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Global transcriptomic response of *Listeria monocytogenes* during growth on cantaloupe slices

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ABSTRACT

Understanding a pathogen's response to food environments is imperative to develop effective control strategies as well as to elucidate the impact of foods on virulence potential. The purpose of this study was to assess transcriptional response of *Listeria monocytogenes* after growth in cantaloupe, as well as its impact on survival in synthetic gastric fluid (SGF). The transcriptional profiles of *L. monocytogenes* grown in cantaloupe or Brain Heart Infusion (BHI) under refrigeration were compared by a custom-designed microarray. A total of 286 and 175 genes were significantly up- and down-regulated, respectively, in *L. monocytogenes* grown in cantaloupe as compared to BHI (fold change ≥ 2.5 and adj. $P < 0.05$). The majority of upregulated genes belonged to functions related to amino acid and nucleotide metabolism, flagellar biosynthesis, and iron acquisition, while most downregulated genes belonged to carbohydrate metabolism. Notably, the branched chain amino acid (BCAA: leucine, isoleucine, valine) biosynthesis operon was shown to be highly upregulated as well as the purine and pyrimidine biosynthesis pathways. Transcript levels of several stress- and virulence-related genes were significantly altered, implying an impact of growth in cantaloupe on the virulence potential of *L. monocytogenes*. Enhanced survival of *L. monocytogenes* in SGF following growth in cantaloupe further demonstrated the impact of cantaloupe-associated growth on the pathogen's subsequent response to a host relevant stress.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that can cause a rare but severe disease (invasive listeriosis) in susceptible populations such as the elderly, pregnant women, and those with a compromised immune system (Vazquez-Boland et al., 2001). Preventing the contamination of ready-to-eat (RTE) foods by *L. monocytogenes* is an important public health goal due to the high mortality and hospitalization rate associated with listeriosis (Scallan et al., 2011). Despite efforts to reduce the contamination of RTE foods, the widespread presence of *L. monocytogenes* in the natural environment (Sauders et al., 2012; Strawn et al., 2013; Chapin et al., 2014) and its well-documented persistence in food processing plants and retail delicatessens (Tompkin, 2002; Carpentier and Cerf, 2011; Ferreira et al., 2014; Leong et al., 2014) underscore the difficulty associated with complete eradication of this pathogen in all stages of food production.

A number of studies have indicated that the association of *L. monocytogenes* in specific food environments can have a profound impact on its gene expression patterns, including virulence gene expression (Liu and Ream, 2008; Mujahid et al., 2008; Rantsiou et al., 2012; Larsen and Jespersen, 2015; Tang et al., 2015; Hadjilouka et al., 2016), and may enhance the pathogen's ability to survive host-relevant defense mechanisms (Wonderling and Bayles, 2004; Peterson et al., 2007; Barmpalia-Davis et al., 2008) and/or its potential cellular invasion capability (Lin et al., 2010). In-depth evaluation of the pathogen's transcriptional responses during adaptation in foods or to food-relevant stresses has also elucidated the mechanisms of survival and growth (Bae et al., 2011; Stasiewicz et al., 2011; Tang et al., 2015), which have subsequently identified potentially promising strategies for development of effective control measures.

Most of the studies on the effect of foods on *L. monocytogenes* transcriptional response have hitherto focused on animal source foods (e.g., deli meats, dairy, and smoked seafood). However, recent listeriosis outbreaks involving fresh produce commodities (e.g., cantaloupe, caramel apples, stone fruit, mung bean sprouts, and leafy greens) highlight that raw agricultural products are also important food

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vehicles for listeriosis (Garner and Kathariou, 2016). Among these outbreaks, a listeriosis outbreak linked to whole cantaloupe in 2011 represents one of the deadliest foodborne disease outbreaks in the United States, causing 147 illnesses and 33 deaths. Although sanitizing options have been suggested to eliminate pathogenic bacteria from the surface of the cantaloupe, cross-contamination of the edible flesh can occur during preparation of fresh-cut cantaloupe slices, after which *L. monocytogenes* levels can increase during subsequent refrigerated storage (Ukuku et al., 2015, 2016; Martinez et al., 2016; Shearer et al., 2016). Additionally, recent studies suggest the ability of *L. monocytogenes* to penetrate the rind of an intact cantaloupe (Macarisin et al., 2017).

In light of an increasing trend in outbreaks and recalls involving fresh produce commodities, it is of pertinent interest to assess the adaptive strategies used by the pathogen for survival and growth in fresh produce, as well as any associated alterations in virulence gene expression during pathogen growth. Thus, the objective of the current study was to assess the transcriptomic response of *L. monocytogenes* after growth in cantaloupe slices at 10 °C, as well as determine the effect of cantaloupe-associated growth on the survival of *L. monocytogenes* in a synthetic gastric fluid challenge. Such information has the potential to identify suitable metabolic targets for effective *L. monocytogenes* control strategies and facilitate improved risk assessment.

2. Materials and methods

2.1. L. monocytogenes inoculum preparation

L. monocytogenes strain LS670 (serotype 1/2b; CFSAN designation 713431-6B-B) used in this study was originally isolated from a farm cantaloupe linked to the 2011 whole cantaloupe listeriosis outbreak (Laksanalamai et al., 2012b). Prior to each experiment, *L. monocytogenes* LS670 was streaked on Brain Heart Infusion (BHI) agar from a −80 °C stock, followed by incubation at 37 °C for 24 h. A single colony was used to inoculate 5 ml BHI broth (in 16 mm tubes) and incubated at 37 °C overnight (16 h) with shaking (175 rpm). This culture was further diluted (1:100) in 5 ml BHI broth and incubated at 10 °C without shaking and allowed to grow to OD 0.1 (~1–2x10^8 CFU/ml), which took approximately 27–28 h.

2.2. Growth of L. monocytogenes in BHI broth and cantaloupe slices

Whole cantaloupes were purchased from a local store on different days. Cantaloupes were selected for equivalent ripeness based on firmness. Whole cantaloupes were cut into crescent shape portions with a sterile knife, followed by the separation of the flesh and the rind. The cantaloupe flesh was cut into 5 ± 0.5g slices using a sterile knife. A *L. monocytogenes* culture grown at 10 °C, to an OD of 0.1 (See section 2.1), was serially diluted in 0.85% saline solution to obtain a cell concentration of approximately 5 × 10^6 CFU/ml. Cantaloupe slices were subsequently spot-inoculated with 20 µl/slice (10 µl per side) of the prepared inoculum culture and transferred into 50 ml Falcon tubes (Thermo Fisher Scientific, Waltham, MA). Similarly, 50 ml Falcon tubes containing 5 ml BHI broth were inoculated with 20 µl of the same inoculum culture. BHI and cantaloupe samples were stored at 10 °C and enumerated daily. All cantaloupe slices were stomached in 5 ml of 0.85% NaCl for 1 min to homogenize the samples. The *L. monocytogenes* population density in BHI and cantaloupe samples was determined by diluting samples in 0.85% NaCl and plating on BHI agar and RAPID' *L. mono* (RLM; Bio-Rad, Hercules, CA). Both BHI and RLM plates were incubated at 37 °C for 24 h prior to counting colonies. The *L. monocytogenes* population density was determined from a total of three biological replicates.

