Meat juice contributes to the stability of ethanol adaptation in Salmonella enterica serovar Enteritidis

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Abstract

Stability assessment of observed tolerance phenotypes is integral in understanding stress adaptation in food-borne pathogens. Therefore, the current work was carried out to determine whether ethanol adaptation induced by exposure to 5 per cent ethanol for 60 min is a stable phenomenon in Salmonella enterica serovar Enteritidis. The capacity of Salmonella Enteritidis (S. Enteritidis) to maintain the acquired ethanol adaptation in the absence of sublethal ethanol stress was investigated at 37 °C, 25 °C or 4 °C in Luria–Bertani broth and two types of meat juice. It was found that ethanol adaptation was completely reversed within 40 min at 37 °C or within 60 min at 25 °C, but was stable at 4 °C for at least 48 h in the broth assay. Ethanol adaptation was retained in chicken juice during 60-min incubation at 25 °C or 48-h incubation at 4 °C. Moreover, exposure to pork juice stored at either 25 °C or 4 °C significantly (P<0.05) increased the ethanol tolerance of ethanol-adapted cells. Collectively, these findings suggest that ethanol adaptation stability in S. Enteritidis under cold conditions and in meat juices should be taken into account when conducting a comprehensive risk analysis during food processing.

Graphical Abstract

Keywords: Salmonella Enteritidis; ethanol adaptation; stability; temperature; meat juice.
Introduction

Food-borne pathogens encounter many stresses (e.g. freezing, heating, pH extremes or chemical disinfection) during food processing, transportation and storage (Spector and Kenyon, 2012; Begley and Hill, 2015). Exposure to a sublethal level of stress can induce tolerance to a subsequent homologous stressing agent in food-borne pathogens (Beales, 2004). This phenomenon, referred to as stress adaptation, has been recognized as a food safety concern because it may compromise currently used food control measures (Cebrián et al., 2012; He et al., 2021). In fact, there is an increasing involvement of stress-adapted pathogens in food-borne outbreaks (Chen, 2017). Therefore, it is of paramount importance to uncover the influence of stress adaptation on food-borne pathogens under food-processing conditions.

Practically, induction of stress adaptation is not usually immediately followed by the same type of inactivation treatment during food processing (Shen et al., 2015). In this scenario, the stability of stress adaptation plays a crucial role in the survival of food-borne pathogens in unfavorable environments, which is impacted by the cellular ‘memory’ or history of previous stresses a particular bacterium has encountered. Stress adaptation stability can be defined as the ability of a pathogen to retain the acquired stress adaptation in the absence of sublethal levels of a given stress (Shen et al., 2014). The stability of acquired adaptation has been characterized for alkali, heat, acid and oxidative stresses in Listeria monocytogenes (L. monocytogenes) (Shen et al., 2014, 2015; Shen Q. et al., 2016; De Abrew Abeysundara et al., 2016). For example, alkali adaptation and heat adaptation in L. monocytogenes were retained at 4 °C, but not at 22 °C in tryptic soy broth supplemented with 0.6 per cent yeast extract (TSB-YE) (Shen et al., 2014; Shen Q. et al., 2016). Moreover, acid adaptation in L. monocytogenes was more stable at 4 °C than at 22 °C or 37 °C in TSB-YE and was well maintained in refrigerated carrot juice or milk (Shen et al., 2015). To date, however, the stability of stress adaptation induced by other factors such as alcohol disinfectants has never been assessed in food-borne pathogens.

Ethanol is a typical alcohol disinfectant that can also be used for other purposes, such as shelf-life extension and colorant extraction in food industries (He et al., 2021). For example, ethanol is employed for hand and conveyor disinfection in meat processing plants in some countries, which creates an opportunity for the induction of ethanol adaptation if bacterial cells are present in this environment (Shen J. et al., 2016; Fagerlund et al., 2017). Adaptation to ethanol has been widely reported in food-borne pathogens such as Salmonella enterica serovar Enteritidis, L. monocytogenes, Cronobacter sakazakii and Vibrio parahaemolyticus (Lou and Yousef, 1997; Chiang et al., 2006; Huang et al., 2013; He et al., 2016). For instance, Salmonella Enteritidis (S. Enteritidis) was found to acquire enhanced tolerance to 15 per cent ethanol (a lethal ethanol concentration) after pre-adaptation to 5 per cent ethanol (a sublethal ethanol concentration) in our previous work (He et al., 2016). Nevertheless, all of the aforementioned studies have focused on how food-borne pathogens respond to lethal ethanol challenge immediately following sublethal ethanol stress adaptation. The stability of ethanol adaptation in food-borne pathogens—namely, the capacity to maintain the acquired ethanol tolerance to a definite time upon removal of the sublethal ethanol stress—has never been evaluated before. Hence, the current work aims to determine the stability of S. Enteritidis ethanol adaptation in both culture medium and food substrates at different temperatures, which will provide insights for subsequent risk analyses associated with this pathogen.

