Antilisterial Effect and Influence on Listeria monocytogenes Gene Expression of Enterocin or Enterococcus faecalis in Sliced Dry-Cured Ham Stored at 78C

Running title: Antilisterial effect of enterocin in sliced dry cured ham

Antilisterial effect and influence on *Listeria monocytogenes* gene expression of enterocin or *Enterococcus faecalis* in sliced dry cured ham stored at 7 °C

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Keywords: biopreservation, enterocin, *Listeria monocytogenes*, gene expression, dry-cured ham

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Abstract

In this study we focused on the effect of an enterocin or an *Enterococcus faecalis* strain added onto sliced dry cured ham that was artificially inoculated with *L. monocytogenes* and stored at 7 °C. The population of *L. monocytogenes* and the expression of 5 genes were monitored throughout the storage period. A persistent and a non-persistent strain were tested and both were influenced by the presence of the enterocin; both populations were reduced by more than 2 Log_{10} CFU/g after 14 days, compared to the control, non-inoculated ham. The presence of *E. faecalis*, a bacteriocin producing lactic acid bacterium, had a much less pronounced effect on the viable counts for both strains. Concerning gene expression, a common trend that was observed for both strains in the presence of enterocin was the downregulation of genes tested after 30 minutes of storage at 7 °C. For the remaining of the storage period the expression fluctuated but was mostly reduced. Similarly, the presence of *E. faecalis* led to an overall downregulation of genes. The effect on gene expression of both the enterocin and the *E. faecalis* was more pronounced on the non-persistent *L. monocytogenes* strain. Although the potential of a bacteriocin and a bacteriocin producing microorganism to control *L. monocytogenes* was confirmed, this study highlights that gene expression may be influenced and needs to be evaluated when considering such biopreservation interventions.

Keywords: biopreservation, enterocin, *Listeria monocytogenes*, gene expression, dry-cured ham

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Highlights

- Viability of *Listeria monocytogenes* in sliced dry-cured ham was greatly influenced by the addition of an enterocin
- Addition of a bacteriocin producing *Enterococcus faecalis* had a less pronounced effect on *L. monocytogenes* viability
- Expression of genes related to *L. monocytogenes* stress response/adaptation was modified in the presence of an enterocin
- The addition of a bacteriocin producing *Enterococcus faecalis* influenced gene expression in one of the two *L. monocytogenes* strains tested

63 The term biopreservation or biological preservation of foods was coined in the mid-90's and
64 refers to the food safety improvement and extension of shelf life through microbial
65 antagonism *(27, 28)*. A strong antagonistic ability is attributed to lactic acid bacteria (LAB)
66 and has been documented for a variety of fermented foods *(16)*. Inhibition of undesirable
67 microorganisms can be due to direct effect of LAB through competition for nutrients, niche
68 occupation or indirect effect through synthesis of bacteriocins and/or production of other
69 metabolites. More than 20 years of research have expanded our knowledge regarding the
70 modes of action of LAB naturally present in the foods or intentionally added as protective
71 cultures. Further, the field of application of LAB and/or associated bacteriocins has been
72 broadened to include non-fermented foods, food plant environment but also employment in
73 non-food sectors *(4)*.
74 Many bacteriocins produced by LAB exert an inhibitory action towards strains of *Listeria*
75 *monocytogenes*, a foodborne pathogen of particular concern for refrigerated ready-to-eat
76 (RTE) foods. For this reason, LAB bacteriocins with antilisterial effect have been the focus of
77 both *in vitro* and *in situ* studies to understand the potential for industrial application to
78 reduce the *L. monocytogenes* risk associated with RTE foods. Efficacy of bacteriocins, or
79 overall LAB competition, in inhibiting or reducing *L. monocytogenes* growth in various RTE
80 foods is well documented and is reviewed by Zilelidou and Skandamis *(35)*. However, most of
81 the studies so far conducted examined how bacteriocins or LAB impact on growth parameters
82 of *L. monocytogenes* not taking into consideration the consequences for the physiology of the
83 microorganism. Therefore there is the need to integrate current knowledge regarding the
84 antilisterial effect with information concerning molecular/cellular response of *L.*
85 *monocytogenes* to LAB and/or bacteriocin presence or addition in foods. A potential first step
86 in appreciating changes in microbial physiology is by looking into changes in gene expression
87 *(9)*.

