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Listeria monocytogenes survival in the presence of malic acid, lactic acid or blueberry extract

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Abstract.
BACKGROUND: *Listeria monocytogenes* represents high risk for consumers, since it can grow under refrigeration and acidic environments by developing acid tolerance response (ATR).
OBJECTIVE: The aim of this work was to study the growth and survival of ATR *L. monocytogenes* strain Scott A in media acidified with malic acid, lactic acid, or blueberry extract.
METHODS: Bacterial growth was evaluated using tryptic soy broth with yeast extract (TSB+YE) combined with the different acid solutions and incubated at 25°C for 24 h. An optical density system measured growth every 15 minutes for 24 h.
RESULTS: Complete inhibition of *L. monocytogenes* occurred in presence of treatments including malic acid pH 2.0 and 3.0; lactic acid pH 2.0, 3.0, and 4.0; and with blueberry extract pH 2.0 in the mixture. No growth was observed in treatments under pH 4.5. Turbidity values of media mixed with blueberry extract at pH 3.0, 4.0, and 5.0 showed no statistical difference at 18 h and growth media had pH of 6.13, 6.53, and 6.78, respectively.
CONCLUSIONS: Lactic acid was more effective inhibiting bacterial growth compared to malic acid. Blueberry extract was not effective acidifying the final pH of the TSB+YE solutions, therefore *L. monocytogenes* survived in media acidified with low pH blueberry extract. Treatments with blueberry extract had the least antibacterial effect in this study.

Keywords: *Listeria monocytogenes*, blueberries, lactic acid, malic acid

1. Introduction

The ability of foodborne pathogenic bacteria to survive under environmental stress, not only inside the host, but also outside in the environment, can lead to an increase in human illnesses. Pathogenic bacteria, including *Listeria monocytogenes*, which has a high fatality rate of approximately 17%, become a threat in products that are not protected from, or properly treated to reduce contamination [1]. For example, in 2011, human listeriosis

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cases attributed to consumption of contaminated cantaloupe, resulted in 33 deaths [2]. The U.S. Food and Drug Administration and U.S. Department of Agriculture Food Safety and Inspection Service have established a zero tolerance for *L. monocytogenes*, meaning no detectable level is permitted in ready to eat foods including minimally processed fresh fruits and vegetables [3, 4]. The pathogen's infectious dose is assumed to be high for most healthy adults, however in the immunocompromised, pregnant women, the elderly and neonates the minimum dose is unknown and those infected are more likely to die [5].

L. monocytogenes is common in fruits and vegetables grown near soil [6]. Moreover, there is an association of this pathogen with acidic fruits and fruit juice products, including unpasteurized apple juice (pH 3.78) and apple/raspberry juice (pH 3.75), cut and whole strawberries, tomatoes, orange juice and orange slices [7–9].

Since 1973 in the US, five bacterial illness outbreaks have been attributed to berries [8, 10] and most of them were related to strawberries contaminated with *Staphylococcus aureus* (1985), *Salmonella* Group B (2003), and enterohemorragic *E. coli* O26 (2006). In this latter case, blueberries were also associated. Although blueberries are considered low risk for foodborne contamination because of their low pH, they were related to a possible outbreak of listeriosis in Connecticut in 1984 [11]. Furthermore, low pH and bacterial acid tolerance are linked to an increased virulence of pathogens [12, 13].

Over the past several years, consumer interest in minimally processed fruits and vegetables has increased because of their freshness, convenience, and healthy attributes. The U.S. per capita food availability of blueberries increased from 0.26 lbs in 2000 to 1.3 lbs in 2011, and the majority are marketed as fresh and not frozen [14]. Fresh blueberries are minimally processed after harvest, refrigerated, and stored for several days, without washing or treatment to inhibit microbes.

