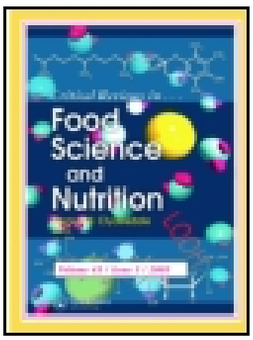

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REVIEW

Ethanol adaptation in foodborne bacterial pathogens

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ABSTRACT

Foodborne pathogens possess the ability to develop adaptive responses to sublethal environmental stresses, leading to increased tolerance to homologous or heterologous stressing agents commonly applied during food manufacturing. This phenomenon may counteract the effectiveness of current intervention strategies to ensure food safety, thus increasing consumer risk. Foodborne pathogens encounter ethanol, a common food component and a widely used food processing agent, in a variety of niches during their life cycles. The present contribution provides an overview of the influence of adaptation to sublethal doses of ethanol on the stress tolerance of major foodborne pathogens (e.g. *Salmonella enterica*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Bacillus cereus*, and *Cronobacter sakazakii*). Fundamental studies on ethanol adaptation mechanisms with a focus on cell membrane properties, gene expression patterns, protein profiles, and mutagenic analyses are discussed. Furthermore, knowledge gaps on effective mitigation of ethanol adaptation in foodborne pathogens are identified and addressed.

KEYWORDS

Ethanol adaptation; pathogenic bacteria; stress tolerance; cell membrane; gene expression; protein profile

Introduction

Foodborne pathogens encounter a vast array of stresses (e.g. heating, freezing, desiccation, osmotic stress, pH extremes, and disinfectants) during food processing and storage (Begley and Hill 2015; Burgess et al. 2016; Esbelin, Santos, and Hébraud 2018; Spector and Kenyon 2012). As such, they must be able to sense and respond appropriately to these environmental signals in order to combat unfavorable conditions (Fong and Wang 2016; Winfield and Groisman 2003). In fact, many pathogens present in foods often survive under stressful environments and retain their pathogenicity, causing subsequent foodborne illness (Yoon et al. 2015). Therefore, much attention has been given to the mechanisms of how foodborne pathogens respond to environmental stresses.

Ethanol (C_2H_5OH) is a small molecule produced through microbial fermentation or by chemical synthesis. It kills microorganisms via membrane damage, cellular dehydration and protein denaturation (Dao and Dantigny 2011; McDonnell and Russell 1999). Ethanol can also be metabolized to form acetic acid and other antimicrobials that are known to inactivate microorganisms. As a food component, ethanol is frequently found in alcoholic beverages, fermented foods, fruits, fruit products as well as baked products at major or minor levels (Table 1) (Alzeer and Hadeed 2016; Chiou et al. 2004; Dao and Dantigny 2011; Logan and Distefano 1998). Furthermore, its use as a direct human food ingredient has been generally recognized as safe in the United States.

There is a long history of employing ethanol for general-purpose sanitation, shelf life extension as well as flavoring and colorant extraction in the food industry. Ethanol and disinfectants containing ethanol are utilized in food processing plants to promote good worker hygiene and to control microbial contamination on equipment and food contact surfaces (Møretrø et al. 2012; Slany, Oppelt, and Cincarova 2017). Direct addition of ethanol (0.5–5%) has a beneficial effect on shelf life extension of some foods (e.g. bread, packed egg-tofu, sponge cake, and soy sauce) (Doulia, Katsinis, and Mougin 2000; Katsinis, Rigas, and Doulia 2008; Shibasaki 1982), while immersion in ethanol solution (2.5–70%) is an effective approach to controlling postharvest decay of fruits such as grapes, strawberries, peaches, lemons, and apples (Dao and Dantigny 2011). Moreover, ethanol is a permitted solvent for the colorants and flavorings used in many foods (Sowbhagya and Chitra 2010). The aforementioned applications of ethanol in the food industry provide opportunities for foodborne pathogens to encounter sublethal concentrations of ethanol, which is defined as exhibiting inhibitory activity but is not lethal to the target bacteria (Andersson and Hughes 2014).

Sublethal ethanol stress can trigger adaptive responses in foodborne pathogens that enhance their tolerance to subsequent stresses. This phenomenon is termed ethanol adaptation and has received much attention in the last two

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Table 1. Ethanol content of various food items.

Food item	Ethanol content	Reference
Beer	3.00–6.00% (v/v)	Wang et al. (2003)
Wine	7.00–21.00% (v/v)	Wang et al. (2003)
Liqueur	20.00–50.00% (v/v)	Wang et al. (2003)
Kombucha	1.12–2.00% (v/v)	Talebi et al. 2017)
Apple juice	0.06–0.66 g/L	Gorgus, Hittinger, and Schrenk (2016)
Grape juice	0.29–0.86 g/L	Gorgus, Hittinger, and Schrenk (2016)
Orange juice	0.16–0.73 g/L	Gorgus, Hittinger, and Schrenk (2016)
White vinegar	2.64 g/L	Gorgus, Hittinger, and Schrenk (2016)
Rye bread	0.17–0.20 g/100 g	Gorgus, Hittinger, and Schrenk (2016)
Wheat and rye bread	0.29 g/100 g	Gorgus, Hittinger, and Schrenk (2016)
Wheat toast	0.18 g/100 g	Gorgus, Hittinger, and Schrenk (2016)
Burger rolls	1.28 g/100 g	Gorgus, Hittinger, and Schrenk (2016)
Sweet milk rolls	1.21 g/100 g	Gorgus, Hittinger, and Schrenk (2016)
Ripe pear	0.04 g/100g	Gorgus, Hittinger, and Schrenk (2016)
Ripe banana	0.02 g/100g	Gorgus, Hittinger, and Schrenk (2016)
Fruit salad	0.01 g/100g	Gorgus, Hittinger, and Schrenk (2016)
Cherry yogurt	0.02 g/100g	Gorgus, Hittinger, and Schrenk (2016)
Kefir	0.02 g/100g	Gorgus, Hittinger, and Schrenk (2016)

