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Original article

Environmental and physico-chemical factors induce VBNC state in *Listeria monocytogenes*

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Abstract – Investigations of bacterial survival in natural environments have indicated that some organisms lose culturability on appropriate media under certain conditions and yet still exhibit signs of metabolic activity and thus viability. This reproducible loss of culturability in many bacterial species led to the description of a "Viable But Non Culturable" (VBNC) state. The purpose of this article is to determine environmental and physico-chemical factors which induce the VBNC state in a food-borne pathogen that has become a public concern: *Listeria monocytogenes*. The factors, i.e. inoculum size, natural sunlight, temperature (4 °C or 20 °C), NaCl concentration (0% or 7%) and pH (5 or 6) were studied on 4 strains (LO28, ATCC 19115, Scott A, CNL 895807). The culturability of the starved cell suspension was determined in each condition tested by the spread plate count, and the cell activity was determined by the Direct Viable Count technique and CTC-DAPI double staining. A strain effect was found in different test conditions. For the LO 28 and ATCC 19115 strains, the VBNC state was very transient in certain conditions. For the other strains tested (Scott A, CNL 895807), the VBNC state was maintained throughout the observation period. In the dark, the incubation temperature was the main factor in the production of VBNC forms in *L. monocytogenes.* However, natural sunlight rapidly produced the VBNC state in *L. monocytogenes* cells in microcosm water. We conclude that because of its ubiquity and the factors studied which are met in the food industry, the presence of VBNC *L. monocytogenes* cells could pose a major public health problem since they cannot be detected by traditional culturing methods. Further investigations are needed to establish virulence before and after resuscitation of VBNC *L. monocytogenes* cells.

VBNC / environmental factors / stress / *Listeria monocytogenes*

Résumé – Les facteurs environnementaux et physico-chimiques induisent l'état VNC chez *Listeria monocytogenes.* Des études sur la viabilité bactérienne dans l'environnement ont démontré l'existence d'un état particulier qualifié d'état Viable non Cultivable (VNC), dans lequel certaines

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bactéries perdent leur capacité à former des colonies sur des milieux de culture, tout en conservant une activité métabolique. L'objectif de ce travail est d'étudier l'influence de facteurs physico-chimiques et environnementaux, intervenant dans l'entrée à l'état VNC de *Listeria monocytogenes.* Les facteurs : taille de l'inoculum, exposition à la lumière naturelle, température (4–20 ºC), concentration en NaCl (0–7%), pH (5–6) ont été étudiés chez 4 souches de *L. monocytogenes* : LO28, ATCC19115, Scott A, CNL 895807. Les cellules ont été placées dans des conditions de privation nutritionnelle (eau distillée filtrée). La capacité à former des colonies a été déterminée par étalement sur boîte de gélose, alors que l'activité métabolique des bactéries a été établie par 2 techniques : le Direct Viable Count et la double coloration CTC-DAPI. Un effet souche a été constaté: pour les souches LO28 et ATCC 19115, l'état VNC semblait très transitoire, alors que pour les souches Scott A et CNL 895807, l'état VNC s'est maintenu pendant toute la durée de l'expérimentation. A l'obscurité, la température d'incubation est apparue comme le facteur primordial d'entrée à l'état VNC, mais l'exposition à la lumière naturelle a entraîné une perte rapide du caractère cultivable. Compte tenu d'une part de l'ubiquité de *L. monocytogenes*, d'autre part du fait que les facteurs étudiés peuvent être rencontrés dans l'environnement des industries agro-alimentaires, les auteurs pensent que l'état VNC chez *L. monocytogenes* pourrait représenter un réel problème de santé publique. Des travaux complémentaires concernant la virulence et le retour à l'état cultivable de ces formes sont en cours afin de préciser le risque lié à l'état VNC chez *L. monocytogenes*.

VNC / facteurs environnementaux / stress / *Listeria monocytogenes*

1. INTRODUCTION

It has been clearly demonstrated that many bacteria can enter the viable but nonculturable (VBNC) state when faced with adverse environmental conditions [24]. Two characteristics make it possible to distinguish the VBNC response from other responses. There is a lack of growth in non-selective media and cells can remain in the VBNC state for long periods of time, sometimes for over a year [24]. The formation of VBNC cells has been regarded by some as a survival strategy [24]. In fact, bacterial populations in the environment are frequently exposed to stresses due to limitations and changes in nutrient availability, temperature, salinity, solar illumi nation and oxygen saturation. The persistence of bacteria in the environment is thus to a large part determined by their ability to endure these stresses.

A number of factors induce the VBNC state, including starvation, which is probably the most predominant factor for most of the bacteria studied [15, 20, 28, 33], temperature [32], NaCl concentration [30, 33] and visible light [8, 16]. The conditions shown to induce nonculturability differ according to the organism [24]. Low temperature is the only factor required to induce the VBNC state in *Vibrio vulnificus* as demonstrated in microcosms containing artificial seawater as well as complex nutrient media [24]. *V. vulnificus* enters the VBNC state [23] when incubated at low temperatures, while at room temperature the organism exhibits a classic starvation response [25]. The kinetics of VBNC formation are affected by the nutritional state of the population. Cells entering into a stationary phase or starvation conditions prior to low temperature incubation delay VBNC formation [23]. Indeed, incubating *V. vulnificus* under starvation conditions for as little as 1 h prior to incubation at 4 ºC significantly decreases the rate at which cells become nonculturable [23, 25].