L. monocytogenes represents a high risk for consumers since it can adapt and grow not only under refrigeration conditions (2–4°C), but also in high acidity foods, high salinity foods and intracellularly [15, 16]. Moreover, due to hand harvesting, field packing, and non-composted manure fertilization use, blueberries are vulnerable to bacterial contamination in the field [17]. Blueberries have a pH of approximately 3.7 when fresh and 3.1–3.35 when frozen [18]. There are no published studies on growth and survival of acid tolerant *Listeria monocytogenes* on blueberries or blueberry extract. The present study's hypothesis presumes that acid adapted *Listeria monocytogenes* can survive and grow in acidified media, therefore we evaluated the growth of this pathogen cultured in the presence of organic acids (malic and lactic) and blueberry extracts. Malic acid concentration in fresh highbush blueberries can average 0.18% fresh weight [19]. Although there is no lactic acid content in blueberries, this acid was used in this experiment because of its well-known antimicrobial properties. Survival and growth in acid environments with blueberry extracts were monitored to assess the potential health risk for consumers that rely on low pH of these fruit products for bacteriological safety.

2. Materials and methods

2.1. Listeria monocytogenes, induction of acid tolerance response

Listeria monocytogenes strain Scott A (Department of Food Science and Technology Culture Collection, Virginia Tech) was propagated in Trypticase Soy Broth (BBL, BD Diagnostics, Sparks, MD, U.S.A.) with 0.6% yeast extract (Acros Organics, Fair Lawn, NJ) (TSB+YE) and incubated for overnight propagation at 37°C. Afterwards the culture was centrifuged at 2000 × g for 10 min at 4°C and the pellets were suspended in an equal volume of TSB–YE with pH adjusted to 5.7 for 3 h at 37°C, to induce the acid tolerance response according to Caggia [7]. The media acidification was carried out using lactic acid [7]. Cells were then centrifuged, suspended in TSB-YE and incubated for 18 h at 37°C for further propagation.

After incubation at 37°C, 10 mL of each culture was centrifuged (Sorvall Legend RT+ centrifuge, Thermo Scientific, Braunschweig, Germany) at 2000 × g for 10 min at 4°C. After centrifugation, the supernatant was discarded and replaced with 10 mL of 0.1% peptone water. Solutions were vortexed (Fisher Mini-Shaker Model

58, Fisher Scientific, Pittsburgh, PA, U.S.A.) and centrifuged again at 2000 × g for 10 min at 4°C. Finally, supernatant was discarded and the remaining pellet was suspended in 10 mL 0.1% peptone water.

2.2. Preparation of acids and blueberry solution

Highbush blueberries [*Vaccinium corymbosum*] were hand harvested from irrigated plots at the Virginia Tech Kentland Research Farm (Blacksburg, VA, U.S.A.) then stored in a cooler (4–8°C) and transported to the Food Science and Technology Building at Virginia Tech. Blueberries were sorted and only fully developed fruits free of visible defects were selected on a visual basis and stored. The measured mean surface pH of fresh blueberries in this study was 3.83 (data not shown). Blueberries were minced in a blender (Hamilton Beach, Glen Allen, VA, U.S.A.) for 30 s, then the sample was passed through a sifter and centrifuged at 9820 × g for 15 min retaining just the liquid phase. The pH of the blueberry supernatants (initial pH 3.06) were adjusted to pH 2.0, 3.0, 4.0, or 5.0, using malic acid 95% (Sigma Chemicals, St Louis, MO, U.S.A.) and 3 M NaOH. These mixtures were stored at 0°C to avoid enzyme degradation. Malic acid was chosen because of its presence in blueberries. The pH of lactic acid 95% (Fisher Chemicals, Fair Lawn, NJ, U.S.A.) (pK_a 3.85) and malic acid (pK_{a1} 3.40 and pK_{a2} 5.20) solutions (both initial pH 1.2) were fixed to pH 2.0, 3.0, 4.0 and 5.0 using the same acid and/or 3 M NaOH. All samples were sterilized using a 0.45 μm syringe filter (PTFE Puradisc, Whatman Inc., Piscataway, NJ, U.S.A.).

