EVALUATING THE EFFECT OF INOCULATION METHOD AND VALIDATING EXISTING COMBASE MODELS FOR *LISTERIA MONOCYTOGENES* ON TEN WHOLE INTACT RAW FRUITS AND VEGETABLES

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ABSTRACT OF THE THESIS

Evaluating the effect of inoculation method and validating existing ComBase models for *Listeria monocytogenes* on ten whole intact raw fruits and vegetables

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US regulatory agencies have established a zero-tolerance policy for *Listeria monocytogenes* in ready-to-eat foods, including fresh fruits and vegetables. Dry inoculation methods can influence microbial behavior in low moisture foods, but few studies have investigated dry inoculation methods for fresh produce. This study elucidates how growth observed on the surface of ten different fresh produce items is influenced by inoculation method, temperature, and food matrix.

Whole, intact blueberry, broccoli, carrot, cauliflower, cherry, mandarin orange, lemon, raspberry, and tomato were investigated. A cocktail of 5 rifampicin resistant *L. monocytogenes* outbreak strains suspended in 0.1% peptone water was used as the wet inoculum. Dry inoculum was prepared by mixing the wet inoculum with sterile sand and drying at 40°C for 24 h before use. Six replicates of each produce type were inoculated (~3.5 log CFU/sample), incubated at 2, 12, 22, 30 and 35°C and enumerated over time. Growth rates were estimated with DMFit and compared with ComBase modeling predictions for *Listeria*.

Carrots did not support *L. monocytogenes* growth regardless of temperature or inoculation method, possibly due to the presence of phytoalexins. Apparent growth was generally observed for all temperatures except at 2°C and on other produce types. Inoculation method had a significant effect on the average maximum increase of *L. monocytogenes* on all commodities and at all temperatures ($P < 0.05$), and wet inoculation often led to greater increases in *L. monocytogenes* concentration.

ComBase gave fail-safe predictions under all conditions except for tomatoes at 30 and 35°C. Inoculation method is a key variable and its effect should be considered in studies on fresh produce. Use of wet inoculation methods may significantly overestimate *L. monocytogenes* growth potential in fresh produce.

Dedication

I would like to dedicate this thesis to my whole family, especially my parents Clara and Albert, who have given everything to me, have pushed me to by best and supported me in my worst. To my brother Edmon, who has always been a source of inspiration. To my grandparents Anna, Pilar and Albert who have always been a source of light in my life, and to my aunt Glòria who was there when I needed her the most. I would also like to dedicate it to my friend Aishwarya, who has been my constant during my time in the U.S. and to all my friends who have made me who I am. Thank you Enric for supporting me in my dreams. I also want to express gratitude to my professor Dr. Schaffner for his immense kindness and understanding.

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1. LITERATURE REVIEW

1.1. *Listeria monocytogenes*

1.1.1. Physiology and pathogenesis

Listeria monocytogenes is a foodborne pathogen in the genus *Listeria*. First described in detail by Murray et al. *(102)* in 1926, it is a gram-positive, non-sporeforming rod-shaped bacterium *(67)*. Initial research indicated that *L. monocytogenes* was able to grow between 3-45°C, with optimal growth between 30-37°C *(67)*, and later work showed that growth could occur over a larger range (-0.4 to 50 °C) varying as a function of strain and/or serovar *(80, 137)*. Heat resistance of *L. monocytogenes* is highly variable depending on strain, growth conditions, environmental stressors and food matrix *(55)*. *L. monocytogenes* can grow between pH 4.0 and 9.6 *(60, 92)*, and survival and growth at different pH values are influenced by temperature and salinity *(48, 125)*. *L. monocytogenes* is facultatively anaerobic, catalase positive, oxidase negative and expresses a β-hemolysin *(60)*, which mediates its virulence.

L. monocytogenes can invade cells of the monocyte-macrophage family and multiply and spread within them *(92)*. It was first isolated from an epidemic in rabbits and guinea pigs *(102)* and many outbreaks in both animals and humans have been reported ever since. The serovars most frequently isolated from foods and humans are the 1/2a, 1/2b, 1/2c, and 4b, with the latter being associated with the majority of the outbreaks related to invasive *L. monocytogenes (54)*. Listeriosis, the disease associated with infection by *L. monocytogenes*, may present itself as a mild-to-severe gastroenteritis, with symptoms like fever, diarrhea, nausea, vomiting or headache *(105)*, but can also lead to meningitis and encephalitis *(141)*. Listeriosis can be very severe and lead to death in immunocompromised individuals and the elderly, and has often been associated with abortions in pregnant woman *(26, 141)*.

1.1.2. Prevalence and outbreaks

L. monocytogenes has been isolated from a wide variety of products all over the world. Recent prevalence studies and meta-analyses (2017-2020) have focused on a wide range of foods and countries, showing that the need for *L. monocytogenes* control is still relevant. The Churchill et al. *(46)* meta-analysis reported a prevalence of *L. monocytogenes* of 2.9% in deli meats, 2.4% in soft cheeses and 2% in packaged salads. The Martinez-Rios and Dalgaard *(97)* meta-analysis found 2.3% prevalence in European cheeses from 2005-2015. The Liu et al. *(90)* meta-analysis reported a prevalence of 8.5% in raw meats and 3.2% in RTE meats. Other prevalence studies reported 2.7% in fish *(12)* and 0.08% in meat products *(84)* from retail markets in Kerala, India, a prevalence of 88% in beef, 64.7% in chicken and 37.7% in fish from local markets in Calabar, Nigeria *(87)* and 2% and 24% *Listeria* spp. in frozen fruits and vegetables respectively from retail and catering premises in England *(144)*. A review by Bangieva and Rusev *(7)* reported prevalence in raw cow milk ranged from 0-12% in the U.S., 26-37% in Colombia, 0-1% in Brazil, 13% in Mexico, 0.04-28% in Europe, 0.7% in Australia and 5% in India. Ruiz-Llacsahuanga et al. *(117)* reported 4.6% *Listeria* spp. on food contact surfaces in Washington State apple packinghouses and Sullivan and Wiedmann *(126)* reported prevalence of *L. monocytogenes* ranging from 0.8-5.8% and <0.4-1.6% on non-food contact surfaces in three packinghouse and five fresh-cut facilities, respectively.

Listeriosis outbreaks were predominantly associated with ready to eat (RTE) processed meat products (deli meats, hot dogs, meat spreads), in the 1990's but more recent cases have been linked to dairy foods (raw-unpasteurized milk, soft cheeses, ice cream), smoked seafood, sprouts, fruits and vegetables *(26, 42, 133)*. Painter et al. *(107)* reported that most (78.1%) of the 21 US outbreaks caused by *Listeria monocytogenes* between 1998–2008 were linked to meat and poultry products, 15.9% of the illnesses were linked to dairy products, and 6% to sprouts. *Listeria spp*. caused 80 foodborne outbreaks between 1998 to 2017, with a pronounced increase in yearly outbreaks over the last decade, including 13 outbreaks in 2014 alone. While *L. monocytogenes*

accounted for 0.08% of the total amount of illnesses during the last two decades (1998-2017), it caused 9.5% of the total deaths during this same period *(42)*. *L. monocytogenes* is estimated to cost Americans over $2.8 billion annually, including medical costs from hospitalizations and deaths *(73, 131)*.

1.1.3. Biofilm formation, antibiotic resistance and use of disinfectants

L. monocytogenes is known to form biofilms, which can be a source of contamination in processing environments. Biofilms can enhance resistance to disinfectants and can lead to repeated episodes of food contamination over months or years *(14, 28)*. *L. monocytogenes* is able to survive on surfaces like stainless steel, and it often shows resistance to acidic conditions, disinfectants and low temperatures frequently used to kill or control other pathogens *(28)*. *L. monocytogenes* biofilm formation has been widely studied *(81, 101)*, but increased ability to biofilm does not appear to be increased ability to cause disease *(20)*.

Hydrogen peroxide and peracetic acid appear to be more effective than sodium hydroxide in a comparison of various disinfectants on *L. monocytogenes* on multiple surfaces, but this effect varied from strain to strain. Four strains isolated from fish, also showed varied resistance to penicillin, ampicillin, meropenem and cotrimoxazole *(124)*. A study of antibiotic resistance of *L. monocytogenes* in retail foods reported that the prevalence of isolates displaying antibiotic resistance to one or more antibiotics was 0.6% *(138)*. Carvalho et al. *(29)* reported that isolates from a chicken processing environment showed high levels of resistance to both antibiotics and disinfectants (specifically peracetic acid, ampicillin and sulfonamides). In a similar study, an isopropanol-based disinfectant was the most efficient, while two formulations containing tertiary alkylamine and dimethyl alamine betaine were the least effective when 11 commercial disinfectants were evaluated by suspension test on clean surfaces or soiled surfaces *(1)*. Fifteen percent of isolates from a Norwegian meat processing plant showed resistance to quaternary ammonium compounds, frequently used in food processing and retail facilities *(70)*. Brauge et al. *(23)* reported that use of hydrogen peroxide or quaternary ammonium-based

disinfectants could not inactivate all *L. monocytogenes* cells on inert surfaces in smoked salmon processing plants, and induced cells in biofilms to become viable but not culturable. Rodríguez-Melcón et al. *(51)* reported that exposure of *L. monocytogenes* to disinfectant doses close to the minimum inhibitory concentration during biofilm formation might inhibit or promote biofilm formation.

1.1.4. International policy

Multiple severe and deadly listeriosis outbreaks in the U.S. in the 1980s led to the establishment of a zero-tolerance policy for cooked, ready-to-eat foods (RTE) in the U.S. *(123)* which is still in place. FDA's "zero tolerance" policy states that RTE foods must contain no *L. monocytogenes* in 25 g *(57)*. While Italy has the same policy, other European countries including Germany, The Netherlands and France have established a maximum detection limit of 100 CFU/g, and Canada and Denmark have different (but still more permissive) policies depending on the foodstuff *(103)*. Although the theoretical *L. monocytogenes* minimum infectious dose is a single cell, high doses (>10^6 CFU) are typically needed for healthy adults to develop listeriosis, and lower doses (10^4 CFU) may be responsible for some illnesses in at-risk groups *(5, 110)*.

1.2. Fresh produce and food safety

Data published by the U.S. Department of Agriculture, Economic Research Service estimates that from 1970 to 2017 fresh produce availability increased by 35% *(132)*. Data on foodborne illnesses from 1998 to 2008 also attributed 46% of total foodborne illness cases to produce, accounting for almost half (4.4 million illnesses) out of 9.6 million total foodborne disease cases *(107)*. A review on fresh produce outbreaks in North America and Europe from 1999-2019 reported a total of 277 outbreaks associated to 44,524 cases *(4)*. *Salmonella* was responsible for the majority of North America outbreaks (52.16%), followed by *E. coli* (18.53%) and *Cyclospora* (15.95%). *Cryptosporidium* (20.45%) was linked to more European outbreaks followed by *Salmonella* and *E. coli* with equal frequencies (18.18%). Other significant organisms were *Listeria* (only in North America), *Shigella* and *Yersinia* (only Europe), *Hepatitis A*, *Norovirus*

and *Campylobacter*. While numbers varied between Europe and North America, food vehicles most frequently associated with fresh produce outbreaks were vegetables, fruits and salads, followed by juices, mixed items and peas in a much lower proportion *(4)*. A massive outbreak in Germany in 2012 led to nearly 11,000 cases of norovirus linked to frozen strawberries *(94)*. The deadliest European produce outbreak during this time period was linked to *E. coli* O104:H4 in vegetable sprouts and resulted in 3842 cases and 53 deaths in Germany, 2011 *(16)*. The largest North America produce outbreak was due to *Salmonella* Saintpaul in jalapeño pepper in 2008, and resulted in 1442 cases and 2 deaths *(11)*, while the deadliest outbreak was linked to *Salmonella* Poona in cucumber, with 991 cases and 6 deaths in 2015-16 *(85)*.

Since fresh produce is often consumed raw, preventing contamination throughout the farm-to-fork chain is of critical importance. Foodborne pathogens can be present in the environment, and contamination can happen through soil, irrigation water, manure, wildlife feces or cross-contamination during handling and processing *(93)*. If contamination does occur, reducing levels or limiting growth become important in reducing risk. Current methods used to decrease levels or prevent growth include rinsing, use of chemical disinfectants, application of biocontrol agents, and temperature control during storage and transport *(148)*. Control of relative humidity is importance since higher relative humidities may be needed to preserve quality but may also enhancing the potential for microbial growth or survival *(45)*.

1.2.1. Raw, whole produce

Research has compared the growth of various pathogens in whole vs. cut produce with risks generally being higher on cut or damaged produce since the cells have more ready access to nutrients and moisture needed for growth *(10, 30, 66, 113, 121)*. While risks are higher in cut produce, multiple outbreaks have been linked to whole fresh produce in the U.S., including *L. monocytogenes* in whole cantaloupe *(32)*, *Salmonella* spp. in cantaloupe *(33)*, *Salmonella* Agona in whole fresh papayas *(31)*, *Salmonella* spp. in mangoes *(34)*, *E. coli* O157:H7 in leafy greens *(37)*, *Salmonella* Stanley in wood ear mushrooms *(41)*, *Salmonella* Enteritidis in peaches *(40)* and *E. coli*

O103 in clover sprouts *(38)*. Between 2013 and 2016, four outbreaks were linked to the consumption of raw cucumbers contaminated with *Salmonella* spp. in the US, causing a total of 1,280 illnesses and 7 deaths *(10)*.

Pathogen behavior on whole and cut produce follows no apparent patterns. Bardsley et al. *(10)* reported that inoculating *Salmonella* and *L. monocytogenes* on the surface of whole or sliced cucumbers did not lead to significant differences in growth and decrease. Castro-Rosas et al. *(30)* found that when inoculating *Salmonella* and *E. coli* on whole and sliced zucchini, decreasing concentrations were found on whole fruits, but significant growth was observed for both pathogens at 25 ± 2°C on sliced samples. The same behavior was observed for *E. coli* on jalapeño and serrano peppers *(66)*, and for mangoes *(113)*, where decrease was observed on whole inoculated foods, but sliced commodities supported growth of the pathogen. Scolforo et al. *(121)* observed that *L. monocytogenes* was equally capable of growing both on the outer rind and the pulp of canary melons at a wide temperature range (5-35°C), while *Salmonella* did not grow on the outer rind at low temperatures.

Since RTE cut produce spoils faster than whole produce, visually apparent food quality losses may reduce food safety risks from cut produce *(65)*. If whole raw fruits and vegetables can support pathogen growth, they may pose a great risk if visual quality is maintained. Recent outbreaks of *L. monocytogenes* linked to whole produce have made it a research priority to investigate those commodities for which *L. monocytogenes* growth and survival potential on intact whole produce is uninvestigated or where previous studies show conflicting data.

