



Survival of *Escherichia coli* and *Acinetobacter junii* at various concentrations of sodium chloride

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Abstract

The survival of two heterotrophic bacteria in conditions of various concentrations of NaCl was tested. Both bacteria can commonly enter marine environments through sewage or wastewater treatment plant effluents; the *Escherichia coli* is a common enteric bacterium and *Acinetobacter junii* is a phosphate-accumulating bacterium inhabiting activated sludge. When cultivated in nutrient rich media (COD 8700 mg O₂ L⁻¹), both bacteria were multiplying during 72 h at concentrations of NaCl up to 5% for *E. coli* and 3.5% for *A. junii*. Total die-off of *E. coli* was detected at 72 h by NaCl concentration of 20%. Total die-off of *A. junii* was detected at 72 h by NaCl concentration of 10%. When the same bacteria were cultured in nutrient depleted media (COD 90 mg O₂ L⁻¹), the multiplication of *E. coli* stopped at 3.5% of NaCl and higher, but the cells were able to survive for longer period of time at extreme NaCl concentrations of 20 and 30%. The negative influence of NaCl to *A. junii* was pronounced in conditions of organic matter shortage and rapid die-off was observed at 3.5% of NaCl and higher. Both bacteria seemed to be osmotolerant when cultured in nutrient-rich media, but not in nutrient-depleted media.

Keywords: *Acinetobacter junii*, bacteria, *Escherichia coli*, sodium chloride, survival.

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INTRODUCTION

The survival of human enteric bacteria in marine environment attracts interest in view of its public health significance. Such bacteria enter the sea through release of untreated sewage. Bacteria inhabiting the activated sludge can also enter marine environment through release of wastewater treatment plant effluents. The highest density of enteric bacteria are usually recorded in the direct vicinity of the outfalls, while at locations at more than 2 km away faecal bacteria are present in low density or are absent (Delille and Delille 2000). Such reduction of faecal bacteria is undoubtedly due to dilution factor, but also to die-off of heterotrophic bacteria in seawater. The natural self-purification of seawater is caused by diverse physical, chemical and biological factors. The period of survival of enteric bacteria in seawater is highly variable, ranging from fractions of an

hour to weeks, depending on the specific characteristics of each organism and on several other factors (Carlucci and Pramer 1960a). Most important factors affecting the survival of enteric bacteria in seawater are salinity, nutrient availability, microbial antagonism and antibiotic substances (Carlucci and Pramer 1959, 1960b). Bacteria which can tolerate high salt concentrations, up to approximately 10% of NaCl, are called osmotolerant. Organisms that require high levels of NaCl to grow are called halophiles. They are generally classified as mildly, moderately and extremely halophilic (Table 1). Many marine bacteria are mildly halophilic, requiring concentrations of approximately 3% of NaCl (Nester et al. 2004).

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Table 1. The tolerance of various bacteria to NaCl (Garrity et al. 2003).

Classification	Genera	Species	NaCl tolerance
Mildly halophile	<i>Clostridium</i>	<i>C. botulinum</i>	0-5%
		<i>C. sporogenes</i>	
		<i>C. perfringens</i>	
Moderately halophile	<i>Bacillus</i>	<i>B. cereus</i>	0-2.8%
		<i>Enterococcus</i>	
		<i>E. faecalis</i>	
Extreme halophiles	<i>Halobacillus</i>	<i>E. faecium</i>	3-10%
		<i>E. avium</i>	
	<i>Staphylococcus</i>	<i>H. halophilus</i>	15%
		<i>S. aureus</i>	
Osmotolerant	<i>Halococcus</i>	<i>S. epidermidis</i>	25-30%
		<i>S. saprophyticus</i>	
	<i>Micrococcus</i>	<i>H. morrhuae</i>	5-15%
		<i>M. luteus</i>	
	<i>Streptococcus</i>	<i>S. thermophilus</i>	4-10%
		<i>Vibrio</i>	
		<i>V. cholerae</i>	
	<i>Salmonella</i>	<i>S. typhimurium</i>	9%
	<i>Leuconostoc</i>	<i>L. mesenteroides</i>	3-6.5%
		<i>L. lactis</i>	

In this study we investigated the survival of two heterotrophic bacteria in conditions of high salt concentration. Both bacteria can commonly enter marine environments through sewage or wastewater treatment plant effluents; the *E. coli* is a common enteric bacterium and *A. junii* is a phosphate-accumulating bacterium inhabiting activated sludge (Hrenovic et al. 2009).

