ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine
www.aaem.pl

Elucidating environmental reservoir of antimicrobial resistance – a phenotypic characterization of gut microbiota from aquatic coleoptera in a low-anthropogenic impact zone

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

Orhan F, Bektaş Me, Barış Ö. Elucidating environmental reservoir of antimicrobial resistance – a phenotypic characterization of gut microbiota from aquatic coleoptera in a low-anthropogenic impact zone. Ann Agric Environ Med. doi:10.26444/aaem/210421

Abstract

Introduction and Objective. This study investigated the antibiotic resistance of bacterial isolates obtained from the gut microbiota of certain insects (Coleoptera: Hydrophilidae and Helophoridae), which were collected from aquatic areas in Erzurum Province, Türkiye. This area is characterised by a low level of human impact, thereby providing a unique opportunity to investigate the baseline microbial diversity and ecological roles within relatively pristine aquatic environments.

Materials and Method. The antimicrobial susceptibility of the isolates was assessed using disc diffusion and minimum inhibitory concentration (MIC) methods. The analysis encompassed 30 Gram-negative bacteria belonging to the genera *Aeromonas*, *Acinetobacter*, *Vibrio*, *Pseudomonas*, *Escherichia* and *Yersinia*.

Results. The results indicated that the most resistant bacteria were *Aeromonas*, *Pseudomonas* and *Acinetobacter*, while enteric bacteria demonstrated greater sensitivity. It is noteworthy that nitrofurantoin, a commonly used antibiotic for treating urinary tract infections, exhibited the highest level of resistance among the antibiotics tested by disc diffusion, followed by cephalosporins and penicillins.

Conclusion. The MIC testing with DKGM and NF kits demonstrated high resistance to cephalosporins, sulfonamides, polymyxins and monobactams. Furthermore, two multidrug-resistant (MDR) isolates exhibited resistance to at least two antibiotic classes. These findings underscore the necessity for expanded antimicrobial resistance surveillance beyond clinical settings, extending into environmental samples, and contributing to ongoing research on resistance mechanisms.

Key words

aquatic and edible insects, antimicrobial resistance, gut bacterial community, microbiome

INTRODUCTION

The loss of biodiversity has long been associated with human overpopulation [1]. Rapid changes in the global environment and calorie-rich foods from cattle will reduce the serious threat they face [2]. Edible insects represent an interesting alternative source of protein for human consumption and animal feed [3]. Eating insects could help solve diet-related health problems [4], and a daily increase in the number of beneficial probiotic bacterial species has been associated with this consumption [5]. In most studies dealing with bacterial communities, the gut microbiomes of various insect species have been extensively characterized, and some insects can be considered as reservoirs of antibiotic resistance genes [6]. Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) in the aquatic environment have become an emerging problem of contamination that has implications for human and ecological health [7]. The presence of acquired antimicrobial resistance in bacteria isolated from aquatic coleoptera. Edible insects may be a potential source for the transfer of antimicrobial-resistant microorganisms and their genes to humans.

The transfer of resistance genes from insects to human pathogens can occur through horizontal gene transfer and the indirect spread of antimicrobial resistance [8]. The impact of reducing antimicrobials on the resistance level of ecological systems, especially on the microbiota, is not known [9]. Novel antibiotics have been developed to combat the insensitivity of Gram-positive bacteria; nevertheless, Gram-negative bacteria acquire resistance mechanisms that lead to multidrug-resistance [10]. However, the gut microbiota of insects is still poorly understood. Insects act as vectors that transfer antimicrobial resistance genes to humans through direct contact, contamination of human food and horizontal gene transfer through the consumption of edible rodents and insects [11].

Food-associated insects play a crucial role between animal farms and urban communities with antibiotic resistance traits. For example, some insects have been shown to carry multidrug-resistant clonal strains of bacteria identical to those found in animal faeces. In addition to the horizontal transfer of resistance genes, resistant bacteria are also transferred

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Received: 04.07.2025; accepted: 03.09.2025; first published: 22.09.2025

from insects to new hosts [12]. There is evidence that the microbiota associated with insects plays a crucial role in the degradation of natural and synthetic organic substances. Insects and bacteria have a long history of co-existence. Moreover, the insect gut microbiota has been shown to be susceptible to community changes in response to insecticides [13]. In recent decades, the widespread and inappropriate use of antibiotics has led to an increase in antibiotic resistance in pathogenic bacteria that cause infections in humans [14]. These insects can be reservoirs for antibiotic resistance (AR) genes and antibiotic-resistant microorganisms [15].