1.3. *Listeria monocytogenes* in produce

1.3.1. Prevalence, incidence and cross-contamination

Weis and Seeliger *(141)* found presence of the *Listeria monocytogenes* ranged from 15.7-27.2% in samples from soil, wildlife feces, wildlife feeding grounds and birds, demonstrating that this bacteria is common in the agricultural environment. While the 1/2b and 4b β-hemolytic serovars were the most frequently isolated, other non-hemolytic serovars were also found.

Presence of *L. monocytogenes* in fruit and vegetable products may be traced to contaminated irrigation water *(63, 111)*, soil *(108)*, livestock manure *(63, 130)* or wild animals *(140)*, as well as cross-contamination in packinghouses or processing plants *(69, 79, 128, 130)*, or by food handling personnel *(79)*. Elevated storage temperatures allow *L. monocytogenes* cells to multiply, increasing risk of illness *(43, 118)*. Since *L. monocytogenes* may be in the agricultural environment *(92)*, it is realistic to assume that whole produce will occasionally be contaminated by the organism. The low frequency of *L. monocytogenes* outbreaks linked to produce and its relatively high median infectious dose indicates that post-harvest handling has a key role to play in managing prevalence and concentration of the pathogen with respect to risk.

1.3.2. Outbreaks

Recent produce-related multistate *L. monocytogenes* outbreaks include those linked to enoki mushrooms *(39)*, packaged salads *(36)*, caramel apples *(35)*, stone fruit *(44)* and whole cantaloupe *(32)*. While *L. monocytogenes* causes relatively few outbreaks linked to fresh produce, the U.S. Food and Drug Administration Recalls, Market Withdrawals, & Safety Alerts web page *(135)* lists more than 60 product recalls occurring between 2017 and 2020 associated with produce including mushrooms, tomatoes, peaches, nectarines, plums, cherries, lemons, limes, oranges, broccoli and cauliflower. The 2011 whole cantaloupe outbreak was linked to 146 cases, 143 hospitalizations and 33 deaths *(32)*, the 2014 caramel apple outbreak resulted in 35 cases, 34 hospitalizations and 7 deaths *(35)*, the 2015-2016 prepackaged salads outbreak resulted in 19 cases and hospitalizations and 1 death *(36)*, and the 2016-2020 enoki mushrooms outbreak lead to 36 reported cases, 31 hospitalizations and 4 deaths *(39)*.

1.3.3. Current research on *L. monocytogenes* in fresh, whole produce

While published articles on *L. monocytogenes* growth kinetics on cut produce are relatively plentiful, Marik et al. *(96)* identified data gaps on for *L. monocytogenes* growth and survival on whole, intact produce commodities where data were available for only 21 commodities. As noted above, two recent multi-state outbreaks on whole cantaloupes (2011)

and whole apples (2014), illustrate that whole fresh produce may pose a food safety challenge for which we have critical gaps regarding risk. Both the cantaloupe and apple outbreaks were linked to cross-contamination in packing facilities *(32, 35)*. Marik et al. *(96)* further recommended that studies were needed to elucidate critical factors influencing *L. monocytogenes* growth including the role of initial inoculum levels, relative humidity, nutrient profile and nutrient availability on produce surfaces. These authors also reported a lack of comparative data on behavior of *L. monocytogenes* at the range of temperatures found along the supply chain and during shelf life.

1.4. Inoculation method

Previous studies have shown inoculation method can influence bacterial growth observed. Variables such as preculture on agar vs. broth *(22, 72, 134)*, diluent choice *(13, 68, 78)*, drying time *(82, 83)* or mode of inoculation *(82, 83)* can impact survival and growth of bacteria on food and food contact surfaces *(53, 71, 95, 112, 129)*. Tokarskky and Schneider *(129)* evaluated the effect of three diluents on survival of *Salmonella* on tomatoes: 0.1% peptone, buffered peptone water and fresh tomato serum, and reported significant differences linked to variations on inoculation method in most cases. Baylis et al. *(13)* compared of two preparations of buffered peptone water from different brands resulted in significantly different recovery of stressed *Salmonella* cells. Igo and Schaffner *(78)* compared survival of *Enterobacter aerogenes* cell populations in distilled water, 1% phosphate-buffered saline and 0.1% peptone broth on different surfaces. These authors observed that when low inoculation levels were used, cell populations increased >1 log CFU in all inocula including distilled water. These results indicate that the minimal nutrients present in water may be sufficient to support growth of bacteria *(2, 75, 86)*. Studies on survival and growth of *Salmonella* or *Listeria* on industrial surfaces including stainless steel, plastic and others *(53, 71, 78, 95)* raise crucial questions about what factors cause apparent bacterial growth in survival studies where surfaces have low nutrient and water availability (e.g., raw fruits and vegetables) and whether growth and/or survival a result of the food matrix or is it facilitated by components of inoculum diluent.

Another inoculation method whose effect on bacterial growth is yet to be fully explored is the technique of dry inoculation. The use of dry inoculation methods for low moisture foods *(19, 59, 146, 147)*, has opened the door to using this method for other foods, such as fruits and vegetables. Dry inoculation may more closely mimic the typical route of cross-contamination in agricultural settings, and thus lead to more realistic results than when wet inoculation is used. Even so, since there are many potential sources of produce contamination, both wet and dry inoculation may mimic different real-life scenarios for contamination *(19)*. Advantages and disadvantages of both methods have been reported in the literature. Wet inoculation methods can change the properties of the food matrix *(3, 146, 147)*, especially for low moisture foods, requiring a longer post-inoculation drying time, and leading to increased variability between batches *(147)*. Dry inoculation methods may not be feasible for solid low moisture foods, e.g. chocolate or dry dog food *(147)* and may make it harder to obtain initial high inoculum levels for inactivation studies *(3)*. While pathogen growth and survival generally decreases when using dry inoculation methods *(19, 61)*, thermal inactivation studies report higher survivability after heat treatment for dry inoculated low moisture food samples *(3, 15, 89)*.

1.4.1. Studies on wet vs. dry inoculation

There are limited data available in the literature where wet vs. dry inoculation methods *(19, 22, 61)* or the influence of inoculum diluent *(78)* have been explored side-by-side in the same study. All such studies have focused on low moisture foods, and not on *L. monocytogenes* and fresh produce, and they also often report disparate results. Feng et al. *(61)* compared wet vs. dry inoculation to assess survival of >7 log CFU *Escherichia coli* O157:H7 on hazelnuts, and reported that survival was significantly reduced under dry inoculation. Blessington et al. *(19)* reported similar survival rates for *Salmonella* Enteritidis PT30 using wet and dry inoculation methods for both almond and walnut kernels. Bowman et al. *(22)* evaluated survivability of *S. enterica* on dry spices as a function of one dry and three wet inoculation methods, and found that method of inoculation had a significant effect on the recoverability of the pathogen and that dry inoculation

promoted *Salmonella* survival. Prior studies have focused on dry inoculation protocols to minimize drying time, obtain a uniform mixture, as well as prevent change in physical characteristics of the food matrix, all of which may influence study results *(59, 74, 146)*.

1.5. Predictive modeling

Predictive food microbiology seeks to describe how microbes behave in food using mathematical model *(25)*. These models help in overcoming the practical limitations of obtaining quantitative data on the vast number of foods available in the market, as affected by numerous variables such as temperature, storage time, relative humidity, additives present, preservation or processing method used, and pathogens strain or serovar, etc.

Predictive models can be classified in many different ways including the kinetics described (survival, growth, inactivation), the model used (linear regression, square root, exponential, etc.), form of model output (probability vs. kinetic) *(24)* or scope of development (primary, secondary, tertiary) *(109)*. Primary models correlate population density with time, and secondary models define the relationship between parameters of the primary model, e.g. growth rate, and an environmental condition of interest, such as temperature or relative humidity *(109)*. Primary growth models include sigmoid functions introduced by Gibson et al. *(64)*, quasi-mechanistic functions by Baranyi and Roberts *(8)* or logistic and linear functions as used by Rosso et al. *(116)*. Primary inactivation models include the linear model by Bigelow *(18)*, the Weibull model or the shoulder/tail models used by Whiting *(142)*. Secondary models commonly used include polynomial models, square root-type models as used by Ratkowsky et al. *(114)*, or the Gamma Model used by Zwietering et al. *(150)*.

Microbial predictive models can provide information for the implementation of hazard analysis and critical control point (HACCP) systems as well as to develop quantitative microbial risk assessment (QMRA). The U.S. Food and Drug Administration states “HACCP is a management system in which food safety is addressed through the analysis and control of biological, chemical,

and physical hazards from raw material production, procurement and handling, to manufacturing, distribution and consumption of the finished product" *(62)*. Microbial models can provide information on the risk associated to a specific HACCP critical control point in regards to microbial growth potential *(58)*. Predictive models can be used in QMRAs to assess the effect of risk management strategies on growth, survival or inactivation of the pathogen of interest, providing quantitative information that would be otherwise difficult to obtain *(27)*. McMeekin and Ross *(99)* conceptualized the relationship between these three systems as "The food safety triangle": HACCP, QMRA and predictive modeling, where the lack of quantitative information needed for HACCP or QMRA is partly overcome by the use of predictive models.

While some models might be complicated to understand and use, numerous tools have been developed integrating such models and combining them with a user-friendly interface, some in the format of Excel add-ins, some as off-the-shelf website tools or with payment restricted access *(17)*. ComBase *(47)* is a database containing large quantities of data on the responses of specific pathogens to specific environmental conditions, obtained from research institutes (Institute of Food Research (UK), Food Standards Agency (UK) and USDA ARS Eastern Regional Research Center (U.S.)) and the scientific literature. The ComBase database, ComBase predictor (which uses data from the database) and other tools like DMFit or the Pathogen Modeling Program can be very useful for risk managers seeking predictions for bacterial growth under certain pH, temperature, water activity and salt content conditions, to introduce the appropriate measures to prevent it *(9)*.

1.5.1. Predictive models for *Listeria monocytogenes*

Predictive models for *L. monocytogenes* behavior are widely available. Many models have been developed focusing on the effect on *L. monocytogenes* kinetics of a wide range of variables including salt concentration *(98)*, pH value *(98, 104, 143)*, temperature *(56, 98, 143)*, $NaNO_2$ concentration *(98)*, presence of organic acids *(104)*, modified atmosphere packaging conditions *(120)* and water activity *(104, 143)* among others.

Early predictive models for *L. monocytogenes* focused on variables like pH, temperature or water activity. These studies evaluated the ranges at which *L. monocytogenes* had been previously reported to grow and tested the interaction between variables *(56, 98, 143)*. A second wave of predictive modeling shifted the focus to specific foods. *L. monocytogenes* growth has been modeled for a wide variety of foodstuffs. The most frequently modeled commodities are RTE foods, especially meats, milk products, seafood and more recently, fruits and vegetables. Sheen and Hwang *(77)* modeled the transfer rate of *L. monocytogenes* between the slicing equipment and deli meat during processing, considering multiple cross-contamination scenarios. Seman et al. *(122)* studied the growth of the pathogen on cured RTE meat products under the presence of different preservatives, reporting that while addition of sodium chloride did not significantly reduce growth rates, both sodium diacetate and potassium lactate did. The authors also reported that predicted growth rates were higher than rates obtained from inoculation studies. Bover-Cid et al. *(21)* reported that higher fat content had a pressure-dependent protective effect when exposing *L. monocytogenes* in dry-cured ham to a high-pressure treatment. Cornu et al. *(50)* focused on the effect of microbial competition in the form of lactic acid bacteria in pork meat products, while Hwang et al. *(76)* modeled the survival of the pathogen in fermented sausages, observing higher decline rates at lower pH and water activities. Xanthiakos et al. *(145)* reported that available models based on laboratory media overestimated growth of *L. monocytogenes* in pasteurized milk, since they did not take into account the effect of inhibitory antimicrobial compounds naturally present in milk. Lobacz et al. *(91)* observed significantly higher growth rates in Camembert rather than in blue cheese, and reported an overestimation of growth by the by ComBase predictor tool *(47)*. Ozer and Demirci *(106)* evaluated the effect of acidic Electrolyzed Oxygen Water (EOW) on growth of *L. monocytogenes* Scott A on the skin of raw salmon fillets and developed a response surface model which demonstrated a 1.09 log CFU/g reduction, indicating that EOW has a potential for fish decontamination. Mejlholm and Dalgaard *(100)* developed a model to predict the effect of many

variables on the growth of lactic acid bacteria on seafood, as well as the effect of this bacteria on inhibition of *L. monocytogenes* growth. Cornu et al. *(49)* modeled and predicted the simultaneous growth of *L. monocytogenes* and spoilage microorganisms in cold-smoked salmon.

Other studies concerning *L. monocytogenes* and predictive modeling have focused on specific model parameters such as the effect of temperature on the growth rate and lag time *(56)* or the effect of strain variability on predicted growth rates *(6)*. Duh et al. compared kinetics of *L. monocytogenes* to those of *Listeria innocua*, often used as a non-pathogenic surrogate of the former *(56)*. This publication, as well as Sant'Ana et al. *(120)*, modeled not only the growth rate, but lag time as well. Multiple papers also compared experimental data to off-the-shelf growth predictor ComBase, validating the tool *(120, 127)*.

1.5.2. Modeling *Listeria monocytogenes* in fresh produce

Recent increase in produce outbreaks linked to *L. monocytogenes* has prompted abundant research on the growth kinetics of this pathogen on fresh fruits and vegetables. Studies modeling the behavior of *L. monocytogenes* in fresh produce have focused on all kinds of fruits and vegetables, outbreak or non-outbreak related; including fresh green coconut, RTE lettuce *(120)*, cut honeydew and watermelon *(52)*, cucumber and zucchini *(127)*, apples *(119)*, fresh-cut celery *(136)*, green asparagus *(115)*, canary melons *(121)* and yellow onions *(88)*.

Predictive modeling of *L. monocytogenes* in coconut water showed the organism would grow *(139)*, and that refrigeration would slow the growth rate. *L. monocytogenes* behavior on lettuce was modeled and predictions were compared to ComBase, the Pathogen Modeling Program and data from the literature, obtaining comparable results *(120)*. Zilelidou et al. *(149)* modeled the risk of multiple cross-contamination scenarios associated to cutting and shredding lettuce. Transfer from knife to lettuce was more frequently observed than the opposite. Danyluk et al. *(52)* modeled growth of a four-strain cocktail of *L. monocytogenes* on cut cantaloupe, watermelon and honeydew. Comparison to previous publications as well as ComBase predictor

showed similar predictions and faster growth of *L. monocytogenes* on these fruits as compared to *Salmonella* and *E. coli* O157:H7. Vandamm et al. *(136)* investigated growth on fresh-cut celery and showed similar results to previous studies, where *L. monocytogenes* showed better growth or survival than *Salmonella* and *E. coli* O157:H7 at lower temperatures (12°C), but lower growth at higher temperatures (22°C). Castillejo Rodríguez et al. *(115)*, modeled *L. monocytogenes* behavior on fresh green asparagus, recommending storage between 2-4 °C, after predicting significant growth at higher temperatures (8, 12 and 20 °C). Scolforo et al. *(121)* reported that a predictive model for *L. monocytogenes* on the rind and pulp of canary melons showed that the pathogen had potential to grow at a wide temperature range (5-35 °C) in both the rind and the pulp.

