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Transcription of *Listeria monocytogenes* Key Virulence Genes on Tomato, Cucumber and Carrot

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Abstract: The aim of the present study was to assess the transcription of *Listeria monocytogenes* key virulence genes, namely *sigB*, *prfA*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* during subsistence on the surface of tomato, cucumber and carrot stored at 4, 10 and 30 °C for 0, 0.5, 6 and 24 h. Gene relative transcription was assessed through reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The results obtained, indicated that the relative transcription of *plcA*, *plcB* and *inlB* was more affected during subsistence on cucumber surface whereas the relative transcription of *sigB*, *prfA*, *hly*, *inlA* and *inlC* was more affected on tomato surface. Subsistence of the pathogen on carrot surface had only marginal effect on the relative transcription of the virulence genes assessed in the present study. In the majority of the cases, the aforementioned effects were dependent on the storage temperature employed.

Keywords: *Listeria monocytogenes*; virulence associated genes; liquid medium; tomato; cucumber; carrot

1. Introduction

Listeria monocytogenes is the etiological agent of foodborne listeriosis, the latest reports on which mention in the United States of America a total of 32 illnesses, 31 hospitalizations and 3 deaths (2017 annual report available at https://www.cdc.gov/fdoss/annual-reports/index.html (accessed on 20 May 2021)) and in the EU a total of 2621 confirmed cases, 1339 hospitalizations and 300 deaths (2019 annual report available at https://www.efsa.europa.eu/en/efsajournal/pub/6406 (accessed on 20 May 2021)). The virulence potential of this foodborne pathogen relies on the ability to cross the intestinal epithelial cells, escape from host defensive responses and disseminate through lymph and blood [1]. This infection requires the coordinated expression of a series of genes, including *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ*. *hly* encodes for listeriolysin O (LLO) a cholesterol-dependent pore-forming toxin that, among several functions, it facilitates internalization into host cells and escape from host phagosomes through a pore-dependent mechanism [2]. *plcA* and *plcB* encode for phosphatidylinositol phospholipase C and phosphatidylcholine phospholipase C, respectively. The cooperation of both phospholipases with LLO is required for escape of the pathogen from the phagocytic vacuole [3]. Internalins InlA, InlB, InlC and InlJ are necessary for effective invasion. InlA and InlB bind to E-cadherin and the hepatocyte growth factor receptor, respectively, which are receptors of the eukaryotic cell membrane. In both cases, bacterial endocytosis is induced [1]. InlC enables cell-to-cell spread of the pathogen by facilitating the formation of protrusions in the cell membrane of apical junctions [4]. InlJ is also important, as suggested by the significantly attenuated virulence capacity of the deletion mutant [5]; however, the mode of action is yet to be identified. The transcriptional control of these genes has been assigned to the alternative sigma factor B (σ^B) and the positive regulatory factor A (PrfA). The alternative sigma factor B is encoded by *sigB* and has a key role in *L. monocytogenes* survival in nature and in

the gastrointestinal tract. It controls the transcription of more than 200 genes, affecting substantially *L. monocytogenes* responses to environmental stimuli and virulence [6]. The latter is achieved through the transcriptional control of several virulence-associated genes, including *prfA*. The latter encodes PrfA that directly regulates transcription of twelve key virulence genes and indirectly a total of 145 genes. Thus, it possesses a key role in directing cellular homeostasis and fine-tuning the virulence machinery of *L. monocytogenes* [7].

The effect that several factors associated with food processing may have on the transcription of genes related to *L. monocytogenes* virulence has been studied to some extent. However, only a few studies address the combined effect that the food matrix, storage time and temperature as well as strain diversity may have. More accurately, Olesen et al. [8], Bae et al. [9], Rantsiou et al. [10,11] and Mataragas et al. [12] studied the transcription of key virulence genes of the pathogen on meat products, Duodu et al. [13] and Pilevar et al. [14] on fish products, Rantsiou et al. [11] and Alessandria et al. [15] on dairy products and Hadjilouka et al. [16] and Kang et al. [17] on fruits and vegetables. The significance of these studies is highlighted by the fact that prior exposure to environmental stimuli affects invasiveness [18–32] and that upregulation of virulence associated genes observed during such exposure is correlated with increased invasion efficiency [13,21].

Tomatoes, cucumbers and carrots are very important vegetables, both in terms of nutritional value (https://fdc.nal.usda.gov/ (accessed on 20 May 2021)) and commercial importance (http://www.fao.org/faostat/en/#data/QV (accessed on 20 May 2021)). Many authors have studied the prevalence of *L. monocytogenes* in these commodities. In cucumbers, it has been reported below 7% [33–35]; however, Arumugaswamy et al. [36], Poonniah et al. [37] and Ajayeoba et al. [38] reported detection of the pathogen in the 80%, 43.8% and 23.4%, respectively, of the samples assessed. A rather high prevalence has been reported for tomatoes. More accurately, Vahidy et al. [34] reported 13.3%, Pooniah et al. [37] 21.9% and Ajayeoba et al. [38] 19.67%. However, many studies have reported its absence [33,39–41]. Similar is the case of carrots, Ponniah et al. [37] reported a prevalence of 24.2% while Ajayeoba et al. [38] of 9.02%. On the contrary, Heisick et al. [33], Vahidy et al. [34] and Odumeru et al. [42] reported absence in the samples assessed.

Outbreaks linked to the consumption of tomatoes and cucumbers usually involve *Salmonella* serovars as etiological agents. On the other hand, illnesses related to carrot consumption have been mostly assigned to Norovirus, *Shigella* ssp. and botulism (data retrieved from outbreakdatabase.com (accessed on 20 May 2021)). However, based on the rather high prevalence values and their widespread and general applicability, their implication in *L. monocytogenes* outbreaks would also be reasonable, either through direct consumption or through cross-contamination. So far, only one recorded outbreak has been partially linked to tomato consumption. More accurately, Ho et al. [43] reported that the *L. monocytogenes* outbreak that took place in 1979 in Boston and involved 23 patients from eight hospitals, could be assigned to the consumption of celery, lettuce and tomatoes.