decades due to the enhanced persistence of these pathogens in the food processing continuum. Many ethanol-adapted pathogens (e.g. *Salmonella Enteritidis*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Bacillus cereus*, and *Cronobacter sakazakii*) become more tolerant to homologous (direct protection) or heterologous (cross protection) stressing agents (Browne and Dowds 2001, 2002; Chiang, Ho, and Chou 2006, 2008; Chiang and Chou 2009; He et al. 2016, 2018; Huang et al. 2013; Lou and Yousef 1997). The acquired stress tolerance may reduce the effectiveness of currently employed measures (e.g. chemical disinfection) for controlling pathogens and increase the persistence of pathogens in the food chain, thus compromising food safety. For instance, a strain of *Salmonella* Agona responsible for two foodborne outbreaks persisted in food processing environments for up to 10 years despite intensive cleaning and sanitation of contaminated equipment (Russo et al. 2013). In this context, it is of paramount significance to evaluate the ability of foodborne pathogens to mount stress tolerance following exposure to chemical disinfectants such as ethanol. Herein this review covers the currently available studies regarding the effects of ethanol on stress tolerance acquisition and adaptive physiological responses in foodborne pathogens.

Ethanol direct protection in foodborne pathogens

Ethanol direct protection refers to the phenomenon where adaptation to sublethal levels of ethanol subsequently protects the bacterium from normally lethal ethanol conditions (He et al. 2016). Recent studies on the development of direct protection as influenced by ethanol adaptation in foodborne pathogens are summarized in Table 2. Ethanol direct protection has been widely reported in the last two decades, stemming from the work on *L. monocytogenes* conducted by Lou and Yousef (1997). In this study, ethanol-adapted cells were prepared through incubation of *L. monocytogenes* in 5% ethanol for 1 h. Ethanol-adapted and non-adapted cells were then exposed to a lethal concentration (17.5%) of ethanol and the viable count of *L. monocytogenes* was determined after 0, 2, 4, 6, 8 and 10 h. It was found that ethanol-adapted cells survived better than non-adapted ones during lethal ethanol challenge, possibly due to the induction of stress proteins by sublethal ethanol stress. Similarly, Browne and Dowds (2001, 2002) reported that pretreatment with 2.5% ethanol conferred protection against a subsequent lethal ethanol stress (12%) in *B. cereus*. Furthermore, previous exposure to 2.5–10% ethanol for 1 h induced tolerance to 15% ethanol in *S. Enteritidis* (He et al. 2016). This direct protection effect was also observed in *V. parahaemolyticus* and *C. sakazakii* (Chiang, Ho, and Chou 2006; Huang et al. 2013). However, the degree of protection is often influenced by factors including the phase of growth, the concentration of ethanol and the duration of adaptation, which will be discussed herein.

Influence of growth phase

The bacterial growth phase is a critical factor affecting the magnitude of the stress response (De Angelis et al. 2004; Jørgensen, Hansen, and Knøchel 1999; McMahon et al. 2000; Yeung and Boor 2004). To our knowledge, the influence of growth phase on the acquisition of ethanol direct protection has only been assessed in *V. parahaemolyticus* (Chiang and Chou 2009). In this study, *V. parahaemolyticus* was harvested at different points of growth (i.e. mid-exponential, late-exponential or stationary phase) and then exposed to an initial stress of 5% ethanol for 1 h. This adaptation resulted in enhanced sensitivity in mid-exponential *V. parahaemolyticus* (ca. 4-fold decrease in survival percentage) to 8% ethanol. For stationary phase cultures, no significant difference in ethanol tolerance was found between non-adapted and ethanol-adapted groups. In contrast, ethanol direct protection occurred in late-exponential cells of *V. parahaemolyticus*; the survival percentage of ethanol-adapted cells (58.68%) was significantly higher than that of non-adapted counterparts (40.50%) after exposure to 8% ethanol for 30 min. Collectively, these results demonstrate the influence of growth phase on the response of ethanol-adapted *V. parahaemolyticus* to 8% ethanol challenge. Variations in the expression levels of stress proteins may account for the growth phase-dependency phenotype in ethanol adaptation (Chiang and Chou 2009).

Table 2. Scientific publications regarding ethanol direct protection in major foodborne bacterial pathogens.

