High pressure sensitization of heat-resistant and pathogenic foodborne spores to nisin

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Short running headline: HP-sensitization of bacterial spores to nisin

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Abstract

Today, there is no effective non-thermal method to inactivate unwanted bacterial spores in foods. High-Pressure (HP) process has been shown to act synergistically with moderate heating and the bacteriocin nisin to inactivate spores but the mechanisms have not been elucidated. The purpose of the present work was to investigate in depth the synergy of HP and nisin on various foodborne spore species and to bring new elements of understandings.

For this purpose, spores of *Bacillus pumilus*, *B. sporothermodurans*, *B. licheniformis*, *B. weihenstephanensis,* and *Clostridium* sp. were suspended in MES buffer, in skim milk or in a liquid medium simulating cooked ham brine and treated by HP at 500 MPa for 10 min at 50 °C or 20 °C. Nisin (20 or 50 IU/mL) was added at three different points during treatment: during HP, during and or in the plating medium of enumeration. In the latter two cases, a high synergy was observed with the inhibition of the spores of *Bacillus* spp.. The evaluation of the germinated fraction of *Bacillus* spp. spores after HP revealed that this synergy was likely due to the action of nisin on HP-sensitized spores, rather than on HP-germinated spores. Thus, the combination of nisin and HP can lead to *Bacillus* spp. spore inhibition at 20°C. And Nisin can act on HP-treated spores, even if they are not germinated.

This paper provides new information about the inhibition of spores by the combination of HP and nisin. The high synergy observed at low temperature has not been reported yet and could allow food preservation without the use of any thermal process.

Keywords

Bacillus, *Clostridium*, psychrotrophic, highly-heat-resistant, germination

1. Introduction

When exposed to unfavorable environmental conditions, several bacterial species, mostly *Bacillus* and *Clostridia*, can enter a metabolically dormant state by forming endospores. Endospores (here after called "spores") have exceptional resistance properties against high temperatures, radiation, desiccation, pressure and toxic chemicals. They can survive under starvation for years before germinating and forming vegetative cells again.

Spore-forming bacteria play an important role in food spoilage and foodborne disease (Wells-Bennik et al. 2016). High-heat treatments, such as sterilization or Ultra-High-Temperature (UHT) treatments, are needed to inactivate spores in food products. Such treatments are usually effective to reduce spores to sufficiently low numbers; however, various species of sporeformers, such as *Bacillus sporothermodurans,* are able to survive commercial wet heat sterilization (André et al. 2013; Huemer et al. 1998). In addition, non-sterilized and low-acid food, such as refrigerated ready-to-eat-food, can be contaminated by bacterial spores, namely, from psychrotrophic species such as non-proteolytic *Clostridium botulinum* species (Mills et al. 2014). As a consequence, preservative ingredients are often required to ensure their safety.

The high-pressure (HP) process is used worldwide as an alternative to heat pasteurization or as an additional decontamination step in packaged food products. However, similar to many inactivation technologies, HP alone is not effective on bacterial spore destruction (Black et al., 2007a). If HP at a temperature lower than 50 °C can hardly inactivate spores, it has long been known that HP initiates germination of the spores of *Bacillus sp.* and *Clostridium sp.* when applied between 200 - 400 MPa (Doona et al. 2016; Reineke et al. 2013).

HP has also been shown to act synergistically with nisin to inactivate spore-forming bacteria of various species. In the abundant literature on the subject, the experimental conditions tested

69 are very different from one study to another. The tested species are different; the nisin
70 concentrations are not always expressed in the same units and vary from 0.2 to more than
71 2000 IU / mL. Finally, the contact between spores and nisin is not performed under the same
72 conditions. In most studies reporting a synergy, nisin is added at concentrations ranging from
73 120 to 500 IU/mL to the spore suspension before a combined heat and HP treatment (Aouadhi
74 et al. 2013; Black et al., 2008; Gao et al. 2011; Kalchayanand et al., 2003; Sokolowska et al.,
75 2012). Rare studies were interesting in adding nisin in the spore recovery medium after HP.
76 Lopez-Pedemonte et al. (2003) show that adding nisin at 15600 UI/mL in two-pressure-cycle-
77 HP treated cheeses increased the inactivation of *Bacillus cereus* and inhibited the regrowth
78 during 15 days. And Roberts and Hoover (1996) found that an HP treatment (400 MPa, 70 °C
79 and low pH) renders spores of *Bacillus coagulans* more sensitive to nisin present in the
80 recovery medium.
81 The objective of the present work was to investigate the effect of the combination of HP and
82 nisin on the inactivation of relevant food pathogenic and spoilage spore species in nutrient
83 media to clarify the conditions allowing the synergy. *Bacillus sporothermodurans*, *B.*
84 *pumilus*, and *B. licheniformis* were chosen for their high resistance to temperature and their
85 frequent implication in UHT and raw milk spoilage (Aouadhi et al. 2014; Klijn et al. 1997;
86 Kmiha et al. 2017). *Clostridium* sp. and *B. weihenstephanensis* were chosen regarding their
87 potential pathogenicity and ability to contaminate meat products and for growing at
88 refrigerated temperatures (Mazuet et al. 2015; Thorsen et al. 2006).
89 For this purpose, low nisin concentrations (≤50IU/mL), which highlight the synergy, were
90 added to the spore suspensions during HP treatments at 20 and 50 °C and/or after treatment in
91 the recovery medium to identify when inhibition occurs. Furthermore, without this being
92 proved, it is generally admitted that HP germination is the underlying mechanism of the

93 synergy between nisin and HP. Thus, our aim was also to clarify whether spore germination is
94 necessary for the action of nisin on HP treated spores.