The aim of the present study was to provide information regarding the transcriptional response of *L. monocytogenes* during subsistence on the surface of tomato, cucumber and carrot, stored at 4, 10 and 30 °C. More accurately, data regarding the relative transcription of *sigB*, *prfA*, *plcA*, *plcB*, *hly*, *inlA*, *inlB*, *inlC* and *inlJ* could improve the accuracy of risk assessment studies and enable the development of commodity-specific control strategies.

2. Materials and Methods

2.1. Bacterial Strain, Inoculum and Sample Preparation

L. monocytogenes strain LQC 15257, serotype 4b, isolated from a strawberry sample [44] was used throughout this study. The strain was stored at −20 °C in Nutrient broth (Lab M, Lancashire, UK) supplemented with 50% glycerol. Before experimental use, the strain was grown twice in Brain Heart Infusion (BHI) broth (Lab M) at 37 °C for 24 h.

The inoculum was prepared as follows: overnight culture of the strain (9 log CFU/mL) was centrifuged (12,000× *g*; 15 min; 4 °C), washed twice with sterile Ringer's solution (Lab

M), resuspended in the same diluent and used to inoculate BHI broth, cucumber, carrot and tomato samples.

Fresh carrots, cucumbers and tomatoes were purchased from local markets and were examined for *L. monocytogenes* occurrence according to EN ISO 11290-1: 2017. Once absence of the pathogen was verified, they were thoroughly washed with tap water and cut in slices. Carrot slices of 2.5–3.0 cm diameter and ca. 0.5 cm thickness, cucumber slices of 4.0–5.0 cm diameter and ca. 0.5 cm thickness and tomato slices of 6.0–7.0 cm diameter and ca. 1 cm thickness were used for the experiment. The slices were placed in sterile containers (volume ca. 500 mL) according to the product type, placed at the intended incubation temperature (i.e., 4, 10 and 30 °C) overnight for temperature equilibration and sprayed with 0.5 mL of pathogen culture previously adjusted to proper concentration. Similarly, BHI broth was also inoculated with 0.5 mL of the diluted pathogen after temperature equilibration. BHI broth, cucumber, carrot and tomato samples were inoculated with 7.00–7.50 log CFU/mL or log CFU/g, respectively. The experiment was performed in triplicate.

2.2. Sampling and Microbiological Analyses

Sampling was performed immediately after inoculation (designated as time 0 h) and after 0.5, 6 and 24 h of incubation at 4, 10 and 30 °C. For microbiological analyses, 10 g of the vegetable sample was aseptically homogenized with 90 mL sterile Ringer's solution with the aid of a Stomacher apparatus (Seward, London, UK). In the case of BHI broth, 1 mL of substrate was aseptically homogenized with 9 mL of sterile Ringer's solution. In both cases, Ringer's solution was previously equilibrated at sample temperature. The homogenates were serially diluted in the same diluent, plated on Polymyxin Acriflavine Lithium chloride Ceftazidine Aesculin Mannitol (PALCAM) agar (Lab M) as well as BHI agar; incubation took place at 37 °C for 48 h. *L. monocytogenes* populations reported refer to enumeration on PALCAM agar.

2.3. In Vitro and In Situ Gene Transcription Assay

After sample homogenization, 10 mL of the homogenate was transferred in sterile Falcon® tubes and centrifuged ($12,000\times g$; 1 min; sample temperature). Then, the supernatant was discarded and the biomass was mixed with 200 μL of RNAlater® solution (Ambion, Whaltham, MA, USA). RNA was extracted with the NucleoSpin® RNA Kit (Macherey-Nagel, Dueren, Germany); cDNA synthesis took place with the PrimeScript™ One Step RT-PCR Kit (Takara Bio, Shiga, Japan) using random hexamers. Two reverse transcription reactions (+RT) and one without the addition of reverse transcriptase (-RT) were performed for each sample, containing ca. 0.5 μg RNA each. The primers and PCR conditions were according to Hadjilouka et al. [45]. Real-Time qPCR was performed using KAPA SYBR qPCR Kit Master Mix (2X) ABI Prism (KapaBiosystems, Boston, MA, USA). No template controls (NTC) were also included to detect reagent contamination. The Ct values of the genes under study were processed only if the difference between +RT and -RT was at least 10 cycles and no amplification was observed in NTC.

2.4. Statistical Analysis

The Ct values of reference and virulence-associated genes were processed according to Hadjilouka et al. [45] and the calculated fold change was converted to the respective $\log_2$ values for further processing. Based on the stability values calculated by NormFinder [46], IGS was used for normalization. The condition used as control is mentioned in each case. The relative transcription of a gene was considered as down- or up-regulated when the $\log_2$ value of the fold change ($\log_2$FC) was below -1 or above 1, respectively, assessed through one-sample *t*-test ($p < 0.05$). The correlation between the $\log_2$FC values of the genes was assessed by the Pearson coefficient. One-way analysis of variance (ANOVA) was performed to assess the differences between the population of the pathogen at each sampling time. All calculations were performed in Statgraphics Centurion XVII.

3. Results

In Table 1, the dynamics of the *L. monocytogenes* population during incubation of BHI broth, carrot, cucumber and tomato at 4, 10 and 30 °C, are exhibited. Increase of the *L. monocytogenes* population was only observed during incubation of BHI broth at 10 and 30 °C as well as during incubation of cucumber at 30 °C.

Table 1. *L. monocytogenes* population dynamics (log CFU mL^{-1} or g^{-1}) in BHI broth, on cucumber, carrot and tomato during incubation at 4, 10 and 30 °C.

