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Review

Role of food sanitising treatments in inducing the 'viable but nonculturable' state of microorganisms

Sergio Ferro [a, *, 1], Tony Amorico [a], Permal Deo [b]

[a] Ecas4 Australia Pty Ltd, 8/1 London Road, Mile End South, SA 5031, Australia
[b] Sansom Institute for Health Research, School of Pharmacy and Medical Science, University of South Australia, Adelaide, SA 5000, Australia

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ABSTRACT

By definition, foodborne outbreaks are illnesses affecting two or more people, correlated in terms of space and time, as a result of the ingestion of etiologic agents present in a common food. Ready-to-eat food products are normally subjected to treatments of sanitisation during processing that aims to miminise the microbial load. However, microorganisms have developed mechanisms to withstand adverse environmental conditions and fight for survival, including the ability to reduce their exposure to external attack through reversible modification of the morphology and physiology of their cell. This results in a significant change of their viability, which becomes undetectable through conventional culture techniques. Subsequent 'resuscitation' of these organisms in favourable food environment can represent a serious public health risk.

This review aims to examine the existing experimental evidence on the role of different sanitising approaches in inducing 'viable but nonculturable' state in microorganism and discuss possible approaches to reduce its occurrence.

Contents

1. Introduction

In recent years, there has been an increasing trend in the incidence of foodborne outbreaks worldwide. In some cases, the occurrences have been associated with food pathogens that are uncommon in food; for instance, recent outbreaks (CDC (Centers for Disease Control and Prevention), 2017; Food Safety News, 2017) have been associated with *Salmonella* spp. in cantaloupes (Jasper, 2016), papayas (FDA, 2017), and prepacked lettuce leaves (Koukkidis, Haigh, Allcock, Jordan, & Freestone, 2017; Lauder, 2016). In addition, the increasing tendency of people to prefer organic food – considered as a safer alternative to conventionally produced food (Harvey, Zachour, & Gould, 2016) – or to consume food ready-to-eat or prepared outside the home is raising concerns about hygiene and food processing conditions. Owing to the possibility of

* Corresponding author.
E-mail addresses: sergio@ecas4.com.au, fre@unife.it (S. Ferro).
[1] On leave from Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17, 44121 Ferrara, Italy.

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direct repercussions of foodborne outbreaks on global public health, sanitisation of food products is becoming a key step in pre-market processing. In most investigations, including recent studies like those by Svoboda et al. (2016), Ukuku, Mukhopadhyay, Geveke, Olanya, and Niemira (2016), and Hilton, de Moraes, and Moraru (2017), the efficacy assessment has been based on conventional enumeration techniques of the target microorganisms, and the results obtained could be questioned because of the possibility that the considered sanitisation approach may not have killed the microorganisms of interest but, instead, induced a 'viable but non-culturable' (VBNC) state.

The VBNC state is a condition of reduced vitality, induced in response to conditions of stress, which can be determined by a number of environmental factors, such as a lack of nutrients, an increase of temperature, or the presence of antibiotics, disinfectants and sanitisers (Dinu & Bach, 2011; Li, Mendis, Trigui, Oliver, & Faucher, 2014; Oliver, 2010; Pinto, Santos, & Chambel, 2015). When a microorganism enters the VBNC state, both the morphology and physiology of the cell are changed to allow its survival in the experienced adverse conditions. Then, once the environmental conditions return to normality, the cell can get back to its vegetative state (culturable), through a reactivation process that is referred to as 'resuscitation' (Ohtomo & Saito, 2001; Pinto et al., 2015; Roszak, Grimes, & Colwell, 1984).

Great attention has been devoted to the VBNC concept, and it is not our aim to rediscuss what has been already examined (at least on the basis of the available knowledge) through a number of reviews. Information on changes in cellular morphology, mechanisms behind the VBNC cell formation, detection of VBNC cells, and factors that stimulate resuscitation and its pathways can be found in the works by Ding et al. (2017), Fakruddin, Mannan, and Andrews (2013), Oliver (2010), Pienaar, Singh, and Barnard (2016), Pinto et al. (2015), Ramamurthy, Ghosh, Pazhani, and Shinoda (2014), and Zhao, Zhong, Wei, Lin, and Ding (2017).

This review presents a summary of available literature on VBNC status of microorganisms, with a focus on physico-chemical factors of induction and highlighting the possible implication of VBNC cells in foodborne outbreaks. In particular, attention is drawn towards the risk of uncritically using some sanitisation procedures, most of which are consolidated, especially in those cases where the outcomes are evaluated using conventional cultivation procedures.

2. Foodborne outbreaks associated with VBNC states in foods: food safety challenges

In food systems, VBNC state is reported to be triggered through conditions such as nutrient starvation, temperature, osmolarity, food preservatives, oxygen availability, heat shock (e.g. pasteurisation), and presence of antibiotics or of a biocide, e.g. chlorination (Asakura, Igimi, Kawamoto, Yamamoto, & Makino, 2005; Dinu & Bach, 2011; Epalle et al., 2015; Fakruddin et al., 2013; Hu & Coates, 2012; Li et al., 2014; Oliver, 2010; Pinto et al., 2015). When the bacterial cells are in a VBNC state, the total number of viable counts in a sample is underestimated if assessed using traditional enumeration techniques, due to the inherent non-cultivability of VBNC cells. In addition, when all the bacteria in the sample are in a VBNC state, assumptions are made that the sample is microbial free due to lack of detection. In food samples, infections due to unexpected outbreaks may be attributed to viruses as no viable bacteria are detected when culture techniques are used.

