Survival of acid-adapted Salmonella typhimurium in fermented millet and acidified broth at different storage temperatures

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Abstract
Salmonella typhimurium KSN 533 was adapted to acid by exposure to lactic acid at pH 5.0 for 18 h. Subsequently, acid-adapted and non-adapted cells were challenged with lactic acid (pH 3.8) in Brain-heart infusion (BHI) at 4°C and 30°C. Acid-adapted and non-adapted cells were also inoculated into already fermented millet broth (pH 3.8) and at beginning of millet fermentation with Lactobacillus fermentum starter culture. Survival curves of acid-adapted and non-adapted cells at pH 3.8 were fitted with the Weibull distribution model. Acid-adapted cells were generally resistant to subsequent acid stress than non-adapted cells. Regardless of adaptation, cells were more sensitive to acid shock (pH 3.8) at 30°C than at 4°C storage. Whereas both acid-adapted and non-adapted S. typhimurium cells survived in appreciable numbers of 5.5 and 3.5 log cfu/ml respectively after 72 h storage at 4°C, no viable cells were detected for both acid-adapted and non-adapted cells after 12 an 9 h respectively at 30°C. Acid-adaptation induced protection against lethal acid and cross protection against cold stresses. Regardless of adaptation, viable population of Salmonella showed a slight increase during the early stages of millet fermentation. Similar inactivation rates were observed for both acid-adapted and non-adapted cells when inoculated at the beginning of fermentation. Thus acid adaptation does not appear to influence survival of S. typhimurium when inoculated at the beginning of the fermentation although acid-adapted cells survived better in already fermented millet.

Keywords: acid-adaptation, millet fermentation, Salmonella, lethal acid challenge

1. Introduction
Cereals, the main sources of macro- and micronutrients for humans in most African countries are often processed through fermentation (Lei and Jakobsen, 2004; Viera-Dalodé et al., 2007; Owusu-Kwarteng et al., 2012, Owusu-Kwarteng and Akabanda, 2013) and forms a vital component of food security in the developing world (FAO, 1996). In Ghana, millet is processed into various fermented foods including koko and koko sour water (Lei and Jakobsen, 2004), fura (Owusu-Kwarteng et al., 2012), maasa (Owusu-Kwarteng and Akabanda, 2013) and foroforo. Generally, fermented foods have a very good safety record even in developing countries where foods may be produced by people without any formal training in microbial food safety and under relatively poor hygienic environments (Steinkraus, 2002). The safety of fermented foods have been attributed to many factors including the production of organic acids and reduction of pH to ≤ 4 where many pathogenic microorganisms will not survive (Adams and Nicolaides, 2008; Gaggi at el., 2011). In spite of the good safety record of fermented foods in general, there are reported cases where pathogens have been detected in certain fermented foods and have been shown to survive and grow in such foods (Gadaga et al., 2004). Amongst the most commonly encountered pathogens in African fermented foods are Bacillus cereus, E. coli, Staphylococcus aureus, Vibrio cholerae, Aeromonas, Klebsiella, Campylobacter, Shigella and Salmonella (Gadaga et al., 2004).

