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COMBATING OF *ESCHERICHIA COLI* STRAINS AGAINST SALINITY BY THE ANTIOXIDANT DEFENSE SYSTEM AND OSMOREGULATION: BIOCHEMICAL AND PHYSIOLOGICAL APPROACHES

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ABSTRACT

The study was designed to elucidate the impacts of salinity on biochemical and physiological properties of two *Escherichia coli* strains (6E and S39) isolated from different sites. The strains were exposed to 600 and 1200 mM NaCl and were harvested after 12th and 24th h of incubation. The lipid peroxidation levels, osmolyte accumulation, H_2O_2 content and antioxidant system [superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase(GR)] of the bacteria were investigated. While thiobarbituric acid reactive substances (TBARS) and H_2O_2 content of S39 has increased steadily with salinity, however, it has not changed at 600 mM NaCl concentration in strain 6E. Accumulation of the osmolytes in 6E increased depending on salinity and application time. In S39 except for glycine-betaine, contents of other osmolytes were either unchanged or decreased. Activities of SOD, CAT, POX, APX, GR and NOX have increased as compared to the control group in 6E exposed to 600 and 1200 mM salt for 12h. In S39, CAT, POX, and GR activities decreased exposed to salinity. Consequently, it can be argued that (i) the different antioxidant responses of 6E in salinity plays a key role which tend to make 6E the more tolerant (ii) this tolerance is closely related to the increased antioxidant capacity against reactive oxygen species and is related to the increased accumulation of osmolytes.

Keywords: *Escherichia coli*, salt stress, antioxidant defense, osmolyte, isozyme

INTRODUCTION

Bacteria are exposed to different environmental stresses such as acidity, pH, NaCl, heat, electrical potential, bacteriocins, and competitive flora found in foods except stress factors such as high salinity, drought, light, O_2, cold, detergent and disinfectants in nature **(Dikici, 2009; McMahon *et al.*, 2007)**. Bacteria can die due to the stresses they encounter, and they can survive by activating adaptation mechanisms. Consequently, bacteria have developed a panoply of mechanisms in response to environmental stresses, in an endeavor to trap, preserve, and transform the energy essential for their biosynthesis and growth. With such adaptive mechanisms, they can strive in stressful environments for a long time or die due to damage to their metabolism as a result of this stress. For decades, researchers have wondered how microorganisms live in extreme environments and the nature of subsequent adaptive mechanisms in extremely stressful environments. According to preliminary research in 1933, it has been reported that heat adaptation of osmotic stress-exposed bacteria increase **(Fay, 1933)**. When bacteria encounter a sub lethal stress called mild stress, their number does not decrease. However, it results in a halt or decrease in the reproduction rate. When the microorganism encounters a moderate stress, microbial growth stops as well as a decrease in the viability of bacteria. Extreme stress, either called extreme or severe stress, is a lethal condition for bacterial cells and results in the death of the majority of the bacterial population **(Neidhardt & VanBogelen, 2000)**.
Oxidative and osmotic stresses are parts of important abiotic stress factors that microorganisms encounter in environment and foods. When bacteria are subjected to oxidative stress, reactive oxygen species (ROS) such as superoxide, hydroxyl radicals and hydrogen peroxide can be formed, and the accumulation of such various oxidants causes damage to cellular proteins, nucleic acid and lipid **(Munna *et al.*, 2013)**. To keep ROS at a non-toxic concentration, enzymatic and non-enzymatic cellular antioxidants regulation the balance between their production and scavenging. Enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR), used to scavenge excess of internal-external ROS. **(Staerck *et al.*, 2017)**. Also, many proteins are known to be activated by oxidative stress, acting as antioxidants. Others are especially necessary to repair oxidative damage, especially nucleic acid damage. Salinity, one of the main abiotic stress factors, affects both agricultural lands and many living things negatively. Salinity causes various disruptions at the cellular, physiological and molecular level as well as various growth processes of bacteria. Bacteria develop various tolerance strategies in response to salt stress. In response to salt stress (i) the activity of various antioxidant enzymes are increased destroying ROS formed as by-product of metabolism, (ii) osmolyte synthesis is promoted, (iii) regulating ion uptake mechanism by gene expression, (iv) activating stress-related genes synthesis of transcription factors, and (v) promoting the production of stress proteins are known to be among the major tolerance strategies. The metabolism best characterized in bacteria under hyperosmotic conditions is the accumulation of so-called intracellular osmolytes. Accumulation of compatible solutes can be achieved *via* synthesis as well as by extracellular transfer. These liquids are polar and are highly soluble in the cell. Even at very high concentrations, they can cope with osmotic pressure without affecting cellular functions. Glycine betaine, proline, ectoine, carnitine, choline and trehalose are the most widely known compatible substances. The accumulation of these compounds in the cytoplasm is regulated either directly by altered enzyme activity or by the level of gene transcription **(Bremer, 2000)**.
Pathogenic and nonpathogenic *Escherichia coli* have previously been reported to show a stress response to sublethal environmental stresses **(Chung *et al.*, 2006)**. The stress responses may enable survival under more negative conditions, cause resistance and increased virulence. Not only microorganisms may come across stress conditions in the environment but also they come across with those conditions in foods, especially minimally processed foods. Temperature, salinity, water content, and pH are the main extrinsic (environmental) factors which effects the survival and growth of *E. coli* in foods, also development of bacterium interacts with food related (intrinsic) factors. One of the factors affecting microbial growth is the inclusion of preservative. To additive and preservative in food, sodium chloride has been used. NaCl, known since time immemorial as antimicrobial agent, has frequently been incorporated as a component in meat and meat products **(Abdulkarim *et al.*, 2009)**. Although members of *Enterobacteriace* do not tolerate high salt concentration, specific strains of *E. coli* are halo-tolerant and able to strive in environments ranging from very dilute aqueous solutions of main nutrients to media including molar concentrations of salts or non-electrolyte solutes. It has always intrigued microbiologists how *E. coli* cope with such exposure and which physiological changes, cause it to survive and at the same time retaining infectivity of the bacterium. Adaptation to the stress both in environmental and food borne microorganisms have been ignored in the past. However, nowadays the importance of this phenomenon in the field of environmental and food safety is increasing. Thus, understanding the impacts of environmental stresses on the physiological tolerance of *E. coli* are urgently needed and are important in order to evaluate and minimize the risk of food-borne illness.

This study was therefore designed to determinate and compare the physiological and biochemical responses of two *E. coli* strains, isolated and identified from the different sites, under oxidative stress by employing different concentrations of salt (NaCl). The specific objectives set for this study are (i) to evaluate the changes in activities of the enzymes taking part in antioxidant defense system such as SOD, CAT, POX, APX, and GR, (ii) to assess the lipid peroxidation levels and hydrogen peroxide (H_2O_2) contents and (iii) to understand and highlight the osmoprotectant metabolism under salt stress conditions.

MATERIAL AND METHODS

Sample collection and isolation of *Escherichia coli* strains

E. coli strains used in the present study were isolated from two different sites in 2014. For the S39 numbered strain, well water sample was taken from the water sampling site as determined with the help of the Konya Public Health Laboratory commissioner. For the 6E numbered strain, water and sediment samples were taken from the watercourse between Cihanbeyli-Golyazi (38° 37.270´N and 33° 08.986´E, 904 m). Sterile water sampling bottles contained 50 mg of sodium thiosulfate to neutralize any residual chlorine in the water were used to collect samples.
The membrane filtration method as described by **Uysal *et al.* (2013)** was used to isolate *E. coli* from water samples. After filtration, the membrane containing the bacteria was placed on a selective differential medium (Standard Lactose Tri Tetrazolium Chloride (TTC) Agar with Tergitol 7) and incubated at 35°C for 2h to resuscitate the injured or stressed bacteria and then at 44°C for 22 h. After incubation, yellow or yellow-brown colonies on TTC agar were chosen to perform oxidase and indole tests. Oxidase (-) and indole (+) colonies were transferred to Eosin Methylene Blue Agar. Finally, Chromocult TBX (Tryptone Bile X-glucuronide) agar medium was used for identification purposes **(Uysal *et al.*, 2013)**.