In aquatic environments, genes responsible for antibiotic resistance can be easily transferred by horizontal transmission between different bacterial communities. Insects can therefore serve as a conduit for the spread of these genes [16]. Consequently, recent studies have shown that a risk assessment of edible insects should include an assessment of AR genes and the prevalence of antibiotic-resistant microorganisms [15]. The digestive tract of arthropods provides an environment that is favourable for DNA alterations [17].

OBJECTIVE

The aim of the study is to analyse the microbiota of insect species from certain families of the order of aquatic beetles (Coleoptera: Hydrophilidae and Helophoridae), which include genera of edible insects. Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria and are responsible for significant morbidity and mortality worldwide [18]. Consequently, the study concentrated on gram-negative bacteria.

MATERIALS AND METHOD

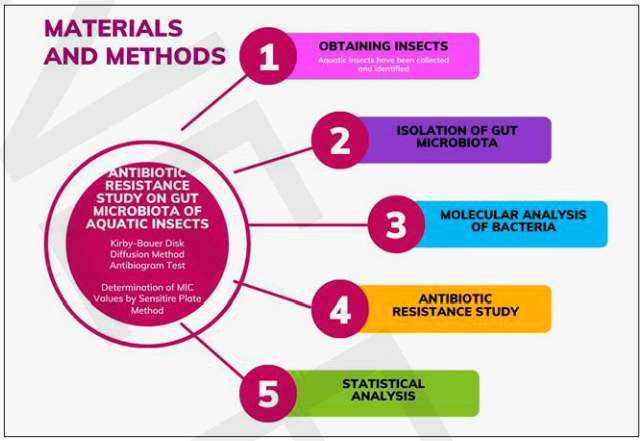


Figure 1. Working general scheme

The general methodology of the study included the procurement, isolation and identification of bacteria (Fig. 1). 17 insects of the families Helophoridae and Hydrophilidae were collected from aquatic environments in different locations in the province of Erzurum and its surroundings in Türkiye. The insects were adult specimens taken from their aquatic environment and transported to the laboratory in a vital state. Each experiment was conducted with a single insect. The insects were first rendered non-viable by placing them in closed boxes with absorbent cotton impregnated with ethyl acetate. The appendages, including the elytra and wings, were then removed and the entire outer surface of the insects was treated with 70% ethanol for five minutes to remove all potentially contaminating microorganisms. The digestive tract of the insects was rinsed in autoclaved, sterile, distilled water to remove the alcohol and then examined under a binocular microscope in the laboratory under aseptic conditions. The samples were dissected, separated and immediately placed in 0.9% sterile physiological water. The samples were then transported to the microbiology laboratory for cultivation. The digestive tracts of the insects were minced in 0.9% sterile physiological water using a sterile glass homogenizer, homogenised by vortexing and serial dilutions were prepared with sterile physiological water. From these dilutions, 100 µl were taken with a sterile pipette and spread on the media Tryptic Soy Agar (TSA) and Nutrient Agar (NA) (Merck), which were then spread on Peer Review Only/Not for Distribution with a Drigalski spatula. The samples were incubated at 30 °C in both aerobic and anaerobic environments. The colonies were differentiated based on their colour, consistency and morphology. The isolates were then transferred to new media, pure cultures were obtained, and the bacterial accession numbers were used as culture codes. The samples were then transferred to Nutrient Broth (NB) containing 16% glycerol for further identification. The samples were stored at -20 °C [19].

Isolation of genomic DNA from isolates. For a more efficient and cost-effective isolation of genomic DNA from the recovered bacteria, an EcoSpin Bacterial Genomic DNA Kit was preferred due to its availability. The commercially available kit was used according to the corresponding protocol. For the fingerprinting analysis of bacterial isolates, the region where 16S rRNA, which is important for bacterial systematics, is generated was selected as the target region. It was then amplified *in vitro* with universal primers and confirmed to contain positive gene bands. The master mix and PCR programme are shown in Table 1.

