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Towards lag phase of microbial populations at growth-limiting conditions: The role of the variability in the growth limits of individual cells

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ABSTRACT

The water activity (a_w) growth limits of unheated and heat stressed *Listeria monocytogenes* individual cells were studied. The a_w limits varied from 0.940 to 0.997 and 0.951 to 0.997 for unheated and heat stressed cells, respectively. Due to the above variability a decrease in a_w results in the presence of a non-growing fraction in the population leading to an additional pseudo-lag in population growth. In this case the total apparent lag of the population is the sum of the physiological lag of the growing cells (time required to adjust to the new environment) and the pseudo-lag. To investigate the effect of a_w on the above lag components, the growth kinetics of *L. monocytogenes* on tryptone soy agar with a_w adjusted to values ranging from 0.997 to 0.940 was monitored. The model of B&R was fitted to the data for the estimation of the apparent lag. In order to estimate the physiological lag of the growing fraction of the inoculum, the model was refitted to the growth data using as initial population level the number of cells that were able to grow (estimated from the number of colonies formed on the agar at the end of storage) and excluding the rest data during the lag. The results showed that for the unheated cells the apparent lag was almost identical to the physiological lag for a_w values ranging from 0.997 to 0.970, as the majority of the cells in the initial population was able to grow in these conditions. As the a_w decreased from 0.970 to 0.940 however, the number of cells in the population which were able to grow, decreased resulting to an increase in the pseudo-lag. The maximum value of pseudo-lag was 13.1 h and it was observed at $a_w = 0.940$ where 10% of the total inoculated cells were able to grow. For heat stressed populations a pseudo-lag started to increase at higher a_w conditions (0.982) compared to unheated cells. In contrast to the apparent lag, a linear relation between physiological lag and a_w was observed for both unheated and heat stressed cells.

1. Introduction

An increased number of studies in quantitative microbiology has shown that lag time is much more uncertain and difficult to predict compared to growth rate (Baranyi, 2002; Niven et al., 2008; Webb et al., 2007). However, the validity of a mathematical model in predicting the conditions that lead to critical levels of pathogenic and spoilage organisms in foods highly depends on its ability to describe the effect of the environment on lag time. This implies that a better understanding of the lag phase and its affecting factors is of great importance for the effective application of predictive models in food safety and quality management.

In the early years of predictive microbiology several deterministic models were developed to describe the process of lag in a more mechanistic way utilizing adjustment functions or compartments (Baranyi and Roberts, 1994; Hills and Wright, 1994; McKellar, 1997). More recently, it became clear that for a complete understanding of lag the behavior of single cells has to be taken into account (Baranyi, 1998, 2002; Baranyi and Pin, 2001) through the development of stochastic models which are also able to deal with more "realistic" low contamination events (Augustin et al., 2015; Couvert et al., 2010; Koutsoumanis and Lianou, 2013; Pin and Baranyi, 2006).

The majority of both deterministic and stochastic models available in the literature consider the cells of a microbial population as uniform in terms of their ability to initiate growth. The classical definition of lag as "the time period needed for bacteria to adapt to a new environment" assumes that eventually all cells in the population will start multiplication. However, recent studies have shown that at growth-limiting conditions the presence of a growing and a non-growing faction in a microbial population is more likely than a uniform ability to initiate growth. Koutsoumanis (2008) reported that as the NaCl concentration increased and the pH decreases, the number of *Salmonella enteritidis* cells in a population that are able to grow gradually decreased and quantified the variability in the growth limits of individual cells. The latter observation is also supported by the findings of several studies which reported that an increase in the inoculum size of microbial populations results to a shift in the position of the growth boundary to

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more extreme inhibitory conditions (Dupont and Augustin, 2009; Koutsoumanis and Sofos, 2005; Masana and Baranyi, 2000; Pascual et al., 2001; Razavilar and Genigeorgis, 1998; Robinson et al., 2001; Skandamis et al., 2007; Vermeulen et al., 2009).

The presence of a non-growing fraction has an important role in the population lag (McKellar, 2001; Koutsoumanis, 2008). Koutsoumanis (2008), using Monte Carlo simulation based on the distribution of individual cell growth limits, concluded that at growth-limiting conditions the presence of a non-growing fraction in a microbial population results in an additional delay of population growth, which he called "pseudo-lag". He also stated that the total "apparent lag" of the population is a sum of the "physiological lag" of the growing cells and the "pseudo-lag". The validity of the above simulation outputs as well as the effect of the environment on the pseudo-lag and the physiological lag needs to be investigated.

The objective of this work was to study the a_w growth limits of individual *Listeria monocytogenes* cells and to investigate the role of the variability in the growth limits of individual cells in the lag phase of microbial populations.

2. Material and methods

2.1. Bacterial strain and inoculum preparation

A *L. monocytogenes* strain (FSL C1-117), serotype 1/2a, was used in this study, kindly provided by Dr. Martin Wiedmann (Cornell University, Ithaca, NY, USA). The stock culture of the strain was stored frozen (−70 °C) onto Microbank™ porous beads (Pro-Lab Diagnostics, Ontario, Canada), while working culture was stored refrigerated (5 °C) on tryptone soy agar (TSA; LabM Limited, Lancashire, United Kingdom) slants and were renewed bimonthly. The strain was activated by transferring a loopful from the TSA slants into 10 ml of tryptone soy broth (TSB, Lab M Limited) and incubating at 30 °C for 24 h to reach late stationary phase. The culture was centrifuged (4629 ×*g* for 15 min) using a model PK120R centrifuge (ThermoElectron Corporation, Waltham, MA). The pellet was washed with 10 ml of quarter-strength Ringer's solution (Lab M, Limited)', centrifuged as previously described and resuspended in 10 ml of fresh Ringer's solution.