Some microbiologists and hygienists have drawn attention to this phenomenon because the VBNC state has been described in a number of pathogens including *Salmonella* [8], *V. vulnificus* [19], *Campylobacter jejuni* [28], *Legionella pneumophila* [17].

Some experiments have shown that VBNC bacteria can recover culturability after digestive tract passage in animals [7, 9, 17, 19] or humans [10]. Some specialized hygienists therefore emphasize the risk presented by pathogenic bacteria in the VBNC state, which cannot be detected by standard analysis procedures usually used in control laboratories, and some of them consider this state to be a public health risk.

The gram positive bacterium *Listeria monocytogenes* is a ubiquitous, intracellular pathogen which has been implicated in the last 2 decades in several outbreaks of food borne disease [5, 14]. Listeriosis is of great concern in the food industry [13] and is distinguished by a high death rate. The predominant risk groups appear to be immunocompromised adults, elderly people, newborn babies and pregnant women, with abortion, neonatal death, septicemia and meningitis [22]. The existence of the VBNC state in this pathogenic bacterium has been described in previous studies [3]. In fact, Besnard et al. [3] reported that *L. monocytogenes* exists and can survive for prolonged periods in nutrient-poor water. However, there is no available information regarding significant factors inducing the VBNC state in *L. monocytogenes*. The aim of this study was to classify the physicochemical and environmental factors inducing the VBNC state in *L. monocytogenes*.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Four *L. monocytogenes* strains (Scott A, CNL 895807, ATCC 19115, LO 28) provided by culture collection in our laboratory were studied. For each test, the strains were grown for 24 h at 37 °C on Plate Count Agar (Biokar, Beauvais, France). Two subcultures were performed under similar conditions and then the cells were placed in microcosm water.

2.2. Starvation experiments

The microcosm water system described by Rollins and Colwell [28] was used to starve *L. monocytogenes* cells by the following three procedures. Most of the experiments were temperature controlled (4 °C and 20 °C) except for experiments performed in natural sunlight (see procedure 3).

Procedure 1. Effect of inoculum size on culturability. After culture, the cells were collected, washed and suspended in bottles containing 500 mL of filtered (Nalgene 0.2 µm, Bioblock, Strasbourg, France), sterilized distilled water, adjusted to pH 6.0 ± 0.1 (NaOH 0.1 M), to obtain a final concentration of about 10^8 bacteria·mL^{-1} (series 1) and about 10^6 bacteria·mL^{-1} (series 2). The bottles were then left in the dark for 100 days at 4 °C or 20 °C with or without NaCl (0% or 7%) according to experimental conditions with gentle shaking at 100 r.p.m. These experiments were performed in triplicate.

Procedure 2. Effect of temperature, pH and NaCl concentration on culturability. In order to study the effects of temperature (4 °C or 20 °C), pH (5.0 or 6.0) and NaCl concentration (0% or 7%), a different microcosm was constituted according to a complete experimental plan (3 factors and 2 levels) (series 3). Microcosms were constituted for each condition. After culture, the cells were collected, washed and suspended in bottles containing 500 mL of filtered (Nalgene 0.2 µm, Bioblock, Strasbourg, France), sterilized distilled water and adjusted to different pH (5.0 or 6.0) and NaCl concentrations (0% or 7%) to obtain a final concentration of about 10^6 bacteria·mL^{-1}. The bottles were then left in the dark for 100 days at a corresponding temperature (4 °C or 20 °C) according to experimental conditions with gentle shaking at 100 r.p.m. This experiment was performed in triplicate.

Procedure 3. Effect of natural light on culturability. A fourth trial was performed (series 4) in order to study the effects of natural light on the production of VBNC *L. monocytogenes* cells. After culture, the cells were collected, washed and suspended in bottles containing 500 mL of filtered (Nalgene 0.2 µm, Bioblock, Strasbourg, France), sterilized distilled water, adjusted to pH 5.0 ± 0.1 (HCl 0.1 M) and different NaCl concentrations (0% or 7%) to obtain a final concentration of about 10^6 bacteria·mL^{-1}. The bottles were then left exposed to natural light with gentle shaking at 100 r.p.m. for 80 days. This experiment was performed in triplicate.

2.3. Cell counts

In each inoculated microcosm, the number of culturable cells, the number of viable bacteria and the number of total bacteria were determined on the first, second, and third days after inoculation and once a week for 80 days (procedure 3) and 100 days (procedures 1 and 2).

2.4. Enumeration of culturable cells

Culturability was assayed by spread plate counts. Serially diluted samples (0.1 mL peptone water) were spread in triplicate on Plate Count Agar. After 48 hours of incubation at 37 °C, colony-forming units (cfu) at appropriate dilutions were counted and referred to the original sample. When culturable cells were less than 300 per mL, 0.1 mL samples of microcosm water were spread on 10 Petri dishes containing Plate Count Agar.

