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Review Article

Progress of viable but non-culturable (VBNC) state foodborne pathogens in milk and dairy products

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Abstract

Foodborne pathogens are an important biological pollution source that affects food quality and safety. Milk and dairy products can induce a viable but non-culturable (VBNC) state of foodborne pathogenic bacteria under "appropriate" conditions such as production, processing, and transportation. At such state, the pathogens cannot grow or reproduce on the plate but still maintain vitality and express virulence and can be recovered to a cultivable state under favorable conditions. VBNC-state foodborne pathogens in milk and dairy products pose a huge threat to the quality and safety of food and dairy products, as well as public health. In this paper, the research progress and formation mechanism of VBNC-state pathogenic are reviewed, and the several common pathogenic bacteria of VBNC-state in milk and dairy products and their inducing and reviving factors and detection methods are introduced. Understanding the importance of pathogenic bacteria in VBNC status provides a reference for detecting pathogenic bacteria in VBNC status in milk and dairy products.

Keywords: Dairy products, Detection method, Foodborne pathogens, Raw milk, VBNC

Highlights

- This article will serve as a valuable source of information on the present status of VBNC pathogenic bacteria in milk and dairy products.
- This review will guide the significance of the risk assessment of pathogenic bacteria in food and public health, and can arouse further research interest in the scientific problems that VBNC bacteria need to solve in the future.
- This review will help to study the development of a standard method or methods for the detection of VBNC pathogenic bacteria.
- It will provide important insights for the prevention of foodborne pathogen outbreaks, and is expected to improve the quality and safety of dairy products to the best level.
- It will provide a research direction to solve the limitations of VBNC pathogenic bacteria detection, so that it can be applied in the dairy industry and promote the high-quality development of the dairy industry.

INTRODUCTION

Since Xu (Xu *et al*., 1982) first reported the induction of bacteria into a live but uncultivable (viable but non-culturable, VBNC) state under adverse environmental conditions, VBNC-state bacteria gradually attracted great concern worldwide as it subverts the traditional understanding of bacterial growth. Compared with normal bacteria, VBNC-state bacteria cannot grow or reproduce on a culture medium,

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making VBNC-state bacteria a source of infection that is not easily detectable.

In recent years, food safety incidents caused by pathogenic bacteria such as *Campylobacter jejuni* (Burakoff *et al*., 2016), *Salmonella* ssp., *Listeria monocytogenes*, *Escherichia coli* O157:H7 (Keba *et al*., 2020) and *Campylobacter* ssp. (Lahti *et al*., 2017) in milk and dairy products have been frequently reported all over the world, indicating that the problem of microbial contamination in milk and dairy products always needs attention. Studies have shown that the production, processing, storage and transportation conditions of milk and dairy products (such as stainless steel pipeline transportation, pasteurization, etc.) can enable various pathogenic bacteria to develop stress adaptability and enter VBNC-state (Gunasekera *et al*., 2002; Zhao *et al*., 2017). Once pathogenic bacteria enter VBNC-state, they cannot be detected by traditional plate culture method, but still have measurable metabolic activity, which can be transcribed and translated, and produce virulence and pathogenicity (Caldera *et al*., 2015), posing a serious threat to the public safety. More importantly, as a survival mechanism for pathogens to resist the external environment, VBNC-state pathogens can be revived under certain conditions and become a "hidden" source of infection. Therefore, VBNC-state pathogenic bacteria in milk and dairy products make the microbiological safety issue increasingly important.

Conventional biological methods are time-consuming and laborious, and cannot detect VBNC-state pathogenic bacteria. However, Quantitative real-time PCR (qPCR) cannot distinguish between live and dead cells, which leads to an overestimation of the number of live cells, resulting in false positives. In the latest research progress, some scholars used Ethidium monoazide bromide (EMA) or Propidium monoazide (PMA) dyes to distinguish between dead and living cells based on cell membrane integrity, the membrane of dead cells is damaged, and the membrane of VBNC and living cells is intact (Lv *et al*., 2020; Truchado *et al*., 2020). For test samples with complex matrices, studies performed specific enrichment utilizing immunomagnetic beads with antigen-antibody reaction or lysozyme with an affinity for cell wall components (Zhao *et al*., 2019; Shi *et al*., 2021). So far, there is no unified detection standard for VBNC-state bacteria, and the related detection system is not yet perfect.

