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# Influence of *Citrobacter freundii* Infection on Ion Levels of Model Organism *Galleria mellonella* Larvae

**Serkan SUGEÇTİ** [1]

**Makalenin Alanı: Biyoloji**

| **Makale Bilgileri** | **Öz** |
|---|---|
| **Geliş Tarihi** 07.10.2021<br><br>**Kabul Tarihi** 17.12.2021<br>**Anahtar Kelimeler** Enfeksiyon modeli<br>İyon dengesi<br>*Citrobacter freundii*<br>*Galleria mellonella* | Son yıllarda *Galleria mellonella* larvaları enfeksiyon modeli olarak kullanılmaktadır. Bu çalışmada memelilerde üriner sistem, solunum sistemi, bakteriyemi, kateter enfeksiyonları ve menenjit gibi birçok patofizyolojik etkiye sahip olan *Citrobacter freundii* enfeksiyonunun *G. mellonella* larvalarının hemolenf dokusundaki iyon seviyeleri üzerindeki etkileri araştırıldı. *G. mellonella* larvalarının hemolenf dokusunda kalsiyum seviyeleri uygulamadan 8 saat sonra kontrol grubuna göre önemli ölçüde azaldı. Demir seviyeleri, bakteriyel enfeksiyondan 2 ve 4 saat sonra önemli ölçüde arttı. *Ancak C. freundii* enfeksiyonundan 8 saat sonra kontrol grubuna göre istatistiksel olarak azaldı. Ayrıca tüm *C. freundii* uygulamalarına bağlı olarak *G. mellonella* larvalarının hemolenf dokusunda potasyum ve magnezyum seviyeleri önemli ölçüde azaldı. *C. freundii* enfeksiyonundan 8 saat sonra *G. mellonella* larvalarının hemolenf dokusunda fosfor seviyeleri arttı, ancak istatistiksel olarak bir fark oluşmadı. Bu çalışmadan elde edilen sonuçlar *C. freundii* enfeksiyonunun *G. mellonella* larvalarının iyon dengesini bozduğu göstermiştir. |

| **Article Info** | **Abstract** |
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| **Received** 07.10.2021<br><br>**Accepted** 17.12.2021<br>**Keywords** Infection model<br>Ion balance<br>*Citrobacter freundii*<br>*Galleria mellonella* | In recent years, *Galleria mellonella* larvae have been used as a model of infection. In this study, the effects of *Citrobacter freundii* infection, which has many pathophysiological effects such as urinary system, respiratory system, bacteremia, catheter infections and meningitis in mammals, on ion balance in hemolymph of *G. mellonella* larvae were investigated. Calcium levels in hemolymph of *G. mellonella* larvae significantly decreased 8 hours after the infection when compared to the control group. Iron levels significantly increased 2 and 4 hours after bacterial infection. However, it was statistically reduced 8 hours after the *C. freundii* infection compared to the control group. In addition, potassium and magnesium levels were significantly decreased in hemolymph of *G. mellonella* larvae due to all *C. freundii* infection. Phosphorus levels increased in hemolymph of *G. mellonella* larvae 8 hours after *C. freundii* infection, but there was no statistical difference. In the present study demonstrated that *C. freundii* infection disrupts the ion balance of *G. mellonella* larvae. |

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## 1. Introduction

*Citrobacter* spp. belong to a group of gram-negative bacteria, facultative and anaerobic, within family Enterobacteriaceae. These bacteria are frequently found in soil, food, sewage water, and the intestines of animals and humans. Citrobacter spp. are opportunistic pathogens and the most frequently isolated strain is *Citrobacter freundii*. *C. freundii* can cause various infections such as urinary system, respiratory system, bacteremia, catheter infections and meningitis (Khorasani et al., 2008; Anderson et al., 2018; Liu et al., 2018).

The great wax moth (*Galleria mellonella*) is an important pest insect that causes economic losses in beekeeping belonging to the Lepidopteran order. *G. mellonella* completes its life cycle in four stages as egg, larva, pupa and adult (Kwadha et al., 2017). *G. mellonella* is used as a model organism in the development of alternative new strategies against pesticides with high toxic effects for the environment and non-target organisms used in agricultural areas (Büyükgüzel et al. 2010; Sugeçti et al., 2016; Kastamonuluoğlu et al., 2020; Tunçsoy et al., 2021; Büyükgüzel & Büyükgüzel, 2021). In recent years, invertebrate infection models have been used to determine the effects of pathogenic bacteria on animals. Especially, *G. mellonella* larvae are the most preferred invertebrate infection models (Asai et al., 2020; Moore & Gitai, 2020; Ochoa et al., 2021). *G. mellonella* larvae are an important infection model that is suitable for injection, can be quickly mass production and can survive at 30-37 °C after injection. In addition, the absence of ethical concerns in the use of this model organism is an important factor (Harding et al., 2012; Ames et al., 2017; Sugeçti & Büyükgüzel, 2018; Sugeçti, 2021).

