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## Targeted gene expression study of *Salmonella enterica* during biofilm formation on rocket leaves



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### ABSTRACT

In the present study, the ability of *Salmonella* Typhimurium to form biofilm community on rocket leaves and rocket extract at 10 °C and 20 °C was investigated. This goal was achieved with the study of expression of genes associated with biofilm formation and other functional roles. The obtained results showed that *Salmonella* growth was inhibited when cultured in rocket extract (liquid and solid state) and when grew directly to rocket leaves. The observed inhibition might be attributed to nutrient starvation to the specific growth media because of plant leaves's variability, cell physiology and antimicrobial compounds of rocket. In addition, gene expression study using Real-Time PCR showed that biofilm was formatted on solid media, while the entrance and adhesion of the microorganism within the plant held more strongly through the stomata of the plant leaves. Furthermore, genes associated with managing stress situations were overexpressed at 20 °C. From these results, it is indicated that further studies are needed to better determine the survival and/or growth of the pathogen as “real” biofilm cells on plants. In addition, the study on development and gene expression of biofilm cells is necessary in order to eliminate the specific pathogen and reduce the food-borne diseases it causes.

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### 1. Introduction

Vegetables are important components of a healthy and balanced diet, so high quantities are consumed worldwide every day. However, their consumption has been associated with foodborne illnesses. According to European Food Safety Authority (EFSA, 2013), highest priority levels worldwide were defined for leafy green vegetables, consumed as raw salad contaminated with pathogenic *Escherichia coli*, while *Salmonella* on/in leafy greens and tomatoes were also most highly graded. However, it was also reported that of in total 394 bacterial pathogen/FoNAO (food non-animal origin) associations indicated in the literature, 109 have been found with *Salmonella* spp., including multiple serovars (i.e. Agona, Durban, Enteritidis, Gustavia, Montevideo, Newport, Typhi, Typhimurium, Umbilo, and unspecified serovars). More specific, *S. Typhimurium* has been identified in association with honeydew, lettuce, basil, salad, vegetables, and salad vegetables (ECDC, 2008; Isara, Isah, Lofor, & Ojide, 2010; Pui et al., 2011; Sagoo, Little, & Mitchell,

2003a,b; Wetzel, Lee, Lee, & Binkley, 2010). Berger et al. (2010) reported that numerous studies have investigated the potential sources of produce contamination in the supply chain both at the pre-harvest (in the field) and post-harvest stages.

During the pre-harvest phase, pathogen populations can establish themselves on growing crops. The risk can be amplified after harvest either by further direct contamination or by proliferation of existing pathogen populations during processing and post harvest handling procedures. It has been reported that, slicing, peeling or damage of plants releases nutrients and thus, facilitates growth of bacteria (Harris et al., 2003). These nutrients could also remain on industrial surfaces and affect bacterial survival, adhesion and biofilm formation, which further will be an important cross contamination source for leafy salads. Olaimat and Holley (2012) reviewed the studies related with the contamination sources and the pathogens survival or growth on fresh produce. The ability of the pathogens to form biofilm on the surface of leaves of different plants is also well reviewed. Although, several studies have been associated with this phenomenon, a few of them include in their study the microbial interactions with autochthonous biota which facilitate biofilm formation. To our knowledge-limited studies if any, have been conducted to investigate the virulence genes

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expression of pathogens during biofilm development on fresh produce. However, it needs to be considered that several factors have to be checked as infection requires the expression of genes not only coding virulence factors but also physiological processes such as stress response and adaptation. In this direction, the aim of this study was to monitor the differential gene expression of *S. Typhimurium* on rocket leaves *in silico* and *in situ*. In order to achieve this, the ability of this bacterium to develop a biofilm community cultivated as biofilm on rocket leaves or solid rocket extract versus planktonic mode were comparatively studied in the present work. Afterward, the expression of genes associated with biofilm formation, pathogenicity and other functional roles was examined.

## 2. Materials and methods

### 2.1. Bacterial strain and preparation of inoculum

In this experimental design, the strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium CDC 6516-60 (ATCC 14028) was used. Cells from a 18 h culture (37 °C) were harvested by centrifugation (5000 × g, 10 min, at 4 °C), washed twice with ¼ quarter Ringer solution and finally resuspended in 1/4 quarter Ringer solution, in order to be used as inoculum (10–20 cfu/mL or cm<sup>2</sup>).