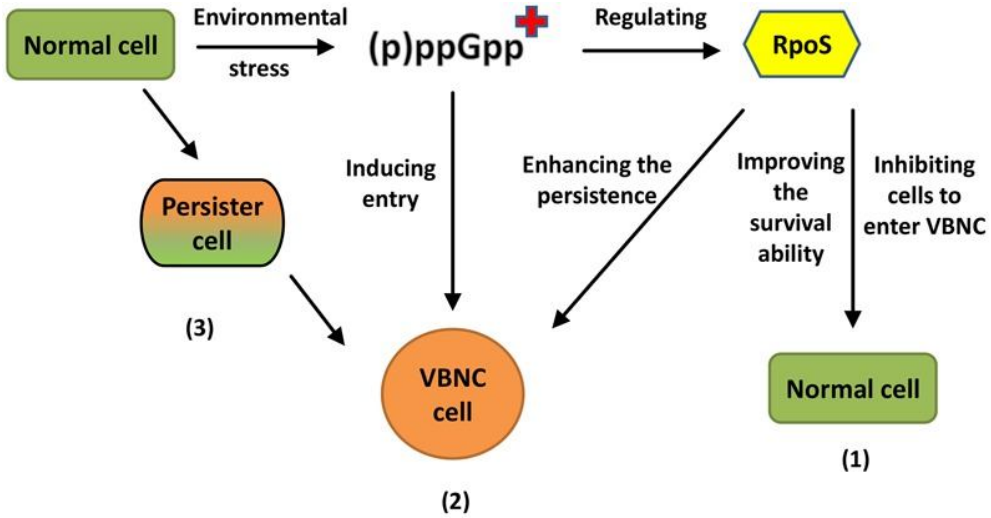
Therefore, discovery of VBNC-state foodborne pathogens greatly increases the difficulty of detection. This article reviews the status quo, induction and detection methods of several common VBNC-state foodborne pathogens in milk and dairy products, highlighting the importance of research on VBNC-state foodborne pathogens. It will provide theoretical basis for the detection of pathogenic bacteria in milk and dairy products, and enhance food safety.

Characteristics and formation mechanism of foodborne VBNC-state pathogenic bacteria

In early 1950s, marine microbiology studies reported significant difference in the number of bacteria measured by direct count under microscopy and plate count (Jannasch *et al*., 1959). Despite the interference of cell aggregation and non-biological particles, these results suggested that the plate counting method based on the culturability of bacteria may underestimate the number of viable cells. Xu *et al*. (1982) first discovered non-culturable *Vibrio cholerae* and *E. coli* in estuarine and marine environments. Colwell and group brought up the concept of VBNC-state (Viable but non-culturable state) in 1985 and reviewed VBNC-state microorganisms in 2000 for the first time (Colwell *et al*., 1985; Colwell, 2000). VBNC-state was extensively studied in the past 30 years. So far, more than 100 species of microorganisms, including bacteria and fungi, have been reported to enter the VBNC state and have been confirmed impacting food safety, environmental applications, and agricultural diseases (Dong *et al*., 2020), most of which are

pathogenic bacteria (Li *et al*., 2014). The morphology and physiology of bacteria cells change upon entering VBNC-state, allowing survival under adverse conditions. Compared with culturable cells, VBNC-state pathogenic bacteria may exhibit dwarfism. The change from a bacillary to a coccoid form was observed in VBNC *Vibrio parahaemolyticus*, with the occurrence of wrinkles and herpes-like structures on the cell surface (Chen *et al*., 2009). Similar structures were reported in VBNC *E. coli* cells, accompanied by enriched intracellular DNA (Sachidanandham *et al*., 2005). In addition, the shapes of *Campylobacter jejuni* and *Edwardsiella tarda* were found to transform from the spiral to coccoid form and from short rods to the coccoid form, respectively (Chaisowwong *et al*., 2012). However, cells can restore cultivable state through the reactivation process, namely resuscitation process, once the environmental conditions become normal.