2.3. RNA isolation and quality checks

Cantaloupe and BHI broth, inoculated and incubated as described in section 2.2, were processed for RNA isolation to examine transcription. Parallel samples were enumerated to verify inoculum level, and counts at day 5 were also determined to verify standard growth. Separately, uninoculated samples were incubated in parallel for RNA isolation, pH evaluation and to control for contamination (data not shown). After 5 days of incubation at 10 °C, *L. monocytogenes* cells in BHI samples were stabilized with 5 ml of RNA Protect (Qiagen, Valencia, CA) whereas cells in four slices of cantaloupe were sequentially stabilized in 10 ml of RNA Protect, accompanied by vortexing between each slice. Stabilized BHI cultures were transferred to 15 ml Falcon tubes and left at room temperature for 10 min. The liquid suspensions containing stabilized *L. monocytogenes* cells from cantaloupe were transferred to 15 ml Falcon tubes and centrifuged at 150 x g for 10 min at 4 °C to remove cantaloupe debris. The supernatants were transferred to clean 15 ml Falcon tubes. Supernatants from the cantaloupe samples and stabilized BHI cultures were centrifuged at 4400 x g for 15 min at 4 °C. *L. monocytogenes* pellets were immediately processed for RNA isolation or stored at −80 °C for future processing. RNA was also isolated from cantaloupe uninoculated with *L. monocytogenes* and processed to generate cDNA to evaluate possible cross reactivity with the *Listeria* microarray and exclude any probe sets on the microarray with cross-reactivity to cantaloupe sequences.

For RNA isolation, pellets containing *L. monocytogenes* cells were resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0; Sigma-Aldrich, St. Louis, MO) containing 50 mg/ml lysozyme (AMRESCO, Solon, OH) and 10 µl of proteinase K (Qiagen Cat No./ID: 19131). The cell suspensions in TE were incubated at room temperature for 30 min. Following incubation, 2% (w/v) polyvinylpyrrolidone (Sigma-Aldrich) was added to each sample to sequester phenolic compounds and reduce interference with nucleic acid isolation procedures (Tong et al., 2012). Total bacterial RNA was isolated using the RiboPure-Bacteria RNA purification kit (Thermo Fisher Scientific), beginning with the addition of 350 µl RNAwiz to each sample. The bead beating step, using a vortex mixer, was extended to 30 min as preliminary trials with 10 min resulted in insufficient RNA yields. Extracted RNA samples were DNase-treated according to the manufacturer's instructions to remove genomic DNA contamination. DNase-treated RNA samples were subsequently quantified with a Nanodrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the integrity of RNA was assessed by the 2100 Bioanalyzer (Agilent, Santa Clara, CA). RIN scores ranged from 7.4 to 9.9. Genomic DNA contamination was assessed via real-time qPCR (qPCR) (iScript™ One-Step RT-PCR kit with SYBR Green; Bio-Rad) using *rpoB* as the target (Sue et al., 2003). Reactions were performed with and without reverse transcriptase with the latter used to verify the absence of gDNA and the former to verify equivalent proportions of *L. monocytogenes* RNA in each sample (data not shown). Specifically, the RT-PCR with reverse transcriptase would evaluate the potential for carryover cantaloupe RNA to boost RNA concentration measurements independent of *L. monocytogenes* RNA processed for microarray analysis. Equivalent Ct values for *rpoB* in both BHI and cantaloupe RNA preparations indicated equivalent *L. monocytogenes* RNA in these samples (data not shown). Each reaction contained 25 µl of 2X SYBR Green mix, 1.5 µl of the *rpoB* forward and reverse primers (10 µM), 1 µl reverse transcriptase for those reactions including reverse transcriptase, 1 µl RNA sample (adjusted to 10 ng/µl), and nuclease free H_2O in a 50 µl reaction. qPCR was performed in the CFX96™ Real-Time PCR detection system (Bio-Rad) using the recommended cycling parameters. RNA was isolated in triplicate over four trials (except one trial with duplicates) for a total of 11 RNA samples per growth condition.

Table 1
Real-time PCR primers used for the validation of microarray data.

Probe set ID	Target gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
LMOf2365_2006_at	*ilvD*	TGTCACAGTTTGGGATGTAGAA	CCTTCCCTCTCGTGAAGTTATC
LMOf2365_2014_s_at	*ilvA*	GTAATCGGGCTCTTTGCTTTG	GCTGTGGAGATGGTGAAAGA
LMOf2365_1790_at	*purH*	CCGTGCCAGGTTCTGTATAA	ACGAACATCACTGGAGAAACT
LMOf2365_1797_x_at	*purC*	CACATTGCGAACAACCACTTC	CATTTAGCAGGAGCGGGAATAG
LMOf2365_1860_at	*pyrF*	GGAAGGTCTGGAAATAGGTTCTT	GCATGTCTGTCTCACTTGTACT
LMOf2365_1864_at	*carA*	CGAGTGGTGCTTGTTGATTATG	CTTCGGCTGTTGTGTTGTAAG
LMOf2365_1989_s_at	lmof2365_1989	GTGCTAAAGACGGCGAGAAA	CGTGTCAAAGTCCATTTGGTAAAC
lmo4a_2165_s_at	*feoB*	ACGAGCAACTACTGGCAAA	CCTGTTGTGATGGGACTGAATA
LMOf2365_0732_at	*flgD*	GCCGTTTGTCGTTTGTTGAA	CGCGGTTAATTTCATCGGTAAAG
LMOf2365_2305_at	lmof2365_2305	CAAGGTTGGGATACAAGGAAGA	ATCACAGGTGCGGTAGTAATG
LMOf2365_1441_at	*bilEB*	TGTGAAGCCGAATATGGGTAAA	GTCTCTAGCACTGTTCCTGTAAAT
LMOf2365_2214_x_at	*srtB*	CTGTGTATGAAACGACGACTGA	TTCGGTCCTTTCCACTAACTTT
LMOSLCC7179_2340_s_at	*hupC*	CTCCGCCTGATAACGAATGTAG	CGGTCATTCACTGGGCTATT
lmo4a_1030_s_at	*gbuA*	TCGTGAGCTGGTTGGTATTG	TTTCTGCAAGTGGCGTATCT
LMOf2365_1150_at	*pduT*	GGCAAATATCGTCGGGAATTTAG	CACCAACTGCATTCATCTCATC
LMOf2365_1155_x_at	*cobS*	GCCCTTACCAGTTGCGATTA	TTTCCAGCATACGCTCTCTTT
LMOf2365_0211_s_at	*prfA*	CCTGACCTATGTGTACGGTAAAG	AGCTGAGCTATGTGCGATAC
LMOSLCC2755_0440_s_at	*inlB*	CCATTTCGGGCTTCTCTATCAA	CGGGCATCTACAAACTTCCA
BN419_2225_s_at	*lpeA*	TGCTGCGGATGCTGATTTA	GCTCCTTGTCGTCTCTTGATT
LMOf2365_2334_x_at	*gadB*	GGCTCTAGTGAAGCGTGTATG	CCAGAGGAGATAACGAGGTTTG
LMOf2365_2744_at	*topB*	CGCTTTATCCGTTACAGAGGAG	GCAGGTAGAGAAGGAGAACTTG