The purpose of this study was dual. First, we compared the antilisterial effect of an enterocin and an *E. faecalis* strain, added to sliced dry-cured ham and incubated at refrigeration temperature. Secondly, we evaluated the expression of genes that are involved in stress response and adaptation, under the same conditions. Two different strains of *L. monocytogenes* isolated from a meat plant environment were tested; one was previously shown to be persistent and the other one non-persistent *(23)*.

Materials and methods

1. Bacterial strains and culture media

Two *Listeria monocytogenes* strains, previously isolated from an Iberian pig processing plant, were used in this study and belonged to the culture collection of INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain). Strain S4-2 was serotype 1/2b and has been characterized as persistent in the environment while strain S12-1 was serotype 1/2c and non-persistent *(23)*. The strains were maintained as stock cultures at -80 °C in Trypticase Soy Yeast Extract Broth (TSYEB, Biolife s.r.l., Milano, Italy) supplemented with 15% glycerol. Before use in experiments, strains were sub-cultured twice onto Brain Heart Infusion agar (BHI, LabM Ltd., Lancanshire, UK) at 37 °C for 24 hours. A bacteriocinogenic strain of *Enterococcus faecalis* was also used. This strain, *E. faecalis* B1, was previously isolated from raw bovine meat, identified to the species level by sequencing of the gene encoding the 16S rRNA and belonged to the culture collection of the University of Turin, Italy. The *E. faecalis* strain was maintained as a stock culture at -80 °C in M17 Broth (Oxoid, Milan, Italy), supplemented with 15% glycerol. Before use in experiments, the strain was sub-cultured twice onto M17 agar at at 37 °C for 24 hours. In addition, an enterocin extract was used in the experiments. The enterocin AB extract was previously obtained from an overnight

culture of *Enterococus faecium* INIA TAB7 *(26)* at 30 °C and semi-purified through ammonium sulfate precipitation (300 g/L) *(8)* and stored at -80 °C until use. The activity of the bacteriocin extract was determined against the two *L. monocytogenes* strains through the agar spot test *(2)* and expressed in arbitrary units (AU) per ml.

2. Dry-cured ham preparation and inoculation

One large piece (~7 kg) of dry-cured ham was purchased from a commercial supplier in Spain and aseptically sliced in the laboratory. A sample was analyzed for the presence of *L. monocytogenes* and resulted negative (absence in 25 g). Subsequently, samples of 10g of dry-cured ham were inoculated by adding a cell suspension in Ringer's solution (Oxoid, Milan, Italy) of *L. monocytogenes* S4-2 or S12-1 to achieve a final concentration of ca. 10^6 cfu/g. Cell suspensions were prepared from overnight cultures in BHI. In a set of samples, the enterocin extract was added on the surface of the sliced dry-cured ham to reach a final activity of 1054 AU/g. For a second set of samples, a cell suspension of *E. faecalis* was added to reach a final concentration of ca. 10^6 cfu/g. Sliced dry-cured ham, inoculated with either of the two *L. monocytogenes* strains but not supplemented with enterocin or *E. faecalis* was used as control. Samples were vacuum packed and maintained at 7 °C for 28 days. This temperature was chosen taking into account literature data that suggest a higher than 4 °C temperature for domestic refrigerators (*12)*. Two biological replicates were considered for each strain of *L. monocytogenes*, in each condition (i.e. enterocin or *E. faecalis* addition). By visual inspection, no color differences were observed between the control and the enterocin or *E. faecalis* supplemented ham during storage. Colour parameters (L*, a* and b*) in sliced dry-cured ham with enterocin were previously studied and no significant changes were detected *(22)*. Average pH and a_w values for this type of ham (as determined in previous experiments) are 5.9 and 0.905, respectively.