Diane and Nystrõm (McDougald *et al*., 1998; Nyström *et al*., 2001) proposed three hypotheses of the formation mechanism of VBNC-state bacteria: (1) "Cell decline theory", which attributes VBNC-state to starvation and other harsh external conditions that lead to cell degradation and intracellular oxidation, causing cell viability. This hypothesis somehow explains the non-cultivation of bacteria in VBNC-state. But it implies that non-cultivable bacteria are dying, which is contrary to the basic concept of bacteria in VBNC-state. (2) "Cell gene regulation theory", which attributes the non-cultivability of VBNC-state bacteria to genetic programming. Aizenman *et al*. (1996) suggested genes regulating mediate programmed cell death of bacteria were activated under stress conditions, allowing bacteria to lose non-cultivability to survive and grow. This hypothesis is very innovative and creative but is not sufficiently convincing since it has never been confirmed. (3) "Physiological adaptation theory", which considers VBNC-state is not dying but a response to harsh environment for survival (Roszak *et al*., 1987; Oliver *et al*., 2010). Although the mechanism of VBNC-state is not fully understood, some genes that play important roles in the formation of VBNC cells have been discovered, and a handful of hypotheses were proposed (Fig. 1).



[(1) RpoS proteins significantly hinder the formation of VBNC cells but enhance the persistence of VBNC. "+" means an accumulation of guanosine polyphosphate [(p)ppGpp]. The activation of the pathway for VBNC production, which involves the RpoS transcription factor that is also essential for the osmotic stress response. (2) (p) ppGpp may be an inducer which can make cells more quickly into VBNC. (3) The normal cell will enter the persister state before entering VBNC state.]

Fig. 1. Different views on the formation mechanism of VBNC cells (Cited from Zhao *et al*., 2017)

Natural survival status of bacteria similar to VBNC-state includes dormancy, spores, tolerance, etc. However, a large number of studies have confirmed that the VBNC-state is an optimal survival strategy adopted by non-spore forming bacteria to resist adverse environments, so it is not a transition state. Furthermore, the relationship between dormant and VBNC-state is still controversial.

Previous studies have shown that VBNC-state does not necessarily mean non-pathogenic. (Yaron and Matthews, 2002) found that VBNC-state *E. coli* O157:H7 still expressed mob A, rfb E, stx 1, stx 2 and some 16s rRNA synthesis-related genes, indicating that virulence genes in VBNC-state cells can still be expressed and the synthesis of metabolites can still be performed. Colwell (2000) demonstrated that injection of VBNC-state *Vibrio cholerae* still causes diarrhoea on testers. Moreover, it still produced cholera toxin after entering VBNC-state for 28 days. Polymerase chain reaction (PCR) results confirmed the presence of cholera toxin related genes; ZhaoFeng's (Zhao, 2014) results showed that VBNC-state *E. coli* O157:H7 can still adhere to host cells and cause rubbing damage. The virulence of VBNC-state *Campylobacter jejuni* carrying *flaA* gene was also confirmed in a similar way. Patrone *et al.* (2013) tested the expression of *CadF* protein of VBNC-state *Campylobacter jejuni* and confirmed that it still can adhere to intestinal mucosa. Lothigius found that although VBNC-state *enterotoxigenic Escherichia coli* (ETEC) maintained the virulence genes *eltB* and *estA* encoding LT and STh entero-toxin (Lothigius *et al.*, 2010), no enterotoxin was detected in GM1-ELISA method. Pinto *et al.* (2015) suggested that VBNC-state bacteria were not pathogenic unless they recovered to normal cells. Nevertheless, VBNC-state cells can recover quickly under suitable conditions, leading to potential danger of fatal diseases (Du *et al.*, 2007).

