Review

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Abstract: Many bacteria have the ability to survive in challenging environments; however, they cannot grow on standard culture media, a phenomenon known as the Viable but Non-Culturable (VBNC) state. Bacteria commonly go into the VBNC state under nutrient-poor environments or under stressful conditions. This review explores the concept of the VBNC state, providing insights into the beneficial bacteria known to employ this strategy. The investigation covers different chemical and physical factors that can induce the latency state, cell features, and gene expression observed in cells in the VBNC state. The revision also covered the significance and applications of beneficial bacteria, methods for evaluating bacterial viability, the ability of bacteria to persist in environments associated with higher organisms, and the factors that facilitate the return to the culturable state. Knowledge about beneficial bacteria capable of entering the VBNC state remains limited, however, beneficial bacteria in this state could face adverse environmental conditions, and return to culturable state when conditions become suitable and continue to exert their beneficial effects. Likewise, this unique feature positions them as potential candidates for healthcare applications, such as the use of probiotic bacteria to enhance human health, applications in industrial microbiology for the production of prebiotics, functional foods, and in the beer and wine industry. Moreover, their use in formulations to increase crop yield and for bacterial bioremediation offers an alternative pathway to harness their beneficial attributes.

Keywords: VBNC state; stress; beneficial bacteria; rhizosphere; latency; survival

1. Introduction

Bacteria in VBNC state exhibit a remarkable phenomenon: they are unable to grow and form colonies on conventional culture media, yet they remain alive and able to restart their metabolic activity [1]. Cells in this status typically display reduced levels of metabolic activity, and undergo significant metabolic alterations, such as: reduction of nutrient transport, respiration rate, and macromolecular synthesis [2]. However, a feature that distinguishes VNBC state is the continuous gene expression within these cells [3].

A typical response of cells entering into the VBNC state is shown in Figure 1, under stressful conditions caused by desiccation in presence and absence of a cytoprotective agent such as trehalose. In presence of trehalose, bacterial cells maintain high avoiding entry into the VBNC state. Conversely, in the absence of the protector a decrease in the number of CFUs/mL is observed under the same environmental stress. After 9 DABD (days after the beginning of desiccation), the cells entered into

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the VBNC state and remained so until 18 DABD; which was confirmed using several methodologies [4]. Interestingly, after prolonged rehydration or rapid rehydration in presence of plant exudates these bacteria reached high numbers indicating that they had returned to the culturable state [4].

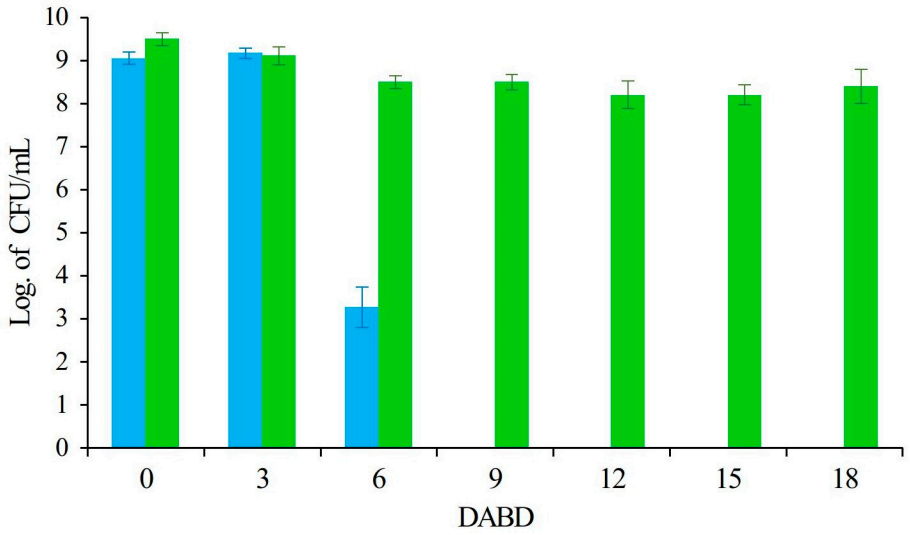

Figure 1. CFU/mL (log) of *Pseudomonas putida* KT2440 under desiccation stress during 18 days after the beginning of desiccation (DABD), in presence (green bars) and absence (blue bars) of trehalose. Adapted from Pazos-Rojas *et al*., 2019 [4].

1.1. Conditions that induce VBNC state

Bacterial cells commonly respond to stressful conditions by losing their ability to form colonies in standard culture media, although cells can remain viable for long periods of time. Cells may enter into the VBNC state in response to natural stresses, such as starvation, extreme temperature, high osmotic or oxygen concentrations, or exposure to white light [1]. Additional stressors that may lead to this status are listed in Table 1. In general, extreme environmental conditions could be lethal unless they adopt a latency status, for example, it is known that one of the most critical factors that plays a crucial role on bacterial survival is the availability of nutrients in the surrounding environment. When bacterial cells are subject to nutrient starvation conditions, they may reduce their size and become more resistant to adverse environmental conditions, alternatively, they could induce a latency estate, forming viable but non-culturable cells or resistance structures such as spores [3]. However, spore-forming bacteria are typically not classified within the VBNC state literature [3]. It is interesting that not only environmental stress can induce the VBNC state, various processes and bactericidal substances are able to induce this state as well. For example, milk pasteurization [5], wastewater chlorination [2], and use of food preservatives such as potassium sorbate and sodium benzoate [6] have been documented as inducers of the VBNC state.

Table 1. Types of stress that can induce VBNC state in bacteria.

Type of stress		Example of microorganism	Reference
Physical parameters	Temperature	*Vibrio cholerae* incubated in a range of 4 to 6°C	[7]
	Visible solar and UV radiation	*Escherichia coli* in microcosm of river water under visible light irradiation	[8]
	Osmolarity	*E. coli* and *Salmonella typhimurium* in freshwater and seawater	[9]

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Chemical parameters	Organic matter		
	Nutritional effects.	*Streptococcus pyogenes* carbon and phosphorus limitation in culture during stationary phase	[10]
	Humic acids	*E. coli* in presence of humic acids significantly decreases the entrance to VBNC status in fresh water but not in seawater	[9]
Aeration		*Campylobacter jejuni* enters faster into the VBNC state in microcosmos incubated under agitation (3 days), compared to immobile microcosmos (10 days)	[11]
Drying		*Sinorhizobium meliloti* part of the population enters into the VBNC state with 22% humidity	[12]
		P. putida KT2440 enters into the VBNC state under desiccation conditions at 30°C and 50% relative humidity	[4]
Biocidal agents	Hypochlorite (100ppm)	*Legionella pneumophila* was detected in culture medium treated with hypochlorite for 10 minutes	[13]
	Cupper (0.6-1mg/mL)	*E. coli* ETEC in buffered water at 4 °C produced VBNC cells that maintained their enterotoxigenic activity when reactivated.	[14]
	Alum $KAI(S0_4)_2$	*V. cholerae* O1 in artificial seawater with alum, a significant number of cells lost their culturable capacity while keeping viability	[15]
	Quinolone and ciprofloxacin	*E. coli*, exposed to 10-100 times minimum inhibitory concentration (MIC), lost the culturable capacity	[16]

2. Bacterial species entering the VBNC state

A substantial portion of bacterial species known to enter the VBNC includes human pathogens such as: *Campylobacter* spp. *Escherichia coli* (EHEC strains), *Francisella tularensis*, *Helicobacter pylori*, *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, several species of *Salmonella* spp., *Shigella* spp., and numerous pathogens from the genus *Vibrio* sp., being *Vibrio vulnificus*, one of the most studied in terms of the VBNC [2]. The medical significance of this phenomenon is multifaceted [17]. For example, it is suggested that the latent phase of *Mycobacterium tuberculosis* infections is caused by bacteria in the VBNC state [18,19] and the recurrence of disease several years later may be linked to their return to the culturable state within the host [20].