1.6. Summary

Recent prevalence studies and meta-analyses (2017-2020) show that the need for *L. monocytogenes* control is still relevant. Recent produce-related multistate *L. monocytogenes* outbreaks include those linked to enoki mushrooms *(39)*, packaged salads *(36)*, caramel apples *(35)*, stone fruit *(44)* and whole cantaloupe *(32)*. While *L. monocytogenes* causes relatively few outbreaks linked to fresh produce, the U.S. Food and Drug Administration Recalls, Market Withdrawals, & Safety Alerts web page *(135)* lists more than 60 product recalls occurring between 2017 and 2020 associated with produce including mushrooms, tomatoes, peaches, nectarines, plums, cherries, lemons, limes, oranges, broccoli and cauliflower.

Marik et al. *(96)* identified that studies were needed to elucidate critical factors influencing *L. monocytogenes* growth including initial inoculum levels, relative humidity, nutrient profile and availability on the surface and their effect on whole intact produce. These authors also reported a lack of comparative data on behavior of *L. monocytogenes* at the range of temperatures found along the supply chain and during shelf life. Our study elucidates the individual and combined effects of temperature, time and food substrate as primary factors influencing *L. monocytogenes* growth on whole intact raw fruits and vegetables. We then build on

this work to contrast the effect of wet vs. dry inoculation as influenced by temperature and food substrate. Our findings should be useful to those seeking to design experiments used to manage risk of *L. monocytogenes* on whole, intact fresh produce.

1.7. References

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2. MANUSCRIPT 1

Running head: Modeling *L. monocytogenes* on whole produce

Research Paper, accepted by the Journal of Food Protection, November 2020

Title: Modeling the fate of *Listeria monocytogenes* on ten whole intact raw fruits and vegetables and validation of existing ComBase models

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2.1. ABSTRACT

L. monocytogenes was associated with more than 60 produce recalls between 2017 and 2020 including tomato, cherry, broccoli, lemon and lime recalls. This study describes the effects of temperature, time and food substrate as factors influencing *L. monocytogenes* behavior on whole intact raw fruits and vegetables. A cocktail of five *L. monocytogenes* strains previously associated with foodborne outbreaks were used. Ten intact whole fruit and vegetable commodities were chosen based on data gaps identified in a systematic literature review. Produce investigated belong to major commodity families: Ericaceae (blackberry, raspberry and blueberry), Rutaceae (lemon and mandarin orange), Roseaceae (sweet cherry), Solanaceae (tomato), brassaceae (cauliflower and broccoli) and Apiaceae (carrot). Intact inoculated whole fruit and vegetable commodities were incubated at 2, 12, 22, 30 and 35 °C with relative humidities matched to typical real-world conditions. Foods were sampled (n=6) for up to 28 days, depending on temperature. Growth and decline rates were estimated using the DMFit for Excel. Growth rates were compared with ComBase modeling predictions for *L. monocytogenes*. Almost every experiment showed initial growth, followed by subsequent decline. *L. monocytogenes* was able to grow on whole intact surface of all produce tested, except for carrot. The 10 produce commodities supported growth of *L. monocytogenes* at 22 and 35°C. Growth and survival at 2 and 12°C varied by produce commodity. The standard deviation of the square root growth and decline rates showed significantly larger variability in both growth and decline rates within replicates as temperature increased. When *L. monocytogenes* growth occurred, it was generally conservatively modeled by ComBase, and growth was generally followed by decreases in concentration. This research will assist in understanding the risks of foodborne disease outbreaks and recalls associated with *L. monocytogenes* on fresh whole produce.

2.2. HIGHLIGHTS

- *L. monocytogenes* grew on intact surfaces of 10 commodities tested except carrot.
- *L. monocytogenes* growth was generally followed by decreases in concentration.
- Fastest rates of increase were generally followed by the fastest rates of decline.
- Variability of growth and decline rates was larger as temperature increased.
- *L. monocytogenes* growth was generally conservatively modeled by ComBase.

Listeria outbreaks were predominantly associated with ready to eat (RTE) processed meat products (deli meats, hot dogs, meat spreads) during the 1990's, but more recent cases have been linked to dairy foods (raw-unpasteurized milk, soft cheeses, ice cream), smoked seafood, sprouts, fruits and vegetables *(15, 30, 128)*. Painter et al. reported that most (78.1%) of the 21 outbreaks caused by *Listeria monocytogenes* between 1998–2008 were linked to meat and poultry products, 15.9% of the illnesses were linked to dairy products, and 6% to sprouts *(97)*. *Listeria spp*. caused 80 foodborne outbreaks between 1998 to 2017, with a pronounced increase in yearly outbreaks over the last decade, including 13 outbreaks in 2014 alone. While listeriosis accounted for 0.08% of the total amount of illnesses during this time period, it caused 9.5% of the total deaths *(30)*. *L. monocytogenes* is estimated to cost Americans over $2.8 billion annually, including medical costs from hospitalizations and deaths alone *(67, 126)*.

Data published by the U.S. Department of Agriculture, Economic Research Service estimates that from 1970 to 2017 fresh produce availability increased by 35% *(127)*. Data on foodborne illnesses from 1998 to 2008 also attributed 46% of total foodborne illness cases to produce, accounting for almost half (4.4 million illnesses) out of 9.6 million total foodborne disease cases *(97)*. Recent produce related multistate *L. monocytogenes* outbreaks include those linked to enoki mushrooms *(29)*, packaged salads *(26)*, caramel apples *(25)*, stone fruit *(32)* and whole cantaloupe *(24)*. While *L. monocytogenes* causes relatively few outbreaks linked to fresh produce, the U.S. Food and Drug Administration Recalls, Market Withdrawals, & Safety Alerts web page *(134)* lists more than 60 product recalls occurring between 2017 and 2020 associated with produce including mushrooms, tomatoes, peaches, nectarines, plums, cherries, lemons, limes, oranges, broccoli and cauliflower.

Presence of *L. monocytogenes* in fruit and vegetable products may be traced back to pre-harvest contamination of crops, i.e. contaminated water used to irrigate fields *(57, 107)*, soil *(98)*, livestock manure *(57, 125)* or wild animals *(138)*, as well as cross-contamination in packinghouses or processing plants *(63, 72, 123, 125)*, or by food handling personnel *(72)*. Elevated storage temperatures can cause any *L. monocytogenes* cell present to multiply increasing risk of illness *(31,*

110). While there is published research on *L. monocytogenes* growth kinetics on cut produce, Marik et al. *(84)* identified growth and survival data gaps on whole, intact produce commodities; in fact, data was only available for 21 commodities. As noted above, two recent multi-state outbreaks on whole cantaloupes (2011) and whole apples (2014), illustrate that whole fresh produce may pose a food safety challenge for which we have critical gaps regarding risk. Both the cantaloupe and apple outbreaks were linked to cross-contamination in packing facilities *(24, 25)*. Since *L. monocytogenes* may be prevalent in the agricultural environment *(83)*, it is realistic to assume that whole produce will occasionally be contaminated by the organism. Fruit surfaces represent an environment for survival and transmission of foodborne pathogens, as emphasized by Scolforo et al. *(113)* on a study modeling the behavior of *L. monocytogenes* on melon rind. Moreover, the low frequency of *L. monocytogenes* outbreaks and its relatively high median infectious dose indicates that post-harvest handling has a key role to play in managing prevalence and concentration of the pathogen with respect to risk.

Marik et al. *(84)* identified that studies were needed to elucidate critical factors influencing *L. monocytogenes* growth including initial inoculum levels, relative humidity, nutrient profile and availability on the surface and their effect on whole intact produce. These authors also reported a lack of comparative data on behavior of *L. monocytogenes* at the range of temperatures found along the supply chain and during shelf life. Our study elucidates the individual and combined effects of temperature, time and food substrate as primary factors influencing *L. monocytogenes* growth on whole intact raw fruits and vegetables. Fruits and vegetables were chosen for which i) *L. monocytogenes* data was completely missing, ii) a sub-set of data was missing or iii) published data showed conflicting results. Fruit and vegetable commodities were selected from within and across major produce families (e.g., Brassicaceae, Rutaceae). We also develop a series of mathematical models to show the conditions under which *L. monocytogenes* growth is expected; as well as, characterize rates of decline, where growth is not expected. The findings and models should be useful to those seeking to manage risk from *L. monocytogenes* in whole fresh produce.

2.3. MATERIALS AND METHODS

Strains and inoculum preparation. A cocktail of five *L. monocytogenes* strains previously associated with foodborne outbreaks were used: A cocktail of five *L. monocytogenes* strains were used: TS14/F.6900, a human isolate from a hot dog associated outbreak, serotype 1/2a *(54)*; G6003, a food isolate from a chocolate milk associated outbreak, serotype 1/2b *(54)*; LIS0234, a food isolate from raw diced yellow onions associated with a recall of pre-packaged diced yellow onions *(135)*; LIS0133, an environmental isolate from a fresh-cut celery processing facility associated with an outbreak of listeriosis *(55)*; and MDD262, an environmental isolate from a cantaloupe packinghouse. All strains were adapted to grow in 80 µg/mL rifampin (R; Alfa Aesar, Ward Hill, MA) by stepwise exposure as described by Parnell et al. *(103)*. *L. monocytogenes* strains from frozen cultures were grown on tryptic soy agar (TSA; Hardy Diagnostics, Santa Maria, CA) supplemented with 80 µg/mL rifampin (TSA-R) for 24 h at 35±2°C prior to each replication. One isolated colony was transferred to 10 mL of tryptic soy broth (TSA; Hardy Diagnostics, Santa Maria, CA) supplemented with 80 µg/mL rifampin (TSB-R) and incubated for 24 h at 35±2°C. A 10 µL loop of this culture was transferred to another 10 mL of TSB-R in a Falcon Tube (Corning, Tewksbury, MA) and incubated for 24 h at 35±2°C. Each culture was centrifuged (Beckman Coulter Allegra X-14R) at 3000 rpm for 5 min to form a pellet. The supernatant for each culture was discarded. A washing procedure was subsequently performed as follows: 5 mL of 0.1% peptone water (Fisher Scientific, Fair Lawn, NJ) was added to each culture and vortexed to break up the pellet. After each washing step, each culture was centrifuged at 3000 rpm for 5 min to form a pellet, and the procedure was repeated twice (two washing steps). Following the second wash step, 5 mL of 0.1% peptone water was added to each culture and vortexed. Each *L. monocytogenes* inoculum concentration was verified by serial dilution and enumeration on TSA-R. All five cultures were combined (cocktail), equaling 25 mL, into a 50 mL falcon tube and vortexed. Equal volumes of each *L. monocytogenes* inoculum were combined to obtain the final inoculum population target of 4-5 log CFU/mL. Inocula was stored on ice no longer than 1 h prior to inoculations.

Fruit and vegetable samples. Ten intact whole fruit and vegetable commodities were chosen based on data gaps from a systematic literature review performed previously *(84)* focusing on the cases where i) *L. monocytogenes* data was absent from the published literature, ii) a specific sub-set of *L. monocytogenes* data was absent or iii) published data showed conflicting results. Produce investigated belonged to major commodity families: Ericaceae (blackberry, raspberry and blueberry), Rutaceae (lemon and mandarin orange), Roseaceae (sweet cherry), Solanaceae (tomato), Brassaceae (cauliflower and broccoli) and Apiaceae (carrot). Produce commodities were obtained from commercial growers/packers in the US and stored at 4±2°C temperature for a maximum of 24 h prior to experimental use.

Inoculation and inoculum populations. Intact whole produce samples were inoculated with the selected *L. monocytogenes* cocktail at 3-4 log CFU/g to allow quantification of either growth or decline, should either occur. Commodities were inoculated with 20 µL of inoculum, distributed in 6-8 drops over the surface. The inoculated samples were dried for 30 min in a biological safety cabinet. Dried samples were placed in sterile containers in growth chambers (with controlled temperature and relative humidity) with microclimate data loggers (HOBO Micro Station Data Loggers, Onset Computer Corporation, Bourne, MA). Relative humidity was maintained between 45-55% throughout experiments.

Enumeration of *L. monocytogenes*. Intact, whole inoculated fruit and vegetable commodities were incubated at 2, 12, 22, 30 and 35±2°C. Commodities were sampled for up to 28 days, depending on the storage temperature (n=6). Intact whole fruit and vegetable commodities were plated in duplicate at each sampling time. All experimental conditions (e.g., temperature, relative humidity, and shelf-life) were confirmed by the Center for Produce Safety industry stakeholder advisory board to be as close to real-world conditions as possible. Only four commodities were studied at 30±2°C (mandarin orange, tomato, cherry and lemon) due to experimental and budgetary constraints, but these data are included in the manuscript, in the interest of full utility.

Each commodity was weighed and placed into a Whirl-Pak sterile filter bag (Nasco, Modesto, CA, USA). Each sample was either homogenized by pummeling for 90 s at low speed (Stomacher 400, Seward, England), or by hand using a 30 s rub, 30 s shake, 30 s rub method with 0.1% peptone. The volume of peptone was adjusted by commodity to use the lowest volume needed to cover the inoculated area and maximize the limit of detection (e.g., 10 g blackberry, 20 mL 0.1% peptone). Serial dilutions were made in 0.1% peptone water and surface plated (0.1 ml) in duplicate onto TSA-R and Modified Oxford agar with 80 ug/uL of rifampin (MOX; Becton, Dickinson and Company, Sparks, MD). Media were incubated for 48 h at 35 and 30±2°C, respectively. Control (non-inoculated) samples were plated onto both agars for each sampling time. An additional 1 mL of the lowest dilution was plated onto four plates each (0.25 mL/plate) of each agar to increase the limit of detection. Colonies were counted, and *L. monocytogenes* population levels expressed for each commodity in log CFU/g (or CFU/commodity).

Predictive modeling. Data for each commodity, replicate and temperature was used to create primary growth or survival models, describing the lag phase and growth or shoulder and decline as a function of time. Since almost every experiment showed initial growth, followed by subsequent decline, a "split point" between growth and decline was determined for each temperature and commodity. The split point was defined as the time point where the average of the results from six replicates shifted from growth to decline. Lag time was generally not observed and was set to be zero under all circumstances for consistency in modeling. Growth and decline rates were estimated using the tool DMFit for excel, version 3.5 *(35)*, based on the Baranyi model *(2)* to describe the effect of temperature on primary model parameters. Growth was defined as >1 log CFU for at least two time points *(92)*. Two secondary models were obtained for the effect of temperature on either initial growth or subsequent decline as a function of temperature. The growth model used the square-root of the bacterial growth rates vs. storage temperature, as illustrated by Dominguez and Schaffner *(45)*. The decline model used the negative square root of the absolute decline rate vs.

storage temperature. Growth rates were also compared with ComBase modeling predictions for *L. monocytogenes* using worst case salt concentration (%NaCl = 0.5) and a pH value of 7.

