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Review

Latest Developments of Research on the Viable Non-Culturable State of *L. monocytogenes* and Implications for Food Safety

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Featured Application: This review article aims to collect updated knowledge on the conditions that induce the viable non-culturable (VBNC) state in the foodborne pathogen *Listeria monocytogenes*, obtain indications on practices that can prevent the occurrence of this form of the pathogen in food, and identify aspects that necessitate further investigation.

Abstract: This descriptive review summarizes the most recent findings on the induction and distribution of viable non-culturable (VBNC) *Listeria monocytogenes* in food production conditions and food. The aim was to obtain information on the factors that favor the transition to the VBNC state in *L. monocytogenes*; its resuscitation capacity; and, according to scientific articles published since 2020, how food contamination by the bacterium in a VBNC state can be prevented. The methods used for VBNC *L. monocytogenes* detection were also reviewed. A few studies reported the presence of VBNC *L. monocytogenes* in food, in which fresh produce and chicken meat were considered. Different physicochemical stresses such as exposure to disinfectants with diverse actions and essential oils, desiccation, low temperatures, absence of nutrients, high NaCl and iron concentrations, and low pH adjusted with acetic acid were reported to induce the VBNC state in *L. monocytogenes*. The VBNC forms of *L. monocytogenes* were able to regain growth and virulence. This could pose a safety risk that cannot be revealed by the standard culture-dependent methods recommended for *L. monocytogenes* detection. Therefore, the presence in food and food production plants of VBNC *L. monocytogenes* should be prevented by the appropriate use of hurdles and cleaning/disinfection procedures. The opportunity to harmonize VBNC cell detection methods for regular use in food safety evaluation also emerged.

Keywords: *Listeria monocytogenes*; VBNC; detection methods; inducing stress factors; occurrence in food; resuscitation

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1. Introduction

The genus *Listeria* comprises Gram-positive facultative anaerobic rod-shaped bacteria with a marked capacity to inhabit a variety of environmental niches and animal hosts, determined by their ability to withstand physicochemical stressors and activate persistence mechanisms. These characteristics make *Listeria* spp. prone to colonizing food production plants and contaminating foodstuff. *Listeria monocytogenes* is an intracellular pathogen that can cause invasive infections, such as septicemia, meningitis, and endocarditis, and is 99% foodborne in immunocompromised persons, the elderly, pregnant women, and newborns.

This pathogen can also cause abortion in humans and animals [1–3]. Upon *L. monocytogenes* infection, healthy adults do not develop symptoms or manifest a gastrointestinal illness that is usually undiagnosed [4]. Pregnancy listeriosis, after a two- to three-week incubation period, can manifest as a self-limiting illness with flu-like symptoms. However, the bacterium can cross the placenta and infect the fetus with consequences such as spontaneous abortion, preterm birth, stillbirth, and newborn infections. These complications were reported in 80% of listeriosis cases in pregnant women [5].

L. monocytogenes is classified in the four phylogenetic lineages I, II, III, and IV, in which 13 serotypes comprising different sequence types (STs) and clonal complexes (CCs) are distributed. About 95% of isolates from food and infection cases belong to serotypes 1/2a, 1/2b, 1/2c, and 4b, among which 1/2b and 4b serotypes belonging to lineage I are more frequently associated with human clinical cases. Strains of lineage II are more common in food and food processing plants, while those belonging to lineages III and IV are isolated mainly from animals. The internalin A, a cell wall-anchored protein encoded by the *inl*A gene, is considered the main virulence factor in the hypervirulent strains of *L. monocytogenes* [6]. A recent systematic review and meta-analysis focused on data from South Korea reported that most foodborne listeriosis outbreaks were caused by serotypes 1/2b and 1/2a [7].

In the European Union (EU), 2738 cases of listeriosis were confirmed in 2022 with a fatality rate of 18.1%, the highest compared to previously reported data, which increased to 2952 confirmed cases, 96.5% hospitalizations and a 19.7% fatality rate in the year 2023 according to the most recent reports on zoonotic diseases in the EU [8,9]. In 2022, 2770 confirmed listeriosis cases, including 17 strong-evidence and 18 weak-evidence foodborne outbreaks that affected 296 persons, with 242 hospitalizations and 28 deaths, were reported by 30 European countries through The European Surveillance System (TESSy). Whole-genome sequencing (WGS) reported to TESSy showed small but long-lasting multi-country epidemiological clusters that persisted for several years. The ECDC-EFSA One Health WGS System, already at the end of 2022, defined that more than one-third of the multi-country outbreak isolates matched non-human isolates, showing the potential of WGS application in identifying the origin of the outbreaks [4].

In the meat, fish/seafood, dairy, and fruit/vegetable sectors *L. monocytogenes* represents one of the main microbiological food contamination threats due to its capacity to persist in the food and feed processing environment (FFPE). Obstacles to eliminating persistent *L. monocytogenes* in the FFPE are insufficient hygiene barriers and inadequate equipment design, cleaning, and disinfection procedures. To identify possible contamination by *L. monocytogenes*, a well-designed environmental sampling program (EMP) must be implemented that also involves the facility personnel. Further, in the case of persistent *L. monocytogenes* detection, a 'seek-and-destroy' approach must be applied, namely, the application of cause analysis and corrective actions to eliminate the contamination at specific sites. Persistence can be assessed by genetically characterizing the food plant isolates e.g., by *sig*B allelic typing (*sig*B AT) and single nucleotide polymorphism (SNP) analysis of whole genomes [10–12].

Beyond implementing the correct use of sanitizers, drainage systems, raw ingredient contamination control, and the improvement of compartmentalization in the manufacturing plant, it should be elucidated as to what extent the genotypes associated with persistence in the production environment are linked to listeriosis outbreaks and cases by making the genetic data of outbreak strains available along with the epidemiological investigation results [10,11].

Stresses to which *L. monocytogenes* is exposed in food, such as suboptimal pH, temperatures and osmotic pressure, heating, and smoking, induce variations in the expression of

genes related to pathogenesis and virulence, such as chemotaxis, flagella motility, transmembrane receptors, and signal transduction. They can also lead to the formation of sub-lethally injured cells that are able to recover by cell repair mechanisms and/or VBNC cells that do not grow in commonly used culture media in the recommended incubation conditions [9,13,14]. *L. monocytogenes* adopts different persistence strategies to tolerate food processing stressors. These are biofilm formation, the development of "persisters", and the transition to the viable but non-culturable (VBNC) state [10,15–23].

L. monocytogenes biofilms develop on the surfaces of food processing environments and withstand stressful conditions such as low temperature and desiccation [18]. *L. monocytogenes* "persisters"—i.e., cells that survive cleaning and disinfection, thus reoccurring in the production plant and final products—are a subpopulation of cells that are slow replicating or non-dividing and transiently multidrug tolerant [15]. Differently, VBNC cells are in a deeper dormancy status with minimal metabolism, active transcription and translation, intact DNA and membrane, and the capacity to resuscitate in particular conditions [16]. Sub-lethally injured cells that remain metabolically active but cannot be resuscitated in the standard culturing conditions may enter the VBNC state [22]. VBNC cell formation was observed in over one hundred microbial species and may occur following starvation, exposure to extreme temperatures, oxygen limitation, high salinity, pH changes, UV light, ultrasound, suboptimal osmotic pressure, and exposure to toxic agents, varying with microbial species and strains. In addition, VBNC formation may be induced even in nutrient-rich environments such as food. The mechanisms by which bacterial cells enter the VBNC state are still largely unknown, but some studies provided evidence of the involvement of stress response proteins, transcriptional regulators, membrane proteins, proteins involved in cell division, and toxin/antitoxin systems [16].

Considering that the detection of culturable cells according to standard methods is carried out in specific culture conditions and defined time frames, nonculturability is defined as the lack of colony formation after the prescribed incubation time. This criterion can be used to distinguish VBNC cells from persister cells [16]. The detection of *L. monocytogenes* in foods is carried out by the culture-dependent standard methods EN ISO 11290–1 and EN ISO 11290–2 [24,25] to accomplish detection and enumeration, respectively, as specified by Regulation 2073/2005 amended by Regulation 2024/2895 for the European Union [26,27]. These methods require several days to obtain results and are unsuitable for detecting VBNC cells [28].

Since VBNC *L. monocytogenes* may threaten consumers' health, its reliable and rapid detection would considerably improve food safety assessment. Therefore, this review summarizes the latest findings on the factors that induce the VBNC *L. monocytogenes* cells and the methods to identify them, with special attention to those occurring in food and food production conditions that could determine the transmission of infectious VBNC forms of the pathogen to the consumers. The aim was to collect indications on how to prevent the formation of VBNC *L. monocytogenes* by applying appropriate disinfection treatments. Scientific publications were retrieved from Google Scholar (https://scholar.google.com/schhp?hl=it (accessed on 29 November 2024)) and Scopus (https://www-scopus-com.bibliosan.idm.oclc.org/search/form.uri?display=basic#basic (accessed on 29 November 2024)) with the search string "viable AND *Listeria monocytogenes* AND food". The aim was to obtain a complete overview of what is currently known regarding the conditions that lead to VBNC formation in *L. monocytogenes*, what is still to be known, and treatments that are effective in preventing the occurrence of this form of the pathogen. Results in the 2020–2024 years window were whole text screened for the occurrence of the words "viable non-culturable", using the words "non culturable" with or without the dash and

also conjoined, and "VBNC" or "VNC". Finally, the scientific articles pertinent to the scope of this review were synthesized.

2. VBNC *L. monocytogenes* Detection Methods

An increase in publications regarding viable pathogen detection in food was reported from the year 2010 to 2022 and the methods used were based on fluorescence microscopy, fluorescence labeling coupled with flow cytometry (FC), viability PCR (vPCR), impedance-based methods, and surface Raman spectroscopy. Articles focused on molecular detection of VBNC *L. monocytogenes* used as targets the genes *hly*, *iap*, 16S rRNA gene, and *prf*A, and were aimed at method optimization and detection from food matrices including powdered infant formula, milk, and shrimp [28].

2.1. Optical Detection Methods

Early studies on VBNC cells compared bacterial cultures with microscopic counting of cells stained with fluorescent dyes, making them able to differentiate viable and dead cells [29]. Immunolabeling with antibodies bound to fluorophores such as fluorescein isothiocyanate (FITC) also allowed the specific detection of bacteria in food. In this case, the antibody and the dye are first incubated in the dark with the target bacteria; then, cells are detected by FC [30].

Among viability staining dyes, fluorescein diacetate (FDA) and the similar dye carboxyfluorescein diacetate (CFDA) can permeate the membrane of metabolically active cells; inside the cells, they are converted by intracellular esterases into fluorescent compounds that remain inside the cells with an intact cytoplasmic membrane [16,30,31]. Propidium iodide (PI) is an indicator of membrane integrity and cell death that enters cells with damaged membranes and binds to DNA at either major or minor grooves with red fluorescence emission. Differential staining with FDA, or CFDA, and PI allows distinguishing viable (green fluorescence), sub-injured (brown fluorescence), and dead (red fluorescence) cells [14,31]. A similar principle is exploited by the commercial staining system LIVE/DEAD BacLight kit (Thermo Fisher Scientific, Waltham, MA, USA) comprising the two fluorescent dyes SYTO9 and PI. Other dyes used for viable cell differential staining are 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), 4′,6-diamidino-2-phenylindole (DAPI), ethidium bromide, Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol (DiBAC4 (3)), calcein, and acridine orange [30,32,33].

The direct epifluorescent filter technique (DEFT) enumerates microbes by filtering samples pre-treated with chemical reagents or enzymes through polycarbonate filters, staining with CFDA or acridine orange dyes, and microscopic analysis. This technique was applied to determine VBNC *L. monocytogenes* cells in hard cheese but showed limitations such as cell injury caused by the chemical reagents or missed detection of weak signals [30].