2.2. Heat treatment

The cells were stressed by a heat treatment of 55 °C for 10 min. Duplicate tubes containing 9.9 ml of sterile TSB were immersed in a temperature-controlled water bath (NB 9, 20, Nüve Sanayi Malzemeleri Imalat Ve Ticaret A.Ş., Ankara, Turkey) and allowed to reach the target temperature before being inoculated with 100 μl of the bacterial suspensions described above. The temperature was monitored using a thermocouple (Onset Hobo data logger, Bourne, MA USA). When the heat treatment was completed, the content of the TSB tubes was mixed with 90 ml of cold TSB in a flask immersed in an ice bath. The flask was kept for 1 min in order to end the heat shock and the possible adverse effects of cold were minimized. Appropriate decimal dilutions were used to adjust the inoculums size for the experiments.

2.3. Study of individual cell growth limits

The water activity (a_w) growth limits of individual *L. monocytogenes* unheated and heat-stressed cells, at 30 °C and pH 7.3 were studied based on the method used by Koutsoumanis (2008). 0.1 ml of an appropriate dilution of the culture solutions was inoculated on TSA plates with a_w adjusted to values ranging from 0.997 to 0.937 to achieve an inoculum of ca. 200 cells per plate. These plates were prepared with several concentrations of sodium chloride (0.5–10% volume) and a_w of the agar was measured at 25 °C using an Aqualab series 3 water activity determination device (Decagon Devices, Inc. Pullman, Washington, USA).

After inoculation, the plates were covered with Parafilm (Parafilm 'M', American National Can, Greenwich, CT, USA) to avoid dehydration and stored at 30 °C in high-precision temperature (±0.2 °C) incubators (model MIR 153; Sanyo Electric Co., Ora-Gun, Gunma, Japan) for 30 days. Three experiments with three replicates for each treatment were carried out. During storage the water activity of the agar was measured every 3 days at 25 °C as described above. The water activity values of the agar for each condition remained constant during the storage period.

The distribution of a_w growth limits of individual cells was estimated from the ratio between the average number of colonies formed at the end of the incubation period for each a_w condition and the average number of cells initially inoculated, based on the assumption that each colony was derived from a single cell. The inoculum size was estimated based on the number of colonies formed on the agar plate with optimum conditions ($a_w = 0.997$, pH = 7.3 and 30 °C for 24 h). For plates in which no colonies were formed at the end of storage period the number of cells on the agar was counted to evaluate the survival of the pathogen. For this, the agar was removed aseptically from the petri dish and placed in a sterile stomacher bag. 100 ml of Ringer's solution (Lab M, Limited) were added to each bag and homogenized in a Bag-Mixer 400 stomacher (Interscience, St. Nom, France) for 2 min. Samples were diluted appropriately with Ringer's solution (Lab M, Limited), surface plated on TSA and the plates were incubated for 48 h at 30 °C before colonies were counted. In all cases where no colonies were formed the number of cells at the end of storage time did not differ significantly ($p > 0.05$) from the number of cells initially inoculated.

2.4. Study of lag phase

The growth response of the unheated and heat-stressed *L. monocytogenes* on TSA with a_w adjusted to values ranging from 0.997 to 0.940 was monitored at 30 °C for the estimation of the lag phase. 0.1 ml of an appropriate dilution of the culture solutions was inoculated on the agar plates (TSA; Lab M Limited) to achieve an inoculum of 10^5 cells per plate. Thirty agar plates for each a_w condition were inoculated. After inoculation plates were covered with Parafilm (Parafilm 'M', American National Can, Greenwich, CT, USA) to avoid dehydration and stored at 30 °C in high-precision temperature (±0.2 °C) incubators (model MIR 153; Sanyo Electric Co., Ora-Gun, Gunma, Japan). At appropriate time intervals 3 plates for each a_w condition were taken and the agar was removed aseptically from the petri dish and placed in a sterile stomacher bag. 100 ml of Ringer's solution (Lab M Limited) were added to each bag and homogenized in a Bag-Mixer 400 stomacher (Interscience, St. Nom, France) for 2 min. Samples were diluted appropriately with Ringer's solution (Lab M Limited), surface plated on TSA and the plates were incubated for 48 h at 30 °C before colonies were counted. The model of Baranyi and Roberts (1994) was fitted to the growth data for the estimation of the kinetic parameters using the DMFit software distributed by the Institute of Food Research (IFR). An additional set of TSA plates (4 plates per a_w condition) were inoculated with 0.1 ml of an appropriate dilution of the same culture solutions to achieve an inoculum of 10^2–10^3 cells per plate. This set of plates was stored at 30 °C until the end of the experimental period in order to estimate the percentage of the initially inoculated cells that are able to grow (Koutsoumanis, 2008). The later percentage was estimated based on the colonies formed on the agar plates at the end of storage period for each a_w condition assuming that each colony is originated from a single cell (Koutsoumanis, 2008). For example, when 200 cells were inoculated on an agar plate and 20 colonies were formed at the end of storage, 10% of the inoculated cells were able to grow.

3. Results and discussion

The a_w limits of *L. monocytogenes* individual cell growth showed a high variability confirming the results reported by Koutsoumanis