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Effect of growth and recovery temperatures on pressure resistance of *Listeria monocytogenes*

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ABSTRACT

Experimental conditions can affect the outcome of bacterial stress-tolerance assays. Growth conditions that optimize microbial recovery should be established to help evaluate the effectiveness of treatment conditions for food safety. The objectives of this study were to determine the effects of growth and recovery temperatures on pressure resistance of early stationary-phase *Listeria monocytogenes* in milk. The tested conditions were the following: (1) *L. monocytogenes* was grown at various temperatures (10, 15, 20, 25, 30, 35, 40 and 43 °C), suspended in ultra-high temperature (UHT) -processed whole milk, pressure-treated at 400 MPa for 2 min at 21 °C and recovered on Tryptic Soy Agar supplemented with 0.6% yeast extract (TSAYE) at 35 °C; (2) *L. monocytogenes* was grown at 35 and 43 °C, pressure treated in milk (400 and 500 MPa, respectively, for 2 min at 21 °C) and recovered on TSAYE at various temperatures (4, 10, 15, 20, 25, 30, 35 and 40 °C); (3) *L. monocytogenes* originally grown at 35 °C, was pressure treated in milk (400 or 450 MPa for 2 min at 21 °C), and recovered on TSAYE at 10 °C for various time intervals (1, 2, 3, 6, 9 and 12 days) then at 35 °C for 5 days. There was no significant difference ($P>0.05$) in pressure-resistance of *L. monocytogenes* grown at 10 to 25 °C with approximately 6.5-log CFU/ml population reductions. At growth temperatures greater than 25 °C, pressure resistance increased with less than 1-log CFU/ml reduction observed for *L. monocytogenes* originally grown at 43 °C. After pressure treatment, regardless of growth temperature and pressure treatment, the greatest recovery of *L. monocytogenes* was within the 4 to 20 °C range; maximum recovery at 10 °C required approximately 24 days. The time for comparable post-pressure treatment recovery could be reduced by incubation at 10 °C for at least 2 days followed by incubation at 35 °C for 5 days. The findings of the present study indicate that growth and recovery temperatures affect the pressure resistance of *L. monocytogenes* and should, therefore, be taken into account when assessing the adequacy of inactivation treatments.

1. Introduction

Ingestion of food contaminated with *Listeria monocytogenes* can result in serious illness affecting the gastrointestinal and nervous systems and can lead to miscarriage in pregnant women (Gandhi and Chikindas, 2007). *L. monocytogenes* is particularly problematic for food safety assurance due to its tolerance of environmental hurdles including its ability to grow at refrigeration temperatures (Gandhi and Chikindas, 2007; Wemekamp-Kamphuis et al., 2002) commonly used to prevent the growth of organisms of significance for food quality and public health. The pathogen has complicated a number of different food systems including those that do not receive a processing treatment lethal to *L. monocytogenes* as well as foods that have been inadvertently contaminated after processing. Milk and dairy products are among the foods that have been associated with *Listeria* contamination (Gandhi and Chikindas, 2007). This can arise from cows with udder infections or contaminated equipment throughout collection and processing (Linton et al., 2008).

Inactivation of *L. monocytogenes* in milk can be achieved with heat pasteurization in conjunction with good handling practices (Farber et al., 1992). High hydrostatic pressure has been evaluated as an alternative method for processing milk and other dairy products (Linton et al., 2008). A notable degree of inactivation of *L. monocytogenes* (Chen, 2007a; Dogan and Erkmen, 2004; Erkmen and Dogan, 2004; Simpson and Gilmour, 1997), and other pathogens (Chen, 2007a) in milk has been demonstrated with high pressure, although some pathogenic bacteria have demonstrated considerable barotolerance in milk (Patterson et al., 1995). Pressure inactivation of *L. monocytogenes* in milk is affected by a number of factors including bacterial strain (Chen et al., 2009), stage of growth (Hayman et al., 2007; McClements et al., 2001; Wen et al., 2009), growth conditions (Bull et al., 2005; Hayman et al., 2007; McClements et al., 2001), pressure level (Erkmen and Dogan, 2004), treatment time (Erkmen and Dogan, 2004), imposition of other stresses (Hayman et al., 2008; Simpson and Gilmour, 1997; Wemekamp-Kamphuis et al., 2002), and recovery conditions (Bozoglu et al.,