Bacterium	Ethanol adaptation conditions	Ethanol challenge conditions	Primary findings	Reference(s)
L. monocytogenes	5% ethanol for 1 h	17.5% ethanol for 10 h	First description of ethanol direct protection (ca. 3 logs of protection after 10 h of ethanol challenge) in foodborne pathogens	Lou and Yousef (1997)
B. cereus	2.5% ethanol for 20 and 40 min	12% ethanol for 20 min	Ethanol direct protection (1–5 logs of protection after 20 and 40 min of ethanol challenge) in *B. cereus*	Browne and Dowds (2001, 2002)
V. parahaemolyticus	5% ethanol for 0.5 and 1 h	8% ethanol for 2.5 h	Influence of adaptation time on ethanol direct protection (ca. 5.8- and 9.2-fold increase in survival percentage after 2.5 h of ethanol challenge) in *V. parahaemolyticus*	Chiang, Ho, and Chou (2006)
	5% ethanol for 1 h	8% ethanol for 0.5 h	Influence of growth phase on ethanol direct protection development in *V. parahaemolyticus*	Chiang and Chou (2009)
C. sakazakii	4% and 5% ethanol for 1 h	15% ethanol for 1 h	Ethanol direct protection (ca. 2,896- and 6,400-fold increase in survival percentage after 40 min of ethanol challenge) in *C. sakazakii*	Huang et al. (2013)
S. Enteritidis	2.5–10% ethanol for 1 h	15% ethanol for 1.5 h	Influence of adaptation concentration on ethanol direct protection (ca. 60- to 442-fold increase in survival percentage after 1.5 h of ethanol challenge) in *S.* Enteritidis	He et al. (2016)

Influence of ethanol concentration used for adaptation

Induction of ethanol direct protection in pathogenic bacteria is also largely influenced by the concentration of ethanol used for adaptation. Trypticase soy broth (TSB) containing 4% and 5% ethanol was employed by Huang et al. (2013) to induce direct protection in *C. sakazakii*. Cells adapted with 5% ethanol exhibited greater tolerance to 15% ethanol than those adapted with 4% ethanol. In *S. Enteritidis*, the tolerance of the cells to 15% ethanol increased when the ethanol concentration utilized for the sublethal treatment was increased from 2.5% to 5% in Luria-Bertani broth. However, no significant difference in ethanol tolerance was found among cells adapted with 5%, 7.5%, and 10% ethanol, respectively (He et al. 2016). These observations suggest that increasing the concentration employed for ethanol adaptation stimulates a more effective ethanol tolerance response, but there exists a limit in the concentration that induces maximal protection.

Influence of exposure duration

The duration of ethanol adaptation plays a role in the development of direct protection in *B. cereus* and *V. parahaemolyticus*. It was shown that greater direct protection in *B. cereus* was achieved by extending the ethanol adaptation time from 20 to 40 min (Browne and Dowds 2001, 2002). Likewise, *V. parahaemolyticus* adapted in 5% ethanol for 1 h exhibited a significantly higher ethanol tolerance than those adapted for 0.5 h (Chiang, Ho, and Chou 2006). However, in these studies, short exposure durations ($\leq$1 h) were used to investigate bacterial ethanol adaptation and it still remains unknown whether long-term exposure (e.g. up to 24 h) to sublethal levels of ethanol is capable of conferring direct protection. Therefore, studies on long-term exposure would be of importance as this is reflective of what commonly occurs in the food industry and related real-world scenarios.

Ethanol cross protection in foodborne pathogens

Foodborne pathogens are exposed to diverse environmental stresses during food processing and storage. The current trend toward production of minimally processed foods has driven combinatorial usage of mild (sublethal) control measures, also known as hurdle technology (Lai and Wong 2013; Leistner 2000; Singh and Shalini 2016). As a result, pathogens must overcome stresses both in parallel and in succession to ensure survival. Cross protection occurs when adaptation to one environmental stress confers tolerance to a subsequent, unrelated stress. This cross-protective effect can be attributed to the overlapping roles of specific stress response genes (Finn, Condell, et al. 2013), posing a threat to the food industry since it can potentially undermine food processing interventions. As shown in Figure 1, cross protection against high/low temperature, high salinity, oxidative stress, and low pH induced by ethanol adaptation has been evaluated in several Gram-negative and Gram-positive bacteria (e.g. *S. Enteritidis*, *V. parahaemolyticus*, *C. sakazakii*, *L. monocytogenes*, and *B. cereus*). Overall, it was found that the

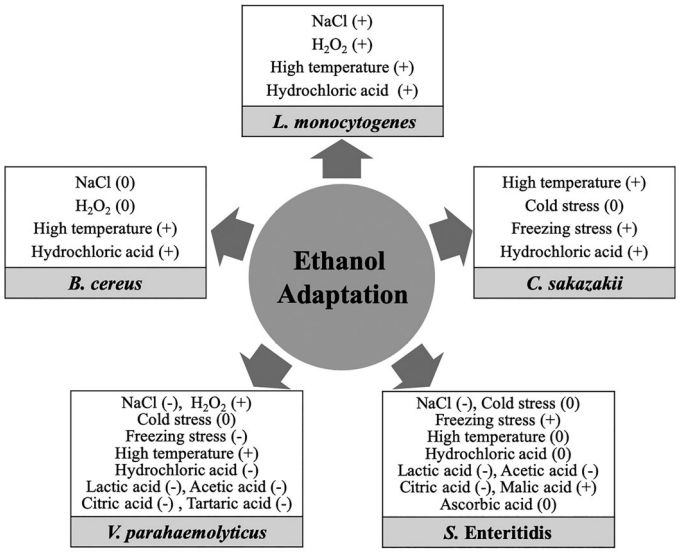

Figure 1. A summary of ethanol cross protection in foodborne bacterial pathogens.
Note: +, protection; −, sensitization; 0, no effect. *B. cereus* was adapted in 2.5% ethanol for 20 or 40 min, while other pathogens were exposed to 5% ethanol for 1 h.

acquisition of ethanol cross protection was dependent on the target bacteria and the subsequent stress.