Temperature (°C)	Time (h)	Substrate			
		BHI Broth (log CFU mL^{-1})	**Cucumber (log CFU g^{-1})**	**Carrot (log CFU g^{-1})**	**Tomato (log CFU g^{-1})**
4	0	7.23 (0.20) [a]	7.15 (0.22) [a]	7.23 (0.25) [bc]	7.11 (0.24) [a]
	0.5	7.35 (0.37) [a]	7.31 (0.31) [a]	7.33 (0.37)[c]	7.17 (0.47) [a]
	6.0	7.34 (0.40) [a]	7.26 (0.33) [a]	6.56 (0.42) [a]	7.23 (0.52) [a]
	24.0	7.42 (0.33) [a]	6.79 (0.20) [a]	6.60 (0.24) [a b]	6.94 (0.59) [a]
10	0	7.20 (0.10) [a]	7.22 (0.31) [a]	7.34 (0.21) [a]	7.13 (0.19) [a]
	0.5	7.36 (0.11) [a]	7.09 (0.38) [a]	7.19 (0.37) [a]	7.36 (0.14) [a]
	6.0	7.69 (0.13) [b]	6.92 (0.14) [a]	7.10 (0.41) [a]	7.36 (0.51) [a]
	24.0	8.12 (0.22) [c]	6.99 (0.15) [a]	6.89 (0.51) [a]	7.21 (0.38) [a]
30	0	7.20 (0.13) [a]	7.52 (0.35) [a]	7.38 (0.27) [a b]	7.34 (0.21) [a]
	0.5	7.65 (0.10) [b]	7.77 (0.12) [ab]	7.51 (0.33) [b]	7.44 (0.27) [a]
	6.0	8.19 (0.27) [c]	8.28 (0.30) [b]	6.73 (0.39) [a]	7.20 (0.37) [a]
	24.0	9.00 (0.12) [d]	8.25 (0.32) [b]	6.86 (0.40) [ab]	7.20 (0.40) [a]

Standard deviation is given in parenthesis. Within a column, for each temperature, different superscript letters denote statistically significant differences ($p < 0.05$).

In Figures 1 and 2, the effect of substrate and temperature on the relative transcription of *sigB*, *prfA*, *plcA*, *plcB*, *hly*, *inlA*, *inlB*, *inlC* and *inlJ* is exhibited. These results are summarized in Table 2.

