Transcription of *Escherichia Coli* O157:H7 Key Virulence Genes During Growth in Liquid Medium and Rocket (*Eruca Sativa*) at 4 and 10°C

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Abstract: The aim of the present study was to comparatively assess the transcription of key virulence genes, during growth of an *Escherichia coli* O157:H7 isolate in liquid medium and rocket (*Eruca sativa*) at different temperatures and time. For that purpose, rocket leaves and BHI broth were inoculated with 7.5 log CFU/g or CFU/mL, respectively, and incubated at 4 and 10°C. Sampling took place upon inoculation and after 0.5, 6 and 24 h of incubation; the RNA was stabilized and the transcription of *stx1*, *stx2*, *eae*, *ehxA* and *etpD* was assessed by RT-qPCR. Downregulation of *stx1* and *ehxA* during growth on rocket surface at 4°C, upregulation of *stx2* and *eae* during growth on rocket surface at both 4 and 10°C and the upregulation of *etpD* and *ehxA* during growth on rocket surface at 4 and 10°C, respectively, were observed. In addition, increase of incubation temperature, i.e. from 4 to 10°C resulted in upregulation of *stx1* and downregulation of *stx2* and *eae*. These results suggest that through differential regulation of genes encoding for virulence factors, the virulence potential of *E. coli* O157:H7 may be altered when it resides on the surface of rocket, at least for short time and at temperature relevant to its storage. However, further research is still necessary in order to improve our understanding of the physiology of this foodborne pathogen and the responses during exposure to food-related stresses.

Keywords: *Escherichia coli*, rocket, *stx1*, *stx2*, *eae*, *ehxA*, *etpD*

1. Introduction

Escherichia coli is an important foodborne pathogen implicated in several outbreaks every year. A total of 20-30% of the *E. coli* infections has fresh produce as etiological agent [1]. In addition, shiga toxin-producing *E. coli* (STEC) was the third most common etiological agent of foodborne disease outbreaks reported in the USA in 2011 and 2012, second in 2013 and fourth in 2014 [2-5], with O157 serogroup being held responsible for more than half of the outbreaks. The most known O157:H7 outbreaks include the 2006 multistate outbreak associated with the consumption of fresh spinach [6] and the 1996 Japan outbreak linked to the consumption of white (daikon) radish sprouts [7].

STEC pathogenicity and virulence factors have been adequately studied and reviewed [8-10]. More specifically, regulation of the genes encoding for the Shiga toxins (*stx1* and *stx2*), intimin (*eae*) and enterohemolysin (*ehxA*) has attracted attention due to their increased importance. In addition, their transcription in actual food matrices has also been assessed. Carey et al. [11] assessed *fliC*, *sodB*, *eae*, *stx1A*, *stx2A* and *rpoS* transcription during growth on romaine lettuce at 4 and 15°C for up to 9 days. Sharma et al. [12] studied the effect of modified atmosphere packaging on *iha*, *rfbE*, *eae*, *ehxA* and *stx2* transcription during growth on shredded lettuce stored at 4 and 15°C for up to 10 days. Mahmoudzadeh et al. [13] studied the transcription of *stx1A* and *stx2A* during growth on ground beef stored at 4°C for up to 7 days. Finally, Van der Linden et al. [14] used a microarray-based screening to assess the transcriptomic response during preharvest survival on butterhead lettuce.

Rocket (*Diplotaxis tenuifolia* (L.) DC., wild rocket; *Eruca sativa* Mill., cultivated rocket) is among the most appreciated vegetables and therefore its postharvest physiology, phytonutrient content, microbiological quality and shelf life have been extensively studied [15-21]. Rocket has been implicated as the food vehicle of two outbreaks, 2001 with *Cyclospora cayetanensis* as confirmed etiological agent and in 2016 with STEC as the suspected agent (data retrieved from www.fda.gov/Safety/Recalls/ArchiveRecalls/default.htm). However, the transcriptional response of STEC during growth on rocket salad has not yet been studied.

The aim of the present study was to assess comparatively the transcription of five key virulence genes, namely *stx1*, *stx2*, *eae*, *ehxA* and *etpD* during growth of *E. coli* O157:H7 in liquid medium and on rocket surface stored at 4 and 10°C.

