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Original article (Orijinal araştırma)

Response of *Rattus norvegicus* (Berkenhout, 1769) (Rodentia: Muridae) to entomopathogenic bacteria infected insect cadavers[1]

Rattus norvegicus (Berkenhout, 1769) (Rodentia: Muridae)'un entomopatojen bakteriler ile enfekte böcek kadavralarına tepkisinin belirlenmesi

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Abstract

Xenorhabdus Thomas & Poinar (Enterobacterales: Morganellaceae) and *Photorhabdus* Thomas & Poinar (Enterobacterales: Morganellaceae) bacteria are mutualistically associated with *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae) and *Heterorhabditis* Poinar, 1976 (Rhabditida: Heterorhabditidae) nematodes, respectively, and are known to produce several secondary metabolites that protect nematode-killed insects from different competitors. One of these compounds called "the scavenger deterrent factor" (SDF) is known to deter different arthropod, bird, and fish species from feeding on insects killed by *Xenorhabdus* or *Photorhabdus* bacteria. The effects of SDF from five different *Xenorhabdus* and one *Photorhabdus* species against the Norway rat, *Rattus norvegicus* (Berkenhout, 1769) (Rodentia: Muridae) were investigated using either a one-choice or two-choice experimental design during 2019-2020 in Aydın Adnan Menderes University. Rats were given four-day-old bacteria-killed *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) larvae and feeding behavior was observed and recorded. The results demonstrate that the Norway rat is deterred from feeding on insects killed by certain *Xenorhabdus* and *Photorhabdus* species and it is likely due to the distastefulness of these cadavers. Ecologically, the data suggest that insects killed by the entomopathogenic nematode/bacterium complex in nature may be protected from attack from insectivorous mammals, especially those that feed on soil-dwelling insects.

Keywords: *Photorhabdus*, scavenger deterrent factor, *Xenorhabdus*

Öz

Xenorhabdus Thomas & Poinar (Enterobacteriales: Morganellaceae) ve *Photorhabdus* Thomas & Poinar (Enterobacteriales: Morganellaceae) cinslerine ait bakteriler sırasıyla *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae) ve *Heterorhabditis* Poinar, 1976 (Rhabditida: Heterorhabditidae) cinslerine ait enotomopatojen nematodlarla mutualistik ilişki içerisindedirler. Bu bakterilerin nematodla enfekte kadavraları rekabetçi organizmalardan korumak amacıyla pek çok sekonder metabolit ürettiği bilinmektedir. Bu sekonder metabolitlerden bir tanesi olan yağmacı uzaklaştırıcı faktörün farklı eklembacaklı, kuş ve balık türlerine karşı uzaklaştırıcı etki gösterdiği bilinmektedir. 2019-2020 yılları arasında Aydın Adnan Menderes Üniversitesi'nde yürütülen bu çalışmada farklı *Xenorhabdus* ve *Photorhabdus* türleri ile enfekte kadavraların *Rattus norvegicus* (Berkenhout, 1769) (Rodentia: Muridae)'a karşı uzaklaştırıcı etkisi tek ya da ikili besin tercihi deneyleri ile test edilmiştir. Ratlara bakteriler ile enfekte edilmiş 4 günlük enfekte *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) kadavraları verilmiş ve ratların beslenme davranışları gözlemlenerek kaydedilmiştir. Yapılan çalışma sonucunda bazı *Xenorhabdus* ve *Photorhabdus* türleri ile enfekte kadavraların ratlara karşı uzaklaştırıcı etki gösterdiği belirlenmiştir. Bu etkinin büyük olasılıkla larvalarda oluşan kötü tattan kaynaklandığı düşünülmektedir. Ekolojik olarak, veriler entomopatojen nematod/bakteri kompleksinin doğada böceklerle beslenen memelilerin saldırılarına karşı korunabileceğini göstermiştir.

