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Betaine osmoregulation functioning against salt stress in methicillin-resistant *Staphylococcus aureus* (MRSA)

Metisiline dirençli *Staphylococcus aureus* (MRSA)'da tuz stresine karşı betain ozmoregülasyon fonksiyonu

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ABSTRACT

Objective: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a clinically common and has a high mortality rate pathogen, as well as a bacterium that causes infection through food. To combat with pathogens, it is commonly preferred to create osmotic stress in their environment but *S. aureus* is able to synthesize some osmoprotectant substances such as betaine. There is limited literature about the synthesis pathways and gene expressions of these substances. This study aims to search the metabolic pathways of osmoprotectan production of *S. aureus* by comparing the bacterial behaviour with the expression of genes responsible for betaine.

Methods: In the current study, different concentrations of NaCl and KCl salts (0.5, 1.0, 1.5, 2.0 and 3.0 M) were applied to *Staphylococcus aureus* ATCC 43300, changes in the number of viable cells of the bacteria were monitored and associated with osmoprotectant production. Bacterial numbers

ÖZET

Amaç: Metisiline dirençli *Staphylococcus aureus* (MRSA), klinik olarak yaygın ve mortalitesi yüksek bir patojen olan ve gıda yoluyla da enfeksiyona neden olan bir bakteridir. Patojenler ile savaşmak için genellikle bulundukları ortamlarda ozmotik stres oluşturmak tercih edilmekte olup bu koşullarda *S. aureus*, betain gibi bazı ozmoprotektan maddeleri sentezleyebilmektedir. Bakterilerin ozmoprotektan madde sentez yolları ve gen ifadeleri hakkında sınırlı literatür bulunmaktadır. Bu çalışma, bakteri davranışını betainden sorumlu genlerin ekspresyonu ile karşılaştırarak *S. aureus*'un ozmoprotektan üretiminin metabolik yollarını araştırmayı amaçlamaktadır.

Yöntem: Çalışma kapsamında, *Staphylococcus aureus* ATCC 43300'e farklı konsantrasyonlarda NaCl ve KCl tuzları (0.5, 1.0, 1.5, 2.0 ve 3.0 M) uygulanmıştır. Bakterilerin canlı hücre sayısındaki değişiklikler izlenmiş ve sonuçlar betain üretiminden sorumlu genler ile ilişkilendirilmiştir. Tuzla muamele edilen bakteri

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treated with salt were counted by culturel and spectrophotometric methods and viability graphs were created. Simultaneously, metabolic pathways under stress conditions were determined by monitoring the expression of genes responsible for betaine in RT-PCR.

Results: When the growth curve of the bacterium is examined in NaCl or KCl salt stress, it was observed that the bacteria exited the logarithmic phase and was more affected by the stress conditions, especially at 12-24 time interval which is critical in the development of bacteria. The most notable results in the RT-PCR trials of the study were obtained when 1.5 M NaCl was present in the growth media at 36th hour of incubation. Under these conditions, a significant decrease of (-2.37-fold) in the betA gene was observed. Contrary to this result, 2.57-fold increase in the betA gene and 3.25-fold increase in the betB-gbsA gene was observed when 2 M KCl was present in the medium at 48th hour of incubation.

Conclusion: During the 48-hour incubation period in which the bacterial growth was followed, it was observed that the bacteria exhibited a fluctuating growth curve against the salt stress and exhibited a different behavior than expected in the growth kinetics.

Key Words: *Staphylococcus aureus*, MRSA, osmotic stress, glycine betaine, osmoprotectant

sayıları kültürel ekim yöntemi ile ve spektrofotometrik olarak sayılarak canlılık grafikleri oluşturulmuştur. Aynı zamanda, betainden sorumlu genlerin ekspresyonu RT-PCR yöntemi ile izlenerek stres koşulları altındaki metabolik yollar belirlenmiştir.

Bulgular: NaCl veya KCl tuz stresinde bakterinin gelişme eğrisi incelendiğinde, bakterinin özellikle gelişimde kritik olan ve logaritmik fazın sonlandığı 12-24 saat zaman aralığında stres koşullarından daha fazla etkilendiği gözlemlenmiştir. Çalışmanın RT-PCR denemelerinde en dikkat çekici sonuçlar, inkübasyonun 36. saatinde büyüme ortamında 1.5 M NaCl bulunduğunda elde edilmiştir. Bu koşullar altında betA geninde (-2.37 kat) önemli bir azalma gözlenmiştir. Bu sonucun aksine inkübasyonun 48. saatinde besiyerinde 2 M KCl bulunduğunda betA geninde 2.57 kat, betB-gbsA geninde 3.25 kat artış gözlenmiştir.

Sonuç: Bakteri gelişiminin takip edildiği 48 saatlik inkübasyon sırasında büyüme kinetiklerinde ortamda bulunan tuz stresine karşı bakterinin dalgalı bir gelişme eğrisi ortaya koyduğu ve beklenenden farklı bir davranış sergilediği gözlenmiştir.

