Running title: Carrot antilisterial activity

Listeria monocytogenes loss of cultivability on carrot is associated with the formation of mesosome-like structures

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Abstract

Raw carrot is known to have antimicrobial activity against *Listeria monocytogenes*, but the mechanism of action has not been fully elucidated. In this study, we examined carrot antilisterial activity against several strains of *Listeria* species (including *L. grayi*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*) and *L. monocytogenes*. A representative strain of *L. monocytogenes* was subsequently used for further characterizing carrot antilisterial activity. Exposure to fresh-cut carrot for 15 min resulted in a similar loss of cultivability, ranging from 2.5 to 4.7 log units, across all *Listeria* strains evaluated. *L. monocytogenes* recovered from the fresh-cut surface of different raw carrots was 1.6 to 4.1 log lower than levels obtained from paired boiled carrot samples with abolished antilisterial activity. *L. monocytogenes* levels recovered from fresh-cut carrot were 2.8 to 3.1 log lower when enumerated by culture-dependent methods than by the culture-independent method of PMAxx-qPCR, a qPCR assay that is performed using DNA pre-treated to selectively sequester DNA from cells with injured membranes. These results suggested that *L. monocytogenes* loss of cultivability on fresh-cut carrot was not associated with a loss of *L. monocytogenes* cell membrane integrity and putative cell viability. Transmission electron microscopy imaging revealed that *L. monocytogenes* rapidly formed mesosome-like structures upon exposure to carrot fresh-cut surface but not upon exposure to boiled carrot surface, suggesting there may be an association between the formation of these mesosome-like structures and a loss of cultivability in *L. monocytogenes*. However, further research is necessary to conclude the causality of this association.

1 Introduction

Listeria monocytogenes is an important foodborne pathogen that remains a significant public health concern due to its high case-fatality rate, which has been reported to range from 12 to 21% (CDC, 2013; EFSA, 2018). Additionally, it was estimated that in 2010 *L. monocytogenes* was responsible for in 23,150 illnesses and 5,463 deaths globally (de Noordhout et al., 2014). From 2009-2018, there were ten confirmed *L. monocytogenes* outbreaks associated with the consumption of ready-to-eat (RTE) fresh produce in the U.S., which resulted in 249 illnesses and 56 deaths (Sheng and Zhu, 2021). Types of fresh produce implicated in these outbreaks included cantaloupe (McCollum et al., 2013), packaged lettuce (Self et al., 2019), stone fruit (Jackson et al., 2015), and celery (Gaul et al., 2013).

The FDA draft guidance for control of *L. monocytogenes* in RTE foods considers RTE foods that have pH < 4.4, water activity < 0.92, or that are formulated with one or more inhibitory substances as "listeristatic formulations". RTE foods lacking the above characteristics are considered to have the ability to support the growth of *L. monocytogenes* and it is recommended that specific time/temperature control measures are implemented for these food products to prevent *L. monocytogenes* proliferation during storage (FDA, 2017). Raw carrot could be considered a high risk food for supporting *L. monocytogenes* growth due to its pH (range of pH 6.43-6.7) and high water activity (a_w >0.98) (Chirife and Fontan, 1982; Sant'Ana et al., 2012; Ziegler et al., 2019). However, several previous studies have shown that raw carrots do not support the growth (Alegbeleye and Sant'Ana, 2022; Girbal et al., 2021, 2020; Lokerse et al., 2016; Ziegler et al., 2019) and in some cases even suppress the survival (Farber et al., 1998; Kakiomenou and Tassou, 1998; Kroft et al., 2022; Sant'Ana et al., 2012) of *L. monocytogenes* under various storage conditions. It has been hypothesized that raw carrots contain intrinsic

antimicrobial properties (henceforth referred to as "antilisterial activity") that can negatively impact the growth of survival of *L. monocytogenes* (Babic et al., 1994; Beuchat and Brackett, 1990; Nguyen-the and Lund, 1992). Despite extensive research establishing the antilisterial activity of raw carrot against *L. monocytogenes*, the causative substance(s) responsible for this effect and the mechanism of action have not been fully elucidated. Studies evaluating the intrinsic characteristics of carrots have suggested that chemicals such as phytoalexins or phenolic compounds could be responsible for carrot antilisterial activity (Babic et al., 1994; Beuchat and Brackett, 1990; Nguyen-the and Lund, 1992, 1991; Parreiras, 1994), while others have suggested the antilisterial activity could be related to antagonistic activities of native carrot microbiota (Liao, 2007; Schifano et al., 2021).

Conventional culture-dependent enumeration methods were used in most of the previous studies establishing carrot antilisterial activity (Alegbeleye and Sant'Ana, 2022; Farber et al., 1998; Girbal et al., 2021, 2020; Kakiomenou and Tassou, 1998; Lokerse et al., 2016; Sant'Ana et al., 2012; Ziegler et al., 2019). It has been well established that microorganisms such as *L. monocytogenes* can enter a viable but non-culturable (VBNC) state under stressful conditions that could render the cells unable to grow on culture media (Highmore et al., 2018; Wideman et al., 2021). This represents a significant public health concern as cells in such a VBNC state could potentially be resuscitated and display virulence characteristics (Highmore et al., 2018). Recently, culture-independent methods such as quantitative PCR (qPCR) have become more frequently used to aid in the detection of VBNC cells (Gu et al., 2020; Truchado et al., 2021; Zeng et al., 2016); in these studies, propidium monoazide (PMA) or other DNA intercalator dyes were used to selectively sequester DNA from lysed cells or cells with injured cell membranes in order to selectively quantify *L. monocytogenes* cells with intact cell membranes.

This study was aimed at assessing *L. monocytogenes* cellular responses following exposure to raw carrot to gain insights on the potential mechanisms associated with carrot antilisterial activity. Transmission electron microscopy (TEM) was used to reveal the ultrastructural changes of *L. monocytogenes* in response to raw carrot exposure (Chen et al., 2003; Gao et al., 2019; Grigor'eva et al., 2020).

2 Materials and Methods

2.1 Bacterial Strains and Inoculum Preparation

Listeria strains were selected from the Environmental Microbial and Food Safety Laboratory (EMFSL) culture collection to include six strains of *L. monocytogenes* representing the three serotypes (1/2a, 1/2b, and 4b), and four non-*L. monocytogenes Listeria* species (*L. innocua*, *L. grayi*, *L. seeligeri*, and *L. welshimeri*) (Table 1). The ten strains were examined for their survival on the cut surface of carrot. In addition, *Escherichia coli* strain TVS354 (Tomás-Callejas et al., 2011) was also included to compare the survival of a Gram-negative bacterium to survival of *Listeria* on carrot. *L. monocytogenes* strain FS2025, associated with a 2011 cantaloupe outbreak, was used as a representative *L. monocytogenes* strain in ensuing experiments. All strains were obtained from the Environmental Microbial and Food Safety Laboratory (EMFSL) culture collection. Single colonies of each strain were inoculated into Tryptic Soy Broth (TSB; Becton, Dickinson and Company [BD]) and incubated for 24 h at 37 °C with shaking at 150 rpm. Bacterial cultures were harvested by centrifugation at 4,500 x *g*, washed three times in 10 % buffered peptone water (BPW; BD), and re-suspended in equal volume of 10 % BPW, unless otherwise specified, for an approximate inoculum suspension concentration of ~9 log CFU/mL.