Halo tolerance of *E. coli* strains

The halo tolerance tests were performed in Luria Bertani (LB) medium supplemented with NaCl in the range of 0.3%–14% (w/v) **(Crognale *et al.*, 2013)**. The bacterial growth was calculated by measuring of absorbance at 600 nm wavelength. Also growth was visualized by the addition of 2,3,5, TTC to culture wells for qualitative evaluation.

Culture conditions and harvesting of the strains

After the determination of halo tolerance of strains, two salt concentrations (600 mM and 1200 mM) were selected for the application of salt stress. Two hundred µl (0.9 at O.D 600 nm) of overnight cultures of strains, grown in standard LB broth medium, were inoculated to LB broth containing 600 mM and 1200 mM NaCl and incubated at 37°C with agitation at 150 rpm for 24 h **(El-Rab *et al.*, 2013)**. At the same time, standard LB broth without salt was used as control. The time-depend bacterial growths were measured at 600 nm wavelength by using UV spectrometer (Shimadzu UV-1800, Japan) as absorbance (bacteria were not counted for logarithmic growth) for every hour and growth curve was drawn (Figure 1). Two time intervals were determined (12th and 24th h) according to this curve for the harvesting of the bacterial cells in the main experiment. In the main assay the volumes of the LB mediums were prepared as 1000 ml for each group and culture conditions applied as above.
Bacterial cells (1000 ml) were harvested from liquid LB medium at 12nd and 24th h of the incubation period. Then cell suspensions were centrifuged at 12000 × g for 10 min, washed twice with ice-cold 0.9% sodium chloride solution, and the specimens were dried in an incubator and stored at -80°C until use **(Lin *et al.*, 2009)**.

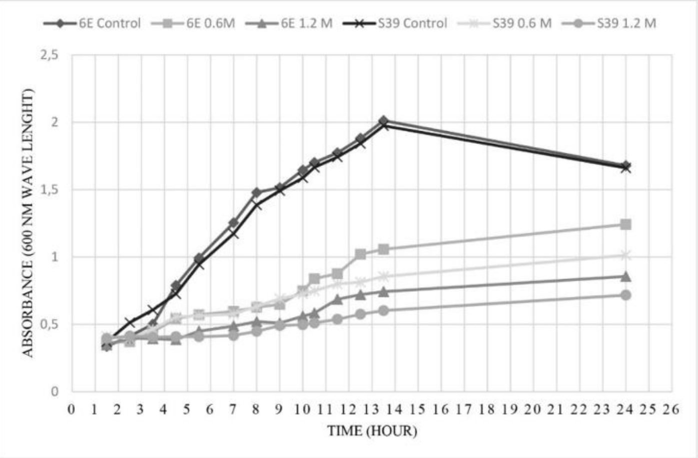

Figure 1 Time-dependent growth curve of strains 6E and S39

Determination of hydrogen peroxide content

Determination of H_2O_2 content was performed according to method described by **Beers and Sizer (1952)**.

Evaluation of lipid peroxidation levels

The level of lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS) according to **Heath and Packer (1968)**.

Changes of osmoregulatory solutes accumulation

Determination of proline (Pro) content was done according to the procedure defined by **Bates *et al.* (1973)**. Total amount of choline (Cho) and glycine betaine (GB) were determined according to **Grieve and Grattan (1983)**.

Enzyme extraction and analysis of isozyme and/or enzyme compositions

Antioxidant enzyme assays were performed on freshly collected cells using the method of **(Beers & Sizer, 1952)**. Homogenization of cells was done in 50 mM Tris-HCl (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol (DTT). The total soluble protein content of the enzyme extracts was determined **(Bradford, 1976)** using bovine serum albumin as a standard.
Samples containing equal amounts of protein (25 µg) were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by **(Laemmli, 1970)** with minor modifications. Superoxide dismutase (SOD) activity was detected by photochemical staining using riboflavin and NBT **(Beauchamp & Fridovich, 1971)**. The different types of SOD were discriminated by incubating gels with different types of SOD inhibitors before staining: Mn-SOD activity was resistant to both inhibitor treatments and Cu/Zn-SOD activity was sensitive to 2 mM KCN. Cu/Zn-SOD and Fe-SOD activities were inhibited by 3 mM H_2O_2 **(Vitória *et al.*, 2001)**. The total SOD (EC 1.15.1.1) activity assay was based on the method of **Beauchamp and Fridovich (1971)** After electrophoresis of samples containing 25 µg protein, catalase (CAT) isozymes were detected according to **Woodbury *et al.* (1971)**.
Total catalase [CAT) (EC 1.11.1.6)] activity was estimated according to the method of **Bergmeyer and Gawehn (1970)**.Total peroxidase [POX (EC 1.11.1.7)] activity was based on the method described by **Herzog and Fahimi (1973)**. Electrophoretic ascorbate peroxidase (APX) separation was performed according to **Mittler and Zilinskas (1993)**.
Total APX (EC 1.11.1.11) activity was measured according to **Nakano and Asada (1981)**. Total glutathione reductase [GR (EC 1.6.4.2)] activity was measured according to **Foyer and Halliwell (1976)**.
Total NADPH oxidase [NOX (EC 1.6.3.1)] activity was measured according to **Jiang and Zhang (2002)**. Gels stained for SOD, CAT and APX activities were photographed with the Gel Doc XR+ System and then analyzed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard amounts of enzymes (0.5 units of SOD and CAT) were loaded onto gels.

Statistical analysis

All data obtained were subjected to a one-way analysis of variance (ANOVA). Statistical analysis of the values was performed using SPSS 20.0. Tukey's post-test was used to compare the treatment groups. Comparisons with $p < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

Changes of Hydrogen peroxide (H_2O_2) contents

One of the main important parameters in determining the effect of salt stress was to evaluate the H_2O_2 level of the bacteria under salt stress condition. The H_2O_2 content is usually low in tolerant species. During the study, the application of 600 mM NaCl did not change the amount of H_2O_2 from the 6E strain (Figure 2A). In this strain, the increase in H_2O_2 occurred only at a high concentration of 1200 mM. In the strain S39, the increase of H_2O_2 was determined in parallel with the application time and concentration of salinity (Figure 2B). As a result of the 24-hour harvest of these bacteria, the amount of H_2O_2 in 600 and 1200 mM NaCl were increased by 77.2% and 62.1%, respectively. When the two strains were compared in terms of H_2O_2 contents, it was observed that the H_2O_2 content of S39 was higher than 6E.