16S rDNA sequence analysis. Automated sequence analysis was outsourced to the BM Lab in Ankara (Türkiye). Base sequence analysis of the 16S rDNA gene of the obtained amplicons was performed using automated sequence analyzers. The 16S rRNA gene was amplified using the forward primer 27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and the reverse primer 1492R (5'- GGT TAC CTT GTT ACG ACT T -3'), followed by bidirectional sequencing. The EcoSpin Bacterial Genomic DNA kit was preferred due to its availability, as it is more efficient and cost-effective for isolating genomic DNA from the obtained bacteria. The commercially available kit was used according to its protocol (Tab. 1). The sequences were compared using BLAST analysis [20]. The isolates of the molecular and conventional data were analyzed together, named, and deposited in the GenBank. Acceptance numbers were recorded (Fig. 3).

Naming and assigning Acceptance Numbers. The 16S rDNA sequence chromatograms obtained from the analyses were manually assembled into a single complete sequence using BioEdit Sequence. The DNA sequences of the strains obtained were aligned with the information in the NCBI database [20], which is available to the public and contains sequence

Table 1. HPCR Program and Base Sequences [19]

Target Locus	Base Sequences of Primers	Master Mix for just 1 sample (50µL)		PCR Programme	
16S rRNA	27F (forward 5′- AGA GTT TGA TCC TGG CTC AG -3′)	EcoTaq master mix	25 µL	Denaturasyon	98°C 30 sn
		Primer 27F (10 µM)	2 µL	Denaturasyon (35 cycles)	94°C 10 sn
		Primer 1492 R (10 µM)	2 µL	Binding	52°C 15 s
	1492R (reverse 5′- GGT TAC CTT GTT ACG ACT T-3′)	Sterile distilled water	20 µL	Extension	72°C 15 s
		Template DNA	1 µL	Extension	72°C 1 min

Table 2. Antibiotic Susceptibility of Gram Negative Bacteria Based on Disk Diffusion Test (Zone Diameter, mm)

Antibiotic name and dosage	*Aeromonas sp.* (n=12)		*Acinetobacter sp.* (n=8)		*Pseudomonas sp.* (n=4)		*Yersinia sp.* (n=2)		*E.coli* (n=2)		*V.cholerae* (n=2)	
	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)	R (%)	I (%)
CTX (30 µg)	3(%3.6)	0	3 (%37.5)	4(%50)	0	0	0	0	0	0		
FEP (30 µg)	2(%16.6)	5(%41.6)	1(%12.5)	0	0	2 (%50)	0	0	0	0		
CAZ (30 µg)	1(%8.3)	2(%16.6)	0	0	0	4 (%100)	0	0	0	0	1(50)	1(%50)
CRO (30 µg)	3(%3.6)	0	2(%1.6)	2(%1.6)	0	0	0	0	0	0		
CZ (30 µg)	3(%3.6)	0	4(%50)	0	4 (%100)	0	0	0	0	0		
DOR (10 µg)	0	1(%8.3	0	0	0	0	0	0	0	0		
ETP (10 µg)	5(%41.6)	1(%8.3)	0	0	0	0	0	0	0	0		
IPM (10 µg)	0	1(%8.3)	0	0	0	4 (%100)	0	0	0	0		
MEM (10 µg)	1(%8.3)	0	0	0	0	0	0	0	0	0		
CN (10 µg)	1(%8.3)	0	0	0	0	0	0	0	0	0		
TOB (10 µg)	0	0	0	0	0	0	0	0	0	0		
AK (30 µg)	0	2(%16.6)	0	0	0	0	0	0	0	0		
TE (30 µg)	0	0	0	0	1(%25)	0	0	0	0	0		
MI (30 µg)	0	0	0	0	0	0	0	0	0	0		
TGC (15 µg)	0	0	0	0	0	0	0	0	0	0		
CIP (5 µg)	0	1(%8.3)	0	0	0	4(%100)	0	0	0	1(%50)		1(%50)
LEV (5µg)	0	0	0	0	0	4 (%100)	0	0	0	0		
SAM (20 µg)	1(%8.3)	0	2(%1.6)	0	4 (%100	0	0	0	0	0		
TPZ (100/10 µg)	1(%8.3)	0	0	0	0	4 (%100)	0	0	0	0		
SXT (25 µg)	4(%33.3)	0	1(%12.5)	2 (%1.6)	1(%25)	0	0	0	0	0		
AM (10 µg)	5(%41.6)	0	0	0	3(%75)	0	0	0	0	0		
F (300 µg)	7(%58.3)	0	8(%100)	0	3(%75)	0	0	0	0	0	0	
ATM (30 µg)	4(%33.3)	2(%16.6)	1(%12.5)	0	0	0	0	0	1(%50)	0	2(%100)	

