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Research Paper

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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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 *Listeria monocytogenes* and stored at 7°C. The population of *L. monocytogenes* and the expression of five genes were monitored throughout the storage period. A persistent and a nonpersistent strain were tested, and both were influenced by the presence of the enterocin; both populations were reduced by more than 2 Log CFU/g after 14 days compared with the control, noninoculated ham. The presence of *E. faecalis,* a bacteriocin-producing lactic acid bacterium, had a less pronounced effect on the viable counts for both strains. Concerning gene expression, a common trend observed for both strains in the presence of enterocin was the down-regulation of genes tested after 30 min of storage at 7°C. For the remainder of the storage period, the expression fluctuated but was mostly reduced. Similarly, the presence of *E. faecalis* led to an overall down-regulation of genes. The effect on gene expression of both enterocin and *E. faecalis* was more pronounced on the nonpersistent *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.

HIGHLIGHTS

- Addition of an enterocin influences *L. monocytogenes* viability in sliced dry-cured ham.
- Bacteriocin-producing *E. faecalis* had less effect on *L. monocytogenes* viability.
- Enterocin modified gene expression related to *L. monocytogenes* stress response or adaptation.
- Bacteriocin-producing *E. faecalis* influenced gene expression in one *L. monocytogenes* strain.

Key words: Biopreservation; Dry-cured ham; Enterocin; Gene expression; *Listeria monocytogenes*

The term "biopreservation" or "biological preservation" of foods was coined in the mid-1990s and refers to food safety improvement and extension of shelf life through microbial antagonism *(10, 27, 28)*. A strong antagonistic ability is attributed to lactic acid bacteria (LAB) and has been documented for various fermented foods *(5, 16)*. Inhibition of undesirable microorganisms can be a direct effect of LAB, through competition for nutrients or niche occupation, or an indirect effect, through synthesis of bacteriocins and/or production of other metabolites. More than 20 years of research have expanded our knowledge regarding the modes of action of LAB naturally present in foods or intentionally added as protective cultures. Furthermore, the field of application of LAB and/or associated bacteriocins has been broadened to include nonfermented foods, the food plant environment, and employment in nonfood sectors *(4)*.

Many bacteriocins produced by LAB exert an inhibitory action toward strains of *Listeria monocytogenes,* a foodborne pathogen of particular concern for refrigerated ready-to-eat (RTE) foods. Therefore, LAB bacteriocins with an antilisterial effect have been the focus of both in vitro and in situ studies to understand the potential for industrial application to reduce the *L. monocytogenes* risk associated with RTE foods. Efficacy of bacteriocins, or overall LAB competition, in inhibiting or reducing *L. monocytogenes* growth in various RTE foods is well documented and is reviewed by Zilelidou and Skandamis *(35)*. However, most studies so far conducted examined how bacteriocins or LAB affect growth parameters of *L. monocytogenes,* not taking

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into consideration the consequences for the physiology of the microorganism. Therefore, there is the need to integrate current knowledge regarding the antilisterial effect with information concerning molecular or cellular response of *L. monocytogenes* to LAB and/or bacteriocin presence or addition in foods. A potential first step in appreciating changes in microbial physiology is to look into changes in gene expression *(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. Second, we evaluated the expression of genes involved in stress response and adaptation under the same conditions. Two strains of *L. monocytogenes* isolated from a meat plant environment were tested: one was previously shown to be persistent, and the other was previously shown to be nonpersistent *(22)*.

MATERIALS AND METHODS

Bacterial strains and culture media. Two *L. monocytogenes* strains, previously isolated from an Iberian pig processing plant, were used in this study and belonged to the culture collection of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA, 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 nonpersistent *(22)*. The strains were maintained as stock cultures at –80°C in Trypticase soy broth supplemented with 0.6% yeast extract (Biolife, Milano, Italy) and 15% glycerol. Before use in experiments, strains were subcultured twice onto brain heart infusion agar (LabM Ltd., Lancashire, UK) at 37°C for 24 h. 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 subcultured twice onto M17 agar at 37°C for 24 h. In addition, an enterocin extract was used in the experiments. The enterocin AB extract was previously obtained from an overnight culture of *E. faecium* INIA TAB7 *(26)* at 30°C, semipurified 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 milliliter.

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 the result was negative (absence in 25 g). Subsequently, samples of 10 g of dry-cured ham were inoculated by adding a cell suspension in Ringer's solution (Oxoid) 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 brain heart infusion broth. 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 1,054 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 packaged and maintained at 7°C for 28 days. This temperature was chosen taking into account literature data that suggest a temperature higher than 4°C 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. Color parameters (L*, a*, b*) in sliced dry-cured ham with enterocin were previously studied, and no significant changes were detected *(23)*. Average pH and water activity (a_w) values for this type of ham (as determined in previous experiments) are 5.9 and 0.905, respectively.