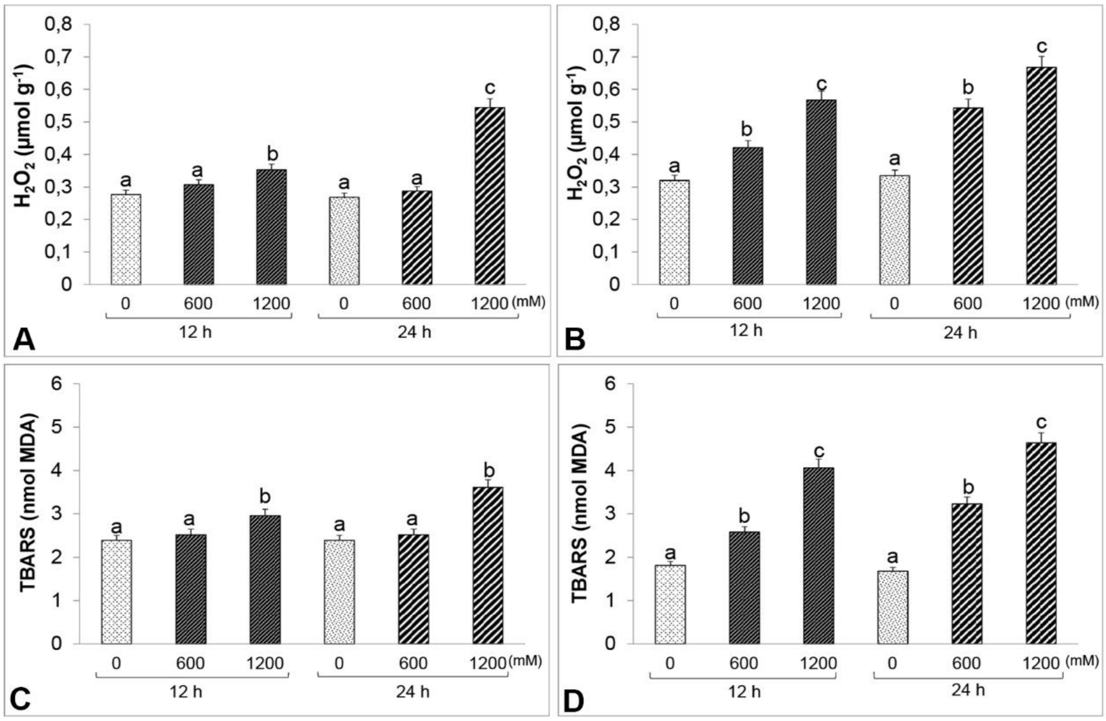

Figure 2 Mean changes in H_2O_2 content and lipid peroxidation levels (TBARS) of *E. coli* strains under salt stress (n = 6). A-C) 6E strain B-D) S39 strain. The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test *p <0.05*).

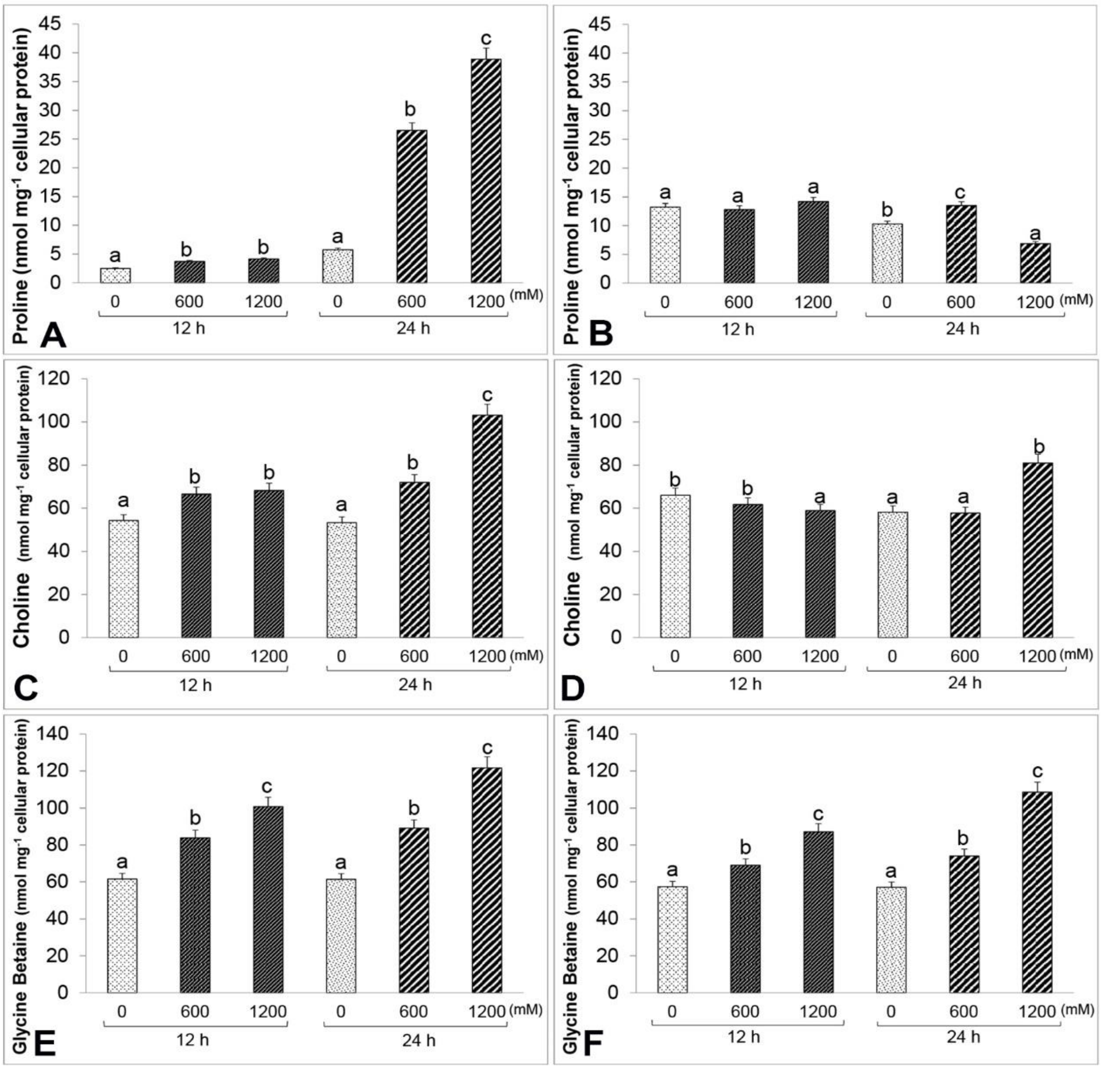

Figure 3 Mean changes in osmolytes of *E. coli* strains under salt stress (n = 6). **A-C-E)** 6E strain **B-D-F)** S39 strain. The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test *p <0.05*).