n: number of isolates.
R:resistant
I: İntermediate

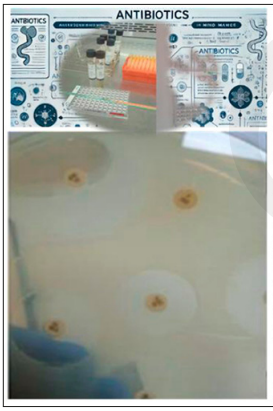
information of various organism genomes. BLAST analysis was performed to facilitate further research.

As a result of analysing the samples with the highest sequence homology, the gene sequences were submitted to Gen Bank (https://www.ncbi.nlm.nih.gov/WebSub/form=history&tool=genbank) and Accession Numbers were obtained. The distribution of isolates included in the study is shown in Figure 3.

Antibiotic susceptibility testing. For Peer Review Only/Not for DistributionTwo? different studies on antibiotic resistance have been conducted – the Sensititre plate method (MIC) and the Kirby-Bauer disc diffusion method.

Kirby-Bauer disc diffusion method Antibiotic susceptibility test. The Kirby-Bauer disc diffusion method was performed in accordance with the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The bacterial inoculum was prepared by suspending the freshly grown bacteria in 4–5 ml of sterile nutrient broth and the turbidity adjusted to a 0.5 McFarland standard. After application of the antimicrobial discs (bioanalysis), the plates were incubated at 37 °C for 24 hours. After this incubation period, the inhibition zones were read and the zone diameters interpreted and classified as sensitive, intermediate or resistant, according to the CLSI (M-45, M100) and EUCAST guidelines. A total of 23 different antibiotics (Fig. 2) discs were tested for each sample using this method [21]. The results of the disc diffusion test are presented in Table 2.

Determination of MIC values using the Sensititre plate method. A liquid microdilution-based Thermo Fisher Sensititre kit containing ready-to-use plates of dehydrated antibiotics at different concentration ranges and growth control wells was used for the study. The minimum inhibitory concentration (MIC) was determined by dilution with



Antibiotic	Antibiotic code	Antibiotic group
Doripenem	DOR10	Carbopenem
Ertapenem	ETP10	
Imipenem	IPM1	
Meropenem	MEM10	Carbonem
Gentamicin	CN10	Aminoglycosides
Tobramycin	TOB10	
Amikacin	AK30	
Ampicillin	AM10	Penicillin
Ampicilin sulbactam	SAM20	Beta-lactam
Ciprofloxacin	CIP5	Fluoroquinolone
Levofloxacin	LEV5	
Nitrofurantoin	F300	Nitrofuran
Piperacilin tazobactam	TPZ110	Beta-lactam
Trimethoprim sulfamethoxazole	SXT25	Phosphate pathway inhibitor
Tetracycline	TE30	Tetracyclin
Minoxidil	MI30	
Tigecycline	TGC15	
Cefotaxime	CTX30	Cephalosporin
Cefepime	FEP30	
Ceftazidime	CAZ30	
Ceftriaxone	CRO30	
Cefazolin	CZ30	
Aztreoneam	ATM30	Monobactam

Figure 2. Used antibiotics and their properties*.
* DOR10 Doripenem, ETP10 Ertapenem, IPM1 Imipenem, MEM10 Meropenem, CN10 Gentamicin, TOB10 Tobramycin, AK30 Amikacin, AM10 Ampicillin, SAM20 Ampicilin sulbactam, CIP5 Siprofloksasin, LEV5 Levofloxacin, F300 Nitrofurantoin, TPZ110 Piperacilin tazobactam, SXT25 Trimethoprim sulfamethoxazole, TE30 Tetracycline, MI30 Minoxidil, TGC15 Tigecycline, CTX30 Cefotaxime, FEP30 Cefepime, CAZ30 Ceftazidime, CRO30 Ceftriaxone, CZ30 Cefazolin and ATM30 Aztreoneam. (supported by Dall-e-3, ai)