Sampling during storage. At time zero (immediately after inoculation), as well as after 6 h and 7, 14, and 28 days of storage at 7°C, a 10-g sample was subjected to microbiological analysis to determine the viable count of *L. monocytogenes*. The sample was transferred to a sterile stomacher bag, and 90 mL of Ringer's solution was added. Then, the sample was homogenized in a stomacher (BagMixer, Interscience, France) for 2 min at normal speed and room temperature. Serial decimal dilutions were prepared in the same solution and plated on Oxford *Listeria* selective agar base (Oxoid). Plates were incubated at 37°C for 48 h before colony count. At time zero, as well as after 30 min and 6, 24, and 168 h (7 days) of storage at 7°C, 10-g samples were used for RNA extraction and for agar well diffusion assay as described by Urso et al. *(31)*. A homogenate was prepared, as described earlier, from each 10-g sample. Then, 2 mL from the homogenate was centrifuged at 13,000 × *g* for 1 min at 4°C. Immediately after centrifugation, the pellet was covered with 0.05 mL of RNAlater (Ambion, Applied Biosystems, Milan, Italy) and stored at –20°C until RNA extraction.

RNA extraction and cDNA synthesis. RNA extraction was performed on the thawed samples by using the procedure described by Rantsiou et al. *(24)*. First, 50 μL of lysozyme (50 mg/mL; Sigma, St. Louis, MO) and 25 μL of proteinase K (25 mg/mL; Sigma) were added to the thawed samples, which were then incubated at 37°C for 20 min in a Thermomixer compact (Eppendorf, Milan, Italy). Samples were then processed using the MasterPure Complete DNA and RNA Purification Kit (Epicentre, Madison, WI), following the manufacturer's instructions. DNA was digested with the Turbo DNase (Ambion), and complete removal of the DNA was verified using an aliquot of the extract as template in a quantitative PCR (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). cDNA synthesis was performed using random hexamers (Promega, Milan, Italy) according to Rantsiou et al. *(24)*. The same quantity of RNA (in nanograms per microliter) was added in the reaction for each sample. Moloney–murine leukemia virus reverse transcriptase (Promega) was used, following the instructions of the manufacturer. An RNase inhibitor (Promega) was added in the reaction, and deoxynucleotide triphosphates were added at a final concentration of 2 mM each. Reverse transcription was performed in a DNA Engine Peltier thermal cycler (Bio-Rad, Milan, Italy) at 37°C for 1 h. The cDNA was stored at –20°C until it was used in qPCR amplification.

qPCR. qPCR amplification was performed using the cDNA, synthesized as shown earlier from each sample, as a template. Five

TABLE 1. Listeria monocytogenes *genes targeted by qPCR in this study to determine the effect of enterocin and* E. faecalis *on stress and virulence gene expression*

Gene name	Function and scope of use	Primer sequence	Reference
tuf	Encoding an elongation factor Tu, housekeeping gene	F: 5′-CTGAAGCTGGCGACAACA-3′ R: 5′-CTTGACCACGTTGGATATCTTCAC-3′	*18*
lmo0669	Encoding for a protein similar to oxidoreductase, acid stress-related gene	F: 5′-TCAAGCTATCAAGGCGCTAATAAA-3′ R: 5′-CCGACCAATTCCGGAGTCT-3′	*30*
lmo2434	Encoding for a glutamate decarboxylase, acid stress-related gene	F: 5′-TGGCGGTTTGGCAATGA-3′ R: 5′-TGCCTGTATATCCAGACCTCGTT-3′	*18*
lmo1421	Encoding for a glycine betaine ABC transporter, osmotic stress-related gene	F: 5′-CCACTGACAACTGGAACCATTTATA-3′ R: 5′-GAAAGAGCGCAATTTGTTGTAAAA-3′	*29*
prfA	Encoding a virulence transcription regulator, virulence gene	F: 5′-CAATGGGATCCACAAGAATATTGTAT-3′ R: 5′-AATAAAGCCAGACATTATAACGAAAGC-3′	*14*
gbuB	Encoding for an ABC transporter, osmotic stress-related gene	F: 5′-TGGTATTTGGATGGCGAA-3′ R: 5′-CAATTACGACCATGGAAAGT-3′	*1*

genes listed in Table 1 were targeted. The amplification took place in a Chromo4 real-time PCR detection system (Bio-Rad) using the SsoAdvanced SYBR Green Supermix (Bio-Rad) 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 interrun experimental variability.