### 2.2. Rocket extract, leaves and gel cassette preparation

Rocket leaves were purchased from a local supplier and transferred to the laboratory. After washing with running tap water, the leaves were dried, cut and mixed with equal volume of dH<sub>2</sub>O. The mixture was left for 4 h under refrigerate temperature, while a pressure was applied every 30 min for better extraction of the rocket compounds. The rocket extract was obtained by filtration with whatman paper and sterilized (121 °C for 15 min) before further use. In the case of solid medium, 15 g/L agar was added before sterilization. Additionally, rocket leaves were transferred in squared petri dishes (10 × 10 cm) in order to cover the whole surface. Samples prepared with leaves surface or stomata to contact the bottom of petri dish. Furthermore, gel cassette system (10 × 10 cm) was prepared according to Brocklehurst, Mitchell, and Smith (1997) and autoclaved at 121 °C for 15 min. After sterilization, gel cassette system was filled with approximately 35 ml sterile LB agar or rocket agar and let to solidify.

All samples were inoculated with 10–15 *S. Typhimurium* cells per mL (for broth media) or cm<sup>2</sup> (for solid media) and incubated at 10 and 20 °C.

### 2.3. Microbiological analysis

All samples subjected to decimal serial dilutions in ¼ quarter Ringer solution for bacterial enumeration. For sampling of solid media (LB or rocket agar on gel cassette system and leaves), samples removed aseptically from gel cassette system or petri dish and placed in sterile stomacher bag along with 20 mL ¼ quarter Ringer solution. For RNA collection, appropriate volume of different samples was centrifuged (5000 g, 10 min, 4 °C) and washed pellet, resuspended in RNAlater solution and stored at –80 °C until further use. In parallel, the population was enumerated on LB agar after 18–24 h at 37 °C. In the case of leaves, XLD medium (incubation for 24 h at 37 °C) was used to differentiate *Salmonella* population from autochthonous biota, while LB agar enumerated after 24 h and 18–24 h at 30 and 37 °C, respectively.

#### 2.3.1. RNA isolation

The manufacturer recommendations of The PureLink<sup>®</sup> RNA Kit (Ambion) were followed for total RNA extraction. The RNA

extraction procedure was performed in duplicate, both for planktonic- and biofilm-derived samples. Each sample replicate was derived from independent bacterial cultures. RNA samples were stored at –80 °C until further use.

#### 2.3.2. Real-time RT-PCR

The protocol described by the Primescript Reverse Transcriptase (Takara) was applied for cDNA synthesis. The cDNA was stored at –20 °C until further use. Real-time PCR amplification was performed in 96-well plates in a StepOnePlus detection System (Applied Biosystems) using double-stranded DNA intercalating fluorescent agent SYBR green for product detection. Each well contained SYBR green Master Mix (Kapa Biosystems), 200 nM of each primer (Table 1) and 1 µL template. PCR amplification was initiated at 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s and 60 °C for 30 s. Control PCRs were included to detect background contamination (no-template control). PCR specificity and product detection were checked post amplification by examining the dissociation curves of the PCR products. These melting curve profiles were generated by first heating the samples to 95 °C and then cooling them to 60 °C and slowly heating them at 1.8 °C min<sup>-1</sup>–95 °C for detection of SYBR green fluorescence. Melting curve profiles were analyzed using StepOne software 2.1 (Applied Biosystems).

