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Article

High susceptibility Among Cloacal Enterococci and *Escherichia Coli* of Free-Living Dalmatian and Great White Pelicans, With Detection of CTX-M-producing *E. coli* ST69

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Abstract: In 2022, an outbreak of H5N1 highly pathogenic avian influenza (HPAI) killed 60% of the largest breeding colony of Dalmatian pelican (DP) in the world, at Mikri Prespa Lake (Greece), prompting a multidisciplinary study on HPAI and other pathogens. This study determines the antimicrobial resistance rates of cloacal enterococci and *Escherichia coli* in DPs. Fifty-two blood and cloacal swab samples were collected from 31 nestlings (20 DP/11 great white pelicans) hatched after the H5N1 outbreak at the Prespa colony and 21 subadult/adult DPs captured at a spring migration stopover. Swabs were inoculated in non-selective and chromogenic selective media. Identification was performed by MALDI-TOF, and antimicrobial susceptibility was tested. The genetic content was characterized by PCR-sequencing and the clonality of extended-spectrum-beta lactamase (ESBL)-producing-*E. coli* isolates by Multi-Locus-Sequence-Typing. Twenty-eight non-repetitive *E. coli* and 45 enterococci isolates were recovered in non-selective media; most of them were susceptible to all antibiotics tested (85.7% *E. coli*/91.1% enterococci). Three of 52 samples (6%, all adults) contained ESBL-*E. coli* isolates (detected in chromogenic ESBL plates), all carrying the *bla*CTX-M-15 gene and belonging to the lineage ST69. Despite the susceptibility of most fecal *E. coli* and enterococci isolates to all antibiotics tested, the identification of *E. coli* of lineage ST69 carrying *bla*CTX-M-15 is of concern. This high-risk clone needs further investigation to elucidate its primary sources and address the growing threat of antimicrobial resistance from an integrated 'One Health' perspective. Furthermore, it is imperative to further study the potential impacts of ESBL-*E. coli* on the endangered DP.

Keywords: Dalmatian pelican; antimicrobial resistance; ESBL-*E. coli*; One Health

1. Introduction

The Dalmatian pelican (*Pelecanus crispus*), hereafter referred to as "DP," is an emblematic Palearctic bird species, ranging from Eastern Europe to China [1,2]. They live mainly in inland freshwater wetlands, rivers, lakes, deltas and estuaries, where they feed almost exclusively on fish [3]. The South-Eastern Europe (SEE) population of DP consists of short distance migrating populations from Greece, Albania, Montenegro, Bulgaria, Romania, Ukraine and Turkey [2,4]. In Greece, the birds belonging to the eastern group of populations (from four breeding colonies: Prespa, Chimaditida, Kerkini and Karla lakes) winter close to their breeding grounds, and usually migrate to wetlands of north/northeastern Greece and western Turkey [4,5]. The largest breeding colony of DPs in the world is located at Mikri Prespa Lake, Greece [6,7], where they breed side by side with the

great white pelican *Pelecanus onocrotalus* (GWP) [8,9], which shows a similar feeding behavior and habitat preferences [3]. However, GWPs are long-distance migrants [10,11] that fly through many important stop-over sites in their southward migration to African wetlands along the Rift Valley for wintering in the eastern Africa region [10-12].

Prior to 2022, due to enormous conservation efforts, the SEE DP population was growing, with an increasing population estimated at 2,831-3,094 breeding pairs, [11]. From February to April 2022, a HPAI outbreak (H5N1, clade 2.3.4.4b) resulted in the death of 1,734 adult DPs only at Prespa Lake (60% of the colony), and a total of 2,286 DPs deceased in Greece, turning this tragic event into the worst ecological disaster for Greek wildlife. Over 40% of the SEE breeding population was lost, corresponding to approximately 10% of the species' global population. GWPs also died in small numbers, leading to the conclusion that DPs are more susceptible to H5N1 than their congener species [13]. Multidisciplinary research into HPAI and other pathogen carriage in DPs, including bacterial diversity and antimicrobial resistance (AMR) rates, was implemented to investigate the underlying reasons.

