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Research article

Effect of co-culture with enterocinogenic *E. faecium* on *L. monocytogenes* key virulence gene expression

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Abstract: The aim of the present study was to assess the expression of key virulence genes during co-culture of *L. monocytogenes* with a bacteriocinogenic *E. faecium* strain in liquid growth medium. For that purpose, BHI broth was inoculated with 7 log CFU·mL^{-1} *L. monocytogenes* and 4, 5 or 6 log CFU·mL^{-1} *E. faecium*. Sampling took place after 8 and 24 h of incubation, corresponding to the maximum and minimum of enterocin production, respectively. The RNA was extracted, stabilized and expression of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ*, was assessed by RT-qPCR. Most of the genes were downregulated during co-culture at 5 °C. Moreover, a statistically significant effect of the inoculum level was evident in most of the cases. On the contrary, no effect on the transcription level of most of the genes was observed during co-culture at 37 °C.

Keywords: *Listeria monocytogenes*; *Enterococcus faecium*; co-culture; bacteriocin; virulence genes

1. Introduction

L. monocytogenes is a pathogenic microorganism whose ubiquitous nature has been well characterized. Indeed, a large amount of studies are currently available reporting prevalence values that may range from 3.6 to 30.2% in meat and meat products [1–5], 3.5 to 39.6% in dairy products [2,6], 0.8 to 80.3% in raw and processed seafood [7] and 0.3 to 36.8% in raw or processed fruits and vegetables [8–13].

Moreover, *L. monocytogenes* has the ability to survive and even proliferate in the human gastrointestinal tract. This intracellular lifestyle requires the coordinated expression of a series of

genes in order to activate an infection cycle that includes a series of stages, namely adhesion, invasion, escape from vacuole, intracellular multiplication and cell-to-cell spread [14]. Most of these genes, namely *prfA, plcA, hly, mpl, actA* and *plcB* are physically clustered in the Listeria Pathogenicity Island 1 (LIPI-1) [15]. Transcription of these genes is principally controlled by the transcriptional activator PrfA that is encoded by *prfA* [16,17,18]. *plcA* and *plcB* encode two phospholipases, namely phosphatidylinositol phospholipase C (PI-PLC) and phosphatidylcholine phospholipase C (PC-PLC). Both phospholipases in collaboration with listeriolysin O, a cholesterol-binding, pore-forming toxin that is encoded by *hly*, promote lysis of the phagocytic vacuole that engulfs the cells of the pathogen [19,20]. *mpl* encodes a zinc-metalloprotease involved in pro-PlcB maturation [21,22] and *actA* encodes for a surface protein that is essential for intra- and inter-cellular motility, having thus a major role in cell-to-cell spread and in epithelial cell invasion [23,24,25]. In addition, a family of surface proteins collectively referred to as internalins are necessary for the active invasion in the host cells. As many as 25 internalins have been so far identified [26]; among them only InlA and InlB have been associated with internalization of normally nonphagocytic cells. The role of the remaining is yet to be discovered; only for some of them, such as InlC and InlJ the importance in virulence has been reported [27].

Application of bioprotective cultures has been in the epicenter of intensive research over the last decades. Bioprotection is achieved through antagonistic interactions with the undesired spoilage or pathogenic microbiota and through the production of antimicrobial compounds. Regarding the latter, bacteriocin production is by far the most widely studied property. Bacteriocinogenic enterococci are very attractive because they are quite widespread in nature and because bactericidal activity against the major foodborne pathogen *L. monocytogenes* is a common property due to their phylogenetic proximity [28]. However, compliance with the EFSA requirements regarding the Qualified Presumption for Safety assessment [29] is still necessary. Thus, the capability of various *Enterococcus* spp. strains as adjunct cultures with bioprotective role has been adequately highlighted [30–34].

Several response strategies to environmental stimuli have been described for *L. monocytogenes* and significantly increased our understanding regarding the physiology of the pathogen. More accurately, the effect of pH, temperature and carbon sources [35–40], interventions associated with food, such as heat treatment, high hydrostatic pressure processing, addition of nisin or disinfectants [41–45], as well as growth in various food-related substrates [46–52] on the expression of the above key virulence genes have been studied to some extent. However, to the best of our knowledge, no studies currently exist addressing the transcriptomic response of *L. monocytogenes* key virulence genes to the co-culture with a bacteriocinogenic *E. faecium* strain. Thus, the aim of the present study was to assess the expression of key virulence genes, namely *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ*, during co-culture of *L. monocytogenes* with a bacteriocinogenic *E. faecium* strain in liquid growth medium.