Anahtar Kelimeler: *Staphylococcus aureus*, MRSA, ozmotik stres, glisin betain, ozmoprotektan

INTRODUCTION

Staphylococcus aureus, a halotolerant facultative anaerobic opportunistic Gram-positive bacterium, is known to be the main cause of foodborne infections (1). In addition to its pathogenic feature in foods, it also causes various infectious diseases such as meningitis, arthritis, pneumonia, soft tissue infections and bloodstream infections (2). Methicillin-resistant *S. aureus*, known as MRSA, is responsible for healthcare-associated infections and causes serious problems such as the failure of antibiotic-based treatment and the need for alternative treatment methods due to its multiple resistance to antibiotics (3).

One of the well-known methods to limit pathogen development is reducing the water activity of the environment and/or exposing the bacteria to osmotic stress. Bacteria protect themselves by synthesizing some substances called “osmoprotectants” against

the stress factors caused by extreme temperatures, high salinity, and drought (4). These substances increase the osmotic pressure of the cell cytoplasm and ensure the adaptation of the cell to the environment. It is known that bacterial cells, like other microorganisms, have positive turgor pressure. Especially under high osmotic pressure, they increase the intracellular osmotic pressure in order to maintain this stress condition (5). This is possible with the accumulation of osmoprotectant substances (6).

Increasing the salt concentration of the environment is a frequently preferred way to combat pathogens and is used especially in the food and leather industries. With some exceptions, the growth of all bacteria at high salt concentration is limited. Bacteria included in halotolerant and halophilic groups can survive even in high salt containing environments, thanks to the osmoprotectant substances they produce and accumulate (1).

NaCl was mostly used salt and has attracted attention in the osmotic stress studies in the literature. This salt, which has been used in applications in the food field, especially in the drying and preservation of meat and fish, has been preferred since ancient times, to ensure long storage times besides to use as an antimicrobial agent (7). The ease of transportation and low cost also make NaCl one of the reasons for preference. KCl is another salt known to have an effect on metabolic pathways in bacteria. There are studies reporting that combined use may be more effective in terms of salt stress compared to a single salt type (8-11).

In cheese samples salted with NaCl/KCl at a ratio of 1:1, the number of *S. aureus* was greatly reduced compared to the samples contained only NaCl which indicated that NaCl applied together with KCl has an higher effect on limiting bacterial growth (8-11). Some *S. aureus* strains showing halotolerant properties are able to survive in high salt or low water activity environments owing to the production and accumulation of osmoprotectant substances (1). The osmoprotectant substances associated with this tolerance include proline, choline, sucrose, taurine, trehalose, and especially glycine betaine (GB) (12).

The three known basic response mechanisms of halotolerant bacteria against stress are hyper-osmotic shock, hypo-osmotic shock, and compatible-solvent-free osmotic responses. These responses are generated in different ways, including membrane regulation and osmoprotectant accumulation (5).

Glycine betaine synthesis in animals and some bacteria occurs by a two-step oxidation pathway. The first step is catalyzed by choline dehydrogenase enzyme (CDH, E.C. 1.1.99.1) which oxidizes choline to betaine aldehyde (BA). The second step of the pathway is the oxidation of BA to GB carried out by betaine aldehyde dehydrogenase (BADH, E.C. 1.2.1.8) using $NADP^+$ as a coenzyme (13).

In the glycine betaine biosynthesis pathway of *S. aureus*, choline dehydrogenase (CDH) is encoded by *betA* gene; betaine aldehyde dehydrogenase (BADH) is also known to be encoded by the *betB* gene. It is stated that another gene named *betT* encodes a choline transporter and these three genes form the enzymes involved in the pathway (14). Osmoprotectants such as GB and proline are taken into the cell by osmotically activated transport systems. However, the uptake of choline into the cell, which is involved in the first stage of the GB synthesis pathway, occurs with a transport system induced in the presence of osmotic stress (15, 16). Choline, which is transported to the bacterial cell, is metabolized in the cell and converted to glycine betaine (15).

In this study, the osmotic stress response of methicillin resistant *S. aureus* ATCC 43300 bacteria at different NaCl and KCl concentrations was investigated. Changes in the number of the bacterial cells were monitored and a link between bacterial growth and osmoprotectant production was searched. Metabolic pathways under stress conditions were determined by monitoring the expression of genes responsible for betaine. In this study, it was aimed to elucidate the behavior and osmoprotectant production of MRSA under salt stress.

MATERIAL and METHOD

In the study, methicillin-resistant *Staphylococcus aureus* ATCC 43300 bacteria was used.

Preparation of media containing different salt concentrations

Tryptic Soy Broth (TSB, Merck, Germany) general medium was used to monitor the growth of bacteria in the presence of salt stress. NaCl (Scharlau, Spain) and KCl (Sigma, Germany) salts were added to the prepared media at final concentrations of 0.5, 1.0, 1.5, 2.0 and 3.0 M. The medium without salt was used as the control group.

Conditions for bacterial growth in salt-containing media

S. aureus was cultivated by 1% inoculation from stock culture of the bacteria into 10 mL of TSB at 37°C under aerobic conditions (17). At the beginning of incubation (0. hour) and at 3., 6., 12., 24., 36., 48. hours of incubation, one of the 10 mL culture-tube was taken from the incubator and then bacterial growth was determined simultaneously by plate culture method and absorbance measurement at 600 nm. Bacterial growth curve was taken into account for monitoring betaine expression; Accordingly, 12., 24., 36., 48. hour samples were included in the trials. The samples were stored at -80°C until used in the further experiments.