2.4. Random priming and cDNA synthesis

Approximately 2.5 μg of RNA was mixed with random hexamers (27 ng/μl final concentration; Thermo Fisher Scientific) in 30 μl, followed by incubation at 70 °C for 10 min, 25 °C for 10 min, and a final hold on ice. The RNA/primer mix was reverse transcribed with the SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific) in the presence of 1X First-Strand buffer, 10 mM DTT, 0.5 mM dNTPs, 0.5U/μl RNasIn (Promega, Madison, WI), and 10U/μl SuperScript III. The assembled reactions were incubated at 25 °C for 10 min, 42 °C for 60 min, 50 °C for 60 min, and 70 °C for 10 min. cDNA was subjected to a RNA degradation step by adding 20 μl 1N NaOH and incubating at 65 °C for 15 min, followed by neutralization by the addition of 20 μl 1N HCl. cDNA was purified using the MinElute PCR purification kit (Qiagen) and quantified by a Nanodrop UV spectrophotometer.

2.5. Listeria microarray hybridization

The *Listeria* microarray (*Listeria* Gene chip) is a custom designed Affymetrix chip (Santa Clara, CA) for use as an expression or genotyping array. The custom array consisted of 57 *L. monocytogenes* sequences (both closed and shotgun) from GenBank including serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4bV, 4c, 4d, 4e, and seven *Listeria* species sequences including *L. fleischmannii* (n = 3), *L. grayi* (n = 2), *L. innocua* (n = 4), *L. ivanovii* (n = 3), *L. marthii* (n = 1), *L. rocourtiae* (n = 1), *L. seeligeri* (n = 3), and *L. welshimeri* (n = 1). The array contains 22,137 probe sets representing 18,726 annotated *Listeria* genes and 3412 intergenic regions from *L. monocytogenes*. Identical or nearly identical alleles of a gene from different genomes were represented with one probe set while each divergent allele was represented by an additional probe set. Each probe set contains 11 perfect match (PM) 25-mer oligonucleotides and 11 mismatch probes (MM) containing a single, central nucleotide mismatch compared to the reference.

cDNA fragmentation, labeling, hybridization, and array washing and scanning were performed as previously described (Patel et al., 2016) with minor modifications. Total cDNA was fragmented in the presence of 0.05 unit of RQ1 RNase-free DNaseI (Promega) at 37 °C for 10 min, followed by DNase inactivation at 99 °C for 15 min. The fragmented cDNA was labeled at the 3' end by the incorporation of biotin-11-ddATP (PerkinElmer, Akron, OH) using 30 units of terminal deoxynucleotidyl transferase (Promega), followed by incubation at 37 °C for 4 h. Labeled cDNA was mixed with the hybridization mix (Affymetrix, Santa Clara, CA) containing 45 μl of Hyb Mix A, 75 μl of Hyb Mix B, and 2.5 μl of Control Oligo B2. The hybridization mix with cDNA was incubated at 96 °C for 10 min and held at 45 °C for 2 min. Each cDNA/hybridization mix (120 μl) was then aliquoted into corresponding wells (4 wells total) of the GeneAtlas hybridization tray, and the prewarmed (45 °C) *Listeria* GeneAtlas array strip (Affymetrix) was fitted into the hybridization tray, which was incubated at 45 °C for 17 h. Array strips were washed and scanned using the GeneAtlas instrument control software.

2.6. Microarray data analysis

Raw data (Affymetrix CEL files) containing the probe set intensity data were preprocessed using the Affy package in R (Gautier et al., 2004). The background correction and normalization were performed using the RMA method of probe level data summarization. Prior to assessing differential gene expression in cantaloupe, probe sets that did not appear to be expressed in any experimental conditions were filtered using the MAS5.0 algorithm with a tau setting of 0.2 (Laksanalamai et al., 2012a). Differentially expressed genes (DEGs) analysis was carried out by the implementation of the LIMMA package in R (Smyth et al., 2005). The final DEGs were summarized to genes showing ≥2.5-fold changes in transcription with adjusted $P < 0.05$. All microarray data was uploaded to NCBI (PRJNA396878; GEO: GSE102153).

For functional categorization of up- and down-regulated genes, the corresponding amino acid sequences were retrieved from NCBI and submitted to the KEGG Orthology and Links Annotation (KOALA) tool, which is KEGG's internal annotation tool (Kanehisa, 2016). Similarly, the pathway mapping tool of KEGG was utilized for a comprehensive visualization of affected biological pathways. Generally Applicable Gene-set/Pathway Analysis (GAGE; [Luo et al., 2009]) was applied to the DEGs to gain further support of biologically interesting pathways identified through DEG analysis and KEGG mapping. To assess the alterations in virulence gene expression, amino acid sequences of virulence-related genes (Toledo-Arana et al., 2009) were queried using BLAST against the amino acid sequences of the entire coding sequences harbored on the genome of *L. monocytogenes* strain F2365 (Nelson et al., 2004).

2.7. Microarray data validation by quantitative real-time PCR (qRT-PCR)

The transcription levels of a subset of DEGs were analyzed by qRT-

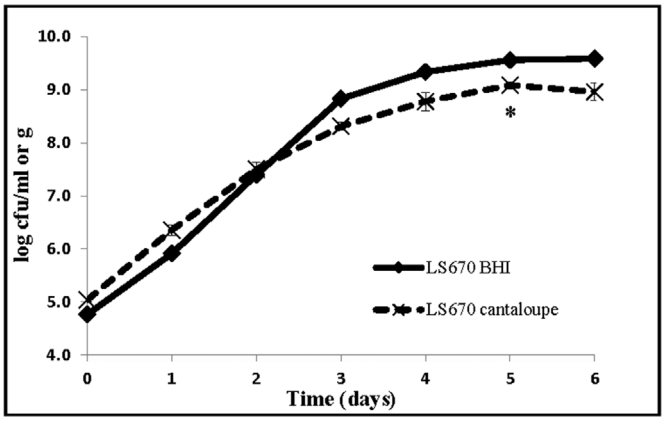

Fig. 1. Growth of *L. monocytogenes* LS670 in BHI and fresh-cut cantaloupe slices at 10 °C. Error bars represent the standard deviation of the mean. The asterisk indicates the time point for RNA isolation for *L. monocytogenes* grown in BHI and cantaloupe slices.