2. Materials and Methods

2.1. Bacterial isolates

L. monocytogenes strain NCTC 10527, serotype 4b, and *E. faecium* strain LQC 20005, isolated from spontaneously fermented sausages were used throughout this study. Long term storage took

place at –20 °C in Nutrient Broth (Biolife, Milan, Italy) supplemented with 50% glycerol. Before experimental use, each strain was grown twice in Brain Heart Infusion broth (Lab M, Lancashire, UK) at 37 °C for 24 h.

2.2. Co-culture conditions, sampling and microbiological analyses

Co-culture took place in test tubes containing BHI broth, and incubation at 5 and 37 °C. Three different inoculum combinations were studied. Co-cultures 1, 2 and 3 consisted of 7 log CFU·mL^{-1} *L. monocytogenes* and 4, 5 or 6 log CFU·mL^{-1} *E. faecium*, respectively. Sampling took place after 8 and 24 h of incubation, corresponding to the maximum and minimum of enterocin production, respectively [53]. One mL from each test tube was used for microbiological analyses; the remaining 9 mL were centrifuged (12,000 × g; 1 min; 5 °C or room temperature when incubation took place at 5 or 37 °C, respectively). The supernatant was discarded; the pellet was mixed with 200 uL of RNAlater® solution (Ambion, Whaltham, MA, USA) and stored at –20 °C. *L. monocytogenes* and *E. faecium* populations were enumerated in each sampling time using Chromogenic Listeria Agar (Oxoid, Whaltham, MA, USA) and Kanamycin Aesculin Azide Agar (Lab M), respectively, according to the instructions of the manufacturer. The experiment was performed in triplicate.

2.3. Gene expression assay

RNA extraction was performed with the PureLink RNA Mini Kit (Ambion) and cDNA synthesis took place using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Whaltham, MA, USA) according to the instruction of the manufacturer. The KAPA SYBR qPCR kit Master Mix (2×) for ABI Prism (Kapa Biosystems, Boston, MA, USA) and the Step One Plus Real-Time PCR System (Applied Biosystems, Whaltham, MA, USA) were used for the RT-qPCR. Primers and PCR conditions are presented in Table 1. *IGS*, *rpob* and 16S-rRNA gene were evaluated as reference genes; *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* were selected due to their significance in *L. monocytogenes* virulence potential. Two RT reactions were performed for each sample containing ca. 0.1 μg RNA each. The resulting cDNA was used for gene expression assessment.

2.4. Statistical analysis

Ct values were processed according to Hadjilouka et al. [52]. The stability of the reference genes was assessed with the NormFinder application for Excel [54]. Then, PCR efficiency correction and normalization with the selected reference gene took place as well as conversion to relative expression and $\log_2$-values (fold change) according to Kubista et al. [55]. Growth of *L. monocytogenes* in BHI broth at the same conditions (i.e. inoculum level, incubation temperature and time) was considered as the control for relative expression of the target genes. The effect of co-culture was assessed by using monocultures as control and the effect of temperature by using growth at 5 °C as control. One-way ANOVA was applied to investigate the effect of temperature and inoculum level on the relative expression of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ*.

Table 1. Primer sequences, amplicon sizes and PCR conditions used for the gene expression assay (Hadjilouka et al. 2016).