MATERIAL AND METHODS

Bacteria

Two heterotrophic bacteria, *Escherichia coli* strain DSM 498 (isolated from polluted water) and *Acinetobacter junii* strain DSM 1532 (isolated from activated sludge with enhanced biological phosphorus removal characteristics), were obtained from Deutsche Sammlung von Microorganismen und Zellkulturen GmbH.

Growth medium

Nutrient Broth (NB) obtained from Biolife, Italy was used for growth of *E. coli* and *A. junii*. This was nutrient rich medium with chemical oxygen demand (COD) value of 8700 mg O₂ L⁻¹. The COD was measured spectrophotometrically (HACH DR/2500 spectrophotometer) using the reactor digestion method (Hach method 8000). To study the survival of *E. coli* and *A. junii* in nutrient depleted media a 100-fold diluted NB (NB:100) was used. Before autoclaving

(121 °C/15 min) appropriate amounts of analytical NaCl (Kemika, Croatia) were added in the flasks to obtain the concentrations of 0, 0.4, 3.5, 5.0, 7.0, 10, 20 and 30% of NaCl. The pH value of both media was adjusted to 7.00 ± 0.10 using 1M NaOH or 1M HCl before autoclaving. The pH was measured with WTW 330 pH-meter.

Experimental procedures

The pure cultures of *E. coli* and *A. junii* were pre-grown on nutrient agar plates (Biolife, Italy) for 16 h at 32 ± 0.1 °C. The biomass was then resuspended in 9 mL of sterile 0.3% NaCl solution; the concentration of bacteria was approximately 10⁸ of viable bacterial cells per mL. A 1 mL of resuspended biomass was inoculated into Erlenmeyer flasks with ranging concentrations of NaCl in 100 mL of NB. The flasks were sealed and incubated for 72 h in a water bath with shaker (70 rpm) at 32 ± 0.5 °C. Starting number of viable cells was determined before incubation, and number of viable cells in each bottle was determined after every 24 h of incubation. The number of viable bacterial cells was determined as colony-forming units (CFU). A 1 mL of bacterial suspension was aseptically taken from each bottle and serially diluted (10⁻¹ to 10⁻⁹). Volumes of 0.1 mL were then aseptically inoculated onto nutrient agar plates (spread-plate method). After the incubation (32 ± 0.1 °C/24 h) the bacterial colonies were counted and the number of cells was reported as CFU L⁻¹. The same procedure was used in second set of experiments except instead of NB, a NB:100 was used as medium for bacterial growth. All measurements were done in triplicate and mean values are presented.

Results were statistically analyzed using the computer program Statistica, version 7.1. The null hypothesis was that the bottles showed no difference in performance. For the evaluation of data ordinary Student's t-tests were performed and the results were considered significant at the 5% level (p = 0.05).

RESULTS AND DISCUSSION

In the first set of experiments nutrient-rich medium was used for bacterial growth. In the control reactors without addition of NaCl both bacteria were intensively multiplying and the numbers of *E. coli* or *A. junii* after 24 h increased for one order of magnitude when compared to starting number (Fig. 1 and 2). In the reactors with *E. coli* the numbers of cells increased until 48 h and started to decrease after 72 h but still remaining significantly higher ($P < 0.05$) when compared to starting numbers (Fig. 1). The bacteria most likely entered the phase of starvation until 72 h of incubation. For *E. coli*, the similar growth trend was obtained in reactors containing 0.4, 3.5 and 5.0% of NaCl (Fig. 1). In the reactor with NaCl concentration of 7.0% the CFUs after 24, 48 or 72 h did not increase significantly when compared to starting number, indicating that at this NaCl concentration there was no cell multiplication. At 10% of NaCl the number of *E. coli* after 24 h was significantly lower ($P < 0.05$) when compared to starting number and decreased further at 48 and 72 h, indicating decay of cells. At 20 and 30% of NaCl the number of viable cells rapidly decreased through time and reached zero at 72 h and 48 h in 20% and 30% of NaCl, respectively.