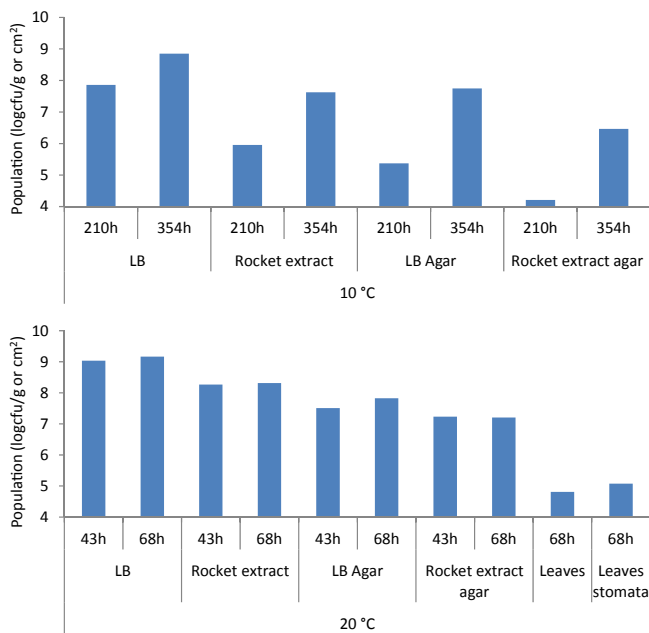
## 3. Results and discussion

The present study aimed to better understanding of biofilm formation of pathogenic bacteria like *Salmonella* on leafy salads. In order to better understand this phenomenon, the differential gene expression of this bacterium cultivated as biofilm on solid laboratory medium, solid plant extract and rocket leaves versus planktonic mode were comparatively studied in the present work. Initially, both type of cells (biofilm and planktonic cultures) were set up at 10 and 20 °C. In order to compare expression of different genes between biofilm and planktonic bacteria, the latter were also grown under exactly the same conditions, while total RNA was successfully isolated from both types of cells. In Fig. 1, *Salmonella* populations under such conditions are presented. From the obtained results, it seems that the growth of the microorganism was inhibited in rocket extract (liquid and solid state) and when grew directly to rocket leaves at both temperatures. Briefly, the final population of *S. Typhimurium* was affected from the rocket extract and rocket leaves as a difference of 1 log cfu/mL and 4 log cfu/cm<sup>2</sup> was observed regarding the laboratory medium. This observed inhibition might be attributed to nutrient starvation to the specific growth media because of plant leaves's variability, cell physiology and antimicrobial compounds of rocket leaves. More accurately, early in Abdou, Abou-Zeid, El Sherbeeney, & Abou-El-Gheat, 1972 reported the antimicrobial effect of rocket extract and seed oil on *E. coli*, *S. typhi* and *Bacillus subtilis*. Later, a similar effect of rocket seeds on *E. coli* was observed (Gulfraz et al., 2011). This antimicrobial effect has been attributed to high concentrations of glucosinolates (GSLs) (Adarsh, Geetanjali, Tarunpreet, & Saroj, 2009; Tiendink et al., 1991).

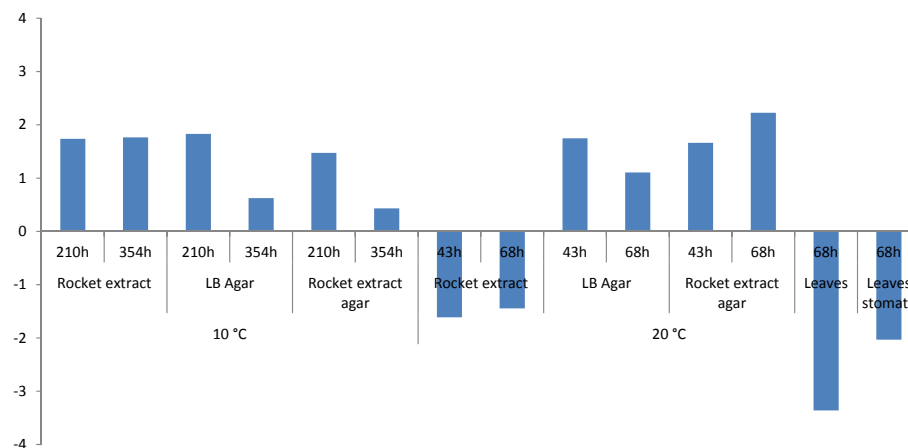
Regarding the gene expression study of the present work was based on 7 different genes (Table 1), which were selected based on previous knowledge on their putative involvement in stress related mechanisms and colonization phenomena. Regarding these seven studied genes i.e. starvation, stress response, adaptation to nutrient deficiency, attachment, pathogenicity and quorum sensing, the differential expression was more associated with the planktonic and biofilm grown cells. In Figs. 2–8, the relative expression levels of these genes are presented. Additionally, it needs to be noted that the reference samples were planktonic cultures on LB broth at 10

