The effect of some disinfectants in inducing the viable but nonculturable (VBNC) state of some pathogenic bacteria

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Abstract

Cells that are physiologically viable but cannot be cultivated on traditional culture media are referred to as viable and non-culturable cells (VBNC), because it cannot grow or divided. Those bacteria enter the (VBNC) state under stressed conditions, as a survival strategy. Although the bacteria that fail to grow on conventional culture media, but are actually alive and can be resuscitated from the VBNC state under suitable conditions. The VBNC state may pose a great threat to food safety and public health. The bacteria such as *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 was entered the VBNC state when exposed to harsh conditions like the addition of preservatives, disinfectants, and food processing steps. In this paper, some bacteria *(Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 are all pathogens) were induced to enter the VBNC state by using sodium hypochlorite and hydrogen peroxide at different contact times. Culturable methods, which include (enrichment and resuscitation methods and the ATPase method, were used for detecting the bacteria used in this study. The result showed that *Salmonella enterica*, *Listeria monocytogenes,* and *Escherichia coli* O157:H7 was lost culturability and were not detected when treated with 0.01% sodium hypochlorite and 5% hydrogen peroxide for 50 minutes when detected by the enrichment method , whereas were detected by the ATPase method. However, the H_2O_2 and NaClO -treated bacteria recovered from a non-culturable to a culturable state in M9 minimal medium with the presence of 30 mM sodium pyruvate, Thus, the results suggested that conventional methods of detection the pathogenic bacteria, like enrichment methods, can not be used and could be caused big problems for health. While, the best method for detecting VBNC bacteria was the ATPase method in terms of speed, accuracy, and ease.

Keywords: Resuscitations, harsh conditions, disinfectants, ATPase, Viability.

Introduction

Many bacteria adopt the "viable but non-culturable" (VBNC) state as a unique survival strategy in response to adverse environmental conditions (26). The VBNC cells of foodborne pathogenic microorganisms are easily missed when using the traditional plate counting strategy, and they can be recovered with pathogenicity under certain conditions, resulting in an immediate threat to human health. VBNC cells were not dead due to several differences, while VBNC cells have an intact membrane that allows them to retain undamaged genetic material, whereas the cells have a destroyed membrane that prevents them from maintaining chromosomal and plasmidic DNA (26). VBNC cells are metabolically active and metabolize oxygen, whereas dead cells do not. *Listeria monocytogenes* showed a high ATP level even a year after entering the VBNC state. Furthermore, VBNC cells continue transcription and mRNA production, while cells do not express genes (17). The VBNC study attracted the attention of researchers in microbiology due to it challenged the standard conception of microorganism growth and development. in contrast to normal cells that cannot be cultured the VBNC cells in conventional culture media, which making difficult through sanitizing technique for removing completely. Some food-borne illnesses maintain their virulence after entering the VBNC state, due to certain conditions, they were resuscitated rapidly into culturable cells (12). Although VBNC pathogens are typically thought to be unable to cause illness, their virulence can be restored or maintained after resuscitation, which might result in disease or infection (11). For example, *Listeria monocytogenes* VBNC cells that were resuscitated by being incubated with embryonated eggs regained virulence that was exactly similarity culturable cells (28). Moreover, a large amount of data suggests that VBNC bacteria may be connected to outbreaks of foodborne illness. In Japan, a foodborne outbreak caused by salted salmon roe contaminated with *E. coli* O157:H7 was recorded (15). A member of the *E. coli* species that causes diarrhea, enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, also known as shigatoxin-producing *E. coli* (STEC), is a food-borne and pathogenic zoonotic disease (2). VBNC pathogens can be a serious threat to both public health and food safety. Some of the most significant human pathogens that cause foodborne diseases (5). Disinfectants like hydrogen peroxide and sodium hypochlorite are excellent anti-biofilm agents through killing both the bacteria cells and the biofilm matrix. as well as was killed the bacteria in biofilms permanently by denaturing proteins in the biofilm matrix and inhibiting the important enzymatic activities for bacteria (18), but hydrogen peroxide causes the VBNC state in *Salmonella enteritis*, according to (13). Also, chlorine dioxide and mono-chloramine (30) have both been shown to produce the VBNC condition in bacteria. VBNC bacteria have a high level of tolerance, which making difficult through sanitizing technique for removing completely (7). Recently, foodborne pathogens are recognized as a potential threat to food safety because they can enter the VBNC state during food processing steps such as high temperatures, high pressure, disinfection processes, preservation, and low-temperature storage. This study aimed to induce *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 into a viable but non-culturable state (VBNC) by exposing to different food disinfectants