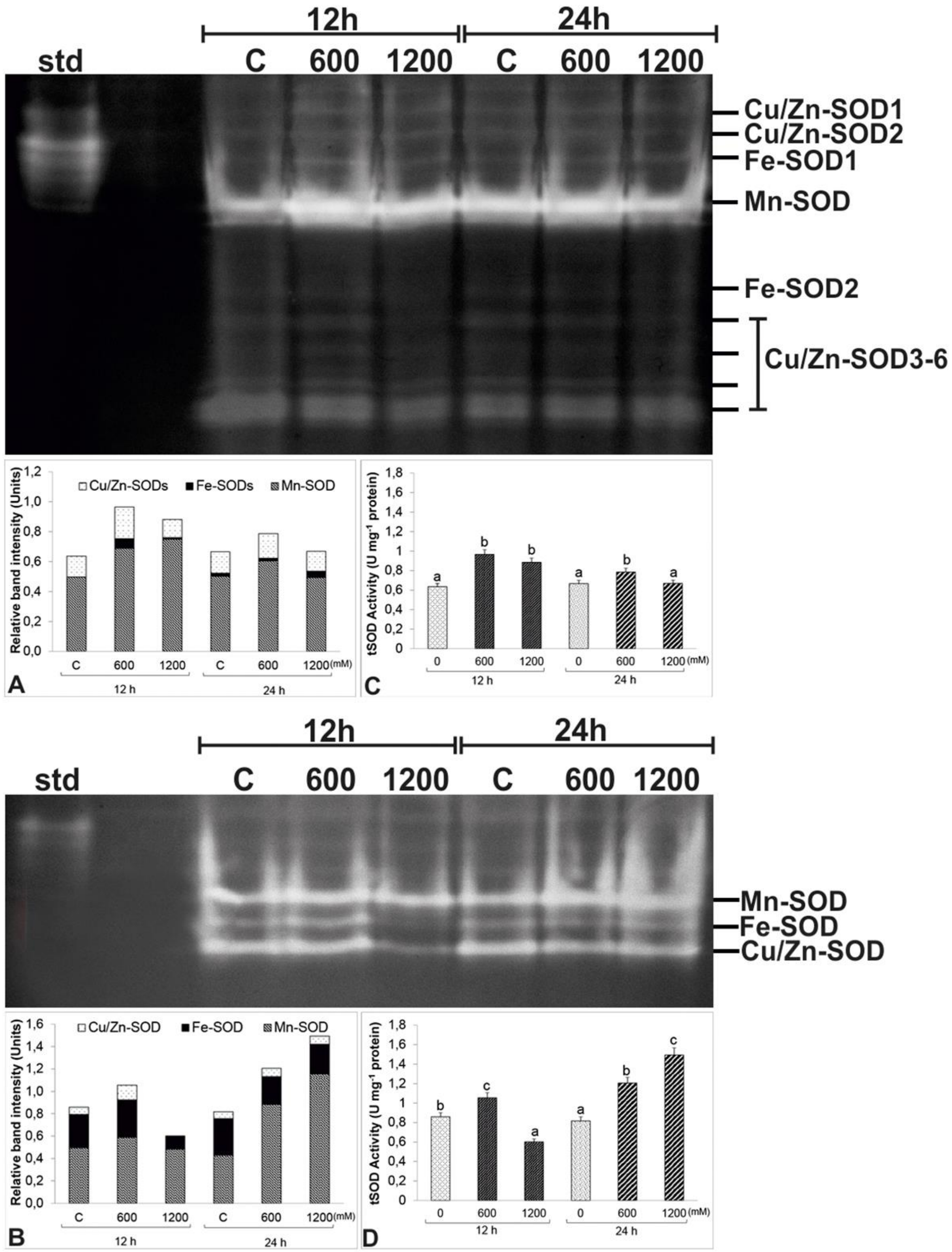

Figure 4 Effects on activity staining of SOD isozymes **(A-B)** and total SOD activity (**C-D)** in *E. coli* strains exposed to 600 and 1200 mM NaCl for 12 and 24 hours (samples containing 25 µg protein of the gel were pipetted; std. 0.5 units of SOD). (Data **A** and **C** belong to strain **6E**; data **B** and **D** belong to strain **S39.** The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test $p < 0.05$).

Lipid peroxidation levels

One indicator of damage caused by salt stress at the cell level is lipid peroxidation (TBARS) occurring at the level of the membrane structures. Measurement of TBARS contents and determination of peroxidation levels of membrane lipids is an important parameter in the assessment of the severity of salinity-induced oxidative stress and bacterial susceptibility and tolerance. Changes in malondialdehyde levels of *E. coli* strains isolated from different localities are shown in Figure 2C-D. While the TBARS of the strain (6E) isolated from Golyazi site did not change in both short (12 h) and long-term (24 h) 600 mM NaCl applications, it increased by 23.9% and 51.0% in the 1200 mM salt treated groups, respectively (Figure 2C). The unchanged TBARS content at 600 mM salinity is one of the most important parameters indicating that the bacteria are not affected by the harmful effects of salinity at this concentration. In the strain isolated from the well water (S39), the increase of TBARS level was determined in parallel with the application time and concentration of salinity (Figure 2D). This increase was 2.2 fold of the control at 1200 mM for 12 h and 2.8 fold at 1200 mM for 24 h under stress. The highest lipid peroxidation increase in both strains was determined in bacteria exposed to 1200 mM NaCl for a long time. When these two strains isolated from different localities are compared in terms of lipid peroxidation contents, *E. coli* samples S39 showed significantly higher malondialdehyde content compared to strain 6E.

Effects of Salinity on Osmoprotectants

One of the most common osmolytes accumulated under environmental stresses as salt stress is proline (Pro). Pro is not only an osmotic regulator, but also serves as an antioxidant and carbon source. The proline contents of strain 6E increased significantly over the entire experiment and treatments when compared with the non-stress (Figure 3A). The highest proline content was 6.76 fold of the control in bacteria subjected to 1200 mM NaCl for 24 h. The proline content of strain S39 did not change as compared to the control in both 600 and 1200 mM salinity applications for a short period (Figure 3B). The proline level of this strain increased by 31.1% in samples at 600 mM NaCl only for 24 h compared to the control. Long-term 1200 mM salinity decreased by 33.5% compared to the control. This decrease in proline content clearly shows that the strain is adversely affected by high salt stress.