95 2. Material and methods

96 2.1 Bacterial strains, growth and sporulation conditions

97 *Bacillus weihenstephanensis* KBAB4 (provided by the Institut National de Recherche
98 Agronomique, Unité SQPOV, Avignon, France), *Bacillus licheniformis, Bacillus pumilus*
99 (both isolated from UHT Tunisian milk*)*, *Bacillus sporothermodurans* (isolated from raw
100 Tunisian milk) (Kmiha et al. 2017) and *Clostridium* sp. BAFF C3DSM 1985 were used as
101 target strains in this study. As reported on the DSMZ website, this strain is, based on a partial
102 16S rDNA sequence, closely related to *Clostridium botulinum* type E, strain ATCC 23387
103 (Curators or the DSMZ n.d.).
104 Table 1 presents the conditions applied to produce the bacterial spores used in this study.
105 For *Bacillus* species, cultures were obtained by inoculating 2-3 fresh colonies into 50 mL of
106 nutrient broth. After incubation (see Table 1), 0.4 mL of the culture was spread on sporulation
107 Petri dishes (∅: 145 mm; h: 20 mm).
108 Sporulation was monitored by phase contrast microscopy and spores were harvested when
109 more than 95% of phase bright spores were observed. Spores were harvested by flooding the
110 agar plates with cold sterile distilled water and scraping the agar surface with a sterile cell
111 spreader. Spore suspensions were centrifuged (3600 *g*, 15 min, 4 °C) and spores were
112 suspended in 0.2 µm filtrated 70% ethanol (filter: cellulose acetate, Sartorius, France; ethanol:
113 Elvetec, France) for 1 h to inactivate vegetative cells (Koransky et al. 1978). Spores were then
114 washed three times by successive centrifugation (3600 *g*, 15 min, 4 °C) and suspension in
115 sterile distilled water at 4 °C. Spores were enumerated by CFU determination on their

respective enumeration media (EM). The concentration of the final spore suspensions was standardized at 10^8 CFU/mL. Suspensions were stored at 4 °C for a maximum of one month. For *Clostridium* sp., a subculture was obtained by adding 0.1 mL of stock cultures previously stored at −80 °C to 9 mL of LYBHI (Table 1) in an anaerobic Hungate tube incubated for 24 h at 30 °C. A culture was obtained by diluting 0.5 mL of subculture into 10 mL of fresh LYBHI in an anaerobic Hungate tube. After incubation, 0.2 mL of this culture was inoculated into sporulation Petri dishes (Table 1) for sporulation. After one month at 30 °C under anaerobic conditions, the spores were harvested, purified and enumerated as previously described for *Bacillus* spp. strains. The concentration of the final spore suspensions was standardized at 10^7 CFU/mL. Suspensions were stored at 4 °C and stored for up to one month.

2.2 Effect of nisin on spore cultivability

Nisin stock solution in 0.02 mol/L HCl was prepared from nisin powder (10^6 IU/g, Sigma-Aldrich, France) and sterilized by 0.2 µm filtration (cellulose acetate, Sartorius, France). Nisin stock solution was stored at 4 °C for a maximum of one week until use. The nisin level of the stock solution was confirmed by a plate diffusion assay using *Micrococcus flavus* DSM 1790, as described by Tramer and Fowler (Tramer and Fowler 1964). Following a protocol adapted from Stewart et al. (2000), nisin stock solution was added to tempered agar enumeration medium (Table 1) to achieve final concentrations ranging from 0 IU/mL to 200 IU/mL (Stewart et al., 2000). Spore suspensions were serially diluted and plated on their respective enumeration media supplemented with nisin (Table 1). Plates were incubated for 48 h (temperatures indicated in Table 1), and colonies were counted. The spore cultivability was plotted against the nisin concentration, and concentrations inducing less than −1.5 logs (CFU/mL) spore count reduction were selected for treatment combining HP and nisin.

Therefore, 50 IU/mL was chosen as the nisin concentration for all the *Bacillus* spp., and 20 IU/mL was chosen for *Clostridium* sp.

2.3 HP treatments in buffer and measurement of germination

HP tests were performed in a 700 MPa vessel (Top Industrie, France) with a double-walled metal pressure chamber of 20 cm^3 (working temperature: −20 °C / 90 °C). Water was used as the pressure-transmission fluid. The internal temperature of the pressure chamber was maintained by a water bath (Minisat 240, Huber, Germany) connected to the double wall of the pressure chamber and monitored by a Pt100 thermocouple (Omega, USA). The temperature of the water bath was set to limit the adiabatic heating to +5 °C above the desired temperature set point. The pressure and temperature during HP treatment were recorded using the instruNet World (iW) software. The compression rate was 3 MPa/s and the decompression was nearly instantaneous (< 3 sec).