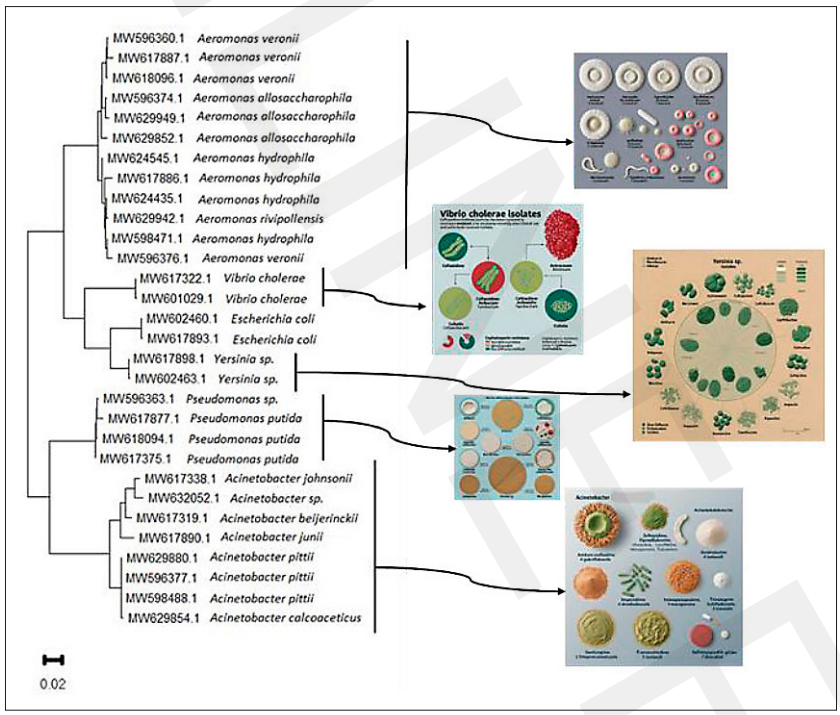


Figure 3. Phylogenetic tree of isolated bacteri and illusionistic demonstration of antibiotic application by bacteria (powered by app.leonardo.ai)

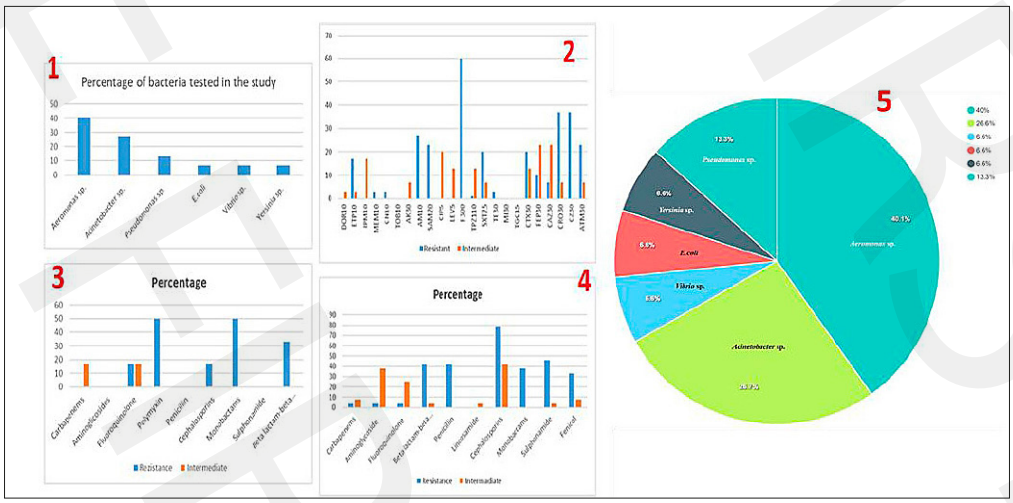


Figure 4. Resistance Rates.
*(1. Species distribution of tested bacteria; 2. Antimicrobial resistance profile based on disk diffusion test method (percentage); 3. Antimicrobial resistance profile of bacteria tested with DKGMN kit (percentage); 4. Resistance rates of bacteria tested with the NF kit; 5. Antibiotic resistance rates of isolated bacteria)

microtiter broth, according to the kit instructions. The lowest concentration that completely inhibited bacterial growth was determined as the MIC value. Considering the biochemical characteristics of the isolates tested, two different plates were used. The DKGMN kit was used for enteric isolates, while the NF kit was used for *Aeromonas*, *Pseudomonas* and *Acinetobacter*. A total of 17 antibiotics were tested with the DKGMN kit and 22 antibiotics with the NF kit using the liquid dilution method. Data were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (EUCAST 2024, CLSI 2023). The antibiotics tested and the dilution ratios are shown in Tables 3 and 4.