111

112 **2.2 Carrots, bacterial inoculation, and culture-dependent enumeration by plating**

113 Carrots (*Daucus carota* L.) were obtained from local retail stores in and around
114 Beltsville, MD, and stored at 4 °C for up to 2 days before being used to carry out experiments.
115 The experimental workflow is schematically presented in supplemental Figure S1. Carrot
116 samples were cut into 0.5 cm transverse slices (referred to as "fresh-cut carrots") and used for
117 inoculation within 30 min. In some experiments, half of the adjoining slices from the same carrot
118 were boiled in sterile distilled water (SDW) at 95-100 °C for 10 min to serve as negative controls
119 for antilisterial activity, as it has previously been shown that the process of boiling can abolish
120 carrot antilisterial activity (Beuchat and Brackett, 1990). Each fresh-cut carrot or boiled carrot
121 sample (one or more slices weighing ~3 g) was inoculated with 100 µL (in ten 10 µL droplets) of
122 individual bacterial strain inoculum suspensions onto the cut surface. Inoculated carrots were air-
123 dried in a biosafety cabinet at ~22 °C for up to 15 min, and then either further incubated for pre-
124 defined timepoints, or immediately retrieved for microbial enumeration.

125 For processing, each sample was sonicated in a 7-oz Whirl-Pak bag with 0.3 mm filter
126 (Nasco, Fort Atkinson, WI) containing 10 mL of 10 % BPW in an ultrasonic water bath for 2
127 min at 40 kHz, along with hand massaging for 30 s prior to and after sonication to recover
128 inoculated bacterial cells. Carrot rinsates were serially diluted in 10 % BPW, and dilutions were
129 plated on the selective agars of Harlequin Listeria chromogenic agar (HLA; Neogen, Lansing,
130 MI) for enumeration of *Listeria*, and MacConkey agar (MAC, BD) for enumeration of *E. coli*. In
131 some experiments, carrot rinsates were also plated on Tryptic Soy Agar supplemented with 50
132 mg/L 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (Chem-Impex, Wood Dale, IL) (TSAG),
133 to achieve differential enumeration of *L. monocytogenes* on a non-selective agar. *L.*

134 *monocytogenes* forms characteristic blue colonies on TSAG due to its β-glucosidase activity,
135 thus allowing for the differentiation of *L. monocytogenes* from native carrot microbiota (Guo et
136 al., 2016). Plating was done by drip plating 20 µL droplets for each dilution onto the surface of
137 the agar plates (Jett et al., 1997). In addition, undiluted carrot rinsates were plated onto HLA,
138 MAC, or TSAG by spread plating 250 µL onto the surface of the agar when the microbial counts
139 were expected to be low. HLA and TSAG plates were incubated for 48 h at 37 °C, and MAC
140 plates were incubated for 24 h at 37 °C prior to enumeration of colonies. The limit of
141 quantification (LOQ) for direct plate count enumeration was 1.1 log CFU/g.
142
143 **2.3 Microbial enumeration using culture-independent methods**
144 Quantitative real-time PCR (qPCR) was used for culture-independent *L. monocytogenes*
145 enumeration. Cells in two 1 mL aliquots of each carrot rinsate were precipitated at 14,000 x *g* for
146 10 min, and then one bacterial pellet was subjected to treatment with DNA cross-linker
147 propidium monoazide (PMAxx; Biotium, Fremont, CA) prior to DNA extraction. PMAxx
148 treatment of bacterial cells was performed as described previously (Gu et al., 2022) to aid in the
149 selective enumeration of DNA from cells with intact cell membranes. After removal of residual
150 PMAxx by centrifugal precipitation and three sequential washings in phosphate buffered saline
151 (PBS; Corning, NY), both PMAxx treated and untreated cells were re-suspended in 150 µL Tris-
152 EDTA buffer (Thermo Fisher Scientific, Waltham, MA) supplemented with 10 mg/mL lysozyme
153 (Epicentre, Madison, WI) and 5 mg/mL proteinase K (Epicentre), incubated for 10 min at 37 °C,
154 followed by DNA extraction using the DNeasy Powersoil kit (Qiagen, Gaithersburg, MD)
155 according to the manufacturer's instructions. As a control to gauge the effectiveness of PMAxx
156 to sequester DNA from cells with compromised membrane integrity, 1 mL aliquots of carrot

157 rinsates were heated to 95 °C for 10 min. These “heat-killed” rinsates were similarly subjected to
158 PMAxx treatment and DNA extraction as described above.
159 qPCR assays were conducted on a CFX96 Touch Real-Time PCR System (Bio-Rad
160 Laboratories, Hercules, CA) to estimate populations of *L. monocytogenes* prepared with and
161 without PMAxx treatment. For both assays, *hly* was targeted using primers
162 (5’GGGAAATCTGTCTCAGGTGATGT and 5’CGATGATTTGAACTTCATCTTTTGC),
163 reagents, and the amplification procedure described previously (Gu et al., 2020), and each
164 reaction was run in triplicate. Standard curves were generated for each independent qPCR run
165 using seven 10-fold serial dilutions of chromosomal DNA extracted from *L. monocytogenes*
166 FS2025 inoculum. Based on the standard curve, the effective range of *L. monocytogenes*
167 quantification for qPCR was 3.7-8.7 log CFU/g.
168
169 **2.4 Transmission Electron Microscopy**
170 The effect of carrot exposure on the ultrastructure of *L. monocytogenes* FS2025 and *E.*
171 *coli* TVS354 was evaluated through Transmission Electron Microscopy (TEM) imaging. Fresh-
172 cut and boiled carrot slices were inoculated as described in section 2.2. After the pre-defined
173 time of incubation, 5 plugs of carrot were excised from each sample using a 1.5 mm sterile
174 biopsy punch and engulfed in 6% agar (at 85 °C) to encapsulate the bacteria on the surface of the
175 carrot through the duration of the embedding procedure. The agar around the carrot was trimmed
176 to 3mm cubes and fixed in 2.5 % glutaraldehyde, 0.05 M NaCacodylate, 0.005 M CaCl2 (pH
177 7.0) for 2 h. Fixed carrot plugs and cell pellets were rinsed with 0.05 M NaCacodylate, 0.005 M
178 CaCl2 buffer and post-fixed in 1 % buffered osmium tetroxide for 2 h at 22 °C. All samples were
179 then rinsed again in the same buffer, dehydrated in a graded series of ethanol (25%, 50%, 75%,

100%), followed by 3 exchanges of propylene oxide, infiltrated in a graded series of LX-112 resin/propylene oxide and polymerized in LX-112 resin at 65 °C for 24 h. Ultrathin sections of 70 nm were cut on a Leica UC7 ultramicrotome with a Diatome diamond knife and mounted onto 100 mesh carbon/formvar-coated copper grids. Grids containing thin sections were subsequently stained with 4 % uranyl acetate and 3 % lead citrate and imaged at 80 kV with a Hitachi HT-7700 transmission electron microscope (Hitachi High Technologies America, Inc., Schaumburg, IL). For experimental controls, cell pellets from *L. monocytogenes* and *E. coli* inoculum suspensions were also evaluated using a similar embedding procedure as described above.