, and to detect the bacteria that were used in this study by different methods (enrichments, resuscitations, and ATPase) to find out the optimize methods for pathogenic bacteria detection to ensure the safety of foods and food contact surfaces.

Materials and Methods:

1. Bacterial strains preparation

The following strains were used: *Salmonella enterica* ATCC 13311, *Listeria monocytogenes* ATCC 19115, and *Escherichia coli* O157:H7 ATCC 700728. Firstly, each strain was activated in 10 ml of nutrient broth, propagated by two sub-culturing steps in nutrient broth, and incubated at 37 ^{o}C for 24 hours. The purity was confirmed by gram staining (27). The pre-cultured bacteria were harvested using centrifugation at 2,850 g for 20 min at 4°C after being twice washed with 10 mL of normal saline and suspended in 10 mL of fresh nutrient broth (bacteria adjusted to about 1×10^{9} cfu.mL^{-1}) according to the McFarland standard.

2. Induction of the viable but non-culturable states (VBNC) in *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 by NaClO and H_2O_2

According to (21), with little modifications, pre-cultured bacteria were induced to enter the VBNC state as follows: Precultured bacteria were suspended in a 50-mL tube of fresh nutrient broth that had been treated with 5% hydrogen peroxide and 0.01% sodium hypochlorite and 10 mL of nutrient broth (23). To expose the cells to the disinfectant, the cells (1×10^{7} cfu.mL^{-1}) according to the McFarland standard were then incubated at 37 °C for 15, 30, 45, and 50 minutes while being shaken at 150 cycles per minute. As previously described, the bacteria were separated by centrifugation and re-suspended at 1×10^{7} cells mL^{-1} according to the McFarland standard in fresh nutrient broth. After serial dilution with normal salin, the bacteria were cultured on Salmonella Shigella agar for *Salmonella enterica*, Oxford agar for *Listeria monocytogenes*, and MacConky sorbitol agar for *Escherichia coli* O157:H7 and incubated at 37 °C for 24 hours. Colony size and morphology were noted. The colonies that resulted were counted for the number of living bacterial cells (cfu), which is a measure of culturability. Also, the viability of bacteria is detected by different methods: enrichments, resuscitations, and ATPase.

3. Effects of NaClO and H2O2 sanitizing treatments on inducing *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in a viable but non-culturable state on celery

25 g of celery intentionally contaminated with pre-cultured *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7. Then the contaminated celery was sanitized by exposing it to 5% hydrogen peroxide and 0.01% sodium hypochlorite, after that incubated at 37 °C for 15, 30, 45, and 50 minutes while shaken at 150 cycles per minute. The bacteria were harvested by centrifugation and re-suspended at 1×10^{7} cells per mL according to the McFarland standard for fresh nutrient broth. After serial dilution with normal salin, bacteria were cultured on Salmonella Shigella Agar for *Salmonella enterica*, Oxford agar for *Listeria monocytogenes*, and MacConky sorbitol agar for *Escherichia coli* O157:H7, and incubated at 37 ^{o}C for 24 hours. Colony size and morphology were noted. The number of live

bacterial cells per colony-forming unit (cfu) was measured. Also, the viability of bacteria is detected by different methods: (enrichments, resuscitations, and ATPase).