Tolerance to high temperature

Similar to sublethal heat shock that resulted in enhanced thermal tolerance (Chang, Chiang, and Chou 2009), ethanol adaptation conferred heat tolerance to all tested pathogens, except for *S. Enteritidis* (Figure 1). For example, ethanol-adapted *C. sakazakii* exhibited a survival percentage of 6.67% after exposure to 51 °C for 2 h, corresponding to a 44.5-fold increase in survival compared with the non-adapted control (Huang et al. 2013). The survival percentage of ethanol-adapted *V. parahaemolyticus* was 0.31% after incubation at 47 °C for 50 min, which was appropriately 10 times higher than that of non-adapted groups (Chiang, Ho, and Chou 2006). Furthermore, *B. cereus* populations were one to three log CFU/ml greater after exposure to 49 °C for 20 min than the controls following pretreatment with 2.5% ethanol for 20 min (Browne and Dowds 2001).

The effect of ethanol concentration used for adaptation on subsequent tolerance to heat stress has also been observed. For instance, no significant effect on the heat tolerance in *L. monocytogenes* exposed to 56 °C was identified with an ethanol concentration of ≤1% (Lou and Yousef 1996). However, adaptation with 2–8% ethanol was sufficient to provide protection to subsequent thermal stress. Maximum heat tolerance, as evidenced by a 4-fold increase in $D_{56°C}$, was achieved when 4–8% ethanol was used. Furthermore, exposure to 12% ethanol decreased the tolerance of *L. monocytogenes* to heat, indicating that this concentration caused cellular damage (Lou and Yousef 1996).

Tolerance to low temperature

None of the tested pathogens shown in Figure 1 altered their tolerance to cold stress at 4 °C following exposure to 5% ethanol for 1 h. The survival percentage of non-adapted and ethanol-adapted *C. sakazakii*, *S. Enteritidis* and *V. parahaemolyticus* exhibited no significant difference during exposure to 4 °C for 7, 9 and 10 days, respectively (Chiang, Ho, and Chou 2006; He et al. 2016; Huang et al. 2013). These findings suggest that cold stress has extensive requirements (e.g. requires induction of a variety of genes unrelated to ethanol stress) for the acquisition of tolerance.

Conversely, the development of cross protection against freezing stress (appropriately −20 °C) appears to be pathogen-dependent. Ethanol adaptation facilitated the tolerance of *C. sakazakii* and *S. Enteritidis* to −20 °C stress (He et al. 2016; Huang et al. 2013). For instance, adapted *S. Enteritidis* demonstrated a 20-fold increase in survival percentage after exposure to −20 °C for 18 h (He et al. 2016). On the contrary, *V. parahaemolyticus* demonstrated enhanced susceptibility to freezing stress following ethanol adaptation as evidenced by the lower survival percentage of ethanol-adapted cultures during storage at −18 °C for 5 days, compared with that of non-adapted controls (Chiang, Ho, and Chou 2006).

Tolerance to sodium chloride (NaCl)

Among the tested pathogenic bacteria, only *L. monocytogenes* was able to mount cross protection against NaCl (Figure 1). After incubation in 25% NaCl for 350 h, ethanol-adapted (5% ethanol for 1 h) *L. monocytogenes* showed higher survival by appropriately 4 log CFU/mL than the non-adapted control (Lou and Yousef 1997). In contrast, cross protection did not occur when ethanol-adapted (2.5% ethanol for 20 or 40 min) *B. cereus* cells were further exposed to 12% NaCl for 20 min (Browne and Dowds 2001, 2002). Furthermore, sensitization to NaCl was observed in *V. parahaemolyticus* and *S. Enteritidis*; exposure to 5% ethanol for 1 h led to reduced survival of *V. parahaemolyticus* subjected to 20% NaCl (Chiang and Chou 2009). Along a similar vein, cell death occurred more rapidly in ethanol-adapted *S. Enteritidis* after exposure to 10% NaCl for 4 h, compared to the non-adapted control (He et al. 2016).

Tolerance to hydrogen peroxide (H_2O_2)

Bacterial tolerance to H_2O_2 following ethanol adaptation has been previously studied in *B. cereus, L. monocytogenes,* and *V. parahaemolyticus* (Figure 1). Adaptation in 2.5% ethanol for 20 min did not afford protection to *B. cereus* against subsequent challenge with 5 mM H_2O_2 (Browne and Dowds 2001). However, ethanol adaptation was found to enhance the tolerance of *L. monocytogenes* to H_2O_2. Exposure of this organism to 5% ethanol for 1 h led to greater survival by appropriately 7.5 log CFU/mL in

ethanol-adapted cells after incubation in 0.1% H_2O_2 for 4 h (Lou and Yousef 1997).

Superoxide dismutase (SOD) and catalase (CAT) enable bacterial cells to scavenge reactive oxygen radicals (e.g. H_2O_2 and superoxide), which oxidize lipids and lethally damage cell membranes. Lower levels of SOD and CAT activities were observed upon ethanol adaptation in *V. parahaemolyticus* (Chiang and Chou 2008). It is reasonable to expect that ethanol-adapted *V. parahaemolyticus* may be more susceptible to oxidative stress. However, ethanol adaptation increased the tolerance of *V. parahaemolyticus* to H_2O_2 (Chiang, Ho, and Chou 2008a). Furthermore, cells adapted to 5% ethanol for 1 h exhibited a higher survival percentage than cells adapted for 30 min after incubation in PBS-3% NaCl containing 20 ppm H_2O_2 for 25 min. This protective effect has been hypothesized to result from the synthesis of stress proteins, which was shown to suppress DNA damage caused by H_2O_2 (Park, You, and Imlay 2005).