2.5. Enumeration of viable cells

In order to detect and enumerate VBNC cells among the bacterial populations, it was necessary to use techniques allowing the detection of metabolic activity in nonculturable cells. Two techniques were used: CTC-DAPI double staining and the Direct Viable Count [4].

2.5.1. CTC-DAPI double staining

Electronic transport chains such as the respiratory chain can be used to detect cellular activity. One compound can be used, i.e. 5-cyano-2,3-ditolyltetrazolium chloride (CTC). Counterstaining with DAPI makes it possible to count active bacteria which are able to reduce CTC (red fluorescent precipitate) and non-viable bacteria stained in blue by DAPI. This staining method has recently been used in many bacteria viability studies [6, 27, 34]. A modified technique of Rodriguez et al. [27] was used to detect cellular activity in *L. monocytogenes* cells.

One milliliter of microcosm water was centrifuged for 15 minutes at 10 000 *g*. After centrifugation, pellets were collected in 0.5 mL distilled filtered water (pH 6.0) with 100 µL at 0.05 g·mL^{-1} solution of pyruvic acid (Sigma, La Verpillere, France), 0.5 mL of brain-heart infusion (Biokar, Beauvais, France), and 100 µL of CTC (5-cyano-2;3-ditolyl tetrazolium chloride; Polysciences Inc., Warrington, Pennsylvania, USA) at 15 mg·mL^{-1}.

After 3 hours of incubation at 37 °C, the mixture was filtered through a 0.2 µm pore size polycarbonate black membrane filter (Bioblock, Strasbourg, France) and stained for 5 min with a DAPI solution (Interchim, Montluçon, France) at 5 µg·mL^{-1}. Microscopy observation was performed as above. Four filters were counted for each sample. Respiring cell counts, showing CTC formazan crystals (red precipitate fluorescent) and total cell counts stained by DAPI, (i.e. viable and non-viable) were determined. The results were expressed as the number of corresponding bacteria per mL of the original sample. The experiments were performed in triplicate.

2.5.2. Direct Viable Count

A modified technique of Kogure et al. [18] was used [4] in order to enumerate viable cells of *L. monocytogenes*. The antibiotic used was ciprofloxacin (Bayer, Puteaux, France) instead of nalidixic acid in order to inhibit cell division in this gram-positive bacterium. Briefly, one milliliter of microcosm water was added to 9 mL of brain-heart infusion (Biokar, Beauvais, France), 100 µL yeast extract at 25 $mg \cdot L^{-1}$ (Difco, Detroit, Michigan, USA) and 5 µL ciprofloxacin solution at 2 $mg \cdot mL^{-1}$. After 7 hours of incubation at 37 °C, the mixture was filtered through a 0.2 µm pore size polycarbonate filter and stained for 5 min with a DAPI solution (Interchim, Montluçon, France) at 5 $\mu g \cdot mL^{-1}$.

Finally, the filter was air-dried and mounted in non-fluorescent immersion oil (Nikon, Paris, France), and a coverslip was added before observation with an epifluorescence microscope (Olympus BX40, Rungis, France), equipped with a BW2-RFL-T3 100-W light source. Counting was performed randomly on the basis of 10 microscope fields per filter.

Four filters were counted for each sample. Only cells elongated at least twice, with respect to cell length in fresh culture, were counted as viable. The experiments were conducted in triplicate.

2.6. Total culturable count

For the DVC technique, total enumeration was performed after staining with DAPI. Bacterial suspensions were filtered on a black polycarbonate membrane (0.2 µm) and the filter was covered with DAPI for 5 min. Finally, the filter was air-dried and mounted in non-fluorescent immersion oil (Nikon, Paris, France), and a coverslip was added before observation with an epifluorescence microscope.

For CTC-DAPI double staining, total culturable count was obtained after enumeration of all the bacteria stained by DAPI on blue on the same preparation used for the viable cell count.

2.7. Resuscitation of VBNC cells in *Listeria monocytogenes*

When no colony was observed in 1 mL, but cellular activity was detected, 10 mL of microcosm were centrifuged for 15 min at 10 000 *g*. After centrifugation, the pellets were collected in 10 mL brain heart infusion (Biokar, Beauvais, France). This solution was then placed at 37 °C. After 24, 48, and 72 hours, 0.1 mL were spread on plate count agar. Petri-dishes were placed for 48–72 hours at 37 °C. If no colony appeared in these Petri Dishes, bacterial cells present in microcosm water were considered to be in Viable But Non Culturable state according to our definition: "VBNC bacteria represent the part of the bacteria population, which is not able to grow in usual culture media and which cannot be resuscitated by traditional resuscitation techniques but which retain metabolic activity detected by various methods in the conditions tested".

2.8. Statistical analysis

Each point on the curves for culturability and enumeration of viable cells presented in this study represents an average of 3 Petri dishes and 3 × 10 microscopy observations. Differences between averages were calculated by analysis of variance. The coefficients of variation between replicate experiments were less than 10%. Probabilities less than or equal to 0.1 were considered significant. Bars marked on curves represent the maximal population variance observed on all performed trials.

3. RESULTS

Since the results obtained for each repeated procedure were quite similar, only one result for each procedure will be presented.

Procedure 1. Effect of inoculum size on culturability.