Genes		Sequence	Concentration (uM)	Amplicon size (bp)	PCR efficiency
reference					
IGS	IGS_f	GGCCTATAGCTCAGCTGGTTA	1.2	135	2.03
	IGS_r	GCTGAGCTAAGGCCCCGTAAA	1.2		
rpob	rpob_f	CCGCGATGCGAAAACAAT	0.9	69	2.04
	rpob_r	CCWACAGAGATACGGTTATCRAATGC	0.9		
16S	16S_f	GATGCATAGCCGACCTGAGA	0.9	114	2.05
	16S_r	CTCCGTCAGACTTTCGTCCA	0.9		
virulence-associated					
hly	hly_f	TACATTAGTGGAAAGATGG	1.2	153	1.98
	hly_r	ACATTCAAGCTATTATTTACA	1.2		
plcA	plcA_f	CTAGAAGCAGGAATACGGTACA	1.2	115	1.94
	plcA_r	ATTGAGTAATCGTTTCTAAT	1.2		
plcB	plcB_f	CAGGCTACCACTGTGCATATGAA	0.9	72	2.00
	plcB_r	CCATGTCTTCYGTTGCTTGATAATTG	0.9		
sigB	sigB_f	CCAAGAAAATGGCGATCAAGAC	1.2	166	2.13
	sigB_r	CGTTGCATCATATCTTCTAATAGCT	1.2		
inlA	inlA_f	AATGCTCAGGCAGCTACAMTTACA	0.9	114	2.12
	inlA_r	CGTGTCTGTTACRTTCGTTTTTCC	0.9		
inlB	inlB_f	AAGCAMGATTTCATGGGAGAGT	0.9	78	2.04
	inlB_r	TTACCGTTCCATCAACATCATAACTT	0.9		
inlC	inlC_f	ACTGGTCAGAAATGTGTGAATGA	0.9	80	2.06
	inlC_r	CCATCTGGGTCTTTGACAGT	0.9		
inlJ	inlJ_f	TGCGTAAATGCTCACATCCAAG	0.9	81	2.03
	inlJ_r	TTGCCCTTCAGCATCCAAGT	0.9		

Thermocycling conditions: initial denaturation at 95 °C for 20 sec and then 40× (95 °C for 10 sec, 60 °C for 30 sec, 72 °C for 30 sec). Melting curve analysis: 95 °C for 15 sec then 60 °C for 1 min and raise to 95 °C at 0.3 °C/sec.

3. Results and Discussion

In Table 2, the population dynamics of *L. monocytogenes* and *E. faecium* during their co-culture at 5 and 37 °C is shown. Populations remained stable in all cases at 5 °C; when growth was noticed it did not exceed 1.0 log CFU·mL^{-1} for both microorganisms. *E. faecium* growth was evident in all sampling points at 37 °C; only in co-culture 1 *E. faecium* population was less than the respective of *L. monocytogenes*. In the latter case, the population of the pathogen remained below 9 log CFU·mL^{-1} that was the population reached in monoculture (data not shown). Moreover, *L. monocytogenes* population in co-culture 2 grew only marginally and diminished below 4 log CFU·mL^{-1} in co-culture 3.

The optimum growth temperature for both species under study is 37 °C. During monocultures at 37 °C, both strains reached late exponential growth phase after 8 h and stationary phase after 24 h (data not shown). On the contrary, growth at 5 °C was much slower; during the 24 h of the current experiment, only marginal population increase was observed, i.e. less than 1 log CFU·mL^{-1}. Co-culture of enterocinogenic strains with *L. monocytogenes* has been studied to some extent, both *in vitro* and *in situ* [31,56,57]. Regarding the former, Izquierdo et al. [57] inoculated both species at 4 log CFU·mL^{-1} and incubated at 37 °C for 48 h. The population of *L. monocytogenes* strain reached 7 log CFU·mL^{-1} and then decreased to 2 log CFU·mL^{-1}, due to the bacteriocin production. Then, growth reinitiated and the population reached 7 log CFU·mL^{-1} by the end of incubation period,

probably due to the detrimental effect of the neutral pH value on the stability of the antibacterial activity [57]. In the present study, no conclusions can be drawn regarding the kinetics of *L. monocytogenes* inactivation since only two sampling times were assessed. However, the effect of *E. faecium* inoculum level on the extent of *L. monocytogenes* inactivation was evident. Growth of the pathogen's population was only observed when *E. faecium* inoculum was 4 log CFU·mL^{-1}.

Table 2. Population dynamics of *L. monocytogenes* and *E. faecium* during their co-culture at 5 and 37 °C.