AMR has become one of the significant challenges to human and animal health globally [14], and multidrug-resistant (MDR) bacteria, particularly *Escherichia coli*, have been detected in the gastrointestinal tract of wild birds living in a wide range of environments, from very anthropized locations like landfills to remote and less human-influenced ecosystems like the Arctic [15-17]. Extended-spectrum beta-lactamase (ESBL)-producing *E. coli* has been designated as critical priority in the World Health Organization's list of AMR "priority pathogens". This is attributed to its resistance to a wide range of antibiotics and its significant clinical relevance, as it is known to cause severe, life-threatening infections. [14]. Wild birds harboring ESBL-producing bacteria have been described by several authors in recent years, including other pelican species [17-20], which puts the spotlight on migratory birds and the role they could play as dispersers due to their ability to connect different ecosystems and remote locations [21-24]. It has been reported that DPs visit many different wetlands along their annual cycle [5,25], so even if their potential role as dispersers remains unclear, these birds could be quite useful as sentinels of AMR in the aquatic environment. From a conservation point of view, AMR in bacteria can be linked to strains with pathogenic potential that could negatively impact pelican health.

It will take decades for the DP to recover from the devastating effects of the recent avian influenza outbreak [13], so determining antibiotic-resistant bacteria that pelicans can carry and excrete is essential to have a better understanding of their epidemiology, besides highlighting other possible threats for these animals. The present study aims to determine the susceptibility of enteric *E. coli* and *Enterococcus* and the prevalence of ESBL and/or carbapenemase-producing (CP) *E. coli* in pelicans, as well as to understand the role of this species as sentinels of AMR.

2. Results

2.1. Recovery of *E. coli* and enterococci from cloacal samples of pelicans in non-supplemented media

After overnight culture on MacConkey agar (MCA) plates, MALDI-TOF identification, and antibiotic susceptibility testing, a collection of 28 non-repetitive *E. coli* isolates were obtained from 6/21 adults (28.6%) and 20/31 (64.5%) nestlings (10 DP/10 GWP). Moreover, after inoculating the cloacal samples in Slanetz-Bartley (S-B) plates, a total of 45 non-repetitive enterococci isolates were obtained from 11/21 adults (52.4%) and 30/31 (96.8%) nestlings (19 DP/11 GWP) (Table 1).

E. coli isolates were frequently retrieved from adult DP (n = 8, 28.6% of samples) and out of all the enterococci isolates (n = 11, 52.4%), *E. faecalis*, *E. faecium* (n = 4, 19% each), and *E. hirae* (n = 3, 14.3%) were the detected species. In the case of nestlings, four enterococcal species were identified with *E. faecalis* being the most frequent one (n = 19, 61.3%), followed by *E. faecium* (n = 10, 32.2%), *E. hirae* (n = 4, 12.9%) and *E. mundtii* (n = 1, 3.2%). *E. coli* was also frequently recovered (n = 20, 64.5%). Detailed information about prevalences and differences found in nestlings of the two pelican species, as well as total prevalences for both age groups of DP, are summarized in Table 1.

Table 1. Occurrence of *E. coli* and *Enterococcus* isolated in non-selective media (MAC and S-B, respectively) in cloacal samples from adults and nestlings.

Isolate	Number/Percentage of positive samples (n.º of non-repetitive isolates ¹)			
	Adults n=21	Nestlings n=31	Nestling DP n=20	Nestling GWP n=11
<i>Escherichia coli</i>	6/28.6 (8)	20/64.5 (20)	10/50 (10)	10/90.9 (10)*
<i>Enterococcus faecalis</i>	4/19 (4)	19/61.3 (19)*	9/45 (9)	10/90.9 (10)*
<i>Enterococcus faecium</i>	4/19 (4)	10/32.2 (10)	9/45 (9)*	1/9.1 (1)
<i>Enterococcus hirae</i>	3/14.3 (3)	4/12.9 (4)	4/20 (4)	-
<i>Enterococcus mundtii</i>	-	1/ 3.2 (1)	1/5 (1)	-
Total <i>Enterococcus</i>	11/52.4 (11)	30/96.8 (34)	19/95 (23)	11/100 (11)
Total non-repetitive isolates	19	54	33	21

¹One isolate of each species and sample or more than one if they presented different resistance phenotype. Significant differences between age groups are marked in red. Significant differences between nestling species are marked in bold. * p < 0.05

2.2. Phenotypes and genotypes Of Non-Repetitive *E. coli* and *Enterococci* Isolates Recovered In Non-Supplemented Media

Most of the *E. coli* isolates recovered in MCA media (n=24, 85.7%) were susceptible to all antibiotics tested (Figure 1), with only one isolate from a DP nestling showing resistance to tetracycline (carrying the *tet*(A) gene) and two isolates from adult DP with resistance to gentamicin. In addition, one isolate from an adult DP showed a multidrug-resistant pattern (MDR, resistant to at least 3 antimicrobial classes) with phenotypic ESBL production; this isolate carried the *bla*_{CTX-M15} gene, encoding the ESBL CTX-M-15 (Table 2).