Materials and Methods

Bacterial strains and storage conditions

S. Enteritidis ATCC 13076 was purchased from the American Type Culture Collection and preserved at –80 °C in Luria-Bertani (LB) broth (Amresco, Solon, OH, USA) supplemented with 25 per cent glycerol. Working stock cultures were maintained at 4 °C by streaking a loopful of frozen culture onto LB agar and incubating at 37 °C for 18 h. Prior to each test, a single colony was inoculated into 5 mL LB broth, followed by overnight incubation at 37 °C. A 500-μL aliquot of bacterial suspension was then transferred to 50 mL LB broth and incubated at 37 °C for 5 h with shaking (200 r/min). This late log phase culture was utilized in subsequent ethanol adaptation assays (He et al., 2018).

Preparation of ethanol-adapted bacterial cells

Ethanol adaptation was conducted by exposure of S. Enteritidis to 5 per cent ethanol for 60 min because this sublethal treatment was identified as an optimal condition to induce a bacterial ethanol tolerance response in our previous study (He et al., 2016). Briefly, a 1-mL aliquot of the late log phase culture (approximately 9 log colony-forming units (CFU/mL) was spun at 8000 g for 10 min, washed with phosphate-buffered saline (pH 7.4; Amresco, Solon, OH, USA) and resuspended in 10 mL LB broth or in LB broth containing 5 per cent ethanol. These samples were then held at 25 °C for 60 min with shaking (170 r/min) to prepare non-adapted and ethanol-adapted S. Enteritidis, respectively.

Stability assay of ethanol adaptation in LB broth

An in vitro stability test was performed according to Shen et al. (2015) with modifications. The stability of ethanol adaptation in S. Enteritidis was determined by assessing the tolerance of ethanol-adapted cells to 15 per cent ethanol challenge when these cells are no longer in contact with the sublethal ethanol stress. Briefly, ethanol-adapted cells (1.5 mL) were pelleted by centrifugation (8000 g, 5 min), resuspended in the same volume of fresh LB broth or milk (Shen et al., 2015). To date, however, the stability of stress adaptation induced by other factors such as alcohol disinfectants has never been assessed in food-borne pathogens.

Ethanol is a typical alcohol disinfectant that can also be used for other purposes, such as shelf-life extension and colorant extraction in food industries (He et al., 2021). For example, ethanol is employed for hand and conveyor disinfection in meat processing plants in some countries, which creates an opportunity for the induction of ethanol adaptation if bacterial cells are present in this environment (Shen J. et al., 2016; Fagerlund et al., 2017). Adaptation to ethanol has been widely reported in food-borne pathogens such as Salmonella enterica serovar Enteritidis, L. monocytogenes, Cronobacter sakazakii and Vibrio parahaemolyticus (Lou and Yousef, 1997; Chiang et al., 2006; Huang et al., 2013; He et al., 2016). For instance, Salmonella Enteritidis (S. Enteritidis) was found to acquire enhanced tolerance to 15 per cent ethanol (a lethal ethanol concentration) after pre-adaptation to 5 per cent ethanol (a sublethal ethanol concentration) in our previous work (He et al., 2016). Nevertheless, all of the aforementioned studies have focused on how food-borne pathogens respond to lethal ethanol challenge immediately following sublethal ethanol stress adaptation. The stability of ethanol adaptation in food-borne pathogens—namely, the capacity to maintain the acquired ethanol tolerance to a definite time upon removal of the sublethal ethanol stress—has never been evaluated before. Hence, the current work aims to determine the stability of S. Enteritidis ethanol adaptation in both culture medium and food substrates at different temperatures, which will provide insights for subsequent risk analyses associated with this pathogen.