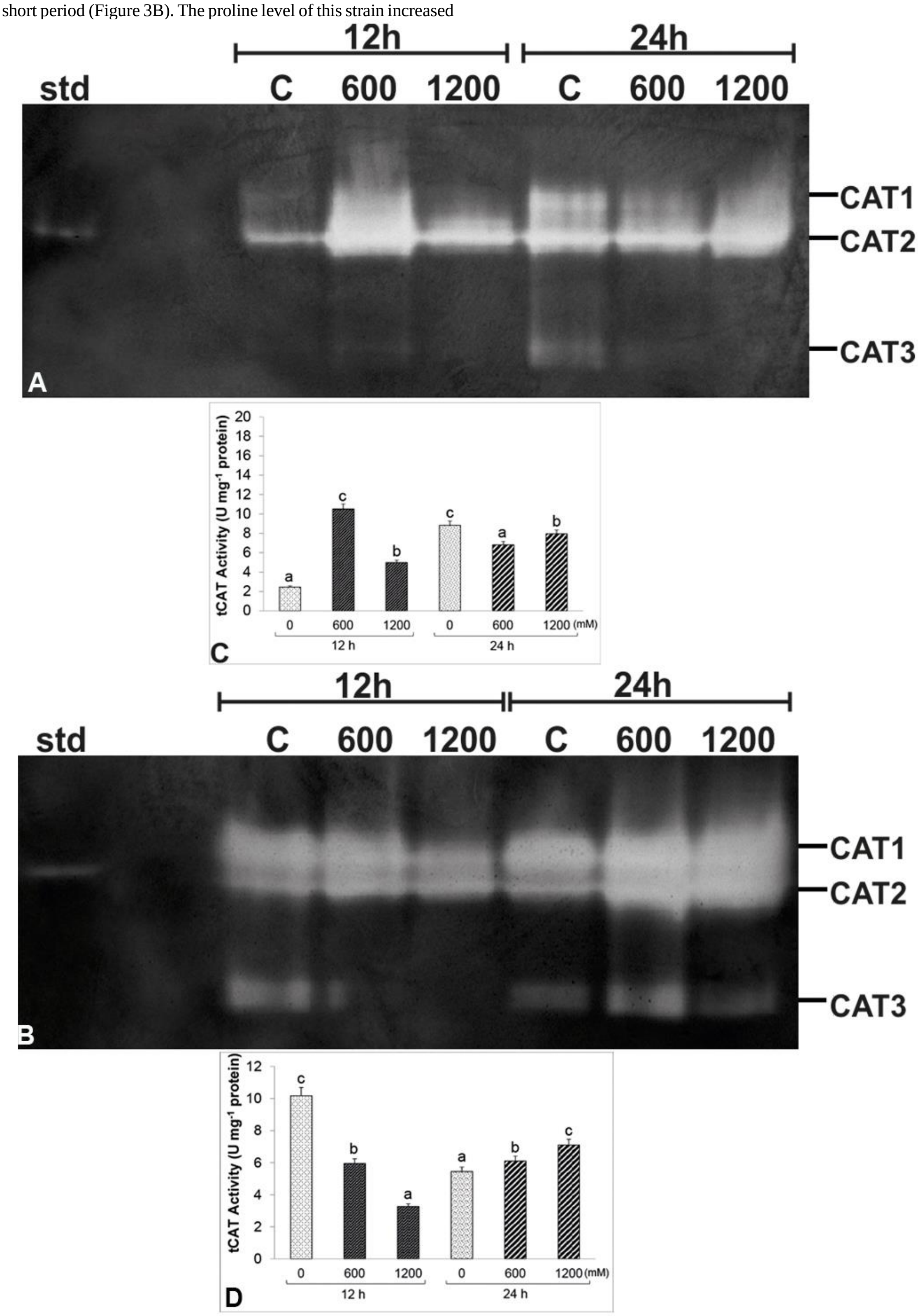

Figure 5 Effects on activity staining of CAT isozymes **(A-B)** and total CAT activity (**C-D**) in *E. coli* strains exposed to 600 and 1200 mM NaCl for 12 and 24 hours (samples containing 25 µg protein of the gel were pipetted; std. 0.5 units of CAT). (Data **A** and **C** belong to strain **6E**; data **B** and **D** belong to strain **S39**). The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test $p < 0.05$).

As in all living organisms under environmental stress conditions such as salinity, osmotic balance between internal and external cellular environment must be maintained. Accumulation of osmolytes such as choline in the cell is a common physiological response to achieve this balance. It was determined that choline contents of 6E strain increased during the whole application period compared to control. The highest choline enhancement was observed in samples exposed to 1200 mM NaCl for 24 h with a ratio of 93.4% (Figure 3C). It can be said that choline accumulation may be a good strategy for 6E strain to cope with high salinity. In strain S39, only 39.2% increase was observed in bacteria under long-term high salinity (1200 mM), and in all other applications the choline content was either decreased or unchanged (Figure 3D).

Glycine betaine increases the osmotic potential of cells and maintained the turgor state by internally increasing the osmotic pressure. In both strains, the increased in glycine betaine content was directly proportional to the salt stress. The highest glycine betaine content in both strains was observed in bacteria exposed to 1200 mM NaCl for 24 h (Figures 3E-F).

Antioxidant enzyme/isozyme composition

Total SOD activity of the strain 6E increased in all applications except 1200 mM NaCl applied group for 24 h (Figure 4C). This increase in SOD activity may reflect an increase in the production of $O_2^{\bullet -}$ radicals in consequence of electron leaks from the electron transport chain. The total SOD (tSOD) activities of the groups treated with 1200 mM salinity for 24 h were not markedly different from the control. The highest increased in SOD was observed in bacteria at 600 mM salinity at both sampling times. These increase rates were 52% and 17.8% respectively. The tSOD activities of these strains, which we considered to be tolerant, were higher under short-term (12 h) salinity than long-term (24 h) treatments. tSOD activities of S39 isolated from well water varied. tSOD activity was significantly increased in all salt stress applications except for the short-term 1200 mM NaCl group. A 29.9% reduction in 1200 mM NaCl was observed for 12 h when compared to the control (Figure 4D).
Nine different SOD isozymes were determined by electrophoretic separation of SOD enzyme from 6E strain (Figure 4A). One of these identified isozymes was defined as Mn-SOD (which is not affected from KCN and H_2O_2); two of them are Fe-SOD (unaffected by KCN but inhibited by H_2O_2) and the other six isozymes were defined as Cu/Zn-SOD according to inhibition by both KCN and H_2O_2. Increased in Mn-SOD and Cu/Zn-SOD isozymes play an important role in increasing SOD enzyme activities.
A total of three different SOD isozymes were determined by electrophoretic separation of SOD enzyme by salt applications from S39 strain. One of these isozymes was defined as Mn-SOD, another one was defined as Fe-SOD and the other isozyme was defined as Cu/Zn-SOD. The 29.9% reduction in bacteria treated with 1200 mM salinity for 12 h was due to the reduction in Fe-SOD and Cu/Zn-SOD isozymes (Figure 4B).
Short and long term salt stress treatments in strain 6E caused different changes in total CAT (tCAT) activity (Figure 5C). tCAT activity was increased when exposed to short-term salinity and decreased in long-term applications. The highest tCAT activity of this strain was determined as 4.3 fold of the control group in 600 mM NaCl for 12 h. In short-term treatments, tCAT activity decreased as opposed to increasing salt concentration in strain S39 (Figure 5D). Both the lowest and highest tCAT activities were detected in bacteria treated with 1200 mM NaCl at both sampling times.
As a result of salt treatments, a total of three different CAT isozymes were determined by electrophoretic separation of CAT enzyme for both strains. Increased CAT2 isozyme plays significantly role in the highest CAT activity of the 6E strain (Figure 5A). Increased in CAT1 and CAT2 isozymes are of great importance in the highest CAT activity of S39 strain (Figure 5B). The lowest CAT activity is due to the decrease in activity of CAT3 isozyme.
Peroxidase (POX) activities of strain 6E increased significantly at both sampling times when compared to the control group (Figure 6A). The highest POX activity under both short and long term salinity was determined to be 3.7 and 2.8 fold of the control in 600 mM NaCl treated bacteria. POX activity of S39 decreased for both sampling times and these reduction rates were determined as 64.9% and 33.6%, respectively (Figure 6B).

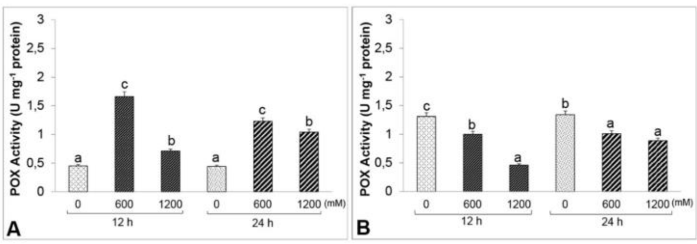

Figure 6 Effects on total POX activity in *E. coli* strains exposed to 600 and 1200 mM NaCl for 12 and 24 hours. (Data **A** belong to strain **6E**; data **B** belong to strain **S39**). The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test *p <0.05*).

In general, APX activity was increased in 6E strain as a result of short and long term salinity (except of 600 mM NaCl for 24 h). The highest induction was detected in bacteria treated with 600 mM NaCl (Figure 7C). Except for 12 h 1200 mM NaCl applied group in S39 bacteria, total APX (tAPX) activity showed a significant increase compared to control in all other applications (Figure 7D). The highest tAPX increase at both sampling times was determined in bacteria under 600 mM NaCl stress with 30.7% and 49.5%, respectively. The highest decrease was determined by 28% in bacteria treated with 1200 mM salinity at 12 h of stress.