Samples were prepared by a 90% dilution of the initial spore suspensions into 0.11 mol/L 2-(N-morpholino) ethane sulfonic acid (MES) buffer at pH 6.1 (Sigma Aldrich, France) to reach a final concentration of approximatively 10^7 CFU/mL for *Bacillus* spp. and 10^6 CFU/mL for *Clostridium* sp.. MES buffer was chosen because its pH varies only slightly with temperature and pressure ($\Delta pKa/\ °C = -0.011$; pH varies from 5.5 to 6.5 between 10 and 1000 MPa at 25°C) (Bruins et al. 2007). Then, 0.5 mL of each spore suspensions in MES was heat-sealed into polyethylene pouches (polyethylene transfer pipet, Dominique Dutscher). For each strain, zero-time samples were taken to determine the initial concentration of the spores before treatment.

Pouches were placed in the HP vessel 5 min before treatment to let the temperature equilibrate. Samples were treated at 500 MPa for 10 min, at 20 °C or 50 °C and immediately immersed in iced water.

After treatment, samples were divided into two equal volumes, one of which was treated at 80 °C for 10 min in a water bath to inactivate germinated spores. The two volumes were then serially diluted and 100 µL of each dilution were plated on the sample enumeration media (Table 1). The fraction of spores induced in germination by HP was calculated as the difference between the logarithmic counts of in pressure- and heat-treated (N_{HPT}) and pressure-treated (N_{HP}) portions of the samples ($\log_{10}(N_{HPT})-\log_{10}(N_{HP})$).

2.4 HP treatment with nisin

Immediately before HP treatment, spore suspensions were diluted to approximately 10^7 CFU/mL for *Bacillus* spp. and 10^6 CFU/mL for *Clostridium* sp. in MES buffer supplemented or not supplemented with nisin at 50 UI/mL or 20 UI/mL for *Bacillus* spp. and *Clostridium* sp. respectively. Immediately after HP treatment, spore suspensions were centrifuged at 11200 *g* for 5 min at 4 °C, and spores were re-suspended in PBS (phosphate buffered saline, Sigma-Aldrich, France). Spores were then plated both on their respective EM (Table 1) and on EM supplemented with nisin at 20 or 50 IU/mL for *Clostridium sp.* and *Bacillus spp.* respectively. After 48 h of incubation (see temperatures in Table 1), colonies were counted. As a control for the effect of nisin alone, non-HP-treated samples were plated on EM + nisin.

2.5 HP treatments in nutrient media

UHT skim milk (Lactel, Lactalis, France) was used as a nutrient medium for the HP treatment of *B. pumilus*, *B. sporothermodurans* and *B. licheniformis* strains.

For *B. weihenstephanensis* and *Clostridium* sp., a liquid medium was formulated to suit the physicochemical properties of cooked ham brine (pH, a_w, sugars and salt contents). The composition of this Medium Modelling Ham (MMH) was adapted from the composition of typical organic ham brine. The MMH is composed of 17.6 g/L of sodium chloride (Sigma

188 Aldrich, France); 10 g/L of peptone from porcine heart (Sigma-Aldrich, France); 5 g/L of
189 autolytic yeast extract (Biokar, France); 2.3 g/L of D-lactose monohydrate (Sigma-Aldrich,
190 France); 0.7 g/L of Sucrose (Sigma-Aldrich, France); and 0.11 mol/L MES buffer *qs* 1 L
191 (Sigma-Aldrich). The pH was adjusted to 6.1 before autoclaving.
192 Nitrites, which are usually added as preservatives in ham brine, were omitted to focus only on
193 the effects of HP and nisin on the spores.
194 For HP treatment without nisin, spores were suspended in their respective nutrient media
195 (MMH for *B. weihenstephanensis* and *Clostridium* sp. and skim milk for *B. pumilus*, *B.*
196 *sporothermodurans* and *B. licheniformis*), treated by HP at 500 MPa at 50 °C or 20 °C for 10
197 min and plated on EM for CFU determination.
198 In the case of HP treatment with nisin, the bacteriocin was present during HP treatment and
199 into the recovery medium..
200

201 ## 2.6 Statistical analysis

202 All experiments were independently performed 3 times. The effect of the factors on spore
203 inactivation after HP treatment was evaluated for each strain by analysis of variance
204 (ANOVA) using R software (R development core team, 2008). Significance was considered
205 to be when the p-value was equal to or less than 0.05. In this case, a Tukey's HSD (Honest
206 Significant Difference) test was performed to observe significant differences among the
207 conditions.
208 In the case of HP treatment in combination with nisin, the sum of the independent effects of
209 HP and nisin was compared with the effect of the treatment combining HP and nisin. When
210 the effect of the combined treatments was significantly higher than the addition of the two
211 independent effects, a synergy was attained.

212 A Student's t-test using a 5% level of significance ($p < 0.05$) was used to detect differences
213 between the sum of the HP-germinated and inactivated spore fractions and the total
214 inactivation provided by the application of nisin during and after HP.

