Effect of high pressure processing in combination with Weissella viridescens as a protective culture against Listeria monocytogenes in ready-to-eat salads of different pH

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1 **Effect of high pressure processing in combination with *Weissella viridescens* as a**
2 **protective culture against *Listeria monocytogenes* in ready-to-eat salads of different pH**

3

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Abstract

This study explored the effect of HPP (400 MPa/1 min) and a *Weissella viridescens* protective culture, alone or in conjunction, against *L. monocytogenes* in ready-to-eat (RTE) salads with different pH values (4.32 and 5.59) during storage at 4 and 12°C. HPP was able to reduce the counts of the pathogen after treatment achieving approximately a 4.0 and 1.5 log CFU/g reduction in the low and higher pH RTE salad, respectively. However, *L. monocytogenes* was able to recover and grow during subsequent storage. *W. viridescens* grew in both RTE salads at both storage temperatures, with HPP resulting in only a small immediate reduction of *W. viridescens* ranging from 0.50 to 1.2 log CFU/g depending on the pH of the RTE salad. For the lower pH RTE salad, the protective culture was able to gradually reduce the *L. monocytogenes* counts during storage whereas for the higher pH RTE salad in some cases it delayed growth significantly or exerted a bacteriostatic effect. exerted a bacteriostatic effect. The results revealed that the increased storage temperature led to an increase in the inactivation/inhibition of *L. monocytogenes* in the presence of *W. viridescens*. The combination of HPP and *W. viridescens* is a promising strategy to control *L. monocytogenes* and can increase safety even when a break in the chill chain occurs.

Keywords: protective culture, *Weissella viridescens*, *Listeria monocytogenes*, high pressure, temperature, pH

40 ## 1. INTRODUCTION

41 *Listeria monocytogenes* is an important foodborne pathogenic microorganism and the
42 causative agent of listeriosis, which has a significant impact on public health and economy
43 (Scallan et al., 2011; Stephan et al., 2015; EFSA 2015). Due to the high mortality rate
44 associated with listeriosis, it ranks in a global scale amongst the most frequent causes of death
45 due to foodborne illnesses (Behravesh et al., 2011; Werber et al., 2013; EFSA, 2014). *L.*
46 *monocytogenes* can survive and grow in a variety of foods, surfaces and equipment under
47 adverse environmental conditions such as low pH, low water activity (a_w) and low temperature
48 (Angelidis, Smith, & Smith, 1999; Hado & Yousef, 2007). Ready-to-eat (RTE) products have
49 been implicated in several listeriosis outbreaks worldwide (Swaminathan & Gerner-Smidt
50 2007; Johnsen, Lingaas, Dag Torfoss, Strøm, & Nordøy, 2010; Shi, Qingping, Jumei,
51 Moutong, Zean, 2015). Therefore, reducing the occurrence of *L. monocytogene*s in RTE foods
52 is an important food safety goal for the food industry. High pressure processing (HPP) has been
53 gaining increasing importance as a potential non-thermal preservation technology for different
54 types of products including RTE foods. In contrast to thermal treatments, HPP results in
55 minimal or no effects on nutritional or quality characteristics and at the same time can lead to
56 microorganism inactivation (Cheftel, 1995). HPP inactivates microbial cells by inducing
57 physical damage and altering the functionality of the cytoplasmic membrane, causing protein
58 denaturation and interfering with genetic mechanisms (Patterson, 2005). In general, studies
59 have shown that vegetative microorganisms present in food are inactivated at pressure levels
60 between 400 and 600 MPa for several minutes (Smith, Mendonca, & Jung, 2009;
61 Patterson, McKay, Connolly, & Linton, 2010; Stratakos & Koidis, 2015). However, as HPP is
62 a batch process and due to the fact that in some cases (i.e. application of high pressure levels)
63 it can result in negative effects on quality (e.g. increased lipid oxidation, texture changes)
64 (Stratakos & Koidis, 2015), from a commercial perspective it is more desirable to use as short