Study of bacterial growth

The cultural counting method was used to examine the growth of bacteria at different salt concentrations and different incubation times. Dilutions were prepared from each sample using phosphated buffered water (PBS) and drop plated on Tryptic Soy Agar (TSA, Fluka, India). The inoculated Petri dishes were incubated for 24 hours at 37°C. Bacterial growths were determined by spectrophotometric measurement in addition to the cultural counting method in order to screen to get a comparable graph and to support plate count results as well. For this purpose, the OD value of each incubated sample was measured at 600 nm (Bio-Rad SmartSpecTM3000).

Bacterial growth at different conditions were evaluated statistically by t-test to determine if there is a significant difference between the means of the groups.

Bacterial RNA isolation and optimization

Bacterial RNA isolation was performed using the "Promega SV Total RNA Isolation System, Z3100" with slight modifications in the protocol specified by the manufacturer. Accordingly, 200 µL (2% inoculation) of passaged bacteria was taken and grown in 10 mL TSB. The cultures incubated at 37°C for 24 hours were transferred to sterile centrifuge tubes and centrifuged at 6000 rpm for 20 minutes at room temperature. Among the salt concentrations included in the assay, bacteria was not able to grow at 3M salt, thus, RNA could not be isolated and 3M NaCl and KCl conditions were not included in qRT-PCR studies.

Primer design, cDNA synthesis and qRT-PCR

Pathway analysis was performed to identify betaine-related genes in the *S. aureus* genome. Two gene sequences were determined as a result of orthology research for *S. aureus* in the betaine pathway (https://www.genome.jp/kegg/) in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (S1, S2, S3, S4). Primers used for qRT-PCR analyzes were designed via web tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the sequences of the primers are given in Table 1.

cDNA synthesis was performed using the "First Strand cDNA Synthesis Kit (Roche, Cat No. 04897030001) and following the manufacturer's protocol. 20 µg of total RNA was used for cDNA synthesis. DNA concentrations obtained at the end of the reaction were measured using the Nanodrop ND-1000 spectrophotometer. DNAs at the appropriate concentration were used to determine the level of target gene expression (18).

qRT-PCR analyzes were performed for expression analyses. rpID-352 was chosen as the housekeeping gene (supplementary file, (19)). Relative expression

levels were calculated according to the 2-ΔΔCt algorithm [Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)] Method (20).

qRT-PCR amplifications were performed using the LightCycler 480 (Roche) instrument. Reactions were carried out in a total of 10 µL reaction mixture, 2 µL of which was cDNA (500 ng/µL). Added 0.8 µL (10 pmol) each of the forward and reverse primers, and 5 µL of LightCycler® 480 SYBR Green I Master (Roche) and ddH2O. A standard curve was prepared by making 10-fold dilutions ratios in control DNAs. The steps applied in the amplifications are as follows: initial polymerase activation at 95°C for 10 min, then at 95°C for 10 s 45 cycles, for 57 °C 10 s, followed by 5 s at 72°C. The specificity of the qRT-PCR amplifications was checked by melting curve analyses. qRT-PCR conditions were optimized to have amplification efficiency >95% for all primer pairs used. The Ct (cycle threshold) values in the amplification curves were calculated.

Table 1. The primer sequences used for qRT-PCR analysis

Gene	Forward Primer	Reverse Primer	Product
betA	5'-GAGGCAGTGCAGGTTCTGTA	3'-ACCGCCCATATGTGGTTCTT	194 bp
betB-gbsA	5'-CGCCATGGGGTGGATACAAA	3'-GTGTTCTTACAATGGCACTCACC	165 bp

RESULTS

Investigation of bacterial growth under salt stress

The improvement in the preferred concentrations of 0.5, 1.0, 1.5 and 2.0 M NaCl was observed in comparison with the sample in which salt-free medium was used as a control. The results of this experiment are shared in Figure 1a and 1b; cultural bacterial count results are given in 1a and spectrophotometric bacterial growth measurement results are given in 1b.

When the growth curve of the bacteria is evaluated in the absence of salt in the media (Figure 1b); it is seen that the first 3 hours of incubation coincides with the adaptation stage of the bacteria. Between the 12-24 hour period, the logarithmic phase ends and the stationary phase begins. On the other hand, the bacteria behaved differently under different salt stresses (Figure 1b).