215 3. Results and Discussion

216 3.1 Effect of HP and nisin on spore inactivation in the buffer

217 Spores were suspended in MES buffer and treated with nisin. Nisin concentrations used were
218 50 IU/mL for *Bacillus* spp. and 20 IU/mL for *Clostridium* sp.
219 Nisin was added at different stages of treatment, creating two groups of samples:
220 - Nisin present only during HP (plating on EM without nisin)
221 - Nisin present during HP and added into the plating medium at the same concentration.
222 The results obtained are shown in Fig. 1. The effect of nisin alone on the spores (striped bars)
223 represents the inhibition of spores by only plating on the EM + nisin. As expected, nisin at 50
224 IU/mL and 20 IU/mL induced approximately 1 log inhibition of *Bacillus* spp. and *Clostridium*
225 sp. spores respectively (that is what the nisin concentration was determined for). Regarding
226 the effect of HP alone, it can be noticed that pressure resistance of spores is strongly related to
227 the temperature of the treatment. While spores treated at 20 °C did not show any significant
228 inactivation (<1 log for all the strains), the viability of the spores of *B. weihenstephanensis*
229 and *B. pumilus* processed at 50 °C was strongly affected (approx. 4 log inactivation for the
230 two strains).
231 Spores of psychrotrophic species, such as *Clostridium sp.* and *Bacillus weihenstephanensis*
232 are poorly heat-resistant (Garcia et al., 2010; Juneja et al., 1995) while *Bacillus pumilus,*
233 *Bacillus licheniformis* and *Bacillus sporothermodurans* are highly heat-resistant spores
234 isolated from UHT milk (Kmiha et al., 2017). Our results confirm the absence of a link

235 between thermo- and baroresistance according to what has been reported in the rare literature
236 on the subject (Margosch et al. 2004; Nakayama et al. 1996). Three groups of spore species
237 clearly appear according to their baroresistance at a temperature ≤ 50 °C, in order of
238 increasing baroresistance: *B. weihenstephanensis*; *B. pumilus* > *B. licheniformis; B.*
239 *sporothermodurans* > *Clostridium sp*. It is well known that inactivation by HP beyond 400
240 MPa was related to the germination induction during HP treatment and the subsequent
241 resistance loss. At this pressure level, this induction was thought to be driven through spoVA
242 channel activation and Ca-DPA release in *B. subtilis* (Reineke et al. 2013). The *B. subtilis*
243 spoVA proteins are composed of 7 subunits, including the SpoVAC subunit, which has
244 recently been identified as a mechanical stress sensitive channel for DPA release (Velásquez
245 et al. 2014). *B. pumilus* and *B. licheniformis* also have genes from the same spoVA subunits
246 as *B. subtilis* (Paredes-Sabja et al. 2011), but it is not known for the other two species of
247 *Bacillus* studied here. Moreover, the lytic enzymes of the cortex can be denatured by HP at
248 500 MPa, which consequently inhibits the second stage of germination (Wuytack et al. 1998).
249 Thus, baroresistance differences could be attributed to varying sensitivities of DPA spoVA
250 channels and lytic enzymes of the cortex in different *Bacillus* species. Regarding *Clostridium*
251 spores, there are important differences in the germination mechanisms of *Bacillus* spores and
252 certain *Clostridium* species. HP has recently been shown to cause the release of DPA by some
253 *Clostridium* species (Doona et al. 2016). However, our results show that the effect of HP up to
254 600 MPa (results not shown) at the moderate temperature on the inactivation of *Clostridium*
255 sp. remains low. This corresponds to what is already reported in the literature on *C. botulinum*
256 type E (Reddy et al. 1999).
257 When nisin was present during HP, a slightly significant increase in spore inactivation was
258 found at 20 °C and 50 °C for *B. sporothermodurans* and B. *licheniformis* ($p < 0.01$). In
259 comparison with the effects of HP alone, the addition of nisin during HP provided a 1 log

supplementary inactivation for both strains. Nisin addition during HP also provided a significant and synergistic increase in the inactivation of *B. pumilus* only at 20 °C (inactivation: 2.5 log; $p < 0.001$). In contrast, nisin addition during HP did not significantly improve *B. weihenstephanensis* and *Clostridium sp.* inactivation, regardless of the temperature. The same applied for *B. pumilus* at 50 °C. However, the presence of nisin during HP and into the plating medium induced a significant, synergistic effect on the reduction of the outgrowth of *B. sporothermodurans*, *B. licheniformis* and *B. weihenstephanensis*, both at 20 °C and 50 °C ($p < 0.001$). For *Clostridium* sp. treated at 50 °C and *B. pumilus* treated at 20 °C, a significant further reduction in spore outgrowth was provided by nisin addition during HP and into the plating medium ($p < 0.01$), resulting in the addition of the independent effects of HP and nisin.

3.2 Effect of HP and nisin on spore inactivation in nutrient media

B. licheniformis, B. pumilus, and *B. sporothermodurans* in suspension in skim milk and *B. weihenstephanensis,* and *Clostridium* sp. in suspension in MMH were treated by HP at 500 MPa for 10 min at 20 °C or 50 °C. Nisin was added to the spore suspensions just before HP treatment to reach 20 IU/mL for *Clostridium* sp. and 50 IU/mL for *Bacillus* spp. to simulate the presence of nisin in HP-treated food products, nisin was also added into the plating medium. Results are shown in Fig. 2.