2.3. Automated growth curve analysis

The effects of lactic acid, malic acid and the blueberry solutions on the growth of *L. monocytogenes* strain Scott A, were tested by a Bioscreen C Microbiology Reader (Growth Curves, Piscataway, NJ, U.S.A.), equipped with an incubator and automated turbidimeter to determine optical density (O.D.) between 420–540 nm. Optical density measurement technique can be quicker, more convenient and more precise than other methods to measure growth changes over time. Each well of a sterilized 100 microwell plate (Growth Curves, Piscataway, NJ, U.S.A.) was filled with 125 μl of TSB+YE pH 7.0 as growth liquid medium, 15 μl of pH fixed prepared acid or extract, and 10 μl of bacterial culture (final concentration of approx. 10^7 CFU/ml). For negative acid controls (uninoculated), wells were filled with 135 μl of growth medium and 15 μl of pH fixed prepared acid or berry solution. For *L. monocytogenes* controls, wells were filled with 140 μl of growth medium and 10 μl of culture. Negative *L. monocytogenes* control wells were filled with 150 μl of growth medium. The mean of optical density (O.D.) of the uninoculated control wells were subtracted from the O.D. of the pathogen inoculated wells for each measurement [20], to obtain only turbidity values due to pathogen growth. Samples were labeled A, B, C or D corresponding to the pH 2.0, 3.0, 4.0 or 5.0 solution used in the mixture, respectively (Table 1). Microwell plates were incubated at 25 ºC for 24 h, and O.D. was measured every 15 min with 10 sec of shaking before each reading. This incubation temperature was set to challenge bacterial growth under common room temperatures rather than 37°C which is an ideal temperature for *Listeria* propagation. Data was generated using EZExperiment software (Growth Curves, Piscataway, NJ, U.S.A.) and exported as a spreadsheet (Microsoft Excel, Seattle, WA, U.S.A.).

2.4. Experimental design and statistical analysis

Optical density data were analyzed using the Generalized Linear Model (GLM) procedure of SAS (V. 9.13, Statistical Analysis Systems Institute, Inc. 2006). The randomized complete block factorial design with three replications was utilized to test the treatments and their interactions on microbial growth (BioScreen turbidity). All the tests used four samples for each treatment (malic acid, lactic acid and blueberry extract) and different mixtures (A, B, C or D). If the interactions between treatments were not significant ($P > 0.05$), the main effects of the treatments were separated by the Least Significant Difference test using the interaction as the error term.

Table 1
Treatment solutions pH before inoculation with 10 μl of *Listeria monocytogenes* in 0.1% peptone water

Treatment	Acid Solution	Growth Media (pH 7.0)	Treatment pH
Malic A	15μl of Malic acid pH 2.0	125 μl of TSB+YE	3.04 ± 0.02
Malic B	15μl of Malic acid pH 3.0	125 μl of TSB+YE	3.67 ± 0.04
Malic C	15μl of Malic acid pH 4.0	125 μl of TSB+YE	4.54 ± 0.04
Malic D	15μl of Malic acid pH 5.0	125 μl of TSB+YE	5.76 ± 0.10
Lactic A	15μl of Lactic acid pH 2.0	125 μl of TSB+YE	3.02 ± 0.02
Lactic B	15μl of Lactic acid pH 3.0	125 μl of TSB+YE	3.53 ± 0.07
Lactic C	15μl of Lactic acid pH 4.0	125 μl of TSB+YE	4.30 ± 0.08
Lactic D	15μl of Lactic acid pH 5.0	125 μl of TSB+YE	6.04 ± 0.11
Berries A	15μl of Blueberry extract pH 2.0	125 μl of TSB+YE	3.85 ± 0.03
Berries B	15μl of Blueberry extract pH 3.0	125 μl of TSB+YE	6.13 ± 0.05
Berries C	15μl of Blueberry extract pH 4.0	125 μl of TSB+YE	6.53 ± 0.02
Berries D	15μl of Blueberry extract pH 5.0	125 μl of TSB+YE	6.78 ± 0.01

3. Results and discussion

Turbidimetry is an established predictive microbiology method used to study bacterial growth based on O.D. measurements, which makes it possible to follow bacterial population growth in real time [20]. Some authors have attempted to derive growth parameters from O.D. measurements and found a linear correlation with viable *Listeria monocytogenes* plate counts, especially in the exponential growth phase [21, 22]. Although O.D. is not directly equivalent to bacterial counts, it can be translated but requires many calibration curves to be accurate.