**Table 1**  
Studied genes and primer synthesis.

Gene	Function	Primer sequence F	Primer sequence R	Reference
dps	DNA starvation/stationary phase protection protein Dps	ATGCGCGGTGCTAACTTTAT	AAGTATCCTGCACGTTATGG	In this study
dppA	Dipeptide transport system substrate-binding protein	TTGACCGTCTGGTCTTCTCC	GACATCATCCAGCGGTTTTT	In this study
rpoH	Starvation	CGTTAAAGTTGCAACCACGA	CCATCTCACGCACGTCTTTA	In this study
sspH2	Pathogenicity	GCACAACCTGGCTGAAGATGA	CGTATTGCCCTTTTCTGCG	In this study
sdiA	Cell division control, quorum sensing	ACGCGCAATGTTGTTACGC	ACCCACGCCGAGGATAAGT	In this study
csgB	Attachment	AGTGCCAGAGTACGCCAGGA	ACCGTAAGCGCTTTCGATA	In this study
osmY	Osmotic stress	GTCACCCCTGAGCGGCTTTGT	CTTCACTGGTCGTGGCCGTA	In this study
16s		GATCATGGCTCAGATTGAACGCTGGCGG	CACCGCTACACCTGGAATTCTACCCCCCTC	Eswarappa, Janice, Balasundaram, Dixit, & Chakravorty, (2009)
rpoD		GTTGACCCGGGAAGCGGAAA	CAGAACCACGCTGAGTTGGC	Quiroz et al. (2011)
rrsG		GTTACCCGAGAAGAAGCAC	CACATCCGACTTGACAGACC	Zheng et al. (2011)



**Fig. 1.** Population of *Salmonella Typhimurium* cultured on different media at 10 and 20 °C.

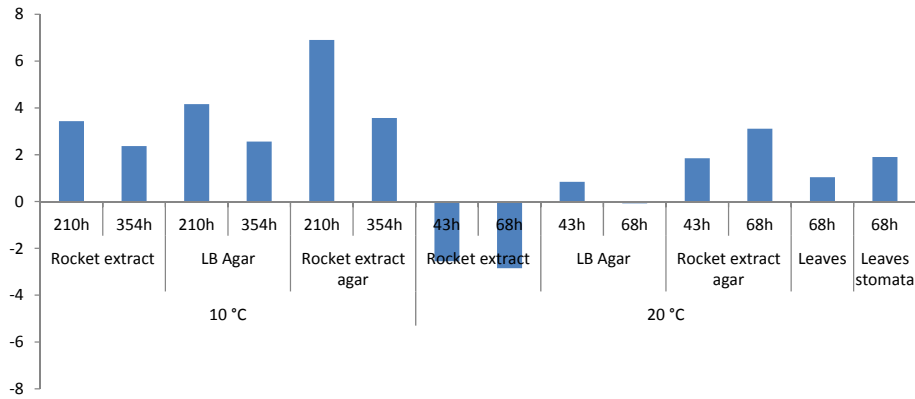


**Fig. 2.** Relative expression level of *dps* gene of *Salmonella Typhimurium* cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while *rrsG* gene was used as housekeeping gene.

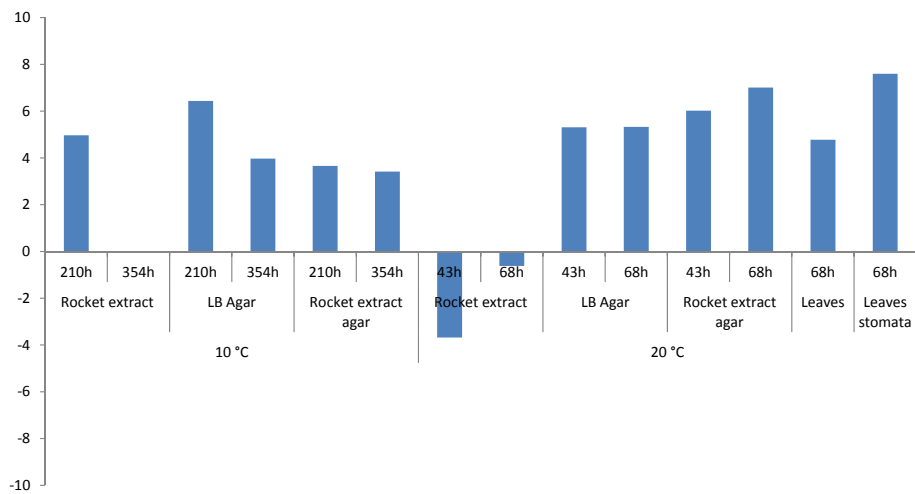
and 20 °C, while *rrsG* gene was chosen from the three tested (16S, *rrsG* and *rpoD*) as the reference housekeeping gene.