2.5 Statistical analysis

Data were analyzed in R, version 4.0.2 (R Core Team). Linear regression models were fit using the lme4 package (Bates et al., 2015) to determine the fixed effects of explanatory variables of bacterial strain (see Table 1), exposure time (in min), and enumeration method used (i.e., plating on selective media, plating on non-selective media, qPCR, or PMAxx-qPCR) on bacterial levels recovered from carrot surfaces. Analysis of variance (ANOVA) was performed on linear regression models, followed by *post hoc* analysis of estimated marginal means with Tukey adjustment using the emmeans package in R (Lenth, 2019). An unpaired t-test was used to compare the levels of *L. monocytogenes* recovered from boiled carrot surfaces to the levels of *L. monocytogenes* recovered from fresh-cut carrots. In addition, two linear mix-effects regression models were fit using the lme4 package to assess for the random effect of the six different brands (i.e., carrot packages) on the outcome of *L. monocytogenes* recovered from the carrot surface, where one model assessed this outcome for carrots that were boiled prior to *L. monocytogenes*

inoculation (boiled carrots), and the other model assessed this outcome for carrots that were not boiled prior to *L. monocytogenes* inoculation (fresh-cut carrots). P values of <0.05 were considered statistically significant.

3 Results

3.1 Survival of *Listeria* and *E. coli* strains on the surface of fresh-cut carrots

Strains of five different species of *Listeria*, including *L. grayi* (1), *L. innocua* (1), *L. monocytogenes* (6), *L. seeligeri* (1), and *L. welshimeri* (1), and one strain of *E. coli* (strain TVS354) were exposed to fresh-cut carrots for 15 min (Table 1). The levels of each bacterial strain following incubation on fresh-cut carrot were compared to the levels of each bacterial strain that were inoculated into 10 % BPW and incubated for the same length of time to evaluate the effect of exposure to fresh-cut carrot on bacterial survival by selective plating. The levels of *Listeria* strains recovered after incubation in 10 % BPW ranged from 6.6 to 7.3 log CFU/mL. In comparison, all *Listeria* strains evaluated showed a population reduction of 2.5 to 4.7 log (levels recovered ranged from 2.0 to 3.4 log CFU/mL) following 15-min exposure to fresh-cut carrot; these levels were all significantly lower than levels recovered in 10 % BPW ($p<0.05$) but were not significantly different from each other across all *Listeria* strains evaluated ($p>0.05$). These results demonstrated that the antilisterial activity of carrot resulted in similar reductions of all strains of *Listeria* evaluated here. Based on these observations, *L. monocytogenes* FS2025, a strain isolated from a cantaloupe associated outbreak, was used as a representative strain for *Listeria* in all ensuing experiments. The level of *E. coli* recovered in 10 % BPW (6.8 ± 0.1 log CFU/mL) was not reduced following exposure to fresh-cut carrot.

3.2 *L. monocytogenes* inactivation on fresh-cut carrots compared to boiled carrots

The recovery of *L. monocytogenes* FS2025 (referred to henceforth as *L. monocytogenes*) after exposure to fresh-cut carrots for 15-min was compared to that from boiled carrot samples evaluated for six different individual packages of carrots obtained from local retailers with different brand labels on each package (with three biological replicates evaluated per carrot package per treatment). The average level of aerobic plate count (APC) on fresh-cut carrots not inoculated with *L. monocytogenes* was 4.3 ± 0.2 log CFU/g, and no enumerable levels of APC were able to be recovered from boiled carrots (LOQ of 1.1 log CFU/g). The recovery of *L. monocytogenes* from boiled carrots ranged from 7.1 to 7.6 log CFU/g. In comparison, *L. monocytogenes* populations were reduced by 1.6 to 4.1 log after 15-min exposure on fresh-cut carrots, with an average of 4.7 ± 0.3 log CFU/g recovered from fresh-cut carrot samples. An unpaired t-test showed that the levels of *L. monocytogenes* recovered from boiled carrot surfaces were significantly higher than levels of *L. monocytogenes* recovered from fresh-cut carrot surfaces ($p<0.05$). Two linear mixed effects models were generated to compare the random effect of carrot package on the recovery of *L. monocytogenes* from (i) boiled carrots and (ii) fresh-cut carrots separately to account for any random variation that could result from the six different carrot packages for carrots that were either boiled or left fresh-cut prior to *L. monocytogenes* inoculation (Table 2). According to these analyses, for (i) boiled carrots the variance in *L. monocytogenes* estimated populations between packages of carrots is 0.2, whereas as for (ii) fresh-cut carrots the variance in *L. monocytogenes* estimated populations between packages of carrots is 1.1. This suggests that predicted *L. monocytogenes* levels will vary by ~1.1 log CFU/g depending on the package of carrots that is challenged with *L. monocytogenes*.

249 **3.3. *L. monocytogenes* shows increased decline on fresh-cut carrots over time**

250 The effect of exposure time on *L. monocytogenes* recovery was examined on fresh-cut
251 carrots (Figure 1). In general, *L. monocytogenes* recovery decreased with increased exposure
252 time on fresh-cut carrots. *L. monocytogenes* levels after 1 min (8.0 ± 0.1 log CFU/g) and 5 min
253 (6.3 ± 0.8 log CFU/g) exposures were significantly higher than levels after 30 min (1.9 ± 0.3 log
254 CFU/g) and 120 min (2.5 ± 0.4 log CFU/g) exposure times ($p<0.05$). *L. monocytogenes* levels
255 recovered after 120 min exposure to fresh-cut carrot were slightly higher than those recovered
256 after 30 min exposure (by ~ 0.6 log units), but these differences were not considered significant
257 based on Tukey's Honestly Significant Difference (HSD) *post hoc* test ($p>0.05$) (Figure 1). *L.*
258 *monocytogenes* levels on fresh-cut carrot were also evaluated following 24 h exposure at both 4
259 °C and 25 °C. After 24 h exposure time, for fresh-cut carrot samples stored at 4 °C, only one of
260 four biological replicates showed levels of *L. monocytogenes* above the LOQ, at 3.6 log CFU/g,
261 and for carrot samples stored at 25 °C, *L. monocytogenes* levels were below the LOQ (<1.1 log
262 CFU/g) for all four biological replicates.
263
264 **3.4 *L. monocytogenes* levels recovered from fresh-cut carrots are higher when enumerated**
265 **using culture-independent enumeration methods compared to culture-dependent**
266 **enumeration methods**

267 After exposure to fresh-cut carrot and boiled carrot surfaces for 30 min, *L. monocytogenes*
268 was enumerated using traditional culture-dependent (i.e., plating on selective and non-selective
269 agar) and culture-independent (i.e., PMAxx-qPCR and qPCR) methods (Table 3). The average
270 level of APC on fresh-cut carrots not inoculated with *L. monocytogenes* was 5.4 ± 0.1 log
271 CFU/g, and no enumerable levels of APC were able to be recovered from boiled carrots (LOQ of