In general, the relative transcription of all genes under study but *hly*, seemed to be unaffected by the substrate, since, in the 66.9% of the cases, the log$_2$(FC) value ranged between -1 and 1 (Table 2), which was not considered as regulation. However, there were notable exceptions. More accurately, transcription of *sigB* and *plcB* were affected on tomato and cucumber, respectively. *sigB* was downregulated on tomato at all temperatures assessed while downregulation of *plcB* was noted at 4 °C and a mixed response, i.e., up- and down-regulation at different sampling times at 10 and 30 °C (Figure 1). Transcription of *prfA* was affected on cucumber and tomato. More accurately, in the former case, downregulation was evident at 4 and 10 °C, while in the latter case a mixed response at 4 °C and upregulation at 30 °C were observed. In addition, transcription of *inlA* and *inlC* was affected on tomato. In the first case, upregulation was observed at 10 and 30 °C and a mixed response at 4 °C (Figure 1). On the contrary, upregulation of *inlC* was observed at 30 °C and a mixed response at 4 °C. Finally, downregulation of *inlB* was observed on cucumber at 4, 10 and 30 °C. Regarding the relative transcription of *hly*, downregulation was observed on cucumber at 4 °C and on cucumber, carrot and tomato at 10 °C. On the contrary, upregulation was observed on carrot at 4 °C and on tomato and carrot at 30 °C. Finally, a mixed response was detected on tomato at 4 °C and on cucumber at 30 °C (Figure 1). In only two cases the same transcriptomic response was observed in all four sampling times, namely downregulation of *hly* and *inlB* that were observed at 10 °C on carrot and cucumber, respectively.
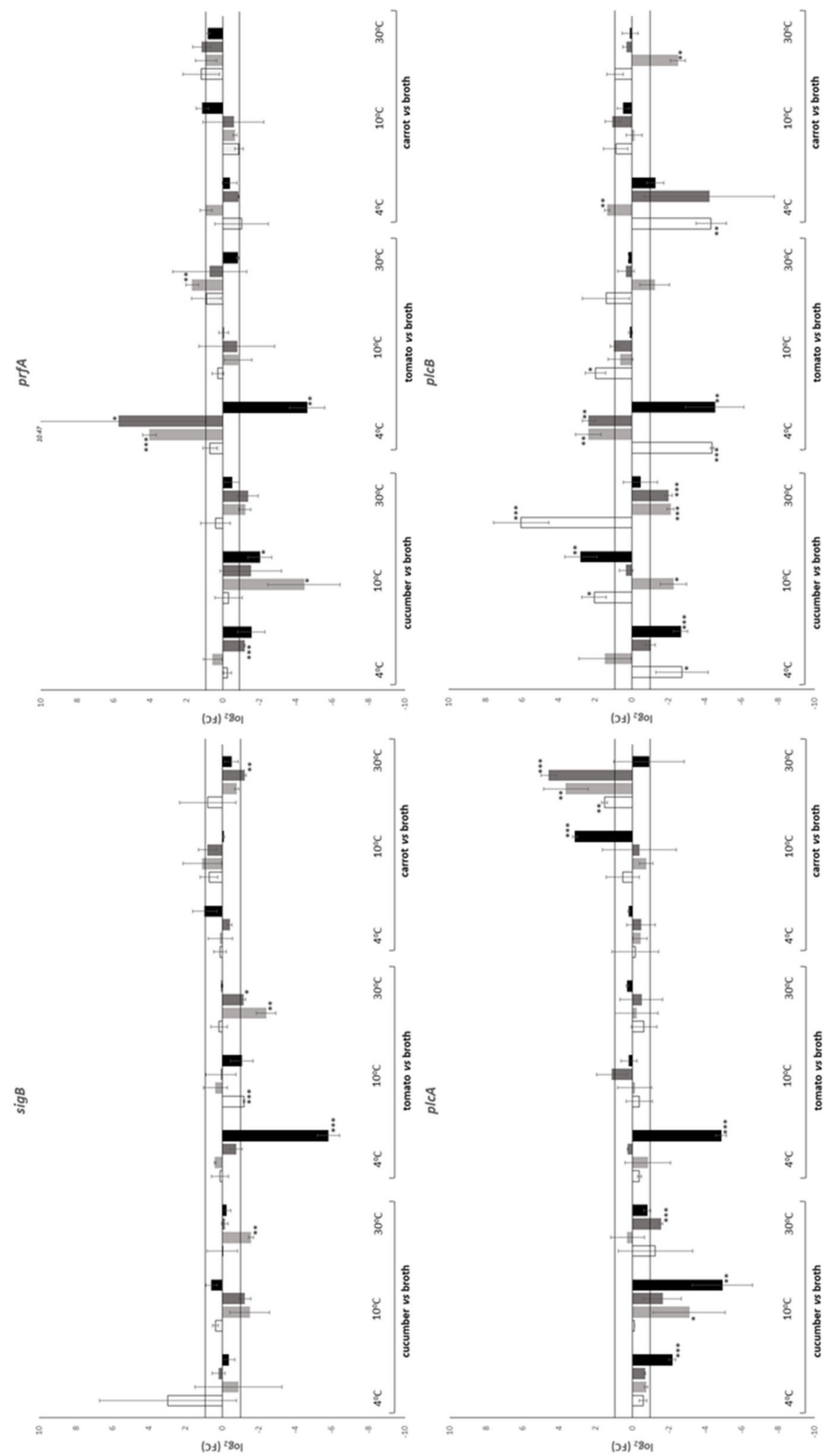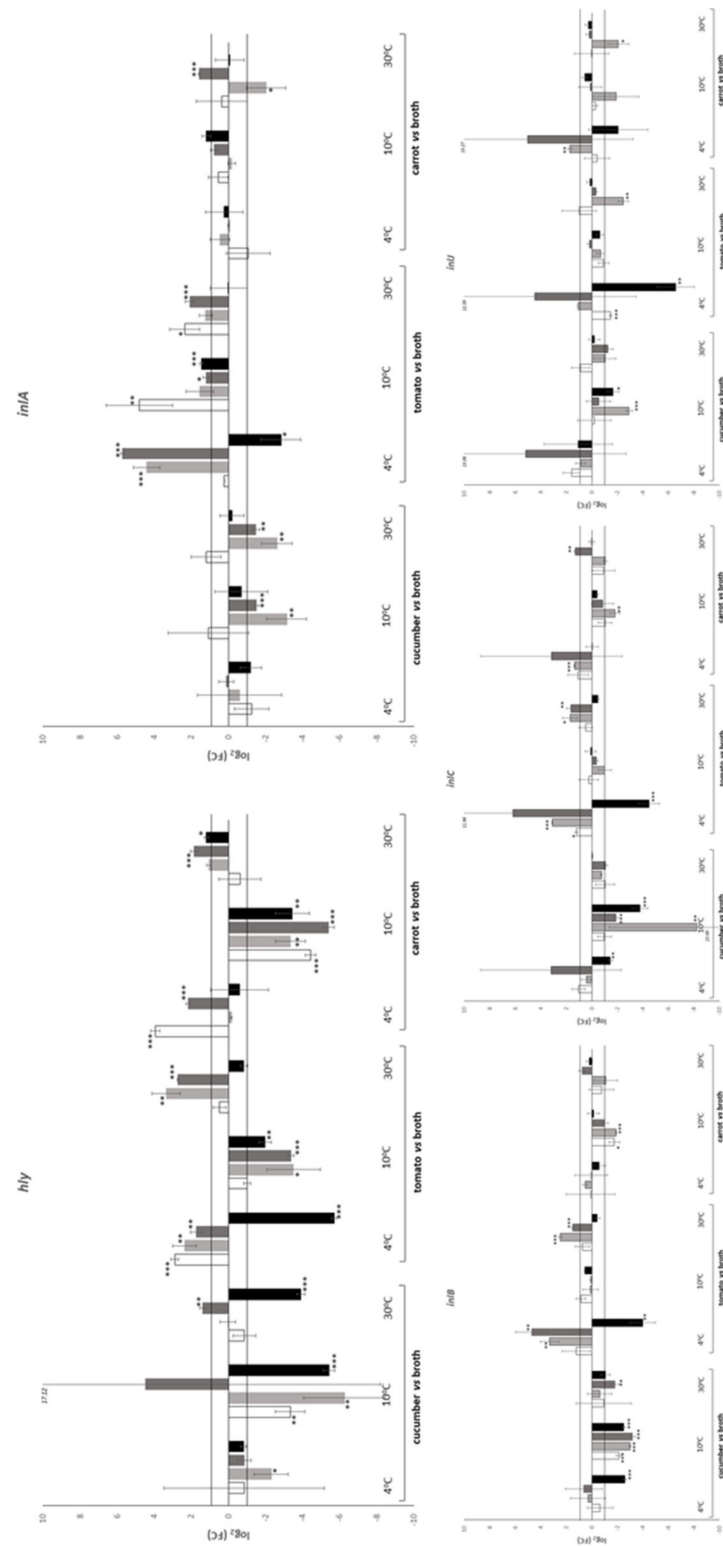
Figure 1. *Cont.*

