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Influence of ethanol adaptation on *Salmonella enterica* serovar Enteritidis survival in acidic environments and expression of acid tolerance-related genes

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ABSTRACT

Cross-protection to environmental stresses by ethanol adaptation in *Salmonella* poses a great threat to food safety because it can undermine food processing interventions. The ability of *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) to develop acid resistance following ethanol adaptation (5% ethanol for 1 h) was evaluated in this study. Ethanol-adapted *S.* Enteritidis mounted cross-tolerance to malic acid (a two-fold increase in minimum bactericidal concentration), but not to acetic, ascorbic, lactic, citric and hydrochloric acids. The population of *S.* Enteritidis in orange juice (pH 3.77) over a 48-h period was not significantly ($p > 0.05$) influenced by ethanol adaptation. However, an increased survival by 0.09–1.02 log CFU/ml was noted with ethanol-adapted cells of *S.* Enteritidis compared to non-adapted cells in apple juice (pH 3.57) stored at 25 °C ($p < 0.05$), but not at 4 °C. RT-qPCR revealed upregulation of two acid tolerance-related genes, *rpoS* (encoding σ^S) and SEN1564A (encoding an acid shock protein), following ethanol adaptation. The relative expression level of the acid resistance gene *hdeB* did not change. The resistance phenotypes and transcriptional profiles of *S.* Enteritidis suggest some involvement of *rpoS* and SEN1564A in the ethanol-induced acid tolerance mechanism.

1. Introduction

Foodborne pathogens are frequently exposed to suboptimal growth conditions due to food preservation and chemical disinfection measures implemented for the control of microbial contamination in the food industry (Møretrø et al., 2012; Yoon et al., 2015). Factors leading to microbial growth inhibition include acidic pH, high osmolarity, low/high temperature and presence of disinfectants (Arroyo et al., 2012). Nevertheless, many pathogens are able to adapt to these stressful conditions, resulting in enhanced resistance to subsequent stresses (Arroyo et al., 2012; Fong and Wang, 2016). Therefore, the adaptive response of foodborne pathogens to food processing-related stresses can undermine food processing interventions, potentially increasing the likelihood of foodborne illness (Xu et al., 2008).

Subinhibitory concentrations of ethanol can be encountered by foodborne pathogens in a variety of niches. Ethanol itself and cleaners containing ethanol are employed in some food processing environments to control microorganisms on equipment and for promoting good worker hygiene (Chiou et al., 2004). The efficacy of low concentrations of ethanol (2–5%) as a preservative in a wide variety of foods such as hamburger, soy sauce, sponge cake and packed egg-tofu was also demonstrated by Shibasaki (1982). Moreover, ethanol can be found in fermented foods/beverages, fruits and fruit products (Chiou et al., 2004). Several Gram-positive and -negative bacteria including *Listeria monocytogenes* (Lou and Yousef, 1997), *Cronobacter sakazakii* (Huang et al., 2013) and *Vibrio parahaemolyticus* (Chiang et al., 2006, 2008; Chiang and Chou, 2009) have been reported to become adapted to a mild ethanol stress and to exhibit a marked induction of direct protection and cross-protection to subsequent stresses, thus potentially increasing microbial food safety risks. Concerning cross-protection against

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acid stress, the majority of previous studies were mainly focused on clarifying the effect of ethanol adaptation on the tolerance of pathogens to inorganic hydrochloric acid (Chiang et al., 2006; Chiang and Chou, 2009; Huang et al., 2013; Lou and Yousef, 1997). Furthermore, none of these studies have examined bacterial survival in acidic food systems as influenced by ethanol adaptation.

The mechanisms involved in ethanol-induced cross-protection against acid stress in foodborne pathogens remain largely unknown; however, previous studies have found changes in gene expression in response to trisodium phosphate, low/high temperature, low-shear modeled microgravity, desiccation and heat shock (Fong and Wang, 2016; Kim and Rhee, 2016; Yang et al., 2014a, b). Many types of shock genes or proteins (i.e. heat shock proteins, acid shock proteins) induced under stressful conditions can protect bacteria against cell damage (Yang et al., 2014a). Specific sigma factors are known to initiate the expression of these stress-related genes. For example, acid shock proteins can be induced by σ^S (encoded by *rpoS*) to protect bacteria against extreme acidic conditions (Yang et al., 2014a, b). Hence, we hypothesized that ethanol adaptation might influence the transcription levels of these acid tolerance-related genes, contributing to cross protection response during subsequent exposure to acid stress in foodborne pathogens.

Salmonella enterica serovar Enteritidis (*S.* Enteritidis) has been reported as the world-leading cause of salmonellosis (Yang et al., 2014b). Eggs, egg-containing foods, poultry and other meat products are the most frequently implicated sources of salmonellosis outbreaks (Harris et al., 2003). Fruit juice products, once excluded from food safety issues due to their inherent acidity caused by naturally occurring organic acids, have also been involved in salmonellosis outbreaks when they are unpasteurized (Gabriel et al., 2015; Yuk and Schneider, 2006). Given that acidification is a commonly employed approach to control contamination with and proliferation of *Salmonella enterica* in food (López-Malo et al., 2012), a fundamental understanding of the development of acid resistance in *S.* Enteritidis assumes great importance. Our previous study showed that *S.* Enteritidis exhibited enhanced tolerance to 15% ethanol and −20 °C after adaptation in 5% ethanol for 1 h (He et al., 2016). The current research was conducted to explore the influence of ethanol adaptation on *S.* Enteritidis tolerance to various acids and on its survival in selected fruit juices. The expression patterns of three acid tolerance-related genes in response to ethanol adaptation were also characterized.