Series 1 (10^8 bacteria·mL^{-1}). After 12 months of incubation of 10^8 bacteria·mL^{-1}, the population of culturable cells was maintained at a bacterial concentration greater than 10^4 UFC·mL^{-1}. Significant variations were observed between 4 strains of *L. monocytogenes* for all conditions used.

Series 2 (10^6 bacteria·mL^{-1}). Whole strains showed a loss of culturable state between 28 and 100 days. In fact, the number of culturable cells dropped below the level of detection (< 0.1 CFU·mL^{-1}). However, only the Scott A strain incubated at 4 °C, CNL 895807 incubated at 20 °C, and CNL 895807 incubated at 20 °C in the presence of NaCl lost the culturable state and retained metabolic activity (10^4–10^5 viable bacteria·mL^{-1}) during the experiment. For CNL 895807 strains, the VBNC state was reached after 4 weeks of starvation in microcosm water incubated at 20 °C without NaCl (Fig. 1) and after 6 weeks of starvation in microcosm water incubated at 20 °C with NaCl (7%) (Fig. 2). For the Scott A strain, the VBNC state was reached after 6 weeks of starvation (Fig. 3).

Procedure 2. Effect of temperature, pH and NaCl concentration on culturability. For each effect h, the standard deviation calculated on whole measurements was 1.27. The results obtained are presented in a matrix of effect (Tab. I). This matrix made it possible to determine the effects of different factors and the interactions between different factors with $\alpha = 10\%$ (Tab. II).

The LO 28 strain. The NaCl concentration and the pH-[NaCl] interaction had a

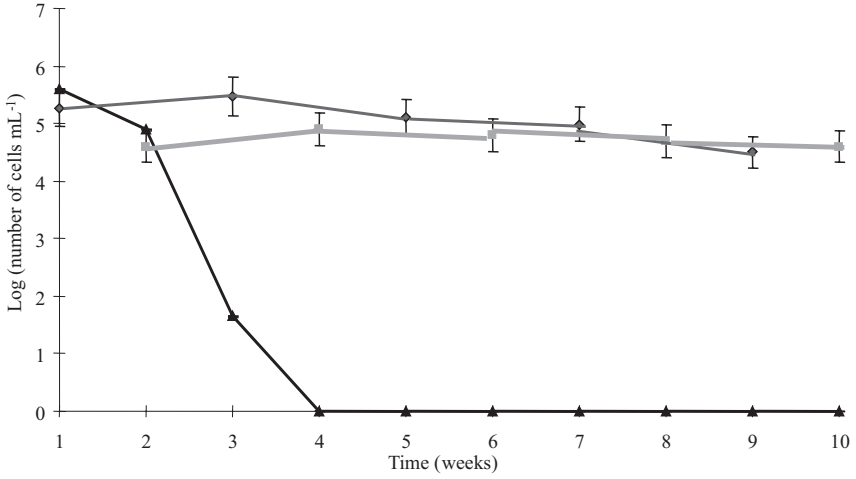

Figure 1. Evolution of the bacterial concentration according to the CNL 895807 strain in microcosm water incubated at 20 °C without NaCl (0%). The culturable cells were enumerated by spread plate count agar. Direct Viable Counts were carried out on brain-heart infusion medium containing yeast extract and ciprofloxacin. Respiring cells were enumerated after the addition of 5 mmol·L^{-1} CTC (5-cyano-2,3-ditolyl tetrazolium chloride); Numeration of viable cells by CTC —◆—, numeration of viable cells by DVC —■—, culturable count —▲—.

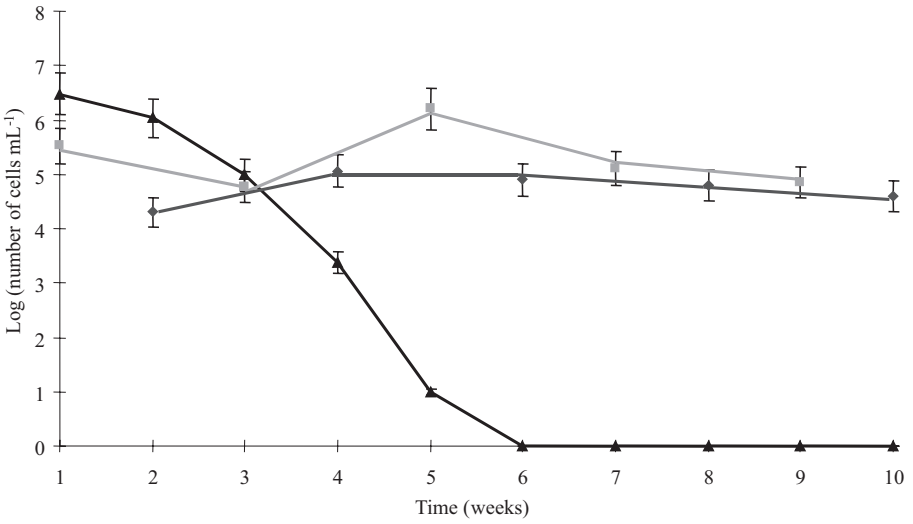

Figure 2. Evolution of the bacterial concentration according to the CNL 895807 strain in microcosm water incubated at 20 °C with NaCl (7%). The culturable cells were enumerated by spread plate count agar. Direct Viable Counts were carried out on brain-heart infusion medium containing yeast extract and ciprofloxacin. Respiring cells were enumerated after the addition of 5 mmol·L^{-1} CTC (5-cyano-2,3-ditolyl tetrazolium chloride); Numeration of viable cells by CTC —◆—, numeration of viable cells by DVC —■—, culturable count —▲—.