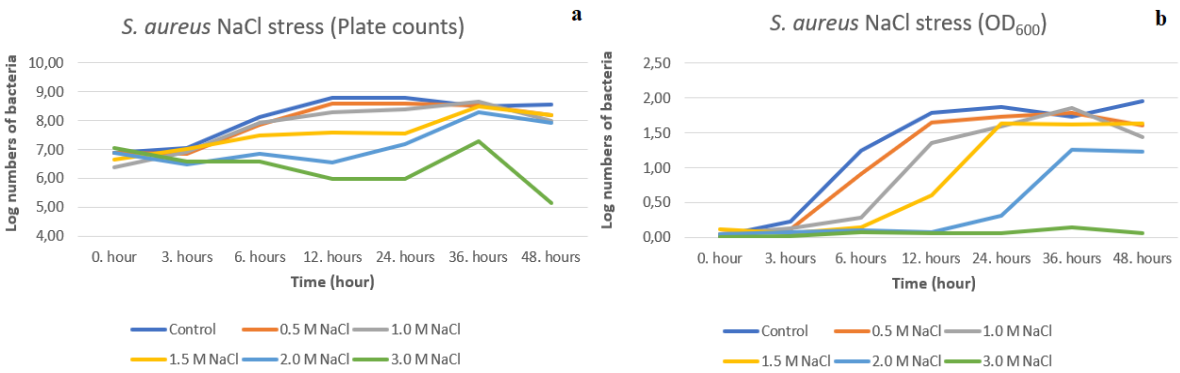

Figure 1. Cultural (a) and spectrophotometric (b) counting results of *S. aureus* under different concentrations of NaCl stress

KCl, the other preferred salt in the study, was also applied at 0.5, 1.0, 1.5, 2.0 and 3.0 M concentrations, and the medium without added salt was used as the control. The results of the KCl trials are given in Figure 2; cultural bacterial counts are given in 2a, and spectrophotometric measurement results are given in 2b.

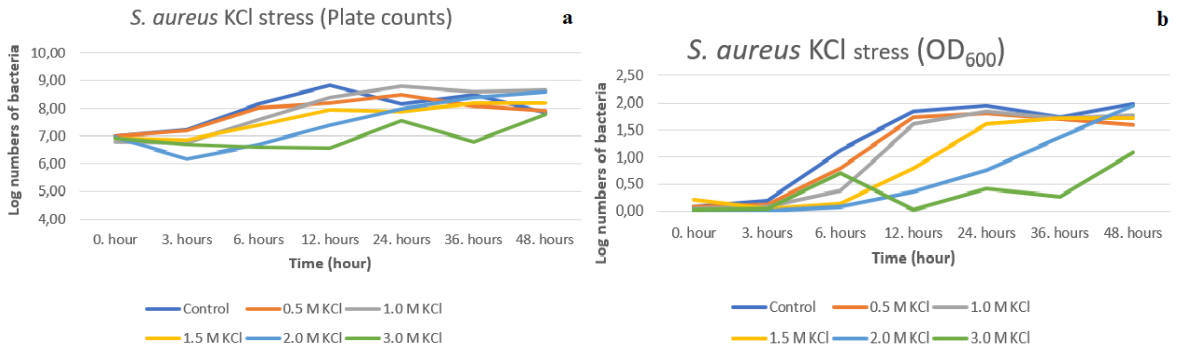

Figure 2. Cultural (a) and spectrophotometric (b) counting results of *S. aureus* under KCl stress

Monitoring of betaine production of *S. aureus* under salt stress condition

The results obtained in this experiment are given in Figure 3. Two important details seen in the chart; the first was observed at 36th hour (the sample exposed to 1.5 M NaCl) of incubation; a significant decrease (-2.37-fold) in betA gene expression was detected. The second one was observed at 48th hour of incubation; an increase of 2.57-fold in the betA gene and 3.25-fold in the betB-gbsA gene was detected in the sample exposed to 2.0 M KCl (Table 2, Figure 3). It was detected that a different behavior from the expected behavior was observed in the growth kinetics during the 48th hour incubation (Figure 4, Figure 5).