Tolerance to acids

Tolerance to inorganic acids

The response of ethanol-adapted pathogens to hydrochloric acid (HCl) varied (Figure 1). We previously demonstrated that ethanol adaptation failed to alter tolerance of *S. Enteritidis* to HCl as determined by minimum inhibitory concentration and minimum bactericidal concentration (MBC) assays (He et al. 2018). Conversely, ethanol adaptation rendered mid- and late-exponential phase *V. parahaemolyticus* more sensitive to pH 4.4 with a decrease in survival percentage by 3.15% and 8.51%, respectively (Chiang and Chou 2009). On the other hand, ethanol adaptation promoted the survival of *C. sakazakii*, *L. monocytogenes,* and *B. cereus* in acidic pH values of 3.3, 3.5, and 4.6, respectively (Browne and Dowds 2002; Huang et al. 2013; Lou and Yousef 1997). For instance, ethanol-adapted *C. sakazakii* exhibited a survival percentage of 13.8% compared to 0.6% in non-adapted cells following challenge in acidified TSB (pH 3.3) for 1 h (Huang et al. 2013). Similarly, the population of ethanol-adapted *L. monocytogenes* was appropriately 5 log CFU/mL higher than that of the non-adapted control after exposure to pH 3.5 for 4 h (Lou and Yousef 1997).

Tolerance to organic acids

Tolerance of foodborne pathogens to organic acids resulting from ethanol adaptation is comparatively understudied. Chiang, Ho, and Chou (2008a) reported that ethanol adaptation resulted in enhanced sensitivity of *V. parahaemolyticus* to 25 mM tartaric, citric, lactic, and acetic acids. Our group found that *S. Enteritidis* acquired tolerance to malic acid after ethanol adaptation solely as evidenced by a two-fold increase in MBC, along with the upregulation of several acid tolerance genes. Enhanced tolerance to citric, lactic, ascorbic, and acetic acids was, however, not observed (He at al. 2018).

Adaptive responses in foodborne pathogens induced by ethanol

Recent studies regarding adaptive responses of foodborne pathogens to ethanol are summarized in Table 3. Most of these research studies have focused on identifying alterations in cell membrane properties, gene expression patterns and protein profiles, with a few studies assessing the function of ethanol stress-related genes and proteins by mutagenic analysis.

The induction of some stress tolerance genes and/or proteins affords protection against cell damage and death (Yang et al. 2014). However, as the initial line of defense, it is equally vital that the integrity and function of the cell membrane be maintained under stressful conditions (Patrignani et al. 2008). For example, bacteria often alter their fatty acid composition (e.g. saturation/unsaturation ratio, *cis*/*trans* isomerization, branched-chain fatty acid synthesis, fatty acyl chain length, and cyclopropane fatty acid level) in response to environmental stresses for maintaining an extent of membrane fluidity compatible with survival (Álvarez-Ordóñez et al. 2012; Hingston et al. 2017; Yoon et al. 2015). These modulatory responses, however, often differ depending on the organism, the physiological state of bacterial cells, and the magnitude and type of the stress imposed.

Cell membrane characteristics

Cell membrane permeability

Adaptation with 2.5–10% ethanol for 1 h did not alter the cell membrane permeability of *S. Enteritidis*; no increase in absorbance at 260 nm (nucleic acids) nor 280 nm (intracellular proteins) was observed, indicating the absence of cellular leakage. Further imaging by scanning electron microscopy (SEM) also confirmed that both non-adapted and ethanol-adapted *S. Enteritidis* cell surfaces were intact (He et al. 2016).

It was observed that the supernatant derived from *V. parahaemolyticus* cultures exhibited a significantly higher absorbance at 280 nm and 260 nm after adaptation in 5% ethanol for 30 and 60 min. Features indicative of cell-surface damage (e.g. cell wall pitting and extensive cell disruption) were additionally noted on the adapted cells through observation with SEM. Interestingly, abundant amounts of irregularly-shaped material, presumably leaked intracellular substances or extracted ethanol soluble cell surface components, were also observed on the surface (Chiang, Ho, and Chou 2006). Taken together, it was evident that ethanol adaptation damaged the cell envelope of *V. parahaemolyticus*, resulting in enhanced cell membrane permeability and the subsequent leakage of intracellular substrates.

Fatty acid composition

Chiang, Ho, and Chou (2008a) assessed the fatty acid profile of ethanol-adapted (5% ethanol for 30 and 60 min) *V. parahaemolyticus.* They found that the proportion of palmitic acid (16:0), a main saturated fatty acid (SFA) was decreased, whereas vaccenic acid (18:1), a main unsaturated fatty acid

Table 3. Scientific publications assessing ethanol stress-induced adaptive responses in major foodborne bacterial pathogens.