The ability of entering the VBNC status could be considered beneficial for bacteria, but represents a serious risk to human health. The presence of VBNC bacteria in food has been suggested and supported by evidence of virulence after resuscitation in food model systems (Dinu & Bach, 2011; Gunasekera, Sørensen, Attfield, Sørensen, & Veal, 2002; Nicolò et al., 2011; Ordax, Biosca, Wimalajeewa, López, & Marco-Noales, 2009). On the other hand, little information is available on the role of a VBNC state in foodborne outbreaks, due to limitation in detection and traceability of the original source (Zhao et al., 2017).

Once the environmental conditions become favourable, resuscitation of VBNC bacteria may take place through two complex phenomena, namely: resuscitation-promoting factors (Rpfs) (Hett et al., 2007; Sheleeva et al., 2004) and auto-inducers of growth, which have been identified as a novel quorum-sensing system and termed as AI-3 (Reissbrodt et al., 2002; Sperandio, Torres, Jarvis, Nataro, & Kaper, 2003). Both these mechanisms are of significance in food safety and in food outbreaks. The resuscitation of VBNC cells in food may take place during shelf-life storage (see e.g. Koukkidis et al., 2017), while the second mechanism is more often associated with the resuscitation of enteropathogenic VNBC bacteria within the human intestinal tract. This could take place at a time when the host may be under significant physiological stress, with the consequent secretion of norepinephrine, a stress-related hormone related to AI-3 (Freestone, Haigh, Williams, & Lyte, 1999; Sperandio et al., 2003).

Nevertheless, there is evidence for both the retention of virulence in VBNC pathogens, and the recovery of virulence together with resuscitation in the host (Ayrapetyan & Oliver, 2016). Nicolò and Guglielmino (2012) indicates that 80% of illnesses are currently caused by unidentified agents, suggesting a large number of outbreaks may be caused by undetectable VBNC pathogens. Makino et al. (2000) reported that salted salmon roe contaminated with enterohemorrhagic *E. coli* O157:H7 (EHEC) in a VBNC state might have contributed to a foodborne outbreak in Japan in 1998. The traditional culture technique estimated only 0.75–1.5 viable EHEC; however, since this number is too low for causing an infection (Tilden et al., 1996), it was suggested that a larger number of EHEC might have been present in a VBNC state in the food. In 2011, the possibility that an initially undetected *E. coli* O104:H4 strain was responsible for a large outbreak (about 3000 cases) causing enterohemorrhagic and enteroaggregative diseases was discussed by Aurass, Prager, and Flieger (2011); they also confirmed that VBNC cells induced by tap water or copper ions were resuscitated after stress relief. In addition, it has been shown that low temperature induces the VBNC state of *E. coli* O157:H7 in lettuce, with detection of verotoxin in samples containing no detectable culturable cells (Dinu & Bach, 2011). The expression of the Shiga-toxin genes from VBNC cells and auto-induced or stimulated resuscitation of bacterial growth have also been reported (Asakura et al., 2005; Reissbrodt et al., 2002). Other common foodborne bacteria such as *Salmonella spp.* (Svoboda et al., 2016; Ukuku et al., 2016) and *Listeria monocytogenes* (Dreux, Albagnac, Carlin, Morris, & Nguyen-the, 2007b, 2007a; Gunasekera et al., 2002; Svoboda et al., 2016; Ukuku et al., 2016) have been shown to evolve towards the VBNC state in the environment and in food processing conditions through the food chain. The induction of the VBNC state of bacteria in food and food model systems suggests that other stresses, including food treatments for disinfection and storage (e.g. desiccation (Vriezen, de Bruijn, & Nüsslein, 2012)), may also play a significant role.

3. VBNC status of human pathogens and limitations on detection

Several bacterial species have been reported to enter a VBNC state and regain culturability after the elimination of the stress (Keep, Ward, Robertson, Cohen-Gonsaud, & Henderson, 2006; Oliver, 2000, 2010). These include human pathogenic species such as: *Campylobacter jejuni* (Cappelier, Magras, Jouve, & Federighi,