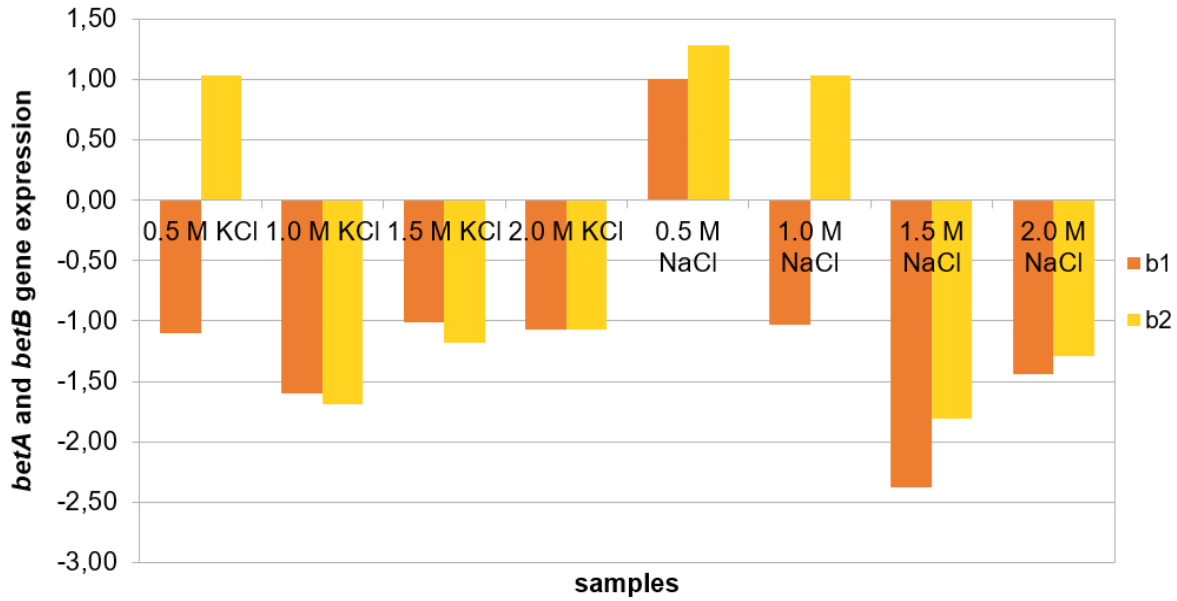

Figure 3. Gene expression analysis of *betA* (b1) and *betB-gbsA* (b2) genes at 36th hours

Table 2. Ct values obtained for *betA* and *betB*-gbsA genes in RT-PCR analysis

Incubation time-gene	Sample							
	0.5 M KCl	1.0 M KCl	1.5 M KCl	2.0 M KCl	0.5 M NaCl	1.0 M NaCl	1.5 M NaCl	2.0 M NaCl
12h-*betA*	1,07	1,33	1,31	1,39	1,67	1,30	1,06	1,41
24h-*betA*	-1,24	1,38	1,07	1,03	-1,27	1,02	1,08	1,45
36h-*betA*	-1,10	-1,60	-1,02	-1,07	1,00	-1,03	**-2,37**	-1,44
48h-*betA*	-1,41	-1,12	-1,10	**2,57**	-1,19	-1,00	-1,59	1,42
Incubation time-gene	**Sample**							
	0.5 M KCl	**1.0 M KCl**	**1.5 M KCl**	**2.0 M KCl**	**0.5 M NaCl**	**1.0 M NaCl**	**1.5 M NaCl**	**2.0 M NaCl**
12h-*betB*	-1,11	1,29	1,13	1,17	1,59	-1,09	1,01	1,13
24h-*betB*	-1,07	1,48	-1,04	1,53	-1,22	-1,06	1,53	1,76
36h-*betB*	1,03	-1,69	-1,18	-1,07	1,28	1,03	-1,80	-1,29
48h-*betB*	1,22	1,41	1,67	**3,25**	1,33	1,38	-1,43	1,53

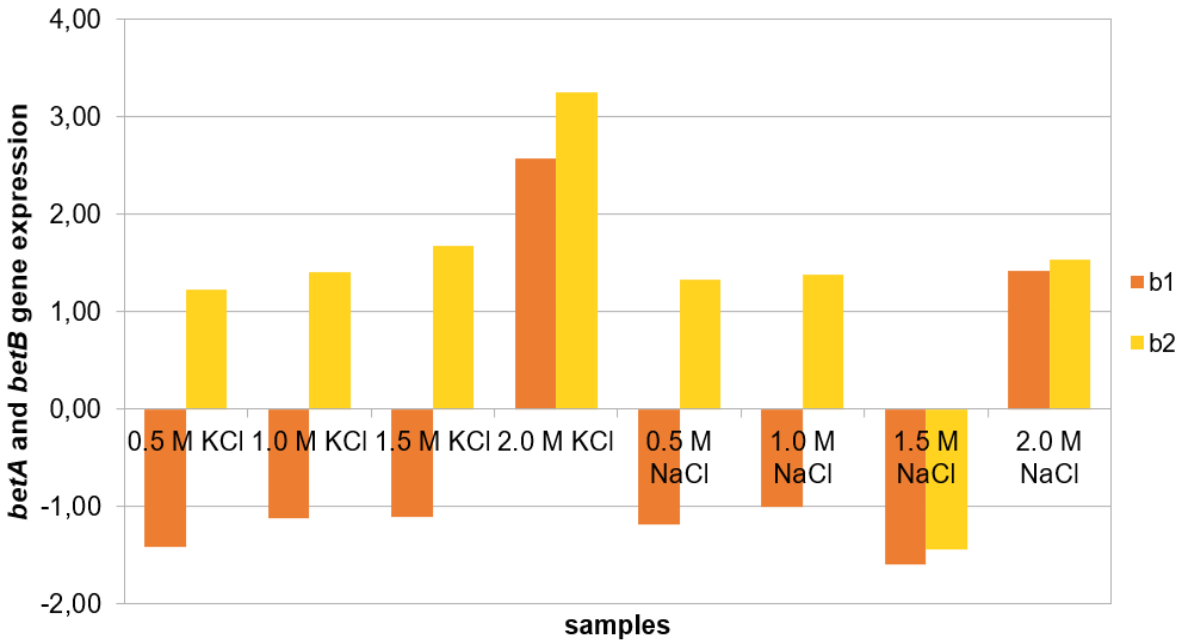

Figure 4. Gene expression analysis of *betA* (b1) and *betB-gbsA* (b2) genes at 48th hours