Stability assay of ethanol adaptation in meat juice

Meat juice, referred to as the exudate of frozen raw meat, has been widely used as a food-based model to mimic nutrients in meat-processing environments (Wang et al., 2013; Ferreira and Domingues, 2016; Pang and Yuk, 2018). Moreover, ethanol has been utilized for chemical disinfection in meat-processing plants in different countries (Shen J. et al., 2016; Fagerlund et al., 2017). Therefore, chicken juice and pork juice were employed to assess ethanol adaptation stability of S. Enteritidis in situ, particularly because chicken and pork meat are important reservoirs for Salmonella (Yang et al., 2010; Zhang et al., 2018). These two kinds of meat juice were prepared according to Birk et al. (2004) and Brown et al. (2014) with slight modifications. Briefly, commercially available chicken drumsticks and streaky
pork were frozen at –20 °C and thawed overnight at room temperature. The exudate was spun at 13 523 g for 5 min to sediment particulate materials. The supernatant was sterilized using 0.22-μm syringe filters (Pall Corporation, Ann Arbor, MI, USA). The filtered chicken juice and pork juice were then divided into aliquots of equal volume, stored at –80 °C and thawed overnight at 4 °C before use in subsequent experiments.

Ethanol-adapted cells of S. Enteritidis were pelleted by centrifugation (8000 g, 5 min), resuspended in temperature-equilibrated chicken juice or pork juice at either 25 °C or 4 °C and then held at the same temperatures. Under these two temperatures, incubation was carried out for 0, 20, 40 and 60 min and 0, 18, 24 and 48 h, respectively. A 100-μL aliquot of chicken juice or pork juice with bacterial suspension was then added to 5 mL LB broth containing 15 per cent ethanol at each time point, followed by incubation at 25 °C with shaking at 170 r/min for 90 min. The cultures were then serially diluted in sterile normal saline (0.85 per cent) and spread onto LB agar. The cell number of S. Enteritidis was determined after overnight incubation at 37 °C. The ethanol tolerance of non-adapted controls was determined as described above.

Statistical analysis
All experiments were conducted in duplicate on at least three separate occasions. Data were shown as the population reduction, which was equal to the cell number (log CFU/mL) before 15 per cent ethanol challenge minus the cell number (log CFU/mL) after this treatment. The resulting data were analyzed by a one-way analysis of variance, followed by Duncan’s test (P<0.05) using SAS version 8.0.

Results
Stability of ethanol adaptation in LB broth was temperature-dependent
The stability of ethanol adaptation was initially assessed in LB broth at 37 °C, 25 °C or 4 °C. Overall, it was observed that ethanol-adapted S. Enteritidis demonstrated reduced ethanol tolerance with increasing incubation time (Figure 1). Following incubation at 37 °C for 40 min or 25 °C for 60 min, the ethanol tolerance of ethanol-adapted cells was reduced to the level of non-adapted cells (Figure 1A and Figure 1B), indicating that ethanol adaptation in S. Enteritidis had been lost. On the contrary, ethanol adaptation was maintained at 4 °C. After 18, 24 or 48 h incubation, a significantly (P<0.05) greater survival by approximately 0.76–1.70 log CFU/mL was observed with ethanol-adapted cells compared to the non-adapted controls (Figure 1C). Overall, these results suggested that ethanol adaptation stability of S. Enteritidis in LB broth was dependent on temperature.

Ethanol adaptation was maintained in chicken juice
Ethanol adaptation stability of S. Enteritidis in chicken juice is presented in Figure 2. It was found that ethanol tolerance of ethanol-adapted S. Enteritidis significantly (P<0.05) increased after incubation in chicken juice at 25 °C for 20 or 40 min, followed by a significant (P<0.05) reduction (Figure 2A). When exposed to chicken juice at 4 °C, ethanol-adapted S. Enteritidis cells exhibited reduced ethanol tolerance with increased incubation time (Figure 2B). Nevertheless, ethanol-adapted cells exposed to chicken juice exhibited a significant (P<0.05) survival advantage (0.26–2.89 log CFU/mL) over non-adapted cells throughout storage, irrespective of exposure temperature (Figure 2). Hence, chicken juice was able to maintain ethanol adaptation in S. Enteritidis.