PCR for technical validation of the microarray data. PCR primers were designed to generate approximately 100 bp amplicons (Table 1) using the PrimerQuest Tool available at the Integrated DNA Technologies (IDT) website (IDT; http://www.idtdna.com/Primerquest/Home/Index). Primer quality checks (self-dimer, hetero-dimer, Tm) were also carried out with the OligoAnalyzer 3.1 tool provided by IDT. Three biological replicate cDNA samples used for microarray hybridization were used as the templates. The reaction mixture was assembled in a 20 µl volume containing iTaq Universal SYBR Green Supermix (Bio-Rad), forward and reverse primers (750 nM each), and cDNA template (40 ng). The template denaturation was at 95 °C for 2 min followed by the target amplification with 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative expression of target genes in cantaloupe samples in reference to BHI samples was determined by the Pfaffl method (Pfaffl, 2001), which calculates the relative expression from the PCR-derived Ct values as well as the real-time PCR efficiencies. *L. monocytogenes topB* (encoding DNA topoisomerase III) was selected as the reference gene due to its constant expression levels across all the microarray data.

2.8. Exposure to synthetic gastric fluid

BHI and cantaloupe, spiked as noted in section 2.2, were collected on the same day as inoculation, referred to subsequently as day 0, and after five days of growth at 10 °C, referred to subsequently as day 5, to assess survival of *L. monocytogenes* in synthetic gastric fluid (SGF), prepared as previously described (Peterson et al., 2008) at pH 2.5. Due to carryover activity of SGF, it was necessary to either dilute aliquots containing SGF 1:100 or wash the cells via centrifugation if cell numbers precluded dilution prior to plating. The former method was chosen whenever possible as exposure times were more tightly controlled. To evaluate survival of the BHI day 0 sample, the initial, undiluted inoculum was added at a 1:50 dilution to either sterile saline or SGF in triplicate. For BHI day 5 cultures, the same procedure was used though with parallel, independent cultures used for the three replicates. In the case of the spiked cantaloupes, on day 0 and day 5, a 5g sample was manually massaged in 10 mL of sterile saline or sterile SGF. To assess whether cantaloupe provided protection (e.g., buffering capacity or matrix protection) against the effect of SGF, 5 mL of this suspension was removed to a separate tube and sampled for bacterial survival independently at each timepoint. This separate sample is referred to as "cantaloupe removed" to distinguish it from the remainder of the sample that still retained the cantaloupe section, referred to as "cantaloupe retained" for ease of reference. Both the "cantaloupe removed" and "cantaloupe retained" treatments were sampled at the same timepoints, using the same sampling and serial dilution scheme. For all the above treatments, aliquots were collected at 0 and 15 min, serially diluted as required, and plated on RLM agar (Bio-Rad) to determine survival. For samples with low recoverable counts that required direct plating of the bacterial suspension without dilution, cells were washed with brief centrifugation (1 min, 15,000 RPM), followed by removal of the supernatant. The pellets were then resuspended in an equal volume of sterile saline. The starting cell density was determined using the average cfu/ml recovered at the 0 min timepoint of the saline treatment for each sample set (e.g. BHI, cantaloupe removed and cantaloupe retained). Counts from each of the replicates were averaged and the percent survival was determined using the average of the cell density from the 0 min saline treatment as the input value. Each trial had three replicates per condition and trials were performed twice independently. Statistical analyses were performed using GraphPad InStat (La Jolla, CA). An ANOVA was performed with a Tukey-Kramer Multiple Comparisons post test to determine significance.

3. Results

3.1. Growth of L. monocytogenes in BHI and cantaloupe slices

Prior to the inoculation of cantaloupe slices for RNA isolation, growth curves of *L. monocytogenes* grown in BHI and cantaloupe were generated to determine the growth pattern over time at 10 °C. Our data indicated a rapid growth of *L. monocytogenes* in cantaloupe slices, which was comparable to growth in BHI (Fig. 1; $p > 0.05$, Mann-Whitney *U* test). After 5 days of growth at 10 °C, *L. monocytogenes* reached the stationary phase in both BHI and cantaloupe slices, with a population density of ca. 9.5 log CFU/ml and 9.0 log CFU/g, respectively. In subsequent experiments, *L. monocytogenes* cells grown for 5 days, representing stationary phase in both BHI and cantaloupe slices, were harvested for RNA isolation, which yielded enough RNA for microarray analysis. Attempts were made to isolate RNA at earlier timepoints; however, yield was insufficient for microarray analysis. RNA isolation in comparable growth phases allowed differences in transcriptomic profiles to be attributed to specific growth environments, and not a result of growth phase-dependent effects (Sue et al., 2003).

3.2. Global transcriptomic response of L. monocytogenes in cantaloupe and validation of microarray data

While similar growth patterns were observed for *L. monocytogenes* grown in BHI and cantaloupe slices, we detected significant alterations in transcriptomic profiles of *L. monocytogenes*, indicating growth matrix-specific transcriptional responses. Equivalent growth for samples processed for RNA isolation was determined via plate counts at day 0 and day 5 (data not shown). Although variations in the transcriptome were observed between different batches of cantaloupe, the overall transcriptome data clustered into two distinct groups that are consistent with the growth matrices (Fig. 2). When comparing genes that are differentially expressed, a total of 286 and 175 genes were identified as being significantly up- and down-regulated (Tables S1 and S2), respectively, in cantaloupe slices as compared to BHI (≥2.5-fold change and adj. $P < 0.05$). The majority of upregulated genes belonged to functional categories of environmental information processing (e.g., ABC transporters and phosphotransferase system), amino acid metabolism, genetic information processing (e.g., transcription and translation), and nucleotide metabolism (e.g., purine and pyrimidine metabolism), whereas the majority of downregulated genes belonged to functional categories of carbohydrate metabolism (e.g., pentose phosphate pathway) (Fig. 3). A subset of 20 differentially expressed genes was tested by qPCR analyses to confirm the microarray results (Table 1) using a housekeeping gene, *topB*, as the reference gene for normalization. When the average fold changes were compared between microarray data and qPCR data, a strong positive correlation was observed ($r = 0.91$; Fig. 4), confirming the validity of the microarray data used in

