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Quantifying growth of cold-adapted *Listeria monocytogenes* and *Listeria innocua* on fresh spinach leaves at refrigeration temperatures

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ABSTRACT

The Baranyi model was used to obtain the initial cell concentration, y_o, lag time, t_{lag}, maximum cell concentration, y_{max}, and maximum growth rate, μ_{max}, using data collected on the growth of cold-adapted (CA) *Listeria monocytogenes* and *Listeria innocua* on fresh spinach leaves under refrigeration temperatures (3, 5, and 8 °C). For L. *monocytogenes*, a dynamic model was validated for y_{max}, t_{lag}, and μ_{max}. Although L. *monocytogenes* grew on the leaves at all refrigeration temperatures, *L. innocua* did not show growth at 3 °C. At 5 and 8 °C, the growth parameters (t_{lag} and y_{max}) for *L. monocytogenes* and *L. innocua* were significantly different ($P < .05$). Therefore, CA *L. innocua* is not a suitable surrogate for CA *L. monocytogenes* in growth studies. Total aerobic mesophilic and psychrotrophic microorganisms restricted growth of *L. monocytogenes* at 3–8 °C on the leaves.

1. Introduction

The consumption of fresh leafy green vegetables as a source of vitamins, minerals, and phyto-nutrients has increased substantially in the last two decades (Taban and Halkman, 2011). However, between 1998 and 2008, fresh leafy green vegetables, including spinach, were the most frequent source of infections (22%) and second most frequent cause of hospitalizations (14%) in the United States (Painter et al., 2013). The Centers for Disease Control and Prevention (CDC) reported 606 leafy vegetable-associated outbreaks, with 20,003 associated illnesses, 1030 hospitalizations, and 19 deaths in the United States between 1973 and 2012 (Herman et al., 2015). Leafy green vegetables are prone to contamination with pathogens such as *Listeria monocytogenes* in the field (Pang et al., 2017; Weller et al., 2016) and during harvesting, post-harvest handling, processing, and distribution (Gil et al., 2015). Because they are minimally processed, these fresh products are not treated in ways that would dramatically eliminate these pathogens (Jung et al., 2014). Hence, contamination of fresh leafy vegetables with *L. monocytogenes* poses a major food safety concern because of its ability to grow at refrigerator temperatures, ubiquity in nature, and its capability to tolerate stresses conditions (Milillo et al., 2012; Buchanan et al., 2017).

Cartwright et al. (2013) reported that during 1998–2008, 24 confirmed outbreaks of listeriosis resulted in 359 illnesses, 215 hospitalizations, and 38 deaths in the U.S. In 2011, an outbreak occurred in 19 different states in the U.S., caused by consumption of contaminated romaine lettuce, in which 84 people were affected and 15 died (Zhu et al., 2017). Stephan et al. (2015) reported a listeriosis outbreak linked to ready-to-eat salad in Switzerland, resulting in 32 people being infected during 2013–2014. Since 2010, 8 recalls have been issued due to possible contamination of *L. monocytogenes* on leafy greens in the U.S. (Brown et al., 2016). More recently, a listeriosis outbreak linked to packaged salads occurred in January 2016 in the U.S., in which 19 infected people were hospitalized and of those, 1 died (CDC, 2016). Several studies have reported the presence of *L. monocytogenes* on leafy greens, including fresh spinach, obtained from the supply chain (Cetinkaya et al., 2014; Gombas et al., 2003; Korir et al., 2016; Oliveira et al., 2010; Omac et al., 2017).

Proper cold chain management is critical for fresh produce after harvesting, during transportation, processing, and storage. Pathogenic microorganisms such as *L. monocytogenes* on produce can be exposed to various stresses, which can induce cellular changes resulting in changed growth behavior (Barbosa et al., 1994; Hong et al., 2014; Moorman et al., 2008). Although, many studies have addressed the cold adaption mechanisms of *L. monocytogenes* and growth of this pathogen in culture media at different temperatures (Arguedas-Villa et al., 2014; Begot et al., 1997; Vail et al., 2012; Cordero et al., 2016), only two studies are available on foods (Hong et al., 2014; Wang and Shen, 2015). In addition, Lou and

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Yousef (1996) found that the heat resistance of starvation-stress-adapted cells of *L. monocytogenes* increased to greater extent than non-starved cells. Therefore, the underestimation of potential growth of *L. monocytogenes* on fresh produce can lead to a significant food safety risk for the consumer (Buchanan et al., 2017).

In a previous study, Omac et al. (2015) showed that newly harvested, non-cold adapted (non-CA), *L. monocytogenes* inoculated on fresh baby spinach leaves grew well at temperatures from 5 °C to 36 °C. The authors reported that the growth model parameters of non-CA *L. monocytogenes* and non-CA *L. innocua* on fresh spinach leaves at the same temperature were not significantly different ($P < .05$). Similarly, Milillo et al. (2012) reported that although *L. innocua* is closely related to *L. monocytogenes*, it does not always respond to stress the same way due to the significant genomic differences. Nufer et al. (2007) found that the growth characteristics of non-CA *L. monocytogenes* and non-CA *L innocua* strains inoculated in BHI (brain heart infusion) broth cultures at 4 °C and 7 °C were significantly different ($P < .05$).

Therefore, the objectives of this study were to (1) compare the effect of cold adaptation on the growth of *L. monocytogenes* and *L. innocua* on fresh baby spinach leaves; (2) use the Baranyi model to predict the growth of these microorganisms under refrigeration temperature changes; and (3) compare their growth kinetics.