Statistical analysis. R statistical software version 3.6.3 (R foundation for Statistical Computing, Vienna, Austria, Europe) *(109)* was used for data analysis. Sets of six replicates obtained for each experimental condition (10 commodities, 5 temperatures and 2240 time points) were compared using the analysis of variance (ANOVA). A post-hoc analysis with Tukey's HSD (honest significant difference) test was used for multiple pairwise comparisons to find significant differences ($P<0.05$). Two separate one-way ANOVAs were used to analyze the effect of time on *L. monocytogenes* population growth and decline on the surface of each commodity and at a specific temperature, using initial and split time points, split and final time points and *L. monocytogenes* population level (i.e, log CFU/g or log CFU/tomato). One-way ANOVA was used to evaluate the effect of temperature on square root growth and decline rates within each commodity. Results were also analyzed for significant difference between estimated secondary growth models among fruits or vegetables belonging to the same family. Blackberry, blueberry and raspberry (Ericaceae), cauliflower and broccoli (Brassaceae), lemon and mandarin orange (Rutaceae), were analyzed within the group using a one-way ANOVA to determine if there was a significant difference between the square root growth and decline rates as a function of commodity for each specific temperature. A one-way ANOVA was used to analyze for a significant effect of temperature on standard deviation as an indicator of variability using the standard deviations from the square root growth and decline rates for each commodity and temperature.

2.4. RESULTS

L. monocytogenes was able to grow on the whole intact surface of all commodities studied (Figure 1.1A-I and Figure 1.2A-I), except for carrot (Figure 1.1J and Figure 1.2J). All other fruits and vegetables supported growth of *L. monocytogenes* at 22 and 35°C, while growth and survival at 2 and 12°C varied by commodity.

Growth and survival of *L. monocytogenes* on berries (Ericaceae). As shown in Figure 1.1A at 2°C, *L. monocytogenes* populations on blackberries decreased significantly (P = 1.33e-06) by 1.13 log CFU/g after 28 days. Blueberries and raspberries (Figure 1.1C and 1B respectively) supported initial small but statistically significant increases of 0.64 (P = 3.52e-06) and 0.42 (P = 0.00121) log CFU/g at 14 and 7 days respectively at the same temperature. This was followed by significant decreases of 1.24 (P = 1.23e-08) and 1.62 (P = 2.47e-09) log CFU/g by day 28 in blueberries and raspberries respectively.

An initial significant increase after 2 days for raspberry at 12°C (Figure 1.1B) and 3 days for blackberry (Figure 1.1A) and blueberry (Figure 1.1C) was followed by a significant decrease for the remaining time up to 21 days. Populations on the surface of raspberries, blackberries and blueberries first increased by 0.88 (P = 4.1e-06), 1.03 (P = 5.8e-06) and 1.2 (P = 9.51e-08) log CFU/g, and then decreased by 1.24 (P = 1.23e-08), 1.32 (P = 4.93e-08) and 1.39 (P = 5.62e-08) log CFU/g respectively. A significant increase after 2 days 22°C was followed by a significant decrease from 2 to 7 days. The population on the surface of blackberries (Figure 1.2A), blueberries (Figure 1.2C) and raspberries (Figure 1.2B) first increased by 1.84 (P = 2.19e-08), 1.4 (P = 5.26e-09) and 1.83 (P = 4.5e-09) log CFU/g and consecutively decreased by 2.2 (P = 9.4e-10), 1.53 (P = 3.66e-09) and 2.28 (P = 2.31e-10) log CFU/g respectively, dropping below the initial inoculation level. An initial significant growth of 1.85 (P = 5.51e-08), 1.41 (P = 1.9e-07) and 1.62 (P = 1.74e-08) log CFU/g was observed after 1 day on blackberries, blueberries and raspberries respectively at 35°C, and was followed by a significant decline by 2.18 (P = 1.41e-09), 1.11 (P = 8.5e-06) and 2.35 (P = 1.8e-08) log CFU/g from 1 to 3 days.

Statistical analysis of the square root growth and decline rates (Figure 1.3A) showed that rates of change on blackberries and raspberries were not significantly different from each other (P > 0.05) at temperatures of 12, 22 and 35°C. Blackberry and blueberry displayed average square root growth rates of 0.130 and 0.146 log CFU/h at 12°C, 0.194 and 0.197 log CFU/h at 22°C, 0.281 and 0.264 log CFU/h at 35°C and square root decline rates of -0.049 and -0.052 log CFU/h at 12°C, -0.0136 and -0.0139 log CFU/h at 22°C and -0.184 and -0.190 log CFU/h at 35°C respectively. Average square

root growth rates on blueberries were not significantly different (P > 0.05) from those on raspberries at 2, 12 and 35°C, with values of 0.047 log CFU/h at 2°C, 0.131 log CFU/h at 12°C and 0.239 log CFU/h at 35°C, while they were similar to growth rates on blackberries at 12°C. Square root growth rates at 22°C and square root decline rates at all temperatures on blueberries were significantly different that rates for blackberries and raspberries, with an estimated average square root growth rate of 0.171 (P = 0.00051) log CFU/h at 22°C and decline rates of -0.041 (P = 2.11e-05), -0.058 (P = 0.005), -0.115 (P = 4.37e-05) and -0.144 (P = 0.005) log CFU/h at 2, 12, 22 and 35°C respectively. ComBase secondary growth models provided fail-safe predictions about growth rates on any of these three berries (Figure 1.3A). Statistical analysis showed that temperature had a significant effect on growth rates observed for all blackberry (P = 4.2e-16), raspberry (P = 1.3e-15) and blueberry (P = 2.09e-14). Decline rates were not significantly different (P>0.05) between 2 and 12°C, but did show a significant difference at these lower temperature vs. 22 or 35°C for blackberry (P = 1.52e-13), raspberry (P = 2.52e-11) and blueberry (P = 2.09e-14).

Growth and survival of *L. monocytogenes* on citrus (Rutaceae). Lemon supported an initial small but statistically significant increase (P = 0.0096) by 0.48 log CFU/sample at 4 days at 2 °C followed by a significant decrease (P = 1.52e-05) of 1.31 log CFU/sample from 4 to 28 days (Figure 1.1D). No significant growth (P > 0.05) was observed on mandarin orange (Figure 1.1E), with an increase of only 0.44 log CFU/sample after 10 days. Mandarin orange supported a small but statistically significant decline (P = 0.00095) by 0.86 log CFU/sample from 10 to 28 days. Growth and decline rates (Figure 1.3B) didn't differ significantly (P > 0.05) between commodities at 2°C, with average square root growth rates of 0.062 and 0.044 log CFU/h and average square root decline rates of -0.046 and -0.042 log CFU/h for lemon and mandarin orange respectively. *L. monocytogenes* populations increased and then decreased significantly on the surface of lemon at 12°C (Figure 1.1D) and mandarin orange (Figure 1.1E) after 10 and from 10 to 28 days. Lemon supported a significant increase (P = 4.24e-06) of 1.63 log CFU/sample and a decline of 2.13 (P = 5.76e-08) log CFU/sample, while on mandarin orange there was an increase of 1.18 (P = 8.67e-07) log CFU/sample followed by a

decrease by 1.55 (P = 6.91e-07) log CFU/sample at 12 °C. While average square root growth rates did not differ significantly (P > 0.05) between lemons and mandarin oranges (0.08 and 0.075 log CFU/h respectively), average square root decline rates were significantly different (P = 0.0054), with value of -0.066 log CFU/h for lemon and -0.047 log CFU/h for mandarin orange (Figure 1.3B). A similar behavior was observed at 22°C where populations on lemon (Figure 1.2D) first increased and then decreased significantly by 1.51 (P = 3.19e-07) and 1.43 (P = 1.6e-07) log CFU/sample after 2 days and from 2 to 21 days respectively. Populations on mandarin orange (Figure 1.2E) also increased significantly (P = 1.71e-05) by 1.15 log CFU/sample after 4 days, followed by a significant decrease (P = 3.22e-05) by 1.07 log CFU/sample from 4 to 21 days. Average square root growth rates (Figure 1.3B) of 0.191 log CFU/h for lemon and 0.116 log CFU/h for mandarin orange were significantly different (P = 8.33e-07), while average square root decline rates of -0.057 and -0.050 log CFU/h were not (P > 0.05). *L. monocytogenes* populations behavior on citrus at 30 and 35°C was generally similar. On lemon surface (Figure 1.2D), a significant population increase by 1.53 (P = 6.1e-07) and 1.45 (P = 4.27e-07) log CFU/sample was observed after 1 day at 30 and 35°C respectively, followed by a small but statistically significant decline by 0.62 (P = 0.00027) and 0.6 (P = 0.0005) log CFU/sample from 1 to 4 days at the respective temperatures. Populations on the surface of mandarin orange significantly increased after 1 day and then showed a small but statistically significant decrease from 1 to 4 days by 0.96 (P = 6.07e-05) and 0.44 (P = 0.0254) log CFU/sample at 30°C, and 1.45 (P = 3.49e-07) and 1.15 (5.65e-05) log CFU/sample at 35°C. Modeled average square root growth rates for lemon and mandarin orange (Figure 1.3B) were significantly different at 30 °C (P = 0.0167), being 0.266 and 0.214 log CFU/h respectively, but not at 35 °C (P > 0.05), with values of 0.258 and 0.252 log CFU/h. Average square root decline rates were not significantly different at 30°C (P > 0.05), -0.089 and -0.062 log CFU/h for lemon and mandarin orange respectively, as opposed to 35°C (P = 0.0001), where average square root decline rate on lemon was -0.085 log CFU/h and on mandarin orange it was -0.128 log CFU/h. Statistical analysis on the effect of temperature on square root growth rates indicated that a similar behavior was exhibited on the surface of lemon at 2 and 12°C, as well as at 30 and 35°C, but

there was a significant difference (P = 1.71e-14) between the lowest (2, 12°C), middle (22°C) and highest (30, 35°C) temperatures. Square root decline rates were significantly different (P = 3.25e-07) at every other temperature, i.e. growth rates at 2 and 12°C or at 12 and 22°C weren't significantly different, but there was a statistical difference between 2 and 22°C or 12 and 30°C. Square root of the growth rate on mandarin orange showed no significant difference between 2 and 12°C, 12 and 22°C and 30 and 35°C, but did among temperature pairs (P = 1.57e-14), while square root decline rates weren't significantly affected by temperature except at 35°C (P = 0.0012).

Growth and survival of *L. monocytogenes* on tomato. Behavior of *L. monocytogenes* on tomato fruit (Figure 1.1F and Figure 1.2F) was different with respect to all other commodities with respect to ComBase prediction (Figure 1.3D) as tomatoes were the only commodity which showed faster growth of *L. monocytogenes* populations than rates predicted by ComBase, specifically at 30 and 35 °C. While square root decline rates at temperatures of 2, 12 and 22°C varied little (Figure 1.3D), the 30 and 35°C replicate rates were quite variable, with some replicates showing increases in the decline phase. This variation is also evidence from the standard deviation of square root decline rates at 30 and 35°C (0.08-0.09) versus the standard deviation for all other commodities at the same temperatures <0.042. All population changes on the surface of tomato at 2 and 12°C were statistically significant but small (< 1 log CFU/sample). There was a decrease by 0.73 log CFU/sample (P= 0.00018) after 28 days at 2°C, and no growth was observed (Figure 1.1F). A small but statistically significant increase by 0.39 log CFU/sample (P = 0.0147) at day 4 at 12°C was followed by a small but statistically significant decrease (P = 0.000109) by 0.72 log CFU/sample from day 4 to 28 (Figure 1.1F) eventually dropping below the inoculation level. A significant growth (P = 1.26e-08) on the surface of tomatoes by 2.17 log CFU/sample at 4 days at 22°C was followed by a significant decline (P = 1.09e-06) by 1.38 log CFU/sample up to 21 days (Figure 1.2F). Growth and decline were similar at the temperatures of 30 and 35°C. An initial significant increase by 2.54 (P=1.45e-07) and 2.87 (P = 2.81e-08) log CFU/sample at 30 and 35°C after 1 day was observed, after which populations remained on the stationary phase from 1 to 4 days, where no significant decline (P > 0.05) was recorded (Figure 1.2F).

Temperature had a significant effect (P < 2e-16) on *L. monocytogenes* square root growth rates on tomatoes. While no growth was detected at 2°C, the average square root growth rate (Figure 1.3D) at 12°C was 0.064 log CFU/h, at 22°C 0.152 log CFU/h and 0.33 log CFU/h at both 30 and 35°C. Square root decline rates were not significantly different (P > 0.05), with average values of -0.03, -0.039, -0.057, -0.023 and 0.0006 log CFU/h at 2, 12, 22, 30 and 35°C respectively.

Growth and survival of *L. monocytogenes* on sweet cherries. *L. monocytogenes* populations on the surface of whole cherries at 2°C significantly declined (P = 1.33e-06) by 1.15 log CFU/sample from time of inoculation up to 28 days (Figure 1.1G). A small but statistically significant increase (P = 5.5e-05) by 0.82 log CFU/sample after 7 days occurred at 12°C (Figure 1.1G), and was followed by a significant decline (P = 1.05e-07) by 1.22 log CFU/sample from day 7 to 28, decreasing below the initial inoculation level. A significant increase (P = 1.22e-08) after 3 days and decrease (P = 2.24e-11) from 3 to 21 days by 1.99 and 2.7 log CFU/sample occurred at 22°C (Figure 1.2G). Behavior on sweet cherries at 30 and 35°C (Figure 1.2G) was similar as noted in other commodities. Significant growth at 30 and 35°C of 2.38 (P = 5.41e-10) and 2.32 (P = 1.2e-09) log CFU/sample respectively was followed by a significant decline by 1.56 (P = 5.51e-09) and 1.82 (P = 2.56e-07) log CFU/sample (Figure 1.2G). Average square root growth and decline rates (Figure 1.3E) at these two temperatures were not significantly different (P > 0.05), with growth rates of 0.321 and 0.316 log CFU/h and decline rates of -0.155 and -0.157 log CFU/h at 30 and 35°C respectively. Temperature did have a significant effect on square root growth and decline rates of *L. monocytogenes* on sweet cherries when the other temperatures were considered (P < 2e-16). No growth was observed at 2°C and the average square root decline rate was -0.034 log CFU/h. Average square root growth and decline rates were 0.073 and -0.049 log CFU/h at 12°C, and 0.168 and -0.079 log CFU/h at 22°C.