65 a treatment time as possible and as low a pressure as possible in order to achieve a high
66 throughput and minimise any potential effects on quality as well as reduce equipment/metal
67 fatigue due to repeated usage (Mertens & Deplace 1993).
68 Therefore, combining HPP with other preservation methods could allow the use of milder
69 pressure treatments to achieve additive or synergistic effects against pathogenic
70 microorganisms. Biopreservation has also been used in recent years as a way of prolonging
71 shelf life and increasing safety of RTE products (Pilet & Leroi 2011; Liu et al. 2012), with
72 various studies showing the synergistic effect between HHP and bacteriocins on inactivation
73 of foodborne pathogenic microorganisms and prevention of microbial spoilage (Jofré, Garriga,
74 & Aymerich, 2008; Chung, Vurma, Turek, Chrism, & Tousef, 2005; Chung & Yousef, 2010).
75 In these studies, the bacteriocin was added or sprayed directly on the food. Direct addition of
76 bacteriocin implies that it has been produced ex-situ by a producer strain with subsequent
77 concentration and purification steps which can be expensive and time consuming (Gálvez,
78 Abriouel, López, & Omar 2007). Moreover, loss of bacteriocin activity might occur due to
79 enzymatic degradation and interaction with food proteins and lipids (Holzapfel, Geisen, &
80 Schillinger, 1995). To circumvent the above problems in-situ bacteriocin production by lactic
81 acid bacteria (LAB) has been also investigated against pathogenic microorgansims (Mataragas,
82 Drosinos, & Metaxopoulos, 2003; Brillet, Pilet, Prévost, Cardinal & Leroi, 2005). The effect
83 of these LAB protective cultures against other microorganisms has been attributed to the
84 competition for nutrients and/or production of antimicrobial compounds such as organic acids,
85 enzymes, bacteriocins and reuterin (Holzapfel et al., 1995).
86 The aim of the study was to explore the possibility of using a *W. viridescens* protective culture
87 and HPP, either alone or in conjunction, to help control the growth of *L. monocytogenes*. This
88 *W. viridescens* strain has been shown to have antimicrobial properties and does not cause
89 spoilage (Patterson et al., 2010). However, the active compound(s) responsible for the

antimicrobial activity has not been identified yet. The efficiency of the above approach was tested on RTE salads with different pH values and during refrigeration and abuse storage temperatures.

2. MATERIAL AND METHODS

2.1 Preparation of *Listeria monocytogenes* inoculum

For each *L. monocytogenes* strain used i) LR102 (Camembert, outbreak isolate; serotype 1/2a, ii) VI 51028 (fish slaughter house; serotype 4), iii) 0227-359 (meatballs; serotype 1), iv) 0113-131 (RTE chicken; serotype 1) and v) VI 51010 (*L. monocytogenes* Scott A; serotype 4b), a loopful of a fresh tryptone soya agar (Oxoid code CM0131, Oxoid, Basingstoke, UK) plus 0.6 % yeast extract (Oxoid code LP0021) (TSAYE) slope culture was transferred into 10 ml of brain heart infusion broth (BHI) (Oxoid code CM1135) and incubated at 37°C for 24 h. Subsequently 100 μl of a 10^{-4} dilution of this broth was transferred into another 10 ml BHI broth and incubated at 37°C for 48 h, in order to reach the stationary phase of growth. The final 10 ml cultures were centrifuged at 3600×g, for 30 min, washed twice in phosphate buffered saline (PBS) and the pellet re-suspended in a final volume of 10 ml PBS to give approximately 10^9 CFU/ml. Subsequently, equal quantities of the 5-strain cell suspensions were mixed well in a plastic 50 ml centrifuge tube to produce the 5-strain cocktail. 100 μl of this suspension were inoculated into the RTE salad samples (10 ± 0.2 g), to give an initial inoculum level of approximately 7 log CFU/g. Samples were massaged for approx. 30 sec to ensure uniform distribution of the inoculum.

2.2 Preparation of *Weissella viridescens* inoculum

115 A *W. viridescens* strain that was previously isolated from cooked chicken in the Agri-Food and
116 Biosciences Institute was used because it is quite salt and pH tolerant (Mol, Hietbrink, Mollen,
117 & van Tinteren, 1971) and was found to have antimicrobial activity and relatively resistant to
118 pressure (Patterson, Mackle, & Linton, 2011). A loopful from a MRS agar (Oxoid code
119 CM1153B) slope culture was transferred into a 10 ml MRS broth (Oxoid code CM0359B) and
120 incubated for 24 h at 30°C under anaerobic conditions. Afterwards, 100 μl of this broth was
121 transferred into another 10 ml MRS broth and incubated at 30°C for 72 h, in order to allow for
122 the microorganism to reach the stationary phase of growth. After the completion of the
123 incubation, 100 μl of this suspension was inoculated into the RTE salad samples (10 ± 0.2 g),
124 at a level of approximately 5 log CFU/g. The inoculation of *W. viridescens* into the RTE salad
125 samples was performed approximately 30 min after the *L. monocytogenes* inoculation.