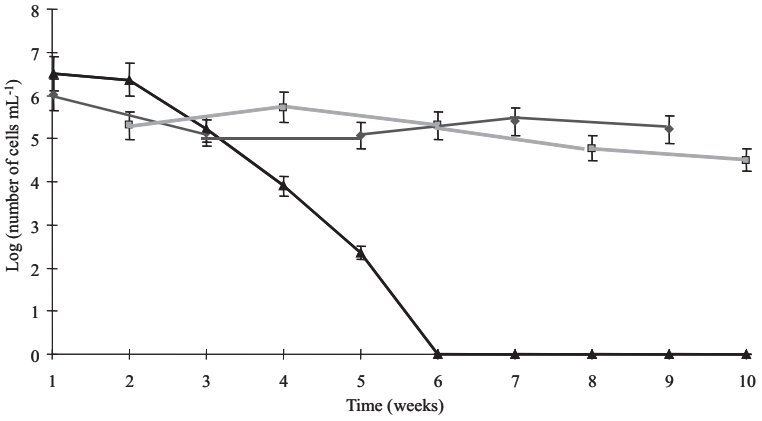

Figure 3. Evolution of the bacterial concentration according to the Scott A strain in microcosm water incubated at 4 °C without NaCl (0%). The culturable cells were enumerated by spread plate count agar. Direct Viable Counts were carried out on brain-heart infusion medium containing yeast extract and ciprofloxacin. Respiring cells were enumerated after the addition of 5 mmol·L^{-1} CTC (5-cyano-2,3-ditolyl tetrazolium chloride); Numeration of viable cells by CTC —◆—, numeration of viable cells by DVC —□—, culturable count —▲—.

Table I. The matrix of effects concerning the variations of conditions of pH, [NaCl] and incubation temperature in different types of microcosms inoculated with the LO 28, ATCC 19115, Scott A, and CNL 895807 strains.

Trials	pH	[NaCl]	Temp.	Interac.	Interac.	Interac.	Interac.	Time[1] (days)			
	(1)	(2)	(3)	(1.2)	(1.3)	(2.3)	(1.2.3)	LO 28	ATCC 19115	Scott A	CNL 895807
1	+1	+1	+1	+1	+1	+1	+1	38*	52*	77	43
2	+1	+1	–1	+1	–1	–1	–1	82*	94*	75	47
3	+1	–1	+1	–1	+1	–1	–1	52*	27*	74	31
4	+1	–1	–1	–1	–1	+1	+1	59*	32*	46	48
5	–1	+1	+1	–1	–1	+1	+1	72	56	89	14.5
6	–1	+1	–1	–1	+1	–1	–1	61*	67*	79	47
7	–1	–1	+1	+1	–1	–1	–1	27	24	72	25.5
8	–1	–1	–1	+1	+1	+1	+1	29*	36*	47	38

[1] Time necessary (days) to the loss of culturable state (< 1 cfu·mL^{-1}) with observation of viability with two techniques (10^4–10^5 viable bacteria·mL^{-1}) and no revivification was observed by traditional resuscitation techniques.
pH (1) : –1 = pH 5 ; +1 = pH 6. [NaCl] (2) : –1 = 0% NaCl; +1 = 7% NaCl. Temperature (3) : –1 = 4 °C ; +1 = 20 °C.
*A loss of viability by two techniques was observed in these conditions after 100 days of incubation of the microcosm.

Table II. The effects of factors and interactions between factors concerning the variation of conditions of pH, [NaCl] and incubation temperature of different types of microcosm inoculated with the LO 28, ATCC 19115, Scott A, CNL 895807 strains. The significant effects are signalized in bold. The P_{value} associated with average effects is given in parentheses.

	LO 28	ATCC 19115	Scott A	CNL 895807
Average effect of [NaCl]	**10.75 (0.0001)**	**18.75 (0.0001)**	**10.125 (0.0001)**	1.125 (0.0077)
Average effect of pH	5.25 (0.0001)	2.75 (0.0001)	–1.875 (0.0002)	5.5 (0.0001)
Average effect of temperature	–5.25(0.0001)	**–8.75 (0.0001)**	**8.125 (0.0001)**	**–8.25 (0.0001)**
Interaction Temp-[NaCl]	–3(0.0001)	–4.5 (0.0001)	–5.125 (0.0001)	–0.875 (0.0252)
Interaction pH-[NaCl]	**–8.5 (0.0001)**	3 (0.0001)	–2.125 (0.0001)	1.625 (0.0009)
Interaction pH-Temp	–7.5 (0.0001)	–3 (0.0001)	–0.625 (0.0252)	3 (0.0001)
Interaction pH-Temp-[NaCl]	–6.25 (0.0001)	–4.75 (0.0001)	–1.375 (0.0015)	4.125 (0.0001)

significant influence with $\alpha = 10\%$. NaCl concentration had a positive effect on the loss of the culturable state. Thus, a low concentration decreased the time necessary to lose the culturable state in the LO 28 strain. Weak pH decreased the time of entry in the VBNC state at a weak NaCl concentration, whereas pH did not influence the time of entry into the VBNC state at a heavy NaCl concentration.