2. Materials and methods
2.1 Bacterial strains and sample preparation

Escherichia coli O157:H7 strain LQC 15263, previously isolated from a cucumber sample was used throughout this study. Long-term storage took place at -20°C in nutrient broth supplemented with 50% glycerol. Before experimental use, the strain was grown twice in Brain Heart Infusion broth (Biolife, Milan, Italy) at 37°C for 24 h. For inoculum preparation, 10 test tubes containing 10 mL BHI broth were inoculated with the *E. coli* O157:H7 culture and incubated overnight at 37°C, resulting in a population of 8.5 log CFU/mL. All test tubes were centrifuged (12,000 x *g*; 10 min; 4°C), the biomass was washed twice with sterile saline, pooled and resuspended in 10 mL of the same diluent and used to inoculate BHI broth and rocket samples at 7.5 log CFU/mL or CFU/g, respectively. More accurately, 50 g of rocket (*Eruca sativa*) were placed in sterile containers (of approx. 500 mL volume) and sprayed with 0.5 mL of the diluted pathogen population to obtain the desired final population. In the case of BHI broth, 50 μL of the diluted pathogen were used to inoculate test tubes containing 5 mL of BHI broth. Before inoculation, all respective materials were placed overnight at the intended incubation temperature for temperature equilibration. Incubation took place at 4 and 10°C.

2.2 Microbiological analyses

E. coli O157:H7 enumeration was performed by spreading serial dilutions on the surface of *E. coli* O157:H7 agar (Merck, Darmstadt, Germany) and BHI agar. The former was used to enumerate the population of the pathogen and the latter to exclude the presence of contaminating bacteria, especially in the case of rocket inoculation. Incubation took place at 35°C for 24 h.

2.3 In vitro and in situ gene transcription assay

Sampling was performed immediately upon inoculation (time 0 h) and after 0.5, 6 and 24 h of incubation. In the case of broth samples, 5 mL were centrifuged (12,000 x *g*; 1 min, sample temperature), the supernatant was discarded and the pellet was mixed with 200 μL of RNAlater® solution (Ambion, Whaltham, MA, USA). In the case of rocket samples, 10 g were first homogenized using a Stomacher apparatus (30 s at 230 RPM) (Seward, London, UK) with 90 mL of sterile Ringer solution (LABM, Lancashire, UK) equilibrated at sample temperature. Then, 5 mL of the homogenate was transferred in sterile falcon tubes, centrifuged and the RNA was stabilized as above. The experiment was performed in triplicate. RNA extraction was performed with the NucleoSpin® RNA kit (Macherey-Nagel, Duren, Germany) and cDNA synthesis with the PrimeScript™ RT reagent kit (Takara, Shiga, Japan) according to the instructions of the manufacturer. Real-Time qPCR was performed with the KAPA SYBR qPCR Kit Master Mix (2X) for ABI Prism (KapaBiosystems, Boston, MA, USA). Primers and PCR conditions are presented in Table 1. *cysG*, *gap*, *rrsA* and *ihfB* were selected as housekeeping genes on the basis of the available literature while *stx1*, *stx2*, *eae*, *ehxA* and *etpD* due to their significance in virulence potential of *E. coli* O157:H7. Approximately 0.5 μg RNA of each sample was used for two RT reactions and the resulting cDNA was used for the assessment of the transcription of the genes under study.

Table 1. Primer sequences and respective amplicon sizes used for the *in vitro* and *in situ* gene transcription assay