		5 °C			37 °C		
Co-culture		0 h	8 h	24 h	0 h	8 h	24 h
1	*E. faecium*	4.10 (0.22)	4.68 (0.16)	5.04 (0.21)	4.02 (0.05)	7.68 (0.27)	7.78 (0.24)
	L. monocytogenes	7.13 (0.14)	7.15 (0.20)	7.45 (0.25)	7.07 (0.11)	8.07 (0.20)	8.32 (0.22)
2	*E. faecium*	5.03 (0.14)	5.30 (0.32)	5.60 (0.27)	5.05 (0.06)	8.42 (0.30)	8.37 (0.26)
	L. monocytogenes	7.08 (0.07)	7.10 (0.18)	7.41 (0.34)	7.08 (0.06)	7.39 (0.18)	7. 34 (0.16)
3	*E. faecium*	6.12 (0.14)	6.39 (0.15)	6.32 (0.24)	6.10 (0.08)	8.41 (0.30)	8.44 (0.28)
	L. monocytogenes	7.10 (0.12)	7.25 (0.27)	7.07 (0.35)	7.12 (0.10)	< 4.00	< 4.00

Co-culture of enterocinogenic *E. faecium* strain with *L. monocytogenes* at 4 °C was studied by Huang et al. [58]. In that study, reduction of *L. monocytogenes* population was already visible from the first day on incubation opposing the results obtained in the current study, in which *L. monocytogenes* population was stable during the 24 h of incubation at 5 °C. This may be due to the lower *E. faecium* inoculum level used and the fact that enterocin production is growth-associated [59].

Based on the above, *L. monocytogenes* virulence gene expression could be assessed in all cases at 5 °C but only in co-culture 1 at 37 °C. Assessment of gene expression in co-cultures may be reliably performed when the extracted RNA originates mainly from the microorganism under study. This can be achieved when the population of the microorganism under study is higher than the background microbiota [51] and the RNA extraction efficiencies are comparable [60].

In Figure 1 the effect of co-culture with enterocinogenic *E. faecium* strain LQC 20005 on *L. monocytogenes* key virulence gene expression after 8 h at 5 and 37 °C is shown. Regarding co-cultures at 5 °C, *sigB* expression exhibited no regulation and concomitantly was not affected by the inoculum level. Downregulation without any effect of the inoculum level was observed for *plcA* and *plcB*. Similarly, the rest of the genes under study were also downregulated but a statistically significant effect ($P < 0.05$) of the inoculum level was recognized. In the case of *hly*, *inlB* and *inlJ*, no regulation in the 1st co-culture and downregulation in the remaining ones were noticed. As far as co-culture at 37 °C was concerned, *prfA*, *sigB*, *plcA*, *plcB*, *inlA*, *inlB* and *inlJ* were not regulated while an increase in the transcription level was observed for *hly* and *inlC*.

The effect of co-culture with enterocinogenic *E. faecium* strain LQC 20005 on *L. monocytogenes* key virulence gene expression after 24 h at 5 and 37 °C is exhibited in Figure 2. As in the previous case, during co-culture at 5 °C, *sigB* exhibited no regulation whereas the rest of the genes under study were downregulated. Moreover, a statistically significant effect of the inoculum level was observed for *hly*, *inlA*, *inlB* and *inlJ*. On the contrary, no such was noticed for

prfA, *plcA*, *plcB* and *inlC*. Regulation was not observed for *plcA*, *plcB*, *inlA*, *inlB* and *inlC*, whereas upregulation for *sigB*, *hly* and *inlJ* and downregulation for *prfA* were detected during co-culture at 37 °C.