Anahtar sözcükler: *Photorhabdus*, yağmacı uzaklaştırıcı faktör, *Xenorhabdus*

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Introduction

Entomoptahogenic bacteria in the genera *Xenorhabdus* Thomas & Poinar, 1979 (Enterobacterales: Morganellaceae) and *Photorhabdus* Thomas & Poinar, 1979 (Enterobacterales: Morganellaceae) are mutualistically asssociated with entomopathogenic nematodes (EPNs) in the genus *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae) and *Heterorhabditis* Poinar, 1976 (Rhabditida: Heterorhabditidae), respectively (Boemare, 2002; Gülcü et al., 2017). In their life cycle, the only free-living stage of these nematodes is the third-stage juvenile called the “infective juvenile” (IJ). The IJ carries bacterial cells in its intestine. When the IJs locate an insect host, they enter it through natural openings (mouth, anus, spiracles) and then penetrate into the insect hemocoel and release their mutualistic bacterium (Gaugler & Kaya, 1990). The bacterium multiplies and releases numerous toxins and enzymes which kill the host within 48 h (Bode, 2009). The IJs initiate their development and the nematodes mature by feeding on both the insect tissues and the multiplying mutualistic bacterium. When the food sources in the cadaver are depleted, the second stage nematodes reacquire their mutualistic bacterial cells and develop into the IJ stage. These new IJs exit the cadaver and enter the soil to search for new hosts (Hazır et al., 2003). The nematode’s developmental time depends on a number of factors such as host size, EPN species, and environmental factors (i.e., temperature and moisture). For example, a given EPN species may have 1-3 generations depending on its host size. Generally, the developmental time from the IJs entering the host until the new generation of IJs exits the insect cadavers usually takes 7-20 days at 20°C to 25°C. During this period, it is crucial that the host cadaver remains intact because the developing EPNs in the cadaver require the intact host for food and protection from the environmental soil conditions (e.g., desiccation, excessive moisture, other microorganisms) (Kaya & Stock, 1997). In addition, the cadavers with the developing nematodes are at risk of being consumed by different foraging omnivores and scavengers before IJ emergence is completed. Thus, *Xenorhabdus* spp. and *Photorhabdus* spp. not only help the EPNs by killing their insect hosts and serving as a food resource for them but also by protecting the cadavers from invasion by opportunistic bacterial and fungal competitors in addition to opportunistic omnivores and scavengers by producing one or more secondary deterrent metabolites (Çimen et al., 2021).

In terms of scavenging arthropods, numerous studies have shown that certain ant species did not feed on EPN-killed insects (Baur et al., 1998; Kaya et al., 1998; Zhou et al., 2002; Gülcü et al., 2018). Interestingly, these ants tended to consume less insect cadavers containing the heterorhabditid/*Photorhabdus* complex compared to cadavers containing the steinernematid/*Xenorhabdus* complex. This avoidance behavior was linked to the presence of a compound(s) produced by the mutualistic bacteria and was called the ‘ant deterrent factor(s)’ (ADF) (Zhou et al., 2002). This compound(s) was renamed as “scavenger deterrent factor” (SDF) as it also deters other scavengers such as wasps and crickets from feeding on cadavers with the mutualistic bacteria (Gülcü et al., 2012). Subsequently, different scavenging and omnivorous arthropod species (i.e., earwigs, cockroaches, beetles, and collembolans) have been reported to be deterred from feeding on insects killed by the EPN/bacterium complex (Uluğ et al., 2014; Mertz et al., 2015; Jones et al., 2016). Besides invertebrate animals, two avian species, *Erithacus rubecula* (L., 1758) (Passeriformes: Muscicapidae) and *Parus major* (L., 1758) (Passeriformes: Paridae) (Fenton et al., 2011; Jones et al., 2017) and two cyprinid fish species *Devario aequipinnatus* (McClelland, 1839) (Cypriniformes: Cyprinidae) and *Alburnoides bipunctatus* (Bloch, 1782) (Cypriniformes: Cyprinidae) (Raja et al., 2017) have also been deterred from feeding on insects killed by the EPN/bacterium complex or by the mutualistic bacterium alone. The objective of this study was to determine if SDF produced by different *Xenorhabdus* and *Photorhabdus* species will have a similar deterrent effect on mammal omnivores/scavengers as it did on birds and fishes. Accordingly, the feeding behavior of the Norway rat, *Rattus norvegicus* (Berkenhout, 1769) (Rodentia: Muridae) exposed to insect cadavers containing *Xenorhabdus* and *Photorhabdus* was tested.