Figure 1. Population reduction of ethanol-adapted (■) S. Enteritidis in 15 per cent ethanol after incubation in LB broth at 37 °C (A), 25 °C (B) or 4 °C (C) for different time periods. The initial bacterial concentration was approximately 6 log CFU/mL. □, population reduction of non-adapted cells in 15 per cent ethanol. Error bars represent standard deviation. Symbols without visible bars indicate the standard deviation smaller than the symbol size. Different letters signify significant differences (P<0.05). LB, Luria-Bertani; S. Enteritidis, Salmonella Enteritidis.

Figure 2. Population reduction of ethanol-adapted (■) S. Enteritidis in 15 per cent ethanol after incubation in LB broth at 25 °C (A), 4 °C (B) and 6 °C (C) for different time periods. The initial bacterial concentration was approximately 6 log CFU/mL. □, population reduction of non-adapted cells in 15 per cent ethanol. Error bars represent standard deviation. Symbols without visible bars indicate the standard deviation smaller than the symbol size. Different letters signify significant differences (P<0.05). LB, Luria-Bertani; S. Enteritidis, Salmonella Enteritidis.

Pork juice enhanced the stability of ethanol adaptation
As shown in Figure 3, the tolerance of ethanol-adapted S. Enteritidis...
to 15 per cent ethanol was significantly (P<0.05) enhanced upon exposure to pork juice at 25 °C for 60 min or at 4 °C for 48 h. Ethanol-adapted cultures exposed to pork juice exhibited a significantly (P<0.05) higher cell number by 0.23–0.98 log CFU/mL, compared with ethanol-adapted cells not exposed to the juice (Figure 3). These findings indicated that pork juice was a promoting factor to the stability of ethanol adaptation in S. Enteritidis.

Discussion

Temperature-dependent stability of acquired adaptation has been reported for acid and alkali stresses in L. monocytogenes (Shen et al., 2013; Shen Q. et al., 2016). Similarly, we found that S. Enteritidis ethanol adaptation was less stable at 37 °C or 25 °C than at 4 °C in LB broth in the current study. The observed reversal of ethanol adaptation after 40 min at 37 °C or 60 min at 25 °C could be attributed to the rapid degradation or downregulation of some ethanol-induced proteins during the growth of S. Enteritidis under these two temperatures. In fact, as bacterial cells recover and resume growth at a steady state, the level of stress-induced proteins is always reduced (Arsène et al., 2000; Barria et al., 2013). For instance, the major cold shock protein CspA accounted for almost 13 per cent of total cell proteins in Escherichia coli under cold conditions, while it was reduced to low levels at 37 °C (Barria et al., 2013). Similarly, translation of the heat shock regulatory gene rpoH was repressed under steady-state growth conditions where sublethal heat stress was absent (Arsène et al., 2000). On the other hand, we observed the upregulation of 56 proteins related to purine metabolism (e.g. HiuH, YaiE) and ABC transporters (e.g. ProX, PstC) in response to ethanol adaptation in S. Enteritidis (He et al., 2019). Elsewhere, ethanol stress induced the expression of nine proteins (PspA, MopA, NmpC, RfaL, PotD, NanA, GldA, Tpx and UcpA) in Salmonella Typhimurium (Shoae Hassani et al., 2009). Thus, it is clear that there are multiple mechanistic shifts in response to ethanol exposure, but
other unrelated factors (e.g., temperature, growth state) must also be considered. Given the extensiveness of proteins upregulated in response to stress, the subsequent downregulation or degradation of these proteins during further incubation at the more optimal temperatures of 37 °C or 25 °C in the absence of sublethal ethanol stress may lead to the loss of S. Enteritidis ethanol adaptation in the current study.

