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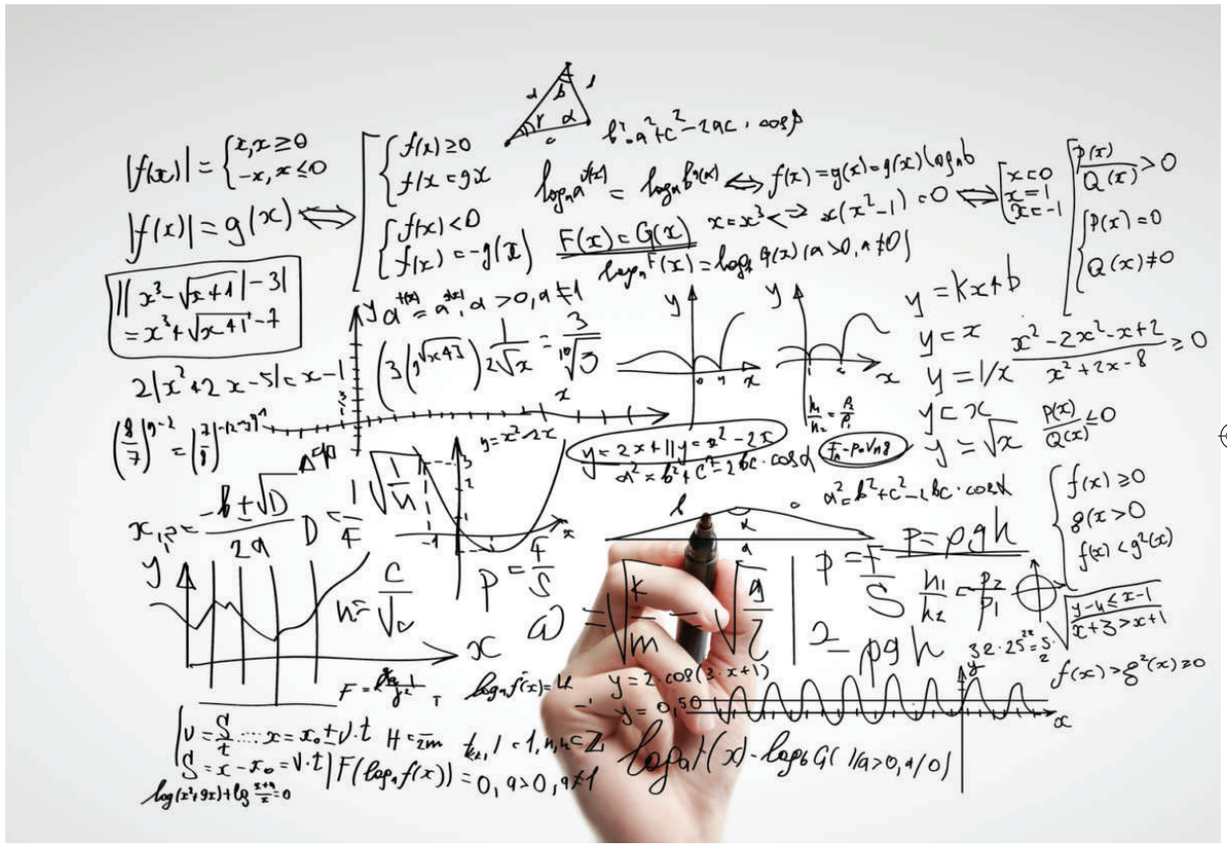
Research and Theory



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Prof. Dr. Rıdvan Karapınar

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Research and Theory

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Prof. Dr. Rıdvan Karapınar



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PREFACE

Research in mathematics and natural sciences covers various fields and forms the foundations of engineering. This book provides a concise, informative and current overview of some topics in the field of mathematics and natural sciences. In particular, the link between theory, research and practice is well developed in the individual chapters. In this book, the authors share their results with the scientific community. The selected articles have been reviewed and approved for publication by referees. It is hoped that this book will be of interest to academics and researchers in the fields of mathematics and natural sciences.

