# Acid Adaptation and Alliin Resistance of *Salmonella Typhimurium* Isolated from Acid Pasteurized Garlic Paste

# Asitle Pastörize Edilmiş Sarımsak Macunundan İzole Edilen *Salmonella Typhimurium*'un Asit Adaptasyonu ve Alliin Direnci

Research Article

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**ABSTRACT**

Garlic is often marketed fresh and without processing but, paste is a potential alternative that would retain the delicate and fresh odor of garlic. Allicin is one of the major antibacterial components of garlic. *Salmonella* was isolated from commercial type garlic paste using ISO 6579 method and identified by serotyping using Kauffman-White Scheme and antibiotic resistance patterns were evaluated using disk diffusion method. The strain was identified as *Salmonella Typhimurium*. Acid adaptation in citric and acetic acids, survival at pH 3 and Alliin resistance of the strain were evaluated. The strain was significantly resistant to lower pH and an increase in numbers of the strain was recorded. The strain was also resistant to Alliin, which may show that this strain would be the survivor of acid pasteurized garlic paste. This study aims to report an extreme Salmonella *Typhimurium* strain which adopts to acid and have Alliin resistance. The study underlines the resistance to natural antimicrobials that may cause a risk in public health.

**Key Words**
*Salmonella*, Alliin, Acid adaptation, Garlic paste

**ÖZET**

Sarımsak genellikle taze ve işlenmeden pazarlanmaktadır ancak, sarımsak macunu lezzetini ve taze kokusunu muhafaza etme açısından potansiyel bir alternatif oluşturmaktadır. Allicin sarımsağın en önemli antibakteriyel bileşenlerinden birisidir. *Salmonella*, ISO 6579 yöntemi kullanılarak ticari sarımsaktan izole edilmiş ve Kauffman-White Şeması kullanılarak yapılan serotipleme ile belirlenmiştir. Disk difüzyon yöntemi kullanılarak antibiyotik direnç modelleri değerlendirilmiştir. Suş *Salmonella Typhimurium* olarak belirlenmiştir. Suşun, sitrik ve asetik asitte asit adaptasyonu, pH 3'te sağ kalımı ve Alliin direnci değerlendirilmiştir. Suşun daha düşük pH'larda önemli derecede dirençli olduğu bulunmuş ve suş sayısında artış kaydedilmiştir. Suş ayrıca Alliin'e de direç göstermektedir. Bu da suşun asitle pastörize edilmiş sarımsak macununda yaşayabileceği anlamına gelmektedir. Bu çalışmada aside adapte olmuş ve Alliin direncine sahip aşırı *Salmonella Typhimurium* suşu rapor edilmesi amaçlanmıştır. Çalışma halk sağlığını riske atabilecek doğal antibiyotiklere direnç kazanma konusunun altını çizmektedir.

**Key Words**
*Salmonella*, Alliin, Asit adaptasyonu, Sarımsak macunu

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## INTRODUCTION

*Allium sativum*, commonly named as garlic, is a widespread found species in the onion genus, *Allium* [1]. It has a history of human use of over 7,000 years, garlic is native to central Asia, and has long been used in the Mediterranean region, as well as a frequent seasoning in Asia, Africa, and Europe [2].

Allicin is the chief component of garlic. This compound is a strong antibacterial, antifungal and antibiotic agent, and released when its bulbs are crushed [3]. Garlic contains 32 additional sulfur compounds and 17 amino acids [4].

Garlic is often marketed fresh and without processing [5]. Paste is a potential alternative that would retain the delicate and fresh odor of garlic. The garlic paste manufacturing process dates back 40 years and stems from the need to find a product with an industrial or semi-industrial process able to compete in the international market. Another advantage is that the surplus from the nonmarketable part of the harvest (broken-up bulbs, loose cloves, etc.) may be used for paste manufacture. The processing of garlic paste consists basically in the peeling of the bulb, separating the cloves and removing the skin; followed by the grinding of cloves and the addition of preservatives and antioxidants [4].