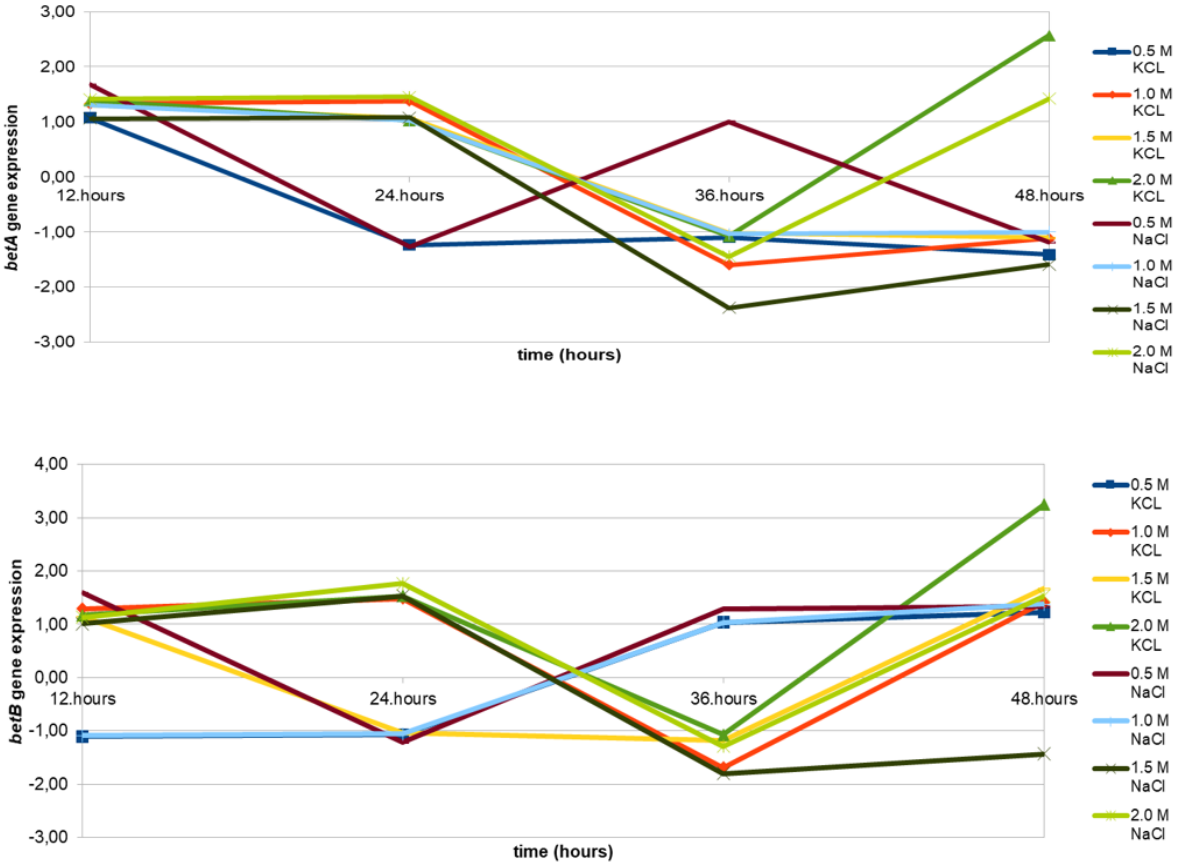

Figure 5. Expression of *betA* (b1) and *betB-gbsA* (b2) genes depending on incubation and salt concentration during *S. aureus* growth in media treated with different ratios of NaCl and KCL

DISCUSSION

Various methods have been used to combat with microorganisms which are especially food pathogens. Low water activity, low temperature, heat treatment and radiation, treatment with antimicrobial agents are the most preferred methods (21). Among those methods, NaCl is usually added to the medium in order to provide low water activity and prevent bacterial growth (22). Salt concentration is the critical point in this application; even in salty environments such as salty food surfaces with low water activity, some bacterial groups can survive called “halophilic” or “halotolerant bacteria”. This is an exceptional case where the application of low water activity cannot be effective (2). This exception arises as a result of the synthesis of some osmoprotectant substances by bacteria, and various studies have been carried out by many researchers about the synthesis and accumulation of these substances. Sleator and Hill (5) were discussed the “compatible solutes” that accumulate by being synthesized in hyperosmotic environments and protect the microorganism. The protection by glycine betaine and proline against osmotic stress has been reported (5). In the review article published by Uğuz and Andiç (23), it was examined how microorganisms are affected by osmotic stress and various other factors. It has

been emphasized that osmoprotectant substances, especially glycine betaine and proline, increase resistance to osmotic stress (23). As mentioned, *S. aureus*, which is known as food pathogen and also a major problem in clinical microbiology due to its antibiotic resistance, can improve defense system against osmotic stress (24). One of the most important osmoprotectant compounds that play a role in the resistance of this bacterium to some extreme conditions is betaine (25). For this reason, glycine betaine transport systems in microorganisms have been another interesting subject. Stimelling et al. (26) investigated the glycine betaine transport system in *S. aureus*. As a result of the study, it was confirmed that there was an increase in the amount of glycine betaine in the presence of osmotic stress condition (26). Another study on the transport of osmoregulatory substances also revealed that glycine betaine and proline transport systems play an important role in the osmoregulation of *S. aureus* (27).