Materials and Methods

Rats

Female *R. norvegicus* (Sprague-Dawley strain) were obtained from Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Laboratory Animals Production and Research Center and rats at ca. 6 months of age were used. These rats were housed in Eurostandard Type IV cages (61X43.5X21.5 cm) with wood shavings as bedding and were given ad libitum access to standard laboratory fodder and water and maintained at 12 h light/12 h dark and 24°C (Gomez et al., 2004).

Bacteria

The *Xenorhabdus* and *Photorhabdus* species were obtained from Helge B. Bode (Max Planck Institute, Marburg, Germany) and their associated EPN species are given in Table 1. They were kept in 20% glycerol at −80°C until they were subcultured and used in the experiments (Hazır et al., 2016).

Table 1. Species of *Xenorhabdus* and *Photorhabdus* used in experiments and their associated entomopathogenic nematode species

Bacteria Species	Associated Entomopathogenic Nematode Species
Xenorhabdus nematophila ATCC 19061 Thomas and Poinar, 1979 (Enterobacterales: Morganellaceae)	*Steinernema carpocapsae* (Weiser, 1955) (Rhabditida: Steinernematidae)
Xenorhabdus cabanillasii JM26-1 Tailliez et al., 2006 (Enterobacterales: Morganellaceae)	*Steinernema riobrave* Cabanillas, Poinar & Raulston, 1994 (Rhabditida: Steinernematidae)
Xenorhabdus kozodoii DSMZ 17907Tailliez et al., 2006 (Enterobacterales: Morganellaceae)	*Steinernema arenarium* (Artyukhovsky, 1967) (Rhabditida: Steinernematidae)
Xenorhabdus ehlersii DSMZ 16337 Lengyel et al., 2005 (Enterobacterales: Morganellaceae)	*Steinernema serratum* Shen & Wang, 1992 (Rhabditida: Steinernematidae)
Xenorhabdus ishibashii DSMZ 22670 Kuwata et al., 2013 (Enterobacterales: Morganellaceae)	*Steinernema aciari* Qiu et al., 2005 (Rhabditida: Steinernematidae)
Photorhabdus kayaii DSMZ 1519Tailliez et al., 2006 (Enterobacterales: Morganellaceae)	*Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae)

Each bacterium from the stock cultures was inoculated onto Luria-Bertani (LB) agar (Merck, Darmstadt-Germany) and incubated at 30°C for 24 h. Then, a loopful of each bacterial species was inoculated into their own flasks containing 10 ml LB broth and incubated at 30°C to obtain an overnight culture. *Bacillus thuringiensis* subsp. *kurstaki* Bulla et al. 1979 (Bacillales: Bacillaceae) (Btk) (Rebound Bioinsecticide® WP, Hektaş), a biological control agent for lepidopterous insect pests, was used as an entomopathogenic bacterial control to compare the effects of *Xenorhabdus* and *Photorhabdus* on the rats.

Obtaining bacteria-killed insects

Each overnight *Xenorhabdus* and *Photorhabdus* bacterial culture was centrifuged at 10000 rpm for 2 minutes. After removing the supernatant, each bacterial pellet was suspended in sterile 0.9% phosphate buffered saline (PBS) solution. A spectrophotometer (Shimadzu UV1280) was used to adjust the optical densities (OD) of each bacterial suspension to OD_{600}=1 (Çimen et al., 2021). A last instar larva of the wax moth *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) was injected with 10 µl of a given bacterial suspension using a 1 ml sterile syringe. The bacterial suspension was injected into the larva through the second or third proleg. All infected larvae were kept at room temperature (23-24°C) for 4 days before the dead insects were use in the experiments.