Similarly, most enterococci recovered in S-B plates were fully susceptible to all antibiotics tested (n=41, 91.1%) (Figure 2), with four isolates belonging to three adults and from one DP nestling showing a resistance pattern to tetracycline (*tet*(M), n=4; *tet*(L), n=2) and to streptomycin (n=1) (Table 2). AMR rates for each antibiotic tested among the *E. coli* and enterococci isolates are detailed in Figure 3.

Table 2. Isolates with resistance phenotypes obtained from non-selective media, with detection of resistance genes.

Isolate	ID	Pelican reference	Age group	Species	Resistance phenotype	Resistance genes
X9689	<i>E. coli</i>	P12	A	DP	CTX-AMC-CAZ-GEN-AMP-CIP-TOB	<i>bla</i> _{CTX-M15}
X9688	<i>E. coli</i>	P12	A	DP	GEN-TOB	
X9686	<i>E. coli</i>	P17	A	DP	GEN-TOB	
X9774	<i>E. coli</i>	P41	N	DP	TET	<i>tet</i> (A)
X9695	<i>E. hirae</i>	P3	A	DP	TET	<i>tet</i> (M)
X9696	<i>E. hirae</i>	P4	A	DP	TET	<i>tet</i> (M), <i>tet</i> (L)
X9701	<i>E. faecium</i>	P9	A	DP	TET	<i>tet</i> (M), <i>tet</i> (L)
X9806	<i>E. faecalis</i>	P38	N	DP	STR-TET	<i>tet</i> (M)

Legend: A, adult; N, nestling; DP, Dalmatian pelican; GEN, gentamicin; TOB, tobramycin; TET, tetracycline; STR, streptomycin.

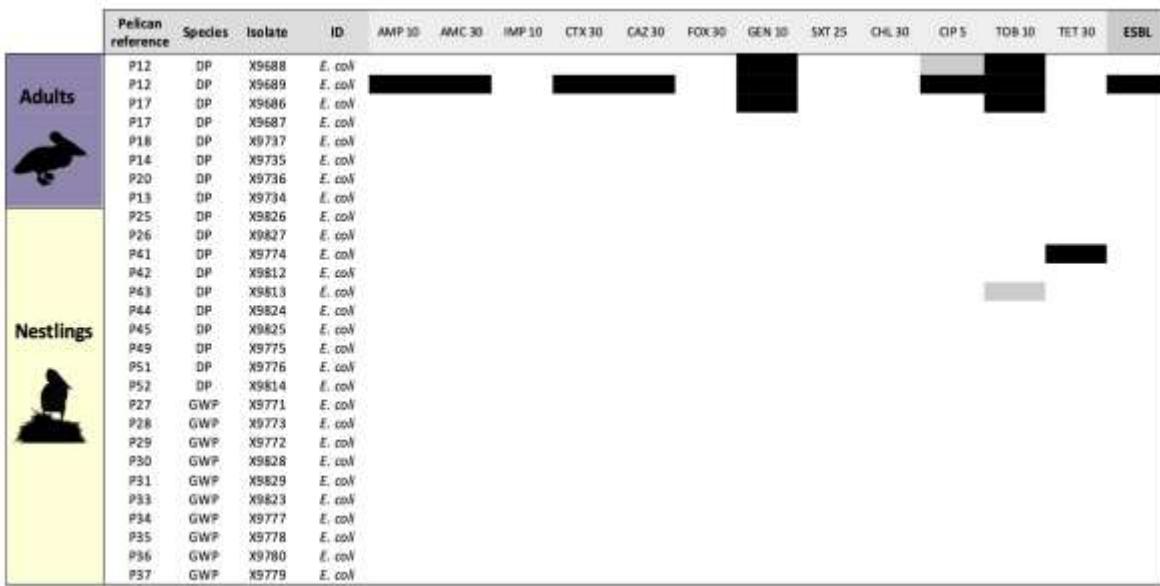


Figure 1. Heat map showing antibiotic susceptibility/resistance of *E. coli* isolated in MacConkey media from cloacal swabs from great white and Dalmatian pelicans. Legend: DP, Dalmatian pelican; GWP, great white pelican; AMP, ampicillin; AMC, amoxicillin/clavulanate; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; GEN, gentamicin; TOB, tobramycin; TET, tetracycline; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole and CIP, ciprofloxacin. Black squares, antibiotic resistant phenotype; grey squares, intermediate resistant phenotype; white squares, wild type/susceptible phenotype.

