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Studying the Cross-Protection between Some Environmental Stressors and Antibiotics Resistance on *Salmonellaspp.*

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Abstract

Salmonella enterica isolates were divided into three groups; high antibiotic resistant (S1 and S2) group, low antibiotic resistant (S3 and S4) group and control according to results of antibiotics susceptibility test. , acid resistance of *Salmonellaenterica* isolates were investigated by exposure of bacteria to 3% lactic acid and calculating the D-value of bacterial population. Results of this experiment showed that D-values of the two strains (S3 and S4) (0.82 and 0.83 respectively) were significantly ($p \geq 0.05$) higher than D-value of control (0.77). While, there was no significant difference between strains ($p \geq 0.05$) (S1 and S2) (0.79 and 0.76 respectively) when compared to control. The relationship between antibiotic susceptibility and gene expression of acid and heat resistant sigma factors rpoS and rpoH genes have been investigated respectively. Results demonstrated no significant difference ($p \geq 0.05$) in levels of transcription between rpoS and rpoH genes after exposure to 3% lactic acid for one minute between antibiotic resistant and antibiotic susceptible groups. Results of rpoD gene expression showed significant increase between both antibiotic resistant and antibiotic susceptible ($p \geq 0.04$) and ($p \geq 0.001$) respectively.

KEYWORDS: Heat acid stress, stress response, stress tolerance , antibiotics resistance

Introduction

Foodborne bacteria are a leading cause of foodborne illness, posing a significant threat to food safety (Abdul mutalib et al.,2015). E coli, Salmonella enterica, Vibrio cholera, S. aureus and Listeria monocytogenes are only a few of the foodborne pathogens that frequently contaminate fruits, vegetables, foods, and seafood. Foodborne pathogens have become an increasing main issue for public health world widely, with high rates of morbidity and mortality [(1)]. In addition, biofilms have been linked to a variety of other pathogen outbreaks. Food is nutrient-dense and conducive to pathogen growth and reproduction (2).

Salmonella are Gram-negative, non-spore-forming, facultative anaerobes that measure between 0.7 and 1.5 meters in diameter and 2 to 5 meters in length [(3)]. They thrive in a temperature range of 35 to 40 degrees Celsius and a pH of 7 to 7.5 (4). Salmonellosis is a symptomatic infection caused by Salmonella bacteria, with diarrhea, fever, stomach cramps, and vomiting being the

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most common symptoms [4]. Symptoms usually appear between 12 and 36 hours after exposure and last between two and seven days.

Bacterial pathogens survive under two entirely different conditions, namely, their natural environment and in their hosts. Response of these pathogens to stresses encountered during transition from the natural environment to human hosts has been described. The virulence determinants of pathogenic bacteria are under the control of transcriptional activators which respond to fluctuations in growth temperature, osmolarity, metal ion concentration and oxygen tension of the environment. The regulation of stress induced genes may occur at the level of transcription or translation or by post-translational modifications. Under certain stress conditions local changes in the superhelicity of DNA induce or repress genes. In addition to their role in survival of bacteria under stressful situations, the stress induced proteins are also implicated in the manifestation of pathogenicity of bacterial pathogens in vivo.. It would therefore be pertinent to evaluate the effects and potential risks associated with the application of interventions used to control pathogens during food processing, including the use of decontamination technologies [5].

Bacteria adapt to their environment by sensing conditions and responding through the co-ordinated expression of gene networks. The alternative sigma factor sE senses perturbations in the outer membrane and periplasm and activates a subset of genes in response to heat stress [6]. sE is required for *Escherichia coli* viability at all temperatures ,implying that the sE play important roles in addition to thermal adaptation. Under non-stress conditions, sE is sequestered by the inner membrane-spanning anti-sigma factor RseA [7].

The first signal to an invading bacteria on entry into the host is an increase in temperature from that of the environment to the physiological temperature of the human body (37°C)[8].