A Btk commercial formulation was suspended in distilled water at the rate of 1.5 g/L and mixed into the *G. mellonella* diet (Han & Ehlers, 2000) to obtain the Btk-killed larvae. Last instar larvae were added to the treated diet, kept at room temperature, and the 4-day-old Btk-killed larvae were removed from the diet and used in the experiment. Healthy last instar larvae were placed at -20°C for 4 h and these freeze-killed larvae were used as negative controls. Freeze-killed larvae were kept at room temperature for at least 1 h before the experiments.

Experimental design

The randomly chosen female rats were individually transferred into 48X26.5X21 cm cages and starved for 12 h before the experiments. In the one-choice experiments, only one larva (4-day-old larva killed by *Xenorhabdus* or *Photorhabdus* or Btk-infected or freeze-killed larva) was given to a rat. In the two-choice experiments, each rat was introduced simultaneously to 4-day-old larva killed by *Xenorhabdus* or *Photorhabdus* and Btk-infected or freeze-killed larva (Aydın Adnan Menderes University Ethics Committee Approval Number: 64583101/2019/026) (Table 2). *Galleria mellonella* larvae were not introduced to the rats prior to experiments. Each rat was given 10 minutes to interact with larvae and replaced with another rat if not. The rat's response and consumption of the insect cadaver were taped. The insect cadavers were recorded as "consumed" (if they were entirely consumed) or "not consumed" (if the rats took a bite but did not continue feeding on larvae or no bites). Each set of experiments had 5 replicates and conducted 3 times on different dates. For each replicate, the rats were only used once. All the experiments were conducted at Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Laboratory Animals Production and Research Center.

Table 2. Experimental design for two-choice tests with the rat with five different *Xenorhabdus* and a *Photorhabdus* species-killed *Galleria mellonella* larvae and control groups (freeze-killed or *Bacillus thuringiensis kurstaki* (Btk)-killed larvae)*

Experiment Number	Two choice tests	
1	*X. nematophila*-killed	Freeze-killed
2	*X. nematophila*-killed	Btk-killed
3	*X. cabanillasii*-killed	Freeze-killed
4	*X. cabanillasii*-killed	Btk-killed
5	*X. kozodoii*-killed	Freeze-killed
6	*X. kozodoii*-killed	Btk-killed
7	*X.ishibashii*-killed	Freeze-killed
8	*X.ishibashii*-killed	Btk-killed
9	*X.ehlersii*-killed	Freeze-killed
10	*X.ehlersii*-killed	Btk-killed
11	*P.kayaii*-killed	Freeze-killed
12	*P.kayaii*-killed	Btk-killed

* The *Xenorhabdus* and *Photorhabdus*-killed larvae were injected with 10 µl of the respective bacterial species and kept at room temperature (23-24°C) for 4 days before exposed to the rat. The Btk-killed larvae were fed food treated with Btk and collected 4 days later. The freeze-killed larvae were held at -20ºC for 4 h and these freeze-killed larvae were kept at room temperature for at least 1 h before the experiments.

Statistical Analysis

The feeding behavior of the rats (consumed vs non-consumed) was analyzed with Chi Square Test of Independence to determine the role of scavenger deterrent factor ($\alpha = 0.05$). Chi Square Test was used to analyze the rat response in the two-choice experiments. (SPSS 22.0 IBM Corp., Chicago, IL, US).

Results and Discussion

In the one-choice experiments, each rat fully consumed freeze-killed, Btk-killed, *Xenorhabdus kozodoii*-, *Xenorhabdus ishibashii*- or *Xenorhabdus ehlersii*-killed larva but took a bite from *Xenorhabdus nematophila*-, *Xenorhabdus cabanillasii*- or *Photorhabdus kayaii*-killed larva and then stopped feeding on each of them (Table 3) (Supplementary videos 1 and 2). The statistical analysis showed that there was a significant difference in the response of the rats to different bacteria-infected larvae and control groups (X^2 = 40, p<.001, α = 0.05). In the two-choice experiments, when the rats were offered *X. nematophila*-, *X. cabanillasii*- or *P. kayaii*- larva and freeze-killed or Btk-killed larvae, they only consumed the freeze-killed and Btk-killed larvae (Figure 1, 2). On the other hand, the rats consumed all the 4-day-old *X. kozodoii*-, *X. ishibashii*- and *X. ehlersii*-killed larvae as well as the freeze-killed or Btk-killed larvae in the two-choice tests. There was a significant difference in the response of the rats to the different treatments offered in the two-choice experiments (X^2 = 36, p<.001, α = 0.05).

