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Bi-phasic growth of *Listeria monocytogenes* in chemically defined medium at low temperatures

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ABSTRACT

The present work reports a novel observation regarding the growth of *L. monocytogenes* in modified Welshimer's broth (MWB) at low temperatures. Specifically, the direct monitoring of the growth of *L. monocytogenes* Scott A using plate count data revealed that the pathogen displays a bi-phasic growth pattern in MWB at 7 °C. This bi-phasic growth pattern is masked (not observed) when optical density (OD) measurements are used to monitor growth due to the inability of OD readings to detect *L. monocytogenes* population density increases up to 10^7 CFU/mL. This bi-phasic growth phenomenon was further investigated as a function of growth temperature (4 °C, 7 °C, 10 °C, 14 °C and 18 °C), medium composition (by altering the MWB composition by ten-fold increases in different sets of medium constituents), inoculum level (10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU/mL) and *L. monocytogenes* strain (10 strains). The growth of *L. monocytogenes* Scott A in MWB at 7 °C, 10 °C and 14 °C was consistently bi-phasic and independent of growth rate; at 18 °C, growth was consistently mono-phasic (single-phase, typical sigmoid growth curves), whereas no growth was observed at 4 °C. The tested modifications in the composition of MWB did not influence the bi-phasic nature of *L. monocytogenes* Scott A growth at 7 °C, and, overall, we could not point out any strain-, or serotype-specific effects. On the other hand, the initial inoculum level appears to affect the form of the growth curve, as there was a shift towards mono-phasic growth in trials with increasing initial inocula. A mathematical model, based on a stepwise response and described through two sequential sigmoid curves, was used to describe bi-phasic growth and estimate the kinetic parameters of *L. monocytogenes* growth. An alternative hypothesis, based on the assumption of the existence of two subpopulations, possessing different growth kinetics, materialized under the stress imposed on *L. monocytogenes* cells due to the combined effect of three factors (defined medium, low temperature and low initial inoculum) was also proposed and formulated.

1. Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen and the causative agent of listeriosis, a disease that can be particularly severe or even fatal for certain high-risk population groups such as neonates, the elderly, and people with a compromised immune system. Contamination of food with low levels of *Listeria* spp. is not an uncommon event (Ryser and Marth, 2007). *L. monocytogenes* is psychrotrophic and as such, it can proliferate at low temperatures both in laboratory media as well as in certain categories of permissive foods. Therefore, considerable research has been conducted in the last decade in order to elucidate the behavior of *L. monocytogenes* under chill stress and reveal the pathogen's underlying physiological adaptive mechanisms (Cacace et al., 2010; Tasara and Stephan, 2006).

Defined media are used in (food) microbiology in order to conduct reproducible experiments and avoid confounding by extraneous, often unknown factors originating from the composition of the growth medium. Modified Welshimer's broth (MWB) is the chemically defined medium that has been most frequently used by researchers working on the physiology and genetics of *L. monocytogenes* (Premaratne et al., 1991). The composition of MWB is rather simple. MWB was developed by modifying Welshimer's medium (Welshimer, 1963) and it consists of three salts, two of which are phosphate salts added for buffering purposes (KH_2PO_4, $Na_2HPO_4{\cdot}7H_2O$, and $MgSO_4{\cdot}7H_2O$), ferric citrate as an iron source, glucose as the sole carbon and energy source, seven amino acids (Leu, Ile, Val, Met, Arg, Cys, and Gln) and four vitamins (riboflavin, thiamine, biotin, and thioctic acid).

The present work reports on a novel observation regarding the growth of *L. monocytogenes* in MWB. Specifically, the direct monitoring of the growth of *L. monocytogenes* by surface-plating aliquots from cultures at selected time intervals on rich, non-selective solid medium

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revealed that the pathogen displays a bi-phasic growth pattern in MWB at low temperatures. In these experiments, *L. monocytogenes* Scott A was inoculated in MWB at an initial inoculum of 10^3 CFU/mL and the observed growth curve displayed five distinct segments. For instance, at 7 °C, the initial lag phase was followed by exponential growth, which, after a *ca*. 2-log increase went into an apparent stationary phase. However, after approximately 20 days of incubation, a second exponential growth phase commenced, resulting in final population densities of about 10^8 to 10^9 CFU/mL.

From a bacterial physiology perspective, the precise knowledge of the phase in the growth curve of *L. monocytogenes* cultures is very important, as the growth phase influences gene expression and consequently also affects the pathogen's adaptive responses to various environmental stresses (Chan et al., 2007; Folio et al., 2004; Schwab et al., 2005; Weeks et al., 2004). Furthermore, the knowledge of the growth characteristics of *L. monocytogenes* at constant temperatures is essential before attempting to predict its behavior in more complex systems under dynamic temperature conditions (Baranyi et al., 1995).

To our knowledge, bi-phasic patterns have not been previously reported for *L. monocytogenes* growth in defined or complex media. The reason for this, to-date, unprecedented phenomenon is likely the fact that, for convenience purposes, most researchers: a) either rely on (manual or automated) optical density (OD) readings as an indirect means of estimating bacterial cell density, or b) when relying on colony counts (*i.e*., direct means of estimating bacterial cell density), obtain data points that are limited either with respect to the extent of the examined time period or in terms of the sampling frequency. Therefore, the aim of the current work was to explore potential factors that could lie behind the bi-phasic growth of *L. monocytogenes* in MWB at low temperatures, and determine the pathogen's kinetic parameters of growth as a function of storage temperature, inoculum level, medium composition and bacterial strain.

