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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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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. monocytogene*s and can increase safety even when a break in the chill chain occurs.

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

1. INTRODUCTION

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

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

2.3 Preparation of RTE salads

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

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

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

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

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

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

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

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

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

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

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

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

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.

4. CONCLUSIONS

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

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5. REFERENCES

Alpas, H., Kalchayanand, N., Bozoglu, B., & Ray, B. (2000). Interactions of high hydrostatic pressure, pressurization temperature and pH on death and injury of pressure-resistant and pressure-sensitive strains of foodborne pathogens. *International Journal of Food Microbiology*, 60, 33–42.

Angelidis, A. S., Boutsiouki, P., & Papageorgiou, D. K. (2010). Loss of viability of *Listeria monocytogenes* in contaminated processed cheese during storage at 4, 12 and 22° C. *Food Microbioliogy*, 27, 809-818.

Angelidis, A. S., Smith, L. T., & Smith, G. M. (1999). Compatible solute accumulation by *Listeria monocytogenes* grown on milk whey. *FASEB Journal*, 13, 1533-1533.

Behravesh, C., Jones, T. F., Vugia, D. J., Long, C., Marcus, R., Smith, K., et al. (2011). Deaths associated with bacterial pathogens transmitted commonly through food: foodborne diseases active surveillance network (FoodNet), 1996-2005. *Journal of Infectious Diseases*, 204, 263-267.

Brillet A., Pilet M. F., Prévost H., Cardinal M., & Leroi F. (2005). Effect of inoculation of *Carnobacterium divergens* V41, a biopreservative strain against *Listeria monocytogenes* risk, on the microbiological, and sensory quality of cold-smoked salmon. *International Journal of Food Microbiology*, 104**,** 309-324.

Bull, M. K., Hayman, M. M., Stewart, C. M., Szabo, E. A., & Knabel, S. J. (2005). Effect of prior growth temperature, type of enrichment medium, and temperature and time of storage on

recovery of *Listeria monocytogenes* following high pressure processing of milk. *International Journal of Food Microbiology*, 101, 53–61.

Cheftel, J. C. (1995). Review: High-pressure, microbial inactivation and food preservation/Revision: Alta-presion, inactivacion microbiologica y conservacion de alimentos. *Food Science and Technology International*, 1, 75-90.

Chung, Y. K., Vurma, M., Turek, E. J., Chrism, G. W., & Tousef, A. E. (2005). Inactivation of barotolerant *Listeria monocytogenes* in sausage by combination of high-pressure processing and food grade additives. *Journal of Food Protection*, 68, 744–750.

Chung, H. J. & Yousef, A. E. (2010). Synergistic effect of high pressure processing and *Lactobacillus casei* antimicrobial activity against pressure resistant *Listeria monocytogenes*. *New Biotechnology*, 27, 403-408.

EFSA (European Food Safety Authority) (2015). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. *EFSA Journal*, 2015, 13:3991.

Faith, N.G., Parniere, N., Larson, T., Lorang, T.D., & Luchansky, J.B. (1997). Viability of *Escherichia coli* O157:H7 in pepperoni during the manufacture of sticks and the subsequent storage of slices at 21, 4 and 20C under air, vacuum and CO2. *International Journal of Food Microbiology*, 37, 47–54.

Gálvez, A., Abriouel, H., López, R. L., & Omar, N. B. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology,* 120, 51-70.

Hado, B. H., Yousef, A. E., (2007). Characteristics of Listeria monocytogenes important to food processors. In: Ryser, E.T., Marth, E.H. (Eds.), *Listeria, Listeriosis and Food Safety* (3rd ed.) (pp. 157-213) Boca Raton, FL, USA: CRC Press.

Holzapfel, W. H., Geisen, R., & Schillinger, U. (1995). Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *International Journal of Food Microbiology*, 24, 343-362.

Ihnot, A. M., Roering, A. M., Wierzba, R. K., Faith, N. G., & Luchansky, J. B. (1998). Behavior of *Salmonella* Typhimurium DT104 during the manufacture and storage of pepperoni. *International Journal of Food Microbiology*, 40, 117–121.