1999; Jones, Sutcliffe, & Curry, 1991; Yang, Dou, Gu, & Wu, 2007), *Escherichia coli* (Gage, Nixon, & Bodine, 2015; Makino et al., 2000; Reissbrodt et al., 2002; Senoh et al., 2012; Zhao, Bi, Hao, & Liao, 2013), *Helicobacter pylori* (Buck & Oliver, 2010), *Klebsiella pneumoniae* (Lappin-Scott, Cusack, MacLeod, & Costerton, 1988), *Listeria monocytogenes* (Busch & Donnelly, 1992; Lavieri et al., 2014; Van Houteghem et al., 2008), *Mycobacterium tuberculosis* (Kana et al., 2008; Shleeva et al., 2002), *Pseudomonas aeruginosa* (Elabed, Bakhrouf, Hamza, Azaiez, & Gaddour, 2012), several *Salmonella* species (Asakura, Watarai, Shirahata, & Makino, 2002; Gage et al., 2015; Reissbrodt et al., 2002; Rengifo-Herrera, Castaño, & Sanabria, 2013; Senoh et al., 2012), *Shigella* species (Rahman, Shahamat, Chowdhury, & Colwell, 1996; Senoh et al., 2012), *Vibrio cholerae* (Binsztein et al., 2004; Mishra, Taneja, & Sharma, 2012, 2011; Senoh et al., 2012; Wai, Moriya, Kondo, Misumi, & Amako, 1996), *Vibrio parahaemolyticus* (Mizunoe, Wai, Ishikawa, Takade, & Yoshida, 2000; Senoh et al., 2012; Wong & Wang, 2004), and *Vibrio vulnificus* (Bogosian, Aardema, Bourneuf, Morris, & O'Neil, 2000). In some cases, resuscitation of VBNC cells was not successful during *in-vitro* tests, but it has been shown that VBNC cells were able to synthesise virulent proteins anyway (Alleron et al., 2013; Yang et al., 2007).

In regards to *Campylobacter jejuni*, VBNC status was obtained through suboptimal growth temperatures (i.e., $T < 20\,^{\circ}C$ or $>50\,^{\circ}C$), starvation conditions, or changes in pH, osmolarity, and pressure (Svensson, Frirdich, & Gaynor, 2008; Yang et al., 2007). Once in the nonculturable state, the bacteria are still capable of producing virulence, although at a significantly lower extent (Yang et al., 2007).

Recently, the effect of ozone and chlorine treatment on *Helicobacter pylori* inactivation has also led to the induction of VBNC status with these bacteria (Orta de Velásquez, Yáñez Noguez, Casasola Rodríguez, & Roman Roman, 2017). Remarkably, it has been possible to prove that both disinfectants, when applied at $C \times t$ values respectively larger than 6 and 20 mg × min/L (where C is the biocide concentration and t the exposure time), led not only to an inhibition of the enzymatic processes, but also to physical damage to the cell membrane, with formation of outer membrane vesicles (Parker & Keenan, 2012) or complete cell lysis (Orta de Velásquez et al., 2017).

When inoculated in grapefruit juice (pH ~3 and low content of carbohydrates), *L. monocytogenes* lost both culturability, viability, and capability to resuscitate by subsequent inoculation in nutrient-rich media. In contrast, *E. coli*, *S. enterica* and *Shigella flexneri* were all resuscitated, with different results depending on inoculum concentration and incubation time (Nicolò et al., 2011).

Interestingly, Serpaggi et al. (2012) have proven that eukaryotic cells are similar to prokaryotic organisms in their ability to maintain viability while losing culturability, when exposed to conditions of stress. *Brettanomyces bruxellensis*, a yeast responsible for wine spoilage (due to the production of off-flavours) were induced to enter a VBNC state by addition of sodium metabisulphite ($Na_2S_2O_5$). However, nonculturable yeast cells have been shown to retain their wine spoilage capacity. *Saccharomyces cerevisiae* and *Candida stellata* were able to survive a treatment with sulphites, owing to the VBNC condition (Divol & Lonvaud-Funel, 2004).

Like wine, beer is also sensitive to damaging microorganisms. Suzuki, Iijima, Asano, Kuriyama, and Kitagawa (2006) investigated two strains of *Lactobacillus*, responsible for beer spoilage, which were shown to enter the VBNC state after a sub-lethal heating treatment. It was shown that the VBNC state was also induced by prolonged adaptation to beer, thus representing a real risk in brewery environments, particularly during fermentation and in maturation tanks.

Heating (up to $70\,^{\circ}C$, for 60 min) has been shown to induce the VBNC status also on *Legionella pneumophila* SG1 strains, and the cells treated for up to 30 min proved still able to resuscitate when incubated in *Acanthamoeba polyphaga* (Epalle et al., 2015).

Table 1 presents a list of microorganisms of concern for public health, owing to their possible presence in contaminated food or water. The table includes the name of the microorganism, the cause of VBNC status, the differences in viability and culturability and indications of success in cell resuscitation.

Whether the organisms under VBNC conditions are alive or not can be proven by using several criteria, including cellular membrane integrity, uptake and incorporation of labelled substances, and quantitation of 16S ribosomal RNA. Kogure at al. (1979) proposed to preincubate samples with yeast extract and nalidixic acid, followed by microscope observation, while Awais, Fukudomi, Miyanaga, Unno, and Tanji (2006) suggested the possibility to detect VBNC *E. coli* O157:H7 thorugh a very specific assay based on the use of properly engineered bacteriophages. In 16S RNA analysis, it is worth noting that it is not possible to differentiate the dead from the live bacteria by normal PCR or qPCR, as both techniques target the DNA (Stokell & Steck, 2012). To bypass the problem, it has been suggested to either operate selectively on DNA from viable cells only, thus including the VBNC bacteria (Inoue et al., 2008; Taylor, Bentham, & Ross, 2014) or make dead cells unavailable to DNA extraction (Li et al., 2017). Zhao et al. (2016) used RNA transcriptomics combined with iTRAQ proteomics to investigate genes and related proteins and assess decreased metabolic activity, repressed cell division, and enhanced survival ability in VBNC *E. coli*. Clearly, these alternative culture-independent methods require using of relatively expensive equipment and trained personnel, with obvious limitation in routine analysis (Fakruddin et al., 2013; McKay, 1992; Rowan, 2004).