Data analysis–statistical analysis. Threshold cycle (C_T) values were exported to Excel for 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 $(C_{T,\text{target}} - C_{T,\text{housekeeping}})_{\text{test condition}} - (C_{T,\text{target}} - C_{T,\text{housekeeping}})_{\text{control condition}}$ *(17)*. Stress or virulence genes were considered targets, while *tuf* was considered a housekeeping gene. The control condition was the sliced dry-cured ham inoculated with *L. monocytogenes* alone, while the test condition was the dry-cured ham inoculated with *L. monocytogenes* and supplemented with enterocin or coinoculated with *E. faecalis* (at the respective time points). The $\log_2$ values of relative expression were calculated and statistically treated using SPSS statistics (IBM Corp., Armonk, NY).

RESULTS AND DISCUSSION

Dry-cured ham is considered an RTE food, and it is known to be prone to *L. monocytogenes* contamination during processing. The main hurdles to *L. monocytogenes* growth during refrigerated storage are low a_w and addition of salt and nitrites. However, these hurdles are not listericidal, and several studies have evaluated alternative approaches with a lethal effect, such as high hydrostatic pressure processing, irradiation, and supercritical carbon dioxide processing *(3, 6, 11, 21)*. Furthermore, the potential of *L. monocytogenes* growth control using bacteriocins has been investigated *(13)*. In this study, we sought to investigate the behavior of *L. monocytogenes* in dry-cured ham supplemented with a bacteriocin extract or coinoculated with a bacteriocinogenic *E. faecalis* during storage. In this context, behavior is intended as population kinetics and gene expression profile during storage. For this purpose, two strains of *L. monocytogenes* were tested: a persistent strain and a nonpersistent strain. The classification of the strains as persistent and nonpersistent was based on previous observations regarding frequency of isolation and occurrence in different areas of a pig processing environment. More specifically, S4-2 was considered a persistent strain found in the environment, equipment, carcasses, and raw and dry-cured products. This genotype was repeatedly isolated. Strain S12-1 was nonpersistent but isolated from dry-cured products *(22)*.

Effect of enterocin and bacteriocinogenic *E. faecalis* on *L. monocytogenes* population. By agar well diffusion assay performed in vitro, it was determined that the enterocin extract and 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 unaltered during the first 7 days and declined by about 0.6 Log CFU/g at 14 days (Table 2). The population then remained stable for both strains for up to 28 days (data not shown). 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. Furthermore, nitrites and salt were added and, during storage, had average concentrations of 2.69 mg/kg and 4.12%, respectively. Altogether, 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 CFU/g for strain S4-2 and by 1.5 Log CFU/g for strain S12-1. A time window of at least 30 min elapsed between the inoculation or 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 CFU/g between time 0 and 14 days for strain S4-2 and by 1.9 Log CFU/g for strain S12-1. At 14 days, the population of strain S4-2 was almost 2 Log CFU/g lower in the dry-cured ham

TABLE 2. L. monocytogenes *counts in dry-cured ham treated with enterocin or coinoculated with bacteriocinogenic* E. faecalis *during refrigerated storage at 7°C (experimental details described in "Materials and Methods")*

L. monocytogenes strain	Treatment[a]	Counts (Log CFU/g)			
		0	6 h	7 days	14 days
S4-2	Control	6.32 ± 0.04 b B[b]	ND[c]	6.65 ± 0.12 c B	5.66 ± 0.13 a B
	Enterocin	5.57 ± 0.14 c A	ND	4.77 ± 0.15 b A	3.74 ± 0.26 a A
	E. faecalis	7.1 ± 0.15 b C	7 ± 0.02	6.9 ± 0.02 a C	ND
S12-1	Control	6.22 ± 0.05 b B	ND	6.29 ± 0.15 b B	5.51 ± 0.09 a B
	Enterocin	4.66 ± 0.10 b A	ND	4.36 ± 0.57 b A	2.72 ± 0.29 a A
	E. faecalis	6.9 ± 0.02 b C	6.8 ± 0.19	6.8 ± 0.04 a C	ND

[a] Control, dry-cured ham inoculated with ca. 10^6 CFU/g *L. monocytogenes*; enterocin, dry-cured ham inoculated with *L. monocytogenes* and supplemented with enterocin; *E. faecalis,* dry-cured ham inoculated with *L. monocytogenes* and *E. faecalis*.

[b] Lowercase letters indicate significant differences ($P < 0.05$) in the counts during time of storage, while capital letters indicate differences between treatments at each sampling point.

[c] ND, not determined.

supplemented with enterocin compared with the control, while the effect was greater for strain S12-1: the enterocin inactivated 2.8 Log CFU/g of the population. Therefore, the enterocin displayed significant listericidal effect. Such effect was strain dependent; it was greater for the nonpersistent strain. RTE meat products may be contaminated by *L. monocytogenes*; therefore, the potential of bacteriocins to control it has been extensively investigated *(33)*. In dry-cured ham, the antilisterial effect has been previously proven for enterocin AB *(13)*. In this previous study, enterocin AB drastically reduced by 2.5 Log CFU/g *L. monocytogenes* in dry-cured ham stored at 4°C for 1 day. The results of our study confirm the potential of enterocin AB to affect the viability of *L. monocytogenes*.