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2004; Bull et al., 2005; Koseki et al., 2008). Unless the conditions for maximal barotolerance and full recovery of injured bacteria are established for treatment of a food system of interest, survivors may not be detected and process requirements underestimated.

With particular interest to growth and recovery temperature effects, Bull et al. (2005) reported that prior growth temperature affects pressure resistance, but interactions of temperature, milk type, and pressure confounded interpretation of the effects. Hayman et al. (2007) reported that *L. monocytogenes* grown at the upper limit of its growth range (43 °C) was more pressure-resistant than when grown at optimal (35 °C) or reduced temperature (4 °C). After growth at temperatures closer to optimal, both short-term cold- (Wemekamp-Kamphuis et al., 2002) and heat-shock (Hayman et al., 2008) treatments increased the barotolerance of *L. monocytogenes* in milk. Temperature during recovery after pressure treatment of milk is also an important consideration as Bull et al. (2005) reported that, of three temperatures evaluated, 15 °C provided better recovery of *L. monocytogenes* than 4 °C or 30 °C, though the specific number of survivors was not determined. Bozoglu et al. (2004) found that detection of injured *L. monocytogenes* occurred more rapidly at 30 °C than 4 °C, though the percent recovery was not determined. Prolonged storage of pressure-treated milk supported the recovery and growth of *L. monocytogenes* better at 4 °C and 25 °C than at 37 °C (Koseki et al., 2008). These studies indicate the importance of growth and recovery temperatures and time in the determination of adequate pressure processing parameters. Although trends are apparent, the variations in findings and approaches also demonstrate the value of a study that compares the magnitude of post-pressure treatment recovery over prolonged time as affected by the broad range of temperatures that support growth and recovery of *L. monocytogenes*.

The study presented herein builds on the findings of previous reports with specific objectives to (1) determine the relationship between pressure resistance and temperature of growth of *L. monocytogenes* throughout its growth range prior to pressure treatment, (2) determine the optimum incubation temperature throughout its growth range for maximal recovery of *L. monocytogenes* after pressure treatment, and (3) minimize incubation time through temperature shift after pressure treatment for maximum recovery of *L. monocytogenes*. The pressure conditions utilized permit *L. monocytogenes* survival such that differences among growth and recovery temperatures could be evaluated and compared to studies previously conducted.

2. Materials and methods

2.1. Determination of time to stationary phase

L. monocytogenes ATCC 19115 was grown on Tryptic Soy Agar (Becton Dickinson and Co., Sparks, MD) supplemented with 0.6% yeast extract (Becton Dickinson and Co., Franklin Lakes, NJ, USA) (TSAYE) for approximately 17 h at 37 °C. A colony was transferred to 10 ml Tryptic Soy Broth (containing dextrose, Becton Dickinson) supplemented with 0.6% yeast extract (TSBYE) and incubated at 35 °C for approximately 17 h. A loopful (10 µl) of the overnight culture was subcultured in fresh TSBYE (100 ml) and incubated at 4, 10, 15, 20, 25, 30, 35, 40 and 43 °C. The time to reach early stationary phase at each temperature was determined by periodic sampling with serial dilution in 0.1% peptone water (BD/Difco Laboratories, Detroit, MI), spread-plating on TSAYE and incubating plates at 35 °C for 72 h.