Statistical analysis. General statistical methods were used to calculate the number of bacteria showing resistance to antibiotics. The data on the number of bacteria showing resistance to antibiotics were subjected to numerical analysis and the resulting data were presented in graphical form with an interpretation.

RESULTS AND DISCUSSION

The study identifies the bacteria and investigates their antibiotic resistance. A total of 27 bacterial isolates were tested, including twelve *Aeromonas*, eight *Acinetobacter*, two *Vibrio*, four *Pseudomonas*, two *Escherichia coli* and two *Yersinia*. *Aeromonas* isolates were most resistant to nitrofurantoin and cephalosporins, with 78% of the distribution isolates showing resistance or moderate susceptibility to at least one antibiotic tested by disc diffusion. *A. hydrophila* was resistant to 10 antibiotics across all methods, while one Aeromonas isolate remained susceptible to all antibiotics. The DKGMN kit was used for Gram-negative enteric isolates, while the NF kit was used for *Aeromonas*, *Pseudomonas* and *Acinetobacter*, for which 17 and 23 antibiotics were tested, respectively (Tab. 2, 3, 4; Fig. 2). The distribution of species among the isolates is shown in Figures 3 and 4.

To ensure a comprehensive recovery of the gut microbiota, the initial cultivation of the samples was performed under strictly anaerobic conditions (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in an anaerobic chamber. Pre-reduced anaerobically sterilised media were used to facilitate the isolation of obligate and facultative anaerobes native to the digestive tract of insects. After initial growth, individual colonies were selected and sub-cultured according to their specific atmospheric requirements for purification and subsequent susceptibility testing.

When the resistance profile of *Aeromonas* isolates tested in the study was evaluated, disc diffusion results showed varying rates of resistance intensity against cephalosporin group drugs. Two isolates were resistant to six drugs (26%) and one – *A. hydrophila,* was resistant to 12 drugs (52%). This isolate was identified as the most resistant by both methods tested. Similarly, *Aeromonas* showed the most resistant antimicrobial profile in the study. Another microorganism in terms of resistance intensity was *Pseudomonas*. Among the four different isolates tested, three were resistant to five different drugs (22%), and moderately susceptible to six drugs (26%). All eight *Acinetobacter* isolates tested showed resistance to at least one drug – nitrofantoin. Two *A. pitti* isolates were more resistant than other *Acinetobacter* species. Among enteric bacteria, *E. coli*, *Yersinia* and *V. cholerae* were more susceptible.

The gut microbiota of aquatic insects consists of diverse microorganisms within their digestive tract [22]. This microbiota assists in the digestion of complex substances [23]. Studies have shown antibiotic resistance in bacteria isolated from aquatic insects, which could pose a risk to public health [24]. In disk diffusion, 1 *E. coli* isolate and two Yersinia isolates were susceptible to all drugs. Aztreonam resistance was observed in *V. cholera* isolates.