272 1.1 log CFU/g). For culture-dependent enumeration, two-way ANOVA and *post hoc* tests
273 showed that *L. monocytogenes* levels recovered from fresh-cut carrots were not significantly
274 different whether enumerated on selective agar (HLA, average of 4.4 ± 0.2 log CFU/g) or non-
275 selective agar (TSAG, average of 4.1 ± 0.4 log CFU/g) ($p>0.05$), but were significantly lower
276 than levels recovered from boiled carrots for both selective (average of 8.1 ± 0.1 log CFU/g) and
277 non-selective (average of 8.3 ± 0.1 log CFU/g) ($p<0.05$) agars (Table 3).
278 For culture-independent methods, *L. monocytogenes* levels enumerated by qPCR for fresh-
279 cut carrots (average of 8.2 ± 0.1 log CFU/g) were not significantly different from levels obtained
280 from boiled carrots (average of 7.8 ± 0.2 log CFU/g) ($p>0.05$). Similarly, for *L. monocytogenes*
281 levels enumerated by PMAxx-qPCR, levels obtained from fresh-cut carrot samples (average of
282 7.2 ± 0.1 log CFU/g) were also not significantly different from levels obtained from boiled carrot
283 samples (average of 7.2 ± 0.1 log CFU/g) ($p>0.05$). These observations indicated that the
284 antilisterial activity of fresh-cut carrots did not result in a loss of membrane integrity in *L.*
285 *monocytogenes* cells. When *L. monocytogenes* cells in the fresh-cut carrot rinsate were "heat-
286 killed" prior to enumeration, both plating on selective and non-selective agars (LOQ 1.1 log
287 CFU/g) and PMAxx-qPCR (LOQ 3.7 log CFU/g) did not yield quantifiable levels of *L.*
288 *monocytogenes*, whereas qPCR without PMAxx treatment yielded levels equivalent to an
289 average of 7.3 ± 0.2 log CFU/g (Table 3). These results demonstrate that the PMAxx treatment
290 used in this study was effective in sequestering *L. monocytogenes* DNA with damaged or
291 otherwise compromised cell membranes.
292 While there was good agreement between the culture-dependent and culture-independent
293 enumerations of *L. monocytogenes* levels recovered from boiled carrots, significant differences
294 between these two types of enumeration methodologies were observed when examining the

295 recovery of *L. monocytogenes* from fresh-cut carrot samples. *L. monocytogenes* levels obtained
296 by plating (on both selective and non-selective agar) were 2.8 to 3.1 log lower than levels
297 enumerated by PMAxx-qPCR ($p<0.05$), and 3.8 to 4.1 log lower than levels enumerated by
298 qPCR without PMAxx treatment ($p<0.05$) (Table 3). These results indicated a loss of
299 cultivability in over 99 % (>2 log decrease) of *L. monocytogenes* cells that were exposed to
300 fresh-cut carrots.
301
302 **3.5 *L. monocytogenes* shows the presence of mesosome-like structures after exposure to**
303 **fresh-cut carrots**
304 Ultrathin sectioning and TEM imaging of *L. monocytogenes* on carrot surfaces was
305 performed to assess ultrastructural changes in *L. monocytogenes* following exposure to fresh-cut
306 and boiled carrot surfaces (Figure 2). *L. monocytogenes* cells, irrespective of carrot surface
307 exposure, did not exhibit obvious morphological changes such as contour distortion, cell
308 wall/membrane disintegration, or cytoplasm leakage. Nevertheless, compared to *L.*
309 *monocytogenes* cells in the inoculum suspension that was used to inoculate carrot samples, or *L.*
310 *monocytogenes* cells that were inoculated onto boiled carrot surfaces, *L. monocytogenes* cells
311 exposed to fresh-cut carrots showed noticeable changes in cellular ultrastructure, characterized
312 by the presence of one or more large spindle-like structures. These structures seemed indicative
313 of lamellar membrane invaginations (Figure 2A-C), which were reminiscent of structures that
314 have been previously described as mesosome-like structures (Greenawalt and Whiteside, 1975).
315 Notably, *L. monocytogenes* cells in the inoculum suspension and those exposed to boiled carrot
316 surfaces did not display these mesosome-like structures (Figure 2D-F). In addition, such

mesosome-like structures were not present in *E. coli* TVS354 cells that were inoculated onto carrot fresh-cut surfaces (Figure 3A-B).

Ultrathin sectioning and TEM imaging was also performed in conjunction with plating (results described in section 3.3) to examine the effect of exposure time to fresh-cut carrot surface on changes in *L. monocytogenes* morphology (Figure 4). The presence of mesosome-like structures in *L. monocytogenes* cells were observed at as early as 1 min after exposure to the surface of fresh-cut carrot (Figure 4B), and at all subsequent sampling timepoints (Figure 4C-I). This suggested that mesosome-like structures formed rapidly following exposure to fresh-cut carrot surface. Additionally, the mesosome-like structures seemed to progressively become denser as the exposure time increased (Figure 4B-I).

4 Discussion

Although the antilisterial activity of carrot has been recognized for over 30 years (Beuchat and Brackett, 1990; Nguyen-the and Lund, 1991), its mechanism of action and its potential of application as an antilisterial tool have remained elusive. In this study, we first showed that carrot's antilisterial activity could result in a significant loss of cultivability in several strains of *Listeria* when using culture-dependent enumeration methods, then we showed that this loss of cultivability was not associated with a loss of *Listeria* cell membrane integrity. Interestingly, this loss of cultivability was found to be correlated with the formation of mesosome-like structures in *Listeria* cells.

The loss of cultivability following brief exposure to carrot fresh-cut surfaces was observed at comparable levels across ten *Listeria* strains representing five unique species, indicating that the antilisterial activity of carrot is not species or strain specific. The differential recovery of *L.*

340 *monocytogenes* after exposure to fresh-cut and boiled carrots was indicative of the heat-labile
341 nature of the carrot antilisterial activity, and was consistent with previous studies that have
342 demonstrated that the antilisterial activity of raw carrots results in a loss of *L. monocytogenes*
343 cultivability when using culture-dependent enumeration methods (Babic et al., 1994; Nguyen-the
344 and Lund, 1992). When six different packages with different brand labels of carrots were
345 evaluated for their antilisterial activity against *L. monocytogenes* FS2025, a wide range (1.6 to
346 4.1) of log reductions were observed after short incubation (15 min) on the fresh-cut carrot
347 surface, which suggests that carrot antilisterial activity can vary considerably. Carrot antilisterial
348 activity has been reported to be soluble in aqueous solutions (Beuchat and Brackett, 1990),
349 which represents a potential explanation for the variance in carrot antilisterial activity observed
350 in this study. While to date the specific compound(s) associated with the antilisterial activity of
351 carrot has not been isolated and causally linked to inactivation of *L. monocytogenes*, several
352 studies have speculated that this antlisterial activity could be associated with antagonistic
353 activities of native carrot microflora (Liao, 2007; Schifano et al., 2021), or antimicrobial
354 chemical compounds produced by plants as a defense mechanism for microbial attacks (also
355 known as phytoalexins) such as 6-methoxymellein and falcarindiol (Kurosaki and Nishi, 1983;
356 Parreiras, 1994). Because the kinetics of *L. monocytogenes* inactivation observed in our study
357 resulted in high log reductions of up to 4.1 log units over a short exposure time (15 min), we
358 hypothesize that the antilisterial activity of fresh-cut carrots observed in this study is more likely
359 to be associated with antimicrobial chemical compound(s) as opposed to the antagonistic
360 activities of native carrot microflora. For example, in (Liao, 2007) the authors observed that *L.*
361 *monocytogenes* levels decreased 2.8-3.9 log when *L. monocytogenes* was co-cultured with native
362 carrot microflora for a long incubation period (48 h at 28 °C), while in (Parreiras, 1994) the