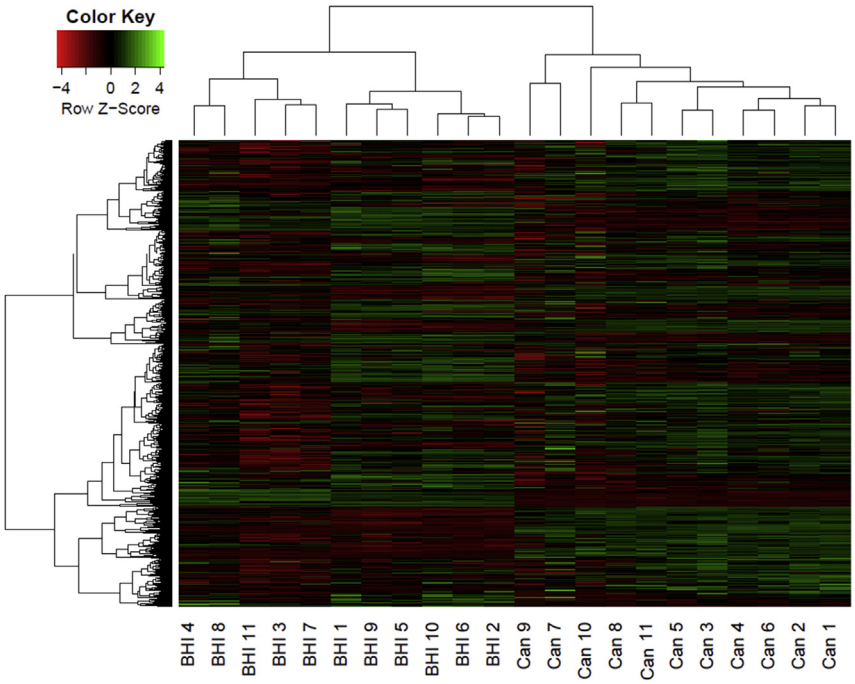

Fig. 2. Heatmap of *L. monocytogenes* transcriptional profiles after growth in BHI or cantaloupe slices for 5 days at 10 °C. Standardized Z-scores of the RMA-normalized probe set intensities (rows) are depicted in a heatmap configuration. Horizontal labels represent the clustering of the expression data collected from 22 L. *monocytogenes* RNA samples (columns; 11 BHI and 11 cantaloupe slices).

this study.

3.3. Upregulation of De novo biosynthesis of amino acids and transporters genes in cantaloupe

A considerable number of upregulated genes in cantaloupe slices encoded protein functions related to amino acid biosynthesis as well as transporters of amino acids and oligopeptides. Among the upregulated genes by *L. monocytogenes* grown in cantaloupe, the transcription of branched chain amino acids (BCAA) valine, leucine, and isoleucine biosynthesis operon (*ilvDBNC-leuABCD-ilvA*) appeared to be highly upregulated (FC 17.09–51.24; Table 2 and Table S1). Transcription of *ilvE* (lmof2365_0999), which encodes branched chain amino acid aminotransferase and is involved in the last step of BCAA biosynthesis, was also found to be upregulated (FC 1.9; adj. $P < 0.05$). The pathway analysis using the GAGE package identified BCAA biosynthesis as the most significantly enriched metabolic pathway in cantaloupe slices, providing further support that BCAA biosynthesis is induced by growth in cantaloupe. Higher transcription levels were detected for several other genes that are involved in the biosynthesis of amino acids such as histidine (*hisDBHAFIE*), threonine (*thrB* and *thrC*), and glutamate (*gltB* and *gltD*). Upregulation of oligopeptide ABC transporter genes, notably some members of the *oppA-F* operon, as well as L-cystine (lmof2365_2316, lmof2365_2317, and lmof2365_2319), glutamine (*glnP* and *glnQ*), and D-methionine ABC transporters (*metI*, *metQ*) was observed for *L. monocytogenes* grown in cantaloupe slices. The increased transcription of genes with a functional role of amino acid biosynthesis as well as amino acid transporters suggests that *L. monocytogenes* adaptation in the cantaloupe environment includes a requirement for increased intracellular biosynthesis of certain amino acids (e.g., BCAA) as well as the ability to scavenge exogenous amino acids and/or oligopeptides present in cantaloupe milieu.

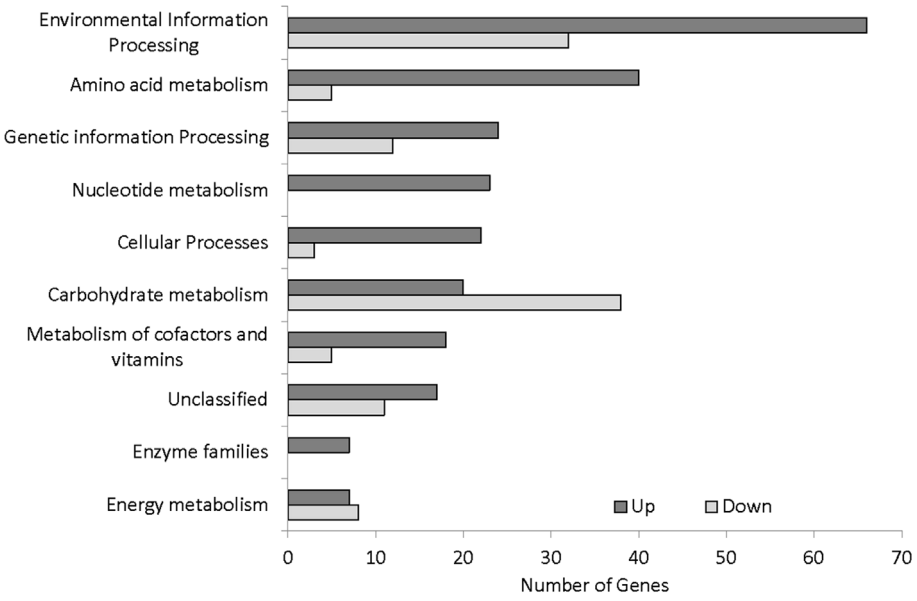

Fig. 3. Functional categories of upregulated (dark grey bars) and downregulated genes (light grey bars) in *L. monocytogenes* LS670 grown in cantaloupe slices for 5 days at 10 °C. The bars represent the total number of genes that are assigned to each functional category by the BlastKOALA annotation server available at the KEGG website (Kanehisa et al., 2016). Functional categories with less than 5 upregulated genes are not shown.

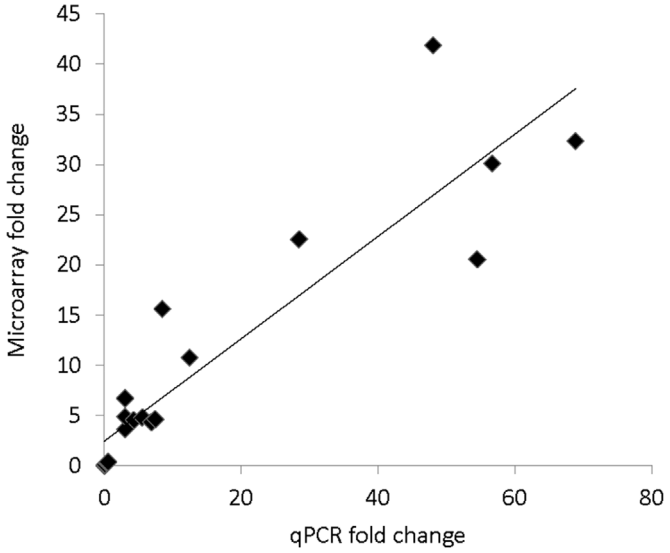

Fig. 4. Microarray data validation via qRT-PCR. Transcription level of a subset of 20 genes was analyzed by qRT-PCR using 3 biological replicate cDNA samples used for microarray hybridizations. Comparison of fold changes for these indicated a positive linear correlation (r = 0.91) between the microarray data and the qRT-PCR data.

Table 2
Selected list of significantly (q < 0.05) enriched pathways among up- and -downregulated gene sets in *L. monocytogenes* LS670 grown in cantaloupe slices for 5 days at 10 °C.