The list of pathogenic bacteria that can adopt the VBNC state as a survival strategy includes not only pathogen affecting humans but also animals such as *Photobacterium damselae* infecting fish [21], *V. vulnificus* eel pathogen [22] and *V. shiloi* that cause bleaching of corals [23,24]. Several plant pathogens have been described, for example, *Ralstonia solanacearum* in tomato plants [25], *Xanthomonas axonopodis* colonizing grapefruit plants [26], *Erwinia amylovora* infecting ripe apples [27], *Pseudomonas syringae* in tomato, cereals, almond, cherry, plum plants, *Acidovorax citrulli* infecting a wide variety of Cucurbitaceae causing bacterial spot disease (Bacterial Fruit Blotch) [28] and *Agrobacterium tumefaciens* which cause tumors in different dicotyledons.

Since the publication of Xu´s *et al*. study [7] more than 30 years ago, a substantial body of research around the world has focused on documenting the occurrence of the VBNC state in different bacterial species [2,4,12,17,29–37].

To date, approximately 101 bacterial species spanning 50 different genera have been reported to exhibit the VBNC phenomenon [2,29,36,38]. In the present review we analyze the beneficial bacterial species where the VBNC status has been reported and the importance of this within their potential in areas such as biotechnology, agriculture, food industry and health.

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3. The VBNC state in beneficial bacteria

Beneficial bacteria play a crucial role in maintaining life on our planet. Some realize nitrogen fixation [39], mineral solubilization [40] greenhouse gas consumption, and are thus viewed as gatekeepers preventing excessive methane emissions from escaping the atmosphere [41]. Due to the several processes in which beneficial bacteria participate, they have been used to increase the crop production [42]. Some beneficial properties of these bacteria include plant growth promotion [43,44], control or inhibition of the activity of plant pathogens [45,46], improvements soil structure, bioaccumulation or microbial leaching of inorganics [47] bioremediation of xenobiotic compounds [48,49], or the production of compounds of industrial interest [50,51].

Certain bacteria that interact with their host establish mutually beneficial relationships. Probiotics for instance, interact with humans to improve overall health. They can eliminate or remove pathogens [52], reinforce the epithelial barrier, induce migration of fibroblasts and epithelial cells [53]. In the immune system probiotics are related to modulation and activation of intraepithelial lymphocytes, natural killer cells, and macrophages through induced production of cytokines [54].

On the other hand, there are beneficial bacteria with industrial applications that favor the production of certain foods, prebiotics and beverages. In the brewing and wine industry, bacteria play a crucial role in the fermentation process. Lactic acid bacteria (LAB) and yeast are instrumental in this context. LAB catalyze the conversion of dicarboxylic malic acid into monocarboxylic lactic acid and carbon dioxide (malolactic fermentation MLF) and yeast convert sugars into alcohol (alcoholic fermentation) [55]. During malolactic fermentation by LAB, no free intermediary products are formed achieving a more palatable wine by reducing the tart taste of malic acid. Additionally, malolactic fermentation reduces the amount of residual nutrients available to support microbial growth, enhances wine aroma, improves microbial stability and reduces the acidity of wine making the wine more stable before being bottled [56].

Despite the importance and benefits of bacteria at different levels, unfortunately, the knowledge about bacteria in the VBNC remains limited. The table 2 show the beneficial bacteria so far reported to enter viable non-culturable state under different conditions. This table displays a variety of conditions that can induce the VBNC state. Factors such as temperature, solute concentration, nutrient limitation, pH, desiccation, and industrial processes are determinants for the formation of VBNC cells. The groups of bacteria with more beneficial species capable to enter VBNC state are Alpha-proteobacteria and Gamma-proteobacteria with nine species each one. The groups with only three species that enter VBNC sate are Actinobacteria and Firmicutes. In the case of Betaproteobacteria only one species has been reported to enter the VBNC state.

Table 2. Main species of beneficial bacteria that enter the Viable non-cultivable state.

Group of bacteria		Species	Conditions that induce VBNC state	References
Proteobecteria	*Alfaproteobacteria*	*Acetobacter aceti*	Treatment with SO_2 at a concentration of 30 and 50 mg/L	[57]
		Acetobacter pasteurianus	High acid stress during fermentation	[58]
		Methylosinus sporium	Freeze drying and Cryopreservation (liquid nitrogen)	[36,41]
		Methylosinus trichosporium		
		Methylocystis hirsuta		
		Methylocystis parvus		
		Methylocella tundrae		

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	Rhizobium leguminosarum	Cupric sulfate to a concentration of 60 ppm	[59]
	Sinorhizobium meliloti	Incubating at 25°C in tap water from The University of North Carolina at Charlote and tap water from The University of Maryland Biotechnology Institute, Center of Marine Biotechnology. Incubating under anoxic conditions in liquid microcosms Incubation in nitrocellulose filters at a relative humidity of 22% for three days at 20°C in the dark.	[12,60,61]
Betaproteobacteria	*Cupriavidus metallidurans*	Incubation into the artificial soil at 30 °C for 12 days, without any C source or H_2O	[60]
Gamaproteobacteria	*Methylomonas methanica*	Lyophilization and Cryopreservation (liquid nitrogen)	[36,41]
	Methylosarcina fibrata		
	Methylocaldum gracile		
	Methylomicrobium alcaliphilum		
	Methylococcus capsulatus		
	Microbulbifer aggregans	Incubation in modified artificial seawater (ASW) by 4 h at 30°C	[61]
	Pseudomonas fluorescens	Incubation in saline solution (NaCl 0.9% w/v) at 37°C	[62]
	Pseudomonas putida KT2440	Desiccation at 30°C and 50% relative humidity	[4]
	Vibrio fischeri	Incubation at 22°C in nutrient-limited artificial seawater (ASW)	[63]