In *salmonellae, shigellae, yersinae, Bordetella pertusis, Borrelia burgdorferi , Listeria monocytogens* and several other pathogenic organisms, the virulence gene cassettes are switched on at 37°C[9]. At lower temperatures, σE is sequestered by the membrane-bound anti-sigma factor RseA. At temperatures above 45 °C, misfolded proteins, destined for the outer membrane and/or periplasm, accumulate in the periplasm triggering the sequential proteolytic cleavage of RseA by the inner membrane proteases DegS and RseP (YaeL). This regulated intra-membrane proteolysis (RIP) of RseA results in the release of an σE -RseA inhibitory complex into the cytoplasm. Interaction with SspB directs this complex to the ClpXP proteasome; resulting in the degradation of the RseA fragment and release of σE into the cytoplasm [10].

Exposure of bacteria to acidic conditions is quite common in the natural environment or within many food processing environments [11]. Acid stress can be recognized as the combined

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biological effect of hydrogen ions (H^+) and weak acids (organic) as a result of fermentation or when added to food as a preservative. Although, inorganic and organic acids affect different mechanisms inside the cell, both will lead to acidification and damage to the cellular contents [(12)].Two pH dependent systems have been identified in *E.coli*; one is induced in the exponential (log) phase while the second is induced during the stationary phase. The log phase acid survival system is also known as acid adaptation, which can be expressed after gradual exposure to sub-lethal acidic conditions and enables cells to tolerate acidic conditions afterwards. Many compounds such as glucose, glutamate, aspartate, FeCl3, KCl, and L-proline can induce acid adaptation. Three acid resistance (AR) systems have been identified in the stationary phase of all *E. coli*[(13).]

Pathogenic bacteria, unlike innocuous commensals alternate between free living and host associated states. The physico-chemical parameters encountered by the bacteria in these two states are very different and exert different demands and stresses on the bacterial cell. Bacterial pathogens have evolved highly sophisticated mechanisms for sensing external conditions and respond by altering the pattern of gene expression with activation of a set of genes whose products assist in survival and turning off those the products of which are not necessary in a particular environment. These sensor-activator systems allow the bacteria to monitor environmental parameters which distinguish host from external environment and adjust gene expression accordingly, particularly by induction of virulence factors [(14)]. The expression of virulence genes is controlled by regulatory systems in such a manner that the virulence factors are expressed at different stages of the infection process dictated by the changing micro-environment of the host as a consequence of the pathophysiology of infection. Accordingly, mutations in some of the regulatory systems attenuate virulence of several bacterial species [(15)].

The aim of this study was to determine whether lactic acid exposure would lead to increased tolerance to a variety of bacterial stresses. This will decide whether such stresses will induce phenotypic and genotypic changes in *Salmonella* strains, which may impair or improve their ability to survive in the environment or in foods as compared to strains that have not been subjected to such stressors

METHODOLOGY

Investigation of Acid shock on salmonella isolate

salmonella strain were isolated from different chicken farms in najaf .xylose lysine deoxycholate agar (Oxoid, Hampshire, United Kingdom) was used for primary salmonella identification. Antimicrobial susceptibility testing of salmonella isolate was carried out against a

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panel of 16 antimicrobial compounds using the disc diffusion technique in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference methods [16]. The antimicrobial compounds included for testing were chloramphenicol (30 µg), gentamicin (10 µg), trimethoprim(5 µg) ,sulphamethoxazole (25 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg) and tetracycline (10 µg) imipenem(10 µg) Ampicillin (10 µg)cefepime(30 µg)cefotaxime (30 µg)cefoxitin (30 µg)ceftazidime (30 µg) azithromycin (15 µg),meropenem (10 µg)

Acid shock was performed by treating of salmonella isolate with lactic acid 3% (Sigma-Aldrich, USA, W261114). salmonella isolates were tested to determine their Lactic acid death LAD value after exposure to 3% lactic acid.

LAD by exposing salmonella isolate to seven time intervals one minute each between intervals .A 40 ml overnight culture of salmonella was prepared by inoculating in nutrient broth (Oxoid, UK, CM0129) incubating tubes at 37oC for 24 h to give an approximate concentration of 109 cfu/ml. Following incubation, the 40 ml cultures were transferred to eight 8 5ml sterile tubes. The tubes were centrifuged at 4000 rpm for 10 minutes and the supernatant was removed.

The bacterial pellet was washed and resuspended in 5 ml phosphate buffered saline (Oxoid, UK, CM0733). A 5 ml volume of 3% lactic acid solution was added to each of the tubes and vortexed for 10 seconds. A 1ml aliquot was removed at one minute intervals for seven minutes, serially diluted, plated on plate count agar (Oxoid, UK, CM0131) and incubated for 24 hours at 37 °C for enumeration.

