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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-Kwarteg *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; Gaggia *et al*., 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 10^9 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-Ordòñez *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 $(GTG)_5$-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. typhumirium* 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* 10^8/ml at 30 °C. Growth and survival of acid adapted or non-adapted *S.*

typhumirium 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.

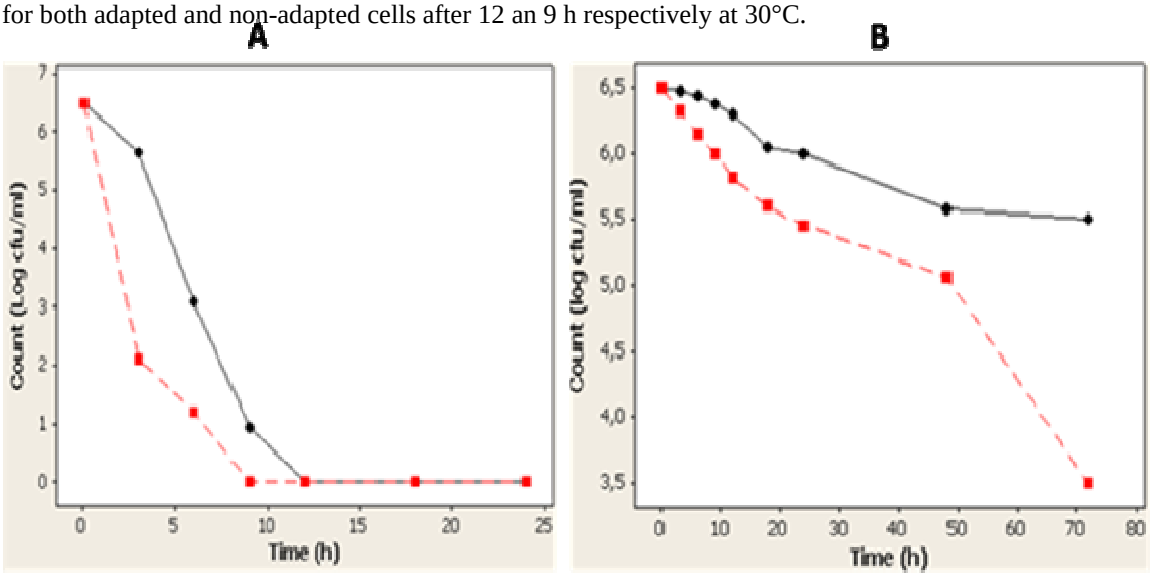

Fig 1. Survival of acid adapted (—◆—) and non-adapted (—■—) *S. typhimurium* in acidified BHI (pH 3.8) during storage at 30 °C (**A**) and 4 °C (**B**).

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.

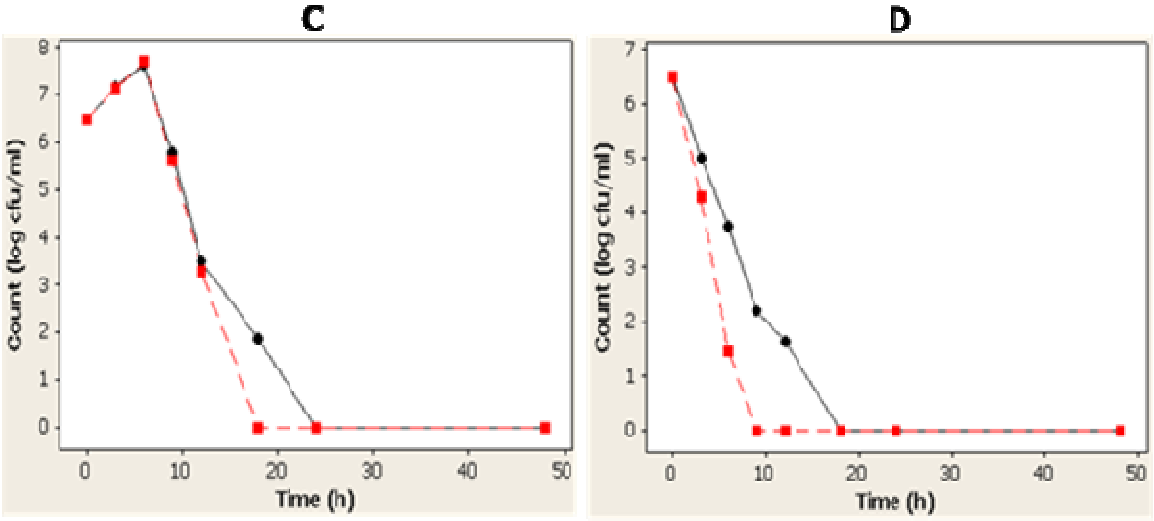

Fig 2. Survival of acid adapted (—●—) and non-adapted (—■—) *S. typhimurium* in millet broth during fermentation (**C**) and in already fermented millet broth (**D**)