Editor

CHAPTER 2

FIRST RECORD OF THE TWO SNAKES MICROBIOTA FROM ÇANAKKALE (TURKEY)

Nurcihan Hacıoğlu Doğru[1] & Çiğdem Gül[2] & Murat Tosunoğlu[3]

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1. Introduction

Conservation of natural life and ecological balance includes protection of wild animals, which are important members of our ecosystem, as well as human health. Many harmful chemicals and anthropogenic wastes that have emerged in parallel with the development of agriculture and industry in recent years cause many negative effects on nature and therefore wildlife. These negative effects can be traced through the microbiota of the animal species and their various resistance factors. All animals harbor complex relationships with their microbial flora living in their gastrointestinal tract. This intestinal microbiota can also influence the ecology and evolutionary processes of their hosts by affecting their behavioral, immune system, nutritional and reproductive isolations. Large-scale microbial diversity inventory studies have been performed mostly in mammals, birds, fish and lower-scale amphibians, but there are limited number of studies in reptiles. Reptiles are represented by about 10,000 species; revealing that the intestinal microbial ecology of these groups is essential to begin to understand the generalized patterns among vertebrate groups (Kohl *et al*., 2017). Snakes are more than 2900 species in the world and various snake species have gained as much popularity as pets in the past few years (Mitchell, 2009). However, although this growing popularity is positive for veterinarians and herpetoculturalists who are concerned with the sale and management of these animals in pet shops, it has increased concerns among public health officials due to the zoonotic potential (eg, *Salmonella*) associated with these animals.

Eryx jaculus (Javelin Sand Boa) belongs to Boidae family is distributed in Southern Balkans, Aegean islands, Transcaucasia, Eastern Caucasus, North of Africa

and Middle East (Sindaco *et al*., 2013). Except for the Black Sea coastal region, it is seen all over the Turkey and prefers arid, sandy and low vegetation habitats and this species is listed as Least Concern (Baran *et al*., 2012). *Montivipera xanthina* (Ottoman Viper) belongs to Viperidae family and is a venomous snake species. This species is distributed in Turkey and Greece. Although it is mostly found in stony rocky areas, it can come to settlements and agricultural areas for feeding purposes (Tosunoğlu *et al.,* 2017). *M. xanthina* is listed in the Least Concern category due to its wide distribution and large population (Mebert *et al*., 2020).

Snakes can be potential reservoirs of infection for other organisms, especially humans and wildlife animals, due to asymptomatically the pathogenic microorganisms they contain. Determination of microbial flora isolated from these animals and antibiotic heavy metal resistance and enzyme production capacities of their flora are important in terms of understanding the extent of anthropogenic and industrial pollution and the effects of potential pathogens to which these animals are exposed (Corrento *et al*., 2004). Most studies in this area have focused particularly on zoonotic infections in commercially available snake species (Schröter *et al*., 2004). However, there is no data related to bacterial flora of freeliving snake -*E. jaculus* and *M. xanthina*- species. This is the first comprehensive report on the microbiological parameters of *E. jaculus* and *M. xanthina* snake species from Çanakkale (Turkey).

The aim of this study is to identify and characterize of Gram negative culturable bacteria of two wildlife snake and to put forth the antibiotic and heavy metal susceptibility and enzymatic activity of the isolates, thus obtained data serve epidemiological studies and conservation programs in *E. jaculus* and *M. xanthina* species.

2. Materials and Methods

2.1. Snake samples

Each individual belonging to *E. jaculus* (n=1) and *M. xanthina* (n=1) snake species caught in the field studies carried out in Çanakkale Center in 2015-2016 within the scope of "Biodiversity Inventory and Monitoring Project of Çanakkale Province" of the Ministry of Agriculture and Forestry. Cloaca, skin, oral swabs and feces samples of two healthy snakes were taken in the field and then the snakes were released back to the field. Samples were stored at 4 ^{o}C until they were brought to the laboratory for microbial analyzing.

2.2. Bacterial identification assay

Cloacal, skin, oral swabs and feces samples of snakes incubated in buffered peptone water for 24 hr 35-37 ^{o}C and then the spread on different culture media like MacConkey agar (MAC), Thiosulfate citrate bile salts sucrose agar (TCBS), Glutamate starch phenol red agar (GSP), Chromogenic *E. coli* agar (CE) at 25– 30 °C for 24–48 h. Identification of isolates were done according to Murray *et al*., (2011). Microgen Gram negative panel (MID-64) was used for verification tests of isolates.