Ions have important roles in the regulation of homeostasis in insects. In addition, this ion supports the functioning of the nervous and muscular system (Southall et al. 2006). In this study, changes in ion amounts such as calcium (Ca), iron (Fe), potassium (K), magnesium (Mg) and phosphorus (PHOS) of model organism *G. mellonella* larvae due to pathogenic bacteria *C. freundii* infection were investigated.

## 2. Materials And Methods

### 2.1. Model insect

The *G. mellonella* larvae used in the experiments were mass-produced under laboratory conditions. Larva, pupa and adult stages of *G. mellonella* were collected from apicultural areas in Zonguldak, Turkey and transferred to glass jars (1000 ml). Newly emerged adults of *G.*

*mellonella* were used for mass-production under laboratory conditions. Adults of *G. mellonella* were transferred to a glass-jar (1000 ml) containing artificial diet. Then, newly hatched larvae were reared artificial diet in incubator (FN 500, Nüve, Ankara, Turkey) set at 28 ± 2 °C and 65 ± 5 % relative humidity, with a photoperiod of 12:12 h (L:D) (Büyükgüzel et al., 2010; Sugeçti et al., 2016). Artificial diet content; 420 g wheat bran, 150 ml filtered honey, 150 ml glycerol (Merck, Darmstadt, Germany), 20 g ground dark old honeycomb and 30 ml pure water (Bronskill, 1961).

### 2.2. *C. freundii* Injection and Collection of Hemolymph

*C. freundii* (ATCC-8090) was obtained from a local company. *C. freundii* dose was prepared in 0.5 McFarland units (approximately $1 \times 10^7$ (CFU/ml) per ml of 0.9% physiological saline. Seventh instar larvae of *G. mellonella* were anesthetized by cooling on ice for approximately 5 minutes. The surface of the larvae was then sterilized with 95% ethanol. Prepared *C. freundii* strains (10 µl) were injected from the *G. mellonella* abdomen of the insect. Hamilton syringe was used for bacteria injection. In the control group, *G. mellonella* larvae were injected with only 10 µl of 0.9% physiological saline.

In the present study, hemolymph from the abdomen of the *G. mellonella* (seventh instar larvae) was obtained by amputating the second pair of prolegs. Samples were collected in Microcentrifuge tubes (1.5 ml) kept in ice. A few phenylthiourea crystals (Sigma Aldrich, Missouri, USA) were placed to prevent melanization. Samples were stored at -80 °C until analysis.

### 2.3. Experimental Design

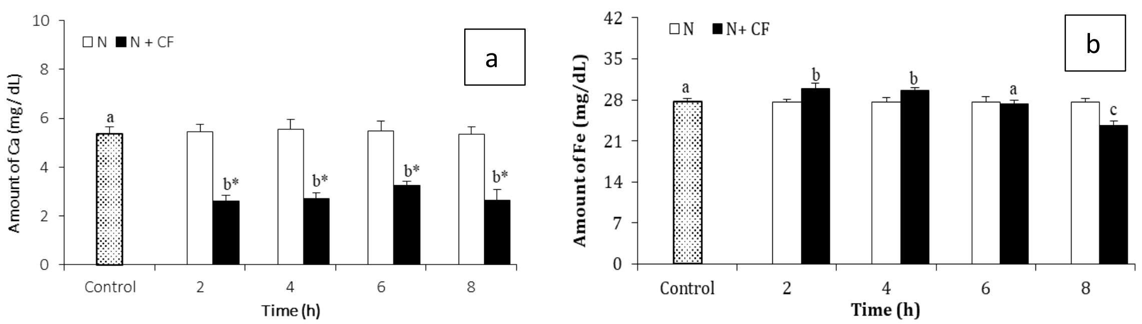
In this study, ion levels were performed with the Roche Hitachi Cobas c501 instrument (Roche, Germany) using appropriate kits. Hemolymph extracts were prepared according to the method of Hyršl et al. (2007). Samples were centrifuged at 10.000 g for 10 min at 4 °C (Nüve FN 800R, Turkey). Calcium (Ca) (Kit no: 45055201), iron (Fe) (Kit no: 44323201), potassium (K) (Kit no: 48497701), magnesium (Mg) (Kit no: 42773901) and phosphorus (P) (Kit no: 44961201). Ion levels assays were performed according to manufacturer’s instructions. All experiments used 20 larvae per treatment. The experiments were repeated four times.