FC allows the detection of viable cells based on cell membrane integrity, energy status, and enzymatic activities such as substrate uptake. In this technique, cells suspended in a liquid pass rapidly through a laser beam and are detected by exploiting light scattering or fluorescence emission. The signals are sent to detectors and converted into digital signals. Before FC, food samples must be pre-treated to decrease background noise. For example, dairy products need pre-treatments such as the dispersion of milk powder in a buffer and the extraction of cheese juice with a hydraulic press. Moreover, proteases and detergents, such as Triton X-100, can be added to degrade proteins and break somatic cells. Filtration and centrifugation are also carried out [30]. FC is not suitable for all food matrices, and optical assays have common disadvantages, such as susceptibility to cell membrane damage, background fluorescence, and the misidentification of dead cells with an intact membrane as viable [29,33].

2.2. Molecular Detection Methods

Molecular methods have been widely used in detecting VBNC bacteria in food matrixes and water for their high sensitivity, specificity, and rapidity. The most commonly adopted method is quantitative polymerase chain reaction (qPCR) applied to cells treated with DNA intercalating dyes before DNA extraction [29,30]. This method is designated as vPCR and allows an accurate quantification of only viable bacteria. It consists of applying a specific qPCR test to DNA extracted from cells treated with dyes, which enter into the cells with a damaged membrane and bind to their DNA blocking amplification after exposure to light. In theory, DNA intercalating dyes cannot penetrate intact cell membranes [18,30,33–35].

Unlike standard culture detection methods applied to bacterial pathogen detection in food, vPCR provides results before products with a short shelf life are sold out [30]. If qPCR is carried out with and without pre-treatment with a DNA intercalating dye, total and viable cells can be quantified separately [36]. This method must be validated for each matrix to avoid the interference of dead cells with intact membranes on VBNC cell quantification [29].

Ethidium monoazide (EMA) was the first DNA intercalating dye to be used to prevent the detection of dead cells [30,34] but presented limitations such as the extent of penetration influenced by the species of microorganisms and cytotoxic effects on viable cells [29,30]. Moreover, the usefulness of EMA is also limited by the ability of the dye to penetrate viable cells with consequent underestimation of their number [18].

Propidium monoazide (PMA) has a lower toxicity than EMA and a higher selectivity for cells with damaged membranes for its double positive charge, differently from EMA, which has only one positive charge [30]. The azide group of PMA allows its crosslinking with the DNA of dead cells, which is permanently modified while the DNA of viable cells remains intact and can be detected in the subsequent PCR reaction. The PMAxx derivative of PMA exerts better discrimination between viable and dead cells in complex matrices [37], and a combination of EMA and PMAxx improves selectivity for viable cells. Indeed, EMA is effective in distinguishing dormant cells that lack active efflux pumps to expel the dye. The mixture of these two dyes is commercially available under the name PEMAX [29].

In the vPCR, dye-treated samples are exposed to light, for example, from the blue light PMA-Lite LED or a 500–650 W halogen lamp, and dye crosslinking to DNA is usually allowed to occur for 5–15 min. Bacterial cells are concentrated by centrifugation and used for DNA extraction. These can also be stored at −20 °C until DNA extraction. The theoretical limit of quantification (LOQ) for *L. monocytogenes* in water and fresh produce by vPCR with PMAxx was 2.4 and 1.0 Log cells/100 mL or gram, respectively, with primers targeting the Listeriolysin O *hly* gene. The numbers of VBNC cells were determined as the difference between viable bacteria quantified by vPCR and plate counts [20,29,30].

Before the vPCR tests, several sample pretreatments must be carried out. For example, solid dairy products are homogenized in phosphate buffer saline (PBS) or trisodium citrate solution, fat and proteins are removed by centrifugation, and the pellet is collected. For liquid samples such as milk and fermented milk, no homogenization is needed. The pellets are then re-suspended in a solution transparent to light mixed with the dye solution, incubated in the dark for a minimum of 5 min at room temperature, and exposed to light while held on ice for 2–5 min to induce dye crosslinking. Finally, pelleted cells are used for DNA extraction and vPCR [29].

It was reported that PMA treatment does not completely abolish amplification from dead cells possibly because cell debris hinders its penetration through the membrane. Therefore, sodium deoxycholate (SD), an anionic surfactant that disrupts the cell membranes of damaged or dead cells, was used to enhance *L. monocytogenes* dead cell permeability. A

PMA concentration of 20 µM and 0.01% SD with an exposure time of 5 min were found to optimally protect the viable cells while at the same time inhibiting amplification from non-viable cells. However, an SD concentration higher than 0.02% caused the inhibition of amplification also from viable cells [38]. In some studies, sodium dodecyl sulfate (SDS) was used to decrease amplification from dead cells before treatment with PMA by enhancing the permeability of the membranes, but this practice is not easily applicable to bacteria at low concentrations and complex food matrices [35].

Immunomagnetic separation (IMS) allows researchers to selectively bind and separate foodborne pathogens from a food suspension, thus eliminating the matrix interference effect. However, its efficiency can be reduced by minor changes in pH, monodispersity, and cross-linking time, which influence the activity of the antibody adsorbed on the immunomagnetic beads. Therefore, an IMS method based on the streptavidin–biotin system was developed to enhance capture capability. However, antibodies were required in high amounts to enhance the capture efficiency. A novel biotin exposure-based IMS assay coupled with SDS and multiplex vPCR with PMA was developed for the rapid detection of viable bacterial pathogens including *L. monocytogenes* in milk. Biotinylated antibodies were used to bind to the cells; then, streptavidin-coated magnetic beads (SA-MB) were used to concentrate the pathogens that were treated with SDS and PMA. Finally, the genomic DNA was extracted and used in multiplex vPCR. The sensitivity of detection ranged between 10 and 10^3 CFU/mL for the target bacteria and the limit of detection (LOD) in artificially contaminated milk was 10 CFU/mL for all pathogens, showing an increased sensitivity compared to the test without IMS [35].

Gold nanoparticles (AuNPs), silver nanoparticles (AgNPs), silver nanowires, and polyethylenimine-coated Fe_3O_4 (PEI@Fe_3O_4) were tested as qPCR signal enhancers able to increase the normalized fluorescence (ΔRn) value. AuNPs with 20 nm particle size and 100-fold dilution displayed the highest signal enhancement with an increase of 20%. The sensitivity of the AuNPs-SD-PMA-qPCR assay was 5×10^2 CFU/mL for *L. monocytogenes* detected simultaneously with *Salmonella enterica* in enrichment cultures from dairy products. Thermal lysis for 15 min for DNA extraction reduced the detection time and the number of needed reagents by improving portability for on-site detection. The use of AuNPs can be easily implementable because it is non-toxic, highly biocompatible, not expensive, and easily produced and modified [38].

Isothermal amplification techniques coupled with intercalating dyes were introduced in recent years as an alternative to vPCR. One of these is loop-mediated isothermal amplification (LAMP), a cyclic strand displacement DNA synthesis process that allows the synthesis of 10^6–10^9 copies of target DNA in less than 1 h at a constant temperature of 60–65 °C. Amplification is visualized in real-time for the formation of a white magnesium pyro-phosphate precipitate that can be measured with a turbidimeter or by adding a fluorescent dye, such as Sybr Green I, to visualize amplification under UV light. The LAMP test is less sensitive to inhibition by the food matrix compared to PCR and can be carried out in water baths or heaters, which makes it a portable method [28,30,33,39]. The specificity is high since up to six specific primers matching the target regions are used. These include forward inner primer (FIP), backward inner primer (BIP), forward outer primer (F3), backward outer primer (B3), forward loop primer (FLP), and backward loop primer (BLP). The sensitivity of LAMP was found to be higher than that of qPCR with an LOD of 6 CFU/reaction for *L. monocytogenes*. LAMP coupled with PMA or EMA proved faster and 100-fold more sensitive than vPCR for food samples [30].

Most LAMP methods do not include an internal amplification control (IAC) due to the difficulty of multiplexing the reaction, and LAMP amplification products are differentiated based on their melting temperatures. However, an *L. monocytogenes*-specific test was

developed to comprise an IAC constituted by a plasmid containing the original target fragment with the inserted sequence CCATCCGACGGCGGGCGGCC, which increased the Tm of the IAC fragment, allowing its distinction by melting curve analysis. This LAMP method effectively blocked amplification from up to 10^6 CFU/mL dead cells, and its LOD_{95} defined by plate counts was 2.7 CFU/25 mL of milk. The performance of the method was tested on a total of 59 spiked milk samples but not on VBNC cells [39].

Competitive annealing mediated isothermal amplification (CAMP) is another isothermal amplification reaction with incubation at 60–65 °C for 60 min and with a simpler primer design compared to LAMP. It was tested for the detection of *L. monocytogenes* in milk and milk powder and was coupled with PMA cross-linking or to PMA and hydroxynaphthol (HNB) to visualize amplification by color change (violet to blue). Visual detection in this method was also achieved by using gold nanoparticle probes (AuNP) whose dispersion and aggregation can be visually detected based on color change. In negative samples, nanoparticles form aggregates conferring a red or blue–grey color to the solution, while in positive samples, the AuNP probe hybridizes with the target and remains dispersed with no color change [30].

A novel technology was developed to detect viable *L. monocytogenes* based on PMA-LAMP with four primers combined with nanozyme strips that are cost-effective and equipment-free with the possibility of visual detection. This technology includes using BIO- and FITC-modified primers targeting the *hlyA* gene and a magnetic nanozyme modified with a biotin-conjugated antibody specifically for *L. monocytogenes*. The peroxidase-like activity of the magnetic nanozyme generated a visually readable color, and quantitative detection could be achieved by measuring the optical intensity of the test line (T line) by ImageJ with a detection limit of 5.4 fg/μL [40].

Fe_3O_4 NPs' S15 nanozyme were tested for viable *L. monocytogenes* detection after mixing with the amplification products of PMA-LAMP and allowed to react with nitrocellulose (NC) membranes on which anti-mouse immunoglobuline G (IgG) and anti-FITC antibodies were immobilized to form C and T lines, respectively. If *L. monocytogenes* was present in the sample, the PMA-LAMP products conjugated with the nanozyme probe by the combination of BIO and anti-biotin antibodies. One side of positive products was labeled with Fe_3O_4 NPs while the other side was labeled with FITC so that the amplification products could be captured by anti-FITC antibody on the T line while the free nanozyme probes stopped in the C line. The color reaction was enhanced by the enzymatic reaction between the nanozyme and 3,3-diaminobenzidine (DAB)/H_2O_2 enzymatic substrate. If viable *L. monocytogenes* was absent, only the C line appeared; if no color appeared, the result was not considered valid. The method allowed the detection of 1.164 × 10 CFU/mL and 1.021 × 10 CFU/mL *L. monocytogenes* in biofilms developed on stainless steel and lettuce, respectively [40].

Other isothermal amplification techniques include polymerase spiral reaction (PSR) and cross-priming amplification (CPA). The CPA assay was applied to the *L. monocytogenes hylA* gene using calcein for product visualization. It involves several primers and probes, with one or more cross primers, and exploits the *Bst* DNA polymerase for strand displacement of the target DNA. The amplification products are detected by turbidimetry and electrophoresis or with fluorescent dyes such as calcein and SYBR Green [33]. A PMA-CPA assay with a sensitivity similar to a PCR-based assay was applied for the direct detection of VBNC *L. monocytogenes* cells in artificially contaminated rice flour. Dead bacterial cells killed by high temperature (121 °C) and high pressure (100 kpa) were included as a negative control and genomic DNA was included to verify no amplification from extracellular DNA. PMA was used in a final concentration of 10 μg/mL, with incubation at room temperature in the dark for 5 min, and exposure to light from a 500 W halogen lamp for 15 min. After

DNA extraction, the CPA assay could quantify bacterial cells in rice flour suspension in a concentration range of 10^4–10^8 CFU/mL [33].

