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Investigating boundaries of survival, growth and expression of genes associated with stress and virulence of *Listeria monocytogenes* in response to acid and osmotic stress

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ABSTRACT

The objective of this study was to correlate the relative transcription of *Listeria monocytogenes* (strains C_5, 6179) stress- (*gad2, sigB*) and virulence- (*prfA*) associated genes following habituation at twenty-five pH (4.8, 5.0, 5.2, 5.5, 6.4) and NaCl (2, 4, 6, 8, 10% w/v) combinations at 7 °C, with the survival against subsequent exposure to severe acid stress (pH 2.0 at 37 °C). Our findings pointed out the inter-strain variation governing growth inhibiting conditions (pH ≤5.0 and NaCl ≥6%), where C_5 was less affected (a reduction of 2.0–3.0 log CFU/mL) than 6179 which was reduced by 4.0–6.0 log CFU/mL at the end of storage. Nevertheless, the higher the habituation at the growth permitting (pH ≥5.5; NaCl ≤4% w/v) or growth inhibiting conditions, the higher the acquired acid resistance or sensitization, respectively. At day 2, *gad2* increased relative transcriptional levels are more related to elevated acid resistance, while at day 6 both *gad2* transcriptional levels and upregulation of *sigB* were correlated to low log reductions and high $D_{pH:2.0}$-values against severe acid stress. Regarding virulence, the increased transcriptional levels of *prfA* at day 2 were correlated to adverse pH and NaCl combinations, while prolonged stay in suboptimal conditions as well as exposure to severe acid stress resulted in general activation of the virulence regulator. Such data could definitely contribute in designing safe intervention strategies and additionally integrate –omics aspects in quantitative microbial risk assessment.

1. Introduction

Listeria monocytogenes is a human foodborne pathogen, responsible for causing "listeriosis", a disease associated mainly to immunocompromised patients and neonetals that comes with a wide variety of symptoms ranging from influenza-like symptoms up to meningitis, with high mortality rates (Farber and Peterkin, 1991). Therefore, failure to control *L. monocytogenes* has negative impact both on human health as well as on food industry and economy, regarding financial losses associated with product recalls.

L. monocytogenes is a rather versatile pathogen, being able to adapt and subsequently overcome adverse conditions frequently encountered in environment or during food processing (Hill et al., 2002). Notably, habituation of the microorganism under suboptimal conditions (i.e., pH, T, and water activity) has been shown to induce adaptive responses, resulting in high tolerance against further lethal homologous or heterologous stresses (Hill et al., 2002; Lou and Yousef, 1996). Specifically, exposure of *L. monocytogenes* at suboptimal pH triggers the acid tolerance response (ATR) mechanism in a growth- dependent manner (Davis et al., 1996) and subsequently renders pathogen resistant not only to further lethal acid exposure (Koutsoumanis and Sofos, 2004; Skandamis et al., 2012), but also imparts cross- protection towards other stresses such as osmotic, heat, and ethanol (Abee and Wouters, 1999; Koutsoumanis et al., 2003; Skandamis et al., 2008; Van Schaik et al., 1999). Moreover, *L. monocytogenes* frequently experiences osmotic stress in foods, since salt is used as preservative as well as flavor and texture enhancer. Considering previous studies, osmoadaptation may induce osmotolerance (Faleiro et al., 2003) and cross-protection towards other stresses, such as peroxide stress (Bergholz et al., 2012). Nevertheless, the exact mechanisms activated during osmoadaptation remain to be elucidated.

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So far, research on *L. monocytogenes* has focused on studying phenotypic and transcriptional responses of this organism under diverse conditions, against short- or long- term stresses, defining growth/no growth boundaries, in response to factors such as pH, a_w and temperature, of different intensity. Nevertheless, in a realistic scenario, the habituation of pathogen at suboptimal growth conditions could range from minutes, hours or even days, prior to consumption. So, in order to adequately understand the mechanisms of habituation under suboptimal growth conditions, the investigation of potential correlation between phenotypic adaptive responses and transcriptional changes which are induced across marginal for growth conditions, over time, is required. Moreover, the subsequent impact of the aforementioned changes on the resistance ability of the pathogen against lethal stresses within the gastrointestinal tract should be investigated. Notably, it has been demonstrated that adaptive acid- or osmo- tolerance of *L. monocytogenes* is linked to increased expression levels of virulence genes *inlA* and *bsh* (Sue et al., 2004) as well as enhanced ability to invade Caco-2 cells (Garner et al., 2006; Werbrouck et al., 2009). However, recent studies have shown that induction of pathogenicity- associated genes and invasion ability during long-term osmoadaptation of *L. monocytogenes*, is strain- dependent (Olesen et al., 2009).

Considering the above, the objectives of the present study were to: i) evaluate the effect of habituation under various combinations of suboptimal and close to optimal pH and NaCl of two *L. monocytogenes* strains of serotypes 1/2a (strain 6179) and 4b (strain C_5) during storage at low temperature, on the resulting growth/no growth interface, ii) estimate the impact of the aforementioned conditions on the survival ability against subsequent lethal pH stress, iii) map the physiological response of the pathogen, as described by the relative transcription of virulence- (*prfA*-positive regulatory factor) and stress- (*gad2*- glutamate decarboxylase 2, *sigB- sigma B factor*) associated genes, during habituation at the aforementioned suboptimal conditions as well as post exposure at lethal pH, and iv) to correlate the latter physiological responses with the resulting acid resistance/sensitization of *L. monocytogenes* against low pH.