138

139 *3. Sampling during storage*

140 At time zero (immediately after inoculation) as well as after 6 hours, 7, 14 and 28 days of
141 storage at 7 °C, a 10 g sample was subjected to microbiological analysis to determine the
142 viable count of *L. monocytogenes*. Briefly, the sample was transferred to a sterile stomacher
143 bag and 90 ml of Ringer's solution were added. Then the sample was homogenized in a
144 stomacher (BagMixer, Interscience, France) for 2 minutes at normal speed and room
145 temperature. Serial decimal dilutions were prepared in the same solution and plated on
146 *Listeria* Selective Oxford Agar Base (Oxoid). Plates were incubated at 37 °C for 48 hours
147 before colony count. At time zero as well as after 30 minutes, 6, 24 and 168 hours (7 days) of
148 storage at 7 °C, 10 g samples were used for RNA extraction and for the Agar Well Diffusion
149 Assay (AWDA) as described by Urso et al. *(31)*. A homogenate was prepared, as described
150 above, from each 10 g sample. Two ml from the homogenate were centrifuged at 13,000 *g* for
151 1 minute at 4 °C. Immediately after centrifugation, the pellet was covered with 0.05 ml of
152 RNA*later* (Ambion, Applied Biosystems, Milan Italy) and stored at -20 °C until the RNA
153 extraction.

154

155 *4. RNA extraction and cDNA synthesis*

156 RNA extraction was performed on the thawed samples, employing the procedure described
157 by Rantsiou et al. *(24)*. Fifty microliters of lysozyme (50 mg/ml, Sigma) and 25 µl of
158 proteinase K (25 mg/ml, Sigma) were added to the thawed samples that were then incubated
159 at 37 °C for 20 minutes in a Thermomixer compact (Eppendorf, Milan, Italy). Samples were
160 then processed using the MasterPure Complete DNA and RNA Purification Kit (Epicentre,
161 Madison, WI, USA), following the manufacturer's instructions. DNA was digested with the
162 Turbo DNase (Ambion) and complete removal of the DNA was verified by using an aliquot of

the extract as template in a qPCR reaction (as described below). When amplification took place, the DNase treatment was repeated until complete removal of the DNA. RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Celbio, Milan, Italy). Complementary DNA (cDNA) synthesis was performed using random hexamers (Promega, Milan, Italy) according to Rantsiou et al. *(24)*. The same quantity of RNA (ng/μl) was added in the reaction for each sample. The M-MLV Reverse Transcriptase (Promega) was used following the instructions of the manufacturer. An RNase Inhibitor (Promega) was added in the reaction and dNTPs were added at a final concentration of 2 mM each. Reverse transcription was performed in a DNA Engine Peltier Thermal Cycler (BioRad, Milan, Italy) at 37 °C for 1 hour. The cDNA was stored at -20 °C until it was used in qPCR amplification.

5. Quantitative PCR

Quantitative PCR amplification was performed using the cDNA, synthesized as above from each sample, as template. Five genes listed in Table 1 were targeted. The amplification took place in a PCR Chromo4 Real Time PCR detection system (BioRad) using the SsoAdvanced SYBR Green Supermix (BioRad) and the amplification conditions described by Mataragas et al. *(19)* with the exception of the *tuf* gene annealing temperature that was adjusted to 55 °C. Each cDNA was amplified in triplicate, in the same amplification run, to reduce inter-run experimental variability.

6. Data analysis – Statistical analysis

Threshold cycle (C_T) values were exported to Excel for further analysis. Mean C_T values, for each cDNA sample, were computed and used to calculate the relative gene expression by the $2^{-\Delta\Delta C_T}$ method, where $\Delta\Delta C_T$ is: $(C_{T,\ target}\text{-}C_{T,\ housekeeping})_{test\ condition} - (C_{T,\ target} - C_{T,\ housekeeping})_{control\ condition}$ *(17)*. Stress or virulence genes were considered as target while the *tuf* as