Genes		sequence	amplicon size (bp)	PCR efficiency
Housekeeping				
cysG	cysG_f [22]	TTGTCGGCGGTGGTGATGTC	105	2.00
	cysG_r [22]	ATGCGGTGAACTGTGGAATAAACG		
gap	gap_f [23]	AGGTCTGATGACCACCGTTCA	65	1.97
	gap_r [23]	TGTGAGACGGGCCATCAAC		
rrsA	rrsA_f [24]	CTCTTGCCATCGGATGTGCCCA	105	1.89
	rrsA_r [24]	CCAGTGTGGCTGGTCATCCTCTCA		
ihfB	ihfB_f [25]	GCGGTTTCGGCAGTTTCT	124	2.01
	ihfB_r [25]	CGCAGTTCTTTACCAGGTTT		
Virulence associated				
stx1	stx1_f [26]	GGATAATTTGTTTGCAGTTGATGTC	107	1.95
	stx1_r [26]	CAAATCCTGTCACATATAAATTATTTCGT		
stx2	stx2_f [26]	GGGCAGTTATTTTGCTGTGGA	130	1.97
	stx2_r [26]	GAAAGTATTGTTGCCGTATTAACGA		
eae	eae_f [26]	CATTGATCAGGATTTTTCTGGTGATA	102	2.02
	eae_r [26]	CTCATGCGGAAATAGCCGTTA		
ehxA	ehxA_f [26]	CGTTAAGGAACAGGAGGTGTCAGTA	142	1.98
	ehxA_r [26]	ATCATGTTTTCCGCCAATGAG		
etpD	etpD_f	CGTCAGGAGGATGTTCAC	84	2.04
	etpD_r	ACCTGTCAGGACTTCCACTA		

Primer concentration was 1uM. Thermocycling conditions were 95ºC for 20 sec
and 40x (95ºC for 10 sec, 60ºC for 30 sec, 72ºC for 30 sec).
Melting curve analysis : 95ºC for 15 sec then 60ºC for 1 min and raise to 95ºC at 0.3ºC/sec.

2.4 Statistical analysis

Pretreatment and data analysis was performed according to Hadjilouka et al. [27]. Relative gene transcription during growth on rocket was assessed by comparison to the respective during growth in BHI broth under identical conditions. Regulation of the gene transcription was only considered when the $\log_2$ value of the fold change ($\log_2$FC) was above 1 and below -1, for up- and down- regulation, respectively, assessed through one-sample t-test ($p < 0.05$). One-way analysis of variance (ANOVA) was used to statistically evaluate the differences between the population dynamics of *E. coli* O157:H7 during growth.

3. Results-discussion

E. coli O157:H7 population presented statistically significant increase only in BHI broth after 24 h incubation at 10ºC; in all other cases it remained at the inoculation level (Table 2).

Table 2. *E. coli* O157:H7 population dynamics (log CFU mL⁻¹ or g⁻¹) in BHI broth and rocket during incubation at 4 and 10ºC