4. Enumeration of VBNC bacteria by enrichment method

4.1. Enrichment of *Salmonella enterica* :

The pre-enriched *Salmonella enterica* were incubated for 24 hours at 37 °C in broth-buffer peptone water. 1 mL of the pre-enrichment inoculum was added to 10 ml of tetrathionate broth, and the mixture was then incubated for 24 hours at 37 °C. Three selective agar media—Salmonella-Shigella agar, Brilliant Green agar, and Deoxycholate agar—were each plated with a 100-L aliquot of tetrathionate broth culture and incubated at 37°C for 24 hours. After incubation, the colony size and morphology were noted (16), and suspected *Salmonella* colonies were sub-cultered to a selective Salmonella-Shigella agar plate and a non-selective Nutrient agar plate and incubated at 37 °C for 24 hours (20).

4.2. Enrichment of *Listeria monocytogenes*:

Listeria monocytogenes were pre-enriched in broth-buffer peptone water and incubated for 24 hours at a temperature of 37 °C.

After incubation, 1 mL of the pre-enrichment inoculum was added to 10 mL of fraser broth, and the mixture was then incubated at 37 oC for 24 hours. A 100-L aliquot of the fraser broth culture was added to two different selective agar media—Oxford agar and Brilliant Green agar—and then incubated at 37°C for a period of 24 hours. After incubation, the colony size and morphology were recorded, and suspected *Listeria monocytogenes* colonies were subcultured on a selective Oxford agar plate and a non-selective nutrient agar plate and incubated at 37 °C for a duration of 24 hours (9).

4.3. Enrichment of *Escherichia coli* O157:H7:

In broth-buffer peptone water, the *Escherichia coli* O157:H7 was pre-enriched and incubated for 24 hours at 37 °C. After incubation, 1 ml of the pre-enrichment inoculum was added to 10 ml of Nutreint broth, and the mixture was incubated at 37 °C for 24 hours. On three selective agar media — MacConkey Agar, MacConkey Sorbitol Agar, and Phenol Red Brilliant Green Agar — a 100-L aliquot of nutrient broth culture was spread on each. The media were then incubated at 37 °C for 24 hours. After incubation, the colony size and morphology were noted, and suspected *E. coli* O157:H7 colonies were subcultered to a selective MacConky agar plate and a non-selective Nutrient agar plate, which have a short generation time (4), and incubated at 37 °C for 24 hours (29).

5. Resuscitation of VBNC (*Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7)

Salmonella enterica, *Listeria monocytogenes*, *and Escherichia coli* O157:H7 that had been exposed to H_2O_2 and NaClO were harvested by centrifuging, suspended in M9 medium at 1×10^9 cfu mL^{-1} with 30 mM sodium pyruvate, and then incubated at 37 °C for 1, 2, and 4 hours, respectively. Bacterial growth was evaluated by the visually monitored turbidity of the bacterial solution and colony-forming unit by using Salmonella Shigella agar for *Salmonella enterica*, Oxford agar for *Listeria monocytogenes*, and MacConkey sorbitol agar for *Escherichia coli* O157:H7. and incubated at 37 °C for 24 hours. Colony size and morphology were noted (21).

6. Detection of *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 by ATPase

6.1. Detection of *Salmonella enterica* :

The InSite™ *Salmonella* test devices were used, and the manufacturing company's instructions were followed. A liquid medium that is specifically designed with growth promoters and chromogenic compounds for *Salmonella* species is contained in each device (24). Neutralizers are included in the wetting solution of each InSite™ test device to help reduce any potential sanitizing side effects and improve sample collection. Simply, 1 mL of each serial dilution was transferred to the InSite™ test device and incubated at 37°C. After 24–48 hours of incubation, a color change can be considered a presumptive positive for *Salmonella* species. Negative samples were cultured for 48 hours before they could be deemed negative. As the chosen population increases, the medium turns acidic, the pH changes, and the color changed from purple to brilliant yellow. After 24 hours at 37°C, a medium's ocular hue changes from purple to bright yellow, indicating a presumed positive test for *Salmonella*, as illustrated in (Fig 1).