It is not clear which level of salt concentration causes osmotic stress in bacteria, and at which level of salt, betaine pathway is activated and what is the maximum concentration of salt that the bacteria can maintain its viability. Within the scope of this study, NaCl and KCl salt stresses were preferred considering 1.0 M, which is the osmotic stress threshold of *S. aureus* declared in the literature (28).

As can be seen in Figure 1b; when the growth curve is compared with the control sample, it was understood that the bacteria was exposed to osmotic stress in the environment with 2.0 and 3.0 M NaCl. Bacteria incubated at these salt concentrations could not reach the desired growth levels in the first hours of incubation; however, it was observed that it can adapt to ambient conditions in the presence of 2.0 M salt concentration, especially at the 12. hour of the incubation. These incubation times indicated the activation of osmoprotectant synthesis in bacteria and thus could play an important role in determining the betaine synthesis pathway. The behavior of the bacteria was also observed at 24, 36 and 48 hours following the determined critical hour.

The bacterial growth curves can provide quite comprehensive information about bacterial growth kinetics of the organism; such as lag phase (adaptation phase), logarithmic phase (exponential phase), stationary phase and death phase (29). According to the curve, the doubling time of the bacteria and the rate of transition to the death phase can be determined. As each microorganism has its own growth kinetics; differences can be observed even in sub-strains of each species. In the current study, the growth curve of methicillin resistant *S. aureus* ATCC 43300 (control sample) was compared with the curve obtained for the cultures propogated at different concentrations of different salts.

When the salt concentration increased from 0.5 to 3.0 M, the bacterial growth slowed down proportionally. As it is seen in the Figure 1, 12th hour of incubation at 1.5 M NaCl concentration, bacterial count was 7.6 log and the number of bacteria was calculated as 7.54 at 24th hour of incubation which was not significant as statistically (p value of t-test is 0.17 ($p>0.05$)) and indicated the stop phase of the growth curve.

When the growth curve in Figure 2b is compared with the control sample, the bacteria entered osmotic stress at the 1.5 and 2.0 M KCl level. Further, when the bacteria were incubated at these salt concentrations, it was observed that the growth does not reach the desired level in the first hours of incubation. However, at the 12th hour of incubation, a significant result was detected; at this condition the bacteria adapted to the environment by initiating osmoprotectant synthesis. Therefore, this time interval is of critical importance in following the betaine synthesis pathway.

When the bacterial behavior in the control culture without any KCl is examined (Figure 2b); it can be reported that the first 3 hours cover the adaptation period of the bacteria. In the 12-24 hour interval, the logarithmic phase ends and the stationary phase begins. However, the behavioral difference of bacteria under 3.0 M salt stress is also clearly noticeable in the graphic. At this concentration, the growth of bacteria

draws a wavy image; the bacteria have difficulty in adapting to the environment and maintaining its viability. As the salt concentration in the media increased from 0.5 to 3.0 M, it was screened as an opposite trend for the bacterial growth. At the 12th hour of incubation the bacterial count was 8.18 log, while it was calculated as 6,54 at 3.0 M KCl at the same incubation time. The differences in the logarithmic bacterial numbers are found statistically important ($p<0.001$) and indicates a dramatical decreasement in bacterial growth. Figure 2a (cultural bacterial counts) and Figure 2b (spectrophotometric count) displayed parallel appearence for the curves. Bacterial growth was not stable during the salt concentration increasment in the media. Bacterial growth was not stable during the salt concentration increasment in the media. It is noteworthy that when the salt concentration reaches 3.0 M, the bacteria could not adapt to the environment easily.

The most striking detail in both NaCl and KCl graphics is that there is an acceleration in bacterial growth around the 12th hour of incubation in the presence of 2.0 and 3.0 M NaCl or KCl. This points out the concentrations and durations that should be considered in terms of osmoprotectant development, which is the main purpose of the study. In the study conducted by Graham and Wilkinson (30) on *S. aureus* osmoregulation, the bacteria was cultured in the presence of NaCl in the media. It was determined that choline was converted to glycine betaine and the amount of glycine betaine increased under osmotic stress conditions. Proline accumulation was also observed, with lower amounts than glycine betaine (30).

As mentioned above, after the stationary phase in bacterial growth curves; transition to death phase due to substrate reduction and accumulation of inhibitory substances in the medium started to cause a decreasement in the number of bacterial cells. In this experiment, after the stationary phase, a period in which an unexpected increase in numbers of bacteria was observed again in the curve around 48 hours. This observation led to thinking about bacterial adaptation to stress conditions and/or some metabolic activities. Miller et al. (31) aimed to investigate the tolerance of *S. aureus* to osmotic stress, which was exposed to high concentrations of NaCl. The results indicated that glycine betaine and proline were the main compatible solutes. In addition, it was determined that the uptake of glycine betaine and proline increased in the presence of osmotic stress (31).

In the literature, there are various studies on the osmoregulatory behavior of betaine in bacteria; however, there are not many studies on gene expressions of *S. aureus* osmoregulators. Csonka (32) stated that osmotic regulation in different microorganisms is realized by glycine betaine and proline and it was determined that these osmoregulators were associated with the presence of stress in the pathways of transport system (32).