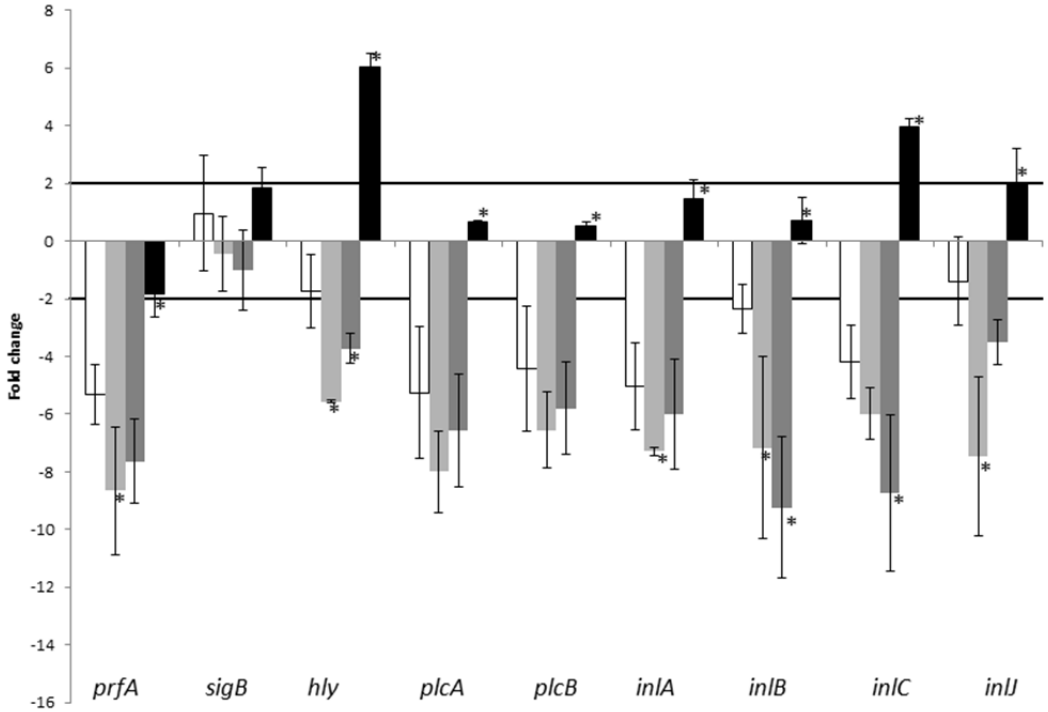

Figure 1. Effect of co-culture with enterotoxigenic *E. faecium* strain LQC 20005 on the relative expression of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* of *L. monocytogenes* strain NCTC 10527 during co-cultures 1, 2 and 3 at 5 °C depicted with white, light grey and dark grey bars, respectively and co-culture 1 at 37 °C (black bars) after 8 h incubation. Error bars represent the standard deviation of the mean value. The asterisk indicates that expression of each gene during co-cultures 2 and 3 at 5 °C and co-culture 1 at 37 ^{o}C is significantly ($P < 0.05$) different from co-culture 1 at 5 °C.

The effect of temperature on *L. monocytogenes* key virulence gene expression during monoculture and co-culture with *E. faecium* strain LQC 20005 is given in Figure 3. Transcription of *prfA* and *sigB* was not affected by temperature, in both mono- and co-culture. On the contrary, temperature affected expression of *inlA*, *inlB*, *inlC* and *inlJ* in both cases, *plcA* and *plcB* only in monoculture and *hly* only in co-culture. More accurately, all internalins under study were downregulated after both 8 and 24 h of monoculture at 37 °C compared to the respective at 5 °C. On the contrary, the internalins were upregulated during co-culture with the exception of *inlB* and *inlJ* after 8 h. Downregulation of *plcA* and *plcB* was evident during monoculture and upregulation of *hly* was only observed during co-culture at 37 °C compared to the respective at 5 °C.

The expression of key virulence genes during growth in various food-related substrates has been studied to some extent. *sigB* and *prfA* possess central role in cellular homeostasis under stressful conditions and virulence [18,61–69]. Regarding their regulation in food-associated matrices, a rather mixed response has been reported [47,48,49,51,52]. In the present study, no regulation of *sigB* was observed during co-culture at 5 °C but an upregulation at 37 °C was evident. On the contrary, *prfA*

was downregulated during co-culture at both temperatures. Interestingly, no effect of the temperature itself on the expression of these genes was noticed. Regarding *sigB*, a differential regulation in various substrates according to temperature has already been reported [48,49,52].