When the bacteriocinogenic *E. faecalis* was coinoculated in the sliced dry-cured ham, the evolution of the pathogen's population showed a reducing trend with time. However, the reduction observed cannot be considered important; in the case of strain S4-2, it was 0.2 Log between time zero and 7 days (statistically significant difference, $P < 0.05$), while for strain S12-1, it was of 0.1 Log. Therefore, the microbial competition exerted by *E. faecalis* resulted in containment of *L. monocytogenes* compared with the control condition. The effective production of bacteriocin by *E. faecalis* in situ, after inoculation in the dry-cured ham, was verified throughout the conservation period by agar well diffusion assay (data not shown). However, the results obtained with the enterocin and the *E. faecalis* cannot be compared. The *E. faecalis* strain used was not the same as the one from which the enterocin was purified. In addition, other variables, such as bacteriocin liberation from the cell and diffusion in the sliced ham, most likely influenced the effect of the *E. faecalis* that was observed. The use of bacteriocinogenic cultures has been largely explored for fermented foods, including fermented meat products. In the case of fermented meat products, the bacteriocin-producing strains act as starter culture and contribute to the safety, by microbial competition and bacteriocin and lactic acid production, and to the development of the desired organoleptic properties of the final product *(7)*. In nonfermented meat products, bacteriocin-producing LAB 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 because of limited diffusion of the bacteriocin or its production at concentrations that may interfere with regulatory mechanisms and therefore contain growth but may not necessarily be high enough to kill *L. monocytogenes (4)*. The observed lack of lethal effect may also be because of limited interaction of the two microorganisms in the solid food matrix, where physical contact, which has been proposed as an interspecies inhibitory mechanism *(33),* does not take place.

Effect of enterocin and *E. faecalis* on *L. 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 strains of *L. monocytogenes*. Strain S4-2 (Fig. 1) is a persistent strain, while strain S12-1 (Fig. 2) is a nonpersistent strain. The genes chosen (Table 1) are representatives of stress response and virulence genes and have been previously employed in studies of *L. monocytogenes* gene expression in situ *(19)*. Relative gene expression was calculated using *L. monocytogenes* artificially inoculated in dry-cured ham as a control condition. Therefore, Figures 1