2. Materials and methods

2.1. Bacterial strains

Ten *L. monocytogenes* strains were used in this work and all were part of the International Life Sciences Institute North America (ILSI NA) *L. monocytogenes* strain collection (Fugett et al., 2006). The strains used were selected in order to a) cover a wide variety of *L. monocytogenes* strains in terms of their original source of isolation (human, animal, food), as well as in terms of the *L. monocytogenes* genetic lineage and serotype and b) include the *L. monocytogenes* strains comprising the recommended five-strain set used for challenge testing of foods (Fugett et al., 2006). The selected strains were the following: FSL J1-225 (Scott A, ILSI NA no 1, lineage I, serotype 4b); FSL J1-177 (ILSI NA no 7, lineage I, serotype 1/2b); FSL C1-056 (ILSI NA no 11, lineage II, serotype 1/2a); FSL J1-094 (ILSI NA no 17, lineage II, serotype 1/2c); FSL J1-158 (ILSI NA no 24, lineage III, serotype 4b); FSL N3-013 (ILSI NA no 29, lineage I, serotype 4b); FSL N3-031 (ILSI NA no 33, lineage II, serotype 1/2a); FSL R2-499 (ILSI NA no 35, lineage II, serotype 1/2a); FSL N1-227 (ILSI NA no 36, lineage I, serotype 4b); and FSL R2-502 (ILSI NA no 39, lineage I, serotype 1/2b). For long-term storage strains were kept frozen at −26 °C in vials containing porous beads (Microbank™, Pro-Lab Diagnostics, ON, Canada).

2.2. Inoculum preparation, inoculum level, growth temperature, and growth monitoring

L. monocytogenes strains stored in the freezer were revived in Brain Heart Infusion Broth (BHIB, Biolife Italiana S.r.l., Milan, Italy) and subsequently streaked onto Brain Heart Infusion Agar (BHIA, Biolife) plates. For inoculum preparation, a single colony from BHIA was used to inoculate BHIB (30 °C, 18 h). In order for the cells to adapt to the MWB medium conditions, fully-grown BHIB cultures (1 mL) were centrifuged, the pellets were washed twice with equal volumes of sterile MWB and the washed cell suspension was used to inoculate (1%) 125-mL flasks containing 50 mL MWB. The cultures were incubated at 30 °C under mild shaking (120 rpm), until early stationary phase to cell densities of *ca*. 10^9 CFU/mL. Stationary phase cultures were then serially diluted in MWB to achieve the target initial inoculum level (*ca*. 2, 3, 4, 5, 6 or 7 log(CFU/mL)) and used to inoculate sets of 125-mL flasks containing 50 mL of MWB. These cultures were incubated aerobically under mild shaking (120 rpm) at constant temperature (4 °C, 7 °C, 10 °C, 14 °C or 18 °C, based on the particular experiment).

Growth was monitored by spreading appropriately diluted culture aliquots in triplicate on the surface of BHIA plates. Plates were incubated at 30 °C for 48 h. BHIA plates containing 10 to 300 colonies were used to calculate the CFU/mL value for each sampling point. The different trials were tested at least in triplicate. OD readings were used in parallel to plate counts to monitor bacterial growth through absorbance measurements at 600 nm (UV–vis spectrophotometer, Heλios Gamma, Thermospectronic, Madison, USA) using non-inoculated MWB as blank.

2.3. Preparation of modified Welshimer's broth and alterations of medium composition

Among several chemically defined media that have been developed over the past decades (Friedman and Roessler, 1961; Phan-Thanh and Gormon, 1997; Premaratne et al., 1991; Tsai and Hodgson, 2003), MWB is probably the chemically defined medium most frequently used by researchers working on the physiology and genetics of *L. monocytogenes*. The choice of MWB is likely a result of its rather simple chemical composition and, consequently, ease of preparation, as well as because its development was published at a point in time when research on *L. monocytogenes* was blooming.

MWB was manufactured as described in its original publication by Premaratne et al. (1991). According to the researchers who developed MWB, certain additional medium components were shown to be stimulatory but not required. These are the four nucleotide precursors, cytosine, guanine, adenine and thymine, at 2.5 mg/L each, as well as hemin at 50 mg/L. The optional components were added in all batches of MWB prepared. However, since during preliminary experiments we found hemin at 50 mg/L to be toxic for *L. monocytogenes* Scott A, hemin was included in the medium at 5 mg/L. All compounds were provided from Fluka (Steinheim, Germany) except ferric citrate and thymine (Sigma, Steinheim, Germany) and guanine (Across Organics, New Jersey, USA). The complete broth was sterilized by filtering through 0.22-μm membrane filters (Millipore Corporation, Bedford, USA) under vacuum.

L. monocytogenes growth in MWB was also evaluated in four separate sets of experiments in which the composition of the medium had been altered by augmenting the concentration of selected groups of constituents. Specifically, in order to investigate whether the growth patterns that were observed in MWB (bi-phasic growth curves; see Results and discussion section) had a nutritional basis, four modifications in the composition of the growth medium were tested during separate experiments at 7 °C: a) MWB with a ten-fold increase in the concentration of branched-chain amino acids (Leu, Ile and Val; termed MWB-BCAA), b) MWB with a ten-fold increase in the concentration of the other four amino acids (Met, Arg, Cys and Gln; MWB-AA), c) MWB with a ten-fold increase in the concentration of the nucleotide precursors (cytosine, guanine, adenine and thymine; MWB-NP), and d) MWB with a ten-fold increase in its vitamin content (riboflavin, thiamine, biotin and thioctic acid; MWB-V).

2.4. Modeling of L. monocytogenes growth in MWB

Among a number of choices (e.g., logistic, Gompertz, Richards equation) used to describe sigmoid microbial growth curves (McMeekin et al., 1993; Zwietering et al., 1990), the model proposed by Baranyi and Roberts (1994), as given here by Eq. (1), was employed, basically