Garlic is accepted to be antibacterial for ages. There are many studies that underline the importance of garlic as an antibacterial and its antibacterial effect on different pathogens. However a garlic borne *Salmonella* epidemic in Australia arose suspection to antibacterial effect of garlic. A sustained increase in *Salmonella enterica* serovar *Virchow* notifications in South Eastern Australia between September 1997 and May 1998 instigated a case-control study and environmental investigations. Cases were defined as having locally acquired culture-confirmed *S. Virchow* phage-type 8 infection and diarrhoeal disease. An exposure and food history questionnaire was administered by telephone. Thirty-two notifications of *S. Virchow* infection met the case definition, 37% reported bloody diarrhoea and *S. Virchow* was isolated from blood in 13% of cases. Twelve patients were admitted to hospital and one died. Fresh garlic (OR 4.1, 95% CI 1.3–12.8) and semi-dried tomatoes (OR 12.6, 95% CI 1.5–103.1) were associated with these cases [6]. Salmonella can survive on the external surfaces of garlic from a recent study of the effects on crops of irrigation with treated wastewater [7]. Produce that is eaten raw is an increasingly recognized vehicle for transmission of pathogens, including salmonella species [8].

Allicin is one of the major antibacterial component of garlic. Pure allicin is a volatile molecule that is poorly miscible in aqueous solutions and which has the typical odor of freshly crushed garlic. Alliin was found to be the stable precursor that is converted to allicin by the action of an enzyme termed alliinase which is also present in the cloves and represents an acidic behaviour [3]. Allicin is reported to be effective over Gram-negative and Gram-positive bacteria species including *Escherichia, Salmonella*, *Staphylococcus, Streptococcus, Klebsiella*, *Proteus*, *Bacillus*, and *Clostridium*. Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic [9]. A very interesting aspect of the antibacterial activity of allicin is the apparent inability of most bacteria to develop resistance to it because the mode of action is completely different from that of other antibiotic substances. It has been proposed that the development of resistance to beta-lactam antibiotics is 1000-fold easier than development of resistance to allicin [3, 10].

Acidity is a commonly used factor for control of growth of pathogens in foods. In addition, acidity is a significant barrier employed by the human body to defend itself against pathogen attack [11]. Data on the acid tolerance response (ATR) are available for *S. Typhimurium* [12-14]. It has been shown that ATR is a complex biological phenomenon as different systems are engaged depending on the organism, growth phase, medium, type of acid stress (i.e. organic/inorganic acid) and other environmental factors [15,16].

This study was designed to determine the acid resistance with two different organic acids, survival at pH 3.0 and Alliin susceptibility of a *S. Typhimurium* strain isolated from acid pasteurized garlic paste.

## MATERIALS AND METHODS

Garlic paste was prepared commercially and has the ingredients as follows: Garlic (79%), water, aalt, modified maize starch, food acid: citric. No preservatives were reported to be added. It was a commercial product in a glass jar approximately 150 g. Five jars were brought to the laboratory under cold chain and analyzed in 2 hours after arrival.

### Isolation, identification and serotyping of *Salmonella* from Garlic paste

For *Salmonella* isolation, ISO 6579 [17] was used. The analysis was accredited by TURKAK (Turkish Accreditation Organization) in all food matrices.

### Isolation and identification of *Salmonella* from Garlic paste

The jar was opened under aseptic conditions and 25 g of garlic paste was weighted into a sterile filtered stomacher bag and 225 mL of buffered peptone water (BPW) was added and homogenized for 45 seconds. The homogenizate was incubated at 37°C for 24 h. After incubation, 1000 μL was transferred to modified Mueller Kauffmann Tetrathionate broth (mMKTT) and 100 μL was transferred to modified Rappaport Vassiliadis broth (mRV). The selective enrichments were incubated at 37°C for 24 hours for mMKTT, and 42°C for 24 h for mRV. After incubation one loopful of each selective enrichment was streaked onto Xylose Lysine Deoxycholate (XLD) agar and Brilliant Green (BG) agar plates in duplicates and incubated at 37°C for 24 h. Five typical colonies from each petri plate were chosen and streaked on Nutrient Agar (NA) plates and incubated at 37°C for 24 hours. From these colonies, API 20 E (Biomerieux) biochemical tests were applied and the strains confirmed to be *Salmonella* spp.

### Serotyping of *Salmonella* strain

*Salmonella* strains were sent to Turkish Public Health Agency, National Enteric Pathogens Reference Laboratory and serotyping of strains were made according to Kauffmann White Scheme.

### Antibiotic resistance of the strain

The strain was sent to Ankara University, Faculty of Veterinary Medicine, Department of Microbiology. The strains were inoculated in Brain Heart Infusion broth (BHI, Oxoid) and 100 μL was transferred to Mueller Hinton Agar (MHA, Oxoid) and spread with drigalski spatules. Amoxicillin + clavulanic acid (30 μg), gentamicin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), cephotaxime (30 μg), ceftazidime 30 μg, ciprofloxacin 5 μg, sulphamide 300 μg, tetracycline (30 μg), trimethoprim (5 μg), trimethoprim sulphametaxazole (25 μg, discs) (Oxoid, England) were applied on the plate and incubated at 37°C for 24 h. All zones were measured with a ruler and the results were calculated with CLSI 2012 standards [18].