From these results, it was revealed that *dps* gene associated with DNA protection was up-regulated in all studied cases at 10 °C, but not at 20 °C. More specific, the expression of this gene was found to be affected from incubation temperature during growth on rocket extract, as up- and down-regulation was observed at 10 and 20 °C, respectively. On the other hand, up-regulation of *dps* gene was observed during growth on solid media (LB agar and rocket extract agar). The opposite results were observed during growth of the pathogen on rocket leaves, while *dps* gene expression was found to be also affected when the pathogen was inoculated on stomata. In upregulation of *dps* gene was been reported previously for *S. Enteritidis* (Doulgeraki et al., (unpublished data); Giaouris, Samoilis, Chorianopoulos, Ercolini, & Nychas, 2013) and for *S. Typhimurium* (Hamilton et al., 2009).

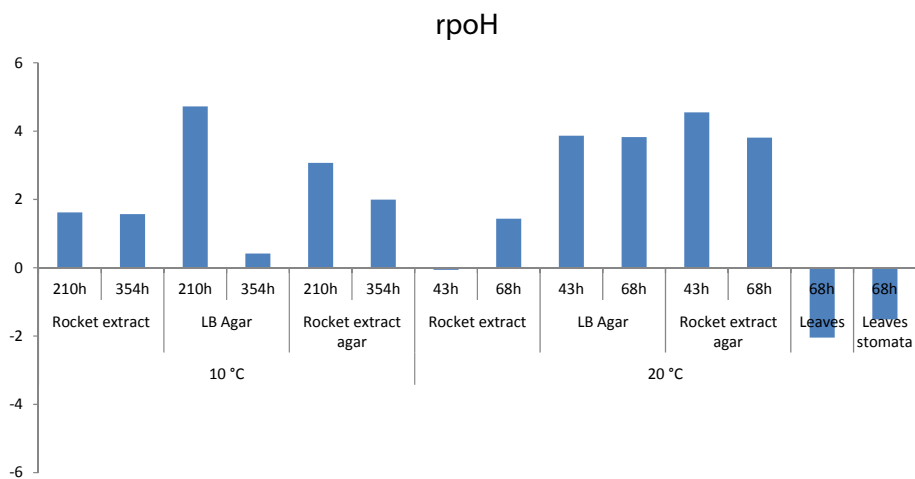
The periplasmic dipeptide-binding protein DppA was over-expressed in all cases but one. In the later case *Salmonella* was grown on rocket extract at 20 °C. The expression of this gene has been associated previously with biofilm growth of *S. Enteritidis* (Giaouris et al., 2013) and *S. Typhimurium* (Hamilton et al., 2009). It is worth noted that, in most of the cases a decreased expression level of this gene was accrued during the incubation time, while the inoculation of the leaves from stomata side resulted in higher expression of *dppA* gene. Similar results were observed in the case



**Fig. 3.** Relative expression level of dppA gene of *Salmonella* Typhimurium cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while rrsG gene was used as housekeeping gene.



**Fig. 4.** Relative expression level of csgB gene of *Salmonella* Typhimurium cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while rrsG gene was used as housekeeping gene.