4. Induction of VBNC state – Role of chemical and physical treatments

After the pioneering work of Colwell et al. (1985) on the topic, Barcina, González, Iriberri, and Egea (1989) documented the loss in culturability of *Escherichia coli* caused by visible light and subsequently proved that the photo-produced hydrogen peroxide (H_2O_2) was responsible for inducing VBNC status (Arana, Muela, Iriberri, Egea, & Barcina, 1992). Idil, Ozkanca, Darcan, and Flint (2010) also showed that red light is as effective as UV-A irradiation in inducing VBNC status of *E. coli*, and stronger than the white, green and blue light. Starvation, i.e. conservation in drinking or river water (Liu, Gilchrist, Zhang, & Li, 2008) as well as sub-optimal growth temperatures (Dinu & Bach, 2011) have also proven to induce the nonculturable state of *E. coli*.

Morishige, Fujimori, and Amano (2015) have confirmed the ability of H_2O_2 (between 1 and 10 mM) to induce the VBNC state in *Salmonella enteriditis*. Furthermore, literature suggests that H_2O_2 is not the only source of reactive oxygen species (ROS) that induces the VBNC state in microorganisms (Asakura et al., 2005), as other ROS such as hydroxyl radicals, atomic oxygen, and ozone are also generated at the gas-liquid interface by an atmospheric pressure plasma jet (Dolezalova & Lukes, 2015). Although these ROS are responsible for the inactivation induced by plasma jet, with efficiencies often higher than standard sterilisation techniques, it has been documented that this inactivation is highly effective (reduction of 7 Logs) only when assayed using conventional enumeration techniques. In contrast, less than 1 Log reduction was found by assessing effects of the plasma treatment when a fluorescent staining technique was used to assess the treatments (Dolezalova & Lukes, 2015). These results suggest that tested bacteria (*E. coli*) may have entered the VBNC state during plasma jet treatment.

The above studies may raise doubts on the action of chemicals

Table 1
List of human pathogenic microorganisms able to enter a viable but nonculturable state. In some cases, evidence for their resuscitation has been also obtained. (ΔC and ΔV indicate differences in culturability and viability, respectively).