Growth and survival of *L. monocytogenes* on brassica (Brassaceae). A significant increase of *L. monocytogenes* populations on both cauliflower (Figure 1.1H and 2H) and broccoli (Figure 1.1I and 2I) was observed at 2°C, where populations increased respectively by 1.61 (P =1.12e-06) and 1.4 (P = 3.21e-08) log CFU/g over a period of 28 days. Average square root growth rates (Figure 1.3C) of 0.052

and 0.046 log CFU/h at 2 °C differed significantly (P = 0.0029) between commodities. No population decline was observed at this temperature. Growth was observed on cauliflower (Figure 1.1H) for a period of 4 days and broccoli (Figure 1.1I) for 10 days at 12°C, and populations increased significantly by 1.9 (P = 6.62e-08) and 1.53 (P = 1.35e-05) log CFU/g respectively. A small but statistically significant decrease of 0.6 (P = 4.44e-05) and 0.66 (P = 0.0073) log CFU/g was observed after 21 days. There was significant difference (P = 2.33e-09) between the average square root growth rates (Figure 1.3C) for the two vegetables, with values of 0.142 log CFU/h for cauliflower and 0.087 log CFU/h for broccoli. Average square root decline rates were not significantly different (P > 0.05) being -0.033 and -0.046 log CFU/h respectively. *L. monocytogenes* populations increased significantly at 22°C by 2.28 (P = 3.33e-08) and 2.87 (P = 2.27e-09) log CFU/g over 3 and 7 days on cauliflower (Figure 1.2H) and broccoli (Figure 1.2I) respectively. Average square root growth rates significantly differed (P = 1.76e-05) between the two commodities (Figure 1.3C), and *L. monocytogenes* increased faster on cauliflower (0.181 log CFU/h) vs. 0.127 log CFU/h on broccoli. A small but statistically significant decrease of 0.3 (P = 0.04) and 0.72 (P = 0.0012) log CFU/g was observed on cauliflower (Figure 1.2H) and broccoli (Figure 1.2I) respectively up to 14 days after the maximum concentration was reached. Average square root decline rates (Figure 1.3C) were not significantly different (P > 0.05) with a rate of -0.032 and -0.058 log CFU/h for cauliflower and broccoli. At 35°C, a significant population increase after 2 days and a small but significant decline from 1 to 4 days by 2.6 (P = 1.72e-08) and 0.9 (P = 0.0002) log CFU/g on cauliflower (Figure 1.2H) and 2.73 (P = 5.02e-10) and 0.71 (0.0005) log CFU/g on broccoli (Figure 1.2I) were observed. Both square root growth and decline rates weren't significantly different for the two commodities (P > 0.05), average square root growth rates being 0.245 and 0.248 log CFU/h and decline rates being -0.132 and -0.108 log CFU/h for cauliflower and broccoli respectively (Figure 1.3C). Square root growth rates were significantly different at 2, 12, 22 and 35°C for both cauliflower (P < 2e-16) and broccoli (P < 6.76e-15). Square root decline rates on cauliflower did not exhibit a significant difference (P > 0.05) at temperatures of 2, 12 and 22°C, while they were statistically different (P = 3.18e-07) at 35°C. Population decline on broccoli did not differ significantly

(P > 0.05) at temperatures of 12 and 22°C, but were significantly different (P = 1.03e-07) when compared to 2 and 35°C.

Survival of *L. monocytogenes* on carrot. Carrot was the only commodity which did not support growth of *L. monocytogenes* populations on its surface at any temperature, and from the time of initial inoculation only population decline was observed. Populations on carrot significantly decreased (P = 5.9e-09) by 1.7 log CFU/sample after 28 days at 2°C (Figure 1.1J). A significant decrease (P = 1.93e-09) by 1.84 log CFU/sample after 28 days was observed 12°C (Figure 1.1J). Populations significantly decreased (P = 5.68e-08) by 1.65 CFU/sample after 21 days at 22°C (Figure 1.2J). A significant decrease (P = 9.77e-07) by 1.25 log CFU/sample by day 4 was recorded at 35°C (Figure 1.2J). Temperature had no significant effect on square root decline rates at 2, 12 and 22°C, where average decline rates were -0.044, -0.048 and -0.051 log CFU/h respectively (Figure 1.3F). Square root decline rates were significantly different (P = 2.71e-07) at 35°C, with an average value of -0.228 log CFU/h.

Replicate variability as a function of temperature. Statistical analysis of the standard deviation of the square root growth and decline rates for each commodity and temperature and data in Figure 1.3 shows a significantly larger variability in both growth (P = 0.0015) and decline (P = 0.0006) rates within replicates as the temperature increases. The average standard deviations across all commodities at 2, 12 and 22°C were not significantly different, with values of 0.009, 0.01 and 0.01 respectively. At 30 and 35°C the average standard deviations across all commodities were 0.026 and 0.020 respectively. The standard deviation value for the square root of the decline rate across all commodities at both 2 and 12°C is 0.006, at 22°C 0.009, at 30°C 0.040 and at 35°C 0.029. It should be noted that the standard deviation values for both growth and decline rates at 30°C (see Figure 1.3) must consider the reduced number of experimental replicates (n=4), with only data for mandarin orange, tomato, cherry and lemon. The number of observations for all other temperatures is n=10 as explained in the methods.

Summary of statistics. Evaluation of fitness of primary growth and decline models showed R^2 values ranging from 0.028-0.999 with an average of 0.785 for growth and 0.003-0.995 for decline, with an average of 0.68. Standard error fit ranged from 0.012-0.839, with an average of 0.297 for growth models and 0.029-0.779 with an average of 0.32 for decline models. Secondary growth models displayed an overall good fitting, with higher linear correlation for square root of growth rates as compared to decline. R^2 values ranged from 0.882-0.953 for the square root growth rate, and 0.612-0.919 for the square root of decline rates. Poor fitting observed in square root decline rates for mandarin orange and tomato (0.317 and 0.0389) were due to high variability.

2.5. DISCUSSION

Listeria spp. are capable of attachment and biofilm formation on produce surfaces at refrigeration temperatures in a strain dependent manner *(46, 60, 61)*. Some factors affecting growth, survival or death of the pathogen on the surface of whole intact produce are background microflora, pH, water activity, relative humidity, surface topography and hydrophobicity, temperature, salt and sugar concentration *(8, 37, 91, 100, 130)*. Intrinsic factors include bacterial cell surface hydrophobicity *(129)*, cell surface charge *(129)*, surface appendages and extracellular polysaccharides *(48, 49, 53)* and temperature-dependent upregulation of virulence and environmental genes *(12, 14, 60, 82)*. Presence of cuts and bruises leads to the release of exudates containing nutrients, organic acids, phytoalexins and other compounds which can either induce or inhibit adherence and growth of microorganisms *(16)*. Such natural defects on fruits and vegetables also increase surface availability and generate spaces which are more difficult to access *(16, 53)*, offering protection from water rinses and antimicrobial treatments to microorganisms and promoting biofilm formation *(21)*. Because *L. monocytogenes* has a hydrophilic cell structure, it is most often found on wounded surfaces where it displays higher affinity *(121)*. *L. monocytogenes* can exhibit decreased adhesion to hydrophobic, waxy food surfaces when compared to other pathogens for the same reason *(53, 129)*. Poimenidou et al. *(106)* reported *L. monocytogenes* cells developed osmotolerance, thermotolerance or acid tolerance

on different degrees if they had been habituated on tomatoes compared to lettuce at low temperatures, indicating a significant effect of food substrate. Previous studies have also shown that efforts to extend shelf life of products can also lead to extended growth of *L. monocytogenes (18)*.

Survival on carrots. All fruits and vegetables besides carrot displayed growth at temperatures at or above 2°C, in agreement with previous studies *(47, 93, 94)*. Beuchat and Brackett *(7)* reported that *L. monocytogenes* growth was significantly reduced in the presence of carrot juice, population decline was observed after inoculation on the surface of whole and shredded raw carrots, but growth was observed on cooked carrots. They hypothesized that the compound 6-methoxymellein, a type of phytoalexin found on carrots *(75)*, may have exerted an anti-*Listeria*l effect by disrupting the membrane structure of the pathogen *(7, 93, 94)*. In a subsequent study, Beuchat et al. *(10)* observed that the anti-*Listeria*l effect was influenced by various factors, including time and temperature, where a temperature increase corresponded to a decreased inhibitory effect on carrots. This contrasts with the data we show in Figure 1.3F, where experiments at 35 °F show a more rapid decrease that at other temperatures. Given the similarities seen in Figures 1J and 2J, this is likely due to the experimental design. Experiments at 35 °C were conducted for only 3 days, while at lower temperature experiments lasted for 21 or 28 days, with a clear change in the rate of decline occurring around the 3-day mark which results in faster modeled rates for only the 35 °C data.

Growth and survival on tomatoes. Tomato was the other commodity which supported an unexpected pattern of *L. monocytogenes* growth significantly affected by temperature. Since recommended storage temperature for tomatoes is ≥ 10°C *(1, 71)*, the ability of *L. monocytogenes* to survive on the surface of whole intact tomatoes at higher temperatures can be considered a potential food safety risk. Beuchat and Brackett *(9)* observed *L. monocytogenes* growth on whole tomatoes stored at 21°C, but not on those either stored at 10°C or cut previous to inoculation. Those authors reported an increase of >2 log CFU/g after 2 days, followed by a stationary phase from 2 to 8 days, with an initial inoculation level of > 3 log CFU/g at 21 °C which is similar to what we observed. They also reported no significant growth on the surface of whole tomatoes at 10°C, which agrees with our

observations, as we saw a statistically significant increase at 12 °C which was not biologically meaningful (i.e. < 1 log CFU). Tomatoes have been implicated in numerous multistate *Salmonella* outbreaks during the last two decades, contamination being traced back to the water used to irrigate the fields or cross-contamination in packaging facilities *(5, 23, 62, 65, 136)*. While *L. monocytogenes* can survive in acidic conditions, its growth is inhibited by the low pH in tomato juice *(105)* and no documented *L. monocytogenes* outbreaks have been linked to tomatoes. The inability of *L. monocytogenes* to thrive on the surface of whole tomatoes at lower temperatures, as opposed to its growth at <20°C could be linked to polyphenol oxidase (PPO) activity of the vegetable at different temperatures. This enzyme, responsible for oxidation of phenols to quinones *(79)*, has been suggested to be part of the defense mechanisms of plants *(4, 78, 85, 86, 118)*. Doğan et al. studied the effect of temperature on PPO activity and observed that, for different eggplant cultivars, maximum PPO activity was reached at temperatures ranging from 20-30°C *(44)*. Li and Steffens *(79)* and Thipyapong et al. *(124)* observed in two complementary studies that, overexpression of PPO on transgenic tomato resulted in increased bacterial resistance to common tomato pathogen *P. syringae* pv. *tomato*, while suppression of PPO activity led to a 55-fold increase of pathogen growth. Zacheo et al. *(140)* reported an increase of PPO activity on tomatoes at 27°C, and a decline at 34°C. As a result, they observed diminished resistance to nematode *M. incognita* at 34°C. These studies point out to the critical role of PPO on bacterial resistance, as affected by temperature.

Growth and survival on berries. Limited growth of *L. monocytogenes* observed on berries can be explained by the combined effect of phenolic antioxidant compounds, low pH and low storage temperatures used in the food industry. Recommended storage temperature for berries ranges from 1-4°C *(1, 71)* and our results show that little to no growth is supported by berries under such conditions. At least twenty foodborne outbreaks between 1973 to 2013 were traced back to berries *(99, 116)*, with outbreaks were linked to raspberry, strawberry, blueberry and blackberry and caused by *Cyclospora*, hepatitis A virus, *Staphylococcus aureus*, *Salmonella*, norovirus, and *E. coli. L. monocytogenes* has been found in blueberries sampled from a fruit processing plant *(96)* but has not

been linked to any outbreaks. Although many studies are available on the inhibitory effect of antioxidant extracts from berries on survival of multiple pathogens *(11, 19, 40, 43, 56, 59, 76, 77, 81, 88, 95, 108, 137, 139)*, there is little information on the growth of *L. monocytogenes* inoculated on whole raw berries. On one of the few available studies on the topic, Molinos et al. *(90)* reported no growth of *L. monocytogenes* populations on the surface of raspberries at 6°C after 7 days, a 1.5 log CFU/g increase at 15°C after 2 days and a 1 log CFU/g increase at 22°C after 2 days which is consistent with our results. Concha-Meyer et al. *(36)* reported a decrease in *L. monocytogenes* by 0.5 log CFU/mL at 4°C and by 1 log CFU/ml at 12°C after 10 days on the surface of whole blueberries. The discrepancy between these growth patterns and those obtained in our study may be due to different initial *L. monocytogenes* populations. Concha-Meyer et al. used an initial inoculum of 5.8 to 6.2 log CFU/mL which were significantly higher than the ~3.5 log CFU/g used in the current study. The significant effect of initial inoculation levels on population growth, survival and death has been widely studied *(58, 87, 104)*. Sheng et al. *(115)* reported no significant growth (<0.5 log CFU/g) of *L. monocytogenes* on the surface of blueberries at 4°C after 14 days, while Thang et al. *(122)* observed an initial non-biologically significant increase of >0.5 log CFU/g at 1-2°C on the surface of blueberries after 5 days, which was followed by a decline below the initial inoculation level after 15 more days. Although no prior studies on behavior of *L. monocytogenes* on whole raw blackberries were identified, Hongshun et al. *(139)* showed that addition of blackberry juice on milk resulted in significant inhibition of *L. monocytogenes* and other foodborne pathogens. A wide arrange of studies have looked into the potential antimicrobial effect of berries' juice *(11, 40, 139)*, lyophilized berries *(95, 108)* and phenolic, anthocyanin and proanthocyanidin extracts from berries *(19, 40, 43, 56, 59, 76, 77, 88, 95, 108)* against a whole array of pathogens, with variable inhibitory effects. Cranberries have the best documented antimicrobial activity of all berries *(43)*, and have repeatedly shown to be effective on suppressing growth of *L. monocytogenes (19, 40, 81, 108, 137)* on multiple substrates, often displaying higher inhibitory power than extracts from other berries.

Growth and survival on cherries. Only one multistate outbreak involving *L. monocytogenes* in stone fruits (peaches, nectarines, plums and pluots) has been recorded in the U.S., which was linked to cross-contamination in a packaging facility *(32)*. A study prior to the outbreak had found rapid decrease of *L. monocytogenes* populations on the surface of peaches at refrigeration temperatures from an initial inoculum of 3-5 log CFU/g *(34)*. The same study showed *L. monocytogenes* was capable of both adhesion and attachment in the trichomes on the surface of peaches and in the smooth surfaces of plums when inoculated at these levels. Multiple studies have found potential antimicrobial effect of sweet and sour cherry extracts against both gram-negative and gram-positive bacteria, including *L. monocytogenes (74, 89)*. Thang et al. *(122)* reported growth of <1 log CFU/g of *L. monocytogenes* on the surfaces of sweet cherries, starting from an initial inoculum of 2.4 log CFU/g during storage at 1-2°C. These results are consistent with our findings that cherries do not support growth of *L. monocytogenes* at 2°C.