2.2. Effect of growth temperature on pressure resistance

L. monocytogenes was grown at 4, 10, 15, 20, 25, 30, 35, 40 or 43 °C to early stationary phase in TSBYE. Thirty-five milliliters were centrifuged for 15 min at 6318×*g* (IEC Centra-4B Centrifuge, International Equipment Co.), washed once with 0.1 M sodium phosphate buffer, pH 7.0 (Fisher Scientific, Fair Lawn, NJ), and resuspended in 35 ml of ultra-high temperature (UHT) processed whole milk. The initial count of *L. monocytogenes* in milk was approximately 10^9 CFU/ml. Milk (12 ml) was transferred to a sterile 3-mil thick polypropylene pouch (VWR International, West Chester, PA, USA), heat-sealed, and sealed in a secondary pouch for added safety. Samples were treated in an Avure PT-1 laboratory-scale pressure unit (Avure Technologies Inc., Kent, WA, USA) monitored with DASY*Lab* ® 7.0 software (DASYTEC USA, Bedford, NH). Samples were treated at 400 MPa for 2 min (not inclusive of come-up time of less than 30 s with almost instantaneous depressurization) at 21 °C with water as the surrounding pressure-transmitting medium. Enumeration of survivors was conducted by duplicate spread plating of appropriate serial dilutions (in 0.1% peptone water) on TSAYE and incubating at 35 °C for 72 h. Three independent trials were conducted.

2.3. Effect of temperature on recovery of *L. monocytogenes* from pressure-treated samples

L. monocytogenes was grown at two temperatures (35 and 43 °C) to early stationary phase in TSBYE, centrifuged, washed and resuspended in UHT whole milk as previously described. Milk samples were treated at 400 MPa and 500 MPa for cultures grown at 35 and 43 °C respectively, for 2 min at 21 °C. Enumeration of survivors was conducted by duplicate spread plating of appropriate serial dilutions (in 0.1% peptone water) on TSAYE and incubating at 4, 10, 15, 20, 25, 30, 35 and 40 °C until no change in counts was observed. Colonies that developed during prolonged incubation on TSAYE were periodically streaked onto modified Oxford agar (Difco Laboratories, Sparks, MD, USA) to check for growth characteristic of *L. monocytogenes*. Three to four independent trials were conducted.

2.4. Effect of incubation temperature shift on recovery time

L. monocytogenes was grown to early stationary phase at 35 °C, centrifuged, washed, and resuspended in UHT whole milk as previously described. Milk samples were treated at 400 and 450 MPa for 2 min at 21 °C. Survivors were enumerated on TSAYE incubated at 10 °C for various time intervals (1, 2, 3, 6, 9 and 12 d), followed by incubation at 35 °C for 5 d. Three independent trials were conducted.

2.5. Statistical analyses

Statistical analyses were conducted using Minitab® Release 15 (Minitab Inc., University Park, PA, USA). One-way analysis of variance (ANOVA) was used to determine the significance of differences between treatments ($P<0.05$). For multiple comparisons, Tukey's One-Way Multiple Comparisons Test was used (family error rate = 0.05).

3. Results

3.1. Determination of time to stationary phase

The time to reach early stationary phase with approximately 10^9 CFU/ml at 4, 10, 15, 20, 25, 30, 35, 40 and 43 °C was 384, 96, 48, 36, 18, 12, 12, 12 and 16.25 h, respectively. These incubation times were used for all subsequent experiments.

3.2. Effect of growth temperature on pressure resistance

There was no significant difference ($P>0.05$) in pressure-resistance of *L. monocytogenes* grown within the range of 10 to 25 °C. With these growth temperatures, approximately 6.5-log CFU/ml population reduction after treatment at 400 MPa for 2 min at 21 °C and recovery at 35 °C (Fig. 1) was observed. Pressure resistance increased as the growth temperatures increased from 25 to 43 °C. Less than a 1-log CFU/ml reduction was observed after pressure treatment when *L. monocytogenes* was grown at 43 °C (Fig. 1).