Metabolic Pathways	
Upregulation	Downregulation
Valine, leucine and isoleucine biosynthesis	Phosphotransferase system (PTS)
2-Oxocarboxylic acid metabolism	Galactose metabolism
Flagellar assembly	Propanoate metabolism
Purine metabolism	Fructose and mannose metabolism
Biosynthesis of secondary metabolites	Pentose phosphate pathway
Alanine, aspartate and glutamate metabolism	Carbon metabolism
Histidine metabolism	Folate biosynthesis
ABC transporters	
Pyrimidine metabolism	

3.4. De novo nucleotide biosynthetic pathways upregulated in cantaloupe

When transcriptomic profiles of *L. monocytogenes* grown in cantaloupe slices were compared relative to BHI, a substantial number of upregulated genes belonged to functions involved in nucleobase (i.e., purine and pyrimidine) biosynthesis. With respect to the purine biosynthesis operon (*purEKBCSQLFMNHD*), transcription of all but two (*purB* and *purK*) genes increased by more than 4-fold relative to the transcription levels in BHI. Higher transcription levels were also observed for *purK* (1.8 FC) and *purB* (1.7 FC). Similarly, the entire repertoire of the pyrimidine biosynthesis operon (*pyrBC-carAB-pyrD*II-*pyrDFE*) appeared to be highly transcribed with relative fold change in transcript levels ranging from 7.23 (*pyrD*II) to 15.62 (*pyrF*). Transcription of two immediate upstream genes, *pyrP* (encoding uracil permease) and *uraA/pyrR* (encoding pyrimidine operon regulatory protein), was also upregulated in cantaloupe slices. The upregulation of purine and pyrimidine *de novo* biosynthesis pathways were further corroborated by the pathway enrichment analysis which identified both pathways as significantly enriched in cantaloupe and strongly suggests that increasing intracellular levels of these nucleic acid precursors is an important metabolic requirement in cantaloupe flesh.

3.5. Flagellar biosynthesis

Our pathway analysis of upregulated genes indicated that flagellar assembly biosynthetic pathway was significantly enriched (Table 2). When looking at individual gene expression levels, the majority of genes involved in flagellar biosynthesis showed higher transcript levels in cantaloupe slices (Table S1). In *L. monocytogenes*, the flagellar biosynthesis pathway is clustered into 6 operons and a monocistronic flagellar structural gene (lmof2365_0726) located between chemotaxis protein-encoding genes *cheV* and *cheY*. When the expression of these genes was analyzed using a lower threshold (fold change ≥ 1.5), 40 genes in the flagellar biosynthesis pathway showed increased transcription. The consistent expression pattern for the members of flagellar biosynthetic pathway suggests that flagellar gene expression is critical for the association of *L. monocytogenes* in the cantaloupe slice environment and clearly demonstrates additional inducing conditions other than the temperature (Peel et al., 1988).

3.6. Iron uptake

A number of genes that play a role in *L. monocytogenes* iron acquisition also showed increased transcriptional activity after growth in cantaloupe. Notably, transcript levels of genes *fhuCDBG*, which encode an iron compound ABC transporter, were significantly increased (FC 7.26–23.69). The Feo iron transport system also appeared to be activated with significant increases in transcription levels of *feoA* and *feoB* (FC 6.03 and 4.56, respectively). The upregulation of another operon (lmo2365_2215, lmo2365_2216, lmo2365_2217), as well as *hupC* (lmof2365_2400), annotated as iron compound ABC transporter, provides further support of iron uptake system upregulation in *L. monocytogenes* as a result of growth in cantaloupe and potentially imply that iron acquisition is an important metabolic requirement of *L. monocytogenes* during cantaloupe-associated growth.

3.7. Stress response and virulence-related genes differentially regulated in cantaloupe

As an extensive reshaping of the transcriptome is known to occur during *L. monocytogenes* transition from saprophytism to a host-associated environment (Toledo-Arana et al., 2009), we also determined changes of virulence gene expression resulting from the pathogen's growth in cantaloupe. Surprisingly, a considerable number of genes with virulence-related functions showed differential gene regulation in cantaloupe slices (Table 3). The majority of these differentially regulated genes belonged to the SigB and/or PrfA regulon(s), transcriptional factors known to be critical for *L. monocytogenes* virulence and stress tolerance (Chaturongakul et al., 2008). Members of the core PrfA regulon with increased transcription included *plc*A, *hly*, *act*A, and *plc*B, whereas *inl*A and *inl*B showed decreased transcription. Members of the SigB regulon that have a role in osmotic and cold stress response and showing increased transcription included *opuCABCD*, *gbuABC*, *betL*, and *bilEAB*. Interestingly, the transcription level of *prfA* was decreased whereas the transcription level of *sigB* was unchanged. Additional virulence related genes with evidence of upregulation included *virR* (FC 1.42), *fbpA* (FC 1.68), *lspA* (FC 1.55), *lgt* (FC 1.72), *ami* (FC 1.83), and lmo0514 (FC 2.06).

3.8. Survival in synthetic gastric fluid (SGF)

To assess whether the growth in cantaloupe slices could influence the ability of *L. monocytogenes* to survive *in vivo*, an SGF survival study was conducted. While initial trials revealed that the vast majority of *L. monocytogenes* was killed within the first 5 min of exposure (data not shown), we elected to focus on a 15 min exposure time as maximal reduction was observed at this timepoint. No significant differences were observed for any of the saline treatments, indicating stable

Table 3
Differential expression of virulence and stress response related genes.

Gene	EGDe locus	FC	Virulence-related function
prfA	lmo0200	0.28	Master virulence regulator
plcA	lmo0201	4.08	Vacuole escape
hly	lmo0202	3.35	Invasion of epithelial cells; vacuole escape
actA	lmo0204	1.82	Cell-to-cell spread via actin polymerization
plcB	lmo0205	2.15	Vacuole escape
inlH	lmo0263	0.60	Invasion
inlA	lmo0433	0.42	Invasion
inlB	lmo0434	0.34	Invasion
flaA	lmo0690	2.14	Invasion
fri	lmo0943	0.51	Intracellular survival and multiplication
dltA	lmo0974	0.47	Adhesion to human and mouse-derived epithelial cells
bilEA	lmo1421	8.21	Bile resistance
bilEB	lmo1422	3.67	Bile resistance
opuCD	1mo1425	1.55	Osmotic stress
opuCC	lmo1426	1.69	Osmotic stress
opuCB	lmo1427	2.15	Osmotic stress
opuCA	lmo1428	2.03	Osmotic stress
mprF	lmo1695	0.66	Invasion of epithelial cells and macrophages
virR	lmo1745	1.42	Adhesion and invasion of epithelial cells and macrophages
fbpA	lmo1829	1.68	Fibronectin-binding protein; adhesion to epithelial cells
lpeA	lmo1847	0.01	Entry into murine hepatocytes and human intestine epithelial cells
bsh	lmo2067	0.59	Bile tolerance
oppA	lmo2196	3.85	Intracellular survival in macrophages and growth in organs of infected mice
gadC	lmo2362	0.16	Acid stress response
gadB	lmo2363	0.15	Acid stress response
hupC	lmo2429	4.89	Iron acquisition
lgt	lmo2482	1.72	Invasion and intracellular survival
ami	lmo2558	1.83	Adhesion to human epithelial cells
betL	lmo2092	1.44	Osmotic stress
gbuA	lmo1014	4.35	Osmotic stress
gbuB	lmo1015	5.04	Osmotic stress
gbuC	lmo1016	5.05	Osmotic stress