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Terrabacteria	*Actinobacteria*	*Bifidobacterium animalis* subsp. *lactis*	1. Storage in fermented foods 2. Refrigerated storage of butter for 4 weeks 3. Microcapsules with full-fat goat milk and inulin-type fructans	[64–66]
		Bifidobacterium longum	Storage in fermented foods	[64]
		Arthrobacter albidus. reclassified as *Sinomonas albida*	absence of proteins Rpf (resuscitation promoting factor) in the culture medium	[65,66]
	Firmicutes	*Bacillus coagulans*	Incubation at pH 2 for 24 hours and subsequent incubation at 140°C for 5 min	[67]
		Lactobacillus plantarum	Treatment for 30 min at 100 °C or with 1 mol/L HCl Incubation in beer at 0°C temperature	[31]
		Oenococcus oeni	Sulfur dioxide and histidine decarboxylase activity in wines	[57]

This review analyzed the phylogenetic relationship between different beneficial bacterial species reported to enter the VBNC state (Figure 2). The sequences were analyzed by comparing them with the National Center for Biotechnology Information (NCBI) database. To evaluate viable but unculturable strains, phylogenetic trees were constructed by the Neighbour-joining method [68] using ClustalX, Bioedit and Mega 4 software. A bootstrap confidence analysis was applied on 1000 replicates to determine the reliability of the topology obtained [69]. The phylogenetic tree show that these bacteria are very diverse. The furthest phylogenetic group corresponds to *Methylosinus*, *Methylocystis* and *Methylocella*. The genus *Rhizobium*, *Sinorhizobium* and *Acetobacter* are groups that are phylogenetically closer to each other. *Methylococcus*, *Methylocaldum*, *Methylomicrobium*, *Methylotuvimicrobium*, *Methylosarcina* and *Methylomonas* are phylogenetically close with *Vibrio*, *Microbulbifer*, and *Pseudomonas*. Another group that can be identified is formed by *Arthrobacter*, *Bifidobacterium*, *Bacillus*, *Lactobacillus*, and *Oenococcus*, with some strains being phylogenetically closely related and others very distantly species. When analyzing the phylogenetic relationships, it can be observed that viable non-cultivable state is not exclusive to any taxonomic group or group of species, and it is a widely distributed strategy in phylogenetically close and distant species.

The beneficial species in which the viable non-culturable state has been reported are described below, highlighting their main applications in different areas such as biotechnology, agro-biotechnology, health and industrial applications.

Within the group of alpha-proteobacteria that enter the VBNC state are organisms belonging to genus: *Acetobacter*, *Methylosinus*, *Methylocistis*, *Methylocella*, *Rhizobium* and *Sinorhizobium* (Table 2).

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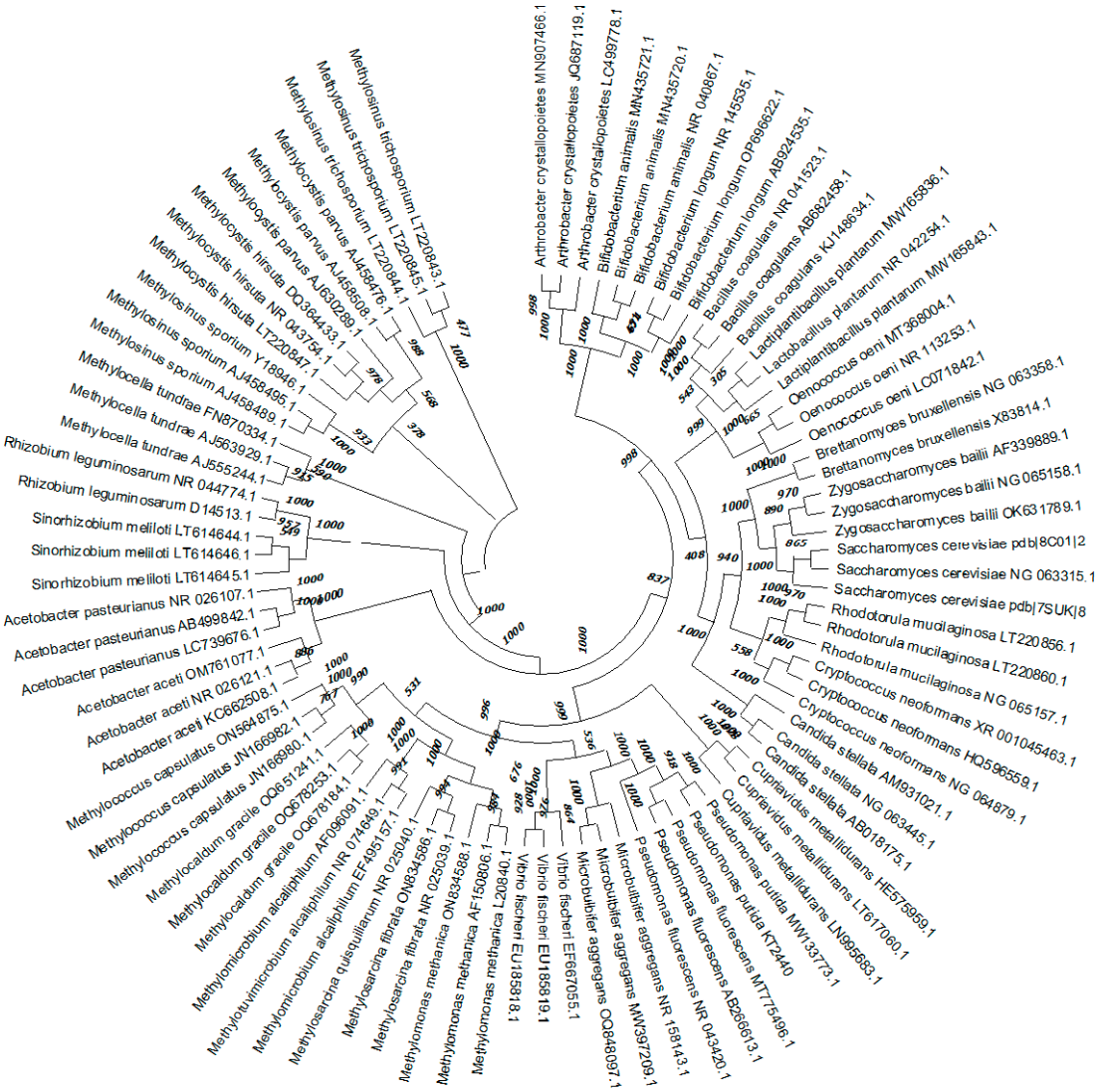

Figure 2. Phylogenetic tree of beneficial bacterial species entering the VBNC state. Phylogenetic trees were constructed by the Neighbour-joining method [68] using ClustalX, Bioedit and Mega 4 software. A bootstrap confidence analysis was applied on 1000 replicates to determine the reliability of the topology obtained.

Acetobacter aceti and *Acetobacter pasteurianus* have a great relevance in vinegar production, since can transform ethanol into acetic acid through oxidative fermentation [70]. The VBNC state in A. aceti has been documented in wine production [57] and *A. pasteurianus* enters to VBNC state under high acid stress generated during the fermentation process [58]. Studies are still lacking that could support the idea that *A. aceti* and *A. pasteurianus* under a non-culturable state can follow the fermentation process, which would help increase production. The ability of these species to persist under adverse conditions represents a challenge to develop novel ways to improve industrial yields, enhancing wine quality and driving vinegar production on a larger scale.