Bacteria that exhibit resistance to MDR bacteria [30]. In the study, one *A. hydrophila* and one *P. putida* isolate were identified as MDR isolates by exhibiting resistance to more than one antibiotic class by both methods. The study tested four bacteria from the Enterobacteriacea class. two *E. coli* bacteria disc diffusion results were evaluated (CLSI); AK30, CAZ30, CIP5, SAM20, ETP10, MI30, CRO30, CZ30, LEV5, TE30, CN10, IPM1, AM10, DOR10. While one isolate was susceptible to FEB30, CTX30, SXT25, For Peer Review Only/Not for DistributionTPZ110, F300, MEM10, TOB10, one isolate was susceptible and the other was moderately susceptible to ATM30. *Yersinia* sp. when the disc diffusion results of the isolates were evaluated; AK30, ATM30, CAZ30, CIP5, SAM20, ETP10, CRO30, MI30, CRO30, CZ30, LEV5, TE30, CN10, IPM1, DOR10, FEB30, CTX30, SXT25, TPZ110, F300, MEM10, TOB10. When the antimicrobial resistance of non-fermentative bacteria, including *Pseudomonas*, was evaluated, the CLSI did not recommend the disc diffusion method for Pseudomonas isolates other than *P. auroginosa*, nor did it include limit values. Therefore, EUCAST limit values were taken into consideration. Of the four Pseudomonas isolates tested, all four were susceptible to TPZ110, AK30, CAZ30, CIP5, LEV5, IPM1, FEB30, MEM10 and TOB10, two were susceptible to ATM30, and one isolate was resistant. AM10 was found to be naturally resistant in all isolates, as expected, and no zone diameter could be measured. Similar results were observed for CZ30, SAM20 and F300. Insects can undoubtedly serve as a source of antibiotic-resistant bacteria [12], in particular, edible insects can naturally carry antibiotic resistance [15]. In this context, the aim of the study was to discover antibiotic resistance properties in isolates derived from aquatic insects (Fig. 3).

Carbapenems are known to be the drugs of last resort in the treatment of clinical infections; therefore, the prevalence of carbapenem resistance genes in insects is alarming. Among the bacteria analyzed in the study, only *Aeromonas* isolates showed carbapenem resistance, while the other isolates did not. The most interesting finding of the study was the discovery of two MDR isolates. The *A. hydrophila* isolate from *Helochares obscurus* (Müller, 1776) exhibited resistance to AM, ETP, TPZ, FEP, CN, SAM, MEM, F and SXT by disk diffusion, as well as moderate sensitivity to two drugs in the carbapenem group (DOR and IMP). The same isolate was tested with a Sensititre NF kit based on the liquid dilution method and was found to be resistant to PIP, FOP, IMI, TIM2, TIC, SXT, A/S2, FOT, AXO and AZT, and moderately sensitive to P/T4. IMP, which was found to be moderately susceptible by disc diffusion, was recorded as resistant by liquid dilution.

Although the DDA methods provided the first, decisive observation of widespread resistance, particularly to

Table 3. Antimicrobial Resistance Profile of Enteric Bacteria Tested With DKGMN Kit

Antimicrobial Class	DKGMN	*Yersinia sp.* (n=2)		*E.coli* (n=2)		*V.cholerae* (n=2)	
	Antibiotic agent and dosage (µg)	R (%)	I (%)	R (%)	I (%)	R(%)	I(%)
Carbapenems	Meropenem (0.12-16)	0	0	0	0	0	0
	Ertapenem (0.12-2)	0	0	0	1(50)	0	0
	Imipenem (0.5-16)	0	0	0	0	0	0
Aminoglycosides	Gentamicin (0.5-8)	0	0	0	0	0	0
	Amikacin (4-32)	0	0	0	0	0	0
	Tobramycin (1-8)	0	0	0	0	0	0
Fluoroquinolones	Ciprofloxacin (0.06-2)	0	0	0	1(50)	1(50)	0
Poliymyxin	Colistin (0.25-8)	1(50)	0	1(50)	0	1(50)	0
Lincosomide	Tigecycline (0.25-4)	0	0	0	0	0	0
Cephalosporin	Ceftazidime (0.5-16)	0	0	0	0	1(50)	0
	Cefotaxime (0.5-8)	0	0	0	0	0	0
Monobactams	Aztreonam (0.5-32)	0		1(50)	0	2(100)	0
Sulfanomide	Trimethoprim /sulfamethoxazole (1/19-8 /152)	0	0	0	0	0	0
Beta-lactam/beta-lactamase inhibitor	Piperacillin / tazobactam constan (1/4- 32/4)	0	0	0	0	0	0
	Ceftazidime/avibactam (0.5/4- 16/4)	0	0	0	0	1(50)	0
	Ceftolozane/tazobactam (0.5/4- 32/4)	0	0	0	0	1(50)	0
	Amoxicillin / clavulanic acid (4/2-64/2)	0	0	0	0	0	0