Jofré, A., Garriga, M., & Aymerich, T. (2008). Inhibition of *Salmonella* sp., *Listeria monocytogenes* and *Staphylococcus aureus* in cooked ham by combining antimicrobials, high hydrostatic pressure and refrigeration. *Meat Science*, 78, 53–59.

Johnsen, B. O., Lingaas, E., Dag Torfoss, Strøm, E. H., & Nordøy, I., (2010). A large outbreak of *Listeria monocytogenes* infection with short incubation period in a tertiary care hospital. *Journal of Infection*, 61, 465-470.

Kalchayanand, N., Sikes A, Dunne C. P, & Ray B. (1998). Interaction of hydrostatic pressure, time and temperature of pressurization and pediocin AcH on inactivation of foodborne bacteria. *Journal of Food Protection*, 61, 425–431.

Jung, L.S., Lee, H.Y., Lee, S.H., Kim, S., & Ahn, J. (2013). Assessment of pressure-induced inactivation of *Listeria monocytogenes* exposed to low pHs. Food Science and Biotechnology, 22, 99-105.

Linton, M., McClements, J. M. J., & Patterson, M. F. (1999). Survival of *Escherichia coli* O157:H7 during Storage in Pressure-Treated Orange Juice. *Journal of Food Protection*, 9, 975-1096.

Liu, G., Wang, Y., Gui, M., Zheng, H., Dai, R., & Li, P. (2012). Combined effect of high hydrostatic pressure and enterocin LM-2 on the refrigerated shelf life of ready-to-eat sliced vacuum packed cooked ham. *Food Control*, 24, 64-71.

Lorentzen, G., Ytterstad, E., Olsen, R.L, Skjerdal, T., 2010. Thermal inactivation and growth potential of *Listeria innocua* in rehydrated salt-cured cod prepared for ready-to-eat products. *Food control*, 21, 1121-1126.

Mataragas, M., Drosinos, E. H. & Metaxopoulos, J. (2003). Antagonistic activity of lactic acid bacteria against *Listeria monocytogenes* in sliced cooked cured pork shoulder stored under vacuum or modified atmosphere at 4±2^{o}C. *Food Microbiology*, 20, 259–265.

Mertens, B., & Deplace, G. (1993). Engineering aspects of high pressure technology in the food industry. *Food Technology*, 47, 164-169.

Mol, J. H. H., Hietbrink, J. E. A., Mollen, H. W. M., & van Tinteren, J. (1971). Observations on the microflora of vacuum packed sliced cooked meat products. *Journal of Applied Bacteriology*, 34, 377–397.

Pal, A., & Ramana, K. V. (2009). Isolation and preliminary characterization of a nonbacteriocin antimicrobial compound from *Weissella paramesenteroides* DFR-8 isolated from cucumber (*Cucumis sativus*). *Process Biochemistry*, 44, 499–503.

Park, S. W., Sohn, K. H., Shin, J. H., & Lee, H. J. (2001). High hydrostatic pressure inactivation of *Lactobacillus viridescens* and its effect on ultrastructure of cells. International *Journal of Food Microbiology*, 36, 775-781,

Patterson, M. F., Mackle, A., & Linton, M. (2011). Effect of high pressure, in combination with antilisterial agents, on the growth of *Listeria monocytogenes* during extended storage of cooked chicken. *Food Microbiology*, 28, 1505–1508.

Patterson, M. F., McKay, A. M., Connolly, M., & Linton, M. (2010). Effect of high pressure on the microbiological quality of cooked chicken during storage at normal and abuse refrigeration temperatures. *Food Microbiology*, 27, 266–273.

Patterson, M. F. (2005). Microbiology of pressure-treated foods. *Journal of Applied Microbiology*, 98, 1400–1409.

Pilet, M. F., & Leroi, F. (2011). Applications of protective cultures, bacteriocins and bacteriophages in fresh seafood and seafood products. In: Lacroix, C. (Ed.), *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation* (pp. 324-347) Zurich, Switzerland: Woodhead Publishing.