survival of the cells from either BHI or cantaloupe throughout the course of the trial. Evaluation of survival in SGF, however, found several levels of improved survival of *L. monocytogenes* in SGF, depending on the reference point chosen. When comparing *L. monocytogenes* cultured in BHI at 10 °C for 5 days to the initial inoculum at day 0, which had been adapted to cold growth for only 27–28 h, an increase in survival was observed ($P < 0.001$)(Fig. 5). Separately, when comparing *L. monocytogenes* grown in cantaloupe for 5 days versus the day 0 sample, an increase in survival was also observed ($P < 0.001$), though it was unclear from this comparison whether this was due to cold growth, as seen with the BHI culture, or something unique to the cantaloupe. A separate comparison of *L. monocytogenes* grown in BHI versus cantaloupe for 5 days found increased protection after 15 min SGF exposure ($P < 0.05$). However, this could have been due to protection from the cantaloupe present in the SGF. In order to determine if the protection observed after growth in cantaloupe was attributable to protection provided by the food matrix, either by chemical or physical buffering, a comparison of the "cantaloupe retained" and "cantaloupe removed" samples was performed for days 0 and day 5. Evidence of protection from the cantaloupe was observed at day 0 between "cantaloupe removed" and "cantaloupe retained" samples as *L. monocytogenes* could be recovered from the latter but not the former ($P < 0.001$)(Fig. 5). However, when comparing day 5 "cantaloupe removed" versus "cantaloupe retained" after 15 min of exposure, while some protection was lost when the bacteria were separated from the cantaloupe, the difference was not significant ($P > 0.01$). Survival was still higher for cantaloupe removed samples than what was observed for bacteria cultured in BHI for 5 days at 10 °C though the difference was significant in only one trial ($P < 0.05$)(Fig. 5). Other trials (data not shown) indicated a

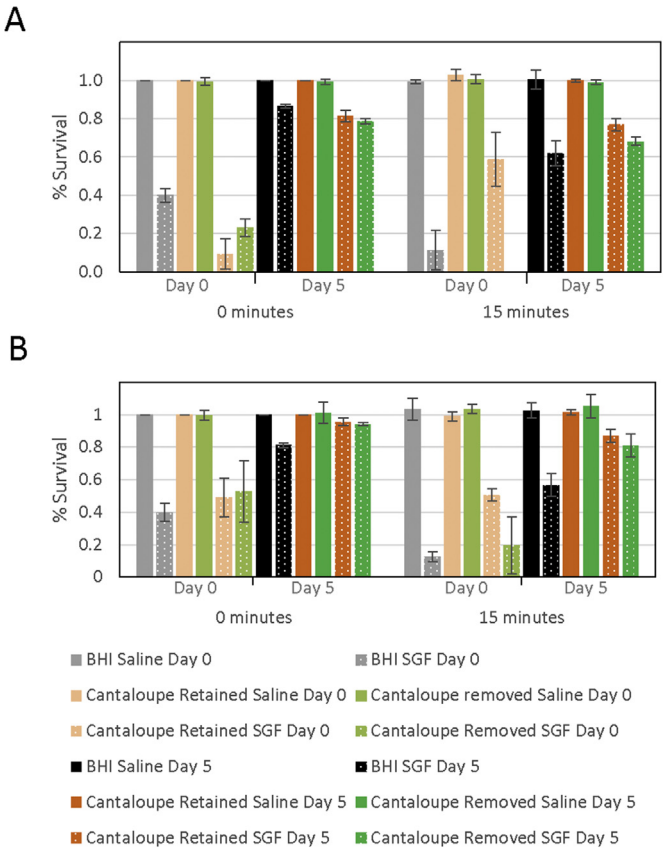

Fig. 5. Survival of *L. monocytogenes* LS670 in SGF. The percent survival of two independent trials evaluating survival in SGF before and after 5 days growth in either BHI or cantaloupe. Cantaloupe retained versus cantaloupe removed refer to the samples processed with and without the cantaloupe as described in the methods. Percent survival is determined for each independent replicate after setting the average of the time 0 for BHI and cantaloupe retained samples as 100%. Standard deviation is shown with the error bars.

similar pattern of significance between BHI and cantaloupe removed samples supporting a trend for increased survival. Taken together the cantaloupe retained samples were not significantly different from the cantaloupe removed samples in both trials.

4. Discussion

The observed increases in the transcription of BCAA biosynthesis genes indicate insufficient levels of exogenous sources of these amino acids in cantaloupe compared to BHI. Interrogation of the frequency of amino acids in the complete set of coding sequences in *L. monocytogenes* revealed that BCAA (i.e., leucine, isoleucine, and valine) are among the most frequently used amino acid constituents in *L. monocytogenes*, comprising approximately 25% of total frequency for the 3 amino acids combined. The upregulation of relatively few (1–2) amino acid biosynthetic genes, and particularly BCAA genes, in *L. monocytogenes* grown in foods of animal origin such as RTE turkey slices (Bae et al., 2011), skim milk (Liu and Ream, 2008), and smoked salmon (Tang et al., 2015) suggests that, unlike in cantaloupe slices, exogenous sources of amino acids and/or peptides are in sufficient quantity in these foods. The available BCAA may be further depleted in cantaloupe due to the conversion of BCAA into volatile aromatic compounds as part of the cantaloupe ripening process (Gonda et al., 2010). Interestingly, *Lactococcus lactis* strains isolated from dairy products are auxotrophic for BCAAs whereas *L. lactis* of vegetable origins are typically prototrophic (Godon et al., 1993), which reflects a food-specific adaptation of this bacterial species based on the nutritional composition of their

associated foods. Although BCAA biosynthesis was shown to be essential for optimal growth of *Streptococcus thermophilus* in milk (Garault et al., 2000), Garault et al. suggested that this observation is likely attributed to the difficulty of obtaining BCAA in milk by *S. thermophiles* (Garault et al., 2000), which can be alleviated by coculture with *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Radke-Mitchell and Sandine, 1986). It may be worthwhile to examine potential differences in the BCAA auxotrophy profiles of *L. monocytogenes* isolates originating from animal-based foods and plant foods.

Aside from BCAA biosynthetic pathway upregulation, several genes involved in the biosynthesis of amino acids, such as histidine, threonine, and glutamate, as well as several oligopeptide ABC transporters, also appeared to be upregulated in cantaloupe indicating that biosynthesis of several other amino acids and transport of peptide precursors are important adaptive process for growing in cantaloupe. While histidine may also play a role in providing amino acid precursors for protein synthesis, it is worth highlighting that the upregulation of this pathway may also serve to provide precursor intermediates that feed into purine and pyrimidine synthesis as the histidine biosynthesis is linked to these nucleotide bases biosynthesis pathways (See below) (Alifano et al., 1996).