126

127 **2.3 Preparation of RTE salads**

128 The RTE salads were prepared in-house using a commercial recipe. Two different salads were
129 prepared with two different final pH values (low and higher). The higher pH RTE salad was
130 prepared by slightly altering the initial formulation to achieve the increase in pH (Table 1). All
131 samples were packaged using polyethylene/polyamide vacuum pouches (Scobie and Junor,
132 Mallusk, Northern Ireland). The following four treatments, all inoculated with a 5-strain
133 cocktail of *L. monocytogenes*, were employed in this study:

134 • Control (C): vacuum-packed RTE salad.
135 • Pressure treatment (HPP): vacuum-packed RTE salad samples pressure treated at 400
136 MPa for 1 min.
137 • *W. viridescens* protective culture (PC): inoculated with *W. viridescens* and vacuum-
138 packed.

- HPP/PC: inoculated with *W. viridescens*, vacuum-packed, and pressure treated at 400 MPa for 1 min.

All samples were stored for a period of 21 days at 4 and 12°C.

2.4 Characterisation of RTE salads

The pH of the salads was determined with the use of a Jenway pH Meter Model 3505, after mixing with deionised water at a ratio of 1:1. Water activity (a_w) was measured by means of a Hygrolab 3 a_w meter (Rotronic instruments, UK). Three replicate samples from different production runs were used for the measurements.

2.5 Irradiation treatment

Packaged samples were sterilised by gamma irradiation after delivering a dose of 25 kGy, using a ^{60}Co Gamma-beam 650 facility (Nordion, Canada). The samples underwent cold sterilisation in order to be able to get a clear picture of the interaction between *L. monocytogenes*, *W. viridescens* and high pressure processing. After irradiation, random samples from all batches were tested to confirm sterility.

2.6 High pressure processing

Pressure treatment of packaged RTE salad samples was performed in a commercial scale high pressure press (Quintus 35L, Avure Technologies, U.S.A.), with a pressure vessel of 35 L volume. The pressure transmission fluid used was potable water. The pressure come-up time was approximately 25 sec per 100 MPa and the pressure release time was approximately 10 s. The initial temperature of the water was approximately 18°C and the temperature increase due to adiabatic heating was approximately 2-3°C per 100 MPa. The processing conditions were 400 MPa with a hold time at that pressure level of 1 min.

164

165 ## 2.7 *Listeria monocytogenes* enumeration

166 Samples were opened aseptically and the contents were transferred to a sterile stomacher bag
167 with a filter insert (Interscience, St. Nom La Breteche, France). A 10^{-1} dilution of the sample
168 was prepared by adding 90 ml of maximum recovery diluent (MRD) (Oxoid code CM733).
169 The dilution was homogenised for 1 min in a Seward stomacher. If necessary, further 10-fold
170 dilutions were prepared in 9 ml MRD. For enumeration of *L. monocytogenes*, 100 µl of each
171 of the 10-fold dilutions were spread plated onto Oxoid chromogenic Listeria agar (OCLA)
172 (Oxoid, code CM1084B) supplemented with OCLA selective supplement (Oxoid code
173 SR0226E) and Brilliance Listeria differential supplement (Oxoid code SR0228E) and
174 incubated at 37°C for 48 h. Each sample was plated in duplicate.

175

176 ## 2.8 *Weissella viridescens* enumeration

177 The enumeration of *W. viridescens* was performed as for *L. monocytogenes*. 100 µl of the
178 appropriate dilutions were spread plated onto MRS agar plates and incubated at 30°C for 48 h
179 under anaerobic conditions. The five strains of *L. monocytogenes* used in this study were not
180 found to grow on MRS agar (Patterson et al., 2011).

181

182 ## 2.9 Statistical analysis

183 The experiment was performed three times on different occasions in order to obtain three
184 independent replicates. Factorial analysis of variance was used to determine the interactions
185 between treatment, storage, storage temperature and pH on the microbial counts obtained. A
186 significance level of 0.05 was used. When microbial counts were below the detection limit (50
187 CFU/g) this was taken as the value for the statistical tests.