A disadvantage of vPCR and other molecular viability tests based on DNA amplification is that sub-injured cells with a damaged membrane are not detected. Colorimetric sensors can aid in vPCR to detect only viable pathogens by using a streptavidin magnetic bead–dual aptamers (SMB-Apts) sandwich coupled with an AuNPs colorimetric sensor [30].

One limitation of PMA-based vPCR was the poor discrimination between viable and dead cells when applied to high levels of *L. monocytogenes* heat-treated at 60 °C for 6 min. False positive results were attributed to the shadowing of non-viable cells in the photoactivation step. In addition, this method might overestimate the number of viable cells since it can misidentify dead cells with an intact membrane as viable cells [41]. To increase vPCR accuracy, the use of a stable internal process control (ISPC), similar to the one developed for *Campylobacter jejuni*, was recommended to normalize and subtract the signal originating from dead cells [42].

An alternative approach to vPCR is the amplification of mRNA by specific reverse transcription followed by qPCR (RT-qPCR) tests [43]. Since mRNAs have only a few minutes of half-life, they are only detectable when cells are viable through reverse transcription to cDNA and PCR amplification [30]. A comparison among commercial RNA extraction kits based on 260/280 and 260/230 optical density (OD) ratios, RNA integrity (RIN) values, and the absence of residual gDNA after DNAse treatment was performed to select the most suitable for the quantitative detection of *L. monocytogenes*. Subsequent multiplex RT-qPCR targeted on *hly*, *act*A, and a non-competitive internal amplification control (NC-IAC) allowed for obtaining LOD_{50} and LOD_{95} values of 1.2 CFU/25 g and 5.1 CFU/25 g, respectively, from enrichment cultures of artificially spiked smoked salmon [43]. Other studies also used *hly* and *inlA*-targeted RT-qPCR to analyze the viability of *L. monocytogenes* in food production conditions [41,44]. Limitations of mRNA usage for viable bacteria detection are its instability and the risk of contamination with DNA. Moreover, RNA extraction from complex food matrices still requires optimization [30].

Figure 1 reports a simplified scheme highlighting the experimental workflows that were applied for the detection of viable *L. monocytogenes* cells.

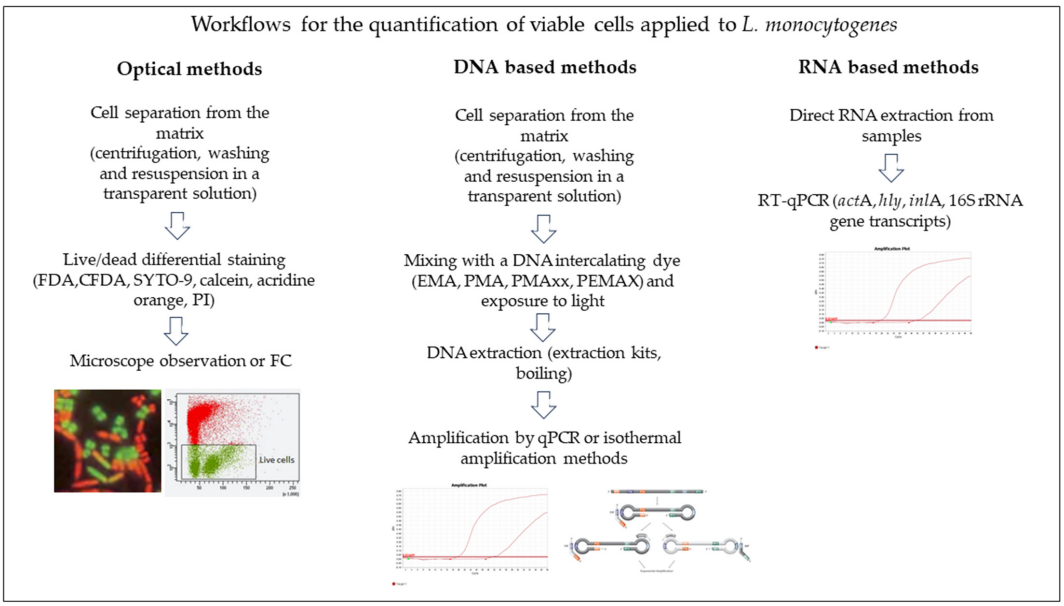

Figure 1. Experimental workflows to detect viable cells of *L. monocytogenes* [14–22,28–44]. The images shown were modified from illustrations available at the following websites: https://www.thermofisher.com;

https://www.raybiotech.com; https://www.neb.com (accessed on 20 January 2025). The amplification curves, used exclusively for explicative purposes, were obtained from an author's experiment.

3. Distribution of VBNC *L. monocytogenes* in Food and Food Production Plants

A few recent studies have investigated the presence of VBNC *L. monocytogenes* in food considered ready-to-eat (RTE) salads and chicken meat [17,36,45]. RTE salads require extensive handling and are exposed to recontamination from processing surfaces and equipment after listericidal treatments. The storage of these products in refrigerated conditions could allow the development of *L. monocytogenes* [17].

Bernardo et al. [17] reported that, based on hygiene indicators and *L. monocytogenes* determination by culture-based methods, an RTE salad considered in the study could have a shelf-life of eight days when stored at 4 °C, according to Regulation (EC) No. 2073/2005 food safety criteria [27]. However, on day 8, the concentration of *L. monocytogenes* obtained by vPCR with PMAxx was at least 3 Log CFU/g higher than that obtained by colony counts. No amplification was observed by vPCR from boiled salad extracts. Therefore, the vPCR results exceeded the food safety criteria of 2 Log CFU/g and showed that the standard culture-based methods might underestimate the number of viable *L. monocytogenes* cells affected by processing treatments that hamper cultivability. However, the potential interference of dead cells was not evaluated [17].

A study on poultry meat investigated the presence of VBNC cells of *L. monocytogenes* in two slaughterhouses in 11 wings, three thighs, 12 drumsticks, and four breast samples. After homogenization of 25 g of sample in 225 mL of 0.1% peptone water, 1 mL was used to determine the total cell concentration and 1 mL was used for vPCR after treatment of cells with PMAxx 25 µM. DNA extraction was performed on 750 µL of the two aliquots, treated and not treated with PMAxx, using a commercial kit with proteinase K. The vPCR technique showed a sensitivity of 100%, a specificity of 66.7%, and an efficiency of 83.3%, with 0.67 kappa coefficient agreement when compared with culturing by enrichment and colony isolation on Oxoid Chromogenic Listeria agar (OCLA) followed by colony identification by species-specific PCR (OCLA-PCR). The rather low specificity and agreement were because five samples were positive on vPCR but negative on OCLA-PCR. These five samples most probably contained VBNC *L. monocytogenes* cells, indicating the occurrence of these forms in poultry meat. Previous studies based on plate counts reported lower contamination levels for poultry, and this can be explained by the ability of vPCR to detect also VBNC cells. The high sensitivity score of vPCR (100%) against OCLA-PCR indicated the absence of false negative results for the method [36].

A second investigation found *L. monocytogenes* in 75.0% of 65 samples from two slaughterhouses by culturing methods and qPCR. The results were not fully concordant since some samples were positive only for one of the techniques. Viability PCR allowed detecting *L. monocytogenes* in half of the carcasses from one slaughterhouse, but the microorganism was not detected by culture-dependent methods. In another slaughterhouse, *L. monocytogenes* was detected in 100% of samples by culture-dependent methods only (38.5%) or both by culturing and qPCR (61.5%). A comparison among culture-dependent detection, qPCR, and vPCR allowed the authors to determine that the contamination levels in the positive samples were 4.01 Log CFU/g for total cells, 3.21 Log CFU/g for viable cells, 1.00 Log CFU/g for culturable cells, 3.20 Log CFU/g for VBNC cells, and 3.93 Log CFU/g for dead cells [45].

4. Factors Inducing the VBNC State in *L. monocytogenes*

According to a review published in 2021, studies carried out before 2020 reported VBNC induction in *L. monocytogenes*, as determined by the detection of respiratory activity by CTC-DAPI staining in seawater and in "microcosm water" (i.e., sterile filtered water with pH 6.0), and, thus, in the absence of nutrients. Those VBNC cells showed metabolic activity for ten weeks, and the VBNC state was induced at a lower extent when a high inoculum was used, probably due to higher nutrient availability within the population. The VBNC state was also induced in biofilms exposed to tap water. Viability PCR highlighted the presence of VBNC *L. monocytogenes* in pig manure, food environments, and on the surface of vegetables such as parsley and spinach [34].

Staining with CFDA showed that *L. monocytogenes* entered the VBNC state in long-ripened hard cheese left for up to 90 days at 6 °C in meat exudate and less in smoked salmon juice. Direct viability count coupled with fluorescence in situ hybridization (FISH) with a 16S rRNA probe and vPCR showed the existence of VBNC *L. monocytogenes* in RTE vegetables [34].

4.1. Role of Disinfecting Agents

Benzalkonium chloride (BC) is a widely used quaternary ammonium compound (QAC) in food production plants and damages the cell membrane. BC was tested on non-adapted and pre-adapted cells of *L. monocytogenes* SLCC2540 serotype 3b and was found to induce the VBNC state, expressed as metabolically active cells, more efficiently in pre-adapted cells. Pre-adaptation was carried out by exposure to BC concentrations from 2 µg/mL to 13 µg/mL, gradually increasing by 1 µg/mL in BHI broth. Pre-adapted cells were also less susceptible than parent cells to 13 antibiotics of different classes except vancomycin and erythromycin. However, when looking only at membrane integrity by LIVE/DEAD BacLight kit staining and FC, non-adapted cultures also showed high numbers of apparently viable cells, about 10^8 cells/mL, at BC concentrations between 4 µg/mL and 13 µg/mL. However, when the metabolically active cells were identified by measuring the uptake of 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG), their number was much higher than for non-adapted cells at concentrations of BC between 5 µg/mL and 11 µg/mL. With BC above 10 µg/mL, no colonies were obtained by plate count. It must be underlined that also for non-adapted cells, low percentages of metabolically active cells were detected at BC concentrations between 5 µg/mL and 13 µg/mL [46].

One of the concerns dealt with in studies regarding VBNC *L. monocytogenes* was the effect of RTE fresh produce process wash water (PWW) in the induction of VBNC cells. PWW is indeed a relevant vector of microbial cross-contamination of clean products from contaminated products that is prevented by adding sanitizers, among which chlorine is the most commonly used. However, it was reported that chlorine induces the VBNC state in *L. monocytogenes* and *S. enterica*, so suitable quantification methods should be applied to detect and control VBNC cells in PWW [29].

An optimized detection/quantification method of VBNC cells in PWW was developed after comparing different combinations of dyes in FC, and of PMAxx, or a combination of EMA and PMAxx in vPCR. The method was validated in industrial settings by using six *L. monocytogenes* isolates from leafy vegetables in a mixture. A PMAxx concentration of 75 µM was tested since a 50 µM concentration added to PWW did not completely inhibit amplification from dead cells, possibly due to the presence of interfering organic matter, while 100 µM appeared toxic for culturable cells. Amplification from heat-treated cells was abolished after 30 min of incubation with the dye at 40 °C but the PCR signal from cells treated with chlorine was not completely inactivated. PMAxx combined with EMA

at concentrations of 75 µM and 10 µM, respectively, and incubation at 40 °C for 40 min followed by 15 min exposure to light, reduced the amplification from dead cells after chlorine treatment [29].