2. Materials and methods

2.1. Bacterial strains and preparation of cold-stress-adapted cells

L. monocytogenes (ATCC 15313, Scott A, Strain A) and *L. innocua* (NRCC B33076) rifampicin-resistant (80 mg/L) were provided from a stock laboratory (Department of Biological and Agricultural Engineering, Texas A&M University) and stored at −80 °C. Frozen cultures were activated in 9 mL Tryptose Phosphate Broth (TPB; Difco, Becton Dickinson, Sparks, MD). Then, for *L. monocytogenes*, single colony isolates were obtained by streaking on Oxford *Listeria* selective Agar (EMD, Gibbstown, NJ) supplemented with Oxford *Listeria* supplement (HiMedia, India) (OLS) and incubated at 36 °C for 24 h through two successive transfers on OLS. For L. *innocua*, single colony isolates were obtained by streaking on Oxford *Listeria*-selective agar supplemented with 80 mg/L of rifampicin (OLR) and incubated at 36 °C for 24 h through two successive transfers on OLR. Colonies of both bacteria were separately stored on Triptic Soy Agar (TSA; Difco, Becton Dickinson, Sparks, MD) slant at 5 °C as working cultures and used within 90 days.

Cold-adapted cells were obtained based on the procedures adopted and modified from Leenanon and Drake (2001) and Moorman et al. (2008) for both *L. innocua* and *L. monocytogenes* strains. A loop of each strain (i.e., three strains of *L. monocytogenes* and one strain of *L. innocua*) at 5 °C was individually transferred to 9 mL TPB test tubes and incubated at 36 °C for 18 h. After that, overnight bacterial cultures were pelleted by centrifugation (3000×*g* for 15 min) and resuspended in 9 mL of TPB and then incubated at 10 °C for 5 days.

2.2. Preparation of inoculum

After incubation for cold-adaptation, each test tube was centrifuged and washed for three consecutive times (3000×*g* for 15 min) with Buffered Peptone Water (BPW; Difco, Becton Dickinson, Sparks, MD) at 5 °C. Then, each pellet was resuspended in 0.1% peptone water (PW; Acumedia, Lansing, MI) at 5 °C. The optical density at 600 nm (OD_{600}) of the cell suspensions was adjusted to 0.5 of absorbance for bacterial preparation. An approximate initial concentration of 10^7 CFU/mL was confirmed by making serial dilutions of the inoculum suspension in 9 mL test tubes of 0.1% PW. The suspension was plated on OLR for *L. innocua* and OLS for *L. monocytogenes* and incubated at 36 °C until visible black colonies could be counted. A cocktail containing all three strains of *L. monocytogenes* was prepared by mixing 1 mL of each strain (10^7 CFU/mL). To prepare the inoculum, serial dilutions of initial population in PW were carried out to reach a target level of 10^2 CFU/mL of *L. innocua* and *L. monocytogenes* cocktail. Prior to each experiment, fresh cultures were prepared.

2.3. Sample preparation and inoculation

Bags of fresh baby spinach leaves (*Spinacea oleracea*) were purchased from a local grocery store. All bags with same expiration date were selected to ensure uniformity of the produce. The bags were stored at 5 °C for no more than 24 h before the experiments. All leaves displaying signs of wilt and decay were discarded and then 5 g weighed and dispensed into sterile stomacher bags (18 oz. Whirl Pak®) prior to inoculation.

Each sample bag was separately inoculated with 0.5 mL of the 2 log CFU/mL bacterial cultures (*L. innocua* and *L. monocytogenes*) without initial flushing of gases. After inoculation, the bags were gently hand-shaken approximately 30 times to disperse the inoculum over the sample. The inoculated fresh spinach leaves samples were then stored under different storage temperatures (3, 5, or 8 °C) to investigate the growth of *L. innocua* and *L. monocytogenes*. Four bags were prepared for each sampling time and the experiment was carried out in duplicate.

2.4. Procedure for enumeration of microorganisms

In each sample bag, 45 mL of BPW was added, and then the bags were pummeled by hand until samples were reduced to small pieces, allowing the internal leaf structure to be exposed. For L. *monocytogenes* and *L. innocua* enumeration, samples of 1 mL from the original Whirl Pak bag and 0.1 mL from serial dilution in 0.1% PW were plated in duplicate on OLS and OLR, respectively, which were incubated 24 h at 37 °C. After incubation, visible colonies were enumerated with the use of a magnifier counter (detection limit was 10 CFU/mL of sample).

Control samples (without inoculation with *L. innocua* and *L. monocytogenes*) were incubated along with the inoculated samples for each growth temperature. The native background microbiota was analyzed using the procedures described above and plated onto TSA plates. For total aerobic mesophilic and psychrotrophic microorganisms, plates were incubated at 30 °C for 3 days and at 5 °C for 5 days, respectively. Moreover, the background microbiota were plated on OLR and OLS to check the presence of naturally contaminated *L. innocua* and *L. monocytogenes*, respectively.

2.5. Model development for bacterial growth

2.5.1. Primary models

The primary model defines the bacterial growth as a function of time under constant environmental conditions. All growth curves of *L. monocytogenes, L. innocua,* total aerobic mesophilic, and total aerobic psychrotrophic bacteria on fresh spinach leaves at 3, 5, and 8 °C were fitted to the Baranyi model except for *L. innouca* at 3 °C as growth was not observed. The DMFit Excel Add-In software (version 2.0, Institute of Food Research, Norwich, UK) was used for the Baranyi and Roberts model (1994) and the growth of these microorganisms was expressed as change in ln CFU as a function of time as

$$y(t) = y_o + \mu_{max}{}^*F(t) - \ln\left(1 + \frac{e^{\mu_{max}{}^*F(t)} - 1}{e^{y_{max} - y_o}}\right) \quad (1)$$