Figure 1. Effect of substrate on the relative transcription of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* during subsistence of *L. monocytogenes* strain LQC 15257 in BHI broth, on tomato, cucumber and carrot at 4, 10 and 30 °C. BHI broth was used as control. If visible, white bars, time 0 h; light grey bars, time 0.5 h; dark grey bars, time 6 h; and black bars, time 24 h. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below −1 (the values that were used as threshold) at *p* value indicated by the number of asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).
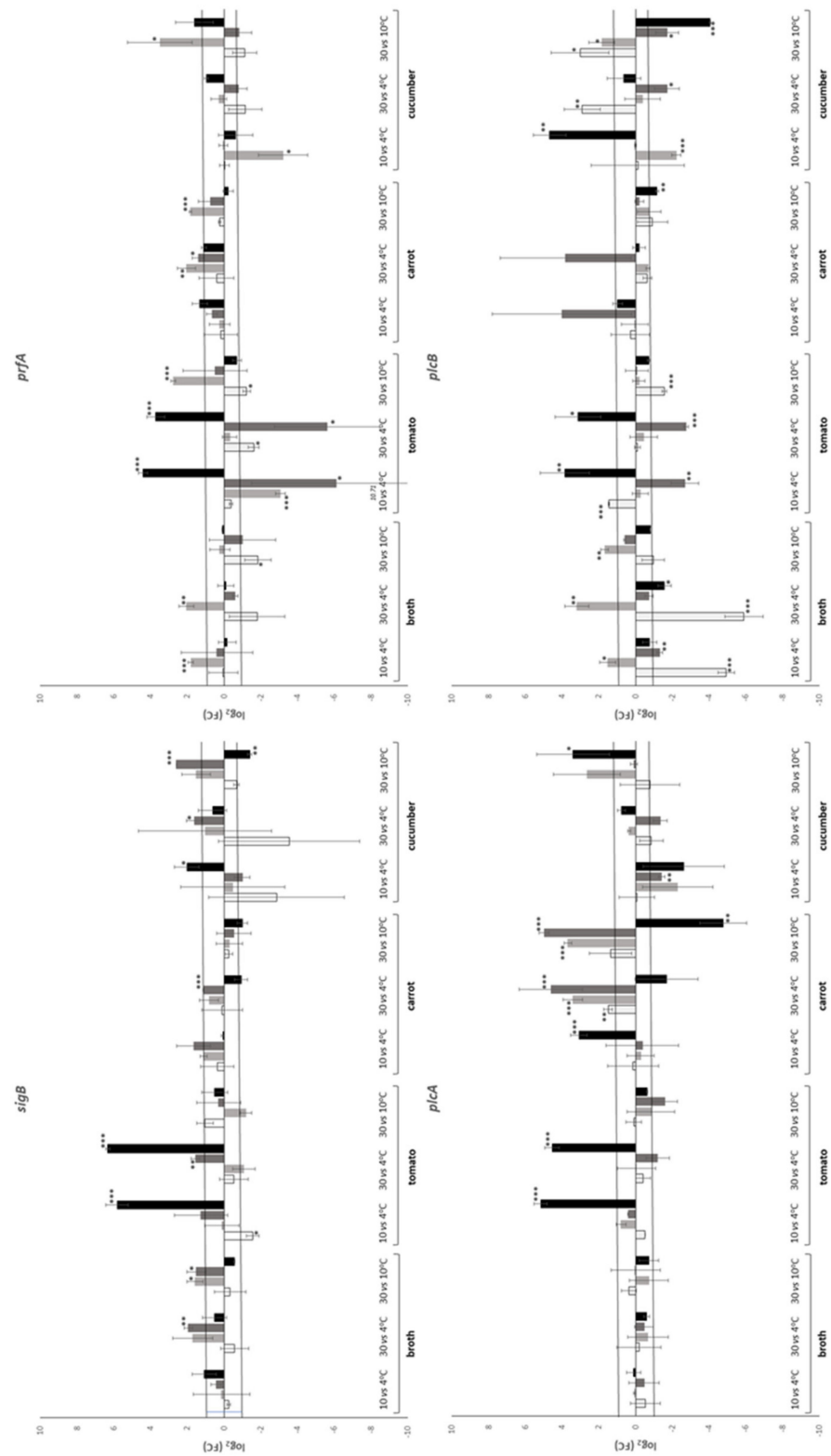
Figure 2. *Cont.*
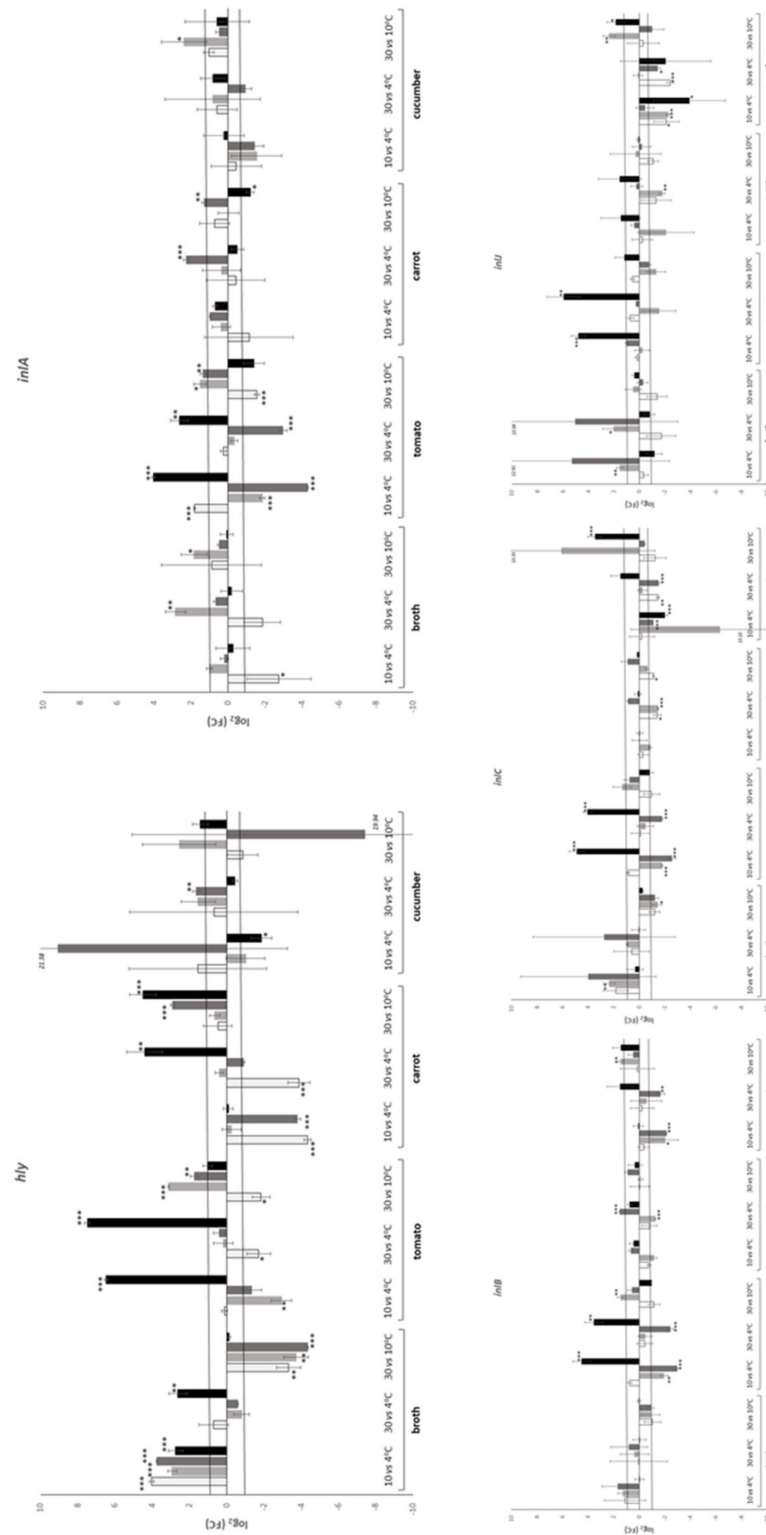
Figure 2. Effect of temperature on the relative transcription of *prfA*, *sigB*, *hly*, *plcA*, *plcB*, *inlA*, *inlB*, *inlC* and *inlJ* during subsistence of *L. monocytogenes* strain LQC 15257 in BHI broth, on tomato, cucumber and carrot at 4, 10 and 30 °C. The lower temperature was used as control. If visible, white bars, time 0 h; light grey bars, time 0.5 h; dark grey bars, time 6 h; and black bars, time 24 h. Error bars represent the standard deviation of the mean value. Presence of asterisks indicates that the relative transcription was above 1 or below −1 (the values that were used as threshold) at *p* value indicated by the number of asterisks (*, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001).