However, a viability count after 100 days of incubation showed the loss of viability in particular conditions (see* in Tab. I). Thus, the VBNC state existed but was transient in these conditions.

The ATCC 19115 strain. Temperature and NaCl concentration had a significant influence with $\alpha = 10\%$. The temperature had a negative effect. Thus, a higher temperature decreased the time necessary to enter the VBNC state.

The NaCl concentration had a greater positive effect. Thus, a weak NaCl concentration was necessary to reduce the time to lose a culturable state.

As observed previously, the VBNC state was fleeting in certain conditions (see* in Tab. I), because a loss of viability was observed after 100 days of incubation.

The Scott A strain. Temperature and NaCl concentration had a significant influence with $\alpha = 10\%$. NaCl concentration and temperature had a positive effect. A temperature of 4 °C and a weak NaCl concentration were therefore necessary to obtain the VBNC forms in the Scott A strain.

A viability of 10^4–10^5 viable bacteria·mL^{-1} was detected after 100 days of incubation in all conditions tested.

The CNL 895807 strain. Only temperature had a significant influence with $\alpha = 10\%$. Temperature had a negative effect. Increased temperature therefore decreased the time necessary to lose the colony forming ability. As for the Scott A strain, 10^4–10^5 viable bacteria·mL^{-1} were detected after 100 days of incubation in all conditions tested.

Procedure 3. Effect of natural light on culturability. After only 9 to 28 days exposure to solar radiation, all strains lost their culturable state in all conditions tested whereas metabolic activity was detected by the DVC and CTC-DAPI double staining techniques (10^4 viable bacteria·mL^{-1}) (Tab. III). For the CNL 895807 strain, 7% NaCl concentration caused rapid loss of the culturable character (14 days against 28 days with an NaCl concentration of 0%). Time of entry into the VBNC state was not significantly different without natural sunlight (Tab. I). For the Scott A strain, 7% NaCl resulted in the loss of the culturable state after 9 days of inoculation against 14 days for trials with 0% NaCl. The same behavior occurred for the LO 28 strain (9 days with 7% NaCl against 21 days without NaCl (0%). However for the ATCC 19115 strain, a loss of the culturable state was obtained more quickly without NaCl (9 days) than with 7% NaCl (14 days). Thus, except for the CNL 895807 strain, natural light rapidly decreased the time necessary to achieve the VBNC state in *L. monocytogenes*, with viability maintained after 80 days of incubation.

Table III. The results obtained for the microcosm incubated under natural sunlight on series 3.

Microcosm	Nature of strain	NaCl Concentration (%)	t (days)
1	CNL 895807	0	28 ± 2
2	CNL 895807	7	14 ± 4
3	Scott A	0	14 ± 4
4	Scott A	7	9 ± 3
5	LO 28	0	21 ± 4
6	LO 28	7	9 ± 3
7	ATCC 19115	0	9 ± 3
8	ATCC19115	7	14 ± 4

t = time necessary for the loss of the culturable state (< 1 cfu·mL^{-1}).

4. DISCUSSION

Culture of bacteria on standard artificial media under laboratory conditions is fundamentally different from the conditions met in a natural environment. In contrast to laboratory conditions, bacteria in nature spend most of their lifetime in a state of starvation or nutrient limitation. To survive extended periods of starvation or other forms of environmental stress, many bacteria have developed strategies for metamorphosis into more or less sophisticated survival forms, where spores may represent an extreme in terms of survival in adverse conditions. Similarly, the formation of VBNC cells by non-differentiating bacteria as a response to adverse conditions has been proposed as a survival strategy [12]. The semantics of critical terms such as viability, vitality, active, alive, nonviable and dead have had to be reviewed yet again, and established methodologies called into question. In spite of substantial activity, there is little evidence to support the view that a single physiologically-defined "VBNC" state exists.

In the present study we confirmed that, as previously described [3], *L. monocytogenes* can enter a state of VBNC after starvation in microcosm water for an extended period. Other bacteria have also been shown to have this capacity after encountering environmental stress such as *V. vulnificus* [24]. Confirmation of the VBNC condition requires the use of viability assays. To study the VBNC state in *L. monocytogenes*, double staining CTC-DAPI and DVC seem to be two techniques of choice in the search for viable bacteria among populations of culturable and non-culturable cells with classical methods of culture [3]. The behavior of our 4 strains studied within microcosm water was quite different according to experimentation and the studied factors. The results obtained in this experimental plan showed that pH is a neglected factor, whereas temperature and NaCl concentration seem to be important factors inducing the loss of capacity to form colonies. For the Scott A strain, a low temperature was necessary to obtain VBNC cells. For all other tested strains, a temperature of 20 °C was necessary to rapidly obtain the VBNC state in *L. monocytogenes*. However, for the Scott A, CNL 895807 and LO 28 strains and the ATCC 19115 strain in certain conditions (pH = 5, [NaCl] = 7%, temperature = 20 °C; pH = 5, [NaCl] = 0%, temperature = 20 °C), the VBNC state was maintained for 100 days after incubation in microcosm water. For other conditions (*), the VBNC state existed but was not maintained after 100 days of incubation. There was therefore a strain effect. A strain effect has been described in other VBNC studies with *C. jejuni* [6, 21]. According to Medema et al. [21], the influence of extrinsic factors must be investigated.