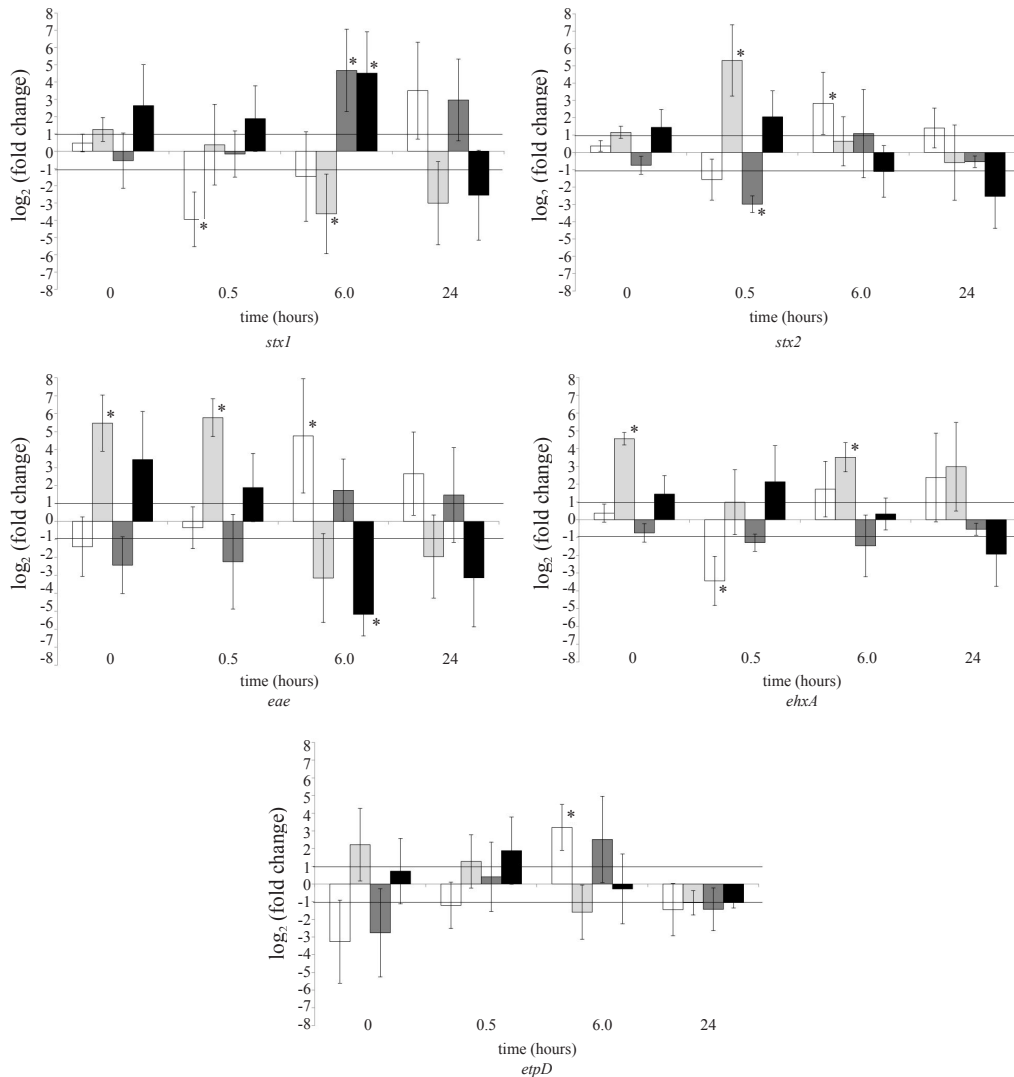| Temperature (ºC)	Time (h)	Growth substrate	
		BHI	rocket
4ºC	0	7.78 (0.35)ᵃ	7.30 (0.42)ᵃ
	0.5	8.15 (0.13)ᵃ	7.51 (0.44)ᵃ
	6.0	8.08 (0.20)ᵃ	7.74 (0.46)ᵃ
	24.0	7.83 (0.05)ᵃ	7.97 (0.38)ᵃ
10ºC	0	7.78 (0.43)ᵃ	7.52 (0.42)ᵃ
	0.5	7.59 (0.19)ᵃ	7.92 (0.54)ᵃ
	6.0	8.08 (0.32)ᵃ	7.39 (0.35)ᵃ
	24.0	8.75 (0.17)ᵇ	7.46 (0.15)ᵃ

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

In Table 1, the PCR efficiency values of the primers, used in the present study to assess the transcription of reference and target genes, as estimated from the slope of the respective linear regression plot is presented. The results showed that the best gene for normalization, producing the lower inter- (between groups) and intra-variation (within each group), was *cysG*.

In Figure 1. the effect of time, substrate and incubation temperature on the transcription of *stx1*, *stx2*, *eae*, *ehxA* and *etpD*, are shown. Studying gene transcription on rocket compared to BHI broth revealed that transcription of all genes assessed was affected. More accurately, growth on rocket surface at 4ºC resulted in downregulation of *stx1* and *ehxA* after 0.5 h growth and upregulation of *stx2*, *eae* and *etpD* after 6 h growth. Similarly, downregulation of *stx1* was also observed

after 6 h growth on rocket surface at 10°C, while upregulation of *stx2* after 0.5 h, *eae* immediately upon inoculation and after 0.5 h and *ehxA* immediately upon inoculation and after 6.0 h growth, were also noticed. Regarding the effect of temperature, relative quantification of gene transcription revealed that *stx1* was upregulated after 6 h growth at 10°C in BHI broth and rocket surface, when compared to growth at 4°C. On the contrary, downregulation of *stx2* and *eae* were noted after 0.5 and 6.0 h growth at 10°C in BHI broth and rocket surface, respectively.