The methylotrophic bacteria *Methylosinus sporium*, *Methylosinus trichosporium*, *Methylocystis hirsuta*, *Methylocystis parvus* and *Methylocella tundrae* are methanotrophic microorganisms also called methane-oxidizing bacteria (MOB) capable of generating energy through the oxidation of methane gas [71]. MOB have different biotechnological applications mainly, biological mitigation of methane greenhouse gas, production of high-value products from methane, and bioremediation of pollutants [72,73]. Because of ecological importance of these microorganisms, it is important understand how different conditions can affect methane consumption. One of the conditions that induces the VBNC state in MOB is freeze-drying and cryopreservation (Table 2). This represents a challenge to

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researchers, because culturable cells are needed whenever beneficial application. Most studies focus on how to avoid loss of cultivability, but is likely that MOB can return to culturable state by being in an environment with methane gas. This have observed with different pathogen species of bacteria that, when find in favorable conditions, leave from the non-culturable state [38]. Research with this group of bacteria in the VBNC state is scarce, which represents a challenge to understand this survival strategy. In the future, this could stimulate these organisms to consume methane, aiding in the degradation of pollutants and production of high-value biomass.

Other species of alphaproteobacteria are *Sinorhizobium meliloti* and *Rhizobium leguminosarum* found mainly in the soil, that have acquired, by horizontal gene transfer, the ability to associate in symbiosis with leguminous plant roots. The association of rhizobia and legumes occurs through a complex signaling process that generates nodules, organs specialized in the fixation of atmospheric nitrogen [74]. Biological nitrogen fixation is a vital process in agriculture, allowing the production of nitrogen through legume-rhizobium symbiosis that contributes to increased nitrogen levels in the soil, resulting in increased plant growth [75]. In addition to the ability to fix nitrogen, *R. leguminosarum* isolated from fava bean root nodules can generate induced systemic response against infection by Bean yellow mosaic virus [76]. The VBNC state in this bacterium is related with presence of cupric sulfate (Table 2). For *S. meliloti* the factors induce VBNC state are temperatures of 20 °C to 25 °C, incubation under anoxic conditions and incubation in nitrocellulose filters at low relative humidity (Table 2). It is interesting that under these conditions the bacteria can persist. This could explain their survival capacity under this types stress in environment. The importance of rhizobia for agriculture represents a major challenge to learn more about their beneficial functions in VBNC state, because it is unknown whether when bacteria are under non-culturable state they can continue performing nitrogen fixation, generate induced systemic response or even develop nodules.

In the group of Betaproteobacteria, the only beneficial bacteria where the VBNC state has been documented is *Cupriavidus metallidurans*. This bacterium is a metallophilic found in environments containing high concentrations of heavy metal, industrial wastes rich in toxic heavy metals, often mixed with recalcitrant organic compounds and hydrocarbons [77]. It is an ideal bacterium for bioaugmentation purposes in environmental applications due to strong resistance to environmental stress factors and its adaptation capacity [78]. Another peculiar application is its ability to synthesize gold of 24-caratgold in one week from gold chloride [79]. These observations suggest that bacteria actively contribute to forming gold grains in surface environments [80]. With this bacterium, the conditions that can induce its loss of cultivability have been studied, but also the conditions that can return to cultivable state. The addition of water and gluconate being sufficient for *C. metallidurans* to be culturable in 24 hours [60]. Due to its interesting applications and ability to persist in toxic environments, it is an excellent model for studying the mechanisms for coping with heavy metal stress. This unique ability to metabolize toxic substances and enter the VBNC state without water and carbon sources could help to understand how the origin of life occurred.

One of the groups with the most beneficial species in which the VBNC status has been demonstrated is Gammaproteobacteria. *Methylomonas methanica*, *Methylosarcina fibrate*, *Methylocaldum gracile*, *Methylomicrobium alcaliphilum* and *Methylococcus capsulatus* are microorganism methylotrophs. This type of bacteria uses methanol and methane as only carbon source [81]. Methylotrophs can habit in soil, water and plants, powering the carbon cycle [82]. When M. fibrate is co-inoculated with species of *Methylomonas* and *Cupriavidus taiwanensis* LMG 19424, its growth is highly stimulated [83]. Also, methane oxidation is elevated when methanotrophs interact with algae and moss [84]. Cryopreservation has been one method that induces the VBNC state in methanotrophs (Table 2). The study of VBNC state in methanotrophs is essential due to its role in the carbon cycle and therefore in the planet's life. It is relevant to consider that interaction with others microorganisms or whit plants could be an alternative for these microorganisms to return to the cultivable state, being able to continue exerting their beneficial effects.

Microbulbifer aggregans, other Gammaproteobacteria is a halophilic, Gram-negative bacterium isolated from sediment in the Matang mangrove forest, Malaysia [85]; its importance is centered in its capability to reduce sulfur by the presence of genes involved in this process [61]. Sulfur is an

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essential element for life, is present in amino acid, proteins, enzymes, vitamins and other biological molecules [86]. *M. aggregans* is a little explored bacterium, recently, it was observed that non-culturable cells exhibit a change in cell shape from rod to coccus, and the genes responsible for sulfate reduction are upregulated in the VBNC state [61]. These findings demonstrate its importance in the environment where it lives, because effective reduction of sulfur in non-cultivable state may occur in response to changes in the sulfur concentration in the environment where it is found, playing a relevant role in the sulfur cycle in marine environments.

Within the Gammaproteobacteria there is also the genus *Pseudomonas*. The species of this genus have a great capacity to use different nutrients as carbon source, which explains their ubiquity. Their enzymatic activity makes them an important group of microorganisms responsible for the aerobic degradation of many compounds in different ecosystems [87]. Some species *Pseudomonas* plant associated promote plant growth by suppressing pathogenic microorganisms, synthesizing growth stimulating plant hormones and promoting increased plant disease resistance [88]. *Pseudomonas fluorescens* and *Pseudomonas putida* KT2440 are bacteria able to colonize plants' root and promote their growth. Some strains of *P. fluorescens* have been shown to degrade a variety of organic compounds, thus important in bioremediation [89]. This bacterium is used for biocontrol to protect plants against soilborne fungal pathogens. One mechanism for the biocontrol by *P. fluorescens* is the ability to produce antibiotics [90]. Also stimulates the systemic resistance induced (ISR) [91] and production of volatile compounds [92].

On other hand *P. putida* KT2440 is a bacterium capable of using different aromatic compounds as a carbon source [93], metabolizing xenobiotic compounds, it can colonize the roots of plants such as corn, wheat, strawberry, sugarcane, sugar and spinach [94] and is capable of promoting the growth and health of the plants [95,96]. *P. putida* KT2440 has been used as part of consortia bacteria formulations to enhance plant growth [96,97].