363 authors observed a 4-log reduction of *L. monocytogenes* when *L. monocytogenes* was cultured in
364 the presence of 1 % ethanolic extract of carrot for 5 h, which is more consistent with the log
365 reductions observed in this study. Moreover, the volatile nature of antimicrobial compounds such
366 as 6-methoxymellein and falcarindiol supports our observations of the wide range of *L.*
367 *monocytogenes* log reductions (1.6 to 4.1) on fresh-cut carrots. Both 6-methoxymellein and
368 falcarindiol have been shown to change in concentration following exposure to processing
369 conditions such as exposure to Ultraviolet-C radiation (Mercier et al., 1993), and washing
370 (Seljåsen et al., 2013). As the previous processing and storage conditions of the six packages of
371 carrots evaluated in this study were not collected prior to obtaining them from local retail
372 facilities, speculation related to the effect of such conditions on the antilisterial activity of carrots
373 is beyond the scope of our investigation here. Regardless, future research should focus on
374 identifying the causal chemical(s) associated with carrot antilisterial activity and explore their
375 potential application for mitigating food safety risks associated with *L. monocytogenes*.
376 To ensure that the loss of cultivability of *L. monocytogenes* following exposure to carrot
377 fresh-cut surfaces was not due to the limitations in our study's recovery methodology, the carrot
378 rinsates of both inoculated fresh-cut and boiled carrots were compared for *L. monocytogenes*
379 enumeration using culture-dependent (plating on selective and non-selective agars) and culture-
380 independent (qPCR and PMAxx-qPCR) enumeration methods. qPCR enumeration without
381 PMAxx pre-treatment showed that rinsates from both fresh-cut and boiled carrots contained
382 comparable levels of *L. monocytogenes* cells, validating the efficacy of the recovery
383 methodology used in this study. It also revealed that, while *L. monocytogenes* in the rinsate from
384 boiled carrot was enumerated at comparable levels using either culture-dependent or culture-
385 independent enumeration methodologies, *L. monocytogenes* in the fresh-cut carrot rinsate was

386 enumerated approximately 2.8 to 4.1 log lower using culture-dependent methodologies compared
387 to culture-independent methodologies, indicating that over 99 % of *L. monocytogenes* cells lost
388 cultivability. *L. monocytogenes* counts were comparable on both selective and non-selective
389 agars, indicating the reduced *L. monocytogenes* recovery by culture-dependent enumeration was
390 not due to cell injuries that could be associated with plating cells on non-selective media (Wu,
391 2008).

392 Treatment of cells with membrane impermeable DNA crosslinking dyes such as PMAxx has
393 become a valuable tool for distinguishing bacterial cells with intact cell membranes, and are thus
394 deemed viable, from cells with compromised cell membrane integrity that are deemed not viable
395 (Dong et al., 2020). Since PMAxx is cell impermeable, only lysed cells or cells with injured or
396 compromised cell membranes can be sequestered by PMAxx, and thus unable to be amplified by
397 subsequent qPCR enumeration (Truchado et al., 2021). In this study, we observed that, despite a
398 2.8 to 3.1 log differential in *L. monocytogenes* exposed to fresh-cut carrots by plating and
399 PMAxx-qPCR, PMAxx-qPCR enumeration from the rinsates of fresh-cut carrots was not
400 significantly different from the rinsates of boiled carrots. This observation suggested that *L.*
401 *monocytogenes* cells in fresh-cut carrot rinsates did not lose cell membrane integrity and could
402 hence maintain viability, despite a significant loss of cultivability on selective and non-selective
403 agars. This may imply that exposure to fresh-cut carrot could lead to the induction of a VBNC
404 state for *L. monocytogenes* cells (Wideman et al., 2021).

405 Regardless of its chemical nature, the substance underlying carrot antilisterial activity
406 seemed to trigger rapid cellular response resulting in the formation of mesosome-like structures.
407 Mesosome-like structures have been previously observed in bacterial cells treated with
408 antibiotics such as amikacin, ciprofloxacin, gentamicin, oxacillin, penicillin G, rifampicin,

409 trimethoprim, and vancomycin (Li et al., 2014; Santhana Raj et al., 2007), and have been
410 hypothesized to form in response to chemical or physical injuries to cells. However, the process
411 through which these mesosome-like structures form is not fully understood (Feng et al., 2022;
412 Morita et al., 2015). Mesosome-like structures have been previously observed in *L.*
413 *monocytogenes* cells that were exposed to linalool (Gao et al., 2019). In this study, mesosome-
414 like structures were absent both in *L. monocytogenes* cells in the inoculum suspension and those
415 exposed to boiled carrots.
416 Several studies have observed the presence of mesosome-like structures in bacteria after
417 treatment with antimicrobial substances at concentrations that inhibit cell cultivability through
418 culture-dependent enumeration, including *Staphylococcus aureus* treated with silver
419 nanoparticles (Krychowiak et al., 2018), *S. aureus* treated with cationic peptides (Grigor'eva et
420 al., 2020), *S. aureus* treated with retinoids (Kim et al., 2018), *Bacillus subtilis* treated with
421 zeylasteral (De León and Moujir, 2008), *B. subtilis* treated with celastrol (Padilla-Montaño et al.,
422 2021), and *S. aureus* cells treated with the bactericidal antibiotics (Santhana Raj et al., 2007).
423 Thus, in this study the presence of mesosome-like structures in ultrastructures of *L.*
424 *monocytogenes* on fresh-cut carrot, and the notable absence of mesosome-like structures in
425 ultrastructures of *L. monocytogenes* on boiled carrot, could provide evidence to support that the
426 antilisterial properties of fresh-cut carrot responsible for reducing *L. monocytogenes* cultivability
427 are also responsible for eliciting the formation of these mesosome-like structures.
428 Interestingly, although membrane invagination was evident in certain cells with such
429 mesosome-like structures, *L. monocytogenes* ultrastructures on fresh-cut carrot did not show any
430 visible damage to the cell membrane. This is in agreement with the PMAxx-qPCR analysis
431 findings in this study, which showed that the majority of *L. monocytogenes* cells exposed to

carrot fresh-cut surfaces likely had intact cell membranes. Two studies have previously reported the presence of mesosome-like structures in *B. subtilis* cells that do not display loss of cell membrane integrity as determined through BacLight live/dead staining assays (De León and Moujir, 2008; Padilla-Montaño et al., 2021). While to date there is limited knowledge on the specific functionality of mesosome-like structures in bacterial cells, results shown here as well as previous observations (De León and Moujir, 2008; Padilla-Montaño et al., 2021) support a hypothesis that the presence of mesosomes is not necessarily associated with a loss of cell membrane integrity. Since the observed mesosome-like structures were closely associated with *L. monocytogenes* populations losing cultivability but retaining cell membrane integrity, it would be of significant scientific interest to determine whether such structures could constitute a physical hallmark indication of cells entering a VBNC state.

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642 # Figure legends

643 **Figure 1. Kinetics of *L. monocytogenes* FS2025 levels recovered from fresh-cut carrot**
644 **surfaces incubated at 22 °C for 120 min.** Each dot is the arithmetic mean level of *L.*
645 *monocytogenes* (reported as log CFU/g), and the error bars represent the calculated standard
646 error means from all biological replicates (n=4). Arithmetic means that do not share an uppercase
647 letter are significantly different based on Tukey's honestly significant difference (HSD) *post hoc*
648 test.