2. Material and methods

2.1. Bacterial strains and inoculum preparation

Two strains of *L. monocytogenes*, 6179 (serotype 1/2a) and C_5 (serotype 4b) isolated from a farmhouse cheese and a farm environment, respectively, were used throughout the study. Both strains were maintained on Tryptic Soy Agar (TSA, LABM, Lancashire, UK) supplemented with 0.6% w/v Yeast Extract (YE, LABM, Lancashire, UK) at 4 °C and sub-cultured once a month. Single colony of each strain was grown independently in 10 mL Tryptic Soy Broth (TSB, LABM, Lancashire, UK) supplemented with 0.6% w/v YE (TSBYE) at 30 °C for 24 h and subsequently 1 mL was transferred to 100 mL of fresh TSBYE and incubated at 30 °C for 18 h in order to reach early stationary phase. Following activation stage, strains were harvested by centrifugation (3600 rpm for 15 min at 4 °C) (Megafuge 1.0R, Heraus, Buckinghamshire, England), washed twice, and finally were resuspended in 10 mL of ¼ strength Ringers' solution (LABM, Lancashire, UK) so as to obtain an approximately 9.5 log CFU/mL inoculum.

2.2. Experimental design

A full factorial design (Fig. 1) was carried out by inoculating both *L. monocytogenes* strains in TSBYE of five pH (4.8, 5.0, 5.2, 5.5, and 6.4) and five NaCl levels (2, 4, 6, 8, and 10% w/v), resulting in 25 experimental combinations, followed by storage at 7 °C for 13 days. Addition of NaCl was performed prior to sterilization of basal medium (TSBYE) which already contains 0.5% w/v NaCl. In the present study though, all the mentioned NaCl concentrations are being referred to the externally added NaCl, therefore TSBYE without additional salt was considered as 0% NaCl. PH adjustment was performed using 6 N HCl post autoclave. Although HCl does not serve as an acidulant to food systems, it was selected in order to simulate: i) the direct effect of low pH, avoiding the additive influence of undissociated organic acid and ii) the low pH conditions that exist in gastrointestinal environment (McClure et al., 1994). Moreover, temperature fluctuation-abuse is often observed during distribution of food products as well as in domestic refrigerators (Kennedy et al., 2005); therefore storage temperature of 7 °C was selected as representative temperature of domestic and retail refrigerators. Growth curves of both strains under the aforementioned conditions were generated and strains' resistance towards subsequent exposure to acid stress conditions for 5 min (TSBYE; pH 2.0, 37 °C) was determined at regular time intervals (every two days) during storage. However, only strain C_5 was selected for further study, taking into account that it was less negatively affected by adverse storage conditions than 6179. More specifically C_5 was used to evaluate the transcriptional changes, regarding key regulatory-, stress-, or virulence- associated genes, across the twenty-five pH and NaCl combinations during storage, in order to provide better correlation between the physiological state of pathogen and the phenotype observed, namely acid resistance or sensitization against subsequently extreme acid stress. Therefore, RNA samples were collected on day 2 and 6 corresponding to exponential and stationary growth phase respectively (under conditions supporting growth), according to the generated growth curves (Fig. 2). Furthermore, at the same time (storage days 2 and 6) $D_{pH\ 2.0}$ values (at 37 °C) for pH-NaCl combinations across growth/no growth boundaries were estimated by two different approaches (turbidimetric method and plating method). Two independent experiments were performed and duplicate samples were used for each trial ($n = 4$).

2.3. Growth and no growth experiments

Aliquots (200 mL) of TSBYE in glass containers were inoculated with the two strains of *L. monocytogenes* separately at the level of 10^7 CFU/mL. All samples were stored at 7 °C for 13 days. Growth curves of both *L. monocytogenes* strains were generated during storage by enumerating bacterial population on days 0, 2, 4, 6, 8, 10, and 13. Enumeration took place by 10- fold diluting growth medium in ¼ strength Ringer's solution and spread on TSAYE followed by incubation at 30 °C for 48 h.

2.4. Acid challenge

At each sampling point (day 2, 4, 6, 8, 10 and 13), survival of both *L. monocytogenes* strains was determined against lethal acid stress (pH 2.0 adjusted with HCl, at 37 °C). Acid challenge temperature of 37 °C was chosen to approximate the temperature that pathogen likely experiences in the human gastrointestinal track. In particular, cells from 10 mL of each bacterial culture (corresponding to different pH and NaCl combinations) were harvested by centrifugation (3600 rpm for 10 min) at 7 °C, so as to minimize the possible impact that temperature shifts may have on the physiology of pathogen. Cell pellets were resuspended in 20 mL of preheated (37 °C) TSBYE pH 2.0 (adjusted with HCl) and incubated for 5 min. RNA samples were collected for further analysis as described in § 2.6.1, while survival of *L. monocytogenes* strains was determined by plating on TSAYE followed by incubation at 30 °C for 48 h.