### Acid adaptation of *Salmonella* Typhimurium

Acid adaptation studies were made according to the study published by Alvarez-Ordonez et al. [19]. The steps are given below.

### Bacterial strain and culture conditions

The *Salmonella Typhimurium* strains from garlic paste (STG) and *S. Typhimurium* ATCC 14028 (STA) were used. The lyophilized cultures were revived in tubes containing 10 mL of BHI and incubated at 37°C for 24 h followed by streaking on Trypticase Soya agar (TSA, Oxoid) plates and incubating under the same conditions. Stationary phase inocula were prepared inoculating 10 mL of fresh BHI with an isolated colony and incubating it at 37°C for 24 h. The suspension was then used to inoculate, approximately to a final concentration of $10^4$ cfu/mL, flasks containing 50 mL of sterile BHI (pH 7.0) non-acidified and acidified at pH values of 6.0, 5.0 and 4.0 with acetic (320099, Cas no: 64-19-7, Sigma-Aldrich, Germany) and citric (251275-100G, Cas no: 77-92-9, Sigma-Aldrich, Germany) acids. These cultures were then incubated at 37°C for the time needed to reach a late stationary-phase of growth.

### Calculation of growth

At appropriate intervals, samples (1 mL) were removed from each culture condition assayed. Number of viable cells in the suspensions was estimated by duplicate plating on TSA with spiral plater (Eddy Jet, IUL Technologies, S.A.) and colonies were counted after 24 h incubation at 37°C. The number of viable cells, expressed as $\log_{10}$cfu/mL.

### Assessment of non-acid adapted cultures acid tolerance

Aliquots of 5 mL of cell cultures obtained as described above were harvested by centrifugation (5000 rpm, 5 min, 4°C) (Eppendorf centrifuge 5804R, Hamburg, Germany) and the pellets were resuspended into flasks containing 50 mL of BHI with pH adjusted to 3.0 with HCl (320331, Cas no: 7647-01-0, Sigma-Aldrich, Germany) and incubated at room temperature. Survival was monitored before incubation (0 h) and hourly plating was made for up to 8$^{th}$ h. The number of viable cells was determined by direct plating on TSA using spiral plater. The plates were incubated at 37°C for 24 h. All experiments were conducted in triplicate on three different fresh cultures.

### Determination of Alliin resistance of the strain

Suspensions of acid adapted and non-acid adapted strains were homogenized and added (10 mL) into 100 mL of sterile Mueller Hinton Agar (MHA) at 45°C were homogenized with magnetic stirrer and poured in sterile petri dishes. After solidifying of the agar holes were opened. (+)L-Alliin (Sigma) which is the pure state of allicin were diluted as follows: 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0% in sterile water. These suspensions were transferred to holes in 200 µL volumes and incubated at 37°C for 24 h. All zones were measured with a ruler and recorded. Zone diameters of 8 mm and over accepted as susceptible.

### Statistical analysis

Statistical analyse was designed as follows Chi Square for growth and survival data ($p < 0.05$) (PASW, SPSS 18) and Fisher's Exact Test for Alliin resistance ($p < 0.05$) [20, 21].

## RESULTS

### Isolation and identification of *Salmonella* from Garlic paste

*Salmonella* was isolated from one jar and pure culture was prepared for serotyping. The serotyping scheme showed that the strain was *Salmonella enterica* serotype Typhimurium (Serotype scheme 4, 5, 12; i; 1, 2). The strain was determined as susceptible to all the antibiotics tested.

### Acid adaptation of *Salmonella Typhimurium*

Acid adaptation analyzes were hold in two different stages. Two different acids were used to determine the growth of two different strains of STA and STG.

### Calculation of growth at different pH values

The growth curves of (STG) in acetic acid (Figure 1) and citric acid (Figure 2) at different pH values are shown below.