According to Hudson [23], to avoid the error in measuring the time to reach a detectable turbidity, the initial inoculum must be high enough to measure the turbidity. In case turbidity is not measurable, the calculated lag time becomes the period taken for the culture to reach detectable turbidity. In this study, the initial bacterial inoculum concentration was approximately 10^7 CFU/ml, while the initial O.D. measured in uninoculated medium was 0.125 for malic acid, 0.133 for lactic acid, 0.277 for blueberry extract and 0.143 for the positive control which are values before background correction.

Growth curves of *L. monocytogenes* in TSB+YE varied among acid treatments (Fig. 1). Malic A, Malic B, Lactic A, Lactic B, Lactic C and Berries A treatments were not included in this graph since they completely inhibited pathogen growth. Pathogen survival and growth in acid treatments Malic D, Lactic D, Berries B, Berries C and Berries D was considerable and included distinct lag, exponential and stationary phases, but were inferior when compared to the *L. monocytogenes* positive control (Control).

Despite published reports indicating that the minimum pH for growth in laboratory conditions is pH 4.4 to 4.5 [24, 25], and from 5.0 to 5.5 when pH of the media is adjusted with lactic acid [26], other studies have described the ability of *L. monocytogenes* to respond and to survive at even lower pH values [27, 28, 29]. Acid tolerance response (ATR) in *L. monocytogenes* can be induced by exposing the organism to mild acidic conditions, such as pH of 5.5 (1 M lactic acid). Such treatments make the cells resistant to severe acidic conditions [30]. The acid response upon exposure of cells to an acidic pH involves several changes in the cell such as protein induction, pH homeostasis and activation of the glutamate decarboxylase system [15]. A study of the role of the glutamate decarboxylase in the acid resistance of *L. monocytogenes* during gastric transit using synthetic human and porcine gastric fluid concluded that addition of glutamate increased the survival of the wild type strain in gastric fluid, creating a concern about contamination in foods containing glutamate [31]. Proteins such as GroEL, ATP synthase, and various transcriptional regulators showed increased synthesis under acid stress growth [32].