A total of five different APX isozymes were determined by electrophoretic separation of the APX enzyme and salt applications of both groups of bacteria. Increased APX1 and APX5 isozyme plays an important role in the highest APX activity of strain 6E (Figure 7A). In S39 strain, the lowest APX activity is due to the decrease in the activities of APX3-5 isozymes (Figure 7B).

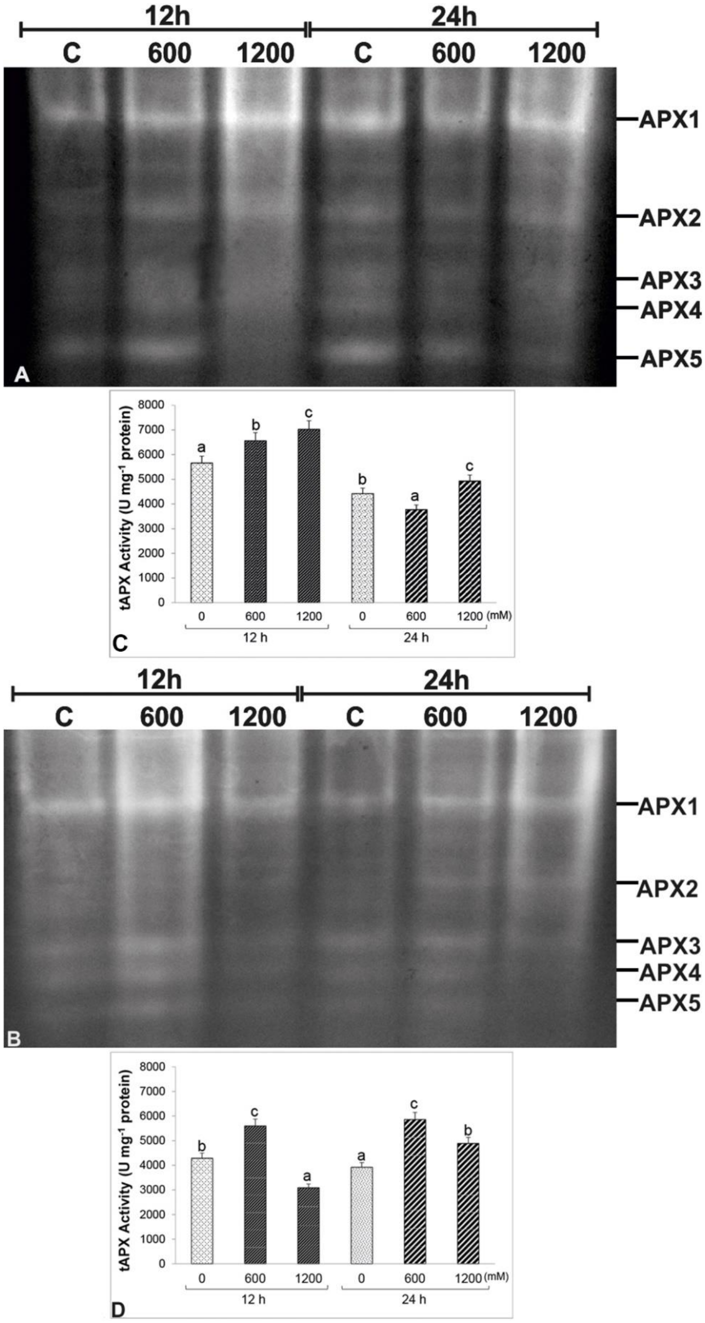

Figure 7 Effects on activity staining of APX isozymes **(A-B)** and total APX activity (**C-D)** in *E. coli* strains exposed to 600 and 1200 mM NaCl for 12 and 24 hours (samples containing 25 µg protein of the gel were pipetted). (Data **A** and **C** belong to strain **6E**; data **B** and **D** belong to strain **S39**). The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test *p <0.05*).

Glutathione reductase (GR) activity of 6E group bacteria induced in both short and long term salt applications. GR activity was also increased in parallel with salt treatment time and concentration. The highest GR increase at both sampling times was determined to be 2.1 and 3.1 folds of the control in bacteria treated with 1200 mM NaCl, respectively (Figure 8A). In the S39 group, the GR activity was either unchanged or decreased significantly with salinity compared to the control. The highest reduction rates are 43.3% at 1200 mM in short-term applications and 43.5% in long-term applications (Figure 8B).

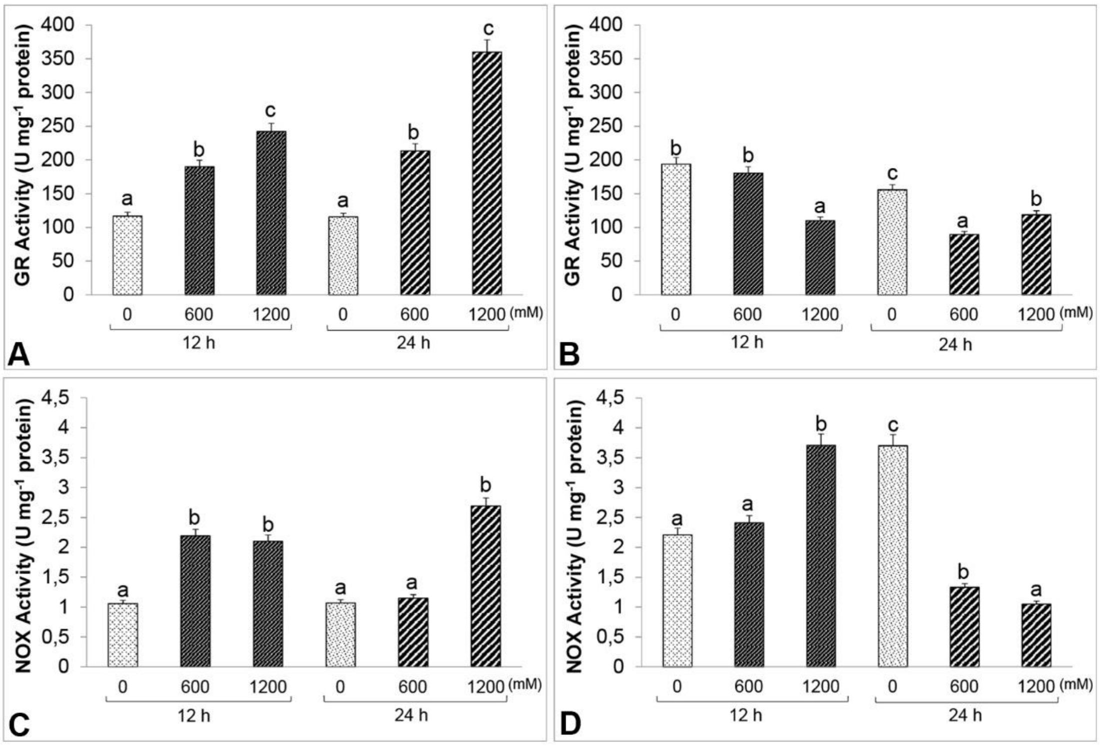

Figure 8 Effects on GR (**A-B**) and NOX (**C-D**) activities in *E. coli* strains exposed to 600 and 1200 mM NaCl for 12 and 24 hours. (Data **A-C** belong to strain **6E**; data **B-D** belong to strain **S39**). The different letters on the columns indicate statistically different values in the same strain (ANOVA, Tukey's post-test $p < 0.05$).