Scallan, E., Hoekstra, R. M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L., Jones, J.L., & Griffin, P.M. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging and Infectious Diseases*, 17, 7–15.

Shi, W., Qingping, W., Jumei Z., Moutong, C., Zean, Y. (2015). Prevalence, antibiotic resistance and genetic diversity of *Listeria monocytogenes* isolated from retail ready-to-eat foods in China. *Food Control*, 47, 340-347.

Smith, K., Mendonca, A., & Jung, S. (2009). Impact of high-pressure processing on microbial shelf-life and protein stability of refrigerated soymilk. *Food Microbiology*, 26, 794−800.

Srionnual S., Yanagida F., Lin L. H., Hsiao K. N., & Chen Y. S. (2007). Weissellicin 110, a newly discovered bacteriocin from *Weissella cibaria* 110, isolated from plaa-som, a fermented fish product from Thailand. *Applied and Environmental Microbiology*, 73, 2247-2250.

Stephan, R., Althaus D., Kiefer, S., Lehner, A., Hatz, C., Schmutz, C., Jost M., Gerber, N., Baumgartner, A., Hachler, H., Mausezahl-Feuz, M. (2015). Foodborne transmission of *Listeria monocytogenes* via ready-to-eat salad: A nationwide outbreak in Switzerland, 2013-2014. *Food Control*, 57, 14-17.

529 Stratakos, A., & Ch. Koidis, A. (2015). Suitability, efficiency and microbiological safety of
530 novel physical technologies for the processing of ready-to-eat meals, meats and pumpable
531 products. *International Journal of Food Science and Technology* DOI: 10.1111/ijfs.12781.
532
533 Stratakos, A. Ch., Delgado-Pando, G., Linton, M., Patterson, M. F., & Koidis, A. (2015a).
534 Synergism between high-pressure processing and active packaging against *Listeria*
535 *monocytogenes* in ready-to-eat chicken breast. *Innovative Food Science and Emerging*
536 *Technologies*, 27, 41–47.
537
538 Stratakos, A. Ch., Linton, M., Patterson, M. F., & Koidis, A. (2015b). Effect of high pressure
539 processing on the shelf-life, safety and organoleptic characteristics of lasagne-ready meals
540 during storage at refrigeration and abuse temperature. *Innovative Food Science and Emerging*
541 *Technologies*. In Press.
542
543 Swaminathan, B., & Gerner-Smidt, P. (2007). The epidemiology of human listeriosis.
544 *Microbes and Infection*, 9, 1236-1243.
545
546 Tahiri, I., Desbiens, M., Kheadr, E., Lacroix, C., & Fliss I. (2009). Comparison of different
547 application strategies of divergicin M35 for inactivation of *Listeria monocytogenes* in cold
548 smoked wild salmon. *Food Microbiology*, 26, 783-93.
549
550 Tholozan, J. L., Ritz, M., Jugiau, F., Federighi, M., & Tissier, J. P. (2000). Physiological effects
551 of high hydrostatic pressure treatments on *Listeria monocytogenes* and *Salmonella*
552 *typhimurium*. *Journal of Applied Microbiology*, 88, 2002–2021.
553

554 Tyrovouzis, N. A., Angelidis, A. S., & Stoforos, N. G. (2014). Bi-phasic growth of Listeria
555 monocytogenes in chemically defined medium at low temperatures. *International Journal of*
556 *Food Microbiology*, 186, 110-119.
557
558 Vermeiren, L., Devlieghere, F., De Graef, V., & Debevere, J. (2004). Evaluation of meat born
559 lactic acid bacteria as protective cultures for the biopreservation of cooked meat products.
560 *International Journal of Food Microbiology*, 96, 149–164.
561
562 Werber, D., Hille, K., Frank, C., Dehnert, M., Altmann, D., Müller-Nordhorn, J., et al. (2013).
563 Years of potential life lost for six major enteric pathogens, Germany, 2004-2008. *Epidemiology*
564 *and Infection*, 141, 961-968.
565
566
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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.