This experiment was conducted to investigate the effect of acid shock on thermal resistance in the salmonella enterica D-values were determined at temperatures (56 °C) 10ml overnight culture of salmonella was prepared by inoculating in nutrient broth (Oxide, UK, CM012) incubating tubes at 37 °C for 24 h to give an approximate concentration of 109 cfu/ml Following incubation, The tube were centrifuged at 4000 rpm for 10 minutes . The bacterial pellet was washed and resuspended in 10 ml phosphate buffered saline (Oxoid, UK, CM0733). Then 1ml was transferred to ten sterile cryotubes (vol .1.5ml) at each10 minute intervals one cryotube was re removed and transferred to ice water, this procedure was maintained up to 120 minutes. External thermocouple (Traceable VWR, USA) was used to measure water temperature. each sample was serially diluted and plated on plate count agar and incubated for 24 hours at 37 °C for enumeration

Table1: Primers used in this study.

Genes	**Primers**	**Primer sequences**	**Annealing temperature (°C)**	**Amplicon size (bp)**

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rpoD	Forward primer Reverse primer	GGCACTGTTGAACTGTTGA GCAGATAGGTAATGGCTTCC	58	118
rpoH	Forward primer Reverse primer	AGATCGCCCTGGTAATGCAG TAGCTTTAGCCCCTGTTGGC	58	118
rpoS	Forward primer Reverse primer	GCCGTATGCTTCGTCTCA TCTTGCGTGGTGTCTTCC	58	127

statistical analysis

Log10 cfu/g was used to convert microbial counts. A 1-way ANOVA(SPSS24) was used to compare mean bacterial counts between treatment groups and controls, followed by a Tukeys Multiple Comparison test. Individual color component measurements were compared using a 1-way ANOVA followed by a Tukeys Multiple Comparison test immediately after treatment and after 24 hours of storage. The significance was calculated at the 0.05 level of significance.the gene expression calculated or analyses by using Microsoft excel 2010

Ethical approval

This study did not include the use of genetically changed organisms or biological materials and was carried out under the supervision and recommendations of the Faculty of Veterinary Medicine, University of Kufa, according to the controls approved by it. All samples that were worked on in this study were collected according to the research protocols for each type, without additional materials or manipulation.

RESULTS.

Antibiotics susceptibility profile of five Salmonella enterica isolates was investigated to determine antibiotics sensitivity between the selected isolates. five strains, results shows that S1 and S2 were resistant to seven antibiotics while, S3 and S4 were resistant to 12 antibiotics and the last strain was resistant to five antibiotics only. According to these results the five selected strains were divided into low antibiotics resistant group (S1 and S2) and high antibiotics resistant group and the final one was considered as control.

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Table (2) D-value of Salmonella entrica colony after exposure to 3% lactic acid

Strain	S1	S2	S3	S4	Control
Acid D-value	0.79 ± 0.32^{a}	0.76 ± 0.24^{a}	0.82 ± 0.38^{b}	0.83 ± 0.33^{b}	0.77 ± 0.31^{a}

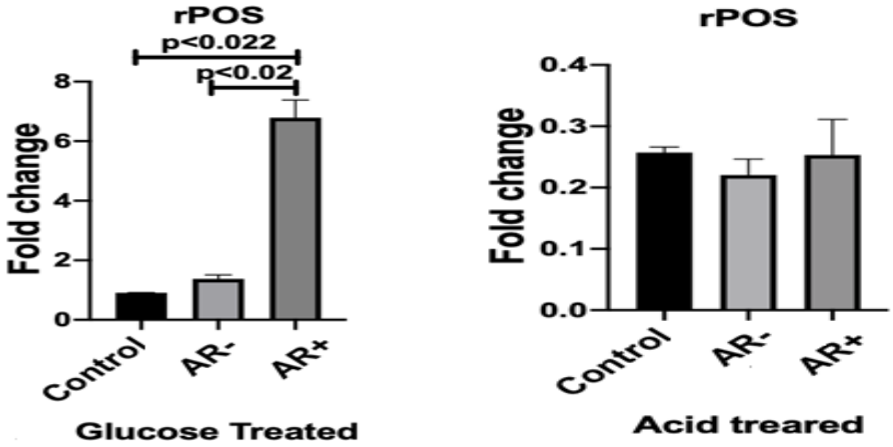
Means followed by the same lowercase superscript within column are not significantly different ($p \geq 0.05$)