In general, NOX activity was increased in 6E strain as a result of short and long term salt applications. The highest increase in the NOX activity was observed at 1200 mM, the highest concentration at both sampling times (Figure 8C). NOX activities of S39 strain differed in short and long term stress treatments. While NOX activity was either unchanged or increased in short-term applications, it was significantly decreased during the 24 h of stress. The lowest NOX activity was determined in bacteria exposed to 1200 mM salinity for 24 h with a decrease of 61.8% compared to the control (Figure 8D).

DISCUSSION

Microbiological stress induced by environmental and biological factors, can individually or together, produce significant changes in the physiological events of microorganisms. Various physiological, morphological, biochemical and molecular reactions occur in microorganisms exposed to stress factors such as salinity, drought, pH, acidity, low and high temperatures, nutrient deficiencies or abundance, heavy metals and radiation. Microorganisms can produce a protective or adaptive response when exposed to stress. When adapting to one stress, resistance to another stress is called cross protection **(Yousef & Courtney, 2003)**.
Exposure of microorganisms to salinity results in an increase in the production of $^1O_2$, $O_2^{\bullet-}$, H_2O_2 and $OH^\bullet$ radicals **(Gill & Tuteja, 2010)**. For the removal of these radicals, bacteria have enzymatic (SOD, CAT, POX, APX and GR) and non-enzymatic antioxidant (glutathione and ascorbate) defense systems. In addition to antioxidant enzyme mechanisms, they are adapted by accumulating or removing osmotic preservatives against these stress factors or by *de novo* synthesis. Osmotically active molecules keep the positive pressure turgor required for cell division. Because most intracellular macromolecules are sensitive to high levels of inorganic ions, most organisms tend to restrict cytoplasmic ion strength to very low levels. Prokaryotes in high osmolality environments have some strategies for survival and development.
High salt concentrations can lead to major problems for living systems. Osmo-adaptation is defined as the physiological and genetic findings of adaptation to low and high water environments **(Galinski, 1995)**. In principle, two strategies have been developed to deal with high osmolarity **(Galinski & Truper, 1994)**. These are salt and organic osmolyte in the cytoplasm.
The salt mechanism in the cytoplasm is known as the accumulation of high intracellular concentrations of ions, a strategy commonly used by halophilic archaea and halotolerant bacteria to overcome osmotic stress. These prokaryotes have the physiology of adaptation to the high salt environment **(Galinski, 1995; Galinski & Truper, 1994)**. In extreme halophilic archaea, predominantly protective potassium ions accumulate **(Brown, 1990; Vreeland, 1987)**. These halophiles provide a sodium-free cytoplasm by expelling sodium ions and accumulating potassium. Internal potassium levels in halophilic prokaryotes can be five times higher than internal sodium ions. The potassium gradient in the cells is regulated by both sodium ion / proton antiporter and potassium ion uniporter. Organisms that follow this strategy also adjust to the cell's internal protein chemistry of the cell to a high salt concentration **(Gülbenzer & Ökmen, 2012)**. This adaptation was first discovered in *Halobacteriaceae* and is typical **(Galinski & Truper, 1994)**. Also fermentation bacteria, acetogenic anaerobes (*Haloanaerobium, Halobacteroides, Sporohalobacter, Acetohalobium*) and sulfate reducing agents are known to develop this strategy **(Oren, 2006)**.
Osmotic preservatives or compatible solubilizers are another mechanism used to adapt to salinity. The term "compatible solutes" (osmotic preservatives) was first described by **Brown (1990)** as "small organic compounds used for osmotic adjustment without disturbing cell function". Non-ionic, water-soluble compounds that do not impair metabolism and remain as osmotic preservatives even at high cytoplasmic concentrations. They show protective effects against denaturing agents such as salt, heat, drought, freezing, thawing and urea **(Brown, 1976; Gülbenzer & Ökmen, 2012)**. Halophilic microorganisms generally synthesize compounds such as nitrogen-containing glycine betaine and ectoine **(Galinski & Truper, 1994)**, whereas salt-tolerant organisms accumulate sugars such as trehalose (Tre, 1-α-glucopyranosyl-1-α-glycopyranoside) or sucrose **(Kempf & Bremer, 1998)**.
In the present study, *E. coli* strains isolated from two different localities were obtained and cultured and are expected to have different salt tolerances. Indeed, the difference in salt sensitivity was revealed and 6E strain was more resistant to salt than S39 strain. Both strains were grown on Luria-Bertani broth media prepared with NaCl at 600 and 1200 mM concentrations for salt treatment according to their maximum salt resistance limits. Previously, the time-dependent growth graph of the strains in NaCl media containing the same concentration was drawn. In line with this graph, it was determined that the strains were in suitable turbidity for harvesting at 12th hour and the first harvest was made in 12th hour cultures. 24th hour was selected as the second harvest time in order to observe whether the physiological responses changed over time. Lipid peroxidation levels were examined as the first indicator of whether the harvested bacteria were affected by the saline environment. The 6E strain did not change the TBARS level at 600 mM salt concentration at both 12 and 24 h, confirming that 6E was not affected by salinity. At 1200 mM, these levels increased in long-term applications. S39 strain was negatively affected by salinity in all assays. This is an expected result according to the salinity of the isolated regions. Thus, it can be proposed that the strain 6E is more salt-tolerant than S39.
It is known that H_2O_2 content increases in microorganisms affected by stressful environments. In this study, the H_2O_2 content of 6E strain increased only in 1200 mM salt applications, and increased in both concentrations over 60% in parallel with the increased salinity of the S39 strain. This shows that S39 is more affected by salinity stress as compared to 6E strain.
The levels of damage products accumulated (estimated as malonyldialdehyde concentration) is a mirror of the density of oxidative stress **(Aubron *et al.*, 2012)**. The 6E strain affected from salinity both sampling times at a concentration of 1200 mM NaCl, while S39 was damaged from salinity both two concentrations of NaCl. Similarly, in a study conducted by **Aubron *et al.* (2012)**, it was determined that *E. coli* strains isolated from urine samples increased TBARS contents due to the

exposing to oxidative stress. Our TBARS results in line with the values reported by **Aubron *et al.* (2012)**.

In the present study, proline levels were measured depending on the salt concentration and reproduction time of the strains exposed to stress. According to the results, 6E strain which is more tolerant to salt increased proline levels in parallel with incrementing salt concentration in short and long term salt treatments. The more susceptible strain S39 may not have increased proline in short-term salt treatment. As a result, it could not fully achieve osmotic stabilization. In prolonged applications, although proline level increased at 600 mM, proline level in the medium containing 1200 mM NaCl decreased when compared to the control and high salinity may cause deterioration in the processes related to proline synthesis of bacteria. A similar situation was observed for choline, another osmolyte. The 6E strain increased choline synthesis at both concentrations at 12 and 24 h, while S39 showed only a slight increase in long-term stress in the treatment of 1200 mM NaCl. In terms of glycine betaine content, both strains produced increased levels of glycine betaine synthesis, but the 6E strain, which was evaluated as tolerant, showed more osmolyte deposition than S39. The higher osmolyte content of the 6E strain than S39 shows that osmolytes are of great importance in salt tolerance in osmotic adaptation. The proline increase has also been shown in microorganisms (*Streptomyces* **(Killham & Firestone, 1984)**, halophilic/halotolerant *Bacillus* strains, *B. subtilis* (some non-halophilic) **(Galinski, 1995)**) exposed to osmotic stress or living in extreme environments for osmotic stability agent. Previous study reported that, glycine betaine have an important role in osmotic equilibrium, particularly in archaea and bacteria **(Santos & da Costa, 2002)**, in *Actinopolyspora halophile*, extreme halophilic bacteria; *Ectothiorhodospira halochloris* and *Aphanothece halophytica*, halotolerant cyanobacteria **(Nyyssl & Leisola, 2001; Reed *et al.*, 1984; Waditee *et al.*, 2003)**. In the present study, the increase in osmolyte due to salinity in the 6E strain tend to corroborate to results reported by previous studies.