In the shredded lettuce PWW, no significant changes in the concentration of free chlorine (10 mg/L) and pH were observed, and the culturable bacteria maintained a total count below 3 Log CFU/mL. However, a higher number of viable cells was detected by using EMA-PMAxx-vPCR, thus showing the induction of VBNC *L. monocytogenes*. FC with double-staining indicated a higher number of viable cells compared to vPCR, possibly for an overestimation caused by the presence of cells with intact membranes but damaged DNA, proteins, and lipids. Therefore, fluorescent markers able to highlight damages to these molecules should be used in FC for improved viable cell discrimination. The VBNC *L. monocytogenes* cells induced by 10 mg/L chlorine could be detected after 1 h and remained stable after 3 h [29].

Residual concentrations in PWW of 20 mg/L for chlorine, 80 mg/L for peroxyacetic acid (PAA), and 3 mg/L for chlorine dioxide (ClO_2), the most commonly used sanitizers in vegetable washing, are recommended by scientific studies, guidelines, and regulations. The operational limits for these sanitizers must be set above these minimum residual levels, and their optimization for different types of fresh-cut produce was the object of many studies [37]. It has been reported that in industrial settings with free chlorine concentrations below the operational limit of 10 mg/L, there was an induction of VBNC bacteria; further, in one study, 100% of 10^6 CFU/mL *L. monocytogenes* cells exposed to 12 mg/L free chlorine entered the VBNC state. The induction of the VBNC state was higher at higher concentrations of organic matter and using the recommended operational limits for chlorine of 20–25 mg/L effectively inactivated *L. monocytogenes* in PWW without the induction of VBNC cells. Therefore, the efficacy of the recommended washing practices was evaluated in a pilot plant [37].

Culturable and VBNC *L. monocytogenes* cells were undetectable in PWW with chlorine after 1 min of contact for all types of washed produce. Instead, PAA at 80 mg/L concentration did not completely inactivate the pathogen in any type of PWW [37]. Significant differences were observed among different types of PWW for ClO_2 used at the 3 mg/L recommended concentration. In particular, the presence of VBNC cells was detected in shredded lettuce PWW. Since the recommended concentrations of PAA and ClO_2 induced the VBNC state, the necessity to revise the current operational limits of these disinfecting agents by increasing the concentration or the time of contact was suggested [37].

The optimized vPCR PMAxx method developed by Truchado et al. [29] was used to evaluate the ability of VBNC *L. monocytogenes* and *E. coli* O157:H7 cells to tolerate sanitizers, cause cross-contamination, and resuscitate in leafy greens during storage [20]. To determine the free chlorine concentration needed in PWW to induce the VBNC state, the concentrations 2.5 and 5 mg/L were tested on a mixture of six *L. monocytogenes* serotype 1/2a isolates from leafy vegetables. With 2.5 mg/L chlorine, VBNC and culturable *L. monocytogenes* cells were detected, while with 5 mg/L chlorine, all cells were inactivated. The VBNC cells in the PWW cross-contaminated the product after 1 min wash with 3 mg/L of ClO_2, and after 5, 10, and 15 days of storage at 7 °C, at least one replicate originated colonies indicating the resuscitation of VBNC cells. On iceberg lettuce and baby spinach during the 15-day shelf life, the levels of inoculated VBNC cells did not significantly change, though these were not culturable [20].

Sterile distilled water (SDW), baby spinach rinse water (SRW), and romaine lettuce rinse water (LRW) were used as media to carry out sanitizer treatments against 8 Log CFU/mL of two foodborne strains of *L. monocytogenes* serogroup 1/2a. In SDW, *L. monocytogenes* became undetectable by selective plating after exposure to chlorine at

a concentration of 0.5 mg/L for 30 s, while in spinach or lettuce rinse water, due to the presence of organic matter, inhibition of growth in the plate was obtained with 10 mg/L of free chlorine. *L. monocytogenes* was undetectable in the plate after treatment with 30 mg/L PAA, except for one colony in one of the four replicates for LRW. For PAA treatment, the effect of organic matter on pathogen inactivation was much less pronounced than for chlorine. After exposure to PAA 30 and 50 mg/L in different media, the pathogens were detected only by vPCR with a decrease of only 1–2 Log from the initial numbers [47].

For treatments with free chlorine in SRW and LRW, selective plating and vPCR were significantly correlated. In SDW, *L. monocytogenes* at an initial level of 7.88 Log CFU/mL became undetectable by selective plating, non-selective enrichment, and vPCR with PMAxx when exposed to 50 mg/L free chlorine for 30 s; however, it was detected by vPCR, but not by selective plating, following treatment with 100 mg/L PAA. Cells treated with free chlorine stained red or brown with the LIVE/DEAD BacLight Bacterial viability kit, while in the PAA-treated samples, most cells stained green, suggesting uncompromised membrane integrity and viability [47].

Negative stain transmission electron microscopy (TEM) showed that most cells were damaged with electron-dense internal aggregations, large electron-lucent areas, and shrunk cytoplasm separated from the cell wall. However, a small proportion of cells resembled untreated cells and were probably VBNC. These cells could not be resuscitated, so the authors hypothesized that they could be damaged though the cell membrane was intact. Nevertheless, since chlorine-induced VBNC *L. monocytogenes* had been previously shown to remain infectious for *Caenorhabditis elegans*, the possibility that these cells pose a food safety risk was not excluded [47].

CFDA/PI differential staining coupled with FC revealed three different subpopulations of *L. monocytogenes* Scott A outbreak strain, serogroup 4b, when exposed to PAA or low pH adjusted with HCl or acetic acid (AA), namely, viable and metabolically active cells with an intact membrane ($CFDA^+PI^-$), dead cells with a damaged membrane ($CFDA^-PI^+$), and metabolically active cells with a damaged membrane classified as sub-lethally injured ($CFDA^+PI^+$). The $CFDA^+PI^-$ cells were considered to be in a VBNC state if they were unable to restart growth in tryptic soy yeast extract broth or agar (TSBYE or TSAYE) [22].

After exposure to 20, 30, and 40 ppm of PAA at 20 °C for 1, 2, and 3 h, $CFDA^+$ metabolically active cells ranged between 40.00% and 47.27%. The percentages of sub-lethally injured cells increased after 2 h of incubation. After exposure for 3 h to 40 ppm PAA at 20 °C, the total culturable and non-injured populations significantly decreased, and the whole population was nonculturable and $CFDA^+$, thus indicating the induction of the VBNC state [22]. Exposure to 20 ppm PAA at 20 °C for 90 min and 180 min resulted in high percentages of $CFDA^+PI^-$ cells, 16.17- and 13.33-fold compared to the control, while exposure to 30 and 40 ppm of PAA for 90 and 180 min increased the proportion of dead cells. However, exposure to higher PAA concentrations for 30 min led to the formation of numbers of 5.8- to 8.5-fold higher injured cells than the control [14].

Exposure to high acidity at pH 3.0 and 2.7 adjusted with HCl determined a small decline in metabolically active cells after 150 and 300 min. The decline was higher when AA was used as an acidifying agent, with a reduced formation of $CFDA^+PI^+$ cells and an increase in dead cells. The number of cells recovering on TSAYE compared to the non-agarized medium was significantly lower in all conditions, except for treatment with 20 ppm PAA for 180 min and 30 ppm PAA for 90 min, interpreted as the ability of PAA to induce the VBNC state in *L. monocytogenes*. After exposure to pH 3.0 adjusted with HCl, a small percentage of cells became non-culturable and the percentage increased to 33% at pH 2.7 after 300 min but only a small fraction of cells seemed to be in the VBNC state. When acidification to pH 2.7 was performed with AA, a significant percentage of $CFDA^+PI^-$ cells

lost culturability, thus indicating VBNC induction. The lag phases for cells able to recover increased to a maximum of 120 h for cells exposed to PAA and to 21–24 h for exposure to pH 2.7 for 150–300 min [14].

The effects of two disinfectants commonly used in the seafood industry, one containing a quaternary ammonium compound and glutaraldehyde (QA) and one containing hydrogen peroxide (HP), were evaluated for the ability to induce VBNC cells of *L. monocytogenes* in single-species and mixed-species biofilms. *L. monocytogenes* Lm1 (serogroup 1/2a-3a), *Carnobacterium maltaromaticum* P1AV5, and *C. divergens* P9AV5, all isolated from food contact surfaces of seafood processing plants, were used to produce biofilms on stainless steel and polyvinyl chloride (PVC) coupons. The biofilms were treated for 20 min at room temperature with 2% (*v*/*v*) solutions of the disinfecting agents according to the recommendations for use [48].

The difference between the number of cells determined by qPCR and vPCR with PMA, expressed as genome equivalents (GE), represented the number of dead cells while the difference between the number of cells determined by vPCR and counts on agar plates corresponded to the number of VBNC cells. *Listeria* cells remained viable and culturable in the biofilm not treated with disinfectants. The difference between qPCR and vPCR measurements in biofilms of stainless steel were 2.06 Log GE/cm^2 and 1.70 Log GE/cm^2 after HP and QA treatment, respectively. However, the decreases in *L. monocytogenes* culturable cell numbers were 5.22 Log CFU/cm^2 and 4.49 Log CFU/cm^2, respectively, indicating the presence of VBNC cells in the biofilms with differences of 2.95 Log GE/cm^2 and 2.12 Log GE/cm^2 compared to the culturable cells. Similar results were obtained for the biofilms on PVC [48].

Sampling of food contact surfaces in four commercial smoked salmon processing plants was also carried out before and after cleaning and disinfection for 8 months to examine the prevalence of culturable and VBNC *L. monocytogenes*. Before cleaning and disinfection, the prevalence of *L. monocytogenes* cells was 3.17% for culturable cells, 10.32% for viable cells, and 15.87% for total cells, while after cleaning and disinfection, the prevalence of *L. monocytogenes* was 1.59% for culturable cells, 9.52% for viable cells, and 14.29% for total cells, thus indicating a high rate of VBNC formation though with differences in prevalence among single plants [48].

Essential oils are natural antimicrobials particularly attractive for use in food production. Those extracted from oregano (*Origanum vulgare* essential oil, OVEO) and rosemary (*Rosmarinus officinalis* essential oil, ROEO), alone or in combination, inhibited *L. monocytogenes* in food matrices. Therefore, their capacity to induce *L. monocytogenes* VBNC formation in culture media and conditions mimicking food matrices was analyzed for a mixture of *L. monocytogenes* strains ATCC 7644 and ATCC 19112 (serotype 1/2c of human origin), and ATCC 19117 (serotype 4d of sheep origin). Exposure to 5 and 2.5 µL/mL of OVEO determined culturability loss after 60 min in PBS and after 180 min in meat broth. Exposure to 10 and 5 µL/mL of ROEO in PBS determined culturability loss after 60 and 180 min, respectively, while in meat broth, culturability loss occurred after 180 min at both concentrations, thus appearing delayed in the presence of nutrients [31].

L. monocytogenes cells that lost culturability did not recover after 6 h, in agreement with previous observations with *L. monocytogenes* Scott A. With a recovery time of 24 h, *L. monocytogenes* cells exposed to OVEO did not regain culturability, while those exposed to 5 µL/mL of ROEO in PBS and 10 µL/mL in meat broth restored culturability, with average viable counts in the range of 3.0–4.8 Log CFU/mL from initial viable counts of about 7 Log CFU/mL. These results indicated that *L. monocytogenes* cells exposed to ROEO in PBS and meat broth entered the VBNC state [31].