Growth and survival on brassica. Broccoli was linked to 25 foodborne outbreaks from 1998 to 2017, with nine linked to Norovirus, two were linked to *Salmonella* and one each linked to *Bacillus cereus*, *Clostridium perfringens* and *Campylobacter jejuni (30)* and none linked to *L. monocytogenes*. Multiple recalls of food products containing broccoli have been associated to *L. monocytogenes*, *E. coli O26* and *Cyclospora (20, 50-52, 131-133)*. While other members of the Brassica family (e.g. cabbage) have been linked to foodborne disease outbreaks *(64, 112, 117)*, cauliflower has seldom been reported as a vehicle for foodborne disease. A recent *Cyclospora cayetanensis* outbreak was linked to pre-packed vegetables trays containing broccoli, cauliflower, carrots and dill dip *(27)*. Cauliflower was also recalled due to possible cross-contamination with *E. coli* O157:H7 from romaine lettuce implicated in another outbreak *(28)*. Studies have also documented the occurrence of *L. monocytogenes* in produce processing plants *(101)*, ready-to-eat prepackaged salads *(117)* and fresh produce *(66)* including that found in supermarkets *(80)*.

Our study shows that *L. monocytogenes* is capable of measurable growth at 2°C on the surface of both broccoli and cauliflower which are normally stored at 1-4°C *(1, 71)*. Berrang et al. *(6)*

reported growth of *L. monocytogenes* at 4°C on the surface of broccoli and cauliflower similar to those we obtained. These authors *(6)* also showed that populations slowly increased by > 1 log CFU/g over 21 days on both commodities from an initial concentration of 2-3 log CFU/g. These authors also reported a ~3 log increase at 15°C versus the 2 log CFU increase we observed at 12 °C. Other studies of *L. monocytogenes* on broccoli florets found a 2 log CFU/g at 4°C after 13 days *(114)*, a 4 log CFU/g increase at 4°C after 12 days *(120)*, both of which are greater than we observed for similar conditions. We suspect this is because of the gamma-irradiation treatment applied to the samples *(114, 120)* which eliminated any competing microflora and may have rendered the produce more susceptible to colonization. A third study found systematic reduction of irradiated *L. monocytogenes* populations on the surface of broccoli and cabbage stored at 4°C during 10 days *(3)*. It's possible they would have eventually detected growth if they had investigated longer time intervals, especially for their control samples which showed little change at 4 °C. Paramithiotis et al. *(102)* observed a similar growth to that in our study when they inoculated cut cauliflower submerged in a brine solution at ambient temperature. *L. monocytogenes* populations reached a maximum concentration of > 6 log CFU/g at 4-7 days, which slowly decreased back to the initial levels after 25 days. This is similar to the maximum growth at 22°C we observed after 8 days, which was followed by a progressive decline. Finally, while studies on the antibacterial effect of *brassica* extracts, e.g. polyphenol extracts *(69, 70)*, leaf juice *(13)* or cauliflower by-product *(111)* show some inhibitory power against *L. monocytogenes* and other pathogenic microorganisms, our research and some of that of some others show these effects may not be evident in whole produce.

Growth and survival on citrus. Lemons have been associated with 23 foodborne outbreaks between 1998 to 2017 in the U.S., 14 of which were linked to lemonade, and none of which were linked to *L. monocytogenes (30)*. Norovirus was the most common causative agent, with other outbreaks linked to *S. sonnei, S. enterica* and *C. jejuni (30)*. Multiple outbreaks have been associated with unpasteurized orange juice *(22, 39, 41, 68)* but none linked to *L. monocytogenes*. Since *L. monocytogenes* can survive and grow under acidic conditions *(33, 38)* and acid-adapted *L.*

monocytogenes (17) was able to grow in orange juice and orange slices with a pH as low as 2.6 and can survive (~1 log CFU reduction) over 7 days in mandarin orange juice, pH 2.8-3.5 *(73)*, citrus cannot be ruled out as a potential vehicle . No previous studies of *L. monocytogenes* on the surface of lemon or mandarin orange were found on the literature. Given citrus storage temperatures of 5-9°C currently used in the food industry *(1, 71)*, our findings of potential growth at 12°C denotes a potential food safety risk.

Comparison with ComBase predictor. Analysis of primary and secondary growth models demonstrated that *L. monocytogenes* populations growth or decline on the surface of all commodities was strongly dependent on temperature (Figure 1.3) and ComBase predictions were generally fail safe. ComBase made fail-safe predictions for all commodities except for tomatoes at 30 and 35°C. Other authors have occasionally reported experimental growth rates higher than those predicted by ComBase *(119)* but such observations are generally the exception rather than the rule *(42)*.

It is encouraging to note that the growth and death patterns of *L. monocytogenes* on a wide range of whole uncut produce items are generally similar. It is also encouraging that when *L. monocytogenes* growth occurs it is conservatively modeled by ComBase, and it is generally followed by decreases in concentration. Those rates of decrease are also temperature dependent, so the fastest rates of increase are followed by the fastest rates of decline. More research is still needed on the roles of inoculation method, environmental relative humidity, background microflora, presence and bioavailability of polyphenols, initial inoculation levels, and produce surface topography on their effect on *L. monocytogenes* behavior on whole intact commodities. These studies assist in understanding the risks foodborne disease outbreaks and recalls associated with *L. monocytogenes* on fresh whole produce.

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2.8. FIGURE LEGENDS

Figure 1.1. Behavior of *L. monocytogenes* inoculated onto whole intact (A) blackberry, (B) raspberry, (C) blueberry, (D) lemon, (E) mandarin orange, (F) tomato, (G) sweet cherry, (H) cauliflower, (I) broccoli and (J) carrot at 2 (▽) and 12 (△) °C over 28 days.

Figure 1.2. Behavior of *L. monocytogenes* inoculated onto whole intact (A) blackberry, (B) raspberry, (C) blueberry, (D) lemon, (E) mandarin orange, (F) tomato, (G) sweet cherry, (H) cauliflower, (I) broccoli and (J) carrot at 22 (▽), 30 (○) and 35 (△) °C over 14 days.

Figure 1.3. Comparison of the square root growth rate predictions of the ComBase Predictor *L. monocytogenes/innoc*ua model at 0.5% NaCl and pH 7 (solid line) or pH 5 (dashed line) and square root of experimentally obtained growth (gray fills) and decline (white fills) rates of *L. monocytogenes* populations on the surface of whole intact (A) berries: blueberries (○), raspberries (△), blackberries (□), (B) citrus: lemon (○), Mandarin orange (△), (C) brassica: cauliflower (○), broccoli (△), (D) tomato, (E) cherry and (F) carrot at different temperatures.

3. MANUSCRIPT 2

Running head: Wet vs. dry *L. monocytogenes* on whole produce

Research Paper, accepted by Journal of Food Protection, June 2021

Title: Wet vs. dry inoculation methods have a significant effect of *Listeria monocytogenes* growth on many types of whole intact fresh produce

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Up to Six Keywords: inoculation method; *Listeria monocytogenes*; dry inoculation; whole intact produce; storage temperature

3.1. ABSTRACT

L. monocytogenes causes relatively few outbreaks linked to whole fresh produce but triggers recalls each year in the US. There are limited data on the influence of wet vs. dry methods on pathogen growth on whole produce. A cocktail of five *L. monocytogenes* strains that included clinical, food, or environmental isolates associated with foodborne outbreaks and recalls was used. Cultures were combined to target a final wet inoculum concentration of 4-5 log CFU/mL. The dry inoculum was prepared by mixing wet inoculum with 100 g of sterile sand and drying for 24 h. Produce investigated belonged to major commodity families: Ericaceae (blackberry, raspberry, and blueberry), Rutaceae (lemon and mandarin orange), Roseaceae (sweet cherry), Solanaceae (tomato), Brassaceae (cauliflower and broccoli) and Apiaceae (carrot). Intact, whole inoculated fruit and vegetable commodities were incubated at 2, 12, 22 and 35±2°C. Commodities were sampled for up to 28 days, and the experiment was replicated 6 times. The average maximum growth increase was obtained by measuring the maximum absolute increase for each replicate within a specific commodity, temperature, and inoculation method. Data for each commodity, replicate and temperature was used to create primary growth or survival models, describing the lag phase and growth or shoulder and decline as a function of time. Use of a liquid inoculum (vs. dry inoculum) resulted in markedly increased *L. monocytogenes* growth rate and growth magnitude on whole produce surfaces. This difference was highly influenced by temperature with a greater effect seen with more commodities at higher temperatures (22 and 35°C), versus lower temperatures (2 and 12 °C). These findings need to be explored for other commodities and pathogens. The degree to which wet or dry inoculation techniques more realistically mimic contamination conditions throughout the supply chain (e.g., production, harvest, post-harvest, transportation, or retail) should be investigated.

3.2. HIGHLIGHTS

- Inoculation method significantly effects *L. monocytogenes* growth on whole produce
- Inoculation method effect varies by commodity and storage temperature
- Carrots never show a significant difference between wet and dry inoculation
- Inoculation method effects often lie on opposite sides of a one log CFU increase
- Inoculation method has a significant effect on growth rate at higher temperatures

Listeria outbreaks have been linked to dairy foods (raw-unpasteurized milk, soft cheeses, ice cream), smoked seafood, sprouts, and fruits and vegetables in recent years *(9, 14, 51)*. While Painter et al. estimated that 46% of all US foodborne illnesses (1998–2008) were linked to fresh produce the number due to *Listeria monocytogenes* in fresh produce was very small and only associated with sprouts *(40)*. Recent produce-related multistate *L. monocytogenes* outbreaks include those linked to enoki mushrooms *(13)*, packaged salads *(12)*, caramel apples *(11)*, stone fruit *(15)* and whole cantaloupe *(10)*. While *L. monocytogenes* causes relatively few outbreaks linked to whole fresh produce, the US Food and Drug Administration Recalls, Market Withdrawals, & Safety Alerts web page *(53)* lists more than 60 product recalls occurring between 2017 and 2020 associated with produce including mushrooms, tomatoes, peaches, nectarines, plums, cherries, lemons, limes, oranges, broccoli and cauliflower.

While there is a growing body of work on *L. monocytogenes* growth kinetics on cut produce, Marik et al. *(38)* identified growth and survival data gaps on whole, intact produce commodities. As noted above, two recent multi-state outbreaks on whole cantaloupes (2011) and whole apples (2014), illustrate that whole fresh produce may pose a food safety challenge for which we have critical gaps regarding risk. Both the cantaloupe and apple outbreaks were linked to cross-contamination in packing facilities *(10, 11)*. Since *L. monocytogenes* may be found in the agricultural environment *(36)*, it is realistic to assume that whole produce will occasionally be contaminated by the organism. Fruit surfaces represent an environment for survival and transmission of *L. monocytogenes (46)*, but the low frequency of *L. monocytogenes* outbreaks and its relatively high median infectious dose *(9)* indicates that post-harvest handling has a key role to play in managing prevalence and concentration of the pathogen with respect to risk. Presence of *L. monocytogenes* in fruit and vegetable products may be traced back to pre-harvest contamination of crops, i.e. contaminated water used to irrigate fields *(22, 43)*, soil *(41)*, livestock manure *(22, 49)* or wild animals *(54)*, as well as cross-contamination in packinghouses or processing plants *(25, 31, 47, 49)*, or by food handling personnel *(31)*.

Marik et al. *(38)* identified that studies were needed to elucidate critical factors influencing *L. monocytogenes* growth including initial inoculum levels, relative humidity, nutrient profile and availability on the surface and their effect on whole, intact produce. These authors also reported a lack of comparative data on behavior of *L. monocytogenes* at the range of temperatures found along the supply chain and during shelf life.

Previous studies have illustrated the significant effect of inoculation method on observed bacterial growth. Variables such as bacterial culture origin, e.g. agar or broth *(8, 27, 52)*, diluent, e.g. 0.1% peptone, buffered peptone water, etc. *(4, 24, 30)*, drying time *(32, 33)* or mode of inoculation, e.g. dip, spot, or spray *(32, 33)* have been studied and found to impact survival and growth of bacteria on food and food contact surfaces *(17, 26, 37, 44, 48)*. Guerini et al.*(24)* evaluated twelve different media for growth and doubling time, while Tokarskky and Schneider *(48)* evaluated the effect of three diluents on survival of *Salmonella* on tomatoes: 0.1% peptone, buffered peptone water (BPW) and fresh tomato serum. Authors reported significant differences linked to variations on inoculation method in most cases. Baylis et al. *(4)* reported that comparison of two preparations of BPW from different brands resulted in significantly different recovery of stressed *Salmonella* cells. Igo and Schaffner *(30)* looked into survival of *Enterobacter aerogenes* cell populations in distilled water, 1% phosphate-buffered saline and 0.1% peptone broth on different surfaces. These authors observed that when low inoculation levels were used, cell populations increased >1 log CFU in all inocula including distilled water. These results may indicate that even the nutrients present in water may be sufficient to support growth of bacteria *(1, 29, 34)*. The effect of diluent should therefore be considered as an important factor promoting growth and survival of microorganisms. These studies as well as research on survival and growth of *Salmonella* or *Listeria* on industrial surfaces including stainless steel, plastic and others *(17, 26, 30, 37)* raise two crucial points: (i) what factors cause apparent bacterial growth in survival studies where surfaces with low nutrient and water availability, such as raw fruits and vegetables are used, and (ii) is growth and/or survival a result of the food matrix or is it facilitated by components of inoculum diluent.

Another inoculation technique whose effect on bacterial growth is yet to be elucidated is the technique of dry inoculation. The use of dry inoculation methods for low moisture foods *(7, 18, 55, 56)*, has opened the door to using this mode of inoculation for other foods, such as fruits and vegetables. One advantage of dry inoculation is that it may more closely mimic the typical route of cross-contamination in agricultural settings, and thus lead to more realistic results than when wet inoculation is used. Even so, since there are many vehicles of produce contamination, both wet and dry inoculation can mimic real-life scenarios of pathogen transmission *(7)*. Advantages and disadvantages of both methods have been reported in the literature; wet inoculation methods can change the properties of the food matrix *(2, 55, 56)*, especially for low moisture foods, requiring a longer post-inoculation drying time, and leading to increased variability between batches *(56)*, while dry inoculation methods may not be feasible for solid low moisture foods, e.g. chocolate or dry dog food *(56)* and may make it harder to obtain initial high inoculum levels for inactivation studies *(2)*. Moreover, while pathogen growth and survival generally decreases when using dry inoculation methods *(7, 19)*, thermal inactivation studies report higher survivability after heat treatment for dry inoculated low moisture food samples *(2, 6, 35)*.