First, regarding the effect of HP alone, we noticed that inactivation was reduced when spores were treated in nutrient media, in comparison with the inactivation obtained after HP treatment in MES buffer. This reduction was significant for *B. pumilus* and *B. weihenstephanensis* at 50 °C for which the inactivation was reduced by 2.5 log and 2 log respectively. The protective effect of MMH on *B. weihenstephanensis* could be due to the presence of sodium chloride and sugars, inducing lower water activity (MMH a_w: 0.97)

284 (Daryaei et al. 2016). A slight protective effect was also noticed for spores treated in milk. A
285 possible explanation for this may be the natural baroprotective effect of milk, due to its high
286 content in calcium and magnesium (Black et al. 2007b; Hauben et al. 1998).
287 Second, the combination of HP and nisin gave similar results in nutrient media and in buffer.
288 Indeed, a significant, synergistic effect was obvious for all the strains when nisin was present
289 during HP and in the plating medium ($p < 0.001$), except for *Clostridium* sp. However, the
290 outgrowth inhibition of *Bacillus spp.* was lower than after HP treatments in buffer.
291 Particularly, the inhibition of *B. licheniformis* at 20 °C was significantly reduced from −5 log
292 in buffer to −3.5 log in milk ($p < 0.01$). This could be due to nisin adsorption on milk
293 proteins, resulting in a decrease in its antimicrobial activity (Lakamraju et al. 1996).
294 Nevertheless, a high synergy was found in both buffer and nutrient media for *B.*
295 *sporothermodurans*, *B. pumilus*, *B. licheniformis* and *B. weihenstephanensis*, leading to a
296 reduction of spore outgrowth of over 4 logs for all the *Bacillus* spp. strains.
297 Recovery of *Bacillus* and *Clostridium* spore growth was inhibited in the presence of nisin
298 (Egan et al. 2016; Hofstetter et al. 2013). The inhibitory effect of nisin occurred rapidly (<5
299 minutes) after the initiation of germination. It appeared that nisin prevented spores from
300 becoming metabolically active by disrupting the establishment of their membrane potential
301 (Gut et al. 2008). Nisin binds to lipid II for both spores and vegetative cells, but for spores,
302 this is not sufficient to induce membrane pore formation and thus inhibits growth recovery
303 (Gut et al. 2011). The formation of pores could also be due to the binding of nisin to the thiol
304 groups of membrane proteins which become accessible only at the time of spore germination
305 (Morris et al. 1984). It is thus commonly admitted that the synergy between HP and nisin is
306 due to the action of nisin on HP-germinated spores which appeared more sensitive to nisin
307 than spore germinated in the growth medium. Some authors also hypothesized HP-induced

physical damages to the spore outer layers (coat and cortex) allow nisin molecules access to their site of action without germination (Aouadhi et al. 2013). We thus measured the number of HP-germinated spores to clarify whether spore germination is a necessary condition for the action of nisin on HP treated spores.

3.3 Effect of HP on spore inactivation and germination induction

We hypothesized that spores, whose germination was initiated by HP, became sensitive to nisin. We thus measured the HP-induced germination rate of the spores. Spores of *B. licheniformis, B. pumilus*, *B. sporothermodurans*, and *B. weihenstephanensis* in suspension in MES buffer were treated by HP at 500 MPa for 10 min at 50 °C or 20 °C. Spores induced in germination (at least first stage) after HP were immediately inactivated at 80 °C for 10 min. Fig. 3 presents the HP-inactivated spore fraction (dark grey bars), the HP-germinated spores fraction (light grey bars) and the total spore inhibition provided by the addition of nisin during HP and into the plating medium (black bars). The cumulated inactivation (dark and light grey bars) represent the inactivation obtained after pressure and temperature treatments.

First, the major phenomenon observed after HP at 50 °C is the spore HP inactivation, while the germination without HP-inactivation represented less than 1 log for all the strains. In contrast, after HP treatment at 20°C, a large proportion of spores was germinated and not inactivated by HP.

Second, the cumulative HP-inactivated and germinated spore fraction are significantly lower than the total inhibition provided by the treatment combining HP and nisin for all the treatments, except *B. pumilus* and *B. licheniformis* at 50 °C ($p < 0.05$). For these two treatments, the spores inhibited by the combination of HP and nisin were equivalent those inactivated by a combination of HP and thermal treatment.

With the hypothesis that heat treatment after HP (80°C, 10 min.) inactivates all the HP induced-germinated spores, i.e. spore having released DPA after HP, the results presented in Fig. 3 clearly indicate that nisin sensitization does not simply come from spores induced in germination by HP. However, our results do not allow concluding if the spores are only reversibly inhibited by the combination of HP and nisin or if they are irreversibly inactivated in the presence of nisin in their growth medium. Nevertheless, HP induced a modification or an alteration of the spores which sensitized spores to nisin without necessarily inducing germination.

HP could target spore coat or cortex. Rare studies show that spores with a damaged cortex are more sensitive to nisin (Delves-Broughton et al. 1996; Rao et al. 2016).

Functionally, the spore coat constitutes an initial barrier to large molecules, such as peptidoglycan-lytic enzymes, which would otherwise have access to the spore cortex (Leggett 2012). The spore coat has also been identified as a critical resistance layer against many chemicals, especially oxidizing agents, such as nitrogen peroxide, ozone, chlorine and hypochlorite. In contrast, smaller molecules, such as the spore germinants, must presumably pass through this barrier. Nisin is a cationic peptide with a molar weight of 3300 Da, and its potential interaction with the spore coat is unknown.

Finally, the inhibition of spores by nisin after HP could be due to the modification of the properties of some inner membrane proteins, resulting in a better binding of nisin to sulfhydryl groups. Indeed, HP acts generally by promoting the unfolded form of the proteins, which are less voluminous than the native form. Pressures between 100 MPa and 500 MPa have been shown to increase the number and reactivity of sulfhydryl groups by unfolding the protein structure and exposing the interior sulfhydryl groups (Funtenberger et al. 1997; Zhang et al. 2015).