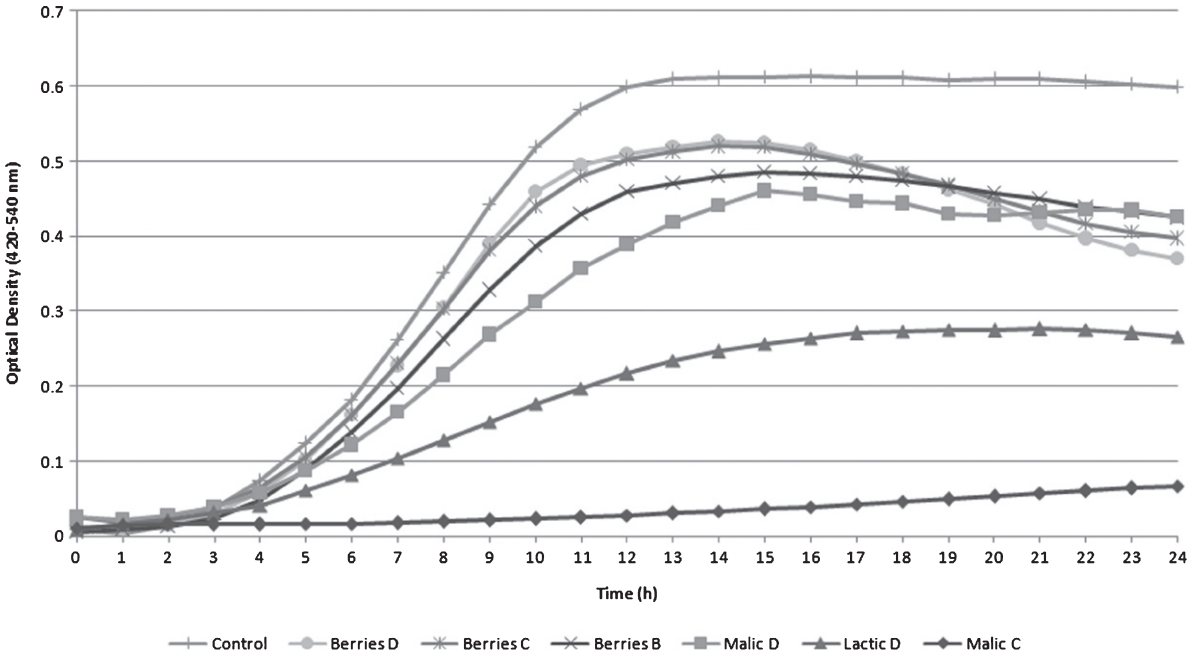

Fig. 1. *Listeria monocytogenes* Scott A optical density turbidity growth curve measured using tryptic soy broth with yeast extract (TSB+YE) under different treatments (B, C, or D) with different acids (malic acid, lactic acid and blueberry extract) incubated at 25°C for 24 h.

F_0F_1-ATPase is a multi-subunit enzyme involved in the acid adaptation of *L. monocytogenes*, serving as an ATP driven proton channel across the cell membrane, helping maintain cellular pH homeostasis [33]. An inadequately maintained intracellular homeostasis can be the cause of loss of cell viability [34]. Gahan [35] compared acid-adapted and non-adapted *L. monocytogenes* survival in a variety of acidic food products using a constitutive acid tolerant mutant isolated by prolonged exposure to pH 3.5 (3 M lactic acid). The acid-adapted strain and the acid tolerant mutant demonstrated better survival in commercial natural yogurt and cottage cheese made in a laboratory. Throughout the ripening of hard cheeses, the acid tolerant mutant showed enhanced resistance and the authors were able to recover pathogens after the 70-day ripening period. Moreover, during milk fermentation by *Streptococcus thermophilus*, the acid-adapted strain demonstrated enhanced survival compared to the non-adapted culture. The *L. monocytogenes* acid tolerant mutant also survived similar to the acid-adapted cells during the first 4 h of fermentation. In the present study no bacteria survived at a pH lower than 4.54.

After 6 h, turbidity measurements were significantly different among the majority of the survival treatments and the *L. monocytogenes* control during the exponential growth phase (Table 2). The positive Control showed the highest O.D. value (0.18) after 6 h of incubation, while Malic C was the lowest (0.02). Turbidities of Berries C and D were not significantly different at 6 h, a trend also observed at 12 and 18 h. Between 12 and 18 h, nearly all *L. monocytogenes* solutions ceased to grow as the stationary phase was reached. The only exception was Malic C, where growth inhibition was observed, without a specific lag and exponential growth phase.

Acid dissociation constants (pK_a) are linked to antimicrobial activity of organic acids against *L. monocytogenes* as well as with greater permeability of weak acids in their undissociated form through the cell membrane [36]. When the solution pH is lower than the pK_a of an acid, the majority of acid in the solution will be undissociated and will be able to pass through the lipid membranes, unlike the dissociated (ionized) form which cannot. In this study, lactic acid solutions were more efficient reducing pathogen growth when compared to malic acid solutions

Table 2
Mean and standard deviation of optical density turbidity growth curve values ($n = 12$) for *Listeria monocytogenes* Scott A in TSB+YE under acid treatments incubated at 25°C for 24 h

Treatment	O.D. $_{420–540\,nm}$			
	6 hours	12 hours	18 hours	24 hours
Control	0.18 ± 0.05^{A}	0.60 ± 0.05^{A}	0.61 ± 0.03^{A}	0.60 ± 0.03^{A}
Berries D	0.16 ± 0.06^{B}	0.51 ± 0.02^{B}	0.48 ± 0.04^{B}	0.37 ± 0.02^{D}
Berries C	0.16 ± 0.05^{B}	0.50 ± 0.04^{B}	0.48 ± 0.04^{B}	0.40 ± 0.02^{C}
Berries B	0.14 ± 0.05^{C}	0.46 ± 0.05^{C}	0.47 ± 0.04^{B}	0.42 ± 0.04^{B}
Malic D	0.12 ± 0.07^{D}	0.39 ± 0.10^{D}	0.44 ± 0.09^{C}	0.43 ± 0.08^{B}
Lactic D	0.08 ± 0.07^{E}	0.22 ± 0.14^{E}	0.27 ± 0.14^{D}	0.27 ± 0.12^{E}
Malic C	0.02 ± 0.01^{F}	0.03 ± 0.02^{F}	0.05 ± 0.03^{E}	0.07 ± 0.04^{F}