The presence of VBNC cells was analyzed by FC and double staining with bis-1,3-dibutyl barbituric acid (BOX) and PI as indicators of membrane potential and membrane integrity, respectively. Exposure to OVEO and ROEO caused the formation of 76.4 to 97.2% of cells with depolarized and damaged cell membranes (BOX^+ PI^+) in PBS and 97 to 98.5% in meat broth. After 24 h of recovery treatment, the cells with a viability phenotype (BOX^- PI^-), especially for cells exposed to ROEO, increased, reaching 69.1–83.5% in PBS and 44.9–75.5% in meat broth [31].

The subpopulations FDA^+ PI^+, i.e., with damaged cell membrane but with enzymatic activity, ranged from 33.5 to 55.8% and 19.7 to 44.8% for cells exposed to OVEO and ROEO in PBS and meat broth, respectively. The number of dead cells (FDA^-PI^+) ranged from 10.8 to 22.7% and 8.6 to 29.5% for cells exposed to OVEO and ROEO in PBS and meat broth, respectively. However, the subpopulations with undamaged cell membranes increased after 6 h of recovery treatment and, for cells exposed to ROEO, reached percentages of 72.6 and 69.7% after exposure to PBS and 99.9 and 86.6% after exposure to meat broth. After 24 h of recovery, cells exposed to ROEO in PBS showed 79.5 and 91% subpopulations with undamaged cell membranes. Subpopulations with enzymatic activity after exposure to OVEO and ROEO in meat broth increased from 58.5 to 71.7% after 24 h of recovery [31].

Cell efflux and respiratory functions, beyond membrane damage, might influence culturability. Therefore, ethidium bromide (EB) staining was performed to investigate efflux activity. This dye can cross intact cell membranes and is pumped out by cells with active efflux systems, so cells with impaired efflux activity are EB+. Cells exposed to OVEO and ROEO originated similar percentages of EB+ subpopulations, namely, 57.2–84.1% and 55.6–81% in PBS and meat broth. Gradual increases in EB− subpopulations were observed after 24 h of recovery from exposure to ROEO in PBS (34.1 and 61.4% for the concentrations 10 and 5 µL/mL). After 24 h of recovery treatment, cells exposed to OVEO increased the efflux activity, whereas those exposed to ROEO increased the EB+ subpopulation. The results of viable counts after recovery diverged for the two essential oils but cells in which the culturability was not restored had improved physiological functions and appeared to be viable. Further investigations should elucidate whether OVEO- and ROEO-induced VBNC cells can restore the ability to multiply in food matrices [31].

Lavender essential oil (LEO) has an attractive aroma and is widely used in the food industry because of its broad antibacterial, antifungal, antiviral, nematocidal, and anti-mite properties. It is also largely used in pharmaceutical products and cosmetics for its multiple health-promoting properties. The effects of LEO against *L. monocytogenes* ATCC19115 were defined toward biofilms of the pathogen by using plate counts and vPCR with PMA. Biofilm formation of *L. monocytogenes* on sterile coverslips in polystyrene microtiter plates was evaluated by the crystal violet staining method [49].

The effect of LEO on *L. monocytogenes* was compared with that of sodium hypochlorite. The minimum inhibitory concentration (MIC) for LEO and sodium hypochlorite against *L. monocytogenes* was 1.6 *v*/*v* and 0.219 mg/mL, respectively. In the treatment with 4 MIC of LEO, viable and culturable cells in biofilm formed at 32 °C (warm biofilm) decreased by 6.35 and 9.01 Log CFU/mL, and those in the biofilm formed at 10 °C (cold biofilm) decreased by 3.16 and 1.93 Log CFU/mL, respectively. In the treatment with 4 MIC of sodium hypochlorite, the proportion of viable and culturable cells decreased by 3.68 and 4.76 Log CFU/mL in the warm biofilm, and 2.88 and 2.91 Log CFU/mL in the cold biofilm, respectively. Consequently, LEO reduced the number of viable cells more effectively than sodium hypochlorite. However, both LEO and sodium hypochlorite-induced VBNC populations that ranged in number from 1.74 to 3.58 and from 2.10 to 3.26 Log CFU/mL in the warm biofilm and cold biofilm, respectively, in the treatment with LEO [49].

After 40 days of exposure to 1/2 MIC of LEO, VBNC cells for planktonic cells and biofilm were 2.51 and 0.50 Log CFU/mL, respectively, while in the 1/2 MIC, the sodium hypochlorite treatment was 4.93 Log CFU/mL for planktonic cells and 1.08 Log CFU/mL for the biofilm, respectively. In the treatments with LEO or sodium hypochlorite at the MIC for 40 days, the numbers of VBNC cells for planktonic cells and biofilm were 2.61 and 0.64 Log CFU/mL and 4.93 and 1.08 Log CFU/mL, respectively. Therefore, a low concentration of LEO induced fewer VBNC cells than sodium hypochlorite [49].

Electrolyzed water (EW) is obtained by electrolysis of diluted NaCl or HCl solutions in a specialized chamber. One type of EW is slightly acidic electrolyzed water (SAEW), with disinfecting properties. SAEW has a pH of 5–6.5 and is gentle on surfaces, so it is suitable for use in the food industry. SAEW contains chlorine, which has a strong oxidizing capacity and covalently binds to proteins, modifying their structure in the chlorination process. To determine the concentration of chlorine required for VBNC induction, *L. monocytogenes* cells were treated with SAEW for 5 min at different concentrations. With SAEW containing 4 and 6 mg/L of available chlorine (ACC), only 0.31 and 0.72 Log CFU/mL reductions were achieved, respectively, while 8 mg/L chlorine completely abolished culturability. Staining with the LIVE/DEAD^{TM} BacLight^{TM} kit showed that 23 to 34% of the cells were viable after treatment with 8 and 10 mg/L of ACC, while standard plating provided negative results, indicating induction of the VBNC state. Most cells appeared to be intact by optical microscopy, but scanning electron microscopy (SEM) showed a shrinkage of the cell membrane and the formation of aggregates in the VBNC cells [32].

Iron is essential for different cell functions, including energy metabolism, cell signaling, gene expression, growth regulation, and cell differentiation, but when the redox reactions catalyzed by iron are poorly controlled, the process of ferroptosis, an iron-dependent nonapoptotic cell death, may occur. This process is also exploited for cancer therapy, in which the Fenton reaction causes lipid peroxidation by hydroxyl radicals production catalyzed by iron in excess and H_2O_2 [19].

L. monocytogenes ATCC19114 was susceptible to inorganic ferrous and ferric iron at a final concentration of 200 µM for 3 h. Cells exposed to $FeSO_4$ lost culturability, as well as 90% of those exposed to ferric citrate (Cit-Fe (III)). *L. monocytogenes* ATCC19114 cells exposed to $FeSO_4$ did not stain with PI nor with SYTOX in FC analysis; so, it was deduced that $FeSO_4$ did not cause cell death. To elucidate if $FeSO_4$ induced DNA degradation, the cells exposed to $FeSO_4$ for 3 h were incubated in a saline solution containing 400 µg/mL thymol for 30 min, and it was observed that thymol led to PI-stained cells, indicating that cells were viable after $FeSO_4$ exposure [19].

The induction of VBNC cells by $FeSO_4$ occurred in a dose-dependent manner, but even at a low concentration of 25 µM, $FeSO_4$ induced the VBNC state in approximately 90% of cells. The process occurred in less than 15 min at temperatures between 5 and 42 °C and for strains belonging to different serotypes. Morphological changes from rod-shaped to spheroids, size reduction, and shrinkage were observed in the $FeSO_4$-induced VBNC cells. These were able to resuscitate in fresh BHI broth after a prolonged lag phase and were virulent for *C. elegans* with survival rates similar to normal *L. monocytogenes* cells after 10 days from infection [19].

Notably, while normal *L. monocytogenes* cells were killed by 240 µg/mL ampicillin, 60 µg/mL tetracycline, and 60 µg/mL chloramphenicol, in BHI broth, VBNC cells developed resistance, except to tetracycline. When resuscitated, the population of VBNC cells was, again, sensitive to ampicillin [19].

Common indicators of ferroptosis—namely, reactive oxygen species (ROS) and lipid peroxidation products—did not significantly increase in *L. monocytogenes* cells exposed to $FeSO_4$, showing that ferroptosis did not occur. Exposure to the intracellular Fe^{2+} chelator

2,2′-Bipyridine almost completely blocked the VBNC state formation by $FeSO_4$. After the addition of 2,2′-Bipyridine, VBNC formed after exposure to Haz-Tab chlorine tablets almost recovered growth as the normal cells, while those formed by exposure to benzalkonium chloride (BC) died. It was concluded that the chelation of intracellular Fe^{2+} hindered VBNC formation, indicating the involvement of Fe^{2+} in VBNC state formation in *L. monocytogenes*. On the other hand, the application of Lip-1 and ascorbate inhibitors of ferroptosis blocked the VBNC state induction and arrested VBNC formation induced by BC or Haz-Tab with similar effects as 2,2′-Bipyridine. This observation suggested that processes related to ferroptosis were involved in VBNC state formation in *L. monocytogenes* [19].

Table 1 summarizes the results obtained on VBNC state induction in *L. monocytogenes* by antimicrobial compounds and the effective concentrations.

Table 1. Antimicrobials that induced the VBNC state in *L. monocytogenes*, their concentration or intensity, and strains studied.

Stress Factor(s)	Intensity–Concentration	*L. monocytogenes* Strain	Reference
BC	5 µg/mL	SLCC2540 serotype 3b	[46]
chlorine	10 mg/L	cocktail of six vegetable isolates, serotype 1/2a	[29]
ClO_2	3 mg/L	cocktail of six vegetable isolates, serotype 1/2a	[20,37]
PAA	80 mg/L	cocktail of six vegetable isolates, serotype 1/2a	[37]
PAA	100 mg/L	Two foodborne strains, serogroup 1/2a	[47]
PAA	40 mg/L	Scott A, serotype 4b	[22]
AA	pH 2.7	Scott A, serotype 4b	[14]
QA	2% *v*/*v*	Lm1 (serogroup 1/2a-3a), seafood production plant isolate	[48]
HP	2% *v*/*v*	Lm1 (serogroup 1/2a-3a), seafood production plant isolate	[48]
OVEO	5 and 2.5 µL/mL in PBS and meat Broth	Mixture of ATCC 7644 and ATCC 19112 (serotype: 1/2c of human origin), and ATCC 19117 (serotype: 4d of sheep origin)	[30]
ROEO	5 µL/mL in PBS and 10 µL/mL in meat broth	Mixture of ATCC 7644 and ATCC 19112 (serotype: 1/2c of human origin), and ATCC 19117 (serotype: 4d of sheep origin)	[30]
LEO	4 MIC (MIC = 1.6 *v*/*v*)	ATCC19115	[49]
Sodium hypochlorite	4 MIC (MIC = 0.219 mg/mL)	ATCC19115	[49]
SAEW	8 and 10 mg/L of available chlorine (ACC)	BCRC14845	[32]
$FeSO_4$	200 µM	ATCC19114	[19]

PAA, peroxyacetic acid; AA, acetic acid; QA, disinfectant containing quaternary ammonium and glutaraldehyde; HP, hydrogen peroxide; OVEO, *Origanum vulgare* essential oil; ROEO, *Rosmarinus officinalis* essential oil; LEO, lavender essential oil; SAEW, slightly acidic electrolyzed water.