Nucleobases (i.e., purines and pyrimidines) are essential biological molecules as they make up the building blocks for DNA and RNA synthesis, facilitate enzymatic reactions as constituents of coenzymes, and play a role in cell signaling and regulation. Our transcriptomic analysis revealed that a large number of genes involved in purine and pyrimidine biosynthesis showed increased transcription levels when grown in the cantaloupe environment, indicating there is a need to increase intracellular levels of these nitrogenous bases, presumably due to insufficient quantities present in the cantaloupe milieu. The induction of the nucleotide biosynthesis pathway has been documented for *L. monocytogenes* grown in RTE turkey meat (Bae et al., 2011) and cut cabbage (Palumbo et al., 2005), whereas pyrimidine biosynthesis was shown to be downregulated in *L. monocytogenes* grown in cold-smoked salmon (Tang et al., 2015) and no differential regulation of these biosynthetic pathways was observed in skim milk (Liu and Ream, 2008). Hence, the requirement for upregulation of nucleobase synthesis appears to be a food-specific adaptive strategy based on available nutrients in each complex food system.

Interestingly, mounting research has provided evidence for co-regulation of microbial metabolism and pathogenesis and this dynamism presumably represents the evolutionary outcome of specific host-pathogen interactions to avoid futile production of virulence gene products (Rohmer et al., 2011). It is particularly worth noting that the metabolic pathways (i.e., BCAA, histidine, purine, and pyrimidine) upregulated in cantaloupe, and hence potentially important for *L. monocytogenes* growth in cantaloupe, have been found to be linked with *L. monocytogenes* virulence function (Lobel et al., 2012). For instance, limiting the concentration of isoleucine can induce virulence gene expression via the involvement of the regulator CodY (Lobel et al., 2012). Nucleotide biosynthesis has been associated with the ability of *L. monocytogenes* and other enteric bacteria to colonize the gastrointestinal tract and cause systemic infection, as well as intracellular growth capability (e.g., macrophage cells, Caco-2 human intestinal epithelial cells) (Samant et al., 2008; Schauer et al., 2010; Vogel-Scheel et al., 2010; Faith et al., 2012; Lobel et al., 2012). Extensive changes in stress response and virulence genes observed in our study may also be the product of an intricate co-regulation of genes involved in metabolism and virulence. Specifically, upregulation of the members of the SigB and PrfA regulons (global regulators of stress response and virulence in *L. monocytogenes*) (Chaturongakul et al., 2008) indicates that the virulence potential of *L. monocytogenes* is altered as a consequence of growth in cantaloupe. While the net effect of the alterations in stress-related or virulence genes expression on infection potential is uncertain, enhanced survival of cantaloupe-adapted *L. monocytogenes* in SGF demonstrates that *Listeria* cells may be 'primed' for the initial stages of infection, which requires passage through the stomach's gastric barrier. This phenotypic alteration in SGF resistance may be partially due to the increased transcription levels of genes involved in bile resistance induced after growth on cantaloupe (*bilEAB* and *bsh*) (Begley et al., 2005; Sleator et al., 2005). Other studies have reported the potential role of food matrix (deli turkey meat and frankfurters) on subsequent host-related stress tolerance in *L. monocytogenes* (Wonderling and Bayles, 2004; Peterson et al., 2007).

When *L. monocytogenes* was grown in cantaloupe, higher transcription of genes encoding iron acquisition systems such as *fhuCDBG*, *feoAB*, and *hupC* (Jin et al., 2006) was observed, indicating the need for iron uptake from the cantaloupe environment. Since the capability of pathogens to scavenge iron from host iron-binding proteins in a host is crucial for a successful infection process (Ratledge and Dover, 2000), *L. monocytogenes* may gain further fitness advantage as a result of the adaptive response to cantaloupe.

Considering the overlapping metabolic requirement for several biosynthesis pathways in the cantaloupe and host environments, pre-adapting *L. monocytogenes* in the cantaloupe environment may confer a fitness advantage during the foodborne transmission of the pathogen. Further, these metabolic demands may also function as signals triggering expression of various virulence genes, thereby providing cross-protection against host-mediated defense mechanisms during the infection stage. Detailed studies involving other disease outcome parameters will be necessary to further shed light on the food matrix effect *on L. monocytogenes* pathogenicity.

When *L. monocytogenes* was grown in cantaloupe slices, we observed that the majority of genes encoding flagella biosynthesis showed increased transcription levels compared to growth in BHI. Aside from providing a physical structure for bacterial locomotion, flagella play a number of important biological functions including attachment to various abiotic surfaces such as glass and stainless steel (Vatanyoopaisarn et al., 2000; Lemon et al., 2007), biofilm formation (Lemon et al., 2007), and host cell invasion (O'Neil and Marquis, 2006). Intriguingly, *L. monocytogenes* strains containing mutations in the flagellar biosynthesis pathway were defective in attachment to fresh-cut radish tissue (Gorski et al., 2003) and, thus, flagella and/or flagella-based motility seems to contribute to a successful interaction between *L. monocytogenes* and cut radish tissues. Moreover, flagella, rather than flagellar-mediated motility, was shown to be critical for colonization of different types of sprouts (alfalfa, radish, broccoli) by *L. monocytogenes*, though the extent of contribution varied by the sprout type and bacterial strain (Gorski et al., 2003). In contrast, a flagellar mutant of *L. monocytogenes* was not impaired in its ability to attach and colonize cut-cabbage though increased expression of flagella assembly genes was observed (Palumbo et al., 2005). The incongruent role of flagellar motility in plant-microbe interactions may be related to distinct differences in surface topology features of plants; however, further investigations are needed to determine the role of flagella and/or flagella-mediated motility in the attachment and colonization of *L. monocytogenes* in cantaloupe slices. Nevertheless, in light of the previously determined role of flagella-mediated motility in *L. monocytogenes* virulence (i.e., colonization of gastrointestinal tract in orally infected mice) (O'Neil and Marquis, 2006; Shen and Higgins, 2006), increased flagella expression in cantaloupe slices may also have a potentially important implication in subsequent interaction of *L. monocytogenes* with human host.

5. Conclusions

In this study, we evaluated the changes in the transcriptomic landscape of *L. monocytogenes* grown in cantaloupe slices. Some of these changes may have overlapping metabolic requirements within the host environment. We specifically report that adaptive strategies of *L. monocytogenes* grown on cantaloupe slices involved upregulation of several biosynthesis pathways including BCAA biosynthesis, nucleotide

biosynthesis (purine and pyrimidine), and flagellar biosynthesis, as well as many uptake systems including iron transport systems and transporters of amino acids and oligopeptides. Further, changes in stress- and virulence-related genes were also observed, demonstrating specific effects of a complex food system on *L. monocytogenes* survival and virulence potential.

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Appendix A. Supplementary data

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