Checked microorganism(s)	VBNC inducer(s)	Effects on culturability (ΔC) and viability (ΔV)	Proof of resuscitation	Ref.
Escherichia coli *Shigella sonnei* *Vibrio cholerae*	Marine environment (up to 19 days)	EC: ΔC ≈ 3 Logs; ΔV ≈ 1 Log (after 19 days) SS: ΔC ≈ 6 Logs; ΔV < 2 Log (after 19 days) VC: ΔC ≈ 4.5 Logs; ΔV ≈ 0 Log (after 7 days)	Not tested	Colwell et al., 1985
Escherichia coli	Visible light (400÷700 nm)	ΔC: between 4 and 5 Logs, after 72 h	Not tested	Barcina et al., 1989
Escherichia coli	1. Visible light (400÷700 nm) 2. H_2O_2 (≤0.2%)	1. ΔC: 5 Logs, after 120 h 2. ΔC: 3–4 Logs, after 15 min of contact time	Not tested	Arana et al., 1992
Escherichia coli	HOCl (0.4÷1.0 ppm, pH 7.1)	ΔC: between 1.5 and 5 Logs, after 20′ of contact time	Yes	Dukan et al., 1997
Escherichia coli	Osmotic shock (7 and 13% NaCl)	ΔC: between 0.5 and 6 Logs, depending on strains; ΔV ≈ 1 Log	Yes	Makino et al., 2000
Escherichia coli	$CuSO_4$ (6–25 mM)	ΔC: 8 Logs, after 7 days; ΔV ≈ 2 Logs	Yes	Grey & Steck, 2001
Escherichia coli *Salmonella typhimurium* *Citrobacter freundii* *Enterobacter agglomerans*	1. Sterile double-distilled water 2. T > 45 °C	ΔC: between 5 and 6 Logs, after up to 5 months	Yes	Reissbrodt et al., 2002
Escherichia coli	1. H_2O_2 (0.05%) 2. Osmotic shock (13% NaCl @ 37 °C) 3. Low pH (Acetic acid, pH 3.0) 4. UV irradiation 5. Heat (56 °C for 6 h)	1. After 6 h ΔC ≈ 100%; ΔV ≈ 50% 2. After 144 h ΔC ≈ 98.3%; ΔV < 20% 3. ΔC ≈ 100%, after 24 h 4. ΔC ≈ 100% @ 12.8 J/cm^2 5. ΔC ≈ 100%, after 6 h	Yes	Asakura et al., 2005
Escherichia coli *Salmonella typhimurium*	NaClO (1 ppm)	After 1 h of contact time: ΔC ≈ 5 Logs; ΔV ≈ 2 Logs	Not achieved	Oliver et al., 2005
Escherichia coli	1. Starvation (drinking water) 2. River water	ΔC ≈ 6 Logs, after 12 weeks; ΔV ≈ 1 Log	Yes	Liu et al., 2008
Escherichia coli	UV-A and different wavelengths of visible light	After 24 h of irradiation: ΔC ≈ 0.66, 0.6, 0.28, 0.18 and 0.12 Log (UV-A > red > white > green > blue)	Not tested	Idil et al., 2010
Escherichia coli (on lettuce leaves)	Low temperature (4–8 °C)	After 7–15 days: ΔC between 6 and 9 Logs; ΔV ≈ 2 Log	Not tested	Dinu & Bach, 2011
Escherichia coli *Salmonella enteritidis* *Shigella flexneri*	Low pH, low carbohydrates (grapefruit juice @ 4 °C)	After 24 h of contact time: ΔC: 6 (EC), 6 (SE) and 3 Logs (SF); ΔV: 1 (EC), 0.5 (SE) and 1 Logs (SF)	Yes	Nicolò et al., 2011
Escherichia coli	High pressure CO_2 (50 bar, T between 25 and 37 °C)	After 25÷40 min of contact time: ΔC ≈ 8 Logs; ΔV < 2 Logs	Yes	Zhao et al., 2013
Escherichia coli	Argon plasma jet (ROS)	After 15 min: ΔC ≈ 100% (7 Logs); ΔV ≈ 45% (<1 Log)	Not achieved	Dolezalova & Lukes, 2015
Escherichia coli *Pseudomonas aeruginosa*	UV irradiation (254 nm)	ΔC: 2 (EC) and 4 Logs (PA) @ 50 mJ/cm^2; 4 (EC) and 6 Logs (PA) @ 100 mJ/cm^2	Yes	Zhang et al., 2015
Escherichia coli *Listeria innocua*	UV light (also pulsed)	ΔC: ≤ 3.5 Logs (@ 1.08 J/cm^2 with 17.5% of UV) Metabolic activity: LI -80%, EC -90%		Kramer & Muranyi, 2013
Salmonella oranienburg *Salmonella enteritidis*	Low temperature (4 °C)	After 24 h: ΔC ≈ 9 Logs; ΔV < 1 Log	Yes	Asakura et al., 2002
Salmonella typhimurium (SEW samples)	PAA ≤ 15 ppm (@ 15 °C)	After 1 h of contact time: ΔC ≤ 7 Logs; ΔV ≈ 1 Log	Yes	Jolivet-Gougeon et al., 2006
Salmonella typhimurium	Photo-Fenton (UV-A), at pH 5.5 (2 ppm Fe^{3+} + 20 ppm H_2O_2)	After 1.5 h: ΔC ≈ 3.5 Logs; ΔV < 1 Log	Yes	Rengifo-Herrera et al., 2013
Salmonella enteritidis	1. Peracetic acid 2. DBDMH	1. 1 Log of VBNC cells 2. no VBNC cells	Not tested	Gage et al., 2015
Salmonella enteritidis	H_2O_2 (1÷10 mM)	After 1 h of contact time: ΔC ≈ 7.5 Logs; ΔV ≈ 6 Logs	Not tested	Morishige et al., 2015
Salmonella enteritidis	1. Acetic acid 2. Citric acid 3. Lactic acid 4. Peracetic acid 5. Hydrogen peroxide 6. DBDMH	1. 0.31 Log of VBNC cells 2. 0.46 Log of VBNC cells 3. 0.43 Log of VBNC cells 4. 1.16 Logs of VBNC cells 5. 0.81 Log of VBNC cells 6. no VBNC cells	Yes	Gage et al., 2016
Shigella dysenteriae type 1	Starvation (sterile water @ 30 °C, for up to 42 days)	After 30 days: ΔC > 7 Logs; ΔV < 2 Logs	Not tested	Rahman et al., 1996
Listeria monocytogenes *Listeria innocua*	Heat (56 °C, for 50 min)	ΔC: between 98.1 and 99.9%	Yes	Busch & Donnelly, 1992
Listeria monocytogenes	Dry conditions (RH between 47 and 69%)	After 15 days: ΔC > 6 Logs; ΔV < 4 Logs	Not achieved	Dreux et al., 2007a
Listeria monocytogenes	1. Intense light pulses (20 flashes) 2. ClO_2 (6 ppm, for 2 min) 3. Lactic acid (0.3M, pH 3, for 10 min) 4. Heat (60 °C, for 23 min)	1. ΔC ≈ 3.5 Logs 2. ΔC: between 3.5 and 5.5 Logs 3. ΔC: between 2.5 and 5.5 Logs 4. ΔC ≈ 3.5 Logs	Yes	Van Houteghem et al., 2008
Listeria monocytogenes	Low pH (~4, 50 mM of acids) @ 37 °C	After 7 h: ΔC ≈ 4 Logs; ΔV ≤ 1 Log	Not tested	Cunningham et al., 2009
Listeria monocytogenes	1. High pressure (4 kbar) @ 12 °C 2. High pressure (6 kbar) @ 12 °C 3. Heat (60 °C, for 3÷9 min)	1. After 6 min: ΔC between 1 and 2 Logs 2. After 2 min: ΔC between 4 and 6 Logs 3. After 9 min: ΔC between 1.5 and 5 Logs	Yes	Lavieri et al., 2014

