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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.