Table (3) D-value of *Salmonella entrica* colony after exposure to heat at 56 C

Strain	S1	S2	S3	S4	Control
Acid D-value	20.16 ± 0.01^{a}	20.01 ± 0.01^{a}	24.15 ± 0.01^{b}	25.25 ± 0.01^{b}	20.92 ± 0.01^{a}

Means followed by the same lowercase superscript within column are not significantly different ($p \geq 0.01$)

Results demonstrated no significant difference ($p \geq 0.05$) in levels of transcription between rpoS and rpoH genes after exposure to 3% lactic acid for one minute between antibiotic resistant and antibiotic susceptible groups. In the other hand, rpoH gene significantly over expressed in the antibiotic resistant strains compared to antibiotic susceptible strains after exposure to 56 heat for 20 minutes.



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Figure(1)show gene expression of rPOS (A) glucose treated rPOS (B) acid treated Of rPOS

Results of rpoD gene expression showed significant increase between both antibiotic resistant and antibiotic susceptible ($p \geq 0.04$) and ($p \geq 0.001$) respectively. While the antibiotic resistant

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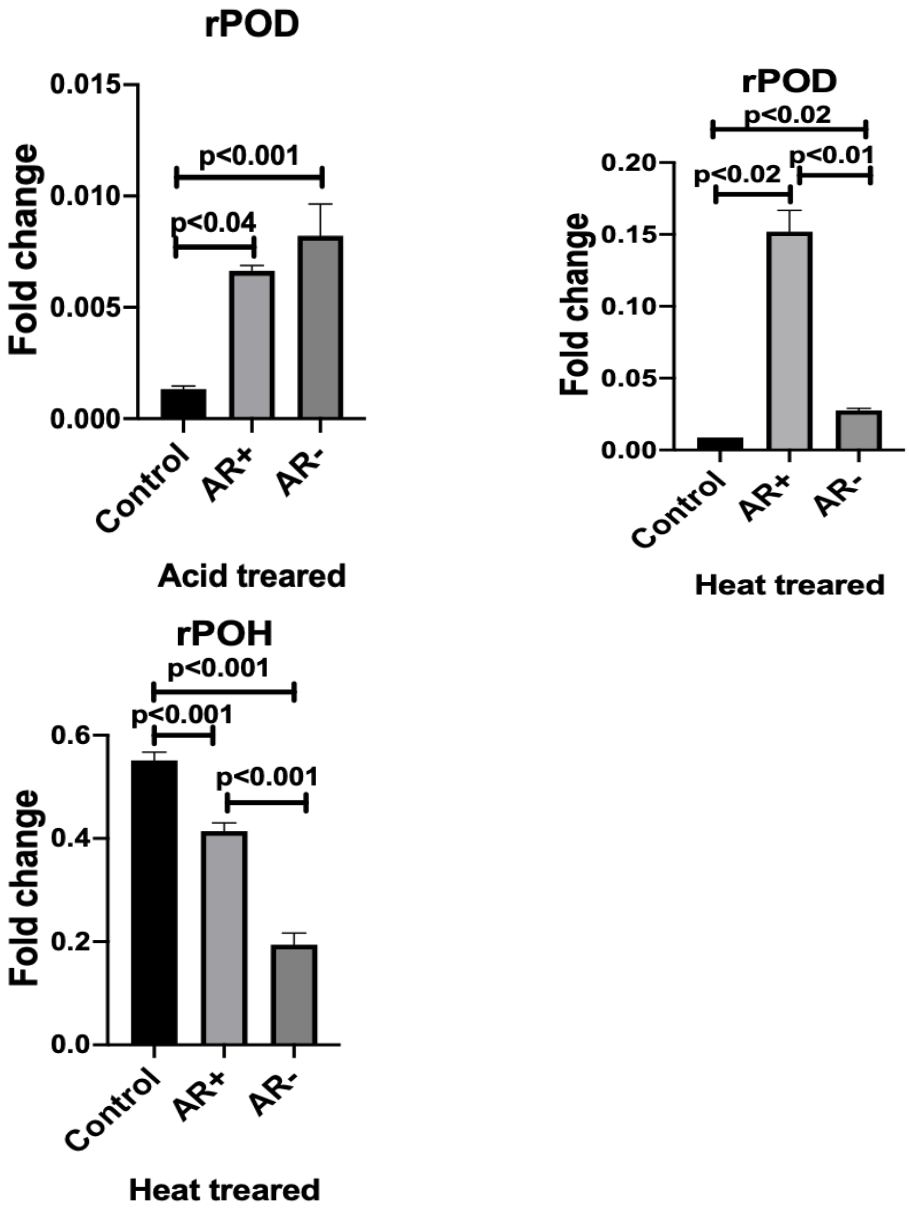
strains were significantly over expressed compared to both antibiotic susceptible and control ($p \geq 0.01$) and ($p \geq 0.02$) respectively.