Superoxide dismutase (SOD), a metalloenzyme, is the key antioxidant enzyme in defense against ROS-mediated oxidative stress in all oxygen-breathing organisms **(Gill & Tuteja, 2010)**. In terms of SOD activity, short-term salt treatments were increased in the 6E strain which was tolerant from the strains exposed to salinity compared with the non-stress group. This increased in SOD activity may reflect an increase in the scavenging of $O_2^{\bullet-}$ radicals produced in consequence of electron leaks from the electron transport chain. In S39 strains, SOD activities decreased more in short term salt treatments. Catalases are enzymes that are absolutely necessary for the detoxification of ROS under stressful conditions. In terms of CAT activities, 6E strain increased CAT activity only in 600 mM concentration in short-term salt treatments when compared to control. S39 showed the lowest activity at 1200 mM concentration in short-term salt applications. The activity decreased 3.1 times of control groups. In the study conducted by **Yao *et al.* (2006)**, the effect of an insecticide acetamiprid on *E. coli* K12, *Pseudomonas* FH2 and *B. subtilis* was investigated. They identified changes in activity of SOD, CAT, ATPase and SOD isozymes. It was observed that SOD and CAT activities increased at different concentrations of acetamiprid depending on the dose. It was emphasized that acetamiprid may cause a certain oxidative stress on the treated bacteria, an increase SOD and CAT activities, and was also argued that new SOD isozymes will be expressed. In a similar study, **Lin *et al.* (2009)** bensulfuron-methyl (BSM) was applied to three bacteria (*E. coli* K12, *Bacillus subtilis* B19, *B. megaterium* L1) for investigating the activity of antioxidant enzymes. All three bacteria were found to have a baseline level of SOD, CAT and ATPase before exposure to BSM. After 24 h of exposure to BSM, enzyme activities were observed to vary depending on the dose. They found that the activity of all the enzymes began to increase 1-1.5 h after exposure to BSM, followed by a decrease, and an increase again at 9 or 14 h, respectively. **Shao *et al.* (2009)** studied the role of antioxidant enzymes in bacterial resistance to nicotine toxicity in their studies and they used nicotine-degrading *Pseudomonas sp.* HF-1 and two standard strains *E. coli* and *B. subtilis*. It was found that SOD and CAT play significant roles in resistance to nicotine stress in *E. coli*. In *Pseudomonas sp.* HF-1, they found that too many enzymes play a role in resistance to nicotine stress. In the study of **Zhang *et al.* (2012)**, the oxidative stress responses of *E. coli* K12 and *B. subtilis* B19 strains exposed to atrazine were examined. It was observed that SOD, CAT, GST and T-AOC enzyme responses were induced by atrazine exposure. In the present study, SOD and CAT activities increased in 6E strain tend to show that these enzymes play an active role in scavenging excessive amounts of stress-induced radicals. However, the constant increase of TBARS and H_2O_2 contents in S39 strain under salinity clearly shows that these enzyme activities are inadequate for adaptation. Peroxidase is one of the important enzymes that play a key role in the scavenging of H_2O_2 produced by SOD scavenging of $O_2^{\bullet-}$ radicals **(Asada & Takashi, 1987)**. In this study, 6E strains increased POX activity by 3.7 and 2.8 fold in contrast to the control at both application times. In S39 strain, POX activity was unchanged at a dose of 1200 mM for 24 h and decreased in other doses compared to control due to stress. At this point, it can be said that the 6E strain is much more effective in scavenging H_2O_2 than S39 strain. APX is involved in the ascorbate-glutathione cycle in scavenging H_2O_2 and has a significantly higher affinity for H_2O_2 compared to catalase and peroxidase. CAT and POX are susceptible to H_2O_2 at the mM level, whereas the APX enzyme is interested in H_2O_2 at the µM level. In the present study, while there was an increase in APX activity in both doses in short-term salt treatments of 6E, there was no significant change in long-term treatments as compared to control. In the S39 strain, APX activity increased against 600 mM salt and decreased at 1200 mM levels in short term treatments. In long-term applications, the highest APX increase was observed at 600 mM concentration compared to the control. Glutathione reductase (GR) activities of 6E strain increased in both treatments compared to control, and GR activity decreased with increasing salt concentration in S39 strain. In general, NOX activity was increased in 6E strain as a result of short and long term salt treatments. NOX activity of strain S39 was either unchanged or increased in short-term applications, but significantly decreased in saline with long-term applications when compared to control. In a study conducted by **El-Rab *et al.* (2013)**, POX, CAT and APX antioxidant enzyme potentials of four *E. coli* strains isolated from heavy metal contaminated area were investigated. In this study, Cr^{6+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} toxicity of heavy metal resistant strains was determined. It has been determined that the key effect of metals, especially chromium, on each enzyme increases in different categories. In addition to CAT, POX, APX enzyme activity; GR and SOD activities increased. Also lipid peroxidation of *E. coli* ASU 7 strain was found to decrease when exposed to Cr^{6+} and Cr^{3+} and this increase was thought to provide definite protection against heavy metals. As a result, researchers have suggested that antioxidant enzymes can be used as biomarkers of heavy metal pollution. In our study, these enzyme assays performed on *E. coli* strains isolated from different localities can be used as a useful tool to show the sensitivity and adaptation mechanisms of the bacteria tested against salinity and to show the time-dependent changes in activities. At this point, results from the present study were consistent with previously published data. Information on microbial salt tolerance can be used not only to characterize the specific adaptations of the strain to ecological niches, but also to produce cells with enhanced tolerance to negative environmental conditions for biotechnological purposes.

CONCLUSION

In this study, *E. coli* strains were isolated from different localities and salt tolerances were compared after purification. Therefore, some physiological parameters in the adaptation of the strains to stress were investigated by being exposed to salinity in certain ratios. According to the results obtained, the following key points can be highlighted: (i) it was found that there was no change in the TBARS level due to salinity of the 6E strain, it was not damaged by the salt, although the increase in TBARS levels by influencing the salt of S39; (ii) it was found that the amounts of proline, choline and glycine betaine, which are intracellular compatible molecules, vary depending on the time and amount of salt treatments and the 6E strain accumulates by synthesizing these osmolytes more than the S39 strain; (iii) antioxidant enzyme levels, such as SOD, CAT, POX, APX, NOX, GR, which scavenge reactive oxygen derivatives produced due to stress, have been shown to differ in both strains depending on salt concentration and treatment times; (iv) SOD, CAT and APX isozyme profiles of these antioxidant enzyme systems and increases and decreases in these isozymes were determined; and (v) it was found that the 6E strain was more tolerant to the increasing salt than the S39 strain.

Investigating and elucidating the responses of *E. coli* at high salt concentrations will provide useful data on how this bacterium is resistant to salt applications in both saline environments and foods. Since *E. coli* is a model microorganism in genetic studies, it can be used to produce cells with enhanced tolerance to negative environmental conditions for biotechnological purposes by elucidating the adaptation mechanisms to the dense saline environment. In conclusion. This study for first time has highlighted the physiological and biochemical changes of *E. coli* strains in different concentrations of salt stress resistance and susceptibility and salt adaptation mechanisms.

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