188

189 ## 3. RESULTS AND DISCUSSION

190 ### 3.1 Fate of *L. monocytogenes* in low-pH RTE salad at 4 and 12°C

191 The formulation, pH and a_w values of the RTE salads are shown in Table 1. Statistical analysis
192 showed that the different formulation of the salads led to significantly different pH values.
193 Figure 1 presents the behaviour of *L. monocytogenes* (A and C) and *W. viridescens* (B and D)
194 during storage at refrigeration and abuse temperature. Although, the initial level of the pathogen
195 (approx. 7 log CFU/g.) chosen to be used is unlikely to occur in real-life scenarios and it might affect
196 the subsequent behaviour of *L. monocytogenes* (Tyrovouzis et al., 2014), its use was deemed necessary
197 to clearly elucidate the potential growth or inactivation of the pathogen during storage. Furthermore,
198 in order to selectively enumerate the target microorganisms and clearly demonstrate the effect
199 of *W. viridescens* against *L. monocytogenes* in a food matrix the samples were cold sterilised.

200 It has been hypothesised that *W. viridescens* produces a small, diffusible compound which is
201 able to inhibit the growth of both Gram-negative and Gram-positive microorganisms (Patterson
202 et al., 2010). *Weissella paramesenteroides* has been found to produce a small (~2.5 kDa) non-
203 proteinaceous compound that shows an antimicrobial effect (Pal & Ramana, 2009). Moreover,
204 a bacteriocin called Weissellicin 110 has been recently identified which is produced by a strain
205 of *Weissella cibaria* (Srionnual, Yanagida, Lin, Hsiao, & Chen, 2007).

206 Statistical analysis for *L. monocytogenes* counts revealed that there was a significant interaction
207 between treatment, pH, storage and temperature ($P=0.009$). For the lower-pH salad, *L.*
208 *monocytogenes* inoculated in control samples remained at the same level during storage at 4°C
209 (Fig. 1A) showing that the pH value of the RTE salad, although it did not result in the
210 inactivation of the pathogen, was able to inhibit its growth.

211 HPP treatment resulted in a significant reduction of *L. monocytogenes* counts. Subsequently
212 the counts remained relatively stable showing the same trend as the control samples. On the
213 other hand, counts of *L. monocytogenes* in the presence of the PC showed a gradual decrease
214 during storage. This decrease became significant ($P<0.001$) after six days of storage and at day
215 21 the pathogen counts were approximately 4 log CFU/g, achieving an approximately 3.37 log
216 average count reduction compared to the controls. Vermeiren, Devlieghere, De Graef, &
217 Debevere (2004) have also found that *Lactobacillus sakei* had antimicrobial activity against *L.*
218 *monocytogenes* inoculated on cooked ham. In an another study, the application of
219 *Carnobacterium divergens* M35 was able to reduce the *L. monocytogenes* counts in cold
220 smoked salmon by 3.1 log CFU/g after 21 days of storage at 4°C (Tahiri, Desbiens, Kheadr,
221 Lacroix, & Fliss, 2009).

222 When the two methods were used in conjunction (HPP/PC) a decrease in the counts of the
223 pathogen were also observed. However in this case *L. monocytogenes* could not be enumerated
224 from the third sampling point (6 days storage) onward and until the end of storage. One of the
225 main sites of damage induced by pressure is the cell membrane (Patterson, 2005). The use of
226 high pressure in conjunction with bacteriocins has been shown to lead to increased
227 antimicrobial activity (Kalchayanand, Sikes, Dunne, & Ray 1998; Chung & Yousef, 2010).
228 The physical damage and/or permeabilisation of the membrane due to HPP could lead to
229 increased entry of the antimicrobial compound(s) produced by *W. viridescens* during its
230 growth. The presence of this antimicrobial(s) could possibly inhibit recovery of sub-lethally
231 pressure-injured cells by interfering with several biological mechanisms (e.g. production of
232 required biological materials). The use of non-selective medium overlaid with selective
233 medium (Lorentzen et al., 2010) or the ISO 11290-2 assay in addition to the selective media
234 used here would have given even more information on the extent of pressure-damaged cells as
235 it would permit their enumeration in damaged cells in contrast to selective media.