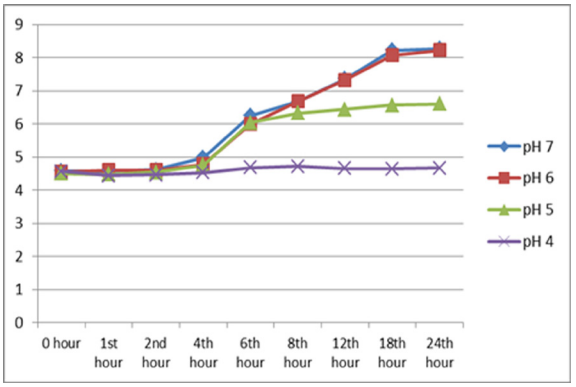


**Figure 1.** Growth curves of *S. Typhimurium* from garlic paste (STG) in acetic acid.

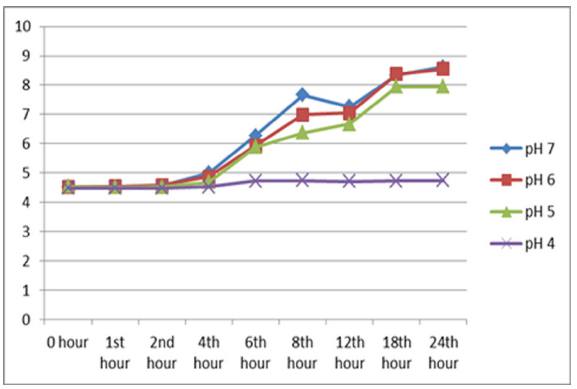


**Figure 2.** Growth curves of *S. Typhimurium* from garlic paste (STG) in citric acid.

STG had reached higher yields in pH value closer to neutral values. In both acids at pH 4, 1 $\log_{10}$cfu/ml increase was recorded. There was a statistical difference between pH values ($p < 0.05$) and a significant increase was recorded in pH 5.0 prepared with acetic acid ($p=0.012$).

The growth curves of STA in acetic acid (Figure 3) and citric acid (Figure 4) are shown below.

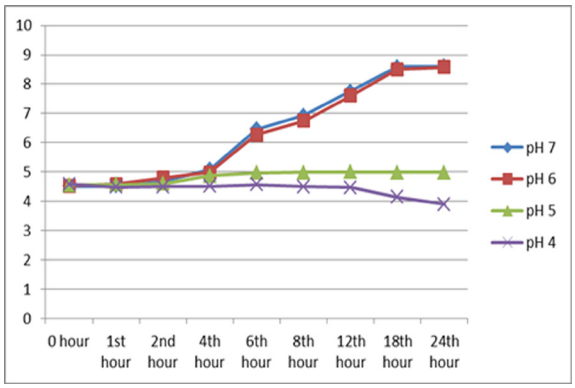


**Figure 3.** Growth curves of *S.Typhimurium* (ATCC 14028) in acetic acid.

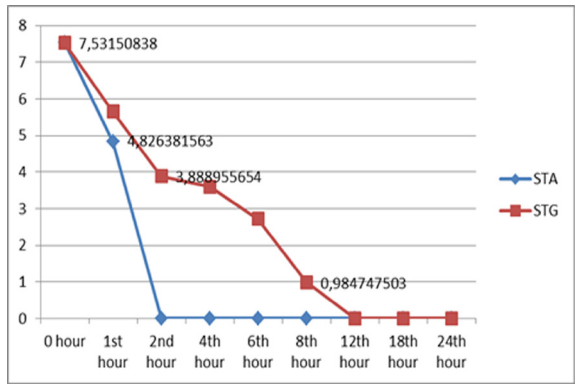


**Figure 5.** Survival of two different *S. Typhimurium* strain at pH 3.0.

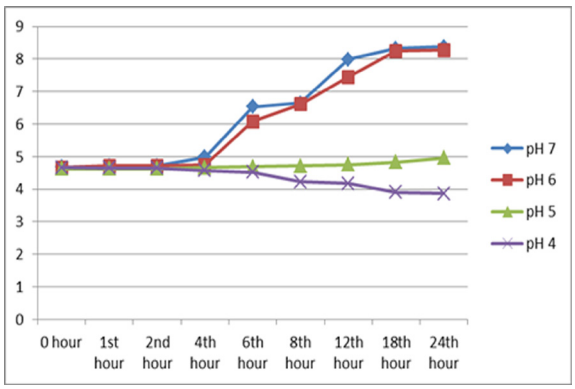


**Figure 4.** Growth curves of *S. Typhimurium* (ATCC 14028) in citric acid.

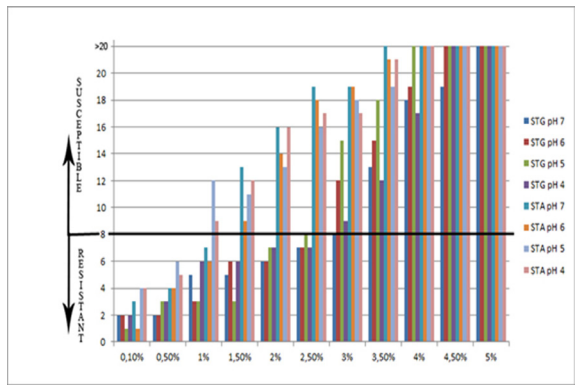


**Figure 6.** Alliin resistance patterns of strains

It can be seen that in lower pH values the growth of STA is suppressed and the counts of bacteria does not represent a significant increase (p=0.180). There is a statistical difference in growth curve obtained at pH 5 of acetic and citric acid (p<0.05).