Induction and resuscitation of VBNC-state pathogenic bacteria in milk and dairy products

VBNC is a response mechanism of bacteria to environmental stress, so many conditions that are not conducive to the growth and reproduction of pathogenic bacteria may induce pathogenic bacteria to VBNC state. Different bacteria and different strains of the same bacteria may be of different conditions and mechanisms for entering VBNC state. Common inducing factors include physical factors, i.e., low/high temperature (Dinu *et al.*, 2013), humidity (Barron *et al.*, 2007), light intensity (Zhang *et al.*, 2015), starvation (Du *et al.*, 2007; Chen *et al.*, 2018), thermosonic (TS) (Liao *et al.*, 2018), pulsed light and high pressure carbon dioxide (HPCD) (Wasfi *et al.*, 2020); chemical factors, i.e. nutrients (Ogane *et al.*, 2019), harmful chemicals (Hamabata *et al.*, 2021) and biological factors (Liu *et al.*, 2009).

During the processing, storage and transportation of milk and dairy products, there are various stress factors, such as high/low temperature, oligotrophic, extreme pH, etc., all of which may induce pathogenic bacteria to enter VBNC-state. Jameelah *et al.* (2018) found that *Enterobacter sakazakii* was induced to VBNC-state under the conditions of simulated milk powder production and processing (1/10TSA, stainless steel pipeline transportation). Ruggirello *et al.* (2018) cultivated cheese samples at 4°C and 12°C for 17 days, and found that pseu domonas was in VBNC-state which could be resuscitated; Gunasekera *et al.* (2002) used labeled *E. coli* with lactose-inducible green fluorescent protein (*gf*) gene, and heated milk at 63.5°C for 30 min. Then the number of viable cells were measured by dye-exclusion based bacteria counting method, which was significantly higher than that measured by plate counting method. The results suggested that *E. coli* in milk after pasteurization was in VBNC-state.

Favorable growth conditions with energy source and ideal carbon to inorganic element ratio can reverse VBNC-state, namely

Table 1. Induction and resuscitation factors of common VBNC-state foodborne pathogens in milk and dairy products

Bacteria	Samples	VBNC-state inducing factors	Resuscitation method	Reference
Staphylococcus aureus	Raw cow's milk	Oligotrophic synergistic low temperature; potassium sorbate	Sterile Tween 80 treatment	Masmoudi *et al*., 2010
Escherichia coli	Pasteurized milk	Oligotrophic synergistic low temperature; chlorides; pasteurization; low temperature	Add intestinal bacterial autoinducer; heating method; add Tween 80 to synergize heating	Lin *et al*., 2017; Orruño *et al*., 2017
Listeria monocytogenes	Pasteurized chocolate milk	Low pH; pressure conditions; weak acidity synergistic low temperature	Add resuscitation promoting factor; embryoculture	Overney *et al*., 2016
Vibrio parahaemolyticus	Milk	Low temperature; low salt synergistic low temperature; oligotrophic synergistic low temperature	Add yeast liquid, Tween 80 and heat up; heating-up	Yoon *et al*., 2020
Campylobacter jejuni	Raw milk	Acidic conditions; oligotrophic synergy with low temperature; artificially simulated seawater environment synergetic low temperature	Mouse intestinal resuscitation	Chaveerach *et al*., 2003; Baffone *et al*., 2006
Enterobacter sakazakii	Milk powder	Pasteurization 63.5°C for 30 min; oligotrophic; low temperature; drying	Add 0.1% sodium pyruvate	Zhong *et al*., 2017

resuscitation. The induction and resuscitation factors of several common VBNC foodborne pathogens in milk and dairy products are shown in Table 1.