4. Conclusion

In conclusion, this work reports an important sporostatic effect of combining an HP treatment at 500 MPa and the addition of nisin into the recovery medium for several food-borne *Bacillus* species. The inhibition is significant even if the HP treatment is carried out at 20 °C and with a concentration of nisin as lower as 50 IU/ml while the regulatory concentration in Europe is 120 to 500 IU/mL. Furthermore, we demonstrated that the inhibition of growth shown by the combination of HP and nisin does not need that spores are induced in germination by HP. The underlying mechanism needs further investigation, which is currently underway with *Bacillus subtilis* to bring new elements of understanding

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Conflict of Interest

None of the authors declare a conflict of interest.

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Legend of table and figures

Table 1. Culture, sporulation and enumeration conditions

Fig 1. Effect of nisin addition on high pressure inactivation of spores (500 MPa, 10 min). Treatment in MES buffer. Nisin concentrations: 50 IU/mL for *Bacillus* spp. 20 IU/mL for *Clostridium* sp. Striped bars: effect of nisin alone; Grey bars: effect of HP treatment at 20°C; Black bars: effect of HP treatment at 50°C. DL: Detection limit. Error bars represent SD calculated from independent triplicates. The letters represent a significant difference ($p<0.05$) obtained with Tukey's HSD (Honest Significant Difference) test.

Fig 2. Effect of nisin addition on high pressure inactivation of spores (500 MPa, 10 min). Treatment in nutrient media. Spores of *B. weihenstephanensis* and *Clostridium* sp. were treated in MMH; spores of *B. sporothermodurans*; *B. pumilus* and *B. licheniformis* were treated in milk. Nisin concentrations: 50 IU/mL for *Bacillus* spp. 20 IU/mL for *Clostridium*

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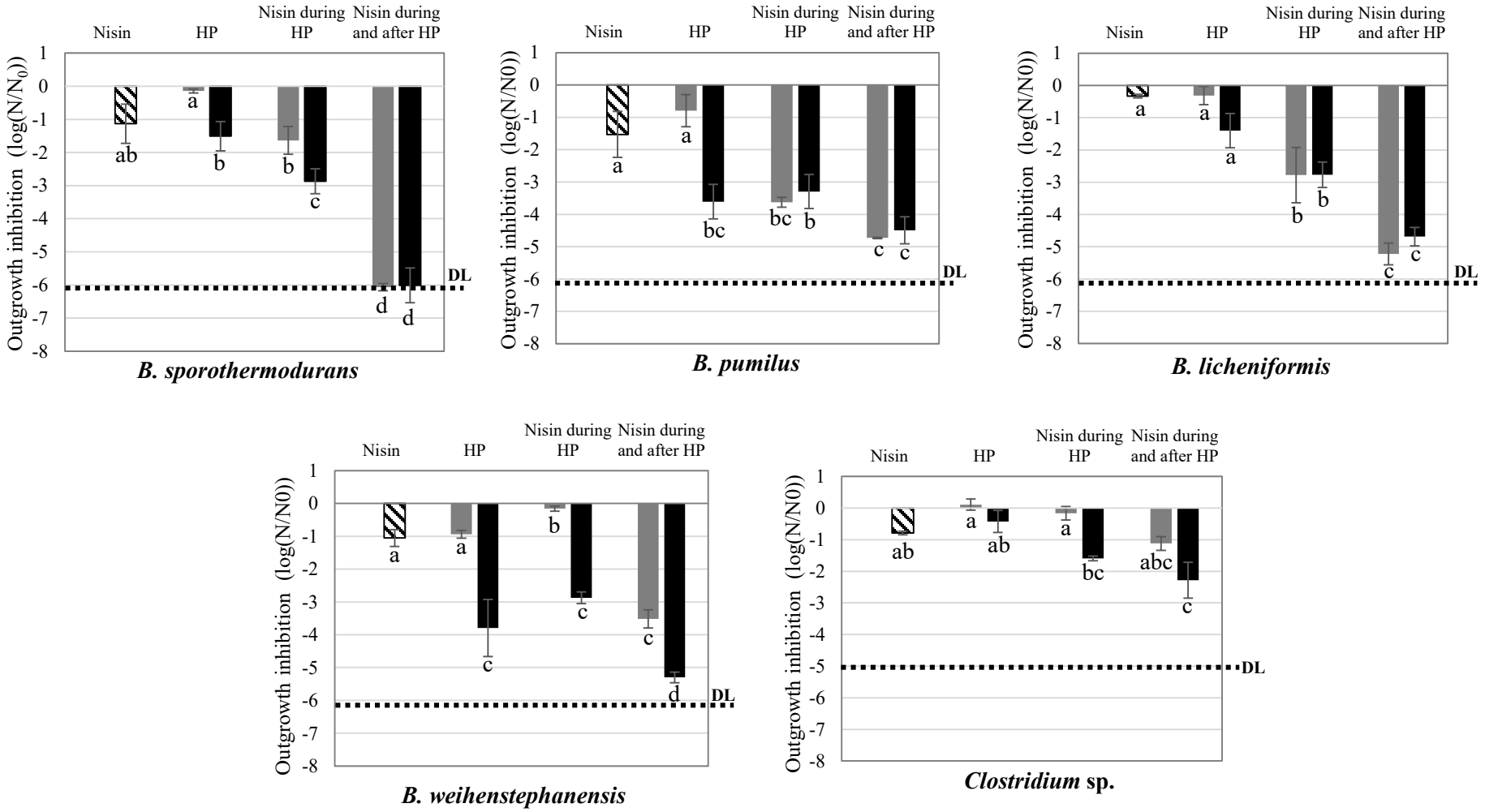
Fig 3. Inactivated and germinated spores after HP treatment at 500 MPa – 10 min: comparison with the reduction of spore outgrowth provided by the addition of nisin during HP and into the recovery medium. Dark grey: HP-inactivated spores fraction; light grey: HP-germinated (heat sensitive) spores fraction; black: inhibition of spore outgrowth induced by the nisin addition during HP and into the plating medium. (a): HP processed at 20 °C; (b): HP processed at 50 °C. Error bars represent SD calculated from triplicates and asterisks represent a significant difference between the sum of HP-inactivated and HP-germinated spore fractions, and the total outgrowth inhibition provided by nisin application during HP and into the plating medium HP (t-test, $p < 0.05$).