Bacterium	Ethanol treatments	Evaluated adaptive response	Main findings	Reference
E. coli O157:H7	5% ethanol for 30 h	Fatty acid composition	Increase in the amount of total fatty acids; *cis* to *trans* isomerization of oleic acid	Chiou et al. (2004)
		Protein profile	Increase in protein content; upregulation of a protein (28 kDa) identical to phage shock protein A	
V. parahaemolyticus	5% ethanol for 0.5 and 1 h	Cell membrane permeability	Increase in cell membrane permeability	Chiang, Ho, and Chou (2006)
	5% ethanol for 0.5 and 1 h	Fatty acid composition	Decrease in saturated fatty acids and increase in unsaturated fatty acids	Chiang, Ho, and Chou (2008a)
	5% ethanol for 1 h	Protein profile	Upregulation of 8 proteins and downregulation of 7 proteins; increase in expression of GroEL-like protein (a well-characterized heat shock protein)	Chiang, Ho, Yu, et al. (2008)
S. Typhimurium	2.5–20% ethanol for 1 h	Protein profile	Upregulation of 9 proteins and downregulation of 10 proteins; induction of phage shock protein PspG	Hassani et al. (2009)
S. Enteritidis	2.5–10% ethanol for 1 h	Cell membrane permeability	Unaltered cell membrane permeability	He et al. (2016)
	5% ethanol for 1 h	Gene expression pattern	Upregulation of two acid tolerance genes (*rpoS* and SEN 1564A)	He et al. (2018)
	5% ethanol for 1 h	Protein profile and mutagenic analysis	Upregulation of 56 proteins related to purine metabolism and ABC transporters and downregulation of 82 proteins associated with enterobactin biosynthesis, ribosome, flagellar assembly, and virulence; deletion of either *hiuH* or *proX* resulted in the development of a higher degree of ethanol direct protection	He et al. (2019)
S. aureus	2.4% ethanol for 24 h	Gene expression pattern	Upregulation of 95 genes and downregulation of 18 genes; differential genes were involved in metabolism, transport, virulence, biofilm formation, gene regulation, and stress response	Korem, Gov, and Rosenberg (2010)
C. botulinum	1–5% ethanol for 28 h	Mutagenic analysis	Mutation in *cspA* assisted bacterial growth under ethanol stress, while growth deficiency was observed in *cspB* or *cspC* mutant compared to the wild type	Derman et al. (2015)
L. monocytogenes	3.5% ethanol for 48 h	Mutagenic analysis	Identification of two-component system genes (e.g. *virS*, *liaS*) and putative DEAD-box RNA helicase genes (e.g. *lmo0866*, *lmo1450*) involved in ethanol stress response	Markkula et al. (2012); Pontinen et al. (2017)

(USFA) was increased by ethanol adaptation. However, the percentage of other identified fatty acids (e.g. palmitoleic acid, stearic acid, and linoleic acid) did not change. The elevated USFA/SFA ratio can lead to enhanced membrane fluidity (Yoon et al. 2015), which correlates well with the increased cell membrane permeability of *V. parahaemolyticus* following ethanol adaptation.

The fatty acid composition of two distinct strains of *Escherichia coli* O157:H7 (30-2C4 isolated from salami and SEA 13B88 isolated from unpasteurized apple juice) was

analyzed following exposure to 5% ethanol for 30 h (Chiou et al. 2004). Significantly higher amounts of dodecanoate, tetradecanoate, 3-hydroxytetradecanoate, *cis*-9-hexadecenoate, hexadecanoate, heptadecanoate, and *trans*-oleic acids were observed compared with those in non-adapted cells, with the exception of nonadecanoic and *cis*-oleic acids. Interestingly, *cis*-oleic acid was found in non-adapted cells of *E. coli* O157:H7 SEA 13B88 (0.32 mg/g) and 30-2C4 (0.36 mg/g), but not in ethanol-adapted cells. In contrast, the proportion of *trans*-oleic acid was 10-fold lower in cells grown in pure TSB than those grown in TSB containing 5% ethanol. Based on these observations, an isomerization of *cis* oleic acid to *trans* oleic acid appears to be induced by ethanol adaptation in *E. coli* O157:H7. Alterations in fatty acid profile may lead to changes in bacterial susceptibility upon subsequent exposure to food processing-related stresses such as acid shock, cold adaptation and heat treatment (Alvarez-Ordóñez et al. 2015). Unfortunately, the stress tolerance of ethanol-adapted *E. coli* O157:H7 has not yet been determined.

Gene expression pattern

He et al. (2018) assessed the expression of acid tolerance genes in ethanol-adapted (5% ethanol for 1 h) *S. Enteritidis*. It was observed that the expression of *hdeB* encoding an acid resistance protein was not differentially expressed. Moreover, the acid shock gene SEN1564A and the general stress response gene *rpoS* were upregulated in response to ethanol. Importantly, RpoS is a global gene regulator which mediates the induction of several acid shock proteins, whose synthesis is essential for *S. enterica* survival at low pH by the prevention and repair of acid-induced damages (Álvarez-Ordóñez et al. 2012). Hence, cross protection against acid stress stimulated by ethanol adaptation in *S. Enteritidis* may occur because some genes (e.g. *rpoS* and SEN1564A) play overlapping roles in the acid and ethanol stress responses (He et al. 2018).

In a separate study, global changes in the gene expression of *Staphylococcus aureus* after exposure to 2.4% ethanol for 24 h was previously determined by microarray analysis (Korem, Gov, and Rosenberg 2010). Of the 7775 genes detected on *S. aureus* GeneChip, a total of 113 genes involved in metabolism, transport, virulence, biofilm formation, gene regulation, and stress response were significantly differentially expressed. Interestingly, the aforementioned pathways were also responsible for the response of *S. aureus* biofilm to 2.5% ethanol as determined by transcriptome sequencing (RNA-seq), indicating a similarity in gene expression pattern between planktonic and biofilm cells of this organism under sublethal ethanol stress (Slany, Oppelt, and Cincarova 2017).