The potential of *P. putida* KT2440 and P. fluorescens to exert its beneficial effects can be affected by their exposure to different stress that induce the VBNC state, such as saline environments for *P. fluorescens* and desiccation for *P. putida* KT2440 (Table 2). However, it has been shown that plant-bacteria interaction is a mechanism that allows these bacterium return to the cultivable state. Therefore, its use in formulation of stable bacterial inoculants that can stimulate plant growth after rehydration [4], or the development of formulations to perform biological control [34] could be an alternative to reduce the overuse of nitrogen fertilizers and pesticides, decreasing the damage caused by those chemical products [98].

Vibrio fischeri, also belonging to the Gammaproteobacteria, is a luminous marine bacterium that lives free or in symbiosis with different species of fish and squid [99]. The most studied interaction in this microorganism is the symbiosis with the Hawaiian squid, *Euprymna scolopes*, inducing bioluminescence that the squid uses to avoid predation during nocturnal activity [100]. In the squid-vibrio symbiosis, bacteria found in a ventral tissue called lumen organ. The relationship between *V. fischeri* and *E. scolopes* is characterized by daily rhythmic cycles that control the population dynamics of bacteria. At night, when squid take feed, the light organ fills with bioluminescent *V. fischeri*. At dawn, the squid bury themselves in the sand and ventilate approximately 90% of bacterial population to environment, the remaining bacteria repopulating the crypts and are ready to produce light at dusk [101]. This example of symbiosis shows beneficial microorganisms' important role in their hosts' health and activities. The role of the VBNC state in *V. fischeri* free in marine environment or symbiosis is still unknown. It has only been discovered that non-culturable cells lose their luminescence in response to fluctuations in salinity [63]. The loss of luminescence could have an application for the diagnosis of marine environments allowing know alterations in salinity, temperature variety and concentration of nutrients relating the number of luminescent bacteria of *V. fischeri*, interpreting that the less luminescent bacteria, the disturbance present in marine environment is greater.

The actinobacteria group includes *Arthrobacter albidus*, reclassified as *Sinomonas albida* is an that was isolated from a seep substrate made of volcanic rock from Niigata, Japan [102]. Among the main applications of this microorganism, it has been demonstrated that it leaves the VBNC state in the presence of RpF proteins, has an efficient flocculant activity [65]. This flocculent activity represents a

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possible application in wastewater treatment and residual sludge dewatering. Until now, little is known about this species, which represents an alternative and challenge to take advantage of the biotechnological properties that *S. albida* can provide.

The presence of bacteria in the human digestive tract has various properties related to health, such as regulation of intestinal microbial homeostasis, inhibition of pathogenic bacteria, modulation the immune response, anticancer effects, production of bacteriocins or bioconversion of diet components in bioactive compounds [103]. Recently, production and consumption of products with beneficial strains for human health has increased considerably [104]. *Bifidobacterium longum* and *Bifidobacterium animalis* subsp. *lactis* are multifunctional probiotic Actinobacteria clinically effective as immunomodulatory, anti-inflammatory, antimutagenic, anticancer and alleviation of gastrointestinal diseases [104]. In both species it has been observed that their storage at low temperatures in fermented foods can cause loss of viability (Table 2). In this respect, determination of the viability and activity of probiotic bacteria is of great economic, regulatory and technological importance to ensure that fermented products or formulations with probiotics carried bacteria capable of exerting their beneficial effect.

Another probiotic, *Bacillus coagulans* is a Firmicute considered safe. This bacterium has the ability to endure high temperatures and genetic stability through several years of commercial production [105]. Its main benefits to human health include modulation of gastrointestinal disorders, immune system stimulation, and lowering cholesterol [106]. This bacterium can form endospores and survive during decades in unfavorable environmental conditions. It was previously thought that their high persistence was due only to ability to form spores, however, it has been observed that *B. coagulans* can enter the VBNC state as a strategy to face adverse conditions [67]. Little is known about the return to viability when humans consume these beneficial microorganisms. Some studies, mainly with pathogens, have shown that bacteria secrete certain specific proteins to come out of the latency state [107], which could be a possibility for the case of *B. longum* and *B. animalis*, this represents a challenge for future research. If we consider that some beneficial bacteria are capable of returning to the culturable state when they interact with their host [4] is very likely think that, although culturable bacteria are not detected in fermented foods, when they are consumed by humans, conditions present in these host may favor its return to cultivable state, being able to exercise its beneficial properties.

Lactobacillus plantarum is a firmicute widely distributed in various environments such as the gastrointestinal, vaginal, and urogenital tracts, in dairy products, vegetables, meat, hay, and wine. This ability to adapt to different conditions demonstrates its metabolic diversity [108] having potential for various applications. The main applications of *L. plantarum* include fermentation of foods such as cheese, kefir, sauerkraut, fermented meat products, fermented vegetables and beverages [109]. It has been reported that *L. plantarum* produces antimicrobial substances, such as plantaricin [110] and can remove microcystins, the main toxins produced by cyanobacteria [111], which makes it an alternative as a food preservative or fighting infections. Studies about VBNC state in *L. plantarum*, reported that can remain latent during beer storage, which contributes to the deterioration of this beverage [31]. Another study showed that *L. plantarum* in VBNC state can inhibit microcystins, which contributes to the preservation of fermented foods [111]. It is interesting to observe that the VBNC state in this specie can be detrimental for beer production, but beneficial for food preservation, reflecting the versatility of non-cultivable *L. plantarum* to continue performing its functions. This leads us to think that when used as a probiotic to improve human health, it can provide its benefits without being culturable. In short, it is a microorganism that should be studied in greater depth. Mainly what happens during the VBNC state to take advantage of its diversity of applications.

In the production of alcoholic beverages, the involvement of microorganisms is crucial for a successful fermentation. A significant challenge in the brewing and winemaking industry is the spoilage of wine and beer by lactic acid bacteria, which have the ability to enter the VBNC state [31]. *Oenococcus oeni* is a firmicute bacterium, belonging to lactic acid bacteria group, adapted to stressful environment of wine. It is widely used as starter microorganism to carry out malolactic fermentation (MLF), where L-malate is converting to L-lactate [112]. The use of starter cultures of *O. oeni* remains

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difficult in some wine regions, due to the hostile environment created by low pH and the presence of SO_2 and ethanol [113]. The ability of *O. oeni* to respond these stress conditions has great relevance for increasing wine production at lower cost. So far, knowledge about the VBNC status of *O. oeni* is limited. It is known that presence of sulfur dioxide induces the loss of viability, and the addition of arginine to medium allows the return to the cultivable state [112]. Due to findings with *O. oeni*, it is interesting to think that, although bacteria are in VBNC state, they may still be capable of carrying out malolactic fermentation, although perhaps not as efficiently as their cultivable counterparts. When cells are present in a rich environment and added arginine, it returns to the cultivable state, increasing its fermentation efficiency. The generation of new knowledge is challenging, but it also involves changing the paradigm and legislation that require all microorganisms used in food production to be culturable. It is feasible to think that using non-culturable cells would decrease production costs because they would not require an expensive infrastructure to preserve bacteria, since they have all the necessary equipment to preserve themselves.