Figure(2)show gene expression of rPOD& rPOH (A) acid treated of rPOD(B) heat treated of rPOD(C) heat treated of rPOH

Results illustrated no significant differences between antibiotic resistant, susceptible and control strains in gene expression of rpoS after acid treatment with 3% lactic acid. However, acid adaptation due to adding 1% glucose to the medium results in over transcription of rpoS gene as a results of releasing of lactic acid as a result of glucose metabolism in the a ntibiotic resistant strains when compared to antibiotic susceptible ($p \geq 0.02$) and control ($p \geq 0.022$) respectively

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Discussion

The most common method has been used to increase the safety of fruit juice is pasteurization this thermal method lead to decrease the outbreak of salmonella. However recently more new spices more resistance to heat has been increased recently.

Heat resistance of bacteria might be affected by other factor such as ph growth temperature water activity[17].some studies showed that heat resistance could be increased according to the growth temperature during this study *S.typhimuruim* increased heat resistance increased at 58 degree when incubation temperature was increased from 10-45 degree [18] investigation the mechanism of heat adaptation and it's relation to acid resistance is an important aspect in food safety, that because microorganisms might exposed to deferent environmental stressor these factors might lead to increase the bacterial adaptation for various stressor example acids are widely used for food preservation or acid could be produced naturally after fermentation.in addition sub lithely heat temperature during food processing may promote possibly heat adaptation of a microorganism [19]

Relation between level of antibiotic resistance and expression of acid and heat stress related genes during acid and heat exposure was investigated in the current study.

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Sigma factor such as σS (encoded with the rpoS gene) and σH (encoded with the rpoH gene) considered related to the expression of stress related gens in the resent study it has been found that σS increases salmonella spices survivability. Studying the expression of more than 50 protein during statuary phase and after changes in the environmental condition like PH and temperature [15],While σH which is encoded with the Rpoh gen play major role in thermal stresses defense mechanism through regulating heat shock protein (HSP) such as protease and chaperones [21].therefore it's more likely that adverse temperature may affect Rpos and Rpoh transcriptions levels which play major role in S. enteritidis stress response[22].

Rpoh sigma factor is responsible of transcription of the gen encoding chaperones and proteases which control the heat resistance throw folding and degrading of the damage polypeptide this mechanism support the heat resistance of bacteria [5].

Rpos sigma factor is also a global regulatory factor which is responsible of expression of varies number of chromosomal gens involve in resistance to stress condition and nutrient deprivation [22]

Conclusion

Results of the current study showed that level of antibiotic resistance may influence the degree of acid resistant of Salmonella enterica strains. Higher antibiotic resistant strains could be more acid resistant.Similarly results of heat resistant study demonstrated an increase in heat resistant in

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relation to the level of antibiotic resistant of Salmonella enterica.The gene expression experiment results showed no increase in rpoS and rpoH genes expression after acid treatment and only rpoH gene was over expressed after heat treatment. Gene expression of rpoS gene of acid adapted cells was significantly higher than those which acid shocked . According to these results a strong relationship between acid and heat resistance and antibiotic resistance has been indicated at the phenotypic level. Acid adaptation could be more significant than acid shock by influencing gene expression of stress sigma factors.

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