4.2. Effect of Physicochemical Stresses in Food Production on VBNC Induction in L. monocytogenes

Some stress factors such as the presence of particular ingredients, sub-optimal temperature, and the presence of inhibitors are inherent to food and food production processes. In a study regarding the use of Raman–deuterium isotope probing (Raman-DIP) microspectroscopy to characterize VBNC cells of *L. monocytogenes*, this technique was evaluated for the analysis of the effects of different food-related stressors, namely, storage temperature, presence of salts, and exposure to disinfectants using strains *L. monocytogenes* Lm1 and EGD-e. Viability was also analyzed by using qPCR, vPCR with PMA, and culturing. An incubation temperature effect was observed with the presence of a VBNC population at 37 °C and at 4 °C. For cells exposed to 2% and 20% NaCl, differences of 1.5 Log UFC/mL and 0.72 Log UFC/mL between viable and culturable cells were observed for *L. monocy-*

togenes Lm1, thus showing the presence of a VBNC population. Results obtained for *L. monocytogenes* EGD-e were similar [50].

The persistence of *L. monocytogenes* in food processing environments is the most important source of contamination of RTE foods. Therefore, a study was conducted to devise the rapid enumeration of viable *L. monocytogenes* cells by vPCR in a food processing environment after heat treatment and desiccation on stainless steel with and without biofilm formation. PMA concentration, incubation time and temperature, light source for photoactivation and exposure time, target genes sequence, and amplicon length were optimized to suppress the signal from dead cells. A 50 µM concentration of PMA was used since it appeared non-toxic to cells. A linear correlation was observed between vPCR and plate count enumeration of *L. monocytogenes* when the difference between sanitizer-killed cells and viable cells did not exceed 4 Log CFU. However, vPCR with a short amplicon gave false positive results from cells killed by heat or isopropanol [18].

A long-amplicon vPCR assay with an estimated LOQ of 1.32 Log CFU/reaction correctly indicated that heat treatment at 90 °C for 15 min reduced the *L. monocytogenes* cell number from 8.02 Log CFU/mL to below the detection limit of 1.22 log CFU/mL, whereas a short-amplicon PMA-qPCR assay used for comparison provided false positive results. Viability PCR slightly but not significantly underestimated the viable cell counts, and the membrane destabilization treatment with SDS before PMA treatment did not significantly improve false positive results, while it was toxic for viable *L. monocytogenes* cells. Similar vPCR results were obtained for two strains, *L. monocytogenes* 568 and 08-5578, throughout an experiment of biofilm desiccation, except for the overestimation of the viable cell concentration at day 10 for *L. monocytogenes* 568. At day 10, both strains exhibited a drastic decrease in culturable cells, which was also observed with vPCR but only for *L. monocytogenes* 08–5578 [18].

In an experiment of biofilm desiccation for 8 days at 15 °C and 33% RH, the plate count quantified a reduction in culturable cells of 1.38 Log CFU/cm^2 for the two *L. monocytogenes* strains. The reduction in viable cells measured by long-amplicon vPCR was 1.40 ± 0.09 log CFU/cm^2 for *L. monocytogenes* 08–5578 and 0.71 ± 0.06 log CFU/cm^2 for *L. monocytogenes* 568. Therefore, the survival to desiccation of *L. monocytogenes* 568 was higher for biofilm cells than non-biofilm cells. The DNA extraction procedure was optimized to obtain a reliable cell number determination by the long-amplicon PMA-qPCR [18].

Zolfaghari et al. [42] evaluated the fate of *L. monocytogenes* ATCC 19115 (serotype 4b) in water microcosm and rainbow trout fillets with 0% or 30% NaCl at refrigeration temperature (4 ± 2 °C). The results showed that bacteria in a water microcosm lost their culturability after 13 days with 0% NaCl concentration and after 27 days of incubation in the presence of 30% NaCl. However, bacteria in rainbow trout fillets remained culturable with and without NaCl. RT-PCR targeted on the 16 S rRNA gene was positive for all treatments during the period of the study, indicating the entering of *L. monocytogenes* into the VBNC state in water microcosm with 0% and 30% NaCl. Moreover, VBNC *L. monocytogenes* expressed *hly* and *inlA* virulence genes, so the authors concluded that VBNC *L. monocytogenes* retains pathogenicity [44].

Another study evaluated the ability of *L. monocytogenes* to form VBNC cells in the presence of high NaCl concentrations at different temperatures and to resuscitate under different nutritional conditions. Viability PCR with PMA was used and compared with the Chinese national standard method for *L. monocytogenes* enumeration. Four strains of *L. monocytogenes* were examined including *L. monocytogenes* ATCC 19115 (serotype 4b) and ATCC 19111 (serotype 1/2a). Single colonies were inoculated into TSBYE and incubated until reaching the exponential phase; then, an induction suspension containing around 10^6 CFU/mL of cells from the exponential phase was prepared with pH adjusted

to 5.0. The suspension was aliquoted and stored at −20 °C to ensure comparability among experiments [51].

At 4 °C, the number of culturable bacteria decreased significantly in the presence of 20% NaCl and 30% NaCl, and was below the LOD after 70 days in the 30% NaCl solution. Culturable *L. monocytogenes* ATCC 19115 was below the limit of detection on day 70 in the presence of 20% NaCl, while *L. monocytogenes* ATCC 19111 and MRL300007 decreased until day 21 or 49 and stabilized later. At 25 °C, a significant decrease in culturable bacteria was observed for all four strains. In particular, *L. monocytogenes* ATCC 19115 at 20% and 30% NaCl and ATCC 19111 at 30% NaCl were below the LOD on day 14. In the presence of 20% NaCl, the loss of culturability occurred on day 21 for some strains, while *L. monocytogenes* MRL300007 lost culturability in the presence of 30% NaCl on day 35 [51].

The VBNC counts of *L. monocytogenes* ATCC 19115 were 4.31 Log CFU/mL at 4 °C and 3.23 Log CFU/mL at −20 °C after 70 days at a high concentration of NaCl, and all cells were in VBNC state. *L. monocytogenes* ATCC 19111 at 20% and 30% NaCl concentration showed a higher number of VBNC cells, 5.08 and 5.38 Log CFU/mL, respectively, compared to the other strains [51].

In the resuscitation experiments, only the strain ATCC 19111 was resuscitated without dilution of the VBNC cell suspension, while all strains recovered in BHI medium from 1000-fold diluted VBNC cell suspensions. Tryptic soy broth (TSB) plus 5% Tween 80 allowed a better resuscitation, except for *L. monocytogenes* MRL300007. TSB added with 2 mg/mL sodium pyruvate also improved resuscitation. Recovery in pasteurized milk at 4 °C was observed only for *L. monocytogenes* ATCC 19115 VBNC cells. Only this strain appeared to enter the VBNC state at 4 °C. When the NaCl stress was removed and pasteurized milk was added in the culture medium, only *L. monocytogenes* MRL300007 did not recover, possibly for the too low number of cells in VBNC state formed during incubation in the stressful conditions [51].

The types of fresh produce implicated in listeriosis outbreaks in the US included cantaloupe, packaged lettuce, stone fruits, and celery [52]. A study regarding the survival of *L. monocytogenes* on fresh-cut products during storage at the temperature values prevailing during commercialization found that for three strains from cantaloupe outbreaks—*L. monocytogenes* FS 2025 1/2b, FS 2030 1/2a, and FS 2061 1/2b—the culturable cells significantly decreased on fresh-cut carrots, fresh-cut pineapple, and fresh-cut celery and broccoli at 4 °C. The populations of *L. monocytogenes* slightly increased on fresh-cut cantaloupe and mango and did not change significantly on fresh-cut apple, cauliflower, romaine lettuce, pepper, and onion [53].

Temperature abuse at 15 °C above the normal storage temperature did not change the overall trends of *L. monocytogenes* survival except for blueberry, on which *L. monocytogenes* showed a lower decline. *L. monocytogenes* population declined at higher rates on fresh-cut carrots, apples, tomatoes, grapes, and avocados while the growth rate on cantaloupe and mango increased. For cauliflower, celery, onion, and romaine lettuce, *L. monocytogenes* growth was observed in temperature abuse conditions, while for fresh-cut broccoli and green peppers, the *L. monocytogenes* populations did not change significantly [53].

Live/dead staining of *L. monocytogenes* cells recovered from carrot showed a ratio of cells with intact membranes comparable to the initial inoculum, indicating that the loss of cultivability of *L. monocytogenes* did not reflect viability. When *L. monocytogenes* recovered from fresh-cut carrot was examined by vPCR with PMAxx, a viable population of 3.84 Log higher than that quantified by selective plating on Day 0 was detected. Moreover, the population determined by plating decreased below the LOD after storage at 4, 10, and 15 °C for 6 and 14 days, whereas vPCR showed a reduction of only 1 Log, indicating the presence of VBNC *L. monocytogenes* remaining after 2 weeks [53].

Ready-to-eat (RTE) foods with a pH lower than 4.4 and water activity lower than 0.92 are considered not permissive for the growth of *L. monocytogenes*, but those lacking the above characteristics can support the growth of *L. monocytogenes* and must be subjected to time/temperature control measures to prevent *L. monocytogenes* development during storage [26]. Raw carrots could be considered a food that supports the growth of *L. monocytogenes* based on pH (6.43–6.7) and water activity (a_w > 0.98) values, but several studies have shown that this food matrix does not support the growth of *L. monocytogenes* and can even suppress its survival under various storage conditions, thus showing intrinsic antilisterial activity by as yet unidentified substances [52].

Strains of the *Listeria* species—*L. grayi*, *L. innocua*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*—exposed to fresh-cut carrots for 15 min in 10% buffered peptone water (BPW) from initial concentrations of about 9 Log CFU/mL showed a recovery range of 6.6–7.3 Log CFU/mL. In addition, *L. monocytogenes* FS2025, a strain associated with an outbreak from cantaloupe, showed a recovery from boiled carrots ranging from 7.1 to 7.6 Log CFU/g against an average 4.7 ± 0.3 Log CFU/g recovery from fresh-cut carrot samples, indicating the inhibition of *Listeria* spp. in fresh carrots [52].

In qPCR without PMAxx pre-treatment rinsates from fresh-cut and boiled carrots contained comparable levels of *L. monocytogenes* cells. Moreover, in the rinsate from boiled carrot, *L. monocytogenes* showed comparable numbers in culture-dependent or culture-independent analysis, while in the fresh-cut carrot rinsate, more than 99% of *L. monocytogenes* cells were non-culturable. On the other hand, vPCR showed similar numbers of *L. monocytogenes* in fresh-cut carrots and boiled carrot rinsates, implying that exposure to fresh-cut carrots could induce the VBNC state in *L. monocytogenes*. Transmission electron microscopy (TEM) showed the formation of mesosome-like structures in cells exposed to the rinsate from fresh-cut carrots, whose role in the formation of VBNC cells should be elucidated [52].

The physicochemical stress factors other than antimicrobials that favored the formation of VBNC cells of *L. monocytogenes* in food or mimicking food stressors are synthesized in Table 2.

Table 2. Physicochemical stress factors that induced the VBNC state in *L. monocytogenes* during real or simulated food production conditions.

Stress Factor(s)	Intensity–Concentration	*L. monocytogenes* Strain	Reference
biofilm desiccation	8 days at 15 °C and 33% RH	568	[18]
starvation	Water microcosm	ATCC 19115 (serotype 4b)	[44]
NaCl	30%	ATCC 19115 (serotype 4b)	[44]
NaCl	2–20%	Lm1 (serogroup 1/2a-3a), EGD-e (serotype 1/2a)	[51]
NaCl	20–30% at 4 and −20 °C	Four strains including ATCC 19115 (serotype 4b), ATCC 19111 (serotype 1/2a)	[51]
fresh-cut carrots		FS 2025 serotype 1/2b, FS 2030 serotype 1/2a, FS 2061 serotype 1/2b (cantaloupe outbreak)	[53]
Raw carrots		FS2025 (serotype 1/2b cantaloupe outbreak)	[52]

5. Ultrastructural, Molecular, and Transcriptomic Changes in *L. monocytogenes* VBNC Cells

A thorough description of the physiological characteristics of *L. monocytogenes* VBNC cells is available for those induced by starvation in water. In the study of Carvalho et al. [21], the *L. monocytogenes* EGDe, serogroup 1/2a, was incubated for 28 days in commercial mineral water with low mineral content and quality-controlled composition at initial concentrations ranging from 10^9 to 10^6 cells/mL. Culturability decreased at a higher rate

and extent at a lower starting bacterial concentration of less than 1 CFU/mL at day 28, while with an initial concentration of 10^8 cells/mL, a reduction of 2 log culturable cells/mL occurred. FC carried out by staining the viable cell population with CFDA showed that the total and viable cell numbers remained nearly unchanged. Moreover, the ATP cell content, which is a marker of cell viability, declined after 7 days and increased later, reaching levels higher than those expected from the culturable cells from day 21, thus indicating a contribution from the VBNC forms [21].

