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Quantifying the impact of biological and experimental variability near the growth boundaries on the stochastic responses of growth, gene transcription and acid resistance of *Listeria monocytogenes*

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Abstract

Hereby, we showed that the experimental and biological variability in culture preparation had little effect on the stochastic outcome of: (i) growth, (ii) relative transcription of stress- (*gad2, sigB*) and virulence- (*prfA*) associated genes and (iii) subsequent acid resistance of *Listeria monocytogenes*, across growth/ no growth boundaries regarding combinations of pH (4.8-7.2) (HCl) and NaCl (0-8% w/v) at 7ºC. Variability of bacterial response, as described by the coefficient of variation (CV) and root mean square error (RMSE) was affected mainly by the previously pH and NaCl conditions that the pathogen had experienced. High biological variability regarding growth potential of the pathogen, was observed in conditions across growth boundaries (at 7ºC), such as pH 5.5-6.4 and NaCl 2-8% w/v, as manifested by the highly ranged growth parameters (CV_{areas} 18.3% - 49%, $RMSE_{areas}$ 8.7-13.9, $RMSE_{lag\ time}$ 9.3-31.2), while acid resistance (pH 2.0, HCl, 37°C) was highly variable when pathogen habituated pH (5.0-5.2) and NaCl (2% w/v) resulting in average D1 (3-6 min) and D2 (14-16 min) of high variability (CV_{D1} 28-35%; CV_{D2} 35-60%). Moreover at the same conditions the highest upregulation of *gad2* was observed with high biological variability of $RMSE_{gad2}$ 2.8, while relative transcription levels of *pfrA* ranged from 0.60 to 4.22, indicating the potential risk derived from the stochastic bacterial response (up- or down- regulation) regarding induction of virulence mechanisms in growth boundary conditions.

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Keywords: Listeria monocytogenes; stochastic response; acid stress; osmotic stress; relative transcription

1. Introduction

Bacterial response has been characterized by high heterogeneity regarding growth of single cells in food-related matrices [1], inactivation against lethal stresses [2] and gene expression [3]. In a previous study of our lab [4] it was shown

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that habituation of *L. monocytogenes* across marginal for growth acid and osmotic conditions has an impact on growth, survival under low acidic conditions and transcription levels of stress- and virulence- associated genes and estimated parameters are highly variable. Therefore the aim of this study was to evaluate the impact of biological and experimental variability during culture preparation of *Listeria monocytogenes* on the stochastic outcome of: (i) growth (ii) acid resistance and (iii) relative transcription of stress- and virulence- associated genes, such as glutamate decarboxylase system (*gad2*) sigma factor B (*sigB*) and positive regulatory factor A (*prfA*), in response to pH and NaCl combinations near and across the growth/no growth interface of the organism at 7°C.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

L. monocytogenes strain C_5 (serotype 4b) isolated from farmhouse environment was kept in Tryptic Soy Agar plates, supplemented with 0.6% w/v yeast extract (TSAYE) at 4ºC. Inoculum was prepared from single colony after double activation of the microorganism in 10 mL Tryptic Soy Broth supplemented with 0.6% w/v yeast extract (TSBYE) at 30°C for 24 h (A' activation) and 18 h (B' activation) respectively. Following activation stage, bacterial cells were prepared as previously described [4].

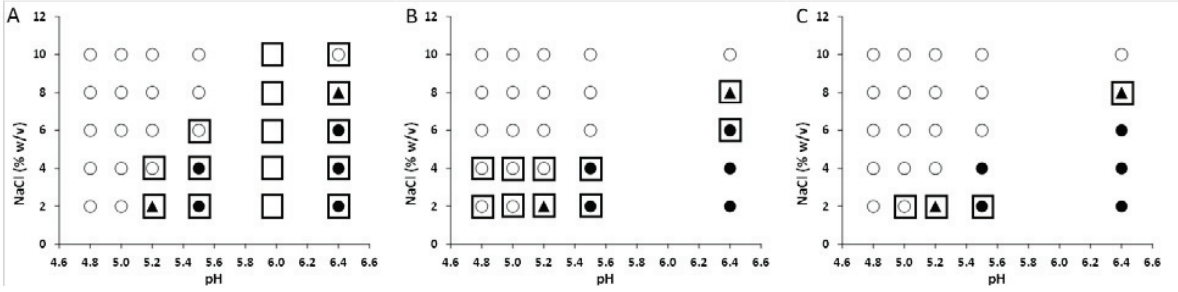

Figure 1 Selection of pH and NaCl (% w/v) combinations (squares) in order to assess variability in growth (A), acid resistance (B) and gene transcription (C) of *L.monocytogenes*, based on previous results indicating growth (close symbols) no growth (open symbols) of the pathogen in response to various pH and NaCl combinations. Data points correspond to the percentage of four biological replicates, in which growth was observed; (●) 100%, (◆) 50%, (▲) 25% and (○) 0% [4].

2.2. Growth of L. monocytogenes

In order to assess biological variability, *L. monocytogenes* inocula were prepared from: i) different single colonies (C) (n=15), ii) same colony and different second activation (B) (n=15) and iii) same colony and same second activation (T, technical) (n=15). In addition, experimental replicates corresponded to independent reproductions (n=3) of the entire experimental set up. TSBYE of various combinations of pH (4.8-7.2) and NaCl (0-10 % w/v) (Figure 1) were prepared as previously described (Makariti et al., 2015), loaded in 96-well microplates (270 µL) and inoculated with approximately 7 log CFU/mL *L. monocytogenes* C_5, followed by storage at 7°C for up to 20 days (n=15x3). Growth was monitored via optical density (620 nm) and OD data were used to estimate growth rate and lag time (DMFit, J. Baranyi, Institute of Food Research, Norwich, UK) as well as the area under the generated growth curve. pH and NaCl combinations were considered to allow growth of the pathogen when OD reached 0.2 [5].

2.3. Acid resistance of L. monocytogenes

In order to assess variability of bacterial acid resistance, TSBYE with selected combinations of pH and NaCl (Figure 1) across growth/ no growth boundaries were inoculated with ca 7 log CFU/mL *L. monocytogenes* (n_B=10; n_C=10) and subsequently stored at 7°C for 24 h, while the whole experimental set up was reproduced three times (n=3). 10 mL of bacterial culture was centrifuged (3600 rpm for 10 min at 7°C) and bacterial cells were resuspended in 10 mL of acidified medium (TSBYE, pH 2.0, HCl) prewarmed at 37ºC, following incubation at 37°C for up to 35 min. Survival of *L. monocytogenes* was determined by plating on TSAYE plates followed by incubation at 30°C for 48 h. Inactivation curves were generated by plotting survivors (log CFU/mL) against time and data were fitted using