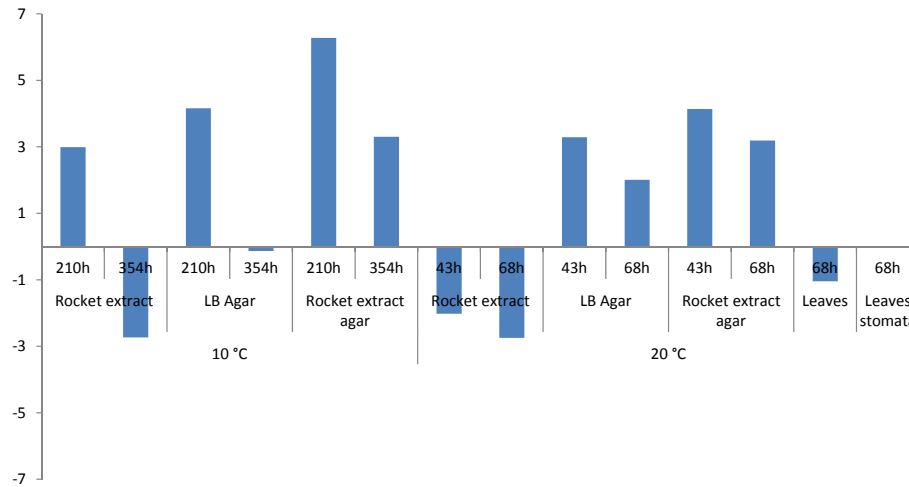


**Fig. 5.** Relative expression level of rpoH gene of *Salmonella* Typhimurium cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while rrsG gene was used as housekeeping gene.

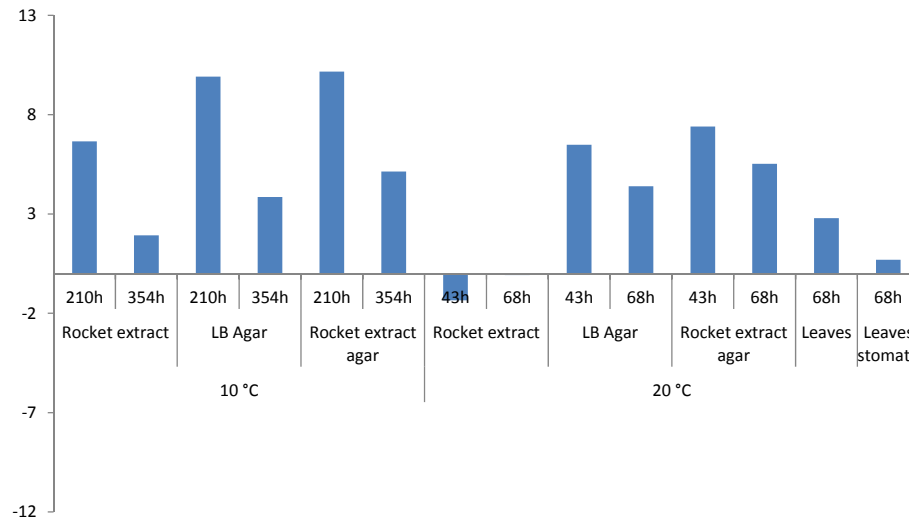
of csgB gene, which is considered to be a regulator for BapA expression in *Salmonella* because a special binding motif, which is very similar to the CsgD-binding sequence in the adrA promoter, is present in the bapA promoter. Additionally, BapA was shown to be

required for pellicle formation at the air–liquid interface and its expression was demonstrated to be coordinated with that of genes encoding curli and cellulose via CsgD (Latasa et al., 2005).

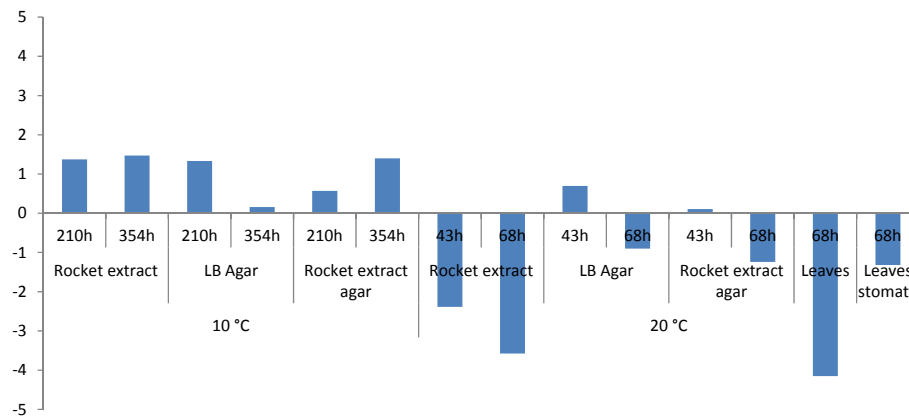
The rpoH gene which encodes the RNA polymerase sigma factor



**Fig. 6.** Relative expression level of *sdiA* gene of *Salmonella* Typhimurium cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while *rrsG* gene was used as housekeeping gene.