188 housekeeping. Control condition was the sliced dry-cured ham inoculated with *L.*
189 *monocytogenes* alone while the test condition was the dry-cured ham inoculated with *L.*
190 *monocytogenes* and supplemented with enterocin or co-inoculated with *E. faecalis* (at the
191 respective time points). Log2 values of relative expression were calculated and statistically
192 treated using the SPSS statistics (IBM Corp., Armonk, NY, USA).
193
194 **Results and Discussion**
195 Dry-cured ham is considered a ready to eat (RTE) food and it is known to be prone to *Listeria*
196 *monocytogenes* contamination during processing. The main hurdles to *L. monocytogenes*
197 growth during refrigerated storage are low a_w, addition of salt and nitrites. These hurdles
198 however are not listeriocidal and several studies have evaluated alternative approaches, with
199 a lethal effect, such as high hydrostatic pressure processing, irradiation and supercritical
200 carbon dioxide processing *(3, 6, 11, 21)*. Furthermore, the potential of *L. monocytogenes*
201 growth control using bacteriocins has been investigated *(13)*. In this study we sought to
202 investigate the behavior of *L. monocytogenes* in dry-cured ham supplemented with a
203 bacteriocin extract or co-inoculated with a bacteriocinogenic *E. faecalis*, during storage. In this
204 context, behavior is intended as population kinetics and gene expression profile during
205 storage. For this purpose, two strains of *L. monocytogenes* were tested; a persistent and a non-
206 persistent. The classification of the strains as persistent and non-persistent was based on
207 previous observations regarding frequency of isolation and occurrence in different areas of a
208 pig processing environment. More specifically, S4-2 was considered as a persistent strain
209 found in the environment, equipment, carcasses and raw and dry cured products. This
210 genotype was repeatedly isolated. Strain S12-1 was non-persistent but isolated from dry
211 cured products *(23)*.

1. Effect of enterocin and bacteriocinogenic Enterococcus faecalis *on* Listeria monocytogenes *population*

By Agar Well Diffusion Assay performed *in vitro* it was determined that the enterocin extract and the bacteriocinogenic *E. faecalis* evenly inhibited both strains of *L. monocytogenes* (data not shown). When *L. monocytogenes* strains were artificially inoculated in dry-cured ham and stored under vacuum at 7 °C, the viable count remained un-altered during the first 7 days and declined by about 0.6 Log_{10} CFU/g at 14 days (Table 2). The population then remained stable for both strains up to 28 days (data not shown). It has to be noted that previous works have determined that both a_w and pH remain essentially unaltered during refrigerated storage of dry-cured ham. The average value of pH for the dry-cured ham was 5.9 while the average a_w was 0.905. Further, salt and nitrites were added and during storage had average concentrations of 2.69 mg/Kg and 4.12 % respectively. Taken together, these physicochemical characteristics render the product a food unable to support the growth of *L. monocytogenes.* Therefore, it is expected that a *L. monocytogenes* population, naturally present or artificially inoculated in such dry cured ham will remain stable or possibly decline with time during storage. Conversely, when the dry-cured ham was supplemented with enterocin, an immediate effect was observed in the population of *L. monocytogenes.* The population was reduced by almost 0.8 Log_{10} CFU/g for strain S4-2 and by 1.5 Log_{10} CFU/g for strain S12-1. It should be noted here that a time window of at least 30 minutes elapsed between the inoculation/enterocin supplementation and the sampling for the determination of the viable count. This time window was sufficient to observe the inhibition of *L. monocytogenes*. *L. monocytogenes* populations further declined at 7 and 14 days; the microbial load was reduced by 1.8 Log_{10} CFU/g between time 0 and 14 days for strain S4-2 and by 1.9 Log_{10} CFU/g for strain S12-1. At 14 days, the population of strain S4-2 was almost 2 Log_{10} CFU/g lower in the dry cured ham supplemented with enterocin compared to the control while for strain S12-1

237 the effect was greater; the enterocin inactivated 2.8 Log_{10} CFU/g of the population. Therefore,
238 the enterocin displayed significant listericidal effect. It has to be underlined that such effect
239 was strain dependent; it was greater for the non-persistent strain. RTE meat products may be
240 contaminated by *L. monocytogenes* and for this reason the potential of bacteriocins to control
241 it has been extensively investigated *(33)*. In dry-cured ham the anti-listerial effect has been
242 previously proven for enterocins AB *(13)*. In this previous study, enterocins AB drastically
243 reduced by 2.5 Log_{10} CFU/g *L. monocytogenes* in dry-cured ham stored at 4 °C for 1 day. The
244 results of our study confirm the potential of enterocins AB to impact on the viability of *L.*
245 *monocytogenes*.
246 When the bacteriocinogenic *E. faecalis* was co-inoculated in the sliced dry-cured ham, the
247 evolution of the pathogen's population showed a reducing trend with time. The reduction
248 observed however cannot be considered important; in the case of strain S4-2 it was of 0.2 Log
249 between time zero and 7 days (statistically significant difference, $P< 0.05$) while for strain
250 S12-1 it was of 0.1. Therefore, the microbial competition exerted by *E. faecalis* resulted in
251 containment of *L. monocytogenes*, when compared to the control condition. It has to be
252 underlined that the effective production of bacteriocin by *E. faecalis in situ*, after inoculation
253 in the dry-cured ham, was verified throughout the conservation period by AWDA (data not
254 shown). However, the results obtained with the enterocin and the *E. faecalis* cannot be
255 compared. Importantly, the *E. faecalis* strain used was not the same as the one from which the
256 enterocin was purified, but also it is clear that other variables such as bacteriocin liberation
257 from the cell and diffusion in the sliced ham most likely influence the effect of the *E. faecalis*
258 that was observed. The use of bacteriocinogenic cultures has been largely explored for
259 fermented foods, including fermented meat products. In the case of fermented meat products,
260 the bacteriocin producing strains used act as starter culture and contribute to the safety, by
261 microbial competition, bacteriocin and lactic acid production and to the development of the