There is limited data available in the literature where wet vs. dry methods *(7, 8, 19)* or the influence of inoculum diluent *(30)* have been explored. These studies, which have focused on low moisture foods, but never on *L. monocytogenes* and fresh produce, often report disparate results. Feng et al. *(19)* compared wet vs. dry inoculation to assess survival of >7 log CFU *Escherichia coli* O157:H7 on hazelnuts, and reported that survival was significantly reduced under dry inoculation. Blessington et al. *(7)* reported similar survival rates for *Salmonella* Enteritidis PT30 using wet and dry inoculation methods for both almond and walnut kernels. Bowman et al. *(8)* evaluated survivability of *S. enterica* on dry spices as a function of one dry and three wet inoculation methods, and found that method of inoculation had a significant effect on the recoverability of the pathogen and that dry inoculation promoted *Salmonella* survival. Prior studies have focused on dry inoculation protocols to

minimize drying time, obtain a uniform mixture, as well as prevent change in physical characteristics of the food matrix, which may influence obtained results *(18, 28, 55)*.

This study builds on prior work *(23)* which evaluated ComBase models for predicting the fate of *L. monocytogenes* on ten whole, intact raw fruits and vegetables using wet inoculation. Here we contrast the effect of wet vs. dry inoculation as influenced by temperature and food substrate. We used the same fruits and vegetables as Girbal et al. *(23)*, which were chosen because *L. monocytogenes* data were not yet published or published data showed conflicting results. Our findings should be useful to those seeking to design experiments used to manage risk of *L. monocytogenes* on whole, intact fresh produce.

3.3. MATERIALS AND METHODS

Prior data and new data. The wet inoculum experimental data (see below) have been published previously *(23)*, but all dry inoculum data are previously unpublished. Both wet and dry inoculation methods, and the methods for assessing growth and decline are included here for readability and understanding.

Strains and inoculum preparation. A cocktail of five *L. monocytogenes* strains previously associated with foodborne outbreaks or recalls were used: TS14/F.6900, a human isolate from a hot dog associated outbreak, serotype 1/2a *(20)*; G6003, a food isolate from a chocolate milk associated outbreak, serotype 1/2b *(20)*; LIS0234, a food isolate from raw diced yellow onions associated with a recall of pre-packaged diced yellow onions *(50)*; LIS0133, an environmental isolate from a fresh-cut celery processing facility associated with an outbreak of listeriosis *(21)*; and MDD262, an environmental isolate from a cantaloupe packinghouse. All strains were adapted to grow in 80 µg/mL rifampin (R; Alfa Aesar, Ward Hill, MA) by stepwise exposure as described by Parnell et al. *(42)*. *L. monocytogenes* strains from frozen cultures were grown on tryptic soy agar (TSA; Hardy Diagnostics, Santa Maria, CA) supplemented with 80 µg/mL rifampin (TSA-R) for 24 h at 35±2°C prior to each replication. One isolated colony was transferred to 10 mL of tryptic soy broth (TSA; Hardy Diagnostics,

Santa Maria, CA) supplemented with 80 µg/mL rifampin (TSB-R) and incubated for 24 h at 35±2°C. A 10 µL loop of this culture was transferred to another 10 mL of TSB-R in a Falcon Tube (Corning, Tewksbury, MA) and incubated for 24 h at 35±2°C. Each culture was centrifuged (Beckman Coulter Allegra X-14R) at 3000 rpm for 5 min to form a pellet and the supernatant discarded. Five mL of 0.1% peptone water (Fisher Scientific, Fair Lawn, NJ) was added to each pellet, and samples were vortexed to break up the pellet. Each culture was then centrifuged at 3000 rpm for 5 min to reform a pellet, and this washing procedure was repeated twice. Five mL of 0.1% peptone water was added to each washed culture and vortexed. Each *L. monocytogenes* inoculum concentration was verified by serial dilution and enumeration on TSA-R. All five cultures were combined into a 25 mL cocktail in a 50 mL falcon tube and vortexed to target a final wet inoculum concentration of 4-5 log CFU/mL. The dry inoculum was prepared as previously described by Blessington et al., 2013 *(7)* by mixing 17.5 mL of the wet inoculum with 100g of sterile sand (Thermo Fisher Scientific, Waltham, MA) in a plastic bag (30 X 30 cm, COM-PAC International, Carbondale, IL), and mixed by hand for 2 min. The inoculated sand was transferred onto filter paper (Thermo Fisher Scientific, Waltham, MA), and spread into a thin layer using a sterile cell spreader (Thermo Fisher Scientific, Waltham, MA). The inoculated sand was dried at 40°C for 24 h, transferred to a bag, and hand mixed again to break up any clumps. The inoculum was stored at 4°C and the concentration confirmed prior to the start of each experiment.

Fruit and vegetable samples. Ten intact whole fruit and vegetable commodities were chosen based on data gaps from a systematic literature review performed previously *(38)* focusing on the cases where i) *L. monocytogenes* data was absent from the published literature, ii) a specific sub-set of *L. monocytogenes* data was absent or iii) published data showed conflicting results. Produce investigated belonged to major commodity families: Ericaceae (blackberry, raspberry and blueberry), Rutaceae (lemon and mandarin orange), Roseaceae (sweet cherry), Solanaceae (tomato), Brassaceae (cauliflower and broccoli) and Apiaceae (carrot). Produce commodities were obtained from commercial growers/packers in the US and stored at 4±2°C temperature for a maximum of 24 h prior to use.

Inoculation and inoculum populations. Intact whole produce samples were inoculated with the selected *L. monocytogenes* cocktail at 3-4 log CFU/g to allow quantification of either growth or decline, should either occur. Commodities were wet inoculated with 20 µL of inoculum, distributed in 6-8 drops over the entire surface. The wet inoculated samples were dried for 30 min in a biological safety cabinet. Commodities were dry inoculated by adding whole produce to a plastic bag (30 X 30 cm, COM-PAC International, Carbondale, IL) containing the inoculated sand and shaking for 2 minutes. Wet and dry inoculated samples were placed in sterile containers in growth chambers (with controlled temperature (± 2 °C) and relative humidity) with microclimate data loggers (HOBO Micro Station Data Loggers, Onset Computer Corporation, Bourne, MA). Relative humidity was maintained between 45-55% for all experiments.

Enumeration of *L. monocytogenes*. Intact, whole inoculated fruit and vegetable commodities were incubated at 2, 12, 22 and 35±2°C. Commodities were sampled for up to 28 d, depending on the storage temperature, and the experiment was replicated 6 times. Intact whole fruit and vegetable commodities were plated in duplicate at each sampling time. All experimental conditions (e.g., temperature, relative humidity, and shelf-life) were confirmed by the Center for Produce Safety industry stakeholder advisory board as either close to real-world conditions or relevant to industry interests.

Each commodity was weighed and placed into a Whirl-Pak sterile filter bag (Nasco, Modesto, CA, USA). Each sample was either homogenized by pummeling for 90 s at low speed (Stomacher 400, Seward, England), or by hand using a 30 s rub, 30 s shake, 30 s rub method with 0.1% peptone. The volume of peptone was adjusted by commodity to use the lowest volume needed to cover the inoculated area and maximize the limit of detection (e.g., 10 g blackberry, 20 mL 0.1% peptone). Serial dilutions were made in 0.1% peptone water and surface plated (0.1 ml) in duplicate onto TSA-R and Modified Oxford agar with 80 ug/µL of rifampin (MOX; Becton, Dickinson and Company, Sparks, MD). Media were incubated for 48 h at 35 and 30±2°C, respectively. Control (non-inoculated) samples were plated onto both agars for each sampling time. An additional 1 mL of the lowest dilution was plated

onto four plates each (0.25 mL/plate) of each agar to increase the limit of detection. Colonies were counted, and *L. monocytogenes* population levels expressed for each commodity in log CFU/g (or CFU/commodity).

Average maximum growth increase. The average maximum growth increase was obtained by measuring the maximum absolute increase for each replicate within a specific commodity, temperature, and inoculation method. The average of the six replicates was calculated and used to compare inoculation methods within a commodity and temperature.

Primary growth and survival models. Data for each commodity, replicate and temperature was used to create primary growth or survival models, describing the lag phase and growth or shoulder and decline as a function of time. Since almost every experiment showed initial growth, followed by subsequent decline, a "split point" between growth and decline was determined for each temperature and commodity. The split point was defined as the time point where the average of the results from six replicates shifted from growth to decline. Lag time was generally not observed and was set to be zero under all circumstances for consistency in modeling. Growth and decline rates were estimated using the tool DMFit for excel, version 3.5 *(16)*, based on the Baranyi model *(3)* to describe the effect of temperature on primary model parameters. Growth was defined as >1 log CFU for at least two time-points *(39)*.

Statistical analysis. R statistical software version 3.6.3 (R foundation for Statistical Computing, Vienna, Austria, Europe) *(45)* was used for data analysis. Four separate one-way ANOVAs were used to separately analyze the effect of inoculation method on the average maximum growth for each commodity at each temperature. Eight one-way ANOVAs were used to identify the effect of inoculation method on square root growth and decline rates at each temperature. A post-hoc analysis with Tukey's HSD (honest significant difference) test was used for multiple pairwise comparisons to find significant differences ($P<0.05$).

3.4. RESULTS AND DISCUSSION

The goal of this study was to elucidate a potential significant effect of inoculation method (wet vs. dry) on growth of *L. monocytogenes* on fresh whole produce, as well as its interaction with temperature and produce type. Our results show that inoculation method does play a significant role on *L. monocytogenes* growth on the surface of whole fresh raw fruits and vegetables, but that this effect depends on the specific commodity and incubation temperature.

Effect of inoculation method and temperature on average maximum growth. Figure 2.1 shows the influence of temperature, commodity, and inoculation method on the average maximum growth increase of *L. monocytogenes*. Each temperature is shown in a different panel: 2 °C (A), 12 °C (B), 22 °C (C), and 35 °C (D), and the asterisks indicate a statistically significant difference ($P<0.05$) between the average maximum growth increase of the wet inoculum (black bars) and the dry inoculum (grey bars). An average maximum growth of one log CFU is indicated by the dashed line. Figure 2.1A shows that at 2°C all commodities had less than a one log average maximum growth, except for the wet inoculum Brassaceae (broccoli and cauliflower). Inoculation method had a significant effect of average maximum growth on broccoli, cauliflower, the Ericaceae commodities (blackberry, blueberry and raspberry) and cutie.

Figure 2.1B indicates that at 12°C six produce types had an average maximum growth greater than one log when a wet inoculum was used (broccoli, cauliflower, blackberry, blueberry, cutie and lemon). Seven produce types had average maximum growth levels that were significantly influenced by inoculation method (broccoli, cauliflower, blackberry, blueberry, raspberry, cutie and lemon) and in all cases the average maximum growth was higher for those items inoculated using the wet inoculation technique.

This same trend continues at 22°C (Figure 2.1C) where all produce items, except for carrots (which never show an average maximum growth above 1 log) show a greater than 1 log average maximum growth when the wet inoculation technique is used. Dry inoculated lemon and tomato samples also show an average maximum growth increase of 1 log CFU or more. In every case, except

carrot (which never shows a significant difference between wet and dry inoculation techniques), all produce items had significantly different average maximum growth increases dependent on inoculation technique. Similar results are also seen at 35°C (Figure 2.1D) as at 22°C, except that only the dry inoculated tomato samples also showed an average maximum growth increase of 1 log CFU or more.

The results in Figure 2.1 make it quite clear that inoculation technique can have a profound effect on the measured average maximum growth, and in many cases those differences lie on opposite sides of the one log CFU increase line. Both the magnitude of the difference as well as the number of commodities affected increased as the temperature increased.

Effect of inoculation method and temperature on growth and decline rates. Figure 2.2 illustrates the relationship between temperature and inoculation method on growth (top panel) and decline (bottom panel) rates for all commodities. These figures are shown as box and whisker plots *(5)* where the shaded box encompasses 25^{th} to 75^{th} percentiles of the data, the whiskers span from 10^{th} to 90^{th} percentiles of the data and outliers are shown as distinct points. The median is represented as a solid line and the mean as a dashed line. The different letters at the bottom of the plot indicate rates that are significantly different ($P<0.05$). The growth rates (Figure 2.2, top panel) at 2°C are different from the rates at the other temperatures, but inoculation technique does not have a significant effect on growth rate at 2°C. The inoculation technique had a significant effect on the growth rate at two other temperatures: 12 and 35°C. The effect of inoculation technique on growth rate at 22°C did not rise to the level of statistical significance selected ($P=0.05$), although the mean and median values of the box plots are quite different. This is likely due to high variability between commodities at 22°C, which could be explored in future studies. The top panel of Figure 2.2 shows that growth rate outliers are also evident at all conditions. High growth rate outliers tend to be from berries, lemons, and tomatoes, while low growth rate outliers are scattered amongst produce types (see supplemental material for details). At the lowest temperature studied (2ºC, top panel) there are

only high growth rate outliers, but as incubation temperature increased, low growth rate outliers become evident. Inoculation method does not have a noticeable effect on the number of outliers. The decline rates (Figure 2.2, bottom panel) showed fewer significant differences by temperature and inoculation type, although a significant difference was clearly observed between decline rates for wet and dry inoculation at 35°C. Several outliers are evident here; as well as, with the fastest decline rates observed for berries, and the slowest decline rates yielded for broccoli, cauliflower, and tomatoes (data not shown). Because of the technique used to determine the transition from growth to decline, some outlying replicates had non-negative decline rates.

Dry inoculation literature. While the use of dry inoculation for low moisture foods has become increasingly common *(7, 18, 28, 55, 56)*, we have not identified any studies where wet and dry inoculation methods were examined for *L. monocytogenes* on whole raw fruits and vegetables. No prior studies have been found that detail the interaction between wet inocula and food matrix, which may be responsible for promoting pathogen growth. While food matrix is an important factor for determining pathogen survival and growth (as illustrated by the differences between carrots and tomatoes shown in our prior work *(23)*, and here in Figure 2.1), the influence of dry vs. wet inoculation techniques can also have a profound effect on observed growth. While temperature did have a significant effect on both inoculation strategies, this effect was exacerbated when using wet inoculation, often with orders of magnitude difference in *L. monocytogenes* multiplication.

The results obtained in this study support our hypothesis that the same commodities, under the same conditions of temperature, relative humidity, and sampling time, supported significantly different growth of *L. monocytogenes* as a function of inoculation method. Use of a liquid diluent inoculum versus a sand carrier inoculum resulted in markedly increased growth of the pathogen on produce surfaces. This difference was also highly influenced by temperature with a greater effect magnitude observed on more commodities at higher temperatures (22 and 35°C), versus lower temperatures (2 and 12ºC). These finding need to be explored for other commodities and pathogens. The degree to which wet or dry inoculation techniques more realistically mimic contamination

conditions throughout the supply chain (e.g., production, harvest, post-harvest, transportation, or retail) should also be investigated.