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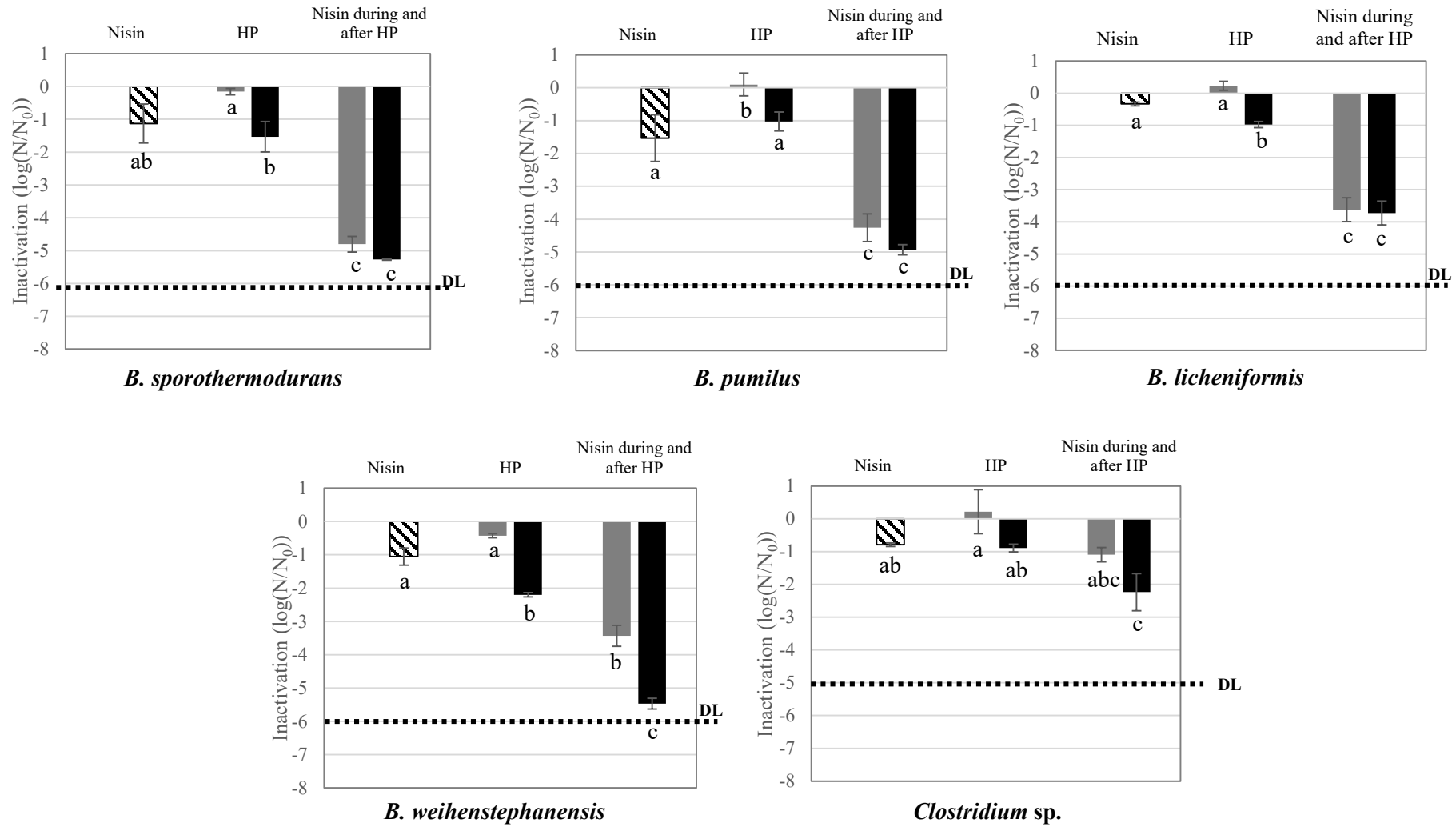