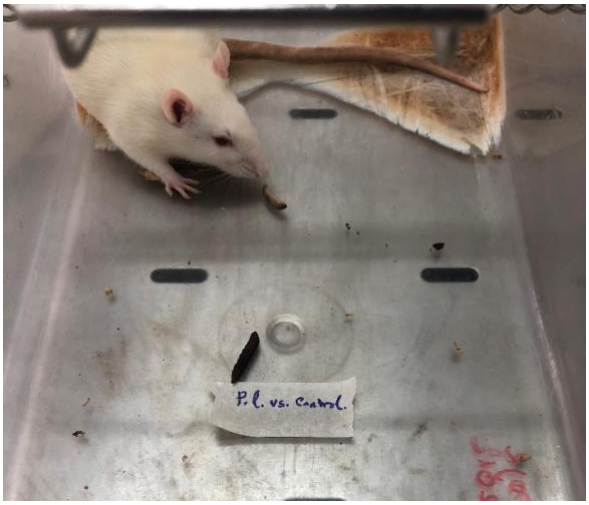

Figure 1. Rat feeding on a freeze-killed larva (control) in the two-choice experiments with *Photorhabdus kayaii*-killed larva.

Table 3. Consumption of *Xenorhabdus*- and *Photorhabdus*-killed *Galleria mellonella* larvae by *Rattus norvegicus* in one-choice experiments

Treatments	Consumed	Not Consumed
X. nematophila-killed		+
X. cabanillasii-killed		+
X. kozodoii-killed	+	
X. ishibashii-killed	+	
X. ehlersii-killed	+	
P. kayaii-killed		+
Freeze-killed	+	
Btk-killed	+	

The response of an omnivore mammalian species to SDF produced by *Xenorhabdus* spp. and *Photorhabdus* spp. was demonstrated for the first time with this study. On several occasions, the rats were observed to approach and attempt to feed on *X. nematophila*-, *X. cabanillasii*- or *P. kayaii*-killed larva, but upon taking a bite, the rats immediately rejected these larvae, whereas both freeze-killed and Btk-killed control groups and other tested *Xenorhabdus*-killed larvae were consumed. After taking a bite from the *X. nematophila*-, *X. cabanillasii*- or *P. kayaii*-killed larva, it was observed that the rats tried to clean their mouths (Supplementary video 2) which appeared to be a response to a distasteful substance.

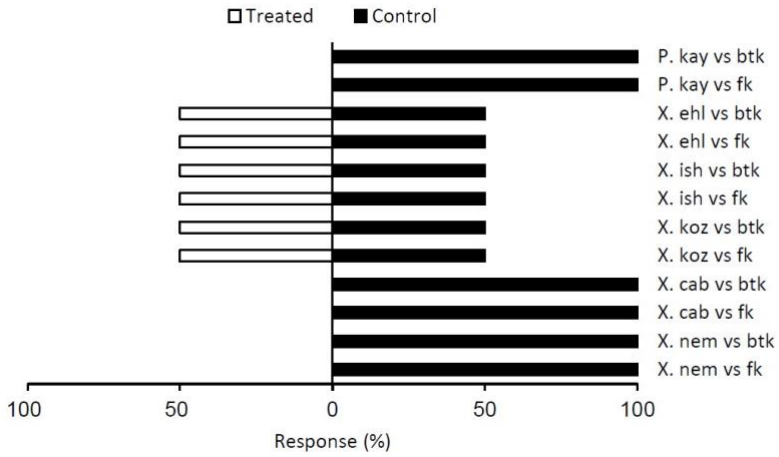

Figure 2. Response of rats in two choice experiments (P.kay=*P.kayaii*; X.ehl=*X.ehlersii*; X.ish=*X.ishibashii*; X.koz=*X.kozodoii*; X.cab=*X. cabanillasii*; X.nem=*X. nematophila*; btk= *Bacillus thuringiensis*-killed; fk= freeze-killed).