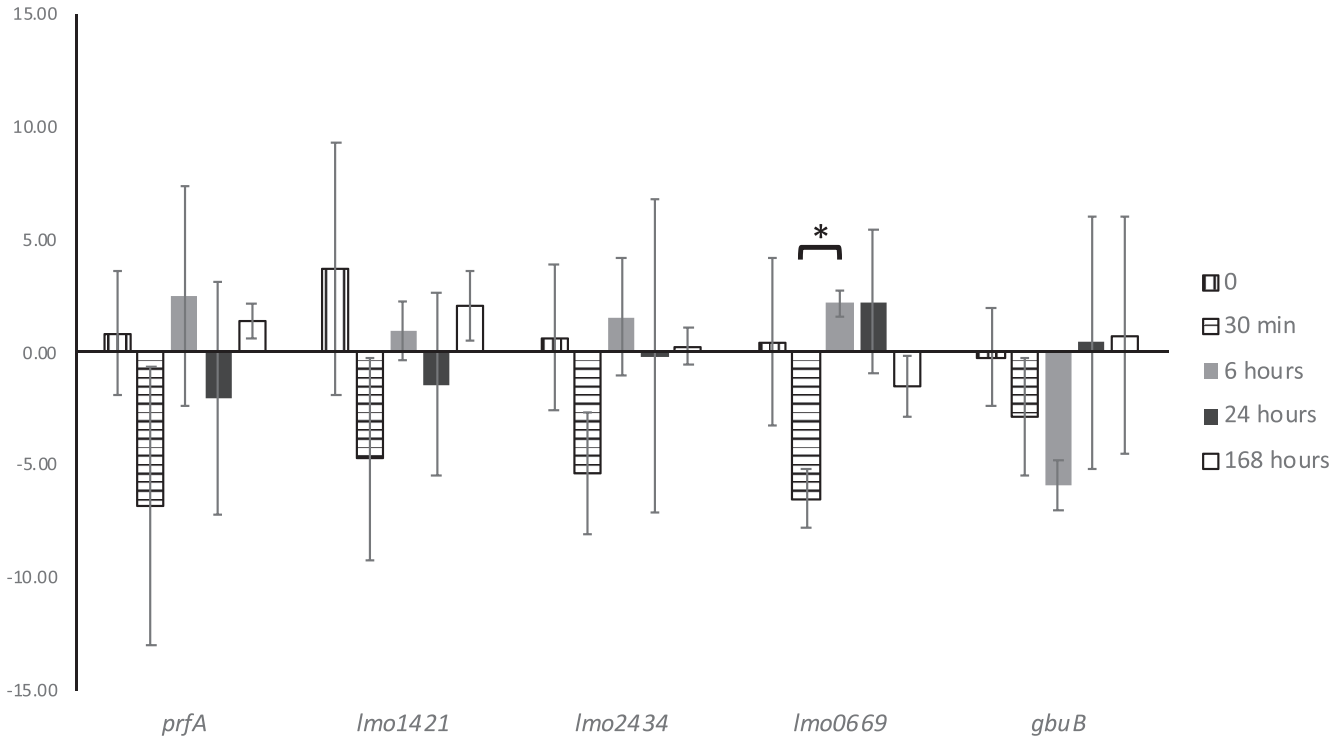

FIGURE 1. *Relative gene expression for genes* prfA, gbuB, lmo1421, lmo2434, *and* lmo0669 *of* Listeria monocytogenes *strain S4-2 inoculated in dry-cured ham and supplemented with enterocin. Relative gene expression was calculated by the* $2^{-\Delta\Delta C_T}$ *method, and* $\log_2$ *values are reported. Error bars indicate standard deviations of two biological replicates. For gene* lmo0669, *the asterisk indicates the significant difference (*$P < 0.05$*) in the expression level between 30 min and 6 h of conservation.*