**Fig. 7.** Relative expression level of *sspH2* gene of *Salmonella* Typhimurium cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while *rrsG* gene was used as housekeeping gene.



**Fig. 8.** Relative expression level of *osmY* gene of *Salmonella* Typhimurium cultured on different media at 10 and 20 °C. 210 h and 42 h cultures on LB broth at 10 and 20 °C, respectively, was used as reference, while *rrsG* gene was used as housekeeping gene.

H is known to play significant roles in stress responses. In a previous study, rpoH expression has been associated with biofilm growth (Hamilton et al., 2009). In the present study, this gene was down-regulated during growth of *Salmonella* on leaves, while a decreased expression related to the incubation time was observed in the case of solid media. However, a different trend was occurred in rocket extract broth, where expression of rpoH was increased during growth of *Salmonella* at 20 °C.

The expression of sdiA gene was also examined in this study (Fig. 6). This single analog protein, which is found in pathogenic bacteria of the intestine, is only known to activate genes that would help a single bacterium to adhere to host tissues, presumably because the presence of AHL-producing bacteria simply indicates that a particular host environment has been reached (Ahmer, 2004; Yao et al., 2006). In the present study, a decreased expression of sdiA gene related to the incubation time and higher temperature was observed in the case of solid media. Additionally, this gene was down-regulated in the case of rocket extract broth at 20 °C and 354 h at 10 °C. In the case of rocket leaves down-regulation of the gene or no expression of the gene has observed, suggesting that the expression of sdiA gene is more obvious *in vitro*. In a previous study, it was also reported that the SdiA system of *Salmonella* is most active *in vitro* during growth in motility agar at 37 °C (Ahmer, 2004). Additionally in accordance with this study, it was determined that *S. Typhimurium* does not respond well to AHLs in liquid LB medium but responds best in LB semisolid agar (0.3, 0.75, or 1.5% agar) (Michael, Smith, Swift, Heffron, & Ahmer, 2001). In the same work, they concluded that, in liquid cultures sdiA-dependent responses were 3- to 5-fold, while on semisolid agar the responses reached 14- to 17-fold. It seems that temperature also affects the sdiA regulon, as it was also mentioned previously (Smith & Ahmer, 2003).

It has been previously hypothesized that quorum sensing may be used by pathogenic species to prevent the expression of virulence factors until the population of bacteria has increased to a point at which victory is ensured (Michael et al., 2001). However, Ahmer (2004) reported that sdiA does not regulate either of the virulence-associated type III secretion systems of *Salmonella*. In this study, the pathogenicity was determined by focusing on the sspH2 effector protein, induced by SPI-2 (Fig. 7). Regarding the observed results similar trends were observed regarding the expression of sspH2 gene and sdiA gene in most of the cases, but leaves. In the later case, sspH2 was up-regulated while sdiA was down regulated.

Furthermore, the RpoS-regulated osmotically inducible gene, osmY, which has been found to be induced by both osmotic and growth phase signals (Yim & Villarejo, 1992), found to be affected from the incubation temperature in the present work. Briefly, this gene was up-regulated and down-regulated at 10 and 20 °C. This gene was found previously to up-regulated during biofilm growth of *S. enterica* (Giaouris et al., 2013; Hamilton et al., 2009).

The findings of the present study could show that *S. Typhimurium* reacts as exposed to different types of stress when inoculated to a heat sterile plant extract at lower temperature. However further studies are needed to better determine the survival and/or growth of the pathogen as “real” biofilm cells on plant leaves. In addition, the study on development and gene expression of biofilm cells is necessary in order to eliminate the specific pathogen and reduce the food-borne diseases it causes.

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