### 2.4. Statical Analyses

In the current study, one-way analysis of variance (ANOVA) test was used to evaluate data on ion levels. Tukey's HSD test was used to determine the importance of the difference between the averages. T-test was used compare to *C. freundii* infection effects at each timepoint to physiological saline treatment at the same timepoint. All analyses were performed in SPSS v.15.0 (SPSS, Chicago, IL, USA). A probability level of 0.05 was used to check the significance of the difference between the averages.

## 3. Results

In this study, Ca levels significantly decreased in hemolymph of *G. mellonella* larvae 8 hours after the *C. freundii* inoculation when compared to the control group. In addition, Ca levels statistically increased 2, 4, 6 and 8 hours after *C. freundii* inoculation when compared to physiological saline treatment for same timepoint (F: 50.547, df: 4, p<0.001) (Fig 1A). Fe levels significantly increased 2 and 4 hours after bacterial infection. However, it was statistically reduced 8 hours after the *C. freundii* infection when compared to the control group (F: 71.193, df: 4, p<0.001) (Fig 1B). Levels of K (F: 246.977, df: 4, p<0.001) and Mg (F: 68.354, df: 4, p<0.001) significantly increased 2, 4, 6 and 8 hours after *C. freundii* infection when compared to the control group and physiological saline treatment for the same timepoint (Fig 1C and Fig 1D). PHOS levels increased in hemolymph of *G. mellonella* larvae 8 hours after *C. freundii* infection, but there was no statistical difference (p>0.05). In addition, 6 hours after *C. freundii* infection, it significantly decreased when compared to the control group (F: 25.295, df: 4, p<0.001) (Fig 1E).



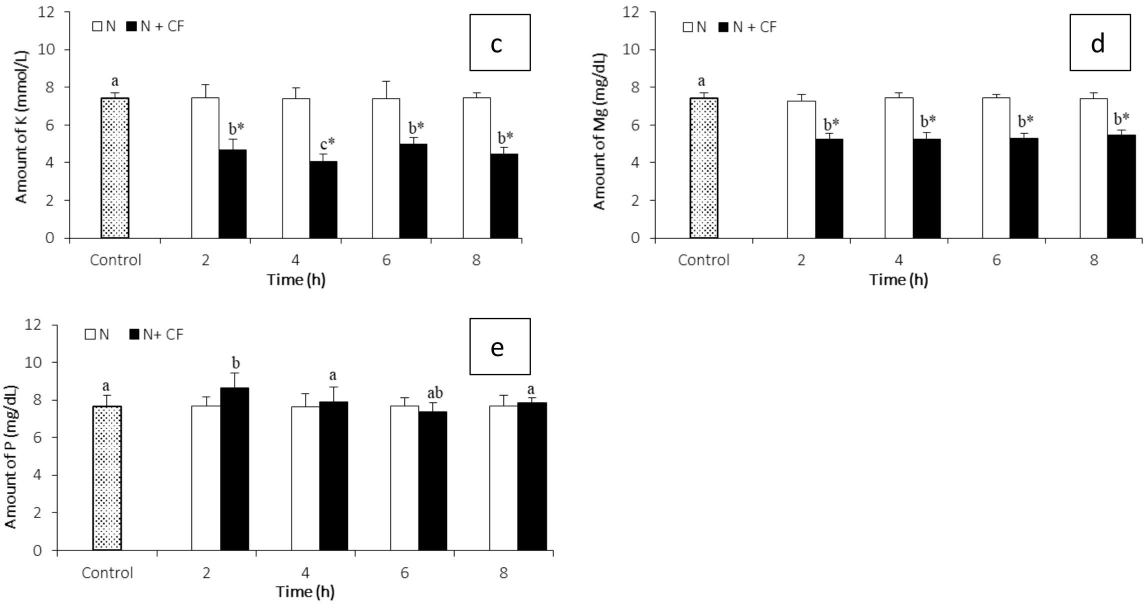



**Fig 1.** Effects of *C. freundii* infection on ion levels of *G. mellonella* (Bars represent the means (± S.D.) of four replicates. Means followed by the same letter are not significantly different ($p>0.05$; * $p< 0.05$: compared with NaCl 0.9% (N) injected for same timepoint of *C. freundii* (CF) infection) (Control: 8 hours after N injection)