For *A. junii*, the growth curves at different concentrations of NaCl were similar to that obtained for *E. coli* (Fig. 2). The differences are that for *A. junii* there was no cell multiplication at 5.0% NaCl, while decay of bacteria was observed at 7.0% of NaCl and zero cells was reached after 72 h in 10 and 20% of NaCl and after 48 h in 30% of NaCl.

Based on the results the *A. junii* seemed to be more susceptible to negative effect of high concentrations of NaCl than *E. coli*. Limiting concentrations of NaCl for bacterial growth and multiplication can be estimated at 5.0% for *E. coli* and 3.5% for *A. junii*. Increased concentrations of NaCl resulted in failure of bacterial multiplication, decay of bacterial

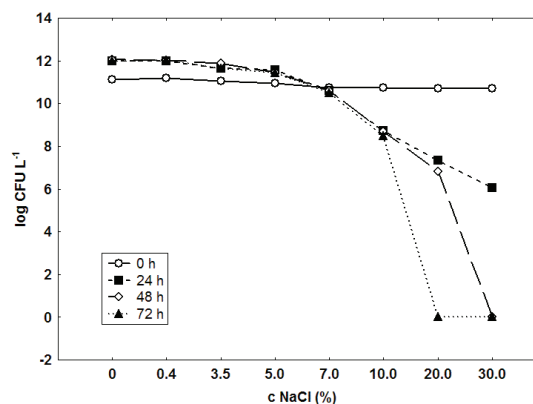


Fig. 1. Logarithm of CFUs of *E. coli* (mean values) after different periods of incubation in nutrient-rich medium with various concentrations of NaCl.

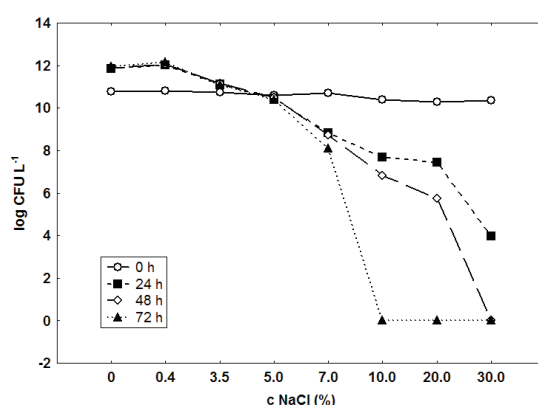


Fig. 2. Logarithm of CFUs of *A. junii* (mean values) after different periods of incubation in nutrient-rich medium with various concentrations of NaCl.

cells and death of all cells in the growth media.

These findings agree with the literature data (Jay 1992, Stein 2000) where salt tolerance levels of up to 8% for *E. coli* O157:H7 growth in culture media was reported. Hajmeer et al. (2006) found bolstered extent of cellular damage for *E. coli* O157:H7 as the NaCl concentration in the growth media increased from 5 to 10%. At 10% NaCl, the damage to bacterial cells was extensive, and the pathogen seemed to have lost its cellular integrity. Carlucci and Pramer (1960b) claimed that salinity contributes significantly to the rapid death of cells of *E. coli* in the seawater. In tests with seawater

and deionised water they concluded that increasing concentrations of salt decreased survival of *E. coli*, but there was no significant difference between the effects of equal levels of sea salts and NaCl. This is opposed to the results obtained in this study where salinity of 3.5% (salt concentration of sea water) did not cause any die-off of bacterial cells, both *A. junii* and *E. coli*. This agrees with the statement of Nusbaum and Garver (1955) that the normal saline constituents of sea water are not antagonistic to enteric organisms. The probable reason for absence of bacterial decay at 3.5% of NaCl in this study is that the bacteria were grown in nutrient rich media, which was opposed to Carlucci and Pramer (1960a) where bacteria were grown in sea water and saline deionised water, where the lack of nutrient needed for these eutrophic bacteria could cause decay of cells. The neutralization of NaCl ions by binding to organic matter in nutrient rich media could also reduce the negative influence of NaCl to bacteria in this study. When Carlucci and Pramer (1960a) added filtered fresh domestic sewage and peptone at concentrations of 100 mg L⁻¹ in the sea water, the survival of the test organism was favoured and *E. coli* multiplied. Vaccaro et al. (1950) showed that 50 mg L⁻¹ of peptone increased the survival of *E. coli* in the seawater. Approximately 10 to 100 mg L⁻¹ of available organic matter are required for active growth and multiplication of heterotrophic bacteria in the sea (ZoBell 1949). The growth of *E. coli* O157:H7 was inhibited at 28% (at 37°C) and at 24% (at 10°C) of NaCl when grown in nutrient rich tryptic soy broth (Conner 1991).