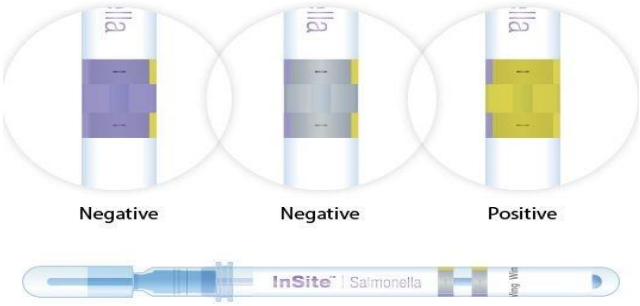

Figure 1. The color changed from purple to bright yellow, indicating a presumed positive test for *Salmonella.*

6.2. Detection of *Listeria monocytogenes*

The InSite™ *Listeria* test devices were used, and the manufacturing company's instructions were followed. A liquid medium that is specifically designed with growth promoters and chromogenic compounds for *Listeria* species is contained in each device (24). Neutralizers are included in the wetting solution of each InSite™ test device to help reduce any potential sanitizing side effects and enhance sample collection. Simply, 1 mL of each serial dilution was transferred to the (InSite™) device and incubated at 37°C. When the media's color changed from yellow to amber to grey to black, *Listeria* species were presumed to be present. Samples presumed to contain *Listeria* species that exhibit green fluorescence when exposed to ultraviolet light (UV 395–400 nm) are presumed to contain *L. monocytogenes*, as shown in (Figure-2).

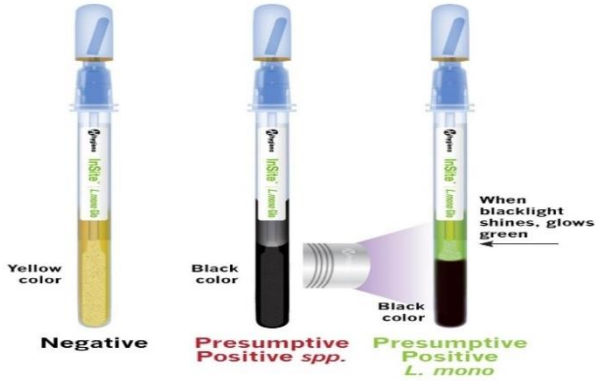

Figure 2.The color changed from yellow to amber to grey then to black, is indication for the presence of *Listeria* species.

6.3. Detection of *Escherichia coli*

The MicroSnap™ *E. coli* test device was used to detect the presence of *E. coli* pathogens. The test uses a special bioluminogenic reaction that generates light when *E. coli*-specific enzymes interact with certain substrates. The EnSURE™ luminometer is then used to quantify the light-generating signal. Results are available in not much more than eight hours. The manufacturing company's instructions were followed for the detection of *E. coli* (24) and as follows:

1 ml of liquid samples directly poured into the enhanced nutrition container and mixed for 10 seconds. Depending on the level of sensitivity required, incubated the sample for 6–8 hours in a Hygiena Digital Dry Block Incubator at 35ºC. Then the test device MicroSnap™ showed in (Figure-3) shaked by firmly flicked it once downward. As a result, the extractant liquid reached the tube's bottom. After that, remove the enhanced nutrient broth tube from the incubator and vortex or hand-shake the sample for 10 seconds to distribute it. Then the bulb was removed, and 0.1 mL of the enriched sample was aseptically transferred into the detection device tube. The detection device returned to its original setting. The snap-valve was broken by moving the bulb back and forth with the thumb and fingers. The detecting device was activated as a result of this. After the detection device started working, all liquid was expelled into the swab tube's bottom, where it was then gently shaken to blend. The results appeared in 15 seconds later.