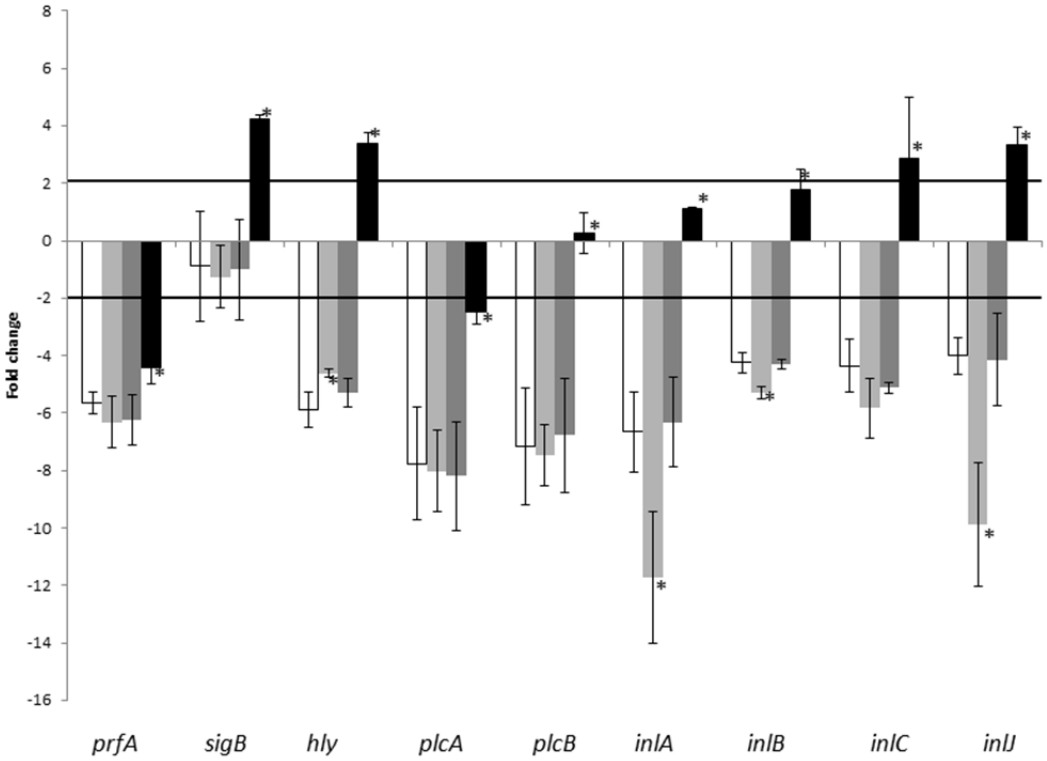

Figure 2. Effect of co-culture with enterotoxigenic *E. faecium* strain LQC 20005 on the relative expression of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* of *L. monocytogenes* strain NCTC 10527 during co-cultures 1, 2 and 3 at 5 °C depicted with white, light grey and dark grey bars, respectively and co-culture 1 at 37 °C (black bars) after 24 h incubation. Error bars represent the standard deviation of the mean value. The asterisk indicates that expression of each gene during co-cultures 2 and 3 at 5 °C and co-culture 1 at 37 °C is significantly ($P < 0.05$) different from co-culture 1 at 5 °C.

Transcription of *prfA* is initiated by the P_{1prfA} and P_{2prfA} promoters as well as the *plcA* promoter through the synthesis of bicistronic *plcA-prfA* mRNA. The latter is thermoregulated; it has been reported that below 37 °C the bicistronic message is absent, *prfA* transcription carries on through P_{2prfA} promoter that is not thermoregulated and therefore the total amount of PrfA is reduced [35]. Thus, regarding the effect of temperature, an upregulation could be expected. Moreover, the downregulation of *plcA* that was observed during monoculture at 37 °C compared to 5 °C indicates that the mechanisms governing regulation, at least regarding LIPI-1, are not yet fully explored [52,70,71]. *plcB* regulation exhibited identical trend to the *plcA* one, i.e. downregulation during co-culture at 5 °C, no regulation during co-culture at 37 °C and downregulation of the monoculture at 37 compared to 5 °C. However, regulation of the two genes can hardly be correlated since the transcription of the latter is initiated through P_{actA}.

Transcription of *hly* is initiated by three promoter sites, namely P_{1hly}, P_{2hly} and P_{3hly}. According to Domann et al. [21], the two former are PrfA-dependent whereas the latter is not. Thus, the downregulation during co-culture at 5 °C may be assigned to any promoter since *prfA* was also downregulated. On the contrary, upregulation of *hly* during co-culture at 37 °C may only be assigned to P_{3hly} since *prfA* was downregulated. To the same promoter the upregulation during co-culture at 37 °C compared to 5 °C may be assigned since *prfA* was not regulated.