2.3. Antibiotic susceptibility test

Standard antibiotics of Trimethoprim (TR10 µg/mL), Tobramycin (TB10 µg/mL), Kanamycin (K30 µg/mL), Amoxycillin (AM10 µg/mL), Oxytetracycline (O30 µg/mL), Cephalothin (CH30 µg/mL), Cefmetazole (CMZ30 µg/mL), Gentamicin (G10 µg/mL), Furazolidone (FR50 µg/mL), Erythromycin (E15 µg/mL), Cefoxitin (CN30 µg/mL), Ampicillin (A10 µg/mL), Cefotaxime (CE30 µg/mL) and Chloramphenicol (C30 µg/mL) were used for determined antibiotic susceptibility of isolated bacteria by Kirby–Bauer Disc Diffusion method (Bauer *et al*., 1966; CLSI, 2009). The multiple antibiotic resistances (MAR) index of isolates was calculated as described by Krumperman, (1983).

2.4. Heavy metal resistance analysis

Analytical grades of metal salts ($CdCl_2.2H_2O$, $CuSO_4.5H_2O$, $CrCl_3$, $Pb(NO_3)$ and $MnCl_2.2H_2O$) were used for determinations of the minimal inhibitory concentration (MIC) of each isolates against heavy metals of Cd^{+2}, Cu^{+2}, Cr^{+3}, Pb^{+2}, Mn^{+2} (100 to 3.200 µg/mL). The isolates were considered resistant if the MIC values exceeded that of the *Escherichia coli* K-12 strain, which was used as the control (Hacıoğlu and Tosunoğlu, 2014).

2.5. Determination of enzymatic activities

Standardized enzymatic activity tests (DNAse, hemolysis, protease, lipase and amylase) were used to evaluate the virulence properties of isolated bacteria (Sokol *et al*., 1979; Collins *et al*., 2003).

3. Results

47 Gram negative isolates from two snake species were identified. 24 bacteria were isolated from *E. jaculus* (cloacal: 6, skin: 5; oral: 6, feces: 7) and 23 bacteria were isolated from *M. xanthina* (cloac: 6, skin: 1; oral: 6, feces: 10). The most frequent isolates were *Aeromonas hydrophila* and *Klebsiella oxytoca* (16.66%) for *E. jaculus* and *Pseudomonas aeruginosa* (34.78%) and *Pantoea agglomerans* (21.73%) for *M. xanthina* (Table 1).

Table 1. Microbiota of ***E. jaculus*** and ***M. xanthina*** snake species

Isolate No	Bacterial species	Source	Isolate No	Bacterial species	Source
EJ1	*A. lwoffii*	Feces	**MX1**	*A.hydrophila*	Feces
EJ2	*S. rubidaea*	Feces	**MX2**	*A. hydrophila*	Feces
EJ3	*E. coli*	Feces	**MX3**	*E. coli*	Feces
EJ4	*E. coli*	Feces	**MX4**	*K. oxytoca*	Feces
EJ5	*E. coli*	Feces	**MX5**	*P. agglomerans*	Feces

EJ6	*K. oxytoca*	Feces	**MX6**	*P. agglomerans*	Feces
EJ7	*K. oxytoca*	Feces	**MX7**	*P. agglomerans*	Feces
EJ8	*A. hydrophila*	Skin	**MX8**	*P. agglomerans*	Feces
EJ9	*A. lwoffii*	Skin	**MX9**	*P. agglomerans*	Feces
EJ10	*A.hydrophila*	Oral	**MX10**	*A. hydrophila*	Feces
EJ11	*P. aeruginosa*	Oral	**MX11**	*P. aeruginosa*	Oral
EJ12	*B. pseudomallei*	Cloac	**MX12**	*P. aeruginosa*	Cloac
EJ13	*A. hydrophila*	Cloac	**MX13**	*A. hydrophila*	Cloac
EJ14	*Actinobacilllus sp.*	Skin	**MX14**	*K. oxytoca*	Oral
EJ15	*K. oxytoca*	Skin	**MX15**	*K. oxytoca*	Cloac
EJ16	*S. rubidaea*	Skin	**MX16**	*P. aeruginosa*	Oral
EJ17	*C. sakazakii*	Oral	**MX17**	*P. aeruginosa*	Cloac
EJ18	*P. mirabilis*	Cloac	**MX18**	*P. aeruginosa*	Oral
EJ19	*A. hydrophila*	Cloac	**MX19**	*A.hydrophila*	Cloac
EJ20	*E. aerogenes*	Oral	**MX20**	*P. aeruginosa*	Oral
EJ21	*C. sakazakii*	Cloac	**MX21**	*P. aeruginosa*	Oral
EJ22	*S. putrefaciens*	Oral	**MX22**	*P. aeruginosa*	Cloac
EJ23	*E. aerogenes*	Oral	**MX23**	*S. arizonae*	Skin
EJ24	*K. oxytoca*	Cloac			