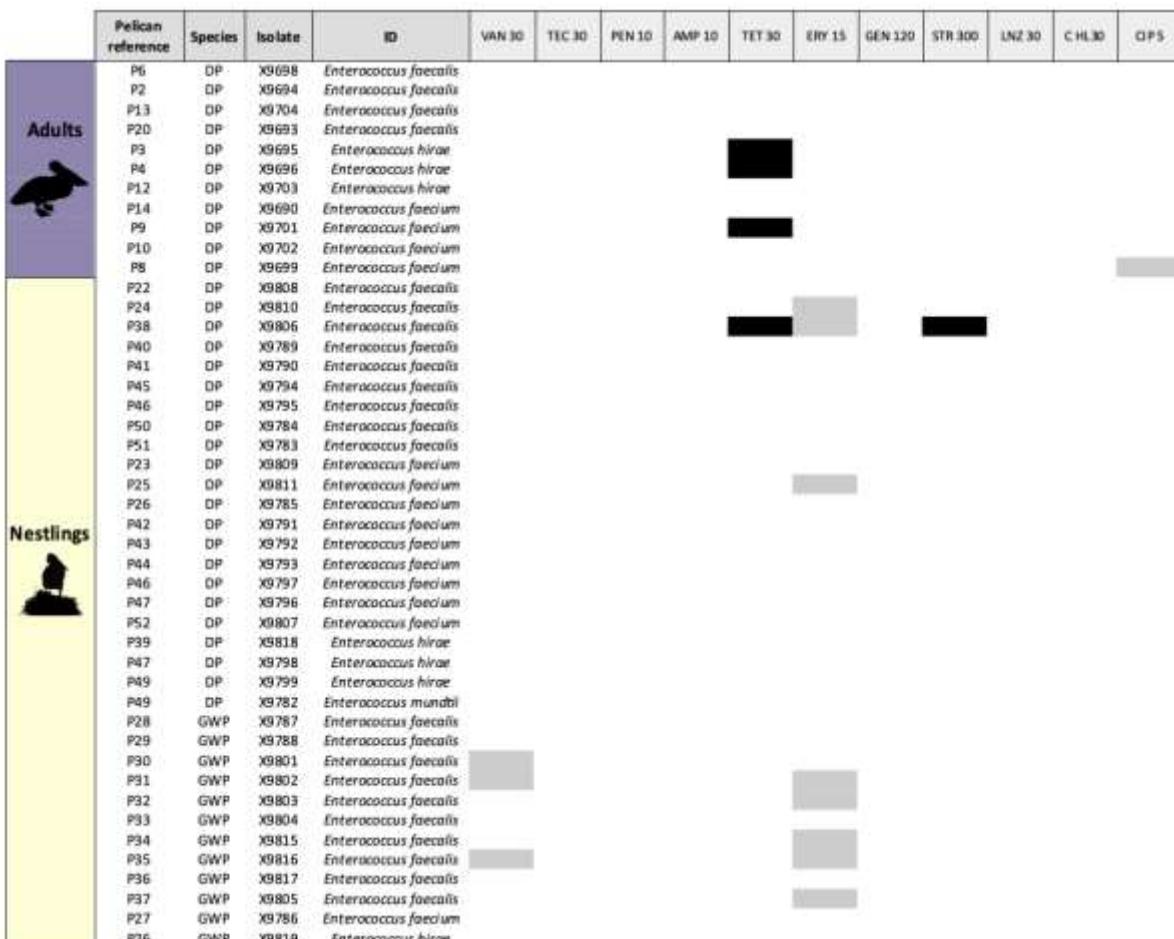


Figure 2. Heat map showing antibiotic susceptibility of all *Enterococcus* strains from Great White and Dalmatian pelican cloacal swabs isolated in Slanetz-Bartley media. Legend: DP, Dalmatian pelican; GWP, great white pelican; VAN, vancomycin; TEC, teicoplanin; PEN, penicillin; AMP, ampicillin; TET, tetracycline; ERY, erythromycin; GEN, gentamicin; STR, streptomycin; LNZ, linezolid; CHL, chloramphenicol; and CIP, ciprofloxacin. Black squares, antibiotic resistant phenotype; grey squares, intermediate resistant phenotype; white squares, susceptible phenotype.