Figure 3. The Micro-Snap™ *E.coli* detecion device

7.Statistical-analysis

The XLSTAT (2016) application's one-way analysis of variance (ANOVA) function was

used to compare the VBNC and the number of culturable cells. Using Duncan's multiple range tests, the variances between the means were calculated. The significance threshold was set at (P0.05), and the data's average and standard error were reported.

Results and Discussions:

Enumeration of VBNC bacteria by culturable and ATPase methods

H_2O_2 and NaClO are good bactericidal agents, and they are widely used for the disinfection of vegetables, fruits, and environmental surfaces. Disenfectants like H_2O_2 and NaClO produce the VBNC condition in bacteria and can cause public health problems (5). As shown in tables (1&2) outlines the results of the effects of two disinfectants tested against *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 during different contact times. by using the viable total count of *L. monocytogenes*, *S. enterica*, and *E. coli* O157:H7 reduced the number of studied bacteria by exposing to 5% of H_2O_2 or 0.01% of NaClO, there was progressive declination in the count of *L. monocytogenes*, *S. enterica*, and *E. coli* O157:H7 in proportion to the duration of exposure to food sanitizer, until no colonies were formed on plates after 50 min when tested by culturable methods. Meanwhile, all bacteria were detected at 50 min when examined by using the ATPase method. theresults suggest that these bacteria changed from culturable state to the nonculturable state VBNC treated with 5% of H_2O_2 or 0.01% of NaClO for 50 min. the bacteria of VBNC state might decrease their metabolic activity by shrinking their surface area and lowering the exchange of elements across their cell surface, as well as it is difficult to detect it by culturable methods (8). These results agreed with (19), who found that the ATPase methods were more accurate than the enrichment methods. Considering ATPase is more accurate and faster than traditional methods(3), it is better to used ATPase method to control pathogenic bacteria in foods. It is important that any direct microbiological test must be rapid to be compatible with HACCP (10).

Table 1. Effects of H_2O_2 (5%) on the culturability cfu/ml of *Salmonella enterica*, *Listeria monocytogenes*, and *E. coli* O157:H7 after (15, 30, 45, and 50 minutes) of contact time.

Bacteria / Time(hrs)	*Salmonella enterica*	*Listeria monocytogenes*	*Escherichia coli*
0 min.(active culture)	1×10^{9}	1×10^{9}	1×10^{9}
15min.	2×10^{3b}	1×10^{3c}	3×10^{3a}
30min.	10^{d}	13^{d}	16^{d}
45min.	2^{d}	3^{d}	2^{d}
50min.	0^{d} (ND)	0^{d} (ND)	0^{d} (ND)

Different letters within each column refer to a significant difference between the means($p<0.05$) according to Duncan's test.

ND:Not Detected

Table 2. Effects of NaClO (0.01%) on the cultureability cfu/ml of *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 after (15, 30, 45, and 50 minutes) of contact time.

Bacteria / Time(hrs)	*Salmonella enterica*	*Listeria monocytogenes*	*Escherichia coli*
0 min.(active culture)	1×10^{9}	1×10^{9}	1×10^{9}
15min.	2×10^{3b}	1×10^{3c}	3×10^{3a}
30min.	10^{d}	10^{d}	11^{d}
45min.	3^{d}	4^{d}	1^{d}
50min.	0^{d} (ND)	0^{d} (ND)	0^{d} (ND)

Different letters within each column refer to a significant difference between the means($p<0.05$) according to Duncan's test.

ND:Not Detected

Resuscitation of VBNC (*Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli* O157:H7)

The resuscitation refers to the culturability of VBNC cells, which occurs when their metabolic functions resume their normal states (14) showed that time-dependent resuscitation of H_2O_2 and NaClO -treated cells in M9 media was induced by adding 30 mM sodium pyruvate, and increased the numbers of viable and culturable bacteria because more cells were able to divide and form colonies after the resuscitation procedures. The creation of big molecules like DNA and proteins is restored by sodium pyruvate, putting the VBNC cells back in a state that allows for culture (26).