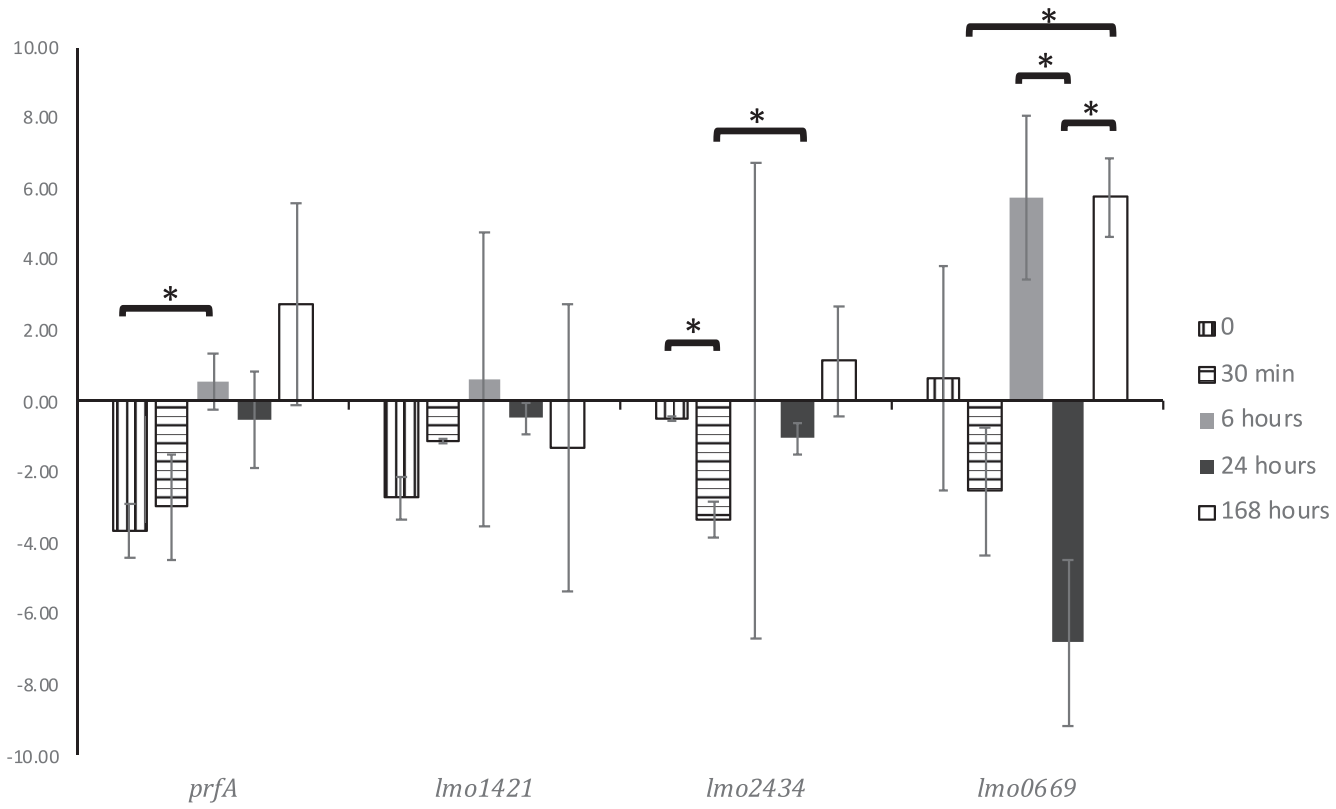

FIGURE 2. *Relative gene expression for genes* prfA, 1mo1421, lmo2434, *and* lmo0669 *of* Listeria monocytogenes *strain S12-1 inoculated in dry-cured ham and supplemented with enterocin. Relative gene expression was calculated by the* $2^{-\Delta\Delta C_T}$ *method, and* $\log_2$ *values are reported. Error bars indicate standard deviation of two biological replicates. For genes* prfA, lmo2434, *and* lmo0669, *the asterisks indicate significant differences (*$P < 0.05$*) in the expression level across time points during conservation.*

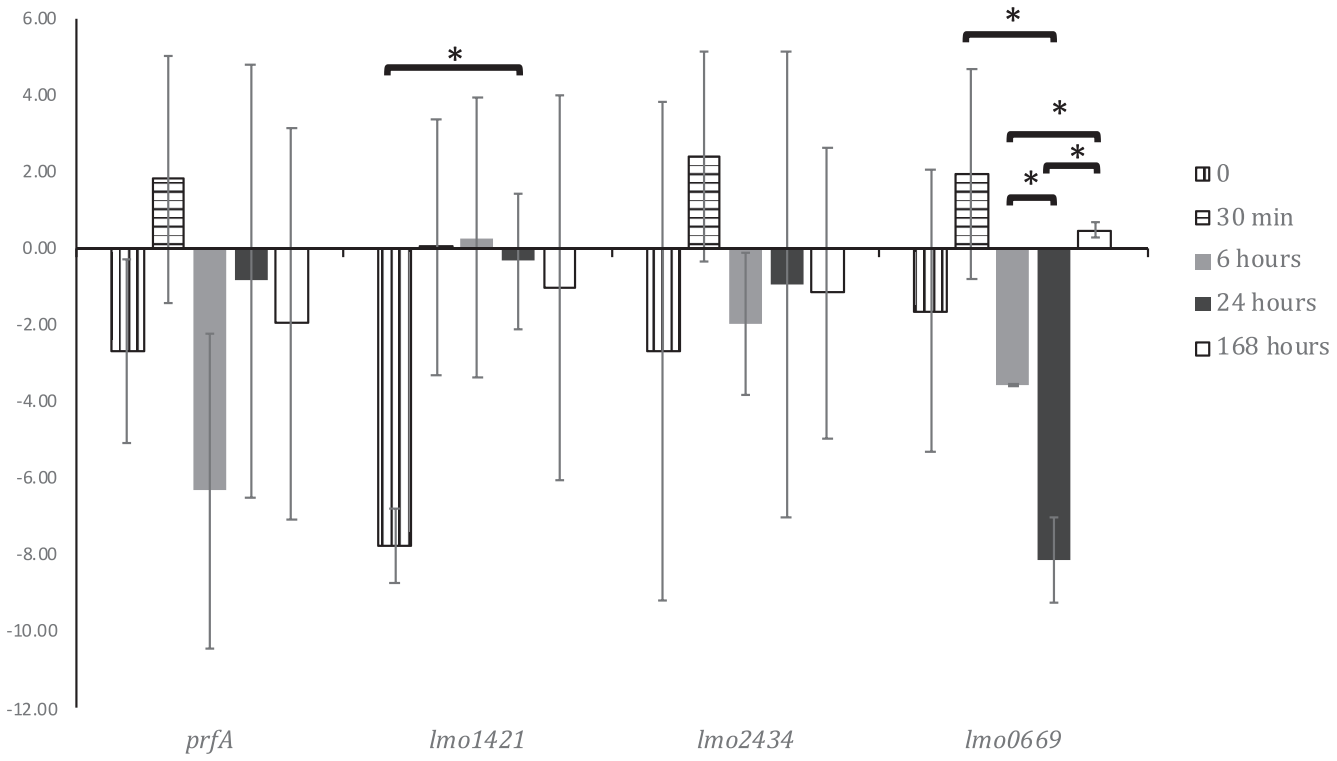

FIGURE 3. *Relative gene expression for genes* prfA, lmo1421, lmo2434, *and* lmo0669 *of* Listeria monocytogenes *strain S12-1 coinoculated in dry-cured ham with* Enterococcus faecalis *B1. Relative gene expression was calculated by the* $2^{-\Delta\Delta C}{}_{T}$ *method, and* log_2 *values are reported.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