Overall evaluation of the data acquired from the study indicates a tendency to adapt acidic environment of STG.

### Assessment of acid tolerance

The results of the acid tolerance hold at pH 3.0 showed that STG is significantly resistant to lower pH than reference strain. The survival and milestones are represented in Figure 5. STG was 7.53 $\log_{10}$ cfu/mL and reference strain was 7.54 log10 cfu/mL at the beginning of the analyses. A dramatic decrease (50%) observed in numbers of STA by 3$^{rd}$ hour and no recover can be made by 4$^{th}$ hour of the study. The same decrease in 4$^{th}$ hour was also observed in STG but an adaptation was observed between 4$^{th}$ and 8$^{th}$ hour. The final count of STG was 0.98 $\log_{10}$ cfu/mL.

### Determination of Alliin resistance of the strain

STG inoculated at pH 7.0, 6.0, 5.0 and 4.0 were found to be resistant up to 2.5% of Alliin. STA inoculated at pH 7.0, 6.0, 5.0 and 4.0 were resistant to 1% of Alliin. The difference was significant (p= 0.038). There is no statistical correlation between pH value and Alliin concentration (p= 0.159) (Figure 6).

## DISCUSSION

*Salmonella* spp. is found in different food matrices. But there is limited data about *Salmonella* contamination in garlic. Bennet et al. [6] reported epidemic importance of fresh garlic as a vehicle of *Salmonella* infections. The authors reported difficulties in isolation of *Salmonella* from garlic. Garlic, through the action of the compound diallyl thiosulphinate (Allicin), possesses antimicrobial properties [22] and the routine *Salmonella* detection requires prior neutralization of these inhibitory substances [23].

In our study, we analyzed 5 jars of garlic paste but we could isolate *Salmonella* from only one jar. This supports the data represented above. Ma et al. [5] reported decrease in *S. Typhimurium* numbers in salsa prepared with fresh garlic. It is reported by Ankri and Mirelman, [3] Alliin quickly converts to Allicin during the smashing process of garlic cloves which is reported to have inhibitoric effect on *Salmonella* spp.

This study designed to determine acid resistance, survival of *S. Typhimurium* isolated from garlic paste. STG was stable at pH 4.0 which was the opposite for STA. Food borne pathogens usually exposed to weak organic acids (lactic acid, acetic acid, etc.) and temperature below 20ºC. As a result, ATR is expressed as a response to these parameters and other parameters not indicated above [24, 25]. The paste was prepared with organic acid and stored at room temperature, this may support the acid resistance of the STG. Foster [15] has reported that organic acids are lethal at moderate concentrations in an acid environment. The pH 4.4 condition itself does not affect viability of *S. Typhimurium* over the examination period. Kwon and Ricke [26] reported that inducible ATR has an importance in gastric passage of the bacteria. In a study acid adaptation of *Salmonella* spp. in cheese was shown to be quicker in acetic acid (60 min) then lactic acid (120 min) which supports the data represented here [27, 28]. Gorden and Small [29] reported that *Salmonella* spp. were extremely sensitive to pH 3.0 and lethality occurs rapidly. This also supports the death of STA in a short time and longest survival of STG.

In a study, *L. monocytogenes, E. coli* O157:H7 and *S. Typhimurium* were exposed to five different pH conditions ranging from 4.0 to 6.0 and their acid resistance to pH 3.5 was compared with non-habituated cultures. Significant differences were observed in the pH range at which habituation resulted in an increased acid tolerance (5.0–6.0, 4.5–5.5 and 4.0–5.0 for *L. monocytogenes, E. coli* O157:H7 and *S. Typhimurium*, respectively). Authors reported a quick decrease in *S. Typhimurium* by the second hour of incubation at pH 3.5. The data of our study and this study are similar for *S.Typhimurium* ATCC 14028 which was also used in our study.

There are differences in the ATR systems of the pathogens or the signal required for activation of the physiological mechanisms that protect the pathogens from acid [14, 16, 30].

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