Table 2. Number of samples under each experimental condition in which the relative transcription of the genes under study was below, above or within the threshold set. Percentage is given in parenthesis.

log$_2$(FC)	Effect of Substrate				Effect of Temperature				
	total	cucumber	tomato	carrot	total	BHI broth	cucumber	tomato	carrot
sigB									
<−1	6 (16.7)	1 (8.3)	4 (33.3)	1 (8.3)	2 (4.3)	0 (0.0)	1 (8.3)	1 (8.3)	0 (0.0)
−1 to 1	30 (83.3)	11 (91.7)	8 (66.7)	11 (91.7)	36 (74.9)	9 (75.0)	8 (66.7)	8 (66.7)	11 (91.7)
>1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (20.8)	3 (25.0)	3 (25.0)	3 (25.0)	1 (8.3)
prfA									
<−1	4 (11.1)	3 (25.0)	1 (8.3)	0 (0.0)	7 (14.6)	1 (8.3)	1 (8.3)	5 (41.7)	0 (0.0)
−1 to 1	29 (80.6)	9 (75.0)	8 (66.7)	12 (100)	32 (66.5)	9 (75.0)	10 (83.3)	4 (33.3)	9 (75.0)
>1	3 (8.3)	0 (0.0)	3 (25.0)	0 (0.0)	9 (18.9)	2 (16.7)	1 (8.3)	3 (25.0)	3 (25.0)
plcA									
<−1	5 (13.9)	4 (33.3)	1 (8.3)	0 (0.0)	2 (4.3)	0 (0.0)	1 (8.3)	0 (0.0)	1 (8.3)
−1 to 1	27 (75.0)	8 (66.7)	11 (91.7)	8 (66.7)	37 (76.8)	12 (100)	10 (83.3)	10 (83.3)	5 (41.7)
>1	4 (11.1)	0 (0.0)	0 (0.0)	4 (33.3)	9 (18.9)	0 (0.0)	1 (8.3)	2 (16.7)	6 (50.0)
plcB									
<−1	9 (25.0)	5 (41.7)	2 (16.7)	2 (16.7)	12 (25.1)	4 (33.3)	4 (33.3)	3 (25.0)	1 (8.3)
−1 to 1	20 (55.6)	4 (33.3)	7 (58.3)	9 (75.0)	26 (54.1)	5 (41.7)	4 (33.3)	6 (50.0)	11 (91.7)
>1	7 (19.4)	3 (25.0)	3 (25.0)	1 (8.3)	10 (20.8)	3 (25.0)	4 (33.3)	3 (25.0)	0 (0.0)
hly									
<−1	13 (36.1)	5 (41.7)	4 (33.3)	4 (33.3)	10 (20.8)	3 (25.0)	1 (8.3)	3 (25.0)	3 (25.0)
−1 to 1	13 (36.1)	6 (50.0)	3 (25.0)	4 (33.3)	24 (50.0)	4 (33.3)	9 (75.0)	5 (41.7)	6 (50.0)
>1	10 (27.8)	1 (8.3)	5 (41.7)	4 (33.3)	14 (29.2)	5 (41.7)	2 (16.7)	4 (33.3)	3 (25.0)
inlA									
<−1	6 (16.7)	4 (33.3)	1 (8.3)	1 (8.3)	6 (12.5)	1 (8.3)	0 (0.0)	4 (33.3)	1 (8.3)
−1 to 1	22 (61.2)	8 (66.7)	4 (33.3)	10 (83.3)	32 (66.7)	9 (75.0)	11 (91.7)	3 (25.0)	9 (75.0)
>1	8 (22.1)	0 (0.0)	7 (58.3)	1 (8.3)	10 (20.8)	2 (16.7)	1 (8.3)	5 (41.7)	2 (16.7)
inlB									
<−1	9 (25.0)	6 (50.0)	1 (8.3)	2 (16.7)	7 (14.6)	0 (0.0)	3 (25.0)	3 (25.0)	1 (8.3)
−1 to 1	23 (63.9)	6 (50.0)	7 (58.3)	10 (83.3)	36 (75.0)	12 (100)	8 (66.7)	6 (50.0)	10 (83.3)
>1	4 (11.1)	0 (0.0)	4 (33.3)	0 (0.0)	5 (10.4)	0 (0.0)	1 (8.3)	3 (25.0)	1 (8.3)
inlC									
<−1	6 (16.7)	4 (33.3)	1 (8.3)	1 (8.3)	10 (20.8)	1 (8.3)	3 (25.0)	3 (25.0)	3 (25.0)
−1 to 1	23 (63.9)	8 (66.7)	6 (50.0)	9 (75.0)	33 (68.6)	10 (83.3)	7 (58.3)	7 (58.3)	9 (75.0)
>1	7 (19.4)	0 (0.0)	5 (41.7)	2 (16.7)	5 (10.6)	1 (8.3)	2 (16.7)	2 (16.7)	0 (0.0)
inlJ									
<−1	5 (13.9)	2 (16.7)	2 (16.7)	1 (8.3)	6 (12.5)	0 (0.0)	5 (41.7)	0 (0.0)	1 (8.3)
−1 to 1	30 (83.4)	10 (83.3)	10 (83.3)	10 (83.3)	37 (77.0)	10 (83.3)	5 (41.7)	10 (83.3)	11 (91.7)
>1	1 (2.7)	0 (0.0)	0 (0.0)	1 (8.3)	6 (12.5)	2 (16.7)	2 (16.7)	2 (16.7)	0 (0.0)
Total									
<−1	63 (19.5)	34 (31.5)	17 (15.8)	12 (11.1)	62 (14.3)	10 (9.3)	19 (17.6)	22 (20.4)	11 (10.2)
−1 to 1	217 (66.9)	70 (64.8)	64 (59.2)	83 (76.8)	293 (67.7)	80 (74.0)	72 (66.7)	59 (54.6)	81 (75.0)
>1	44 (13.6)	4 (3.7)	27 (25.0)	13 (12.1)	78 (18.0)	18 (16.7)	17 (15.7)	27 (25.0)	16 (14.8)