Means within each column followed by the same letter are not significantly different ($P > 0.05$) from each other. Abbreviations: Positive *Listeria monocytogenes* Scott A Control (Control), Blueberry extract (Berries), Malic acid (Malic) and Lactic acid (Lactic).

at the same pH. At same pH lactic acid has a pK_a of 3.85, while malic acid has a pK_{a1} of 3.40. Since lactic acid has the higher pK_a, it would have higher amounts of undissociated molecules. Their accumulation in the cells will lead to lower intracellular pH values and deregulation of metabolic activity of the cell, causing higher inhibitory activity. In aerobic bacteria, the active transport of protons is associated with the electron transport process in respiratory chains with three major constituents, dehydrogenases, quinines and oxidoreductases. In anaerobic bacteria, proton transport occurs through a specific *H+* channel in the F_0F_1-ATPase molecule using energy from ATP hydrolysis. As a facultative bacterium, *Listeria* may use both processes to control its intracellular pH homeostasis [32].

Eswaranandam et al. (2004) studied the effectiveness of partial replacement of glycerol with citric, lactic, malic, and tartaric acids on the antimicrobial activities of nisin (205 IU/g protein) incorporated soy protein film against *L. monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Gaminara [37]. In that study, organic acids such as malic and lactic acids had greater antimicrobial activity than citric acid. Moreover, Phan-Thanh [32] also noted that organic acids were more lethal at low pH than inorganic acids. This appeared to be due to their ability to alter the internal pH of the cell (pH_i) of L. monocytogenes to a lower pH value than that observed with inorganic acids. Miller [38] indicated that weak organic acids are more efficient against *L. innocua* than a stronger acid, such as HCl, at the same pH. The effectiveness of malic and lactic acid against *L. monocytogenes* has been previously demonstrated [6] and confirmed in this study.

Except for Berries A, *L. monocytogenes* grew in all blueberry extract treatments. Turbidity of Berries C and D solutions showed no significant difference until 24 h. At 24 h, the O.D. growth between the Malic D and Berries B treatments were not significantly different ($P > 0.05$).

Wang et al. (2008) reported a total phenolic of 319.3 and 190.3 mg/100 g of fwt (fresh weight) blueberries from organic and conventional cultures, respectively. And a total anthocyanin content of 131.0 and 82.36 mg/100 g fwt blueberries from organic and conventional cultures, respectively [39]. The antimicrobial activity of phenolic compounds and anthocyanins from berries has been demonstrated [40]. However, several studies show that the growth of *Listeria* species are not inhibited by berry phenolics [41, 42, 43, 44]. In this study, blueberry extract showed antimicrobial activity against *L. monocytogenes* only when the pH was low, such as in Berries A (pH 3.85), while pathogen growth was observed in treatments Berries B, C and D with pH 6.13; 6.53 and 6.78, respectively (Table 2). These acidic solutions containing blueberry extract resisted the decrease of pH expected, unlike what occurred with the organic acids. Moreover, considering that blueberry extract contains acids, nutrients, minerals and water, it is understandable why the same effects were not seen when compared with malic and lactic acid, which were concentrated acids.

4. Conclusions

Since consumers are demanding more natural food products, the use of natural food ingredients such as acidic juices may be an attractive option to control pathogen growth and prevent foodborne illnesses and outbreaks. However, in this study blueberry extract was ineffective in lowering pH of growth solution when compared to malic and lactic, hence presenting a limited inhibition of *Listeria monocytogenes*. Additionally, this research confirms that *Listeria monocytogenes* can adapt to acid-stress conditions and survive at pH 4.5. More research is required to understand the effectiveness of blueberry extracts as a natural antimicrobial for food, not considering inhibition due to low pH of the solutions. Even though current research results showed that blueberry extract exhibited poor antibacterial effectiveness on media, for future work this could be evaluated on a food for confirmation.

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