The use of *L. monocytogenes* cells expressing the Green fluorescent protein (GFP) allowed observing the formation of coccoid cells, sometimes near rod-shaped structures resembling empty cell walls, which increased in number from day 7 to day 28. The coccoid cells did not stain as Gram-positive because of the peptidoglycan loss that was complete on day 28, as shown by Ultra-High-Performance Liquid Chromatography (UHPLC) analysis [21].

Differential staining of the cell wall with a fluorescent lectin able to bind teichoic acids and of the cell membrane with an antibody specific for *L. monocytogenes* of the 1/2 serogroup showed that rod-shaped cells, observed by cryogenic electron tomography (cryo-ET), presented cell wall gaps from which spherical protoplast egressed. This phenomenon was also observed for three other *L. monocytogenes* strains and other *Listeria* sensu stricto species, *L. ivanovii*, *L. innocua*, *L. marthii*, *L. seeligeri*, and *L. welshimeri*, thus suggesting that this is a common mechanism in *Listeria* species [21].

Known and putative autolysins, namely, *lmo0394*, *p60*, *aut*, *lmo1215*, *lmo1521*, *lmo2522*, *ami*, and *namA*, were strongly downregulated after 7 days. Therefore, the cell wall degradation was probably carried out by a pre-existing pool of autolysins. However, 90% of NamA-deficient mutants of *L. monocytogenes* EGDe still maintained the cell wall at day 14, compared to 44% of the wild-type. A similar behavior was observed in deletion mutants of the *secA2* component of the Sec system ATPase that mediates the secretion of the NamA autolysin [21].

Gas chromatography coupled with mass spectrometry highlighted a decrease in the relative abundance of anteiso- branched-chain fatty acid species (a-BFA) a-C15:0 and a-C17:0, key regulators of membrane fluidity, and an increase in linear saturated (SFA) and unsaturated (UFA) fatty acids between day 0 and day 28. The use of a ratiometric probe that detects local membrane fluidity changes suggested a decrease in membrane fluidity during the first 14 days. Diffusion of the fluorogenic dye Nile red detected by total internal reflection fluorescence correlation spectroscopy (TIR-FCS) showed a reduction in the diffusion coefficient in rod-shaped cells between 7 and 14 days, the period of most severe cell wall damage, while coccoid cells showed a similar diffusion coefficient after 7 and 14 days, indicating an adaptation to a wall-less status [21].

Transcriptome analysis showed the downregulation of genes associated with nucleotide and coenzyme biosynthesis, transcription regulation, uptake of phosphate and some carbohydrates, cell envelope biosynthesis and maintenance, cell division, energy production, and protein secretion at day 7. Upregulated genes included those encoding amino acid metabolism; uptake of glucose, mannose, and metal ions; protein translation and folding; transport of carnitine/glycine betaine compatible solutes, which protect from osmotic stress; and glutathione metabolism, which protects from oxidative stress. Moreover, nearly half of the regulon controlled by the stress-response sigma factor SigB was induced. However, a SigB-deficient *L. monocytogenes* EGDe mutant became VBNC faster than the wild-type, indicating for SigB a modulation rather than an essential role for survival during starvation. The genes encoding the synthases *relA*, *relP*, and *relQ* of the alarmone guanosine pentaphosphate ((p)ppGpp) activator of the stringent response were not upregulated but a Δ*relAPQ* strain switched to the VBNC status faster than the wild-type, indicating that the stringent response plays a role in the early phase of VBNC formation [21].

In another study on VBNC cells generated by SAEW treatment, global proteome analysis with tandem mass tags (TMT) labeling showed that RpsZ, RpmI, Lmo1306, BetL, Lmo2564, RplX, RpmD, RplT, RplO, RnpA, and Lmo0208 associated with ribosomal functions were significantly upregulated in VBNC cells, while proteins Lmo1608, FloA, TrpD, GadB, and GlyS related to aminoacyl-tRNA biosynthesis were significantly downregulated. Therefore, it was suggested that protein synthesis was initiated but peptide chain elongation was impeded. A down-regulation of LuxS in VBNC *L. monocytogenes* was observed that might indicate the inactivation of autoinducer-2 (AI-2) mediated quorum sensing (QS) and consequent inhibition of processes such as biofilm formation, production of virulence factors and toxins, motility, and drug resistance [32].

The comparative transcriptome profile showed that upregulated genes in $FeSO_4$-induced VBNC cells were significantly more numerous than in the control group. Significantly enhanced processes included stress response and ribosome binding with upregulation of transcription factors *rpoC*, *rpoB*, and *rpoD*; genes associated with the VBNC state formation *lmo0668*, *trxA*, *trxB*, and *lmo0593*; and stress response genes *lisK*, lmo0539, lmo1502, lmo0521, lmo0913, rsbX, spxA, metK, tig, and *htrA* [19].

Table 3 summarizes the upregulated and downregulated gene clusters and proteins in VBNC cells of *L. monocytogenes* induced in different conditions.

Table 3. Upregulated and downregulated gene clusters and proteins in VBNC cells of *L. monocytogenes*.

VBNC Inducing Condition	Gene(s) or Protein(s)		Reference
	Upregulated	Downregulated	
Starvation	amino acid metabolism; uptake of glucose, mannose, and metal ions; protein translation and folding; transport of carnitine/glycine betaine compatible solutes; glutathione metabolism; half SigB regulon	autolysins *lmo0394*, *p60*, *aut*, *lmo1215*, *lmo1521*, *lmo2522*, *ami*, *namA* nucleotide and coenzyme biosynthesis, transcription regulation, uptake of phosphate and some carbohydrates, cell envelope biosynthesis and maintenance, cell division, energy production, protein secretion	[21]
SAEW exposure	proteins associated with ribosomal functions RpsZ, RpmI, Lmo1306, BetL, Lmo2564, RplX, RpmD, RplT, RplO, RnpA, Lmo0208	aminoacyl-tRNA biosynthesis proteins Lmo1608, FloA, TrpD, GadB, GlyS, quorum sensing activator LuxS	[32]
$FeSO_4$ exposure	transcription factors *rpoC*, *rpoB*, and *rpoD*	*lmo0668*, *trxA*, *trxB*, *lmo0593*, response genes *lisK*, *lmo0539*, *lmo1502*, *lmo0521*, *lmo0913*, *rsbX*, *spxA*, *metK*, *tig*, and *htrA*	[19]

SAEW, slightly acidic electrolyzed water.

6. Conditions for VBNC *L. monocytogenes* Resuscitation

The process of resuscitation is defined as the conversion of non-culturable cells into culturable cells without any change in cell numbers [14]. The resuscitation of VBNC *L. monocytogenes* was found to occur at relative humidity close to 100%, but not in a dry environment and possibly on cheese rind, but this was not unequivocally demonstrated. Regain of virulence was excluded by an experiment with human colon cells (HT-29) and in a murine model, but pre-incubation in embryonated chicken eggs restored pathogenicity. Models of VBNC *L. monocytogenes* formation in human epithelial cells have suggested that subpopulations of unculturable cells regained active growth [34].

Some studies used conventional culture media to demonstrate resuscitation of VBNC cells, such as TSAYE and TSBYE, BHI broth, TSB added with pyruvate or Tween 80, pasteurized milk, and half Listeria Fraser broth supplemented with 0.1% sodium pyruvate with long incubation times [14,19,20,51]. However, in one study, resuscitation of chlorine and PAA-treated *L. monocytogenes* did not occur in TSB, TSB supplemented with 0.3% of sodium pyruvate or 100 U/mL of catalase, and synthetic medium M9 salts (Becton Dickinson) supplemented with 0.4% glucose and 0.5 mM of each of the 20 amino acids previously reported to stimulate resuscitation. In addition, cell-free supernatants from logarithmic phase cultures were tested as resuscitation media without success [47].

Resuscitation of VBNC *L. monocytogenes* induced by disinfectants was obtained by incubating 1 mL of lettuce homogenate in BPW in 9 mL of half Listeria Fraser broth, supplemented with 0.1% of sodium pyruvate, incubating for 20 h at 37 °C and then transferring 100 µL of the culture to 9 mL of Fraser supplemented with 0.1% sodium pyruvate incubated for 24 h at 37 °C. Samples were considered positive when the medium turned black. To confirm resuscitation, positive samples were streaked onto agar *Listeria* Ottaviani Agosti (ALOA) and OCLA, and at least two typical *L. monocytogenes* colonies were tested by *hly* PCR after DNA extraction by boiling [20].

It was also reported that nutrient supplementation in culture media and non-embryonated chicken eggs did not allow the resuscitation of the VBNC forms of *L. monocytogenes* EGDe generated by prolonged starvation in water. Instead, inoculation into embryonated chicken eggs of the isogenic GFP-expressing VBNC variant exhibited growth two days after inoculation. The resulting cells showed the presence of a cell wall, were rod-shaped, and could infect human trophoblasts (JEG-3) and hepatocytes (HepG2) after 6 h of contact with polymerization of host actin tails as efficiently as vegetative cells. This demonstrated that VBNC forms of *L. monocytogenes* with prolonged survival under nutritional limitation can revert to infectious cells [21]. Regaining of virulence in animal models was observed in some studies [19]. The conditions that led to VBNC *L. monocytogenes* resuscitation and the induction stress factors are shown in Table 4.

Table 4. Conditions for VBNC *L. monocytogenes* resuscitation and inducing stress factors.

VBNC State Inducing Stress Factor	Cultivation Condition *	Reference
Exposure to PAA and AA	TSAYE or TSBYE	[14]
Exposure to $FeSO_4$	BHI broth	[19]
Sanitizers in PWW	Half Fraser broth supplemented with 0.1% (*w*/*v*) sodium pyruvate	[20]
Starvation in water	embryonated chicken eggs	[21]
Exposure to 20% and 30% (*w*/*v*) NaCl at low temperatures	TSB with added 2 mg/mL sodium pyruvate or 5% (*v*/*v*) Tween 80 or 0.6% (*w*/*v*) yeast extract, BHI, pasteurized milk	[51]

* All incubations were carried out at 37 °C; PWW, fresh produce process wash water; PAA, peroxyacetic acid; AA, acetic acid; TSAYE, tryptic soy agar added with yeast extract; TSB, tryptic soy broth; TSBYE, TSB added with yeast extract; BHI, brain hearth infusion broth.

Resuscitation factors (Rpfs), initially identified in the bacterium *Micrococcus luteus* are involved in the process. In dormant cells, peptidoglycan has a low turnover and its degradation is necessary to reinitiate active growth. An Rpf at a certain pM concentration can increase the number of dormant cells by at least 100-fold. Proteins similar to Rpfs are widely distributed among high G+C Gram-positive bacteria, such as corynebacteria, mycobacteria, and streptomycetes, and in some firmicutes. Rpfs have been extensively employed to promote bacterial growth or resuscitation and isolate species that are difficult to

culture. However, their mechanism of action is not completely elucidated. *L. monocytogenes* encodes two Rpf orthologs, Lmo0186 and Lmo2522, that comprise a lytic transglycosylase domain and exert muralytic activity on crude preparations of *L. monocytogenes* cell walls. These enzymes stimulated growth in minimal media but have not yet been tested for the ability to resuscitate VBNC cells [34].