To further investigate how availability of organic matter influences the multiplication and survival of *E. coli* and *A. junii* in saline water, we incubated both bacteria in nutrient depleted media (NB:100). The COD of this medium was 90 mg O₂ L⁻¹. The *E. coli* in this medium multiplied at NaCl concentrations of

0% and 0.4% (Fig. 3). This multiplication was significantly lesser ($P < 0.05$) when compared to nutrient-rich media at same NaCl concentrations. At concentrations of 3.5% of NaCl the number of *E. coli* after 72 h of incubation was approximately half of the starting number (Fig. 3) indicating decay of bacteria. This was even more pronounced at 5, 7 and 10% of NaCl. However, at 10% NaCl the numbers of *E. coli* after 72 h of incubation was about 10-fold higher when compared with reactors containing nutrient-rich media. At 20 and 30% NaCl the number of bacteria in reactors after 72 h of incubation was 1×10^7 and 2×10^7 CFU L⁻¹, respectively. This is opposite to zero viable cells obtained in the reactors with NB. A decreased amount of organic matter in the media decreased the multiplication of *E. coli*, but enabled longer survival of cells at high concentrations of NaCl.

In control reactors (0% NaCl) with nutrient depleted media *A. junii* multiplied during the first day of incubation; the CFUs after 24 h were significantly higher ($P < 0.05$) when compared to starting number. After 48 h and 72 h the numbers of viable bacterial cells were lower ($p > 0.05$) when compared to 24 h indicating the start of bacterial decay (Table 7). The negative influence of NaCl to *A. junii* was pronounced in conditions of organic matter shortage. At 0.4% NaCl the cell decay was observed after 48 h and 72 h of incubation (Fig. 4). In the reactors with 3.5% of NaCl and higher a decay of *A. junii* was noticeable after 24 h of incubation. At 3.5% of NaCl numbers of *A. junii* decreased by 99.99% after 72 h of incubation as opposed to reactors with nutrient-rich media where the number of bacteria increased by 206%. At 5.0% of NaCl the number of bacteria after 72 h of incubation decreased by 99.99% and at 7, 10, 20 and 30% of NaCl there was no viable cells in the reactors after 72 h of incubation. In nutrient-depleted media the number of *A. junii* rapidly decreased through

Table 2. Survival of *E. coli* and *A. junii* during 3 days of incubation at various NaCl concentrations (0-30%) in regard to starting CFUs.

NaCl (%)	Time (days)	Survival (%)			
		Nutrient rich medium		Nutrient depleted medium	
		<i>E. coli</i>	<i>A. junii</i>	<i>E. coli</i>	<i>A. junii</i>
0.0	1	761.94	1210.48	129.22	153.46
	2	857.46	1294.02	195.58	108.74
	3	727.61	1425.06	173.46	102.64
0.4	1	596.87	1686.88	206.68	100.36
	2	689.97	1736.88	136.74	51.08
	3	657.68	2368.83	212.94	20.43
3.5	1	387.88	273.30	36.02	4.64
	2	656.28	253.06	31.73	0.08
	3	374.46	205.78	44.06	0.00
5.0	1	419.32	75.32	37.23	3.29
	2	345.45	84.42	13.38	0.03
	3	317.61	66.36	16.59	0.00
7.0	1	70.41	1.38	8.83	0.95
	2	70.23	1.09	7.68	0.00
	3	57.66	0.27	6.80	0.00
10.0	1	1.01	0.20	15.16	0.01
	2	0.91	0.03	7.50	0.00
	3	0.57	0.00	13.20	0.00
20.0	1	0.04	0.15	3.13	0.00
	2	0.01	0.00	0.18	0.00
	3	0.00	0.00	0.04	0.00
30.0	1	0.00	0.00	0.59	0.02
	2	0.00	0.00	0.08	0.00
	3	0.00	0.00	0.04	0.00

time at high NaCl concentrations.