Food storage usually requires low temperature conditions. The low temperature was the most important inducing factor of VBNC, and oligotrophic factors were used as synergistic factors. However, previous studies (Gunasekera *et al*., 2002) also showed that sterilization at high temperature could result in VBNC-state, such as pasteurization of milk. VBNC conditions can occur not only at low temperatures, but also at elevated temperatures below lethal temperatures. Therefore, it is necessary to strengthen the monitoring of foodborne pathogens via control of storage temperature (Zhong *et al*., 2017).

VBNC-state bacteria can only resuscitate to cultivable state under suitable conditions, but cells in the dying stage do not have this capability. It is worth noting that VBNC-state can only be maintained for some time. Once the appropriate time or conditions for resuscitation are missed, the cells will enter apoptosis process. So far, quite a few studies have focused on resuscitation of VBNC-state mechanisms of bacterial proposed Scout cells, or "exploring factors", which was considered the key to resuscitation of VBNC-state bacteria (Epstein, 2009; Epstein, 2013). ''Exploring factors" first respond upon adverse changes from environment, and secrete signal molecules to other cells, prompting other cells to enter VBNC-state and resist the unfavorable environment. Once the stress factor is relieved, "exploring factors" secretes another signal molecule, such as Rpf protein, promoting the recovery of some VBNC-state cells, while VBNC-state cells that fail to sense the signal

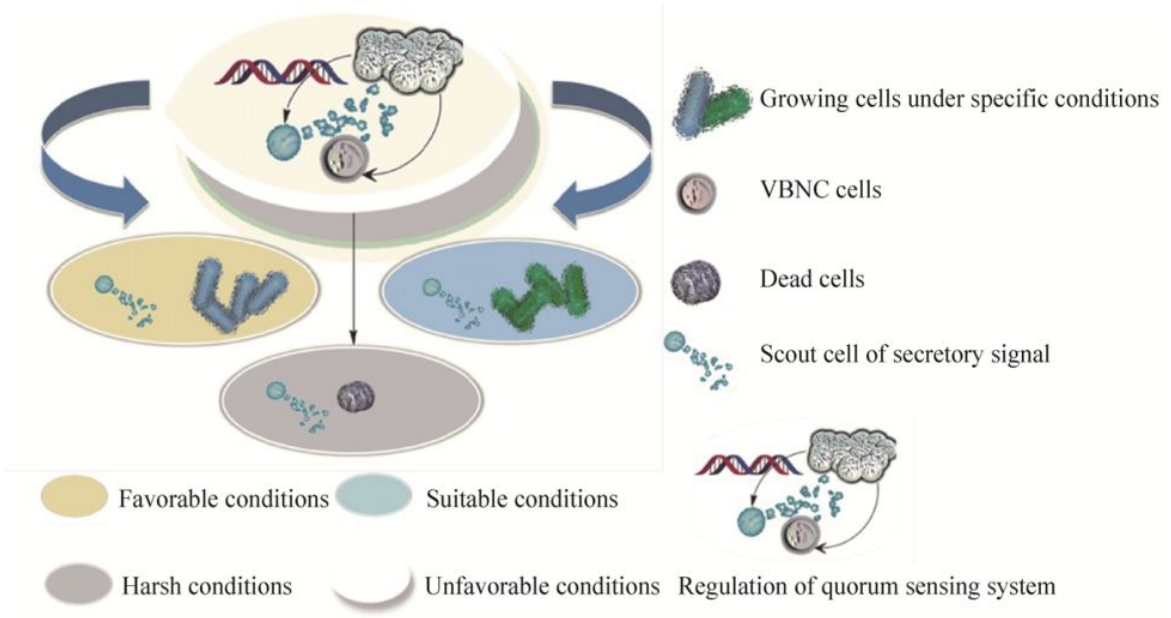


Fig. 2. Schematic diagram of mechanism of formation and resuscitation of VBNC cells (Zhang *et al*., 2018)

molecule decline until death (Fig. 2).