649

650 **Figure 2. Transmission electron microscopy images of *L. monocytogenes* FS2025 incubated**
651 **on raw vs. boiled carrots for 30 min showing mesosome like structures**. (A, B): *L.*
652 *monocytogenes* on the surface of fresh-cut carrot, shown in cross-section and longitudinal views,
653 with arrowheads indicating the presence of mesosome-like structures. (C): High magnification
654 image of *L. monocytogenes* with an arrow indicating a continuous connection between the
655 mesosome-like structure and the cell membrane, likely forming initially as an invagination of the
656 cell membrane. (D, E): *L. monocytogenes* on the cut surface of boiled carrot, shown in cross-
657 section and longitudinal views. (F): Control *L. monocytogenes* inoculum suspension not exposed
658 to carrot.

659

660 **Figure 3. Transmission electron microscopy images of *E. coli* TVS354 exposed to the**
661 **surface of fresh-cut carrot surface for defined time intervals**. (A) *E. coli* exposed to fresh-cut
662 carrot for 30 min at 22 °C, showing no mesosome-like structures. (B) *E. coli* exposed to fresh-cut
663 carrot for 24 h at 25 °C, showing no mesosome-like structures after an extended duration

664 exposed to fresh-cut carrot. (C) Control *E. coli* inoculum suspension that was not exposed to
665 carrot.
666
667 **Figure 4. Transmission electron microscopy images of *L. monocytogenes* FS2025 exposed to**
668 **the surface of fresh-cut carrot surface for defined time intervals showing kinetics of the**
669 **formation of the mesosome-like structures.** (A-F): *L. monocytogenes* exposed to fresh-cut
670 carrot for 0, 1, 5, 10, 30 min, and 2 h at 22 °C, showing an increase in size and abundance of
671 mesosome-like structures over time. (G): *L. monocytogenes* exposed to fresh-cut carrot for 24 h
672 at 4 °C. (H, I): *L. monocytogenes* exposed to fresh-cut carrot for 24 h at 25 °C. Arrowheads
673 indicating the presence of mesosome-like structures, and arrows indicating an apparent
674 continuous connection between the mesosome-like structure and the cell membrane.
675

676 **Table 1.** Bacterial strains used in this study and their log reductions after exposure to fresh-cut
677 carrot surface for 15 min.
678

Strain ID	Species	Serovar	Isolation Source	Average Reduction (Log)[a]
FS2001	*L. grayi*	NP [b]	NP	4.6 ± 0.4 A
FS2022	*L. seeligeri*	NP	NP	3.8 ± 0.2 A
FS2023	*L. welshimeri*	NP	NP	4.2 ± 0.1 A
FS2025	*L. monocytogenes*	1/2b	Cantaloupe	4.3 ± 0.1 A
FS2030	*L. monocytogenes*	1/2a	Cantaloupe	2.5 ± 0.2 A
FS2061	*L. monocytogenes*	1/2b	Cantaloupe	4.3 ± 0.2 A
FS2063	*L. monocytogenes*	4b	Cheese	4.5 ± 0.1 A
FS2064	*L. monocytogenes*	1/2a	Celery	4.7 ± 0.1 A
FS2065	*L. monocytogenes*	1/2b	Cantaloupe	4.0 ± 0.2 A
FS2066	*L. innocua*	6a	Bovine	4.3 ± 0.1 A
TVS354	*E. coli*	NP	Lettuce	0.0 ± 0.1 B

679

680 [a] Log reduction was calculated by subtracting individual target bacterial counts recovered on fresh-cut
681 carrot surface (in log CFU/mL) from those recovered in 10 % BPW (in log CFU/mL) after 15-min
682 incubation under the same condition. Values were represented as arithmetic mean ± standard error (n=3).
683 Different uppercase letters within the column indicated a significant difference ($p<0.05$) based on
684 Tukey's honestly significant difference (HSD) *post hoc* test.

685 [b] NP: Information not provided.

686

687
688 **Table 2.** Results of linear mixed-effects models that characterized the variance associated with
689 the random effect of carrot package on the level of *L. monocytogenes* FS2025 recovered after 15-
690 min incubation on the cut surface of carrots from fresh-cut and boiled carrots.
691

Model	Estimate[a]	95% Confidence interval	P-value	Variance of random effect of carrot package (SD[b])
I: Boiled carrot[c]				
	7.4	7.3, 7.6	<0.001	0.02 (0.1)
II: Fresh-cut carrot[d]				
	4.7	3.8, 5.7	<0.001	1.1 (1.1)

692 [a] Parameter estimate of expected level of *L. monocytogenes* recovered from carrot in each linear mixed-
693 effect model
694 [b] Standard deviation
695 [c]Model I tests for the random effect of carrot package (i.e., the individual package with different brand
696 labeling from which carrot samples were obtained) on the outcome of *L. monocytogenes* levels on boiled
697 carrots. No fixed effects were evaluated in this model.
698 [d]Model II tests for the random effect of carrot package (i.e., the individual package with different brand
699 labelling from which carrot samples were obtained) on the outcome of *L. monocytogenes* levels on fresh-
700 cut carrots. No fixed effects were evaluated in this model.
701