## 4. Discussion

The cytotoxic effects of chemical and biological agents on organisms can cause oxidative damage and disruption of ion balance (Sugeçti 2021; Sertçelik et al., 2021; Akbaba et al., 2021). In recent years, model insect *G. mellonella* has been used to investigate the deterioration of ion balance, homeostasis and oxidative stress due to infections (Asai et al., 2020; Sugeçti, 2021). In the current study, it was provided information about the effects of *C. freundii* infection on ion levels in *G. mellonella* larvae. The results of this study showed that ion levels were altered in the hemolymph of *G. mellonella* larvae due to *C. freundii* infection.

Ions have important physiological roles in living organisms, including insects (Aci et al., 2020; Sugeçti, 2021). Ions have physiological effects such as osmoregulation, homeostasis, and contribution to oxidative stress in insects. Calcium acts as a second messenger in all cells. In addition, this ion supports the functioning of the nervous system and muscular system (Southall et al., 2006). In this study, the amount of calcium significantly decreased 8 hours after *C. freundii* infection. The reason for the decrease in the amount of calcium may be as a result of cell damage due to infection. In another study, it was reported that there were changes in free calcium and hydrogen ions in the hemocytes of *Bombyx mori, Exolontha* *serrulata* and *Spodoptera litura* SL-1 cell line due to the toxic effects of destruxins. This study reported that hemocytes to destruxins tend to rapidly increase and then decrease calcium levels (Chen et al., 2014). Fe, which is an important transition metal, causes an increase in reactive oxygen derivatives and oxidative stress in organisms (Gaete et al., 2017). Hydrogen peroxide ($H_2O_2$), which causes oxidative stress, is the result of Fenton reactions and the interaction of Cu (II) and Fe (II) ions with hydrogen. In this study, although the amount of Fe increased 2 and 4 hours after *C. freundii* infection, it decreased 8 hours after infection. When Cu and Fe bind to proteins in organism, these ions become less susceptible to participating in Fenton reactions. Therefore, non-enzymatic antioxidants such as albumin can bind metals and protect the organism from oxidative stress (Roche et al., 2008). The decrease in the amount of Fe ion in the hemolymph of *G. mellonella* larvae 8 hours after infection may be a result of antioxidant defense. Additional experiments are needed to support this hypothesis. In the present study, K and Mg ions were significantly decreased in the hemolymph of *G. mellonella* larvae due to *C. freundii* infection. Magnesium plays important role in the physiological function and intermediary metabolism of insects (Clark et al., 1958). K plays a key role in important physiological functions, including maintenance of membrane potentials and cell volume, acid/base balance, and nerve impulse transmission [Dunham, 2004; Marshall & Grosell, 2006) In another study, Sowers et al. (2006) reported that K, Ca and Na levels in the *Litopenaeus vannamei* exposed to artificial sea salt or mixed ion solution were adversely affected by salt stress. Another study was reported that Na and K levels significantly increased in *G. mellonella* larvae exposed to dichlorvos (8 µg/100 g diet). In the study was reported that there were significant changes in ion levels and impaired homeostasis in *G. mellonella* larvae due to dichlorvos (Kayış et al., 2015). In this study, the amount of P ion increased significantly 2 hours after infection. In addition, although the amount of P ion increased 8 hours after infection, there was no statistical difference. Sugeçti (2021) reported that 8 hours after *Klebsiella pneumoniae* infection, the amount of P ion increased, while the amount of Mg, K and Ca ions significantly decreased. These results show that pathogenic bacteria disrupt the ion balance in insects.

In conclusion, this study showed that *C. freundii* infection disrupts the ion balance and homeostasis of *G. mellonella* larvae. In addition, *G. mellonella* can be used as a model organism in determining the effects of infections on the ion balance.

**Conflict of interest:** The authors declare no conflict of interest.

**Author Contributions:** SS analyzed the ion levels. SS was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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