Table 1 (*continued*)

Checked microorganism(s)	VBNC inducer(s)	Effects on culturability (ΔC) and viability (ΔV)	Proof of resuscitation	Ref.
Pseudomonas aeruginosa	Starvation (sterile seawater @ RT, for 14 years)	Not tested	Yes	Elabed et al., 2012
Klebsiella pneumoniae	Starvation (PBS, at 23–25 °C, for 24÷63 days)	After 9 weeks: ΔC ≈ 82%	Yes	Lappin-Scott et al., 1988
Campylobacter jejuni	Starvation (sterile pond water @ 4 °C)	After up to 4 weeks: ΔC ≈ 7 Logs	Yes	Jones et al., 1991
Campylobacter jejuni	Starvation (sterile surface water @ pH 6.0 and 4 °C)	After 15 days: ΔC ≈ 7 Logs; ΔV < 1 Log	Yes	Cappelier et al., 1999
Helicobacter pylori (on spinach leaves)	Sunlight (>350 nm)	ΔC ≈ 6 Logs, after 12 h ΔV ≈ 95% (5% of the cells in VBNC state)	Not tested	Buck & Oliver, 2010
Legionella pneumofila (SG1)	NH_2Cl (≤10 ppm)	ΔC ≈ 100% for [NH_2Cl] ≥ 1 ppm; ΔV ≈ 50%, after 20 days @ 10 ppm	Yes	Alleron, Merlet, Lacombe, & Frère, 2008
Legionella pneumofila (SG1)	NH_2Cl (≤2 ppm)	ΔC: between 2 and 3 Logs, after 24 h	Not tested	Türetgen, 2008
Legionella pneumofila	NH_2Cl (≤50 ppm)	VBNC cells for [NH_2Cl] > 20 ppm; after 24 h of contact time @ 20 ppm: ΔC ≈ 70%, ΔV ≈ 35%	Not achieved	Alleron et al., 2013
Legionella pneumofila	ClO_2 (0.1÷10 ppm)	VBNC cells for [ClO_2] > 4 ppm (VBNC cells up to 10 ppm of ClO_2)	Yes	Mustapha et al., 2015
Legionella pneumofila (SG1)	Heat shock @ 70 °C for 30 min	Between 15 and 40% of VBNC cells, depending on strains	Yes	Epalle et al., 2015
Vibrio cholerae O1	M9 salt or PBS solution @ 15 °C for 3 months (no shaking)	After 3 weeks: ΔC ≈ 7 Logs	Yes	Wai et al., 1996
Vibrio cholerae O1	Marine water	VBNC cells were found in the aquatic environment	Yes	Binsztein et al., 2004
Vibrio cholerae O1	Starvation (sterile natural fresh lake water @ 4 °C)	After 3 weeks: ΔC ≈ 8 Logs; ΔV ≈ 3 Logs	Yes	Mishra et al., 2012
Vibrio parahaemolyticus	Starvation (modified PBS solution @ 4 °C)	After 12 days: ΔC ≈ 7 Logs; ΔV < 1 Log	Yes	Mizunoe et al., 2000
Vibrio parahaemolyticus	Starvation (slightly saline medium @ pH 7.8 and 4 °C)	After 35 days: ΔC ≈ 7 Logs; ΔV ≈ 0 Log	Not tested	Wong & Wang, 2004
Vibrio vulnificus	Starvation (sterile seawater @ 5 °C)	After 10÷16 days: ΔC ≈ 7 Logs; ΔV < 1 Log	Yes	Bogosian et al., 2000
Brettanomyces bruxellensis	$Na_2S_2O_5$ (0.8 ppm of SO_2)	After 2 days: ΔC ≈ 7 Logs; ΔV ≈ 1 Log	Yes	Serpaggi et al., 2012
Saccharomyces cerevisiae *Candida stellate*	SO_2 (200÷250 ppm)	After 12 days: ΔC ≈ 8 Logs; ΔV ≈ 1 Log	Yes	Divol & Lonvaud-Funel, 2004
Lactobacillus lindneri *Lactobacillus paracollinoides*	Sublethal heat treatment (LL: 10' @ 60 °C; LP: 5' @ 52 °C)	ΔC: between 2 and 3 Logs	Yes	Suzuki et al., 2006
Enterococcus faecium *Bacillus subtilis*	Peracetic acid (≤3000 ppm)	EF: ΔC ≈ 5 Logs; ΔV < 1 Log (5 ppm, for up to 60 min) BS: ΔC ≈ 3 Logs; ΔV < 1 Log (3000 ppm, for 60 min)	Not tested	Park et al., 2014
Mycobacterium tuberculosis *Rhodococcus rhodochrous*	Incubation in modified Sauton's minimal medium	MT: ΔC ≈ 7 Logs RR: ΔC ≈ 5 Logs	Yes	Shleeva et al., 2002
Mycobacterium tuberculosis	Starvation without oxygen	Not stated	Yes	Kana et al., 2008