In the light of the literature knowledge, *S. aureus* has the ability to synthesize components such as glycine betaine, proline and choline, which are effective in adaptation to stress conditions. In this regard, Kaenjak et al. (15) investigated the effect of choline transport on osmotic stress in *S. aureus*. It has been found that choline uptake increases in the presence of hyperosmotic stress at low phosphate concentrations and accumulates by converting choline into glycine betaine (15). Similarly, Ko et al. (33) revealed that glycine betaine supports osmotolerance and cryotolerance in *Listeria monocytogenes* (33). Peddie et al. (12) conducted a study investigating the protection of betaine analogs of *S. aureus* against osmotic stress. The researchers stated that betaine accumulates in the absence of osmotic stress and the amount of accumulation increases up to 10 times under hyperosmotic conditions (12). Further, the accumulation of glycine betaine was faster at high salt concentration such as 4% NaCl. It is a general agreement that the osmotolerance and also Na^+ tolerance of *Staphylococcus aureus* are seriously high which make hard to combat this pathogen in human colonization, pathogenesis, and growth in

food. But, the molecular basis of this tolerance is still not enlightened in detail to date. Price-Whelan et al. (34) performed a study to put forth the Na+ tolerance mechanism of *S. aureus* and reported that Ktr transporters play a significant role in high Na+ tolerance.

Glycine betaine is more prominent in studies than other synthesized osmoprotectant components. In our study, the genes responsible for glycine betaine production of bacteria exposed to stress were investigated. Boch et al. (35), similar to our study, investigated the genes responsible for glycine betaine synthesis and synthesis in *Bacillus subtilis*. *B. subtilis* in high osmolarity environment synthesized glycine betaine from choline; gbsA and gbsB genes have been reported to play a role in the synthesis pathway (35). Betaine production was examined with primers that amplify 165 and 194 bp regions in RT-PCR experiments conducted in our study. As seen in the literature, in the review of Clements and Foster (36), the similar results were notified. In this review, the responses of *S. aureus* to environmental stress were evaluated. It has been emphasized that when bacteria are exposed to osmotic stress, they are protected against stress by increasing the amount of betaine, proline and glycine (36). Similarly to our study, Gutierrez et al. (37) investigated the responses of *S. aureus*, *Escherichia coli* and *Listeria monocytogenes* bacteria to osmotic stress. As a result of this research, betaine, proline, taurine and choline play a role in the osmoregulation of *S. aureus* and provide resistance against stress (37).

An in silico study of osmotolerance in *Staphylococcus aureus* was performed by Casey and Sleator (38). The regulation of osmotolerance in ST772-MRSA-V was mediated at the transcriptional, translational, and post-translational levels and 17 distinct putative hyper and hypo-osmotic stress response systems, comprising 78 genes, were identified in the study. OpuD, nhaK, mrp/mnh, and opp were found related with osmotic stress system.

Besides *S. aureus*, many bacteria can synthesize glycine betaine against osmotic stress and catabolize this component as a growth substrate. In *Pseudomonas syringae* B728a, the expression of betaine genes was investigated; Within the scope of that study, it was determined that while the amount of betaine accumulated in the medium was stable under moderate osmotic stress, the expression of betaine-catabolizing genes decreased, whereas the expression of these genes increased under high osmotic stress. Similarly, an increase in betaine degradation was detected under this high osmotic stress. In the study, the mutant lacking the *gbc*AB gene, which is responsible for betaine catabolism, showed low osmotolerant profile. It has been determined that high amount of betaine accumulates in the environment, whereas endogenous solutes are screened at low amounts (39). These results show that bacteria support betaine production under osmosis and try to balance the environment with betaine degradation when the stress level increases (39).

In the current study, the gene level expression of betaine synthesized as an osmoprotectant by *S. aureus*, which can cause infection in food and clinical environments, was investigated. The fact that this bacterium has resistance mechanisms against high salt concentrations, which aim to reduce water activity in the environment, and this makes it difficult to struggle with this pathogen. Contrary to the aim of our study, it is sometimes preferred for technological purposes to gain this feature to bacteria that do not have the genes responsible for betaine synthesis. For example, it is commercially important for probiotic cultures to be resistant in extreme situations such as high salt concentration, low temperature application, high pressure application. Sheehan et al. (40) used the nisin-controlled expression system for heterologous expression with the listerial betaine uptake system BetL in a probiotic strain, *Lactobacillus salivarius* UCC118 (40). Based on the results, strains expressing BetL following nisin induction showed increased resistance to spray and freeze-drying with osmo-, cryo-, baro- and cold tolerance.

According to the results of the current study, it is noteworthy that there are fluctuations in the growth

curve of MRSA exposed to NaCl and KCl stress. These fluctuations most likely occurred because the bacteria sought to maintain their survival in the stress state and therefore resorted to the production of a range of osmoprotectants. The expression of two betaine-related gene regions was investigated in RT-PCR experiments, and it was noted that there was up and down regulations of these gene regions at different incubation hours. There was a differentiation in the expression of betaine genes in the later hours of the incubation; It can be interpreted as the fact that the bacterial numbers show fluctuating graphics in the salt-stressed environment, and that they have undergone adaptation phases due to betaine production in the environment.

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ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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