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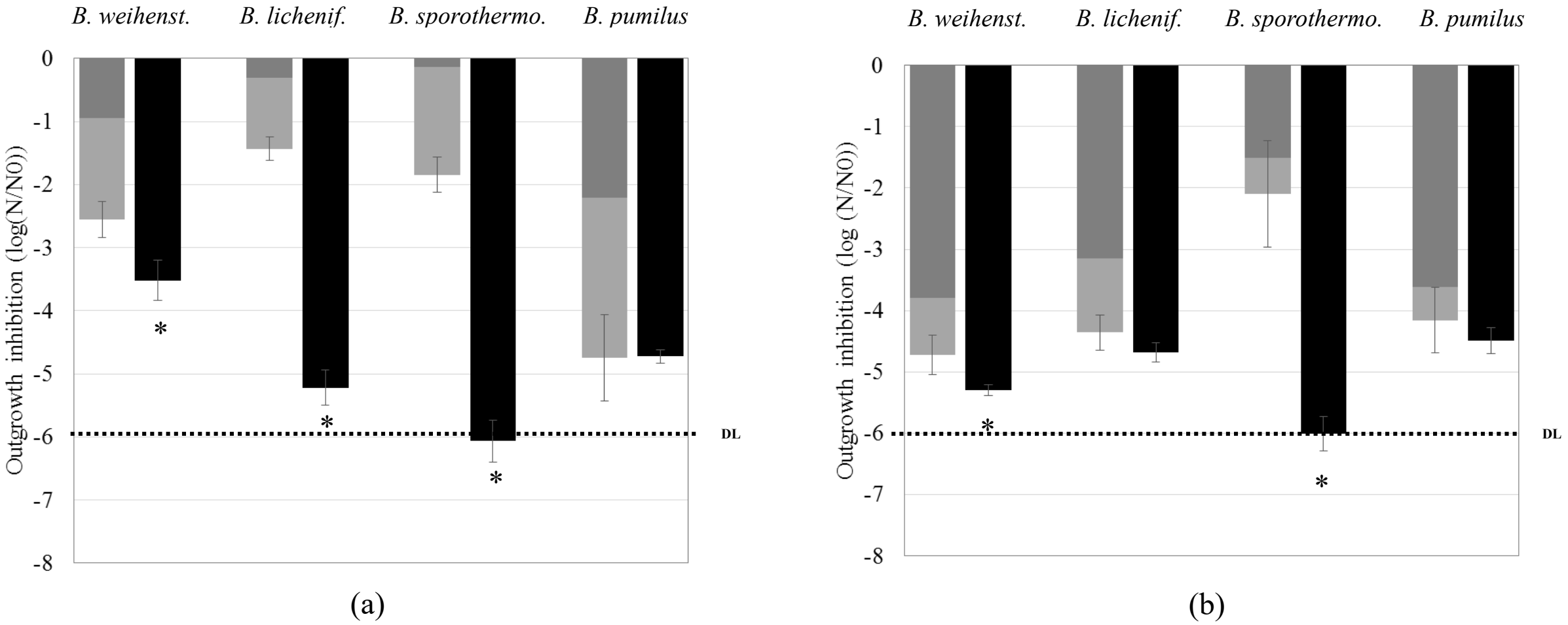

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Table 1. Culture, sporulation and enumeration conditions

Strain	Culture		Sporulation		Enumeration	
	Medium	**Incubation**	**Medium**	**Incubation**	**Medium**	**Incubation**
B. sporothermodurans	BHI vit [a]	37 °C 24 h	CMA [d]	37 °C 7 days	BHI vit + agar [a]	37 °C 24 h
B. licheniformis	BHI vit [a]	37 °C 24 h	CMA [d]	37 °C 7 days	BHI vit + agar [a]	37 °C 24 h
B. pumilus	BHI vit [a]	37 °C 24 h	CMA [d]	37 °C 7 days	BHI vit + agar [a]	37 °C 24 h
B. weihenstephanensis	CM [b]	30 °C until 0.4 < OD_{600} < 0.6	CMA [d]	30 °C 7 days	BCP [f]	30 °C 48 h
Clostridium sp.	LYBHI [c]	30 °C 24 h	VSFR [e]	30 °C 1 month	VSFR [e]	30 °C 48 h

a: **BHI vit**: Brain Heart Infusion (Sigma-Aldrich, France) 37 g/L supplemented with1 mg/L of vitamin B12 (Sigma-Aldrich, France) (Klijn et al. 1997). **BHI vit + agar**: BHI vit supplemented with 15 g/L of agar (pH: 7,2 ±0,2).
b: **CM** (Complex Medium): meat extract (Biokar, France) 10 g/L; yeast extract (Biokar, France) 2 g/L, MnSO4 (Sigma-Aldrich, France) 0.04 g/L (André et al. 2013).
c**: LYBHI**: Brain Heart Infusion (Sigma-Aldrich, France) 37 g/L, supplemented with 5 g/L of yeast extract (Biokar, France), 5 g/L of hemin (Sigma-Aldrich, France), 1 g/L of cellobiose (Sigma-Aldrich, France), 1 g/L of Maltose (Sigma-Aldrich, France); 0.5 g/L of L-cysteine (Sigma-Aldrich, France). The pH was adjusted to 7.4 before autoclaving.
d: **CMA** (Complex Medium Agar): CM supplemented with 15 g/L of agar (Biokar, France).
e: **VSFR**: meat liver glucose agar with 2 g/L yeast extract (Biokar, France) (pH: 7.4±0,2).
f: **BCP:** Bromocresol Purple Dextrose Tryptone Agar (Biokar, France) (pH: 7.0±0,2).