EJ: *E. jaculus*; MX: *M. xanthina*

The antibiotic susceptibility test showed that the most effective antibiotics G120 and C30 for the bacterial isolates of *E. jaculus* and *M. xanthina*, respectively. However, *E. jaculus* and *M. xanthina* bacterial isolates showed the highest resistance against CMZ30 and CN30 antibiotics, respectively (Figure 1). The MAR Index of isolates was found *P. aeruginosa* (*M. xanthina*) (0.64) > *S. putrefaciens* (*E. jaculus*) (0.57) > *B. pseudomallei* (*E. jaculus*) (0.5) = *P. mirabilis* (*E. jaculus*) (0.5) (Figure 2).

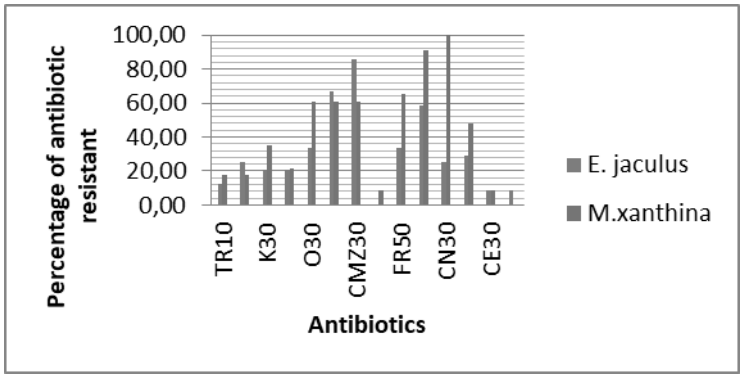

Figure 1. Antibiotic susceptibility profile of isolates

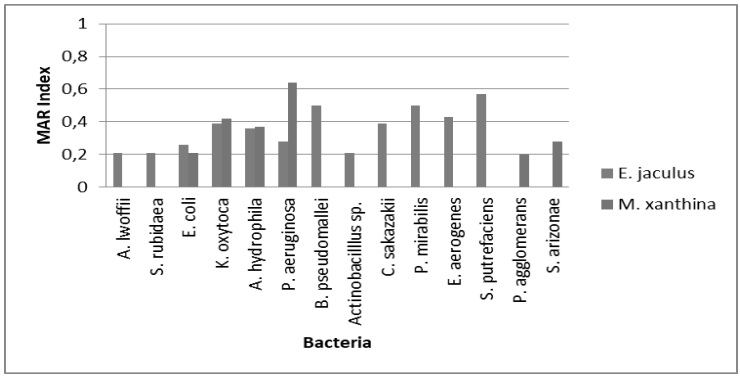

Figure 2. MAR index of isolates from *E. jaculus* and *M. xanthina*

The heavy metal resistance profiles of isolates are shown in Table 2 from *E. jaculus* and *M. xanthina*, respectively; Cd (91.66%) > Pb (87.5%) > Mn (83.33%) > Cu= Cr (66.66%); Cr (100%) > Mn (82.6%) > Pb (78.26%) > Cd (60.86%) > Cu (66.66%).

Table 2. Heavy metal resistance profile of isolates

Heavy metal	*E. jaculus* / *M. xanthina*	Metal concentrations (µg/mL) with number of tolerant isolates					Resistant isolates	
	N=24/23	400	800	1600	3200	>3200	n	%
Cadmium			*					
				13/5	9/9	0/0	22/14	91.66/60.86
Copper		*						
			13/8	2/2	1/1	0/0	16/11	66.66/47.82
Chromium			*					
				10/11	5/11	1/1	16/23	66.66/100
Lead			*					
				18/17	2/1	1/0	21/18	87.5/78.26
Manganese				*				
					15/17	3/2	20/19	83.33/82.60

* MIC values of the *E. coli* K-12

The enzymatic activity findings of isolates obtained from *E. jaculus* and *M. xanthina* snake species against 5 different enzymes used in the study are given in Table 3. DNase, amylase, lipase, haemolysin and protease activity of *E. jaculus* and *M. xanthina* isolates were 25%, 66.66%, 45.83%, 20.83%, 95.83% and 69.56%, 73.91%, 86.95%, 47.82%, 56.52%, respectively. In addition, it was determined that EJ9, EJ16, EJ23 and MX23 isolates carry only amylase enzyme.