Salmonella continue to persist as pathogens implicated in many food related outbreaks including fermented foods, making the specie a major public health concern (Hall, 2002; CDC, 2003, 2004; Boccia et al., 2004; Mazurek et al., 2004). As a facultative anaerobic bacterium, Salmonella does not usually require strict conditions for its growth and is therefore able to grow and survive in diverse environmental niches, including food production and processing systems, and the intestinal tracts of the host organisms. During its life cycle, Salmonella periodically encounters various environmental stress conditions, such as nutrient starvation, oxidative stress, osmotic shock, extreme temperatures and acidity/pH extremes (Foster and Hall, 1990; Foster and Hall, 1991). As a neutralophile, S. typhimurium normally requires a pH environment above 5.5 for growth but can survive down to pH 4 for extended periods of time. However, the limits of endurance can be stretched if
the organisms are first adapted to a moderate acid pH before exposing them to acidity below pH 4.0. Several studies have shown that Salmonella spp. growth in a moderately acid environment induces an adaptive response which results in an enhanced resistance to more extreme acid conditions (Bacon et al., 2003; Yuk and Schneider, 2006; Álvarez-Ordóñez et al., 2009; Álvarez-Ordóñez et al., 2010). Cells adapted to mild acid stress may also survive other different lethal stress conditions such as cold storage and higher temperatures, known as multiple adaptive response or cross-protection (Rodriguez-Romo and Yousef, 2005; Xu et al., 2008). An important consequence of these phenomena is successful pathogenesis, the possibility that acid-adapted cells could be more resistant in fermented foods with reduced pH and subsequently to the strong acidic gut system, increasing the risk of human salmonellosis (Wilmes-Riesenberg et al., 1996; Yuk and Schneider, 2006). The purpose of this investigation therefore, was to assess the fate of both acid-adapted and non-adapted S. typhimurium SKN 533 in acidified Brain Heart Infusion (BHI) and in fermented millet broth.

2. Materials and Methods

2.1 Bacteria culture conditions and acid adaptation

Salmonella typhimurium SKN 533 (Obtained from the University of Copenhagen, Food Microbiology) was maintained on Brain Heart Infusion Agar (BHIA; Oxoid) plates at 4°C and revived by transferring an isolated colony from BHIA to Brain Heart Infusion Broth (BHI; Oxoid) and incubated at 37°C for 24 h to give a stock suspension of 10^8 cfu/ml. Flasks containing 50 ml of sterile BHI (pH 7.4) non-acidified and acidified with lactic acid (Merck) (pH 5.0) were inoculated with the subculture to a final concentration of 10^5 cells/ml and incubated at 37°C for 18 h. For acid non-adapted cell, buffered BHI was maintained at pH 7.2 by addition of 0.2 M phosphate buffer (Álvarez-Ordóñez et al., 2010). Cells were then harvested by centrifugation at 5000 g for 10 min at 4°C. The acid-adapted and non-adapted cell pellets were washed twice in 0.1 M phosphate buffer (pH 7.0), centrifuged, and re-suspended in 100 µl of phosphate buffer.

Lactobacillus fermentum f-26 used as starter for millet broth fermentation in this study was previously isolated from traditional millet fermentation during fura production in Ghana. They were identified by (GTG)_2-based rep-PCR fingerprinting and 16S rRNA gene sequencing as described elsewhere (Owusu-Kwarteng et al., 2012). For starter preparation, L. fermentum f-26 stock culture was subcultured into 10 ml MRS broth and incubated at 30°C for 24 h. About 100µl of the 24 h old culture were transferred into 10 ml MRS broth and incubated at 35°C for 18 h. Subsequently, the cells were harvested by centrifugation at 5000 g for 10 min (4 °C), washed twice with 20 ml sterile diluent [0.1% (w/v) peptone (Merck), 0.8% (w/v) NaCl (Merck), pH 7.2 ±0.2], and finally suspended in 10 ml of sterile diluent. This suspension served as the starter inoculums for millet broth fermentation and was sampled for viable cell count on MRS agar.

2.2 Preparation of millet broth

Whole millet grains were cleaned to remove husks, stones and damaged or discolored seeds by winnowing, hand-picking and thorough washing with distilled water. The washed millet grains were wet milled using a laboratory plate attrition mill (Grinding mill 4E, Phoenixville, PA 19460, USA). Millet broth was prepared as an aqueous suspension 10% (w/v) in distilled water, dispensed into conical flasks (200 ml per flask) and autoclaved at 115°C for 10 min.

2.3 Survival of S. typhimurium in acidified BHI broth

Acid-adapted and non-adapted S. typhimurium cells were separately inoculated into 5 ml of BHI broth pre-acidified (pH 3.8) with lactic acid (Merck) to yield a cell concentration of cal 106/ml. The inoculated tubes were then statically incubated at 4 and 30°C, and viability determined at various time (0, 3, 6, 9, 12, 18, 24, 48 and 72 h) intervals by serial dilution and plating on BHI agar.