desired organoleptic properties of the final product *(7)*. In non-fermented meat products, bacteriocin producing lactic acid bacteria may be added as protective cultures and they are not expected to grow significantly or to produce large amounts of lactic acid. This approach has not yet been explored for dry-cured ham and the results of the present study imply that the *E. faecalis* strain used cannot by itself reduce the population of *L. monocytogenes.* This may be due to limited diffusion of the bacteriocin, or to its production at concentrations that may interfere with regulatory mechanisms and therefore contain growth, but not necessarily high enough to kill *L. monocytogenes (4)*. Further the observed lack of lethal effect may be due to limited interaction of the two microorganisms in the solid food matrix, where physical contact, which has been proposed as an inter-species inhibitory mechanism *(33)*, does not take place.

2. Effect of enterocin and Enterococcus faecalis *on* Listeria monocytogenes *gene expression*

Although the effect of bacteriocins and bacteriocinogenic microorganisms on growth and inactivation behavior is widely investigated, the consequences on the physiology of the microorganisms have not been adequately addressed. The outcome of a given environmental condition on the physiological state can be inferred from the transcriptome, proteome or metabolome of microorganisms. Studies so far have primarily focused on the transcriptome under *in vitro* conditions *(9, 25)* to describe the impact of food-related environmental factors on the physiology and behavior of foodborne pathogens. The purpose of the present study was to explore the effect of an enterocin and a bacteriocin producing *E. faecalis* on expression of selected genes of *L. monocytogenes,* artificially inoculated in dry-cured ham.

Figures 1 and 2 present the relative gene expression for two different strains of *L. monocytogenes;* strain S4-2 (Figure 1) is a persistent strain while strain S12-1 (Figure 2) is a non-persistent strain. The genes chosen (Table 1) are representatives of stress response and

287 virulence genes and have been previously employed in studies of *L. monocytogenes* gene
288 expression *in situ* (*19).* Relative gene expression was calculated using as a control condition *L.*
289 *monocytogenes* artificially inoculated in dry-cured ham. Therefore, figures 1 and 2 depict the
290 sole impact of enterocin or *E. faecalis* addition while the stressful conditions (low a_w,
291 refrigeration temperature, nitrites) that are known to have influence on gene expression, are
292 leveled out. During the long-term storage of vacuum packed dry cured ham, changes in the
293 physicochemical or microbiological parameters are not significant and therefore gene
294 expression is not expected to be influenced. Therefore, the gene expression was monitored up
295 to the 7^{th} day of refrigerated storage while a time point very close to the inoculation (30
296 minutes) was considered in order to capture the response of *L. monocytogenes* upon
297 inoculation. As can be seen in the two figures, the expression of the target genes fluctuated
298 during refrigerated conservation. Notably, for both strains of *L. monocytogenes* an overall
299 downregulation tendency for all the genes was observed after 30 minutes of storage. For
300 strain S12-1, this downregulation was already evident immediately after inoculation (time 0).
301 For strain S4-2, statistically significant variation in expression was observed for gene
302 *lmo0669*. This gene, encoding for a protein similar to an oxidoreductase and likely involved in
303 acid stress response, was downregulated at 30 minutes and then significantly upregulated at
304 6 hours while expression leveled off throughout the rest of the storage period. Similar pattern
305 was observed for this gene in the strain S12-1; downregulation at 30 minutes, upregulation at
306 6 hours followed in this case by significant up regulation at 168 hours. Upregulation at 168
307 hours was also observed for gene *lmo2434*, encoding for a glutamate decarboxylase and
308 involved in acid stress response. The virulence gene *prfA*, encoding for a major virulence
309 transcriptional regulator, displayed fluctuating expression with a tendency for reduced
310 expression as compared to the condition of dry-cured ham.