236 For lower-pH samples stored at 12°C (Fig 1C) a similar trend was observed for the control
237 samples in which the populations of *L. monocytogenes* remained relatively stable during
238 storage. For HPP samples, *L. monocytogenes* counts remained at the same levels after the initial
239 reduction despite of the elevated temperature showing again the inhibitory effect of the low
240 pH. PC treatment at 12°C also led to a significant gradual reduction of the pathogen counts.
241 However at this temperature at the end of the storage the average pathogen counts were 2 log
242 CFU/g indicating that the protective culture had an increased bactericidal effect at the abuse
243 temperature. Statistical analysis showed that after day 3 for the PC treatment the *L.*
244 *monocytogenes* counts at 12°C were always statistically significantly lower compared to the
245 counts for the PC treatment during storage at 4°C. The combination of HPP and PC resulted in
246 a reduction below the enumeration limit throughout storage in the abuse temperature too.
247 However, it should be noted that *L. monocytogenes* decreased below the enumeration limitfrom
248 the second sampling point (3 days storage) forward, three days sooner compared to the
249 refrigeration temperature, again revealing a more pronounced antimicrobial effect. From these
250 results it is obvious that the storage at the abuse temperature was more detrimental to the
251 survival of *L. monocytogenes* when *W. viridescens* was present. The results of this experiment
252 are consistent with the study of Angelidis, Boutsiouki, & Papageorgiou, (2010) that showed
253 that increased inactivation rates were observed for *L. monocytogenes* counts inoculated in
254 cheese, during storage at 22°C compared to storage at 12 and 4°C. Fig. 1B and Fig. 1D present
255 the behaviour of *W. viridescens* in RTE salad (PC and HPP/PC treatments). *W. viridescens*
256 grew rapidly in the low-pH RTE salad with the counts reaching approximately 7 log CFU/g
257 after 6 days during of storage at 4°C. However, when samples were pressure treated (HPP/PC)
258 a prolongation of the lag phase was evident. Following the end of the lag phase average counts
259 increased rapidly reaching 6.43 log CFU/g at day 9. The observed extension of the lag phase
260 can be attributed to the sub-lethal injuries induced to the microbial cells due to pressure

261 (Tholozan, Ritz, Jugiau, Federighi, & Tissier, 2000). On the other hand, *W. viridescens* grew
262 faster in the abuse temperature ($P<0.001$) and reached 8.13 log CFU/g at day 6. It is noteworthy
263 that a shorter or no lag phase was observed for pressure-treated samples (HPP/PC) at the abuse
264 temperature which allowed average counts to reach 8.06 log CFU/g at day 6. Due to the limited
265 initial sampling we could not determine precisely the absence or length of the lag phase. The
266 increased storage temperature enabled the cells to recover fast from the sub-lethal injuries and
267 commence growth (Bull, Hayman, Stewart, Szabo, & Knabel, 2005; Stratakos, Delgado-
268 Pando, Linton, Patterson, & Koidis, 2015a; Stratakos, Linton, Patterson, & Koidis, 2015b).

269

270 **3.2 Fate of *L. monocytogenes* and *W. viridescens* in higher pH RTE salad at 4 and**
271 **12°C**

272 The results on the behaviour of *L. monocytogenes* and *W. viridescens* in RTE salads with a
273 higher initial pH are illustrated in Figures 2 (A-D). The higher pH of this version of the RTE
274 salad permitted the growth of *L. monocytogenes*. For the control samples stored at 4^0C the
275 counts of *L. monocytogenes* increased rapidly during storage reaching >8 log CFU/g after 6
276 days. With regards to the PC treatment at day 3 a reduction was observed in the counts of the
277 pathogen compared to the control. However, after this initial reduction, growth commenced
278 again and after day 9 of storage there were no statistically significant differences in *L.*
279 *monocytogenes* average counts between the control and PC treatments. For the HPP trials a
280 lower immediate reduction was observed after treatment (see 3.3) and the exponential stage of
281 growth started after the end of a 6-day lag phase. *L. monocytogenes* counts for the HPP and
282 HPP/PC trials showed no statistical significant differences ($P>0.05$) until day 6 of storage.
283 Beyond this point significant differences were found between the two treatments. The presence
284 of *W. viridescens* (HPP/PC) was able to delay the growth of pathogen which reached 6.95 log
285 CFU/g at the end of storage, which was significantly lower compared to all the other treatments.