The VBNC state could be an important reservoir of beneficial bacteria species, as this state constitutes a survival strategy in response to harsh environmental conditions. The capability of bacteria to enter the VBNC state in response to stress could have important biotechnological applications. It is possible consider that VBNC state may reduce negative selection and regulate microbial dominance in soil, as the rhizosphere, where plants could induce a selective bacterial revival by releasing selected organic compounds, directly influencing the diversity present in these environments. In the same way, the hosts themselves could be exerting a selection by releasing compounds that determine the viability of certain bacteria necessary in a given physiological state.

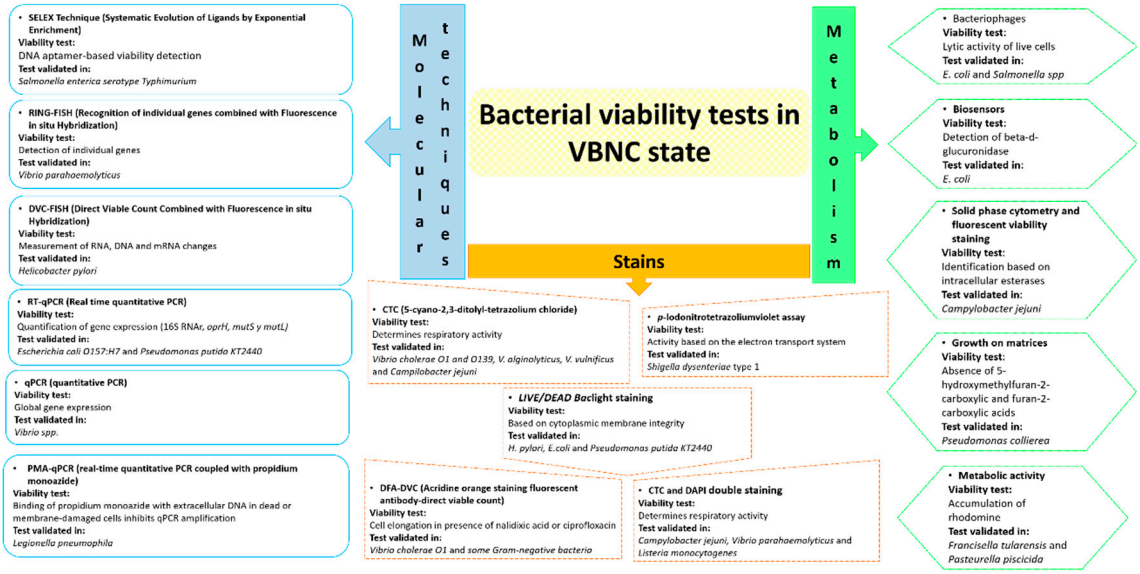

Figure 3. Methodologies to evaluate bacterial viability in the VBNC state.

4. Techniques to evaluate bacterial viability in the VBNC state

One challenge that researchers who study bacteria in the VBNC state face is verifying the viability of these cells. Currently, several methodologies have been reported that can be used to determine the viability of a bacterium (Figure 3). The methodologies are divided into three groups: molecular techniques, techniques focused in metabolism and staining techniques. The molecular techniques are based on detecting individual genes or individual and global gene expression in non-culturable cells. According to the literature, the use of transcripts is an excellent alternative, given that mRNA half-lives are typically in the range of seconds to minutes [114,115] and the identification of the mRNA is evidence that cells remain metabolically active. In addition, its detection providing an essential insight into factors that may be regulated the VBNC sate. The techniques focused in metabolism search metabolites or enzymatic activity, generally used methodologies with colorimetric results, biosensors or matrices. The staining techniques mainly are based in detect activity in electron

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transport system and integrity of cytoplasmatic membrane. Cellular respiration allows quickly identifying metabolically activity cells, only using a compound fluorescent or a compound that reacts and forms a fluorescent compound. The BacLight® Live/Dead kit, is a method frequently used in which, by differential staining and fluorescence microscopy, bacteria in the VBNC state stain green indicating that they have largely intact membranes and thus can be considered to be alive [12]. However, in the case of bacteria during desiccation stress, membranes suffer an apparent sublethal damage during the VBNC state and they stain red [4]. Is important to mention that there is not a decisive test for bacterial viability in the VBNC state, and it is usually proposed that, when trying to prove this fact two or more methodologies should be performed.

5. What happens during the VBNC state?

There are different characteristics shown by cells in the VBNC state. Several bacterial species decrease their size, producing several metabolic changes, including depletion of energy reserves, altered gene expression and DNA replication [3]. Biosynthesis is a process that does not stop during this state; with cells forming new proteins related to starvation and cold shock [116,117]. ATP levels rapidly decrease in dead cells, however, in VBNC cells these levels are high [118]. Other studies have shown that *E. coli* O157: H7 cells in the VBNC state show continuous gene expression of *mob*A, *rfb*E, *stx*1 and those responsible for synthesizing 16S rRNA [119]. Similarly, in *V. vulnificus* VBNC cells, a continuous production of messenger RNAs for different genes was observed for 4.5 months [120]. Cells of *Shigella dysenteriae* in the VBNC state could actively capture methionine to incorporate it into several proteins during 4-8 weeks [121]. Pazos-Rojas *et al*., (2019) detected the expression of *opr*H, *mut*S, *mut*L genes and 16S RNA in *P. putida* KT2440 VBNC cells under desiccation stress [4]. These studies suggest that some genes may be effective monitors for viability, but whether they are involved in the entrance or exit from VBNC state is still unknown.

At the structural level, it has been described that VBNC cells may suffer damage to the plasmatic membrane and modifications in the composition of fatty acids, strongly suggesting that changes may be essential for the entrance into this state [4,122]. Cells may undergo biochemical changes in cell walls; in example, the peptidoglycan cell wall of *E. coli* in the VBNC state suffer some changes. An increment of 3-fold of DAP-DAP crosslinking was observed in peptides carrying covalently bound lipoproteins, as well as a shortening of the average length of glycan chains [123]. Hence, genes involved in peptidoglycan biosynthesis could be a required characteristic of VBNC cells to create a more rigid wall than in dividing cells [124].