Conditions shown to induce the VBNC state include such diverse factors as starvation, salinity, visible light and temperature [24]. In our experiment, inoculum size seemed to be an important factor. In procedure 1, with an inoculum of 10^8 to 10^9 UFC·mL^{-1}, the culturable bacterial concentration in microcosm water was maintained for 12 months after the beginning of experimentation whereas with an inoculum of 10^6 UFC·mL^{-1} all strains showed the disappearance of a culturable character from 28 to 80 days. The ability of cells to cope with starvation or stress, to maintain essential processes and to repair damage will obviously depend on conditions prior to and during recovery. Thus, when no nutritive substance is added to the medium, the observed recuperation could be due to cryptic growth resulting from the multiplication of a few cells which utilise the nutrients released by dead cells [31].

Moreover, the results presented clearly show the deleterious effect of solar radiation upon *L. monocytogenes* released into the microcosm water. Thus, a loss of culturable character (< 1 UFC·mL^{-1}) was

obtained after only 2 weeks of starvation whereas metabolic activity (10^4–10^5 bacteria·mL^{-1}) was always detected after 80 days of starvation. The most harmful effect was found when bacteria behavior was analyzed according to the culturability pattern. The effect of sunlight on culturability has been reported by numerous authors in both fresh water and seawater [16]. Barcina et al. [1] concluded that one of the effects of visible radiation on *Escherichia coli* cells in fresh water is the progressive loss of their capacity to multiply in standard bacteriological media. It is well known that microorganisms can enter a VBNC state, and therefore the loss of culturability of an organism should not be equated with its death [2, 24, 29]. The results reported here are compatible with the findings of other researchers. Davies and Evison [11], working with *E. coli* and *Salmonella* spp. exposed to natural sunlight, found that direct counts by the acridine Direct Viable Count method decreased much more slowly than the culturable counts in seawater. Likewise, Pommepuy et al. [26], demonstrated that an enterotoxigenic *E. coli* strain entered a VBNC state, although the cells retained the ability to produce enterotoxin after exposure to natural sunlight in an estuary. However, our results differ from those of Caro et al. [8]. In fact, the exposure of *Salmonella* cells to UV-C and seawater led to a progressive change in cell physiology. This change was characterized by a rapid loss of culturability and metabolic activity, followed by a reduction in respiratory activity and structural integrity and finally by a slight degree of damage to genome integrity [8]. Finally, the VBNC state is important if VBNC cells are capable of recovering a culturable state. One disadvantage of using culture medium for resuscitation is that resuscitation of viable cells can result in the growth of viable cells which escape detection.

Animal systems provide a complex and dynamic nutritional environment, the essential features of which may be difficult to determine or to replicate in vitro. When apparent recovery occurs, it is often assumed that this is due to the resuscitation of previously "nonculturable" cells in animals.

In this context, the recovery medium through which cells apparently return to culturability becomes critical. Perhaps man is a particularly good recovery medium [10], better in practice than any available set of laboratory media. Further studies are therefore needed to research resuscitation of VBNC *L. monocytogenes* cells after animal passage.

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REFERENCES

[1] Barcina I., Gonzales J.M., Iriberri J., Egea L., Effect of Visible Light on progressive dormancy of *Escherichia coli* cells during the survival Process in natural fresh water, Appl. Environ. Microbiol. 55 (1988) 246-251.

[2] Barcina I., Lebaron P., Vives-Rego J., Survival of allochthonous bacteria in aquatic systems: A biological approach, FEMS Microbiol. Ecol. 23 (1997) 1-9.

[3] Besnard V., Federighi M., Cappelier J.M., Evidence of VBNC state in *Listeria monocytogene*s by DVC and CTC-DAPI double staining, Food. Microbiol. 17 (2000) 697-704.

[4] Besnard V., Federighi M., Cappelier J.M., Development of a direct viable count procedure for the investigation of VBNC state in *Listeria monocytogenes*, Lett. Appl. Microbiol. 30 (2000) 1-6.

[5] Bhunia A.K., Antibodies to *Listeria monocytogenes*, Crit. Rev. Microbiol. 23 (1997) 77-107.

[6] Cappelier J.M., Lazaro B., Rossero A., Fernandez-Astorga A., Federighi M., Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable *Campylobacter jejuni* cells, Vet. Res. 28 (1997) 547-555.

[7] Cappelier J.M., Magras C., Jouve J.L., Federighi M., Recovery of viable but non-culturable *Campylobacter jejuni* cells in two models, Food Microbiol. 16 (1999) 375-383.

[8] Caro A., Got P., Lesne J., Binard S., Baleux B., Viability and virulence of Experimentally stressed Non culturable *Salmonella typhimurium*, Appl. Environ. Microbiol. 65 (1999) 3229-3232.