286 *L. monocytogenes* growth was faster at 12°C storage, as expected, reaching >8 log CFU/g after
287 3 days storage. In this case the PC treatment did not result in reduction but exerted a
288 bacteriostatic effect until day 9 after which growth of the pathogen commenced again. The
289 HPP treatment was not sufficient to control the growth of the pathogen with the exponential
290 stage of growth starting only after 3 days storage. When the two methods were combined
291 (HPP/PC) a clear bacteriostatic effect was observed until the end of storage (5.39 log CFU/g at
292 end of storage). In this case as well, the elevated storage temperature allowed for a better
293 control of the growth of *L. monocytogenes* even in the RTE salad with a more favourable pH
294 for its growth. *Carnobacterium divergens* V41 inoculated on cold smoked salmon has also
295 been found to be able to inhibit the growth of *L. monocytogenes* during storage for 28 days at
296 4 and 8°C with minimal effects on the quality of the product (Brillet et al. 2005).

297 *W. viridescens* counts showed a similar trend to the low-pH RTE salad stored both at 4 and
298 12^0C (Fig 2B and 2d). *W. viridescens* grew well in this product too and reached at day 6 of
299 storage 7.34 and 6.56 log CFU/g for PC and HPP/PC, respectively for samples stored at 4^0C
300 and 8.15 and 8.06 log CFU/g, respectively for samples stored at 12^0C. A shorter or no lag phase
301 was also evident for the HPP/PC treatment at 12°C.

302 In general, the increased antimicrobial effect observed at the abuse temperature for the PC and
303 HPP/PC treatments can be attributed to the higher counts of the *W. viridescens* due to the
304 absence of the lag phase and possibly to the increased metabolic activity of the *L.*
305 *monocytogenes* due to higher temperature which led to an enhanced lethal effect of the
306 antimicrobial compound(s) produced by *W. viridescens*. An increased death rate at abuse
307 temperatures compared to refrigeration temperatures has also been observed for *E. coli*
308 O157:H7 and *Salmonella Typhimurium* DT104 inoculated on pepperoni (Faith, Parniere,
309 Larson, Lorang, & Luchansky, 1997; Ihnot,, Roering, Wierzba, Faith, & Luchansky, 1998).
310

311 ## 3.3 Effect of pH on *L. monocytogenes* and *W. viridescens*

312 ### *3.3.1 Effect of pH on HPP inactivation of L. monocytogenes and W. viridescens*

313 The level of pressure induced inactivation was found to be significantly different (P<0.05)
314 depending on the pH of the RTE salad, for both microorganisms. The low-pH resulted in a
315 much higher average reduction (P=0.01) in the *L. monocytogenes* counts (approx. 4 log CFU/g)
316 compared to the salad with the higher pH, which showed a 1.51 log CFU/g average reduction.
317 Jung et al., (2013) found that pressure treatment at 300 MPa for 5 min resulted in >6 log
318 reduction in *L. monocytogenes* counts in PBS adjusted to pH 4 compared to <1 log reduction
319 at pH 7.2. Pressurization (345 MPa) at pH 4.5 increased inactivation of *L. monocytogenes* in
320 PBS by an additional 1.2 - 3.9 log cycles at pH 4.5 compared to a pH 6.5 (Alpas, Kalchayanand,
321 Bozoglu, & Ray, 2000). This increase in pressure induced inactivation as the pH decreases has
322 also been confirmed in studies with real food. Specifically, the survival of *Escherichia* coli
323 O157:H7 in orange juice was increasingly reduced as pH values of the juice decreased
324 (Linton, McClements, & Patterson, 1999). With regards to *W. viridescens*, HPP treatment
325 (HPP/PC) resulted in statistically significant reductions in counts for both RTE salads (<0.5
326 and 1.12 log CFU/g for the higher and lower pH salads, respectively). However, these relatively
327 low reductions did not prevent it from exerting its protective effect against *L. monocytogenes*
328 during storage. Park, Sohn, Shin, & Lee, (2001) found that HPP treatment of 600 MPa for 5
329 min resulted in approximately 4 log CFU/g reduction of *W. viridescens* counts in ham. In
330 general, lactic acid bacteria are tolerant to low pH, so it is not surprising that *W. viridescens*
331 was able to tolerate low pH values and therefore a relatively small reduction was observed in
332 the low pH product.