that are commonly considered as effective disinfectants. Indeed, the scientific literature provides further experimental evidence, which may well justify growing concern on the topic. It has been reported that the VBNC state is induced on *E. coli* and *Pseudomonas aeruginosa* by UV irradiation, even at a dose as high as 100 mJ/cm^2 (Zhang, Ye, Lin, Lv, & Yu, 2015). Comparable results were obtained by Kramer and Muranyi (2013) by applying pulsed UV light (UV content up to 189 mJ/cm^2) to *E. coli* and *Listeria innocua*. Likewise, *E. coli* (Dukan, Lévi, & Touati, 1997) and *S. typhimurium* (Oliver, Dagher, & Linden, 2005) enter the VBNC status when treated with a sub-lethal dosage of chlorine (less than 1 mg/L). Furthermore, VBNC cells were obtained after treatment with peroxide-based disinfectants, like peracetic acid (PAA; Park, Lee, Bisesi, & Lee, 2014). While 5 ppm of PAA led to a 5-Log reduction on *Enterococcus faecium* after only 5 min of contact time, qPCR proved that only 1-Log reduction was actually obtained, even extending the contact time to 1 h. Results obtained on *Bacillus subtilis* spores, by using up to 3000 ppm of PAA, proved even worse: a 4-Log reduction through plate counts but no reduction based on qPCR. Similar outcomes were obtained on *S. typhimurium* (Jolivet-Gougeon, Sauvager, Bonnaure-Mallet, Colwell, & Cormier, 2006) and *S. enteritidis* (Gage et al., 2015). On the latter microorganism, the VBNC state is readily induced not only by PAA and H_2O_2 but also by citric, lactic and acetic acid (Gage, Nixon, & Bodine, 2016). A low pH environment, such as that caused by benzoic and propionic acid, proved to have the same effect on *Listeria monocytogenes* (Cunningham, O'Byrne, & Oliver, 2009). The viability of VBNC cells was confirmed through resuscitation experiments (Cunningham et al., 2009; Gage et al., 2016).

Regarding the biofilm produced by *E. coli*, some commercial formulations, based on PAA, have proven not only to be ineffective in removing the biofilm under investigation but even to improve its adhesion to the glass support material (Loukili, Becker, Harno, Bientz, & Meunier, 2004).

Among chlorine-related disinfectants, chlorine dioxide (Mustapha et al., 2015) and monochloramine (Alleron et al., 2013, 2008; Türetgen, 2008), at dosages up to 5 and 10 mg/L respectively, have proven to induce the VBNC state on *Legionella pneumophila*. A subsequent contact with ROS scavengers (pyruvate and glutamate) showed to stimulate resuscitation, helping the injured cells to recover after the stress (Ducret at al., 2014). Similarly, Zeng et al. (2013) obtained resuscitation of *Salmonella typhi* by adding catalase.

In water disinfected with ozone, *Helicobacter pylori* was found able to survive by transforming its helical bacillary form (normal conditions) into the coccoid form (under stressful environmental conditions). A $C \times t$ value as high as 15 mg × min/L was required to obtain a disinfection higher than 99.9% (Casasola-Rodríguez, Orta de Velásquez, Luqueño-Martínez, & Monje-Ramírez, 2013).

In terms of non-thermal pasteurization techniques, the use of high-pressure carbon dioxide at 5 MPa (50 bar) is able to induce the VBNC state on *E. coli*; while plate counts indicated a complete

inactivation (8-Log reduction), culturability was almost completely recovered by incubating the cells at 37 °C in tryptic soy broth for at least 6 h. Increasing the temperature of the CO_2 treatment from 25 to 37 °C, the time required for the cells to enter the nonculturable state was shortened (Zhao et al., 2013, 2016). Lavieri et al. (2014) showed that high hydrostatic pressure (4 kbar for at least 4 min, or 6 kbar for ≥1 s) significantly affects culturability of *Listeria monocytogenes*, which can be recovered in the presence of selective agents.

Finally, some chemicals with a preventative or sanitising effect have historically been applied on vegetable crops for controlling pathogen-related diseases. Copper salts at low pH have always been considered an effective approach. A recent work by Jiang et al. (2016), however, has shown that this practice may also require some reconsideration. Grey and Steck (2001) reported similar results obtained from their research on *E. coli* cells exposed to toxic concentration of copper.