7. Discussion and Future Perspectives

From the recent scientific studies, it emerged that most investigations were aimed at identifying the stress factors that induce *L. monocytogenes* VBNC formation in conditions mimicking those existing in food and food production plants, especially analyzing the effects of the most common sanitizers [20,29,37,47]. The evidence that four of these, i.e., chlorine, PAA, BC, and ClO2, led to the formation of VBNC populations appears particularly worrisome for the consequences that can arise from the presence of viable *L. monocytogenes* in RTE products that go undetected by the standard culturing procedures. Those findings indicated that the VBNC issue should not be ignored and that the highly sensitive molecular methods of VBNC cell detection, such as FC coupled with viability staining, vPCR, isothermal amplification methods coupled with DNA intercalating dyes, and mRNA detection, should be optimized and validated to develop shared procedures for routine application together with the standard methods for the control of this pathogen.

In particular, the methods based on mRNA detection, which are those requiring a minimum number of phases compared to the DNA-based methods, were applied only in a few instances and have not yet been compared to the DNA-based methods for the sensitivity and specificity parameters [41,43,44]. In addition, a highly sensitive detection method not prone to the presence of amplification inhibitors—namely, Droplet digital PCR (ddPCR)—though being widely used to detect foodborne pathogens with low concentration in samples, has not yet been applied for the detection of VBNC *L. monocytogenes*. In this technique, the PCR mixture is divided into nanodroplets, and the target DNA copy number in the sample can be absolutely quantified according to the number of positive droplets without the need for a standard curve [28]. The application of ddPCR coupled with PMA treatment has already been tested with *Salmonella* Typhimurium with promising results in terms of sensitivity [54].

Beyond mRNA, rRNA precursors (Pre-rRNA), which account for more than 25% of total rRNA in the cells, are used as targets in a technique named molecular viability test (MVT). In this technique, Pre-rRNAs are detected when pathogens are growing actively. The quantification of viable cells is achieved on the basis of quantification cycle values produced by RT-qPCR before and after nutritional stimulation. MVT successfully detected viable *L. monocytogenes* and other pathogens in milk [30]. Also, for RNA detection, an isothermal amplification method named nucleic acid sequence-based amplification (NASBA) is available. This method requires three enzymes—i.e., T7 RNA polymerase, RNase H, and the Avian myeloblastosis virus (AMV) reverse transcriptase—and two primers—i.e., one including the T7 promoter sequence and one complementary to the target sequence—and a constant temperature of 41 °C. The entire reaction can be completed in 2 h through cycles of reverse transcription, RNA degradation, double-stranded DNA synthesis, and target transcription [30].

Sequencing-based techniques are increasingly being applied to detect foodborne pathogens in microbial communities, also allowing source-tracking in the food industry. Direct metatranscriptome RNA-seq and multiplex RT-PCR were compared in detecting viable *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* in Romaine lettuce and juice extract with nanopore MinION sequencing. Metatranscriptome analysis, which includes the steps of RNA extraction, library preparation, and sequencing, allowed the

defining of the physiological state of the cells by mapping the sequences to metabolic databases (e.g., Kyoto Encyclopedia for Genes and Genomes (KEGG) database), although the sensitivity was lower than for multiplex RT-PCR. Moreover, the technique presents limitations such as a complex sample pre-treatment, the need for enrichment steps, and advanced bioinformatics expertise, requiring improvements for its application to the study of VBNC cell physiology [55].

Limitations of VBNC molecular detection methods are the loss of some bacteria in the pretreatment steps and the interference of background noise. Therefore, IMS with immunomagnetic beads (IMB) conjugated with antibodies to capture target microorganisms was introduced but not applied to detect VBNC cells. The use of bentonite-coated activated carbon (BCAC) instead of IMB to remove DNA inhibitors also proved successful in sensitive detection of bacteria in milk. Other tools used to target foodborne pathogens were streptavidin magnetic beads–dual aptamers with high binding affinity, structural stability, ease of storage, and ease of synthesis in vitro and vancomycin-modified poly-l-lysine magnetic beads [30]. All these strategies should be evaluated in the ability to detect VBNC cells.

Among the natural compounds that attract interest as substitutes for chemical preservatives in food, OVEO, ROEO, and LEO were tested and shown to favor VBNC *L. monocytogenes* induction. Therefore, beyond attempting concentration and exposure time optimization of their usage in different foods to prevent this hazard, other natural compounds need to be tested for the capability to induce VBNC formation in *L. monocytogenes*. An example is luteolin, a flavonoid that showed efficacy in destroying the cell membrane integrity, with an increase in the number of non-viable cells, producing modifications in cell morphology, inhibitory effects on biofilm formation, and enhanced antibiotics diffusion within biofilms. This molecule was found to damage three tested strains of *L. monocytogenes* treated with the MIC (32 to 64 μg/μL), with an increase in non-viable cells identified by confocal laser scanning microscope combined with the Live/Dead BacLight Kit. A 2 MIC concentration of luteolin induced remarkable cell membrane damage, as demonstrated by field emission scanning electron microscopy (FESEM), and an increase in dead cells based on fluorescence staining, thus proving suitable for preventing VBNC formation [56].

Green sanitizer formulations, such as one comprising the natural phenolic compound gallic acid, hydrogen peroxide, and DL-lactic acid, which induced the VBNC state in *L. innocua*, should be evaluated for VBNC formation also in *L. monocytogenes* strains, as well as erythorbyl laurate (EL), which showed promising results in the inactivation of *L. innocua* when coupled with mild heating [57,58]. Other antimicrobials to be tested in the capacity to inhibit VBNC induction are bacteriocins, particularly those inhibiting listerial growth such as nisin, enterocin CRL35, and lactocin [59]. To date, only nisin has been tested for death induction in two *L. monocytogenes* strains in stationary phase by the BD Cell Viability Kit coupled with FC. It was observed that after 4 h of treatment with 75 μg/mL nisin, 25% of the cells were viable for the *L. monocytogenes* A1 strain, a food isolate, but after 24 h, 91.7% of the cells were dead or VBNC/injured. For the collection strain *L. monocytogenes* M5, 16.2% of cells were viable after 4 h and 95.2% of cells were dead after 24 h. Therefore, the capacity of nisin to leave a residual number of VBNC cells should be better investigated [54].

In addition, the effects on VBNC formation of alternatives to heat exposure to improve food microbiological safety should be defined. As an example, high-pressure technology (HPP) or pascalization, a low-temperature treatment of food that improves microbiological safety with minimal effects on the nutritional, functional, and/or sensory properties, and is largely applied to extend the shelf life of many liquid and solid foods at refrigeration temperature, should be tested for induction of the VBNC state in *L. monocytogenes*. Indeed, some studies reported that immediately after HPP with different combinations of pressure

values (200–600 MPa), times of exposure (3–30 min), temperatures (4–45 °C), and storage duration and temperature (14–75 days; 4–37 °C), culturable *L. monocytogenes* was not detected but grew during storage, suggesting a possible VBNC state induction. However, none of those studies utilized appropriate methods to demonstrate this hypothesis [60]. Other physical treatment methods, e.g., radiofrequency and microwave applied to control microorganisms in a variety of products, must still be evaluated for the ability to induce the VBNC status [59].

A few studies reviewed here regarded the detection of VBNC cells of *L. monocytogenes* directly in food and on contact surfaces in food production plants after cleaning and disinfection, highlighting the presence of VBNC cells of the pathogen [17,36,45,48]. The findings strongly suggested that many more food categories and processes still need to be evaluated for the induction of VBNC *L. monocytogenes* and the levels of these forms of the pathogen. Indeed, it is important to note that in a study regarding RTE salad, the number of VBNC cells was higher than the law limit of acceptability for the pathogen at the end of the shelf-life period fixed on the basis of the standard culture method [17]. This result, together with different confirmations that food matrices such as fresh-cut carrots can favor *L. monocytogenes* VBNC formation [52,53], clearly indicated the necessity to use methods alternative to the culture-dependent ones to determine the presence of viable *L. monocytogenes*. An example is given by apples with or without wax coating, on which a decrease in count for three major disease-causing serotypes, 1/2a, 1/2b, and 4b of *L. monocytogenes*, was observed at 1 °C and at room temperature, but it was not elucidated if the reduction was due to death or to the transition to the VBNC state [61].

The strong capacity to form biofilms is a characteristic of some *L. monocytogenes* strains such as *L. monocytogenes* EGD-e, and it is favored by endotoxin proteins for initial adhesion and the SecA2 protein export pathway for structure formation and cell–cell interactions. In food processing environments, *L. monocytogenes* can form mixed biofilms with *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Lactiplantibacillus plantarum*, and *Vibrio parahaemolyticus* that cause food spoilage and cross-contamination. Biofilm formation is one of the main mechanisms for *L. monocytogenes* persistence in food production plants; so, applying effective cleaning and disinfection treatments to destroy and prevent biofilm formation in food manufacturing plants is a necessity. In addition, the presence of VBNC cells in the biofilm was reported [49]. These insidious persistent forms of *L. monocytogenes* can be efficiently contrasted only by adopting multiple hurdles, since physical treatments provide insufficient sterilization effects on biofilms, biological techniques are expensive and poorly stable, and chemical sanitizers induce pathogen tolerance and leave residues [33]. Baicalin, the 7-O-glucuronide flavonoid derived from the roots of *Scutellaria baicalensis* and an inhibitor of AI-2 in biofilm-forming bacteria, and postbiotics, which are metabolites derived from probiotics and showed great potential against biofilm-forming bacteria [62], bacteriophages, or their recombinant endolysins [59], should be tested also against *L. monocytogenes* biofilms and in the prevention of VBNC formation.

The studies analyzing resuscitation came to different conclusions on the regain of culturability by VBNC *L. monocytogenes* even when the same non-selective culture media were used [19,47]. The discrepancies could be due to the different stages of the VBNC state for the cells tested, the different lengths of incubation time, and differences in the medium composition that can depend on the preparation procedures and ingredient quality. Moreover, the resuscitation capacity of VBNC *L. monocytogenes* in foods was little investigated by methodologies unequivocally demonstrating the VBNC status of the inoculated cells, except for a study carried out earlier than the year 2020 [34] and the experiments with pasteurized milk and leafy green by Zhao et al. [51] and Truchado et al. [20], respectively. Thus, further investigations are required on this aspect.

8. Conclusions

This review highlighted that the study of VBNC cell formation in response to environmental factors in *L. monocytogenes* has mainly been devoted to evaluating the effect of common sanitizers in the washing process of RTE salads, while other food sectors have been little considered. Only three studies regarded the natural detection of VBNC *L. monocytogenes* cells in foods during storage, indicating that more food categories known to be possibly contaminated by this pathogen, especially those dedicated to fragile consumers, should be investigated in this respect.

It must be remarked that, to date, no consensus strategies have been established to combat VBNC pathogen occurrence in food, and an improvement of the VBNC cell detection methods is necessary to improve sensitivity and reduce laborious sample pretreatment steps. To this aim, mRNA detection and ddPCR methods deserve particular attention for evaluation and validation in food safety assessment.

Variability of the ability to form VBNC cells among *L. monocytogenes* foodborne strains should be better addressed, and resuscitation methods should be developed to allow the culture-dependent detection of *L. monocytogenes* VBNC cells induced by different stress factors.

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