Tables(3&4) showed the effects of using M9 medium with 30 mM sodium pyruvate in resuscitating pathogenic bacteria used in this study after exposure to H_2O_2 (5%) and NaClO (0.01%) for 50 min. The tables also showed that all bacteria slowly regained their ability to form colonies after one hour and began to increase rapidly. The efficiency of resuscitation appears to be in detecting pathogenic bacteria that were exposed to harsh conditions, as long as the bacteria in processed foods are often under stressed conditions and cannot be detected by traditional methods, which poses a great danger to the public health of an individual (22).

Table 3. Resuscitated VBNC bacteria after exposure to H_2O_2 (5%) in M9 medium with sodium pyruvate for (1, 2, and 4 hours) and examined their ability to form cfu.

Time / Bacteria	1 hour	2 hours	4 hours
Salmonella enterica	664	1761	TNTC
Listeria monocytogenes	631	941	TNTC
E.coli O157:H7	TNTC	TNTC	TNTC

TNTC: too numerous to count.

Table 4. Resuscitated VBNC bacteria after exposure to NaClO (0.01%) in M9 medium with sodium pyruvate for (1, 2, and 4 hours) and examined their ability to form cfu.

Time / Bacteria	1 hour	2 hours	4 hours
Salmonella enterica	532	1011	TNTC
Listeria monocytogenes	589	927	TNTC
E. coli O157:H7	TNTC	TNTC	TNTC

TNTC: too numerous to count.

These studies illustrated that M9 medium with 30 mM sodium pyruvate was a powerful medium to enhance the resuscitation of VBNC states of pathogenic bacteria due to an increase in the number of cells able to divide and form colonies. Additionally, the VBNC cells were restored to a culturable state after the mixture of M9 with sodium pyruvate was added, which helped restore DNA and protein synthesis (21).

Effects of NaClO and H_2O_2 sanitizing treatments on inducing a viable but non-culturable state of *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 on celery

Ready-to-eat Celery was intentionally contaminated with *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica* and then sanitized by disinfectant (5% H2O2 and 0.01% NaClO) at different times (15, 30, 45, and 50 minutes). All pathogenic bacteria were not detected after 50 minutes by the culturable method (19). While by using the ATPase method, all bacteria were detected after 50 min, as seen in (Table-5), chlorine and hydrogen peroxide that were used to sanitize vegetables induced all pathogenic bacteria to enter the VBNC (6). This data shows the risk that VBNC pathogenic bacteria can pose to public health.

Table 5. Effects of NaClO and H_2O_2 sanitizing treatments on inducing a viable but non-culturable state of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* on celery were examined by enrichment methods and ATPase methods.

Pathogenic bacteria	Disinfectant	Enrichment-methods	ATPase methods
S. enterica	H_2O_2 5%	Not. Detected	Detected
	NaClO 0.01%	Not. Detected	Detected
L. monocytogenes	H_2O_2 5%	Not. Detected	Detected
	NaClO 0.01%	Not. Detected	Detected
E. coli O157:H7	H_2O_2 5%	Not. Detected	Detected
	NaClO 0.01%	Not. Detected	Detected