5. Precautions for food disinfection

Based on the above-reported body of evidence, the choice of a method for food disinfection is a critical decision, yet the risks discussed in this current contribution may be reduced by selecting a disinfectant that is able to exert its action through more than one mechanism. The exact mechanism by which disinfectants act on microorganisms has not been completely elucidated yet. Depending on their molecular structure and physico-chemical properties, the different biocides are generally able to perform different actions, either by targeting the cell membrane, the molecules responsible for energy metabolism, or directly the cell nucleous at chromosomal level (Maris, 1995). Finnegan, Denyer, McDonnell, Simons, and Maillard (2010) investigated the activity of some oxidative biocides, namely chlorine dioxide, peracetic acid (as such, as well as within a few commercial formulations) and hydrogen peroxide, highlighting that oxidation of cellular components is a common mechanism, yet with different degree of effectivness. In liquid form, H_2O_2 proved to be a good oxidant against all amino-acids tested, but no degradation of proteins was observed. In contrast, 2.5% ClO_2 was able to completely degrade bovine serum albumin, however no oxidation products were found after exposure to aminoacids, at a 1:5 aminoacid:biocide ratio. PAA formulations showed conflicting results, even though their overall effects were better than the other investigated chemicals. Peroxide-based oxidants are thought to take advantage of the catalytic activity of iron present in *in-vivo* systems (DNA-associated iron), thus reproducing the Fenton reaction or leading to powerful ferryl radicals (Linley, Denyer, McDonnell, Simons, & Maillard, 2012). Regardless, there is a significant difference between *in-vitro* experiments, with specific molecules or planktonic cells, and real systems, typically characterised by microbial contamination in sessile form. In the presence of a biofilm, the biocidal activity is highly reduced, mostly because the target cells are protected within a slimy matrix of exopolymers, predominantly composed of carbohydrates (ReAlco, 2014). Biofilm consists of sugars such as glucose, but also contains a variety of charged moieties such as carboxyl groups, uronic acids, pyruvate groups, or sulphated sugars. At the physiological pH, these ionogenic groups are dissociated and a negative surface charge is present that electrostatically limits the approach and hence the activity of negatively-charged biocides. Additionally, the antimicrobial agent is almost completely consumed in the outer layers of the biofilm (Costerton, Stewart, & Greenberg, 1999).

As a biocide, chlorine, especially in the neutral form of hypochlorous acid (HOCl), does not seem to have significant competitors (Fukuzaki, 2006) since it is able to perform its activity not only as an oxidising agent, which comprises acting as a source of hydroxyl radicals like H_2O_2 (Dukan & Touati, 1996), but also through reactions of addition and electrophilic substitution, especially on unsaturated substrates (Deborde & von Gunten, 2008). Contrary to hypochlorite, which only allows a partial oxidation of sugar molecules (Bonfatti et al., 2000), HOCl is able to completely mineralise the various components of the biofilm, thanks to reactions of chlorinolysis (Whistler, Mittag, Ingle, & Ruffini, 1966). By comparing the biocide efficacy of HOCl against *E. coli* vs. that of H_2O_2, Dukan and Touati (1996) pointed out that the latter needs to be dosed at concentrations 200 times higher. Alasri, Roques, and Michel (1992) investigated the biocidal activity of some disinfectants against *E. coli, P. aeruginosa* and *Staphylococcus aureus*, and found that chlorine (also as sodium hypochlorite) is more effective than PAA, even when the latter is considered in combination with H_2O_2. In turn, H_2O_2 alone is better than formaldehyde, but both require to be used at concentrations significantly higher than the former products.

In recent years, recourse to chlorine-based disinfectants has been discouraged because of the possible formation of chlorinated by-products; however, it has also been stressed that the risk depends on the specific nature of the formed molecules. Moreover, when the chlorine dose is limited, only small variations are expected with respect to the parent compounds (Dukan & Touati, 1996). In any case, a compromise must be sought, since the use of disinfectants alternative to chlorine may produce health repercussions potentially even more dangerous than a few traces of chloroform.

'Electrochemically activated solutions' (ECAS, also called 'oxidising waters' or simply 'anolytes') have gained a lot of interest in recent years (Hricova, Stephan, & Zweifel, 2008; Huang, Hung, Hsu, Huang, & Hwang, 2008; Rahman, Khan, & Oh, 2016). In hospital environment, ECAS are normally dosed in water networks to fight against waterborne pathogens like *Legionella*, and proved to be effective in controlling a variety of microorganisms (Ferro, Amorico, & Donner, 2016; Migliarina & Ferro, 2014; Robinson, Thorn, & Reynolds, 2012; Thorn, Lee, Robinson, Greenman, & Reynolds, 2012). Owing to their high oxidation-reduction potential (>850 mV), and considering that pH-neutral anolytes are non-hazardous and non-corrosive, investigations have recently been extended to the food sector where these solutions can be exploited to control bacterial contaminations, especially those responsible for rapid spoilage of fresh food (Rahman et al., 2016). Preliminary results look very promising: a significant increase of shelf life (between 2 and 4 days, depending on the fish) has been recorded for fish fillets washed with ECAS and then stored in ice, without affecting the overall raw and cooked qualities of the fillets (Khazandi et al., 2017). Positive results have also been obtained in the sanitisation of minimally processed foods such as carrots, potatoes, and lettuce where ECAS showed to compare well with, and sometimes better than sodium hypochlorite and PAA-based disinfectants (Ecas4 Australia, internal reports; Khazandi et al., private communication; Premier, 2013), thus creating the possibility of replacing the latter products to the benefit of: operators, infrastructure, and consumer health (Khazandi et al., private communication). Although anolytes have proven to be effective in reducing the microbial load, their ability to trigger the VBNC status or lead to complete elimination of bacteria requires further investigation.

6. Concluding remarks

There is a call for detailed knowledge on the mechanism of induction and resuscitation of VBNC states, improved detection methods, and of the food safety risk associated with the non-culturable response to develop effective intervention strategies. Furthermore, a better understanding of these organisms and of

their impact on food safety could ultimately lead to consideration of the VBNC state during investigation of foodborne outbreaks.

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