We further observed that ethanol adaptation in S. Enteritidis was maintained during the 48-h refrigeration at 4 °C in the LB broth assay. In agreement with this finding, heat adaptation in L. monocytogenes was still stable after 24 h of storage at 4 °C (Farber and Brown, 1990; Shen et al., 2014). It is currently unknown why S. Enteritidis is able to retain ethanol adaptation at 4 °C. A postulation may be that the absence of S. Enteritidis growth at 4 °C slows down the degradation of ethanol adaptation-induced proteins, because metabolism is less active at colder temperatures. In addition, an increase in cell membrane fluidity was observed in S. Enteritidis ATCC 13076 (the same strain used in this work) upon decreased growth temperature (Yang et al., 2014). Interestingly, ethanol-adapted (5 per cent ethanol for 30 min and 60 min) cells of Vibrio parahaemolyticus with evaluated membrane fluidity as suggested by a diminished saturated fatty acid/unsaturated fatty acid ratio were able to withstand subsequent lethal ethanol challenge better than non-adapted cells (Chiang et al., 2006, 2008); thus, membrane fluidity may be another factor contributing to the maintenance of ethanol adaptation at 4 °C. Collectively, it is suggested that cold environments during food processing delay the growth of S. Enteritidis while maintaining the acquired ethanol adaptation.

It is known that stress-adapted bacteria, a potential contaminant in food-related environments, may come into contact with raw food matrices instead of being exposed to lethal inactivation treatments immediately after adaptation (Shen Q. et al., 2016). In this scenario, the stability of stress adaptation in situ plays a role in bacterial tolerance to further elimination treatments (Shen Q. et al., 2016). In the current study, ethanol adaptation in S. Enteritidis was maintained better in pork juice and chicken juice compared to in LB broth at either 25 °C or 4 °C, highlighting the importance of thorough elimination of meat residues during food processing. The high stability of ethanol adaptation in meat juice may result from the maintenance or further induction of ethanol adaptation-related genes. Meat juice (e.g. chicken juice, fermented sausage juice) was found to induce transcriptional changes in pathogenic bacteria; genes involved in stress response, biofilm formation, quorum sensing and virulence were differentially expressed (Ligowska et al., 2011; Rantsiou et al., 2012; Wang et al., 2016; Lamas et al., 2018). It should be noted that meat juice and LB broth differ in their properties such as pH value; meat juice has an acidic pH of 5.87–5.91, whereas LB broth has a neutral pH of 7.00. Interestingly, adaptation to mild acidic pH has been demonstrated to increase the ethanol tolerance of log-phase S. Enteritidis, possibly by upregulation of ethanol adaptation-related genes in our previous study (Ye, 2019). This could be a reason for the better maintenance of S. Enteritidis ethanol adaptation in meat juice than in LB broth. We also observed that pork juice was more effective in retaining ethanol adaptation than chicken juice, potentially due to variations in juice constituents (e.g. quorum sensing signaling molecules, types of proteins). Quorum sensing signaling molecules such as acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) have been observed in meat and are thus likely to be present in the juice of meat (Bruhn et al., 2004; Blana and Nychas, 2014). Salmonella can respond to AHLs present in the environment, although this bacterium does not produce these molecules due to lack of the luxI gene (Dourou et al., 2011; Vanetti et al., 2020). Moreover, AI-2 production by Salmonella in milk and chicken juice differed by food type (Dourou et al., 2002). Thus, it is speculated that quorum sensing signaling molecules may be a factor influencing the stability of S. Enteritidis ethanol adaptation in different kinds of meat juice.

In summary, the stability of ethanol adaptation in S. Enteritidis was influenced by incubation temperature and meat juice. Ethanol adaptation was completely reversed at 37 °C or 25 °C but was relatively stable at 4 °C when incubated in LB broth in the absence of sublethal ethanol stress. Moreover, ethanol adaptation stability was maintained in chicken juice and was promoted by pork juice. These findings highlight the maintenance of acquired ethanol tolerance in S. Enteritidis by meat residues in food-related environments, which potentially increases microbial food safety risks. Further investigations could focus on the interplay between meat juice components and ethanol adaptation stability in order to provide indispensable information for the development of effective mitigation strategies against ethanol tolerance in S. Enteritidis. More importantly, ethanol adaptation stability in other food-borne pathogens can be assessed in a similar way to gain more information for the development of effective measures to combat bacterial ethanol tolerance.

**Author Contributions**
Conceptualization, Siyun Wang and Xianming Shi; methodology, Shoukui He and Siyun Wang; investigation, Shoukui He; data curation, Shoukui He and Karen Fong; writing—original draft preparation, Shoukui He; writing, review and editing, Karen Fong, Siyun Wang and Xianming Shi; supervision, Siyun Wang and Xianming Shi. All authors have read and agreed to the published version of the manuscript.

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**Conflict of Interest**
The authors declare no conflict of interest.

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