As far as the effect of temperature on the relative transcription of the genes under study was concerned, it was not significant in the majority (67.7%) of the cases. More accurately, transcription of *sigB*, *inlB* and *inlC* seemed to be unaffected by temperature. Transcription

of *prfA*, *hly* and *inlA* on tomato exhibited a mixed response, i.e., up- and down- regulation at different sampling times. The same response was observed for *plcB* transcription in BHI broth and on cucumber. Transcription on *plcA* was mostly affected on carrot; in the majority of the cases temperature increase resulted in upregulation. Transcription of *hly* in BHI broth and *inlJ* on cucumber presented a similar trend. In the first case, the relative transcription at 10 and 30 °C compared to the one at 4 °C indicated upregulation but the relative transcription at 30 °C compared to the one at 10 °C indicated downregulation. In the case of *inlJ*, the opposite was evident; the relative transcription at 10 and 30 °C compared to the one at 4 °C indicated downregulation but the relative transcription at 30 °C compared to the one at 10 °C indicated upregulation. In only one case, the same transcriptomic response was observed in all four sampling times, namely *hly* upregulation in BHI broth at 10 °C compared to 4 °C.

The correlation between the transcriptomic responses of the genes under study were collectively assessed by the Pearson correlation coefficient (Table 3). A total of 36 assessments were performed; no statistically significant correlation was observed in seven cases and positive correlation was observed in the remaining 29 cases.

Table 3. Pearson product moment (r) correlations between the relative transcription of each pair of genes.

		prfA	*sigB*	*plcA*	*plcB*	*hly*	*inlA*	*inlB*	*inlC*	*inlJ*
prfA	r									
	p									
sigB	r	**0.2548**								
	p	0.0193								
plcA	r	**0.4970**	**0.3361**							
	p	0.0000	0.0018							
plcB	r	**0.4347**	**0.2836**	0.1373						
	p	0.0000	0.0089	0.2128						
hly	r	**0.4430**	0.0654	0.2050	−0.0811					
	p	0.0000	0.5545	0.0614	0.4631					
inlA	r	**0.7528**	**0.2293**	**0.3497**	**0.6363**	0.1316				
	p	0.0000	0.0359	0.0011	0.0000	0.2327				
inlB	r	**0.7959**	**0.2581**	**0.4265**	**0.3001**	**0.4618**	**0.7264**			
	p	0.0000	0.0178	0.0001	0.0056	0.0000	0.0000			
inlC	r	**0.7218**	**0.2803**	**0.4250**	0.1368	**0.5274**	**0.5850**	**0.8322**		
	p	0.0000	0.0098	0.0001	0.2147	0.0000	0.0000	0.0000		
inlJ	r	**0.4045**	**0.4817**	**0.3182**	0.1934	**0.3311**	**0.3646**	**0.5134**	**0.6898**	
	p	0.0001	0.0000	0.0032	0.0780	0.0021	0.0006	0.0000	0.0000	

Statistically significant correlations ($p < 0.05$) are presented in bold.

Weak correlation ($0.00 < r < 0.29$) was indicated in 5 cases, moderate correlation ($0.30 < r < 0.49$) was indicated in 14 cases and strong correlation ($0.50 < r < 0.99$) in 10 cases. The strongest correlation ($r > 0.70$) was indicated between *prfA* and *inlA*, *inlB*, *inlC*, between *inlA* and *inlB*, as well as between *inlB* and *inlC*.