2. Materials and methods

2.1. Acids

Acetic (99.5%) and ascorbic (99.7%) acids were procured from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Lactic (85%), citric (99.5%), malic (98%) and hydrochloric (36%–38%) acids were obtained from Sinoreagent Co., Ltd. (Shanghai, China).

2.2. Test microorganism

S. Enteritidis ATCC 13076, provided by the Shanghai Entry-Exit Inspection and Quarantine Bureau of China, was stored in 25% glycerol at −80 °C in our laboratory. Prior to each experiment, this bacterium was streaked onto Luria-Bertani (LB) agar and incubated overnight at 37 °C. A single colony was transferred to 5 ml LB broth and incubated at 37 °C for 24 h. Five-hundred μl of this culture was then inoculated into a flask containing 50 ml LB broth, followed by incubation at 37 °C/200 rpm for 5 h. This late log phase culture was utilized in ethanol-adaptation experiments.

2.3. Ethanol adaptation treatment

Ethanol-adapted cells of *S.* Enteritidis were obtained as previously described (He et al., 2016). Briefly, a 1-ml aliquot of a late log-phase culture was harvested by centrifugation at 8000*g* for 10 min, the pellet was washed with PBS (phosphate buffered saline, pH 7.4) and resuspended in 10 ml fresh LB (control) or in LB containing 5% (v/v) ethanol (Changshu Yangyuan Chemical Co. Ltd., China). The sample was then incubated at 25 °C/170 rpm for 1 h. This adaptation condition was used because it induced the highest direct protection against subsequent lethal ethanol challenge in *S.* Enteritidis (He et al., 2016).

2.4. Acid tolerance test in vitro

The induction of cross-protection against acid stress was initially investigated *in vitro* by comparing the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values of different acids against ethanol-adapted and non-adapted *S.* Enteritidis. The microtiter plate test modified by Monte et al. (2014) was employed to determine the MIC of acids. Solutions of acids (160–0.156 μl/ml for acetic, hydrochloric and lactic acids; 160–0.156 mg/ml for citric, ascorbic and malic acids) were prepared in sterile distilled water. One-hundred μl of water or acid solutions were pipetted into the wells of 96-well plates (Corning Incorporated, Corning, NY, USA) containing 100 μl of 2 × LB broth. Afterwards, 2 μl of ethanol-adapted or non-adapted *S.* Enteritidis cells (approximately 10^8 CFU/ml) was inoculated into each well. Plates were statically incubated at 37 °C for 24 h. The MIC was recorded as the lowest concentration of acid that completely inhibited the visible growth of *S.* Enteritidis (Jordán et al., 2013). MBCs were determined following the MIC assays (Dung et al., 2008). An aliquot of 50 μl from each well without visible bacterial growth was spread onto the surface of LB agar plates and incubated at 37 °C for 24 h. The lowest concentration of acid at which *S.* Enteritidis completely failed to grow was defined as MBC. Acid tolerance was considered to be significantly induced when the MIC or MBC of the ethanol adapted cells was at least two-fold higher than that of the non-adapted cells (Hammer et al., 2012).

2.5. Survival studies in fruit juices

To evaluate the survival of *S.* Enteritidis in acidic beverages, commercially available orange juice (pH 3.77) and apple juice (pH 3.57) (with no declared added preservatives) were obtained from a local supermarket. These two kinds of juices were selected because they are most commonly reported as beverage vectors of foodborne diseases (Gabriel et al., 2015). Plating the fruit juices onto LB agar revealed no microbial growth after 24 h incubation at 37 °C. When the acid tolerance test was performed, 0.1 ml of ethanol-adapted and non-adapted *S.* Enteritidis cells (approximately 10^8 CFU/ml) was inoculated into 10 ml orange juice or apple juice. These juices were stored at 4 or 25 °C for 48 h. Samples were taken after 12, 24, 36 and 48 h and serially diluted with sterile normal saline (0.85%). Subsequently, 0.1 ml of appropriate dilutions was plated onto LB agar. Colonies were enumerated after 24 h incubation at 37 °C.

2.6. RNA extraction and RT-qPCR analysis

Ethanol-adapted and non-adapted *S.* Enteritidis cells were centrifuged at 12000*g* for 1 min, resuspended in 200 μl TE buffer containing lysozyme (10 mg/ml), and incubated at 37 °C for 3 min. The treated cells were harvested by centrifuging at 12000*g* for 1 min. Total RNA was extracted with Trizol reagent based on the manufacturer's protocol (Invitrogen, Carlsbad, USA). The quantity