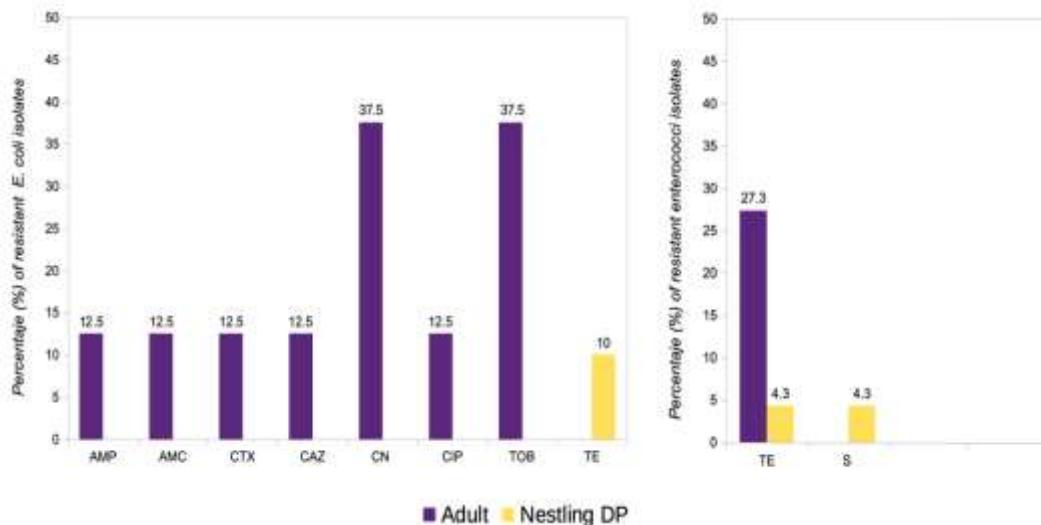


Figure 3. Frequency of antimicrobial resistance exhibited by *E. coli* isolated in MAC (left) and enterococci isolated in S-B (right). Antibiotics with no resistance detected among the isolates are excluded.

2.3. Prevalence of ESBL-producing *E. coli* (ESBL-Ec)

Subsequent seeding of the samples in ESBL- and carbapenem-resistance chromogenic media, resulted in the detection of ESBL-Ec isolates in three of the 21 adult pelicans (14.3%); as described above in one of these pelicans, ESBL-Ec had also been detected in the non-supplemented MCA media. After Multilocus-Sequence-Typing (MLST) of ESBL-Ec isolates all of them were assigned to the lineage ST69 (Table 3). None of the nestling samples were positive for ESBL-Ec. None of the pelicans tested (adults or nestlings) gave a growth in the carbapenem-chromogenic media, indicating the absence of Enterobacterales with this specific resistant phenotype.

Table 3. Characteristics of ESBL producing *E. coli* isolates recovered in cloacal swabs of adult DP.

<i>E. coli</i> isolate	Pelican reference	Resistance phenotype	ESBL resistance genes	ST
X9682	P11	CTX-AMC-CAZ-GEN-AMP-CIP-TOB	CTX-M15	69
X9684*	P12	CTX-AMC-CAZ-GEN-AMP-CIP-TOB	CTX-M15	69
X9706	P15	CTX-AMC-CAZ-GEN-AMP-SXT-CIP-TOB	CTX-M15	69

*Isolated in both MAC and chromogenic media. Legend: CTX, cefotaxime; AMC, amoxicillin/clavulanate; CAZ, ceftazidime; GEN, gentamicin; AMP, ampicillin; SXT, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin and TOB, tobramycin.

2.4. Sexing

From a total of 21 adult pelicans, molecular sexing determined 12 males and 9 females. DP nestlings were identified as 9 males and 11 females while GWP were 5 males and 6 females.