Table 3. Extracellular enzyme profile of isolates from snakes

Isolates	Enzyme					Isolates	Enzyme				
	DNase	Haemolysin	Protease	Lipase	Amylase		DNase	Haemolysin	Protease	Lipase	Amylase
EJ1	-	-	+	-	+	**MX1**	+	+	+	-	+
EJ2	-	-	+	-	+	**MX2**	+	+	+	-	+
EJ3	-	+	+	-	+	**MX3**	+	+	+	-	+
EJ4	-	+	-	-	+	**MX4**	+	+	+	-	+
EJ5	-	+	-	+	+	**MX5**	+	-	+	-	+
EJ6	-	+	-	+	+	**MX6**	-	-	+	-	+
EJ7	-	+	-	-	+	**MX7**	-	-	+	-	+
EJ8	-	+	-	-	+	**MX8**	-	-	+	-	-
EJ9	-	-	-	-	+	**MX9**	+	-	+	-	+
EJ10	-	+	-	-	+	**MX10**	+	+	+	+	+
EJ11	-	+	-	-	+	**MX11**	+	+	+	+	-
EJ12	+	-	+	-	+	**MX12**	+	+	+	+	-
EJ13	+	+	+	-	+	**MX13**	+	+	+	+	-
EJ14	-	+	+	-	+	**MX14**	-	+	-	+	-
EJ15	-	+	-	-	+	**MX15**	-	+	-	+	+
EJ16	-	-	-	-	+	**MX16**	+	+	+	+	-
EJ17	-	+	-	-	+	**MX17**	+	+	+	+	-
EJ18	+	+	+	+	+	**MX18**	+	+	+	+	-

EJ19	+	+	+	-	+	**MX19**	+	+	+	+	-
EJ20	-	-	-	+	+	**MX20**	-	+	+	-	+
EJ21	+	+	+	-	+	**MX21**	+	+	+	+	-
EJ22	-	-	+	+	-	**MX22**	+	+	+	-	+
EJ23	-	-	-	-	+	**MX23**	-	-	-	-	+
EJ24	+	+	+	-	+						

EJ: *E. jaculus*; MX: *M. xanthina*

4. Discussion

Canakkale (Turkey), which is an important element of the ecological balance of amphibians, reptiles, etc. animals are very rich in terms of geography. This preliminary study, carried out in two different snake species, is very important in terms of examining the possible threats of the both species in the region. Researching of this topic is so important in terms of public health because of the intense human contact with these two snake species, which can be easily found in nature. There are many researches with different snake species that includes numerous microbial floras, pathogen epidemiology and antibiotic resistance profiles all over the world (Correnti *et al*., 2004; Schröter *et al*., 2004; Kobolkuti *et al*., 2009; Kobolkuti *et al*., 2013; Hacıoğlu and Tosunoglu, 2014; Iqbal *et al*., 2014; Schmidt *et al*., 2014; Hacıoğlu *et al*., 2015; Pawlak *et al*., 2020) and our study confirms their findings that snake species carry Gram negative bacteria.