Table 1. Inactivation kinetic parameters obtained from the fitted Weibull model for the survival curves of acid-adapted and non-adapted *S. typhimurium* after challenge in acidified BHI broth and fermented millet*

	Challenge in BHI (pH 3.8)				Challenge in fermented millet	
	4 °C		30 °C		30 °C	
Treatment	*b*	*n*	*b*	*n*	*b*	*n*
Acid-adapted cells	0.12±0.06^a	1.05±0.80^a	1.41±0.06^a	0.96±0.15^a	1.25±0.08^a	0.94±0.16^a
Acid non-adapted cells	0.65±0.05^b	0.81±0.14^a	3.16±0.22^b	0.15±0.04^a	2.96±0.11^b	1.13±0.21^a

*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 septicaemia (D'Aoust, 2000), with *Salmonella typhimurium* accounting for about 35% of reported human isolates (Wilmes-Riesenberg *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

ambient temperature (30±2 °C) during spontaneous cereal fermentations in tropical Africa (Lei and Jakobsen, 2004; Viera-Dalodé *et al*., 2007; Owusu-Kwarteng *et al*., 2012; Owusu-Kwarteng and Akabanda, 2013). During food processing such as spontaneous fermentations, pathogens such as *S. typhimurium* can be injured under food processing-related stresses, and sublethally injured cells may repair the damage and regain viability and pathogenicity under favourable environmental conditions (Wong *et al*., 1998; Liao and Fett, 2005). Acid adaptation responses have been reported for several pathogenic bacteria including *Salmonella* species (Greenacre *et al*., 2003; Yuk and Schneider, 2006; Álvarez-Ordóñez *et al*., 2009; Álvarez-Ordóñez *et al*., 2010), and represents a great concern for food processing and safety, since during processing, microorganisms encounter several mildly acidic conditions which may induce acid tolerance response and may subsequently survive other lethal stresses, including the low pH of the stomach and intracellular environments (Wilmes-Riesenberg *et al*., 1996; Tosun and Gönül, 2003). Moreover, studies have shown an increased virulence and invasion for acid adapted cultures of *Salmonella* spp. (Humphrey *et al*., 1996; Wilmes-Riesenberg *et al*., 1996; Gahan and Hill, 1999), which is important in disease pathogenesis. Thus studies regarding the fate of acid-adapted cells would help not only understand the behaviour of injured cells during food processing but also in designing preservation and safety control measures.

In the present study, acid-adapted cells of *S. typhimurium* were more tolerant to acid stress at both 4°C and 30°C than non-adapted cells. Thus pre-exposure to mild acid conditions, offered protection to *S. typhimurium* in subsequent lethal acid as well as attaining cross protection in cold stress. Acid adaptation appears to provide protection against other stresses such as heat (Ingham and Uljas, 1998; Duffy *et al*., 2000; Mazzota, 2001), Osmotic and oxidative stress (Lee *et al*., 1995; Wilde *et al*., 2000), ionising radiation (Buchanan *et al*., 1999) and cold stress (Xu *et al*., 2008). Although the mechanisms involved in *Salmonella* stress and cross protection responses are not fully unravelled, some progress have been made and there is evidence for the involvement of several genes such as *rpoS*, and proteins like the acid shock proteins (ASP) and heat shock proteins (HSP) (Foster and Spector, 1995; Foster, 2000; Dodd and Aldsworth, 2002). Additionally, the involvement of internal pH maintenance has been found. Thus, the differences in the bacterial acid tolerance response as a function of the acid used was associated with the different acids ability to alter the internal pH of the cells (Foster and Hall, 1991; Greenacre *et al*., 2003). Furthermore, the induction of modifications in membrane fatty composition and consequently membrane fluidity has also been implicated in *Salmonella* acid tolerance response (Chang and Cronan, 1999; Kim *et al*., 2005; Álvarez-Ordóñez *et al*., 2008). Álvarez-Ordóñez *et al*., (2008) showed that acidification of the growth medium caused a decrease in unsaturated to saturated fatty acid ratio (UFA/SFA) and an increase in the cyclic fatty acid content in *Salmonella typhimurium*, which in combination with other factors may account for the observed resistance to subsequent acid and cold stresses.

In already fermented millet broth, the sharp inactivation of both acid-adapted and acid non-adapted cells could be attributed to a variety of factors. According to the hurdle concept (Leistner, 2000) the inhibition of microorganisms in food products is a result of the combined effect of factors such as pH, water activity, oxidation-reduction potential, and antimicrobials naturally occurring. Traditional fermented cereals (millet) may combine from their very nature a powerful set of factors, which enables them to inhibit the growth of microorganisms. Although acidity is the most important intrinsic characteristic in determining the survival and growth of pathogenic bacteria (Smittle, 2000), the contribution of other antimicrobials during millet fermentation may play a role in the sharp decline in population of both acid adapted and non-adapted *S. typhimurium*, as the *Lactobacillus fermentum* starter used has previously been found to produce some bacteriocins, although to lesser extent (Owusu-Kwarteng *et al*., 2013). 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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