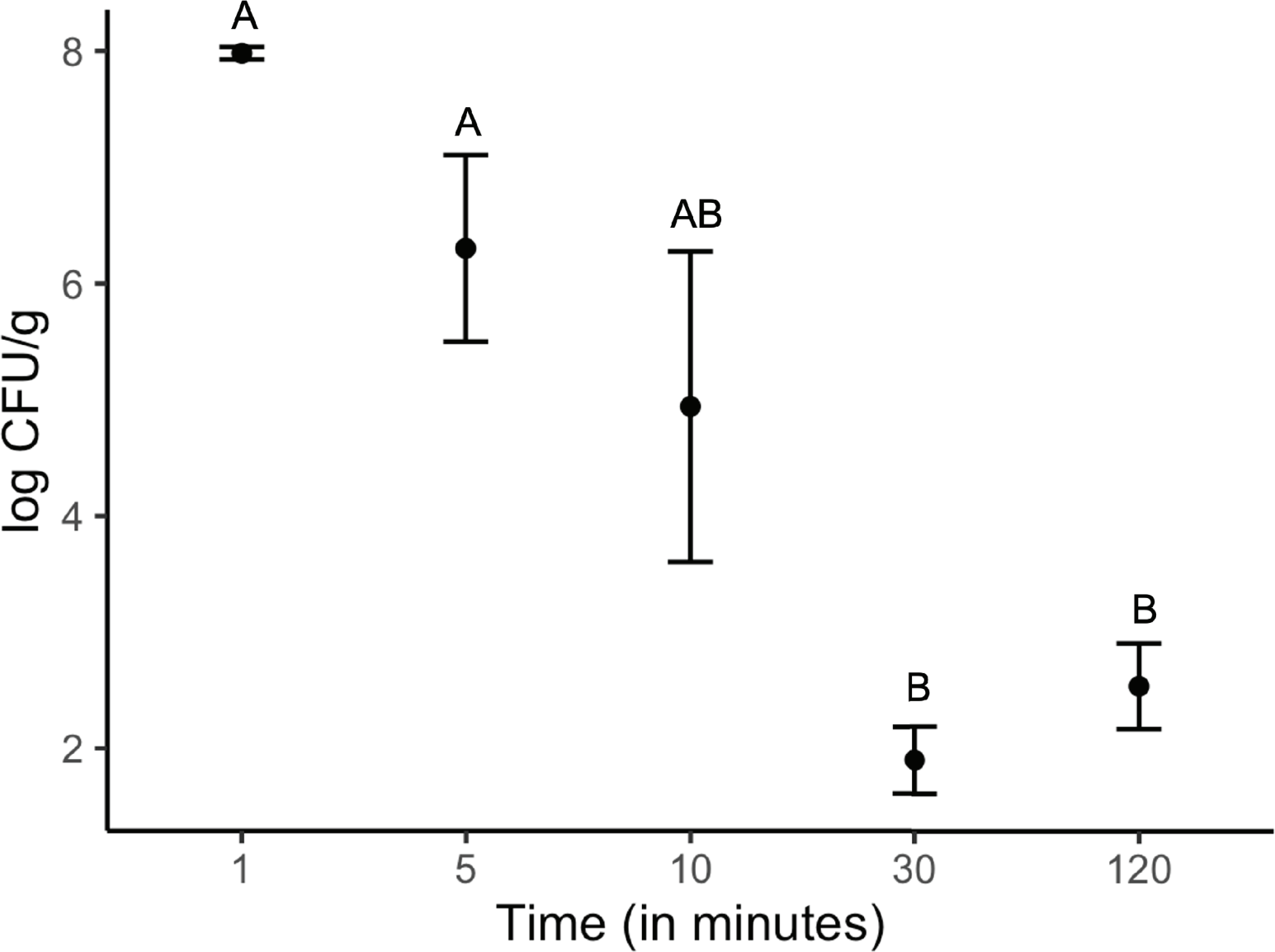
Table 3. Levels of *L. monocytogenes* FS2025 recovered from the cut surface of carrot enumerated using traditional culture-dependent and culture-independent methods.

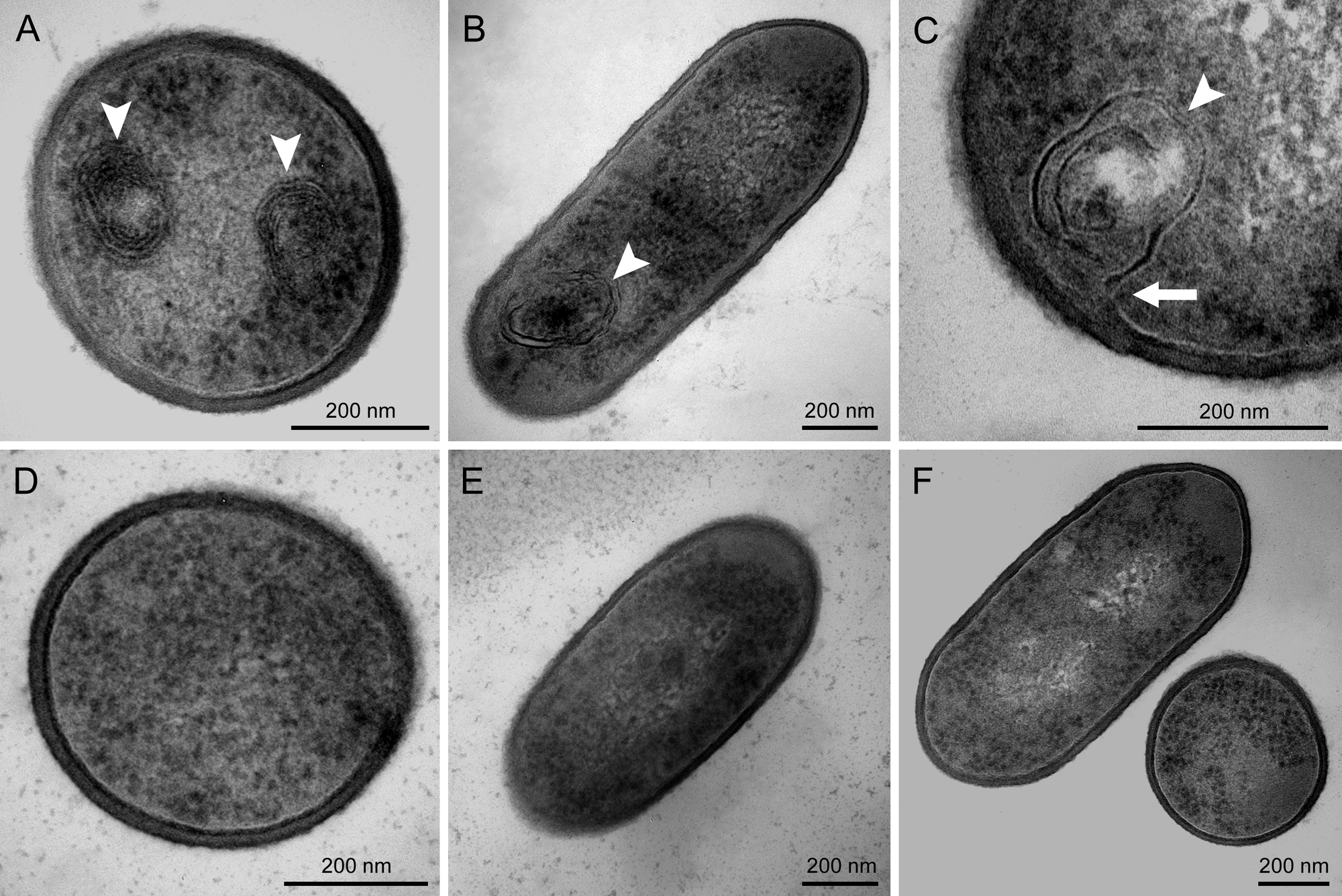
Enumeration method[a]	Average *L. monocytogenes* (log CFU/g) on		
	Fresh-cut carrots	Boiled carrots	Heat-killed rinsates
Culture-dependent enumeration			
Selective agar	4.4 ± 0.2[b] Bc	8.1 ± 0.1 Aa	<BLOQ[c] Cb
Non-selective agar	4.1 ± 0.4 Bc	8.3 ± 0.1 Aa	<BLOQ Cb
Culture-independent enumeration			
qPCR	8.2 ± 0.1 Aa	7.8 ± 0.2 Aa	7.3 ± 0.2 Aa
PMAxx-qPCR	7.2 ± 0.1 Ab	7.2 ± 0.1 Aa	<BLOQ Bb