Many snake species oral and cloaks mixed with especially Gram(-) bacteria. These species are opportunistic pathogens and detection of these species is accepted as an indicator of important clinical conditions (Rosenthal and Mader, 1996; Iqbal *et al*., 2014). In our research, member of Moraxellaceae (n=2), Enterobacteriaceae (n=14), Aeromonadaceae (n=4), Pseudomonadaceae (n=1), Burkholderiaceae (n=1), Neisseriaceae (n=1), Shewanellaceae (n=1) from *E. jaculus* and Enterobacteriaceae (n=10), Aeromonadaceae (n=5), Pseudomonadaceae (n=8) were isolated from *M. xanthina*. The isolated bacterial species diversity is similar to the data in the literature (Aviles *et al*., 2008; Iqbal *et al*., 2014; Schmidt *et al*., 2014; Artavia-Leon *et al*., 2017; Pawlak *et al*., 2020). Although there are some differences between the two species in terms of their natural flora, it can be said that all the isolates are opportunistic pathogens (Artavia-Leon *et al*., 2017; Liu *et al*., 2017). Shek *et al*. (2009) stated that these discrepancies may be caused by habitat diversity, different predation strategies and type of prey. The Gram negative bacteria which were found in *E. jaculus* and *M. xanthina* appear to be the natural microbiota of these snakes. Human who are the natural hosts of these isolated pathogens should be especially careful at contact with these animals. As stated in previous studies about different snake species that immunocompromised people, young children (under 5 years old), the elderly, and pregnant women are at risk of infection and can be augmented by pets as additional vectors that come into contact with free-living snakes (Schmidt *et al*., 2014; Pawlak *et al*., 2020). The most common bacterial species is *A. hydrophila* and is found in the skin, oral and cloacal microbiota of *E. jaculus*; it was previously obtained from the oral cavity, skin and internal organs of snakes such as anacondas, cobras and vipers (Shek *et al.,* 2009; Schmidt *et al*., 2014; Hacıoğlu-Doğru *et al*., 2018). In snakes, *P. aeruginosa* which is also the most common isolate of *M. xanthina*, is considered a normal component of the microflora of oral and intestinal tract as well as an opportunistic pathogen causing diseases in susceptible animals *(*Colinon *et al*., 2010; Foti *et al*., 2013; Iqbal *et al*., 2014). These isolated pathogen bacteria may be also a significant threat in people who share the same habitat or are in close contact with these snake species.

Antibiotic resistance is a growing threat to public health around the world. Undoubtedly, not writing proper prescriptions is one of the biggest reasons and

this is a big problem, but also in agriculture and livestock this resistance deserves more focused study. Moreover, the increasing number of antimicrobial agents in veterinary medicine creates animal's potential habitat of resistant bacteria (Hacıoğlu and Tosunoglu, 2014). In the present research, the high resistance rates of isolates to different antibiotics and high MAR indexes are proof that the habitats of both *E. jaculus* and *M. xanthina* species are contaminated with antibiotic residues. Our findings on high multi resistance incidences, similar to other investigations about different snake species except *E. jaculus* and *M. xanthina* (Colinon *et al*., 2010; Foti *et al*., 2013; Hacıoğlu and Tosunoglu, 2014; Hacıoğlu *et al*., 2015; Artavia-Leon *et al*., 2017; Hacıoğlu-Doğru *et al*., 2018). This is also the first report about frequency of antibiotic resistant of isolated bacteria from these two snake species. Additionally, the tested G120, CE30 and C30 antibiotics worked well against the isolates.

Heavy metal resistance may have been observed in this study as a result of heavy metals contamination of snake species habitats. Cu, Cr and Mn etc. metals have an important place in the biological systems, but it is also known that uninterrupted exposure to high doses causes toxic effects (Nies, 1999). Heavy metals entering the environment in various ways can cause significant changes in the structure and function of microbial communities. Over the past decade, a number of studies have reported that antibiotic-resistant bacteria can emerge in the environment through common or cross-resistance or co-regulation of resistance pathways to metals (McArthur and Tuckfield, 2000; Akinbowlale *et al*., 2007). As the increase in heavy metal and antibiotic resistance to pathogens continues, the ongoing global war against infectious diseases will become more difficult. No studies on heavy metal susceptibility of bacterial isolates obtained from both snake species were found in the literature. However, high resistance to the same heavy metals was found similar to the studies with other reptile species in the region (Hacıoğlu and Tosunoglu, 2014; Hacıoğlu *et al*., 2015; Hacıoğlu-Doğru *et al*., 2018).

Bacterial pathogenicity is highly positively correlated with some virulence factors such as extracellular enzyme production (Soler *et al.,* 2002; Hacıoğlu-Doğru *et al.,* 2018), so some enzymatic activities of the isolates have been. In our study, the high enzymatic activities of the isolates indicate that both snake species naturally spreading in Çanakkale and people in contact with them may be exposed to bacterial flora with high virulence.

5. Conclusion

Our research is the first study to reveal the bacterial flora (also the antibiotic-heavy metal susceptibility and extracellular enzyme profile) of venomous and non-venomous snake species from Çanakkale (Turkey). Our results revealed that the majority of the 14 bacterial species could bring health complications especially after a snake bite. While these data determine the importance of epidemiological surveillance and microbiological monitoring, they once again reveal the need to implement environmental protection programs for free-living snakes.

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