Bacteria can detect stressors through histidine kinases bound to the membrane, mediating cellular response through differential expression of the target genes [125]. Therefore, early signaling could be a determining factor for VBNC state induction in bacteria. As mentioned above, cellular energy is one of the key characteristics of the VBNC state, triggering the induction of genes codifying subunits of proton pumps such as NADH:ubiquinone oxidoreductase, a protein essential for processes requiring energy in VBNC cells as well as under normal conditions [126]. This suggests that when bacterial cells enter the VBNC state in response to an environmental change, the activity of complex I of the respiratory chain and NADPH-generating systems are critical for the maintenance of cell viability [126]. In addition, selective permeability to nutrients and metabolites provided by ABC transporters may be a prerequisite for the VBNC state [127]. It is possible to consider that this behavior is widely distributed among bacteria. What has been described above in pathogenic microorganisms may also occur in beneficial bacteria. Nevertheless, further studies are needed to better understand it

6. Proteomics and transcriptomics of bacterial cells in VBNC state

When bacteria enter the VBNC state, various cellular changes may occur. Studying changes at the post-transcriptional and post-translational levels could elucidate which genes could be important for bacteria to perform this phenomenon; however, knowledge is still limited under the VBNC state. *H. pylori* produce more alkaline phosphatase and α-ketoglutarate oxidoreductase (KOR), while CoA transferase levels decrease in the Krebs cycle. There is also a decrease in urease, leucine arilamidase

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and naftol-AS- β -1-phosphohidrolase [128]. Based on these observations, it has been proposed that it would be possible to eliminate multi-drug resistant strains of *H. pylori* by activating the KOR enzyme, preventing cells from entering the VBNC state [129]. On the other hand, latent *E. coli* (EHEC) O157 isolated from food, had low levels of AhpCF and AceF proteins, but showed an increase in OmpW external membrane proteins [130]. Transcriptomic studies in *V. vulnificus*, showed a high expression of glutathione S-transferase, suggesting that when the VBNC state is induced by oxidative stress, this enzyme may prevent damage to different cellular components [131,132]. Under saline stress at low temperature several proteins related to transcription, translation, ATP synthesis, gluconeogenesis and antioxidants were over-expressed in *V. parahaemolyticus* [133]. On the other hand, *V. parahaemolyticus* in VBNC state induced by low temperature, an over-expression of gene *dac*B which encodes D-alanyl-D-alanyl carboxypeptidase was observed, resulting in the interruption of cell wall synthesis, and consequently the production of amorphic cells [134]. It is interesting that under saline stress at low temperatures, the protein AhpC (Alkyl hydroperoxide reductase subunit C) of *V. parahaemolyticus* can delay the entrance into the VBNC state [135]. The protein AhpC is responsible for the detoxification of reactive oxygen species that form in bacterial cells and facilitates the survival of pathogenic bacteria under environmental stresses, suggesting the importance of antioxidative activity during the induction of the VBNC state [135,136]. In VBNC cells of *V. cholerae* by cold stress an over-expression of different genes was detected, such as *pol*B corresponding to DNA polymerase II, *fli*G codifying for proteins of the flagellar motor, *fla*C for protein C in the flagellar subunit and ABC iron transporter genes [137]. It has been observed that the *tcp* gene which encodes the pilus toxin and is required for bowel colonization in lactating mice, is expressed in coccoid cells of *V. cholerae* in the VBNC state. The transition from bacillary to coccoid morphology is thought to be a mechanism of survival that *V. cholerae* uses in response to environmental stress and the expression of the *tcp* gene in coccoid cells in the VBNC state suggests that this could be very important for the adaptation and survival of *V. cholerae* during the transition between the host and aquatic environment [138]. In *C. jejuni*, the expression of the external membrane protein CadF was detected after entering the VBNC state. This protein functions as a mediator of fibronectin binding of Caco2 cells. The expression of this type of proteins suggests that even when bacteria enter the latency state, they can maintain their virulence [139].

The expression of the RpoS sigma factor in the VBNC state has been demonstrated in several bacterial species, with guanosine 3', 5'-bispirophosphate (ppGpp) acting as a positive regulator during the synthesis and function of this factor [140], interestingly it has been observed that RpoS mutant strains quickly lose culturability and cannot return to the culturable state with existing resuscitation methods [132,140]. Considering that the absence of this protein can mean imminent cell death, it could be one of the key genes involved in the entrance and exit from the VBNC state in bacteria.

The activity in the ribosomes is one of the most important processes in all living cells. A low expression level for RaiA proteins (Ribosome-associated inhibitor A) has been observed in the VBNC state, for several bacterial species [132]. This could mean that bacteria decrease their translational activity during the latency status, thereby saving energy required to face stress conditions. As described in this review, different bacterial groups modulate genes responsible for cellular processes such as cell wall reordering, detoxification, transporters and virulence genes.

Transcriptomic studies of *P. syringae* in VBNC state, with presence of acetosyringone synthesized in some plants in response to bacteria, showed that a complex network of biochemical reactions related to oxidative phosphorylation, ABC transporters, peptidoglycan biosynthesis, Krebs cycle, chemotaxis, two-component system and bacterial secretion systems, are important for keeping cells in the VBNC state. Likely the entrance into this state begins with metabolic pathways that control stimulus-response mechanisms (two-component system) and bacterial movement (chemotaxis), determined by the presence of chemicals in the environment where the cells are located. In addition, bacterial survival could depend on multi-drug resistance efflux pumps, which could give them tolerance to the presence of oxidative residues caused by stress [127]. The same research also found a positive regulation of genes involved in peptidoglycan biosynthesis, genes of the transcriptional

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factors LysR, Lrp/AsnC, and MarR. It is proposed that keeping cells in VBNC state may be under the control of LysR, which is the most abundant transcription factor in bacteria that regulates several genes (virulence, metabolism, quorum sensing and motility) [141]. Furthermore, the VBNC state could also be regulated by the proteins Lrp/AsnC (leucine-responsive regulatory protein/asparagine synthase C products) and MarR [127]. The MarR protein controls genes involved in the degradation of toxic compounds (including phenols), virulence, export of harmful chemicals and resistance to oxidative stress [142]. Another interesting finding was the repression of genes related to pathogenesis pathways, for example, Type III Secretion System (T3SS) and type VI secretion system proteins, which could be an additional requirement or consequence of the VBNC state in *P. syringae*.

Within our scant knowledge on beneficial bacteria, there are studies on *P. putida* KT2440 and *C. metallidurans*. Transcriptomic studies of *P. putida* KT2440 in VBNC state caused by desiccation stress [143], shown that 6 genes related to transmembrane transport and oxidation-reduction processes were upregulated. Ethylene glycol porin (PP_ 2662) and substrate-binding protein (PP_2676) genes could transport and degrade polyhydric alcohols, which are accumulated during desiccation stress as compatible solutes. The upregulation of TonB dependent receptor (PP_1446) gene could be a strategy used by *P. putida* KT2440 to provide ferrous-iron required for vital functions during the VBNC state. *P. putida* KT2440 cells return to cultivable sate upon 24 h of rehydration, after this return, 148 genes related to transport, oxidation-reduction, regulation of transcription, and biosynthetic process were upregulated while 42 genes related with translation, oxidation-reduction, and the regulation of transcription were downregulated. During prolonged rehydration of *P. putida* KT2440 cells the catabolism of phenylalanine/tyrosine is activated, maybe to provide energy and carbon source for ubiquinone biosynthesis while maintaining reduced protein synthesis [143].

Studies in *C. metallidurans* showed that during transition from the culturable state to VBNC state, there is a strong decrease in the expression of proteins involved in different mechanisms such as: basal bacterial metabolism, cellular processes, signaling, information storage, protein synthesis pathways, energetic processes and cell shape regulation [60]. The proteins that showed an increase in their regulation are involved in producing energetic processes and redox reactions. Proteomic analysis of return to culturable state showed that gluconate increased the expression of several proteins related to fundamental bacterial metabolism when used. While, when only water was added to promote the return to the culturable state, the expression of only six proteins was increased. These results suggest that reduced soil carbon or water availability could initiate bacterial VBNC state in soil-like environments. This limitation could induce gene expression and protein synthesis leading to the VBNC state.