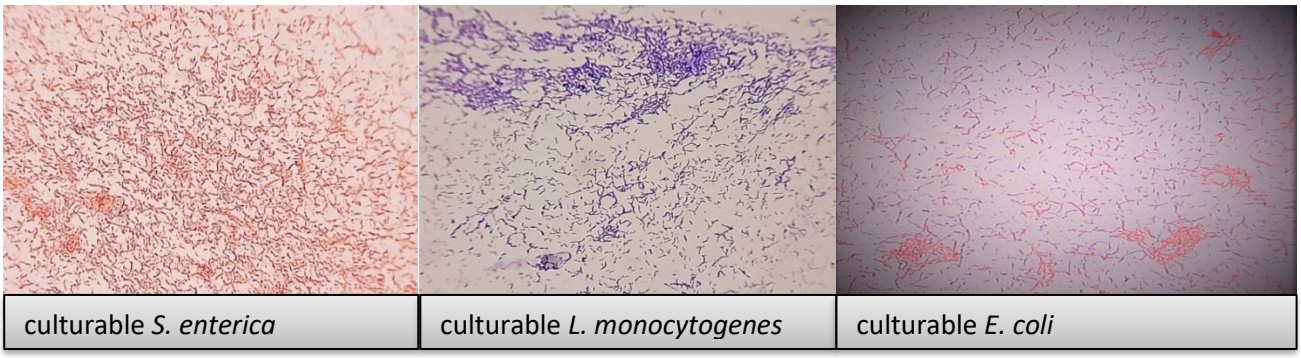

Figure 4. Gram staining morphological observation of culturable *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 (magnification: 100X)

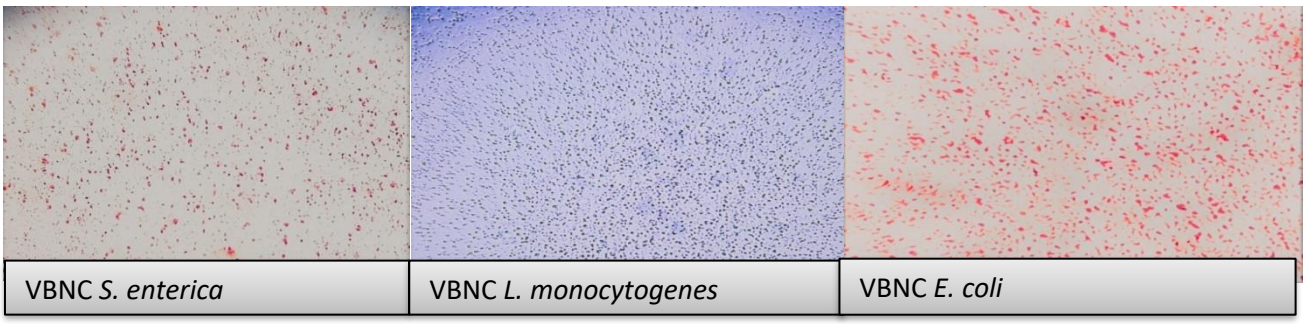

Figure 5. Gram staining morphological observation of VBNC *S. enterica*, *L. monocytogenes*, and *E. coli* O157:H7 (magnification: 100X)

During this study, *Listeria monocytogenes*, *Salmonella enterica*, and *Escherichia coli* O157:H7 were changed morphologically from their characteristic rod to cocco-bacilli shape, while a transition to the VBNC state under a microscope was illustrated in (Figure-4&5),

bacterial cell shapes were examined (1). Cellular morphology significantly influences the phenotype of a cell. Any variations in the cell wall composition may impact the cellular morphology since the cell wall/peptidoglycan dictates the cell's shape and provides the tensile strength and diffusion barriers required to achieve a specific shape. Some VBNC bacterial cells exhibit this since many have different cellular shapes, including cell dwarfing and rounding. It is believed that a technique to reduce the energy requirements of VBNCs is a reduction in cell size. For instance, it was noted that during the transition to the VBNC, *Salmonella enterica*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 cells morphology changed from their typical rod to cocco-bacilli form (25).

Conclusion

Food sanitizers like (5% hydrogen peroxide and 0.01% sodium hypochlorite) cause *Salmonella enterica* ATCC 13311, *Listeria monocytogenes* ATCC 19115, and *Escherichia coli* O157:H7 ATCC 700728 to enter the VBNC states. Enrichment methods are inaccurate in detecting VBNC pathogenic microorganisms in processed food, and instead of enrichment, That better to utilize resuscitation or ATPase methods to detect the pathogenic organism in foods. ATPase assay enrichment highlights the importance of microbial assays in areas such as the HACCP system where necessary rapidly results.

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Conflict of interest

The authors have no conflict of interest.

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