Figure 1. Effect of substrate type and temperature on the relative transcription of *stx1*, *stx2*, *eae*, *ehxA* and *etpD* during growth of *Escherichia coli* O157:H7 strain LQC 15263 in BHI broth and on rocket at 4 and 10°C. Relative transcription of the genes under study is presented as follows: if visible, white bars, transcription levels during growth at 4°C on rocket compared to the transcription levels during growth at 4°C in BHI broth; light grey bars, transcription levels during growth at 10°C on rocket compared to the transcription levels during growth at 10°C in BHI broth; dark grey bars, transcription levels during growth at 10°C on BHI broth compared to the transcription levels during growth at 4°C in BHI broth; and black bars, transcription levels during growth at 10°C on rocket compared to the transcription levels during growth at 4°C on rocket. Error bars represent the standard deviation of the mean value. The asterisk indicates that transcription is significantly (p < 0.05) above or below the values of 1 and -1, respectively, that were used as threshold

Assessment of gene transcription under various food-related conditions offers improved understanding of the physiology of the microorganism and the responses upon exposure to food-related stresses. Under this perspective, several studies have been performed.

stx1 and *stx2* consist of two subunits each, designated A and B, and encode the production of two types of Shiga toxins. Their structure and function have been extensively studied and comprehensively reviewed by Melton-Celsa et al. [28]. *eae* is located on the locus of enterocyte effacement (LEE) and encodes for intimin, a protein that is necessary for the intimate contact between the pathogen and the host cell. *ehxA* is a part of the hemolysin operon (*ehxCABD*) located on pO157; it is the structural gene of the hemolysin while *ehxB* and *ehxD* encode the specific secretion machinery and

ehxC the necessary modifying factor [29]. *etpD* is a part of the *etp* gene cluster that is located on the pO157 plasmid of EHEC O157:H7 [30, 31]. This gene cluster encodes for a type II secretion system through which StcE, an extracellular zinc metalloprotease, is secreted. StcE has been characterized as a potential virulence factor due to the cleavage of C1 esterase inhibitor as well as gp340 and MUC7, two salivary glucoproteins, contributing thus to intimate adherence of the pathogen to the HEp-2 cell surface [32]. All these genes, but mostly *stx1*, *stx2* and *eae*, have been extensively used as an indicator of virulence potential of isolates [33-37].

In the present study, growth on the rocket surface resulted in downreguation of *stx1* and upregulation of *stx2* and *eae* at both temperatures, downregulation of *ehxA* at 4°C, as well as upregulation of *etpD* and *ehxA* at 4 and 10°C. In addition, growth at 10°C resulted in upregulation of *stx1* and downregulation of *stx2* and *eae*. However, these observations were not consistent throughout all sampling times.