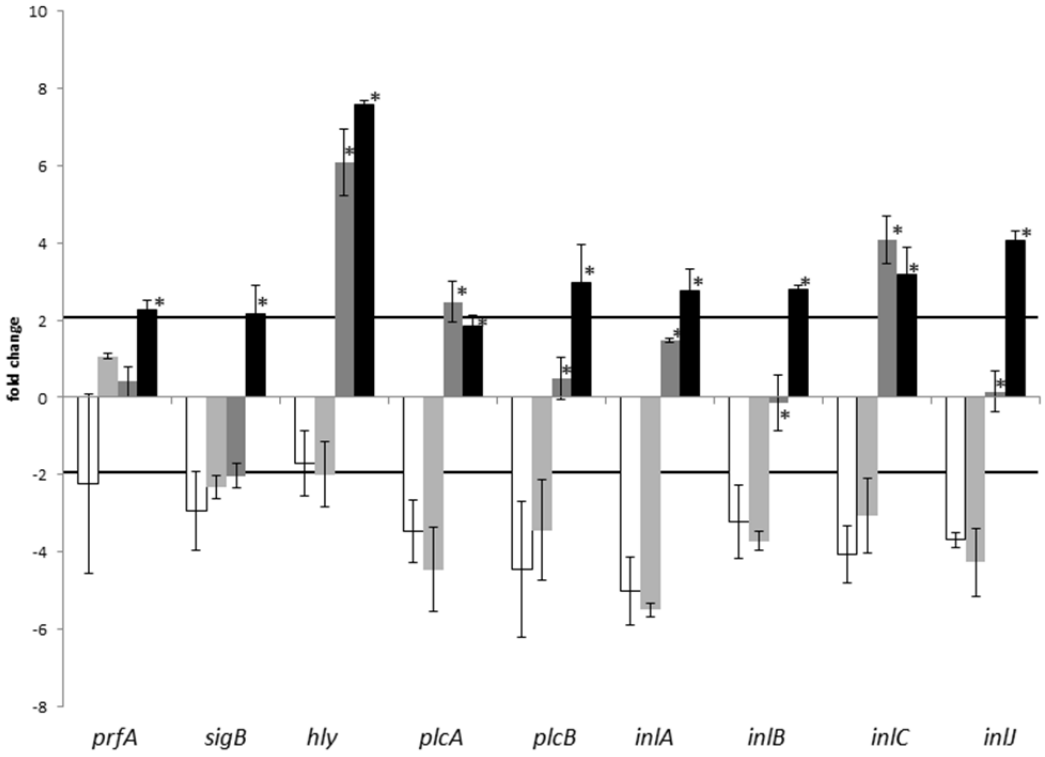

Figure 3. Effect of temperature on the relative expression of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* of *L. monocytogenes* strain NCTC 10527 during growth of monoculture (8 h white bars; 24 h light gray bars) or co-culture with enterotoxigenic *E. faecium* strain LQC 20005 (8 h dark grey bars; 24 h black bars) Error bars represent the standard deviation of the mean value. The asterisk indicates that expression of each gene in co-culture is significantly ($P < 0.05$) different from the expression in monoculture during the same sampling time.

Transcription of internalins was uniform in most of the cases, i.e. with the exception of co-culture at 37 °C, in which *inlC* and *inlJ* were downregulated. Their downregulation during co-culture at 5 °C may be explained by the partial PrfA-dependence of their expression [72]. However, in the rest of the cases, existence of PrfA-independent regulation is suggested [52].

4. Conclusion

The transciptomic response of *L. monocytogenes* key virulence genes to the co-culture with a bacteriocinogenic strain of *E. faecium* at 5 and 37 °C was successfully assessed for the first time.

Co-culture at 5 °C resulted in the downregulation of the majority of the genes under study accompanied in most of the cases by a statistically significant effect of the inoculum level. On the contrary, co-culture at 37 °C had no effect on the transcription level of most of the genes under study.

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Conflict of Interest

All authors declare no conflicts of interest in this study.

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