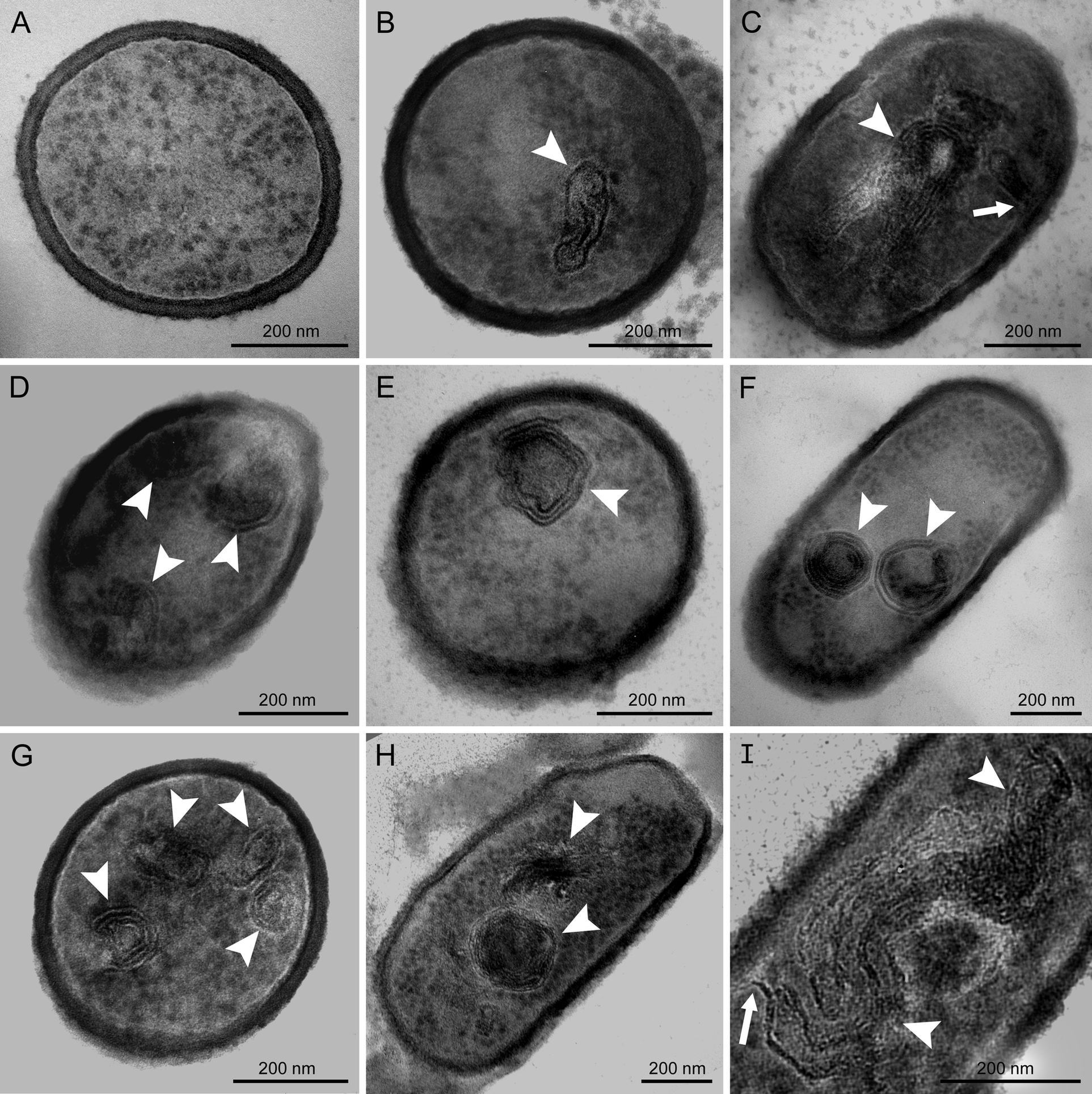
[a] Selective agar: samples were plated on Harlequin Listeria chromogenic media, Non-selective agar: samples were plated on Tryptic Soy Agar supplemented with 50 mg/L 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (TSAG), qPCR: quantitative PCR assay carried out on DNA lysates from carrot rinsates, PMAxx-qPCR: quantitative PCR assay carried out on DNA lysates from carrot rinsates subjected to pre-treatment with propidium monoazide (PMAxx) before DNA extraction.

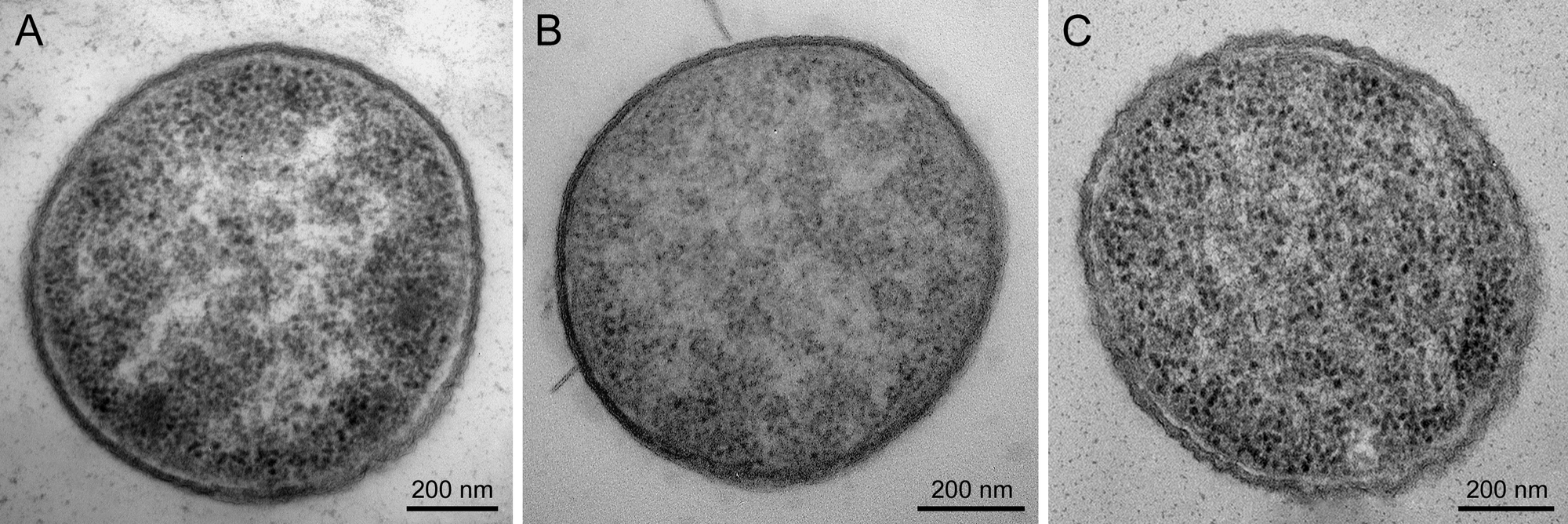
[b] Values are represented as arithmetic mean ± standard error (n=3). Different uppercase letters in the same row indicate a significant difference ($p<0.05$) in *L. monocytogenes* levels for different sample types, and different lowercase letters in the same column indicate a significant difference ($p<0.05$) in *L. monocytogenes* levels for different enumeration methods based on *post hoc* multiple-comparison adjustment with Tukey's honestly significant difference (HSD) test.

[c]BLOQ: Below the limit of quantification. For culture-dependent enumeration, the limit of quantification was 1.1 log CFU/g, and for culture-independent enumeration, the limit of quantification was 3.7 log CFU/g.


A
A
AB
B
B
8
6
4
2
log CFU/g
1
5
10
30
120
Time (in minutes)


A
200 nm
B
200 nm
C
200 nm
D
200 nm
E
200 nm
F
200 nm


A
200 nm
B
200 nm
C
200 nm
D
200 nm
E
200 nm
F
200 nm
G
200 nm
H
200 nm
I
200 nm


A
200 nm
B
200 nm
C
200 nm