As a complex process, resuscitation cannot be achieved simply by removing the inducing factors. So far, it is not possible to restore all bacteria to cultivable state. There are still some controversies about resuscitation. It is difficult to prove whether the number of cultivable bacteria after resuscitation is due to cell resuscitation or regeneration of some undetected cultivable bacteria. However, the uncertainty and diversity of the resuscitation conditions did not prevent further exploration of resuscitation.

Nevertheless, VBNC-state pathogen poses a major potential risk in food safety since it is an important part of microbial contamination. It is urgent to further study VBNC-state pathogens. In particular, it is worth noting the virulence and pathogenicity of cells resuscitated from VBNC-state in milk and dairy products under processing, storage and transportation. Therefore, the exploration of reasonable and effective methods for detecting bacteria in VBNC-state is of great importance for prevention and control of pathogenic bacteria in milk and dairy products.

Detection methods of VBNC-state in milk and dairy products

The detection of VBNC-state bacteria includes total bacteria count detection, viable cell count detection and cultivable viable cell count detection, as the VBNC state can only be declared when there is zero cultivable cell count but the viable cell count is not zero. Currently, there are many detection methods for determining VBNC-state pathogens in milk and dairy products. In addition to the "gold standard" Plate count method (PC), there are also Acridine orange direct count (AODC), Direct viable count (DVC), LIVE/DEAD backlight bacterial viability kit (LIVE/DEAD), Fluorescent antibody staining (FAC), molecular biology methods, breath detection methods and immunological methods, etc. The research status of each detection method is shown in Table 2.

Our previous (Zhao *et al*., 2019) research developed a simple and specific method for the direct detection of *Streptococcus agalactiae* in dairy products. Propidium monoazide (PMA) and sodium dodecyl sulfate (SDS) were used to eliminate the interference of dead and damaged cells in qPCR. Lysozyme (LYZ) was

Table 2. Several common detection methods of VBNC pathogenic bacteria in milk and dairy products

Method category	Mechanisms	Method application	Features	Pros and cons	Reference
Traditional method	Nalidixic acid inhibits cell division and makes it elongate	PC	Changes in bacterial morphology	Advantages: convenient and direct; Disadvantages: poor accuracy	Kogure *et al.*, 1979
Optical method	Stain with acridine orange or Live/Dead kit and determine the survival status of bacteria using fluorescence microscope	1) AODC; 2) DVC; 3) LIVE/DEAD	Use nucleic acid dye distinguishes dead and live bacteria	Advantages: fast and accurate; Disadvantages: small measuring range	Tian *et al.*, 2013
Immunological methods	Microbiological analysis using specific reactions of antigens and antibodies	1) Enzyme-linked immunosorbent assay 2)Indirect immunofluorescence	Bind to its specific antibody	Advantages: simple operation, strong specificity and anti-interference, low detection limit; Disadvantages: low accuracy	Razzuoli *et al.*, 2018
Molecular Biological methods	Amplify specific DNA for qualitative or quantitative detection	Quantification of 16S ribosomal RNA; membrane integrity	1) EMA/PMA-qPCR 2) LYZ*-SDS#-PMA-qPCR 3) Large-capacity IMS-PCR** 4) qPCR 5) RT-qPCR##	Advantages: sensitive, specific, and rapid results; Disadvantages: mRNA is easily degraded, and the extraction process is complicated	Zimmermann *et al.*, 2014; Luo *et al.*, 2017; Zhao *et al*, 2019
Loop-mediated thermostatic nucleic acidAmplification (LAMP)	Design specific primers for the target region of the target gene, and utilize Bst DNA polymerase with strand displacement activity to complete the amplification reaction under constant temperature conditions	Fluorescent dyes, DNA amplification	1) LAMP direct detection; 2) IMS+LAM 3) PMA+LAM	Advantages: high specificity, sensitivity and rapidity, low cost, easy operation; Disadvantages: few applications	Li *et al.*, 2017; Yan *et al.*, 2017
Biological sensor	An instrument that is sensitive to biological substances and converts its concentration into	Lytic activity of living cells	1) Electrochemical biosensor targeting DNA recognition	Advantages: Convenient production, low price, strong specificity, and durability;	Xu *et al.*, 2018

Cont. Table 2.