Multiple defense mechanisms or the combination of different mechanisms such as color and odor, increases the chances of survival of the EPN-bacterium complex in the insect cadavers. Previous studies with avian predators have suggested that color, especially with *Photorhabdus*-killed insects, and odor can both play a role in the protection of the *Heterorhabditis/Photorhabdus* killed insects at different stages of the infection against scavenger attacks (Fenton et al., 2011; Jones et al., 2017). *Photorhabdus*-killed larvae generally turn red 2 to 4 days after death, bioluminesce and produce an odor (Ffrench-Constant & Bowen, 2000). The color change in the dead larvae containing the *Photorhabdus* is most likely an indication of the presence of a distasteful chemical. Avian foragers usually rely on visual cues when they encounter a new diet. After their brief aversion (neophobia), in some cases, they completely reject this diet, which is called dietary conservatism (Marples et al., 1998, 1999). This behavior recently has also been shown to be present in some individuals of different fish species (Thomas et al., 2010; Richards, 2014).

Depending on the species, *Xenorhabdus*-killed insects generally turn ochre, brown or black (Hazır et al., 2022). There has been no study where color or odor plays a role in feeding deterrence of *Xenorhabdus*-killed insects by omnivores and scavengers. In this study, *Photorhabdus*- and *Xenorhabdus*-killed larvae were attacked almost every time (data not shown-personnel observation) indicating that the color change in *Xenorhabdus*- or *Photorhabdus*-killed larvae did not play a role in deterring the rats from feeding on the cadaver. In this experimental design, the control groups and bacteria-killed larvae were placed closely together in the cages, so it is highly unlikely that odor itself had a significant effect on selection by the rat to feed on a given larva. Based on the feeding behavior of rats, the main reason why the rats rejected the *P. kayaii*-, *X. nematophila*-, and *X. cabanillasii*-killed larvae appears to be due to the distastefulness of the cadavers (Supplementary video 1). The rats reject these cadavers only after taking one or two bites and exhibited cleaning behaviors of attempting to get rid of cadaver material from their mouths (Supplementary video 2).

Interestingly, not all EPN-associated bacteria had SDF activity on the rats. It is known that different *Xenorhabdus* and *Photorhabdus* species or even strains produce different secondary metabolites (Bode 2009), and these metabolites probably act differently against different scavengers. Recently, two volatile compounds (hexadecanal and 2-heptadecanone) isolated from *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae)-infected larvae were found to be highly deterrent to the ant species *Lasius niger* (L., 1758) (Hymenoptera: Formicidae) (Jaffuel et al., 2021). These two compounds were highly active when the ants were very close to the bait area (“touching”).

EPNs sold as biological control agents are usually mass produced in vivo or in vitro and the IJs are applied in an aqueous suspension for the control of insect pests. However, recent studies have showed that they can be also successfully applied as “infected cadavers” (i.e., larvae killed by an EPN species and the IJs allowed to remain in the cadaver) with a superior infectivity, persistence and pest control (Gulzar et al., 2020; Perez et al., 2003; Shapiro-Ilan et al., 1999, 2003). “Infected cadavers” protect the IJs from desiccation and UV inactivation. Ecologically, soil insects naturally infected with the EPN/bacterium complex are important in allowing the IJs to persist the soil. However, consumptive and destructive actions of ground foraging omnivores and scavengers can have a top-down impact on EPN populations in soil and can reduce the success of biological control applications as well as natural biological control of pest insects. It will be interesting to determine whether insectivorous mammals such as shrews, moles, raccoons, skunks, etc. that feed on soil insects are adversely affected by SDF and assess their impact on natural biological control of insect pests. In addition, future studies should endeavor to identify whether there is one or a complex of deterrent compound(s) that affects the feeding behavior of scavengers and omnivores.

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