Protein profile

Chiang, Ho, Yu, et al. (2008) assessed the protein expression pattern of *V. parahaemolyticus* subjected to 5% ethanol for 1 h. SDS-PAGE analysis indicated that 4 protein species with a molecular mass of 100, 86, 46, and 30 kDa were reduced 0.50- to 0.83-fold in ethanol-adapted cells, compared to the controls. On the other hand, the expression of 93-, 77- and 58-kDa proteins was enhanced 1.24- to 1.46-fold by ethanol. Subsequent analysis of the protein profile by 2-D electrophoresis found that the expression of 7 proteins was reduced 0.22- to 0.64-fold while the expression of 8 proteins was enhanced 1.11- to 1.94-fold in ethanol-adapted cells. The GroEL-like protein (a molecular chaperon involved in the proper folding of heat stress-damaged proteins) was also upregulated in ethanol-adapted cells as revealed by immunoblot, possibly accounting for the acquisition of cross protection against heat stress after ethanol adaptation in *V. parahaemolyticus* (Chiang, Ho, and Chou 2006). Further investigation, however, is required regarding protein identification and functional analysis.

Ethanol-mediated shifts in protein profile were further observed in *S.* Typhimurium. Differential protein expression was noted following exposure to varying concentrations (2.5–20%) of ethanol as revealed by SDS-PAGE (Hassani et al. 2009). Notably, phage shock protein (Psp) G of 9.02 kDa was induced by 10% ethanol, exhibiting maximal expression in 12.5% ethanol. However, this protein was not produced in the presence of 20% ethanol. Subsequent analysis by LC-MS demonstrated that the expression of 9 proteins (GldA, MopA, NanA, NmpC, PotD, PspA, RfaL, Tpx, and UcpA) increased and 10 others (YbdQ, AnsB, DeoD, FilC, LuxS, NanE, STM4242, YciF, YgaU, and OsmY) decreased after ethanol adaptation.

The proteome of *E. coli* O157:H7 30-2C4 and SEA 13B88 exposed to 5% ethanol for 30 h was determined by Chiou et al. (2004). More than 40 protein bands were observed by SDS-PAGE analysis. In both strains, protein expression was slightly lower in non-adapted cells than in ethanol-adapted cells. Moreover, an ethanol-induced protein of 28 kDa, identical to PspA, was uniquely detected in ethanol-adapted cells. The Psp operon, first described in *E. coli* by Brissette et al. (1990), is highly conserved in Gram-negative bacteria. Interestingly, ethanol induced the expression of Psp in both *S.* Typhimurium and *E. coli* O157:H7 in the aforementioned studies (Chiou et al. 2004; Hassani et al. 2009), suggesting its involvement in bacterial ethanol adaptation. Indeed, the Psp system has been linked to response to phage infection, resistance to membrane damaging agents, virulence as well as other significant phenotypes in bacteria (Flores-Kim and Darwin 2016).

Traditional gel-based methods (e.g. SDS-PAGE and 2-D) used in the aforementioned studies suffer from their lack of proteome coverage, sensitivity and reproducibility (Wu et al. 2006). Recently, He et al. (2019) employed the iTRAQ technique to overcome these shortcomings and successfully obtained the protein expression profile of *S. Enteritidis* in the presence of 5% ethanol. A total of 138 differential proteins were identified, with 56 unregulated and 82 downregulated. These proteins were principally involved in ABC transporter, flagellar assembly, enterobactin biosynthesis, metabolism, transcriptional regulator, ribosome, and virulence, suggesting that *S. Enteritidis* responded to ethanol

adaptation by coordinated regulation of multiple pathways. This iTRAQ-based proteomics approach contributes to the identification of more proteins compared to the traditional 2-D electrophoresis and to a better understanding of bacterial ethanol adaptation mechanisms.

Mutagenic analysis

Mutagenic analysis of candidate genes and/or proteins for ethanol stress response has been conducted on several foodborne pathogens. Jiang et al. (2019) found that five DEAD-box RNA helicase genes (i.e. *csdA*, *dbpA*, *rhlB*, *rhlE*, and *srmB*) were not essential for the growth of *Yersinia pseudotuberculosis* in 3% ethanol. Mutagenic analysis also confirmed that *sigB* encoding a key stress response regulator played no role in ethanol direct protection of *L. monocytogenes*, whereas RNase III *rncS* gene, putative DEAD-box RNA helicase genes (e.g. *lmo0866,* and *lmo1450*) and two-component system genes (e.g. *virS*, *virR*, *liaS,* and *degU*) were crucial for the response to sublethal ethanol stress (Ferreira, O'Byrne, and Boor 2001; Markkula et al. 2012; Pontinen et al. 2017; Wang et al. 2020). On the other hand, three cold shock genes (*cspA*, *cspB*, and *cspC*) were demonstrated to be involved in ethanol stress response of *Clostridium botulinum*; a mutation in *cspA* assisted the growth of *C. botulinum* under sublethal ethanol stress, while growth deficiency was observed in *cspB* or *cspC* mutant compared to the wild type (Derman et al. 2015). Moreover, HiuH (5-hydroxyisourate hydrolase) and ProX (glycine betaine-binding periplasmic protein) played a negative regulatory role in ethanol direct protection of *S. Enteritidis* by an as-yet unidentified mechanism (He et al. 2019).

It is noteworthy to mention that ethanol stress response mechanisms have been studied most intensively in nonpathogenic bacteria. For instance, systems-level analysis of ethanol adaptation in biofuel-producing *E. coli* suggested: (i) ethanol disrupted cell wall and membrane integrity, decreasing cross-membrane proton potential; (ii) multiple stress types and ROS production were induced, reducing intracellular O_2 level and aerobic respiration for ATP production; (iii) decreased ATP generation resulted in several cellular alterations (e.g. decreased macromolecule biosynthesis and increased fatty acid oxidation); (iv) ethanol could directly bind over 200 proteins and alter their functions, affecting extensive biological processes; (v) these functionally-linked changes resulted in a reduction in cell viability; and (vi) evolved *E. coli* became ethanol-adapted and regained cell growth via selection of specific genomic mutations and alterations in stress response (Cao et al. 2017). There may be some overlaps in regulatory networks of ethanol stress response in pathogenic and nonpathogenic *E. coli*, which warrants investigation in future studies.