and 2 depict the sole impact of enterocin or *E. faecalis* addition while the stressful conditions (low a_w, refrigeration temperature, and nitrites) that are known to have influence on gene expression are leveled out. During the long-term storage of vacuum-packaged dry-cured ham, changes in the physicochemical or microbiological parameters are not significant, and gene expression is not expected to be influenced. Therefore, the gene expression was monitored up to the seventh day of refrigerated storage, while a time point close to the inoculation (30 min) was considered, to capture the response of *L. monocytogenes* upon inoculation. As can be seen in the two figures, the expression of the target genes fluctuated during refrigerated conservation. For both strains of *L. monocytogenes,* an overall down-regulation tendency for all genes was observed after 30 min of storage. For strain S12-1, this down-regulation was evident immediately after inoculation (time zero). For strain S4-2, statistically significant variation in expression was observed for gene *lmo0669*. This gene, encoding for a protein similar to an oxidoreductase and likely involved in acid stress response, was down-regulated at 30 min and then significantly upregulated at 6 h while expression leveled off throughout the rest of the storage period. A similar pattern was observed for this gene in strain S12-1: down-regulation at 30 min and upregulation at 6 h, followed in this case by significant upregulation at 168 h. Upregulation at 168 h was also observed for gene *lmo2434,* encoding for a glutamate decarboxylase and involved in acid stress response. The virulence gene *prfA,* encoding for a major virulence transcriptional regulator, displayed fluctuating expression with a tendency for reduced expression compared with the condition of dry-cured ham.

Apart from the effect of a bacteriocin extract, we sought to investigate how the presence of a bacteriocinogenic *E. faecalis* strain would influence gene expression of *L. monocytogenes* in sliced dry-cured ham. The goal was to mimic a situation (i.e., copresence in food of *L. monocytogenes* and a competitive lactic acid bacterium) frequently verified during food production and storage. For strain S4-2, no significant differences in gene expression were observed during the time window (data not shown). In contrast, for strain S12-1, gene expression varied with time. As can be seen in Figure 3, the main outcome observed from the presence of *E. faecalis* in the dry-cured ham is down-regulation for all genes throughout time with the exception of the 30-min time point, at which all target genes were upregulated. Variation in gene expression through time was significant for genes *lmo1421* and *lmo0669*. Limited information is available in the literature concerning the effect of bacteriocins or bacteriocin-producing microorganisms on *L. monocytogenes* gene expression. Winkelströter and De Martinis *(32)* registered down-regulation of the expression of the *inlA* gene, an important virulence gene, in in vitro tests with 10 strains of *L. monocytogenes* in the presence of three bacteriocins, produced by *E. faecium, Leuconostoc mesenteriodes,* and *Lactobacillus sakei*. Genes *inlA* and *prfA,* encoding for a major virulence gene regulator, were down-regulated in *L. monocytogenes* in