4. Discussion

The pathogenic potential of *L. monocytogenes*, assessed almost exclusively by invasion to cell lines, such as Caco-2, THP-1 and HT-29, is affected by abiotic and biotic stimuli that include exposure to temperature, acid and osmotic fluctuations as well as the food

matrix and co-existing microorganisms [18–32]. Correlation between the transcriptomic response of the pathogen to the above stresses with invasiveness, in some cases was successful [13,21]. Several studies have assessed the transcription of virulence associated genes during growth of *L. monocytogenes* on a food matrix at various storage temperatures and times [8–13,15–17]. The basic conclusions of these studies were that their transcription was, in the majority of the cases, affected by the experimental conditions (time, temperature, food matrix) and this effect was strain dependent. Indeed, when more than one strain was included in a study, substantial differences in gene transcription were reported [8,10–13,15]. In the present study, only *L. monocytogenes* strain LQC 15257 was employed. The relative transcription of virulence associated genes of this strain was also assessed on the surface of rocket and melon [16], on the surface of BHI agar under the effect of lemongrass essential oil [47], in co-culture with *Aspergillus flavus* strain 18.4 [48] as well as after in vitro exposure to human gastric and duodenal aspirates [45]. Thus, a database of responses to various stimuli has been established, which is enriched with the transcriptomic responses assessed in the present study.

sigB and *prfA* possess very important role in homeostasis and virulence capacity of the pathogen [1]. Moreover, transcription of *prfA* may be initiated by three promoter sites, one of which is partially σ^B-dependent [48]. In the present study, only weak association of the relative transcription between *sigB* and *prfA* was revealed. In addition, relative transcription of both genes seemed to be unaffected by substrate and temperature, with a few notable exceptions. Among them, downregulation of *sigB* was observed on tomato at all temperatures. However, this was accompanied by up- and down- regulation of *prfA* at 4 °C (at different sampling times) and upregulation at 30 °C. Downregulation of *prfA* could be explained through the effect of the σ^B-dependent promoter P2$_{prfA}$, while upregulation through the effect of the σ^B-independent promoter P1$_{prfA}$. The bicistronic *plcA-prfA* mRNA, which is initiated by the *plcA* promoter, should be absent at temperatures below 37 °C [49], i.e., the temperatures employed in the present study. This seems to be partially verified, considering the only moderate correlation between *prfA* and *plcA*, which was indicated by Pearson correlation coefficient.

Internalins InlA, InlB, InlC and InlJ are necessary for effective invasion. Indeed, deletion mutants exhibited significantly attenuated virulence capacity [5,50–52]. In the present study, based on the times that log$_2$(FC) value indicated lack of regulation, the relative transcription of *inlA*, *inlB*, *inlC* and *inlJ* was generally not affected by substrate and temperature. However, in several cases, effect of substrate and temperature on internalin genes relative transcription was observed. The substrate mostly affected *inlA*, *inlB* and *inlC* relative transcription and only marginally the one of *inlJ*. Subsistence on tomato affected *inlA* and *inlC* relative transcription more than subsistence on cucumber did. On the contrary, cucumber affected *inlB* relative transcription more than tomato did. In nearly all cases, subsistence on cucumber resulted in downregulation, while subsistence on tomato in upregulation; carrot had only marginal effect. Regarding the effect of temperature, this was more pronounced on tomato and cucumber and the genes mostly affected were *inlA*, *inlB* and *inlC* in the first case, and *inlC* and *inlJ* in the second. All the above suggest that transcription regulation of *inlA*, *inlB* and *inlC* is related. Indeed, *inlA* and *inlB* form the operon *inlAB*, the transcription of which is controlled by both σ^B and PrfA; transcription of *inlC* is controlled by PrfA, while transcription of *inlJ* by none of the above [53]. The effect of *prfA* on the relative transcription of *inlA*, *inlB* and *inlC* was also verified by the high (above 0.7) Pearson correlation coefficient. On the contrary, only a moderate correlation was exhibited between *prfA* and *inlJ* as well as between *sigB* and all four internalins.

The role of *hly* in virulence has been adequately highlighted; it encodes for (LLO), the absence of which has been reported to result in avirulence [54,55]. However, Phelps et al. [52] reported that LLO-dependent internalization did not occur in the case of cytotrophoblasts and endothelial cells, which indicates that LLO deficient strains avirulence is tissue dependent. Relative transcription of *hly* seemed to be substantially affected by substrate and temperature. More accurately, subsistence on tomato seemed to affect more

than carrot, which in turns affected more than cucumber. This effect was mostly observed at 4 and 10 °C. In the latter temperature, *hly* was downregulated in all cases, while in the former, *hly* was upregulated in the majority of the cases. Transcription of *hly* may be initiated by three promoter sites, two of which are PrfA-dependent since they contain a PrfA box [56]. In the present study, activation of the third promoter site is indicated, taking into consideration the moderate correlation between *prfA* and *hly* transcription revealed by Pearson correlation coefficient.

plcB also holds a very important role in pathogenesis [57]. Subsistence of the pathogen on cucumber affected *plcB* relative transcription more than tomato and carrot did. In the majority of the cases, a mixed response, i.e., up- and down-regulation at different sampling times, was observed. Regarding the effect of temperature, this was more pronounced on cucumber, followed by BHI broth and tomato. The effect of temperature on carrot was only marginal. As in the previous case, a mixed response was evident. Transcription of *plcB* is PrfA-dependent [58]. However, the data obtained in the present study revealed only a moderate correlation between *prfA* and *plcB* transcription.

5. Conclusions

Subsistence of the pathogen on carrot surface had only marginal effect on the relative transcription of the virulence associated genes assessed in the present study. On the contrary, the relative transcription of *sigB*, *prfA*, *hly*, *inlA* and *inlC* was more affected on tomato surface, while the relative transcription of *plcA*, *plcB* and *inlB* was more affected on cucumber surface. In the majority of the cases, this effect was dependent on the storage temperature employed. These results constitute a significant input to the ongoing effort to elucidate the physiology of the pathogen during subsistence in food matrices, which may concomitantly enable targeted virulence-attenuation approaches.

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