The percentage of survival of bacteria during 3 d of incubation at various NaCl concentrations (0-30%) in regard to starting CFUs is shown in Table 2. Both bacteria incubated in nutrient-depleted media started to die-off after shorter period of incubation and at lower NaCl concentrations when compared to nutrient-rich media. The *E. coli* was actively multiplying up to 5.0% of NaCl in nutrient-rich and up to 0.4% of NaCl in nutrient-depleted media. The *A. junii* was actively multiplying up to 3.5% of NaCl in nutrient-rich media and only in control reactors (0% NaCl) in nutrient-depleted media.

The *E. coli* managed to survive for 72 h in nutrient-depleted medium at 20 and 30% of NaCl as opposed to zero CFU obtained after 72 h of incubation in nutrient-rich media at the same concentrations of NaCl. Shortage of nutrients probably slowed down the bacterial multiplication, enabling the longer survival of *E. coli* even at extreme concentrations of NaCl.

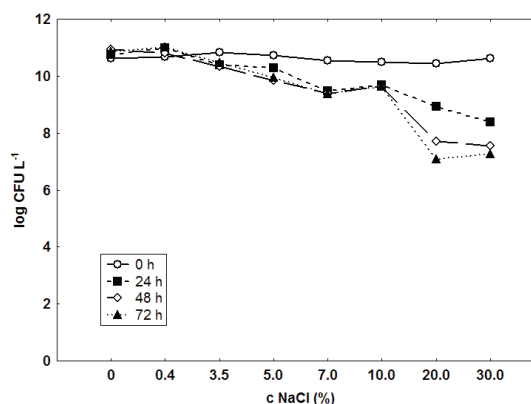


Fig. 3. Logarithm of CFUs of *E. coli* (mean values) after different periods of incubation in nutrient-depleted medium with various concentrations of NaCl.

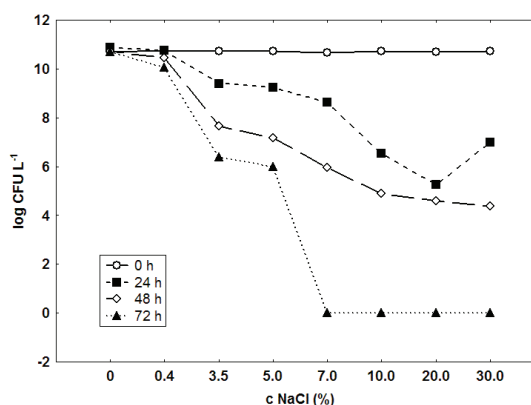


Fig. 4. Logarithm of CFUs of *A. junii* (mean values) after different periods of incubation in nutrient-depleted medium with various concentrations of NaCl.

Rosenfeld and ZoBell (1947) pointed out that the death of bacteria in seawater was greater than accounted for by salinity or osmotic pressure. Burke and Baird (1931) showed that many fresh water bacteria grow in seawater supplemented with organic matter. Similar studies by Krassilnikov (1938) and Vaccaro et al. (1950) demonstrated that the addition of organic nutrients to seawater, such as glucose or peptone, decreased the rate of death of *E. coli*. It was reported that lowered nutrient availability reduced the activity of heterotrophic bacteria in the seawater (Delille et al. 1988, Delille and Perret 1989). Ogawa (1974) stated that in natural seawater *E. coli* was not extinct by "self-

purification" or "anti-biosis" action of seawater and showed that this organism decreased mainly because of their starvation caused by lack of nourishment.

In our preliminary experiment (data not shown) *A. junii* was incubated in autoclaved Adriatic seawater. After 72 h of incubation the starting number of cells was reduced by 99.46%. At the same concentration of NaCl (3.5%) in nutrient-depleted medium the reduction of starting number of *A. junii* was 99.99%. The die-away of *A. junii* in seawater can thus be linked with low amount of organic matter in such medium. It seems that the die-off of both bacteria in seawater is not due to the concentration of NaCl itself, but rather to the combination of high salt content and shortage of nutrients. In paper by Anderson et al. (1979) the percentage of survival of *E. coli* after 48 h was 1.7% in autoclaved seawater containing 3.0% of NaCl, while in our study at similar NaCl concentration of 3.5%, *E. coli* evermore multiplied by 656% in nutrient-rich and had 32% survival in nutrient-depleted media. The difference in survival indicates that factors other than salt concentration and nutrient availability are also involved in die-off of heterotrophic bacteria in seawater.