3.5. ACKNOWLEDGMENTS

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3.6. SUPPLEMENTAL MATERIALS

Raw data will be available at data.mendeley.com at http://dx.doi.org/10.17632/kh2txp8v7v.1 once the manuscript is accepted.

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3.8. FIGURE LEGENDS

Figure 2.1. Influence of temperature, commodity, and inoculation method on the average maximum growth increase of *L. monocytogenes*. (A) 2°C, (B) 12°C, (C) 22°C and (D) 35°C. Grey bars represent dry inoculation technique and black bars represent wet inoculation technique. Error bars are one standard deviation. Asterisks indicate a significant difference ($P<0.05$) between the two inoculation techniques. An average maximum growth of one log CFU is indicated by the dashed line.

Figure 2.2. Box and whisker plots for square root of growth rates (top) and decline rates (bottom) for all observations across all ten commodities separated by inoculation technique (wet and dry) and incubation temperature. Different letters indicate a different rate between treatments where each unique inoculation technique and temperature represents a different treatment. A small number of decline rates are greater than zero due to the way the rates were calculated.

APPENDIX. Figures

Figure 1.1. Behavior of *L. monocytogenes* inoculated onto whole intact (A) blackberry, (B) raspberry, (C) blueberry, (D) lemon, (E) mandarin orange, (F) tomato, (G) sweet cherry, (H) cauliflower, (I) broccoli and (J) carrot at 2 (▽) and 12 (△) °C over 28 days.

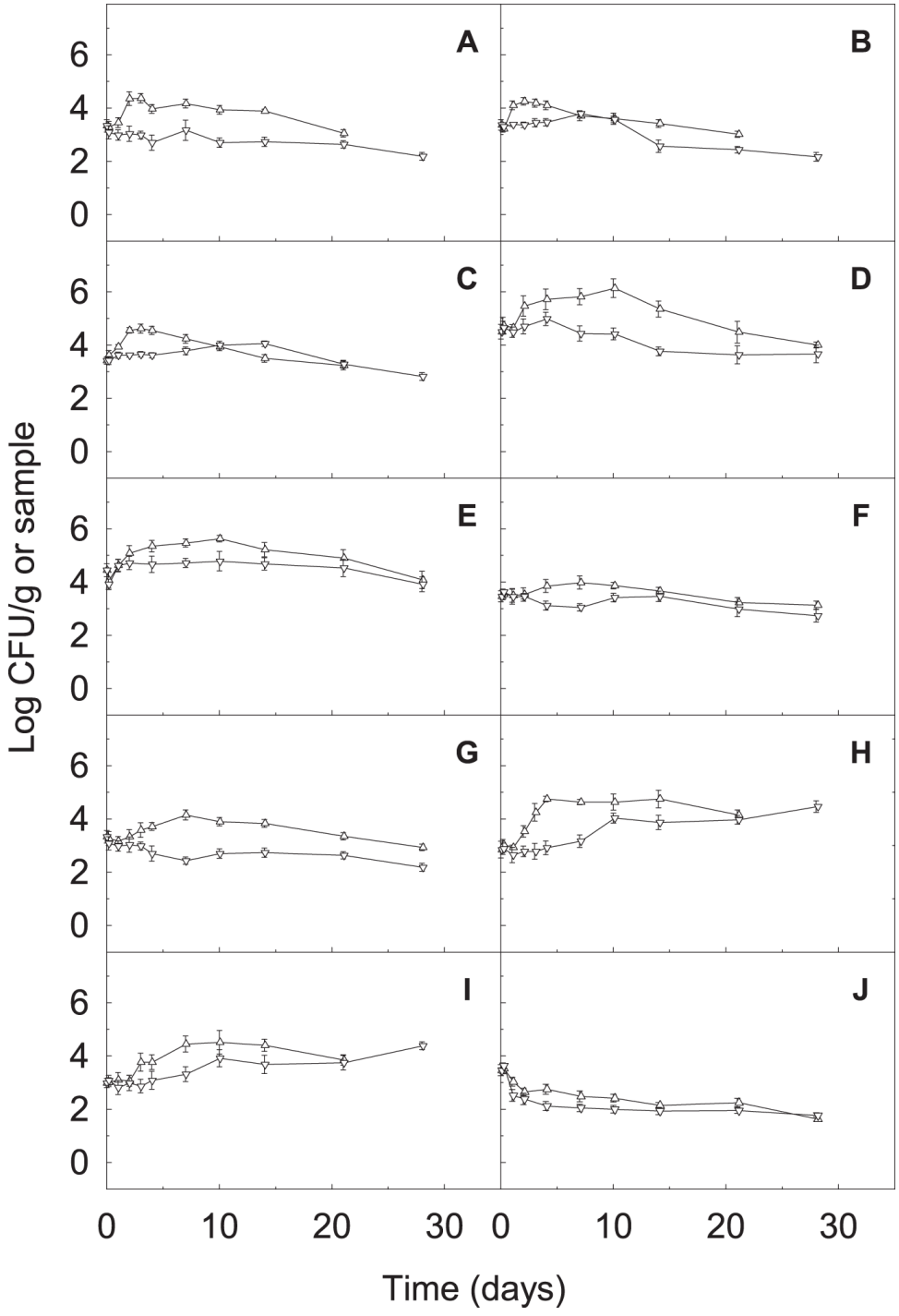


Figure 1.2. Behavior of *L. monocytogenes* inoculated onto whole intact (A) blackberry, (B) raspberry, (C) blueberry, (D) lemon, (E) mandarin orange, (F) tomato, (G) sweet cherry, (H) cauliflower, (I) broccoli and (J) carrot at 22 (▽), 30 (○) and 35 (△) °C over 14 days.

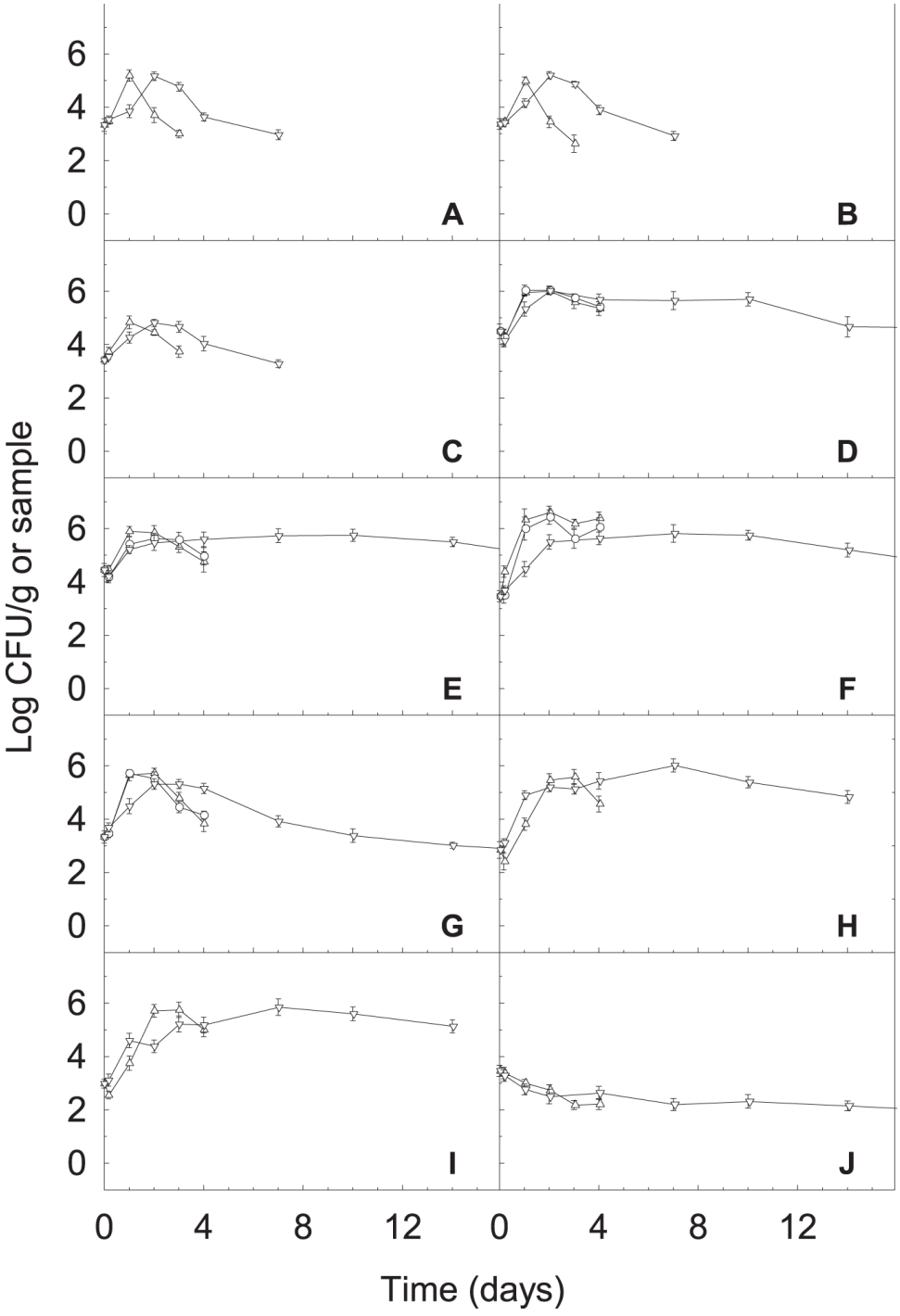
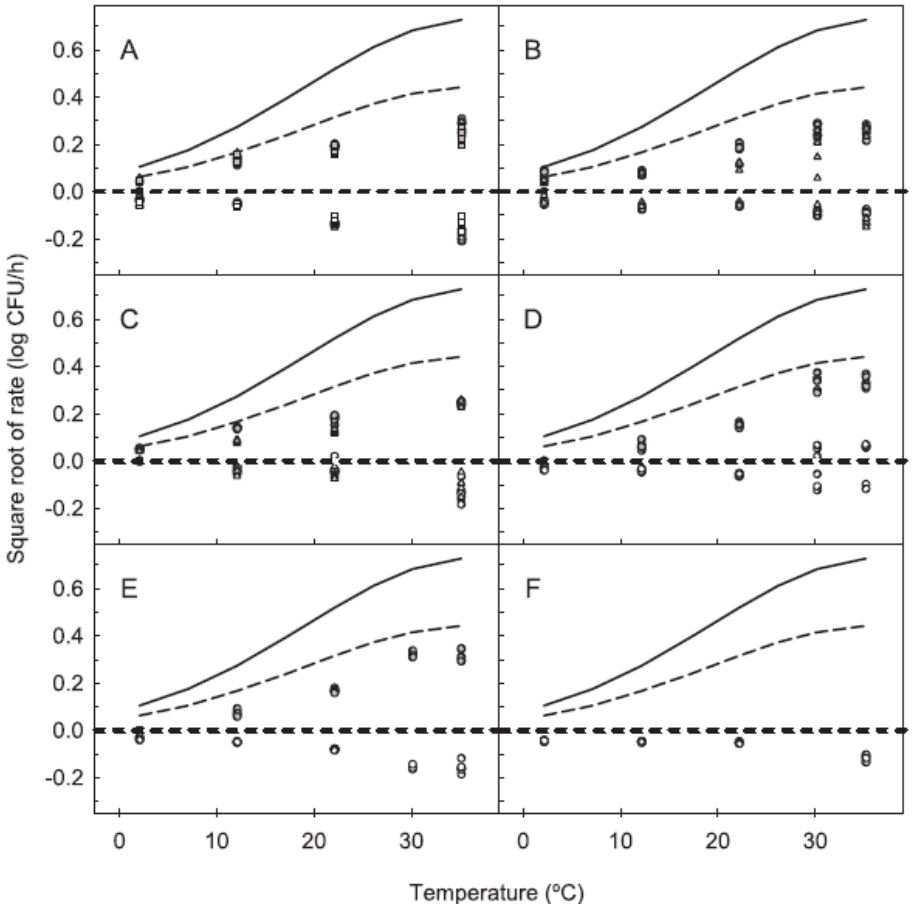


Figure 1.3. Comparison of the square root growth rate predictions of the ComBase Predictor *L. monocytogenes/innoc*ua model at 0.5% NaCl and pH 7 (solid line) or pH 5 (dashed line) and square root of experimentally obtained growth (gray fills) and decline (white fills) rates of *L. monocytogenes* populations on the surface of whole intact (A) berries: blueberries (○), raspberries (△), blackberries (□), (B) citrus: lemon (○), Mandarin orange (△), (C) brassica: cauliflower (○), broccoli (△), (D) tomato, (E) cherry and (F) carrot at different temperatures.



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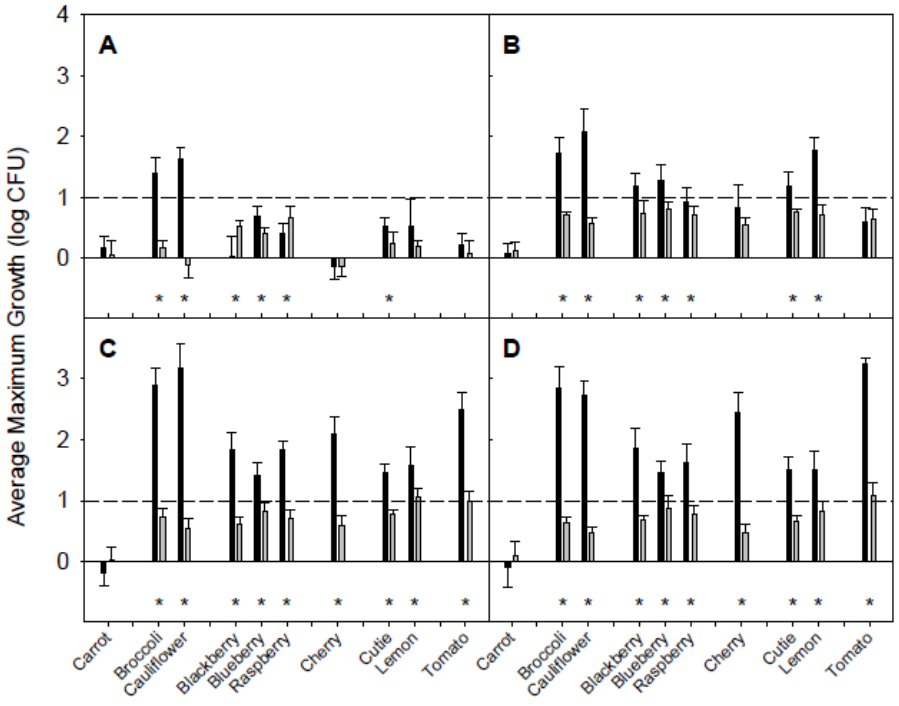

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