7. Resuscitation of bacteria under VBNC state

As mentioned in previous sections, bacteria in the viable but non-culturable state do not grow in routine bacteriological media. However, they are still alive, which may constitute a survival strategy under stressful conditions. The cells must be able to increase their metabolic activity to return to the culturable state [2]. It has been a topic of discussion and a great challenge to demonstrate that cells can return to the culturable state and that observed cells which grow again on media do not simply correspond to the growth of other surviving bacteria. *V. vulnificus* is one of the best studied microorganisms able to return to the culturable state. This bacterium enters the VBNC state in response to low temperatures (10 °C) [117]. Numerous studies have reported that a simple reversal of stress is sufficient to return VBNC cells to the culturable state, for example, by changing the temperature to that for optimal growth [144–146]. Similarly, it was observed for *V. parahaemolyticus* temperature changes control the VBNC state [147].

In *P. putida* KT2440 cells in VBNC state induced by desiccation, the colonization of rhizosphere of maize plants, short rehydration in presence of root exudates and prolonged rehydration with only distilled sterile water were shown to allow the return to a culturable state [4].

Interestingly, it has been observed that the interaction with higher organisms can function as a biological mediator to return to the culturable state. For example, in *L. pneumophila* which enters the VBNC state under starvation and hypochlorite treatment, it can return to the culturable state in

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presence of protozoa *Acanthamoeba polyphaga* and *Acanthamoeba castellanii* [148,149]. In a different system, the association of VBNC cells of the coral pathogen, *Vibrio shiloi*, with the seafire worm *Hermodice carunculate* is a strategy for remain and infect coral [150]. The resuscitation of the pathogen *L. monocytogenes* was observed after inoculation of pathogen free embryonic chicken eggs [151]. Regarding beneficial bacteria, a recent study has shown that VBNC cells of *P. putida* KT2440, can colonize the rhizosphere of maize plants and this interaction allows the return to the culturable state [4]. Furthermore, root exudates of maize can return these bacteria to the culturable state [98], thus, it is of interest to study bacterial associations with their host since, this influences the survival of the bacteria even in the VBNC state.

Other factors that could be decisive for the return to the culturable state, are based on the study of extracellular proteins Rpfs (resuscitation-promoting factors). Rpf was discovered in *Micrococcus luteus* as a cytokine, that promotes resuscitation and growth of dormant cells [152]. It was reported that a picomolar concentration of Rpf could increase the viable cell number of dormant *M. luteus* [153]. Other study reported that Rpf and RipA proteins work synergistically to remodel the cell wall promoting cell division and resuscitation. The cell wall fragments digested by Rpf and RipA serve as signaling molecules binding with a receptor to trigger resuscitation [154]. RpF-like proteins have been reported with the ability to induce return to cultivable state. The YeaZ protein with protease activity present in *V. parahaemolyticus*, *V. harveyi*, *S. typhimurium*, and *E. coli* has been shown to promote return to culturable state [155]. The promoting effect of YeaZ may be correlated with its protease activities, but mechanism that help recovery cultivability lacks further investigation.

Return to the culturable state in *M. tuberculosis* and *M. smegmatis*, is promoted by activity of a peptidoglycan hydrolase, involved in the digestion of the cell wall and cell division. In this way, reorganization of peptidoglycan plays an important role in the VBNC state [124,153,156]. Another type of proteins involved in the return to the culturable state are the so called "autoinducer of growth", which are stable to heat and are secreted by Gram-positive and Gram-negative bacteria growing in culture media with the hormone norepinephrine [157]. Humans release this type of hormone after the occurrence of an injury, thus in humans it is considered a stress-related hormone [157,158]. These findings are of great relevance since it was observed that in the presence of autoinducer proteins in culture media other bacteria such as *S. enterica* serotype *Typhimurium*, and two *E. coli* O157:H7 strains were able to return to the culturable state [159]. These investigations provide a breakthrough in understanding the return to the culturable state of enteropathogens, found in the intestinal tract when the host (human) suffers tissue damage and is under significant physiological stress.

Molecules such as sodium pyruvate have been studied in their role in return to culturable state. This molecule is an intermediate key metabolite in glycolysis and H_2O_2 degrading compound [160]. It was observed that VBNC cells can return to culturable state in a media supplemented with sodium pyruvate [161]. It was suggested that sodium pyruvate, catalase and superoxide dismutase, due to their H_2O_2 or reactive oxygen-degrading effect can induce return to cultivable state [144].

The Quorum sensing (QS) is a mechanism widespread communication system in bacteria that induces global gene expression changes [162]. It has been observed that QS can induce an adaptation to stressful conditions and play a role in return of VBNC state. Studies in *P. aeruginosa* indicated that QS system has a role in activating superoxide dismutase or catalase to regulate the antioxidation activities [163]. In *S. typhimurium*, QS triggered the expression of catalase to restore the culturable state [164]. It is proposed that QS may help the cell to express genes related to oxidative stress, which as mentioned above play an important role in allowing return to the culturable state of different bacteria. Being certain that the VBNC state is reversible opens up a range of possibilities for using microorganisms in different areas that may offer some benefit.

8. Conclusions

The study of the VBNC state in bacteria remains controversial, because its verification does not completely satisfy all researchers worldwide, even though, many scientists have concluded that this is a survival strategy under stressful conditions. Regardless of the role of the VBNC state in the life

cycle of bacteria, it has been fully proven that many bacterial species, especially human pathogens, can carry out this survival strategy. During the VBNC state bacteria maintain their cellular structure and biological functions, such as cellular respiration, and continuous gene expression, having the ability to leave this state and return to a culturable state when conditions become more favorable. Despite the advances made over the last thirty years, it remains a challenge to investigate this state's physiology, biochemistry and genetics, because it is unknown what gene or genes could directly be involved with the loss and recovery of culturability. The study of beneficial bacteria in the VBNC state is still little explored. Expanding knowledge in this field could represent an opportunity for future biotechnological applications, for example, for the biological control of plant pathogens, since even when bacteria enter the VBNC state under stressful conditions, when returning to the culturable state they can continue exercising their biocontrol over species harmful to their hosts. Microbial biocontrol technology avoids the use of pesticides that are highly toxic to the environment. Another application is the formulation of bacterial inoculants with beneficial species capable of entering the VBNC state, to increase its shelf life, so that when applied to the plant rhizosphere, bacteria will be able to return to the culturable state and carry out their beneficial activity on the growth of plants of agricultural interest. The impact of beneficial bacteria on human health represents a challenge to understand the role of the VBNC state and how it can affect or benefit hosts. Changing the topic on the elaboration of consumable biotechnological products using only culturable microorganisms is a real challenge for the industry, however we must understand that bacteria have their preservation system such as the VBNC state that can represent a significant decrease in production costs. This highlights the importance of more precisely understanding the transition between the culturable and non-culturable state.

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