333

334 ### *3.3.2 Effect of pH on L. monocytogenes and W. viridescens during storage*

335 The pH played an important role in the fate of *L. monocytogenes* during storage. According to
336 European Regulation (EC) No. 2073/2005, the lower-pH RTE salad falls under the category of
337 products (pH≥4.4) that do not support the growth of *L. monocytogenes*. Therefore, a very
338 different trend in the behaviour of *L. monocytogenes* was observed during storage between the
339 two types of salad stored at the same temperature both at 4ºC (Fig. 1A and Fig. 2A) and 12°C
340 (Fig. 1B and 2B) since the low pH was able to inhibit the growth but not lead to the inactivation
341 of the pathogen. For the low-pH RTE salad the pathogen counts of the PC and HPP/PC
342 treatments dropped during storage (Fig. 1A and 1B) whereas for the higher pH salad (Fig. 2A
343 and 2B) the counts remained in the same levels or increased.

344 The *W. viridescens* counts (PC treatments) did not show any significant differences during
345 storage between low (Fig. 2B) and higher pH samples (Fig. 2D) (P>0.05). On the other hand,
346 HPP/PC treatments did show significant differences in the counts of *W. viridescens* during
347 storage between the two different types of RTE salad. These significant differences observed
348 in the latter case, were attributed to the different initial HPP-induced inactivation levels (see
349 3.3.1.). Overall, these results reveal that the *W. viridescens* protective culture showed similar
350 growth potential in the two different pH values tested.

351 In this study, the low pH acted as an extra hurdle which in combination with the potential
352 production of antimicrobial compound(s) probably contributed towards the metabolic
353 exhaustion of the *L. monocytogenes* cells. When the low pH hurdle was removed (in the RTE
354 salad with the higher pH) the bactericidal effect was substituted with substituted in most cases
355 with a significant delay in growth or exerted a bacteriostatic effect. The results showed that *W.*
356 *viridescens* alone or in combination with HPP, is able to be used as a protective culture in RTE
357 salads with different pH values, as it can grow well and exhibit antilisterial activity and thus
358 offer an increased safety margin.

359

360 ## 4. CONCLUSIONS

361 The application of this strain of *W. viridescens* has significant potential as a protective
362 culture/biopreservation method because it can successfully be used to help control the growth
363 of *L. monocytogenes* in low and higher pH RTE salads due to the possible antimicrobial(s)
364 produced during its growth. The specificity of bacteriocins is not linked to LAB origin but is
365 more likely linked to the bacterial species that produces them (Pilet & Leroi, 2011) which
366 means that *W. viridescens* could probably be used as a preservation method in other food
367 products of plant and animal origin. HPP was able to significantly reduce the counts of *L.*
368 *monocytogenes* in both types of RTE salads but the pathogen was able to recover during storage
369 at abusive temperature. A relatively mild HPP treatment in conjunction with the *W. viridescens*
370 protective culture exhibited a synergistic effect against *L. monocytogenes* which was even more
371 pronounced during storage at abusive temperature. The combination of HPP and *W. viridescens*
372 is a promising strategy to control *L. monocytogenes* is RTE salads of different pH and can
373 increase safety even in a cold chain break scenario.

374

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FIGURE LEGENDS

Figure 1

Changes in the *L. monocytogenes* counts (A and C) on low-pH RTE salads during storage at 4 and 12°C, respectively. Control (■): untreated vacuum-packed samples, HPP (▲): pressure treated vacuum-packed samples (400 MPa for 1 min), PC (**X**): vacuum-packed samples inoculated with *W. viridescens*, HPP/PC (♦): combination of high-pressure and protective culture. Changes in the *W. viridescens* counts (B and D) on low-pH RTE salads during storage at 4 and 12°C, respectively. PC (**X**): vacuum-packed samples inoculated with *W. viridescens*, HPP/PC (♦): combination of high-pressure and protective culture. Each point in the figure represents the mean of three separate trials. The error bars represent ± S.D. Dashed line represents the limit of enumeration.

Figure 2

Changes in the *L. monocytogenes* counts (A and C) on higher pH RTE salads during storage at 4 and 12°C, respectively. Control (■): untreated vacuum-packed samples, HPP (▲): pressure treated vacuum-packed samples (400 MPa for 1 min), PC (**X**): vacuum-packed samples inoculated with *W. viridescens*, HPP/PC (♦): combination of high-pressure and protective culture. Changes in the *W. viridescens* counts (B and D) on higher pH RTE salads during storage at 4 and 12°C, respectively. PC (**X**): vacuum-packed samples inoculated with *W. viridescens*, HPP/PC (♦): combination of high-pressure and protective culture. Each point in the figure represents the mean of three separate trials. The error bars represent ± S.D.