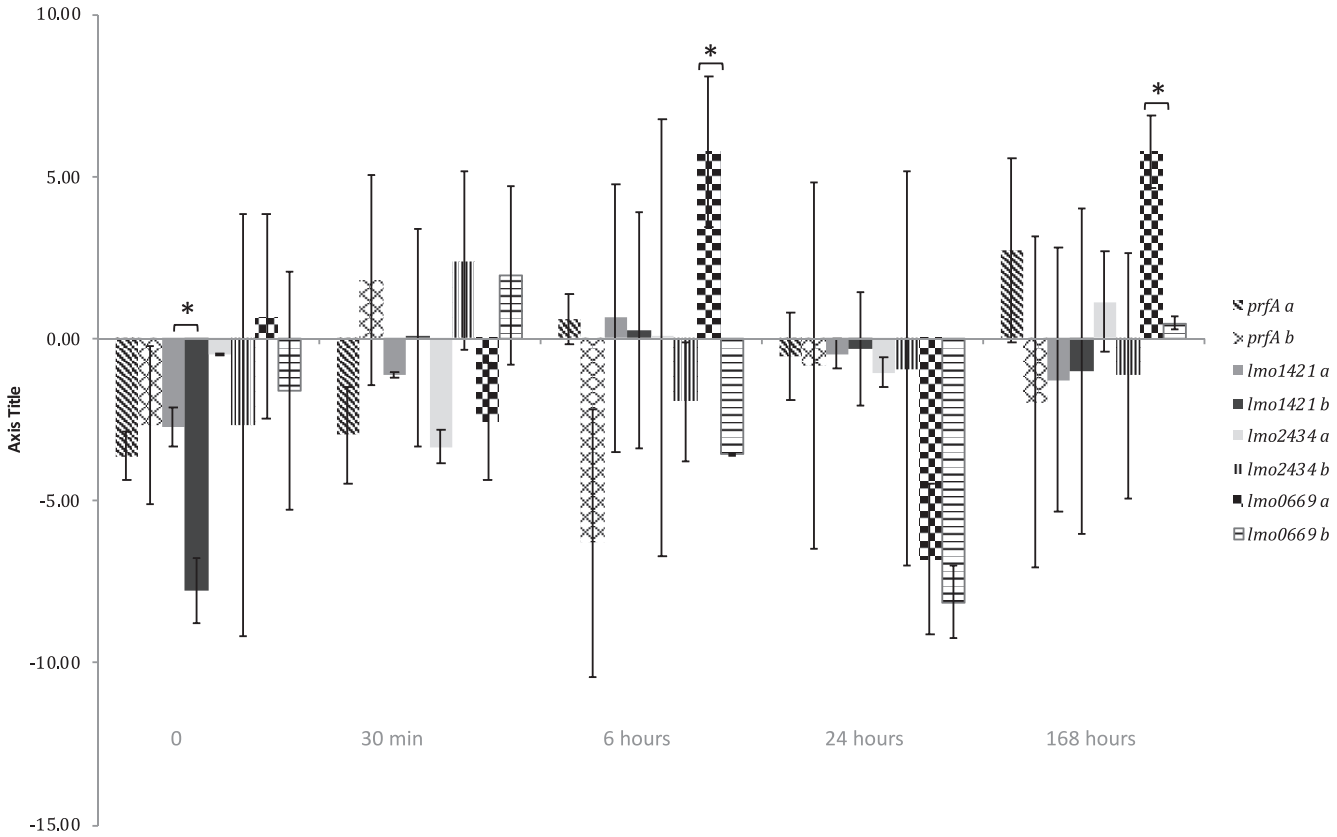

FIGURE 4. *Relative gene expression for genes* prfA, lmo1421, lmo2434, *and* lmo0669 *of* Listeria monocytogenes *strain S12-1 inoculated in dry-cured ham supplemented with enterocin (condition a) or coinoculated in dry-cured ham with* Enterococcus faecalis *B1 (condition b). Relative gene expression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

the presence of metabolic products of two strains of *E. faecium (34)*. The results of the present study are in agreement with these previous reports: gene *prfA* was down-regulated in both *L. monocytogenes* strains in response to the presence of the enterocin or the *E. faecalis* strain (for *L. monocytogenes* S12-1). Apart from *prfA,* other genes (involved in virulence and stress response or adaptation) tested in the present study but also by Ye et al. *(34)* were down-regulated in the presence of a bacteriocin, a metabolic product of *E. faecium,* or *E. faecalis*. Although this general trend was identified in both studies, the effect on gene expression depended both on the strain of *L. monocytogenes* tested and 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 coinoculated with a nisin-producing *Lactococcus lactis*. Of the four genes tested, *gadD2* consistently showed increased expression in the milk containing *L. lactis* compared with milk without *L. lactis*. Genes *sigB* and *groEL* were also investigated, and expression varied with time, showing down-regulation as incubation proceeded. However, gene *gbu* was down-regulated by the presence of *L. lactis*. The incubation temperature was different from that found in the current study (20 or 30°C, as opposed to 7°C here), a liquid food matrix was used rather than a solid one, and the antagonistic microorganism was different. Results concerning the *gbu* gene appear to be consistent: in both studies, the gene was essentially down-regulated by the presence of a bacteriocin-producing microorganism.

When the two tested conditions (i.e., the presence of enterocin and the presence of *E. faecalis*) were compared (Fig. 4), it was evident that the effect on gene expression was similar. With the exception of the 30-min time point when most genes were upregulated by the presence of *E. faecalis,* at the remaining time points, expression went down. *E. faecalis* exhibited a higher, mostly negative impact on gene expression of *L. monocytogenes* compared with enterocin. In most cases, *E. faecalis* accentuated the down-regulation of genes or inversed the pattern (from upregulated to down-regulated). Expression of *prfA* was reduced in the presence of *E. faecalis* at three time points (6, 24, and 168 h). Similarly, gene *lmo0669* showed decreased expression at four of five time points, and this reduced expression was significant at 6 and 168 h. Gene *lmo1421* was further down-regulated because of 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 because of differences in the experimental approaches adopted: different strains of *L. monocytogenes* tested, different media or types of food, different temperature or time regimes considered, and a

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

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 nonpersistent strain was more prominent compared with 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 whether the persistence phenotype is associated with higher resistance to a bacteriocin. Further studies are needed to elucidate this aspect. However, a common pattern regarding the expression of the five tested genes could be delineated for both strains; in the presence of enterocin, the 30-min time point determined down-regulation of the genes, and this trend was essentially maintained throughout the storage period, up to 168 h. 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 nonpersistent strain, differences were highlighted during storage, with an important shift from time zero (down-regulation), to 30 min (upregulation), and to the remaining period (down-regulation). Based on the data of this study, we cannot correlate the persistence phenotype with the behavior observed; additional strains (both persistent and nonpersistent) should be tested under in situ conditions. 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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