attention. Nonetheless, given the surreptitious characteristic of this state, little is known regarding the induction and resuscitation of VBNC cells. Fast and accurate recognition of VBNC pathogens is of great significance in tackling possible contaminants and foodborne outbreaks. Owing to the distinct features of VBNC cells, conventional microbiological plate count techniques might result in

underestimating the abundance of viable microorganisms. Despite the development of innovated culture-independent methods, strain-specific characteristics and induction conditions extremely challenge food safety and public health. Therefore, a universal classification system and platform, as well as general biomarkers are critical to be established to accelerate further investigations.

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Conflict of Interests

None declared.

Ethical Approval

Not applicable.

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