Some investigators have reported the existence of sublethal stress in coliforms exposed to unfavourable conditions such as those found in aquatic environments (Anderson et al. 1979). Olson (1978) noted that the enumeration process itself may be responsible for apparent die-away of coliforms in natural waters. He suggested that a proportion of the fecal coliforms isolated may have been sublethally damaged and, therefore, would be undetected by usual enumeration procedures such as growth and gas production. Anderson et al. (1979) observed a maximum loss in *E. coli* viability during the first two days of exposure to seawater at salinities of 1.5 and 3.0%, whereas at salinity of 1.0% a death rate was appreciably lower. They detected a sublethal

stress in *E. coli* in various media after in vitro exposure to seawater of various salinities. Stressed cells were identified by their ability to grow on a nonselective, but not on a selective medium (Bissonette et al. 1975). Since in our experiments *E. coli* and *A. junii* were cultivated on nonselective nutrient medium, the sublethally stressed or damaged cells could be safely ignored.

CONCLUSION

The conducted experiments showed that both bacteria tested in the study were able to actively multiply in nutrient rich media such as Nutrient Broth at concentrations of NaCl up to 5% for *E. coli* and 3.5% for *A. junii*. Total die-off of *E. coli* was reached after 48 h and 72 h at concentrations of 30% and 20% of NaCl, respectively. Total die-off of *A. junii* was reached after 48 h at 30% of NaCl, and after 72 h at 20% and 10% of NaCl, respectively. When the same bacteria were cultured in nutrient depleted media, the growth of *E. coli* stopped at 3.5% NaCl and higher, but bacteria were able to survive for longer period of time at extreme concentrations of 20 and 30% of NaCl, since total die-off of bacteria was not observed even after 72 h of incubation. The negative influence of NaCl to *A. junii* was pronounced in conditions of organic matter shortage. Rapid die-off of bacterial cells was observed at 3.5% of NaCl and higher. Both bacteria seemed to be osmotolerant when cultured in nutrient-rich media. In nutrient-depleted media both bacteria did not actively multiply at concentration of NaCl higher than 0.4%, so we cannot classify them as osmotolerant.

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Farkli Sodyum Klorür Konsantrasyonlarında *Escherichia coli* ve *Acinetobacter junii*'nin Dayanıklılığı

Özet

İki heterotrof bakterinin farklı NaCl konsantrasyonlarında hayatta kalmaları test edildi. Her iki bakteri de yaygın olarak, kanalizasyon ve atıksu arıtma tesislerinin atıkları yoluyla deniz ortamlarına girebilmektedir. *Escherichia coli* yaygın enterik bir bakteridir, *Acinetobacter junii* ise aktif çamurlarda bulunan ve fosfat biriktiren bir bakteridir. Besin maddelerince zengin ortamda (COD 8700 mg O₂ L⁻¹) kültive edildiklerinde, her iki bakteri de *E. coli* için %5 ve *A. junii* için %3.5'e kadar NaCl konsantrasyonlarında 72 saatte çoğaldılar. *E. coli*'nin tamamen ölmesi, %20 NaCl konsantrasyonunda 72 saatte gerçekleşti. *A. junii*'nin tamamen ölmesi, %10 NaCl konsantrasyonunda 72 saatte gerçekleşti. Aynı bakteriler besince fakir ortamda (COD 90 mg O₂ L⁻¹) yetistirildiklerinde, *E. coli*'nin çoğalması %3,5 ve daha üzeri NaCl konsantrasyonlarında durdu, fakat hücreler %20 ve %30 gibi son derece yüksek NaCl konsantrasyonlarında daha uzun süre sağ kalabildiler. NaCl'ün *A. junii* üzerindeki olumsuz etkisi, organik madde eksikliğinde belirgin bir şekilde göze çarpmaktaydı ve %3.5 ve daha yüksek NaCl konsantrasyonlarında hızlı ölüm gerçekleşti. Besin maddesince zengin ortamda kültive edildiklerinde her iki bakterinin de ozmotolerant oldukları görüldü, fakat besince fakir ortamda ozmotolerant değillerdi.

Anahtar Kelimeler: *Acinetobacter junii*, bakteri, *Escherichia coli*, hayatta kalma, sodyum klorür.