2.4 Survival of S. typhimurium during millet fermentation

Flasks containing 200 ml of sterile millet broth (initial pH 7.0) were inoculated with L. fermentum f-26 starter culture to obtain initial cell concentration of ca 10^8/ml, and fermentation was allowed to proceed at 30 °C until the millet broth pH was 3.8, as often reported for traditional spontaneously fermented millet products (Lei and Jakobsen, 2004; Owusu-Kwarteng et al., 2012). Acid adapted or non-adapted S. typhimurium SKN 533 was then inoculated into the already fermented millet broth to a final cell concentration of cal 10^6/ml, and viable salmonellae were enumerated at various time intervals by serial dilution and plating on BHI agar. In a separate experiment, acid-adapted or non-adapted S. typhimurium SKN 533 were independently inoculated at initial cell concentration of cal 10^6/ml into millet broth at the beginning of a controlled fermentation with L. fermentum f-26 (initial cell concentration of cal 10^6/ml at 30 °C. Growth and survival of acid adapted or non-adapted S.
typhimurium SKN 533 as fermentation proceeded was determined by serial dilution and plating on BHI agar.

2.5 Inactivation and growth kinetics

The Weibull model (Chen, 2007) was used to describe acid resistance of S. typhimurium cells in BHI broth acidified with lactic acid (pH 3.8) and in fermented millet broth (pH 3.8, 30 °C): log \( N = \log N_0 - b * t^n \), where \( N_0 \) and \( N \) represent the count of initial inoculum and the count of survivors respectively after being exposed to acid challenge for a given time (t). The \( b \) and \( n \) values are the scale and shape factors, indicating a measure of resistance and a degree of curvilinear, respectively. Microbial inactivation and growth curves were analysed using Nonlinear Curve Fitting Function of Microcal Origin® 7.5 (Microcal Software Inc., Northampton, MA, USA). Data were analysed by the generalized linear model (GLM) procedure of the Statistical Analysis System (SAS). Least significant difference (LSD) was used to determine significant differences among acid adaptation treatments at 5% significance level.

3. Results

3.1 Acid tolerance response of S. typhimurium during storage at 30°C and 4°C

Acid adapted and non-adapted Salmonella typhimurium inactivation in BHI broth acidified (pH 3.8) with lactic acid were assessed during storage at refrigeration temperature (4°C) and at ambient (30°C). Acid adapted cells of S. typhimurium were generally more tolerant to acid stress than their non-adapted counterparts at both storage temperatures (Fig 1). There was however, a similar pattern of reduction in cell counts for both acid adapted and non-adapted S. typhimurium cells during storage in acidified BHI broth. During storage at 30°C, acid-adapted decreased in counts from 6.5 to 5.7 log cfu/ml (a one log cycle reduction) whereas acid non-adapted cells decreased from 6.5 to 2.1 log cfu/ml (approximately 4 log cycles reduction) within the first 3 h of storage. No viable counts were detected for the acid adapted and non-adapted S. typhimurium cells after 12 h and 9 h respectively, during storage at 30°C (Fig 1A). Thus acid adapted cells survived substantially longer than non-adapted cells during storage at 30°C. Regardless of adaptation, cells were more sensitive to acid shock (pH 3.8) at 30°C than at 4°C storage. Whereas both acid adapted and non-adapted S. typhimurium cells survived in appreciable numbers of up to 5.5 and 3.5 log cfu/ml respectively after 72 h at 4°C, no viable cells were detected for both adapted and non-adapted cells after 12 an 9 h respectively at 30°C.

![Fig 1](image1.png) Survival of acid adapted ( ) and non-adapted ( ) S. typhimurium in acidified BHI (pH 3.8) during storage at 30 °C (A) and 4 °C (B).