[9] Colwell R.R., Brayton P.R., Grimes D.J., Roszack D.B., Hug S.A., Palmer L.M., Viable but non-culturable *Vibrion cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms, Biotechnology 3 (1985) 817-820.

[10] Colwell R.R., Brayton P., Herrington D., Tall B., Huq A., Levine M.M., Viable but nonculturable *Vibrio cholerae* 01 revert to a cultivable state in the human intestine, World J. Microbiol. Biotechnol. 12 (1996) 28-31.

[11] Davies C.M., Evison L.M., Sunlight and the survival of enteric bacteria in natural waters, J. Appl. Bacteriol. 70 (1991) 265-274.

[12] Effendi I., Austin B., Dormant/unculturable cells of fish pathogen *Aeromonas salmonicida,* Microb. Ecol. 30 (1995) 183-192.

[13] Farber J.M., Losos J.Z., *Listeria monocytogenes*: a food-borne pathogen, Can. Med. Assoc. J. 138 (1988) 413-418.

[14] Farber J.M., Peterkin P.I., *Listeria monocytogenes*, a food-borne pathogen, Microbiol. Rev. 55 (1991) 476-511.

[15] Flint K.P., The long-term survival of *Escherichia coli* in river water, J. Appl. Bacteriol. 63 (1987) 261-270.

[16] Gourmelon M., Cillard J., Pommepuy M., Visible light damage to *Escherichia coli* in seawater: oxidative stress hypothesis, J. Appl. Bacteriol. 77 (1994) 105-112.

[17] Hussong D., Colwell R.R., O'Brien M., Weiss E., Pearson A.D., Weimer R.M., Burge W.D., Viable *Legionella pneumophila* not detectable by culture on agar media, Biotechnology 5 (1987) 947-950.

[18] Kogure K., Simidu U., Taga N., A tentative direct microscopic method for counting living marine bacteria, Can. J. Microbiol. 25 (1979) 415-420.

[19] Linder K., Oliver J.D., Membrane fatty acid and virulence changes in the viable but non-culturable state of *Vibrio vulnificus*, Appl. Environ. Microbiol. 55 (1989) 2837-2842.

[20] McKay A.M., Viable but non-culturable forms of potentially pathogenic bacteria in water, Lett. Appl. Microbiol. 14 (1992) 129-135.

[21] Medema G.J., Schets F.M., Van de Giessen A.W., Havelaar A., Lack of colonization of one day old chicks by viable non-culturable *Campylobacter jejuni*, J. Appl. Bacteriol. 72 (1992) 512-516.

[22] Menudier A., Bosgiraud C., Nicolas J.A., Virulence of *Listeria monocytogenes* in pregnant mice, Pathol. Biol. 42 (1994) 510-515.

[23] Oliver J.D., Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state, Appl. Environ. Microbiol. 57 (1991) 2640-2644.

[24] Oliver J.D., Formation of Viable But Non-culturable cells, in: Kjelleberg. S. Ed., Starvation in Bacteria, Plenum, New York, 1993, pp. 239-272.

[25] Paludan-Müller C., Weichart D., McDougald D., Kjelleberg S., Analysis of starvation conditions that allow for prolonged culturability of *Vibrio vulnificus* at low temperature, Microbiology 142 (1996) 1675-1684.

[26] Pommepuy M., Butin M., Derrien A., Gourmelon M., Colwell R.R., Cormier M., Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight, Appl. Environ. Microbiol. 62 (1996) 4621-4626.

[27] Rodriguez G.G., Phipps D., Ishiguro K., Ridgway H.F., Use of a fluorescent redox probe for direct visualization of actively respiring bacteria, Appl. Environ. Microbiol. 58 (1992) 1801-1808.

[28] Rollins D.M., Colwell R.R., Viable but non-culturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment, Appl. Environ. Microbiol. 52 (1986) 531-538.

[29] Roszak D.B., Colwell R.R., Metabolic activity of bacterial cells enumerated by Direct Viable Count, Appl. Environ. Microbiol. 53 (1987) 2889-2983.

[30] Roth W.G., Leckie M.P., Dietzler D.N., Restoration of colony forming activity in osmotically stressed *Escherichia coli* by betaine, Appl. Environ. Microbiol. 54 (1988) 3142-3146.

[31] Weichart D., Oliver J.D., Kjelleberg S., Low temperature induced non-culturability and killing of *Vibrio vulnificus*, FEMS Microbiol. Lett. 100 (1992) 205-210.

[32] Wolf P.W., Oliver J.D., Temperature effects on the viable but non-culturable state of *Vibrio vulnificus,* FEMS Microbiol. Ecol. 101 (1992) 33-39.

[33] Xu H.S., Robert N., Singleton F.L., Attwel R.W., Grimes D.J., Colwell R.R., Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment, Microbiol. Ecol. 8 (1982) 313-323.

[34] Yu W., Dodds W.K., Banks K.M., Skalsky J., Strauss E., Optimal staining and sample storage time for direct microscopic enumeration of total and active bacteria in soil with two fluorescent dyes, Appl. Environ. Microbiol. 61 (1995) 3367-3372.