Knowledge gaps and future directions

Although ethanol adaptation in foodborne pathogens has been explored in the past two decades, many areas remain understudied. The impact of strain variability on bacterial stress response has been demonstrated to be significant (Lianou and Koutsoumanis 2013a, 2013b; Lianou, Nychas, and Koutsoumanis 2017). Nevertheless, single strain-based studies were widely conducted for the characterization of ethanol adaptation in foodborne pathogens. In this sense, future survey of ethanol adaptation targeting more strains should be carried out to confirm adequate representation of the observed tolerance phenotypes. On the other hand, diverse species of dominant residential bacteria have been isolated from food industrial surfaces after cleaning and disinfection (Møretrø and Langsrud 2017). Thus, ethanol stress may be encountered simultaneously by several species of bacteria coexisting in food related environments. Therefore, a major research question yet to be addressed is: how will the ethanol adaptation response occur in the presence of multiple bacterial species?

Appropriate and relevant experimental conditions are also integral to deepening our understanding of bacterial adaptive response to ethanol. Ethanol has been used as a disinfectant to control microbial contamination on food contact surfaces. In particular, equipment surfaces used during food processing, handling and storage have been identified as an important source of microbial contamination (Bae, Baek, and Lee 2012). Stainless steel, an ideal material for work surfaces due to its high corrosion resistance and physicochemical stability, has been extensively employed to determine the stress response of pathogenic bacteria (Finn, Händler, et al. 2013; Huang, Ells, and Hansen 2015; Takahashi et al. 2011). As such, studies utilizing stainless steel should be undertaken considering the application value of ethanol on food processing equipment. To the best of our knowledge, however, there is no available information regarding ethanol adaptation of bacteria on equipment surfaces.

To date, a limited number of investigations have been conducted using food matrices. It was previously demonstrated that ethanol stress (12% ethanol for 1 h) decreased the thermal tolerance of *C. sakazakii* in infant milk formula (Osaili et al. 2008). More recently, we assessed the survival of ethanol-adapted (5% ethanol for 1 h) *S. Enteritidis* in fruit juices stored under ambient or refrigerated conditions (He et al. 2018). Ethanol-adapted *S. Enteritidis* survived better in apple juice at 25 °C, compared with the non-adapted control. Nevertheless, the survival of this organism in apple juice stored at 4 °C and in orange juice stored under 25 °C or 4 °C was not influenced by ethanol adaptation. Diversity in the survival patterns of *S. Enteritidis* in the aforementioned juices could be associated with differences in their acid characteristics (e.g. the type of acids and the pH). Hence, microbial behavior in foods as influenced by stress adaptation may be more complicated than expected, highlighting the significance of studying bacterial ethanol adaptation in various food systems.

Overall, research in understanding ethanol adaptation mechanisms of foodborne pathogens is largely lacking at the molecular level. The key genes, sRNAs and proteins responsible for ethanol-induced direct protection and cross protection in major foodborne pathogens such as *C. sakazakii* and

B. cereus have not yet been identified and characterized. Multi-omics approaches (e.g. genomics, transcriptomics, and proteomics) should be employed to understand in detail why ethanol adaptation provides bacterial stress tolerance. Comparative genomic analysis is a powerful tool used for many genomics-related aspects of modern food microbiology (Wang et al. 2016) and will thus be of great benefit in future studies of stress tolerance mechanisms, as stress loci may be identified by genome homology in previously and subsequently characterized systems. Further, transcriptomics analysis should be employed to evaluate global gene expression and sRNA landscape. Combined proteomics and mutagenic approaches for comprehensive profiling of the regulatory networks would also be of great importance to reveal bacterial ethanol adaptation mechanisms. It is worth noting that selection of ethanol-tolerant mutants by parallel evolution or other approaches would also be useful in assessing the response of foodborne pathogens to ethanol. Genomic, transcriptomic, and proteomic profiling of ethanol-tolerant mutants is expected to provide useful insights into bacterial ethanol tolerance mechanisms.

Conclusions

Sublethal stress imposed by ethanol in foodborne bacterial pathogens is considered high importance in the food industry. Generally, ethanol adaptation results in the development of bacterial direct protection, potentially due to adaptive responses in cell membrane, gene expression and protein profile. Protection, sensitization or an absence of either effect has been observed when assessing ethanol-induced cross tolerance in foodborne pathogens. The acquisition of cross protection depends largely on the induction of genes and/or proteins responsible for bacterial tolerance to other types of stress during the course of ethanol adaptation. The ultimate challenge is how to translate information on ethanol adaptation in foodborne pathogens into intervention techniques that can be adopted by the food industry to ensure food safety. For example, knowledge of the ethanol adaptation phenotype is fundamental in the development and optimization of food control measures; combinations of mild ethanol stress and lethal factors such as heat applied sequentially should be carefully considered or avoided due to the development of cross protection. More importantly, some key genes, sRNAs, and proteins responsible for bacterial ethanol adaptation, which will be identified in future studies, can be promising novel targets for food preservation and safety. Hence, it is necessary to continue and deepen investigations in the field of ethanol adaptation in foodborne pathogens.

Disclosure statement

The authors declare that there are no conflicts of interest.

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