Table 4. MIC Results for NF Bacteria

Antimicrobial Class	NF	*Aeromonas sp.* (n=12)		*Acinetobacter sp.* (n=8)		*Pseudomonas sp.* (n=4)	
	Antibiotic agent and dosage (µg)	R (%)	I (%)	R (%)	I (%)	R(%)	I(%)
Carbapenems	Imipenem (1-8)	1(8)	0	0	0	0	2(50)
Aminoglycosides	Gentamicin (1-8)	0	3(25)	0	4(50)	0	0
	Tobramycin (1-8)	0	0	1(13)	0	0	0
	Amikacin (4-32)	0	2(17)	0	0	0	0
Fluoroquinolones	Ciprofloxacin (0.25-2)	0	1(8)	0	0	0	2(50)
	Levofloxacin (0.12-0.5)	0	0	1(13)	0	0	3(75)
	Lomefloxacin (0.5-4)	0	0	0	0	0	0
	Ticarcillin (8-64)	2(17)	0	1(13)	0	2(50)	0
Lincosomide	Tetracycline (1-8)	0	1(8)	0	0	0	0
Cephalosporin	Ceftazidime (1-16)	4(33)	0	0	1(13)	0	1(25)
	Cefotaxime (4-32)	5(42)	0	0	1(13)	0	1(25)
	Ceftriaxone (4-32)	3(25)	0	0	2(25)	0	0
	Cefepime (2-16)	1(8)	3(25)	0	0	0	0
	Cefoperazone (4-32)	1,1	0	5(63)	1(13)	0	0
Monobactams	Aztreonam (2-16)	5(42)	0	1(13)	0	3(75)	0
Sulfanomide	Trimethoprim/sulfamethoxazole (0.5/9.5-4 /76)	5(42)	0	4(50)	1(13)	2(50)	0
Beta-lactam/beta-lactamase inhibitor	Piperacillin / tazobactam constant (8/4- 64/4)	0	1(8)	2(25)	0	0	0
	Ticarcillin/clavulanic acit constant(16/2-128/2)	2(17)	0	0	0	3(75)	0
	Ampicillin/sulbactam(2/1-16/8)	1(8)	0	0	0	2(50)	0
Amfenicol	Chloramphenicol (2-16)	0	2(17)	6(75)	0	2(50)	0
Penicillin	Carbenicillin (32-256)	0	0	1(13)	0	3(75)	0
	Piperacillin (8-64)	1(8)	0	0	0	0	0

nitrofurantoin and important β-lactam classes (penicillins, cephalosporins, and carbapenems), they offered only a qualitative insight into the resistome of these environmental isolates. Subsequently, the quantitative precision of MIC methods not only confirmed cephalosporin resistance, but also unmasked a more alarming and broader resistance profile encompassing clinically critical agents, such as sulphonamides and polymyxins. It is important to emphasise that this methodological escalation from qualitative screening to quantitative analysis was essential for the final

identification of multidrug-resistant (MDR) isolates. This process revealed a more complex and worrying level of resistance in this almost pristine aquatic environment.

A study found that exposure to CN10 caused the mealworm's gut microbiota to collapse and reduced its ability to degrade plastic. It is important to test the gram-negative bacteria isolated from aquatic insects in the presented study. Cutting-edge approaches have begun to unravel how pathogen interactions and antibiotic resistance behave within natural microbiome communities [25].

CONCLUSION

This study is the first to investigate antibiotic resistance in Gram-negative bacteria from different types of edible insects using two different methods. The results show that even in environments far removed from clinical waste, antibiotic-resistant bacteria pose a significant risk to public health. The microbiota of aquatic insects, containing potential *Aeromonas*, *Acinetobacter*, *E. coli*, *Vibrio* and *Yersinia*, may serve as a reservoir for AR genes, including multidrug-resistant strains. Phylogenetic analysis revealed close genetic relationships between isolates, although resistance profiles did not correspond to genetic similarity, suggesting the need for broader studies to further understand antimicrobial resistance in invertebrates and its implications for AMR research.

Acknowledgements
The study was supported financially by Atatürk University Scientific Research Unit (Project No: TSA-2022–10866).

Ethical considerations. The study did not involve the use of human or animal subjects. The experiments were carried out under controlled conditions in a laboratory and performeded in compliance with relevant institutional biosafety and biosecurity protocols.

All authors read and approved the final manuscript and declare that they have no conflicts of interest.

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