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Method category	Mechanisms	Method application	Features	Pros and cons	Reference
	electrical signals for detection, which is an analysis tool or system formed by immobilized biologically sensitive materials as identification elements (enzymes, antibodies, antigens, etc.) and appropriate physical and chemical transducers (oxygen electrodes, photosensitive tubes, etc.) and signal amplifying devices		2) Biosensor based on the infection rate of bacteriophages	Disadvantages: potential inhibitory effect of inherent substances in bacteria.	

*, LYZ: Lysozyme; **, SDS: Sodium dodecyl sulfate; ***, IMS-PCR: Immunomagnetic separation-PCR; ##, RT-qPCR: Reverse Transcription-polymerase chain reaction

adopted to increase the extraction efficiency of target bacteria DNA in milk matrix. The established LYZ-SDS-PMA qPCR method has good sensitivity and specificity for the detection of live *Streptococcus agalactiae* in milk.

In the detection of dairy products, traditional national standard detection methods (such as isolation and culture, biochemical identification) cannot detect pathogenic bacteria that are difficult to cultivate or cannot be cultured. They have disadvantages such as poor specificity, complicated operation, and time-consuming, and cannot achieve timely and effective detection. For VBNC-state pathogenic bacteria in dairy products, both optical and immunological methods are prone to missed detection, and there are many factors influencing ELISA technology among immunological methods. Structural analogs can also cause different degrees of cross-reactions, leading to false positive result. In addition, the cost of PCR and biosensor methods are very high and unsuitable for large-scale promotion. LAMP technology and RPA technology have good specificity and sensitivity in detecting VBNC-state bacteria, and only simple thermostatic equipment can be used for LAMP reaction at a relatively low cost. Due to the interference of dead bacteria and protein and fat in the food matrix, LAMP or RPA detection method alone still has their shortcomings. Current research usually combines active staining, immunomagnetic bead enrichment, sensor technology and molecular biology methods. Such practice only provides new detection ideas, but also can effectively avoid the shortcomings of a single method, thereby improving the detection accuracy of foodborne VBNC-state pathogens.

Future studies

There are still many limitations of VBNC-state bacteria research. To remove these limitations following problems are required to be solved through further studies:

1. Improve the detection standard system of VBNC-state foodborne pathogens.

2. Value evaluation and application development of VBNC-state bacteria in food fermentation, agriculture, environmental protection and other fields.
3. To study the antimicrobial resistance of VBNC-state bacteria.
4. More integrated multiomics analysis of the universal mechanisms of the VBNC state can be carried out in future studies.

Conclusion

With the development of modern biotechnology, a variety of molecular biology research methods provide the possibility to understand more VBNC-state bacteria. VBNC research not only has important significance in epidemiology and public health, but people can also use the VBNC state of bacteria to prolong the shelf life of bacteria. For certain probiotics, it can avoid damage during the production process, simplify the preparation process, and develop new long-acting micro-ecological live bacteria preparations. In addition, it also has high value in the animal husbandry industry, such as research on foodborne VBNC-state pathogens, establishment and improvement of corresponding animal product testing systems, etc. Therefore, cultivable methods and technologies of VBNC-state bacteria will become the focus of related research in the future, which can provide a new scientific basis for re-understanding and evaluating the role of microorganisms in the fields of food fermentation, agriculture, environmental protection, etc., and endow it with broader development and application prospects.

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Author's contributions: YZ, HL: Wrote the manuscript; HC, FW, SW, YW: Jointly established the detection method of LYZ-SDS-PMA-qPCR, contributed to the writing of "Detection methods of VBNC-state in milk and dairy products"; XF: Revised the format of the paper and completed the submission; CW, JL: Completed the assumption of the overall framework of the review and revised the article many times; YZ, HL: Contributed equally in this work.

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