311 Apart from the effect of a bacteriocin extract we sought to investigate how the presence of a
312 bacteriocinogenic *E. faecalis* strain would influence gene expression of *L. monocytogenes* in
313 sliced dry-cured ham. The goal was to mimic a situation, i.e. co-presence in food of *L.*
314 *monocytogenes* and a competitive lactic acid bacterium, which is frequently verified during
315 food production and storage. For strain S4-2 no significant differences in gene expression
316 were observed during time (data not shown). Contrarily, for strain S12-1 gene expression
317 varied with time. As can be seen in figure 3, the main outcome observed from the presence of
318 *E. faecalis* in the dry-cured ham is downregulation for all genes throughout time with the
319 exception of the 30 minutes time point in which all target genes were upregulated. Variation
320 in gene expression through time resulted to be significant for genes *lmo1421* and *lmo0669.*
321 Limited information is available in the literature concerning the effect of bacteriocins or
322 bacteriocin producing microorganisms on *L. monocytogenes* gene expression. Winkelströter
323 and Martinis *(32)* registered dowregulation of the expression of *inlA* gene, an important
324 virulence gene, in *in vitro* tests with 10 strains of *L. monocytogenes,* in the presence of 3
325 different bacteriocins, produced by *E. faecium, Leuconostoc mesenteriodes* and *Lactobacillus*
326 *sakei*. Gene *inlA,* as well as *prfA,* encoding for a major virulence gene regulator, were
327 downregulated in *L. monocytogenes* in the presence of metabolic products of two strains of *E.*
328 *faecium (34)*. The results of the present study are in agreement with these previous reports;
329 gene *prfA* was downregulated in both *L. monocytogenes* strains, in response to the presence of
330 the enterocin or the *E. faecalis* strain (for *L. monocytogenes* S12-1). Apart from *prfA*, also other
331 genes (involved in virulence and stress response/adaptation) tested in the present study but
332 also by Ye et al. *(34)* were downregulated in the presence of either a bacteriocin, a metabolic
333 product of *E. faecium* or *E. faecalis*. Although this general trend was identified in both studies,
334 it should be noted that the effect on gene expression depended both on the strain of *L.*
335 *monocytogenes* tested but also on the strain of *E. faecium* used to control *L. monocytogenes.* In

a similar study, Miranda et al *(20)* investigated gene expression of *L. monocytogenes* in milk co-inoculated with a nisin-producing *L. lactis*. Out of the 4 genes tested, *gadD2* consistently showed increased expression in the milk containing *L. lactis* compared to milk without *L. lactis*. Genes *sigB, groEL* were also investigated and expression varied with time, showing a downregulation as incubation proceeded. On the other hand, gene *gbu* was downregulated by the presence of *L. lactis*. Although the incubation temperature was different than in the current study (20 or 30 °C as opposed to 7 °C here), a liquid food matrix rather than a solid, and importantly, the antagonistic microorganism was different. Results concerning the *gbu* gene appear to be consistent; in both studies the gene was essentially downregulated by the presence of a bacteriocin-producing microorganism.

When the two tested conditions, i.e. presence of enterocin and presence of *E. faecalis*, were compared (Figure 4), it was evident that the effect on gene expression was similar. With the exception of the 30 minutes time point when most genes were upregulated by the presence of *E. faecalis*, in the remaining time points expression went down. It is interesting to note that *E. faecalis* exhibited higher, mostly negative impact, on gene expression of *L. monocytogenes* compared to the enterocin. In most cases *E. faecalis* accentuated the downregulation of genes or inversed the pattern (from upregulated to downregulated). Expression of *prfA* was reduced in the presence of *E. faecalis* in 3 time points (at 6, 24 and 168 hours). Similarly, gene *lmo0669* showed decreased expression in 4 out of 5 time points and this reduced expression was significant at 6 and 168 hours. Gene *lmo1421* was further downregulated due to the presence of *E. faecalis* at the first time point.