3.2 Survival of S. typhimurium in fermented millet broth

The purpose of this experiment was to examine whether acid adaptation would promote the survival of S. typhimurium in already fermented millet (pH 3.8) or during millet fermentation with L. fermentum starter culture. In general, both acid adapted and non-adapted cell had similar survival patterns when inoculated at the beginning of millet fermentation (Fig 2). Regardless of adaptation, there was an initial increase in counts of S. typhimurium at the early stages of the fermentation process before declining in population. No viable cells were detected after
18 and 24 h of fermentation in millet broth for non-adapted and acid-adapted cells respectively (Fig 2C). In already fermented millet broth (pH 3.8), acid-adapted cells showed relatively higher tolerance than non-adapted cells. However, there was a sharp decline in Salmonellae counts and no viable cells of either non-adapted or acid-adapted cells were detected after 9 and 18 h of fermentation respectively (Fig 2D).

3.3 Inactivation kinetics

The inactivation kinetic parametric values as estimated from the Weibull model is shown in Table 1. The survival curves for acid-adapted cells showed linearity ($n = 1$) in acidified BHI broth (pH 3.8) at both 4 and 30 °C, whereas those for non-adapted cells exhibited nonlinear pattern, showing a noticeable upward concavity ($n < 1$). The survival curves for the non-adapted cells declined sharply within the first 3 h of incubation at 30 °C of incubation as compared with 4 °C, which is described by a sharp decline followed by a slight tail. In already fermented millet however, both acid-adapted and non-adapted cells showed linearity ($n = 1$). In general, there were also significant differences in the scale factor ($b$) between non-adapted and acid-adapted cells. Thus, pre-adaptation at pH 5.4 resulted in an increase in resistance, as shown by smaller $b$ values for acid-adapted *S. typhimurium* cells.

![C](image1) ![D](image2)

Fig 2. Survival of acid adapted (−→) and non-adapted (−−−) *S. typhimurium* in millet broth during fermentation (C) and in already fermented millet broth (D)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Challenge in BHI (pH 3.8)</th>
<th>Challenge in fermented millet</th>
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<tbody>
<tr>
<td></td>
<td>4 °C</td>
<td>30 °C</td>
</tr>
<tr>
<td>Acid-adapted cells</td>
<td>0.12±0.06$^a$</td>
<td>1.05±0.80$^b$</td>
</tr>
<tr>
<td>Acid non-adapted cells</td>
<td>0.65±0.05$^a$</td>
<td>0.81±0.14$^b$</td>
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*Means (±SD) with same superscripts in a column are not significantly different (p<0.05)

4. Discussion

*Salmonella* spp. as leading cause of bacterial foodborne diseases all over the world, cause several illnesses including typhoid fever, gastroenteritis and septicemia (D’Aoust, 2000), with *Salmonella typhimurium* accounting for about 35% of reported human isolates (Wilmes-Riesenber et al., 1996). Here, the tolerance of acid-adapted and non-adapted *S. typhimurium* in lethal acid challenge at 4°C and 30°C, as well as in lactic acid fermented millet broth was investigated. These experimental conditions represent possible real scenarios occurring during food processing and storage, including refrigeration (4°C), pH gradient of 3.5 to 5.5, and
et al. 2003; 2008). When S. typhimurium was inoculated together with L. fermentum starter at the beginning of fermentation however, regardless of adaptation, viable population of S. typhimurium showed a slight increase during the early stages of the fermentation. This was due to the fact that acidity and the production of other antimicrobials attributable to fermentation had not been achieved at such early stages of the fermentation process.

5. Conclusion

Acid adaptation enhances the survival of S. typhimurium KSN 533 in lethal acid conditions. Acid-adapted S. typhimurium KSN 533 survives better than non-adapted cells in already fermented millet broth. However, acid adaptation does not appear to influence survival of S. typhimurium KSN 533 when inoculated together with L. fermentum starter culture at the beginning of millet broth fermentation. Contaminating pathogens in an acid-adapted state may thus, persist for longer periods in fermented cereals (millet) with significant health implications and should therefore be considered in designing food preservation and quality control measures.
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References