The transcriptomic response of these genes to a variety of challenges has been assessed to some extent. Rashid et al. [38] studied the transcription of *stx2* and *ehxA* from fecal samples derived from naturally infected humans and experimentally infected calves and reported that these genes were not differentially transcribed. Bergholz et al. [39] reported downregulation of *eae* at early stationary stage during growth in MOPS minimal media at 37°C but no differential transcription of *stx1A*, *stx1B*, *stx2A* and *stx2B*. These results oppose the ones presented in the current study. More accurately, upregulation of *eae* was observed during growth on rocket surface, which may be considered as less nutritious than BHI broth. Moreover, *stx1* was downregulated and *stx2* was upregulated. Olesen and Jespersen [23] reported that long term adaptation to conditions representing acidity and salinity of 3 EHEC strains did not change significantly the relative transcription levels of *eae*, concurring with the studies of Allen et al. [40] and Bergholz et al. [41]. In the first case acid shock (pH 3.5) had no effect on the transcription levels of *eae* while in the second a model apple juice system was employed to apply the same acid shock. On the contrary, transcription levels of *stx1A* and *stx2A* were affected but in a strain-dependent manner. More accurately, only strain EDL933 exhibited induction of *stx1A* relative transcription after long term salt stress. On the contrary, *stx2A* was upregulated after long term adaptation to salt and acid stresses applied individually in EDL933 strain but downregulated in STEC2 and c196-01 strains [23]. Saitoh et al. [29] reported that Ehx levels increased with the overexpression of GrlA, indicating that GrlA is responsible for *ehxA* regulation. Carey et al. [11] assessed the transcription of *stx1A*, *stx2A* and *eae* during growth on romaine lettuce incubated at 4 and 15°C for up to 9 days in aerobic conditions while Sharma et al. [12] *eae*, *ehxA* and *stx2* during growth on shredded lettuce stored at 4 and 15°C under modified atmospheres. In both studies upregulation was reported as an effect of storage time in both cases and storage under near atmospheric conditions in the latter case. However, direct comparison with the results obtained in the present study is not possible due to the different quantification strategies. Upregulation of *stx1A* was reported by King et al. [42] as a result of exposure to HCl at pH 5.5. Increase of Stx2 production by two *E. coli* O157:H7 strains grown in Luria-Bertani broth supplemented with 2% NaCl was reported by Harris et al. [43]. In the same study addition of 3% NaCl resulted in the decrease of Stx2 production. Downregulation of *stx1A* and *stx2A* on the 7th day of growth on ground beef stored at 4°C was reported by Mahmoudzadeh et al. [13]. Similarly, downregulation of *eae* was reported under sublethal lactoperoxidase stress [44], concurring with the results presented by Huang et al. [45], which demonstrated repression of *eae* and *stx* genes expression through proteomic analysis. On the contrary, upregulation of *eae* was reported by Slanec and Schnidt [46] and Kocharunchitt et al. [47]. In the first case the upregulation was detected after heat treatment of inoculated ground beef at 48°C for 10 min but not after heat treatment at 37°C for 10 min. In the latter study, the upregulation was reported 80 min after an abrupt downshift in water activity obtained by NaCl in BHI broth. Landstorfer et al. [48] assessed *eae*, *stx1A*, *stx1B*, *stx2A* and *stx2B* transcription after growth in a series of different growth conditions. Downregulation of *eae* was reported after growth in ten-fold diluted lysogeny broth at pH 4 or pH 9, with the addition of 200 mg/L sodium nitrite, during growth at 15 or 37°C, on the solidified medium, as well as spinach juice, radish sprouts and cattle feces. Downregulation was also reported regarding *stx2A* after growth in spinach juice and cattle feces. Regarding *stx1A* and *stx1B*, upregulation was detected after growth in lysogeny broth at pH 9.0, the solidified medium, the minimal medium but downregulation on radish sprouts. Finally, *stx2B* was not regulated under any growth condition assessed. Heat shock (47.5°C for 10 min) of *E. coli* O157:H7 in dairy compost resulted in upregulation of *stx1* but downregulation of *stx2* and *eae* [49]. Similarly, transient cold shock in romaine lettuce leaf lysate resulted in upregulation of *stx1* but downregulation of *eae* [50]. However, *eae* was not differentially regulated during preharvest survival on butterhead lettuce [14] concurring with the results presented by Xicohtencatl-Cortez et al. [51] who reported that this gene was not involved in attachment to fresh produce. Again, no direct comparison can take place with the results obtained in the present study due to different quantification strategies. Regarding *etpD* regulation, it has not been previously assessed during growth of *E. coli* O157:H7 in any food-related substrate, at least as far as the authors were concerned.

4. Conclusion

The differential transcription of *E. coli* O157:H7 key virulence genes during growth on rocket surface was successfully assessed for the first time. The effect of growth substrate, temperature and time on the transcription of the genes under study can be summarized into the downregulation of *stx1* and *ehxA* during growth on rocket surface at 4°C, upregulation of *stx2* and *eae* during growth on rocket surface at both 4 and 10°C and the upregulation of *etpD* and *ehxA* during growth on rocket surface at 4 and 10°C, respectively. In addition, increase of incubation temperature, i.e. from 4 to 10°C resulted in upregulation of *stx1* and downregulation of *stx2* and *eae*. Although the above trends were detected, further research is still necessary in order to improve our understanding of the physiology of this foodborne pathogen and the responses during exposure to food-related stimuli.

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