Previous studies have addressed the effect of bacteriocins on gene expression of *L. monocytogenes* however data comparison is not plausible due to differences in the experimental approaches adopted. Different strains of *L. monocytogenes* tested, different media or types of food, different temperature/time regimes considered and a range of genes

361 targeted. Concordant conclusions though have been reached and are also supported by the
362 present study. Bacteriocins or bacteriocin producing microorganisms have an effect on gene
363 expression of *L. monocytogenes*, both *in vitro* and *in situ*, and gene expression varies with time
364 *(20, 15, 34, present study)*. These concordant outcomes suggest that *L. monocytogenes* senses
365 and responds by adapting its expression and therefore there is a need to go beyond viable
366 counts when biopreservation approaches are investigated and explore global physiological
367 response of the target microorganism.
368
369

Viability of *L. monocytogenes* in dry-cured ham was greatly influenced by the addition of an enterocin while the effect of the addition of *E. faecalis* was less pronounced. Differences were detected between the two strains of *L. monocytogenes*; inhibition of the non-persistent strain was more prominent compared to the persistent strain. The results obtained suggest that addition of a bacteriocin is a more effective measure to control *L. monocytogenes* than addition of a bacteriocinogenic protective culture, in sliced dry-cured ham. It remains to be seen if the persistence phenotype is associated with higher resistance to a bacteriocin. Further studies are needed to elucidate this aspect. On the other hand, a common pattern regarding the expression of the 5 tested genes could be delineated for both strains; in the presence of enterocin, the 30 minutes time point determined a downregulation of the genes and this trend was essentially maintained throughout the storage period, up to 168 hours. For the persistent strain, no significant differences could be observed in gene expression during storage, in the presence of *E. faecalis*. On the contrary, for the non-persistent differences were highlighted during storage, with an important shift between time 0 (downregulation), 30 minutes (upregulation) and the remaining period (downregulation). Based on the data of this study we cannot correlate the persistence phenotype with the behavior observed; additional strains (both persistent and non-persistent) should be tested, under *in situ* conditions, to respond to this query. The gene expression results, although not conclusive, underline the need to broaden our understanding of *L. monocytogenes* behavior in foods by integrating phenotypic description with transcriptomic data.

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Figure legends

Figure 1. Relative gene expression for genes *prfA, gbuB, lmo1421, lmo2434, lmo0669* of *Listeria monocytogenes* strain S4-2 inoculated in dry-cured ham and supplemented with enterocin. Relative gene expression was calculated by the 2 $^{-\Delta\Delta C_T}$ method and log2 values are reported. Error bars indicate standard deviation of two biological replicates. For gene *lmo0669,* the asterisk indicates difference ($P < 0.05$) in the expression level, between 30 minutes and 6 hours of conservation.

Figure 2. Relative gene expression for genes *prfA, gbuB, lmo1421, lmo2434, lmo0669* of *Listeria monocytogenes* strain S12-1 inoculated in dry-cured ham and supplemented with enterocin. Relative gene expression was calculated by the 2 $^{-\Delta\Delta C_T}$ method and log2 values are reported. Error bars indicate standard deviation of two biological replicates. For genes *prfA*, *lmo2434* and *lmo0669*, the asterisks indicate differences ($P < 0.05$) in the expression level, across different time-points during conservation.

Figure 3. Relative gene expression for genes *prfA, lmo1421, lmo2434, lmo0669* of *Listeria monocytogenes* strain S12-1 co- inoculated in dry-cured ham with *Enterococcus faecalis* B1. Relative gene expression was calculated by the 2 $^{-\Delta\Delta C_T}$ method and $\log_2$ values are reported. Error bars indicate standard deviation of two biological replicates. For genes *lmo1421* and *lmo0669,* the asterisks indicate differences ($P < 0.05$) in the expression level, across different time-points during conservation.

Figure 4. Relative gene expression for genes *prfA, lmo1421, lmo2434, lmo0669* of *Listeria monocytogenes* strain S12-1 inoculated in dry cured ham supplemented with enterocin (condition *a*) or co- inoculated in dry-cured ham with *Enterococcus faecalis* B1 (condition *b*). Relative gene expression was calculated by the 2 $^{-\Delta\Delta C_T}$ method and $\log_2$ values are reported. Error bars indicate standard deviation of two biological replicates. Asterisks indicate statistically significant differences (ANOVA, $p < 0.05$) in the expression between conditions *a* and *b.*