2.5. Differences Between Sex, Age Groups and Species

A significantly higher prevalence of *E. faecalis* ($\chi^2 = 6.304$, d.f. = 1, $p = 0.012$) and *E. coli* ($\chi^2 = 5.188$, d.f. = 1, $p = 0.023$) was found in GWP nestlings, while the occurrence of *E. faecium* was significantly higher in DP nestlings ($\chi^2 = 4.188$, d.f. = 1, $p = 0.041$) (Table 1). The occurrence of AMR bacteria in nestlings did not differ between the two pelican species. To compare age groups, both nestling species were considered as a whole group without distinction. The prevalence of *E. faecalis* was significantly higher in nestlings ($\chi^2 = 9.057$, d.f. = 1, $p = 0.03$) and the prevalence of *E. coli* showed also a trend to be higher in this age group ($\chi^2 = 3.516$, d.f. = 1, $p = 0.061$). ESBL-Ec isolates were found only in adults (two males and one female), these being also the only isolates with a MDR phenotype, a finding that was statistically significant ($\chi^2 = 4.7$, d.f. = 1, $p = 0.03$) and the prevalence of resistant *E. coli* showed a trend to be higher in adults than in nestlings ($\chi^2 = 3.606$, d.f. = 1, $p = 0.058$) (Table 1). No differences were found between sexes in adults or nestlings from both species for prevalence of AMR/MDR bacteria, nor for the occurrence of any of the bacterial species isolated.

3. Discussion

This is the first general microbiological screening performed for AMR *E. coli* and enterococci in cloacal samples of free-living Dalmatian pelicans; so to our knowledge, all our findings can be potentially considered novel for this species. Except for the MDR isolates found in adults (carrying ESBL-Ec), most of the *E. coli* and enterococci recovered were pan-susceptible, showing low rates of resistance for the other antibiotics screened. Differences between the two species of nestlings were scarce, with similar occurrence of AMR and a higher prevalence of *E. coli* in GWP. The reason or clinical significance of the difference in the predominant species of enterococci has not been elucidated, with *E. faecalis* more present in GWP while *E. faecium* was more common in DP. Few significant differences were observed between adults and nestlings, highlighting the ESBL strains mentioned above and the tendency ($p < 0.1$) for *E. coli* with any resistant phenotype, to be more prevalent in adults.

As previously mentioned, one important result obtained in this study is the high proportion of pan-susceptible *E. coli* and enterococci isolates (susceptibility to all antibiotics tested) obtained when non-supplemented media were used. This is relevant data that could reflect a relatively limited environmental contamination with MDR/AMR bacteria where the animals live. Nevertheless, despite this fact, another remarkable result obtained in this study was the isolation of ESBL-Ec with the *bla_{CTX-M-15}* gene and belonging to the lineage ST69 in three adult birds.

Third-generation cephalosporin-resistant *Enterobacteriaceae* are included in the critical group of the WHO Bacterial Priority Pathogen List [14] and, therefore, are considered a highly important finding due to their clinical impact on public health. Among the CTX-M family, CTX-M15 ranks first in prevalence in most parts of the world [26], except Greece, where SHV-type was most frequent [27]. Regarding ST69, it is considered a pandemic lineage like ST131, ST95 or ST73, and it has been frequently reported from both community and nosocomial infections [28,29]. After ST131, this lineage is the most frequent clone, present in both humans and animals [29,20], and its effective transmission was highlighted in a household transfer of CTX-M group 9 *E. coli* ST69 strain between pet dogs and humans [31]. The dissemination of this high-risk clone can contribute to an accelerated spread of CTX-M15 beta-lactamase globally and could additionally be spread through avian migration, as was suggested for wintering rooks (*Corvus frugilegus*) [32].

In 2022, ECDC reported that in Greece, antimicrobial consumption was nearly twofold the average total consumption of European Union (EU) countries [33]. Having one of the highest rates of antibiotic consumption explains rates of antibiotic resistance consistently higher than EU average [34]. ESBL in human isolates have been frequently described in Greece [27]; regarding food and food-producing animals, EFSA/ECDC described an ESBL prevalence of 33.8% in fattening pigs in Greece in 2021/2022, and lower prevalences in pig and cattle meat (5.3% and 4%, respectively) [35].

Considering the aforementioned AMR prevalences, it could be reasonable to think that these bacteria and genes could transfer to Greek aquatic ecosystems, where pelicans and other wildlife are exposed to them. Antibiotic-resistant bacteria (ARB) reach rivers and lakes through many sources, such as hospital effluents and wastewater treatment plant discharges and leaching from proximate

farms [36-38]. Once in the aquatic ecosystem, ARB can persist and spread despite the natural processes of dilution and degradation [37]. Moreover, antibiotic-resistant genes (ARGs) remain and reach other bacteria of public health concern through horizontal gene transfer [39]. In both freshwater and seawater from Greece, several ARBs and ARGs have been described, even in uninhabited coastal sites with presumptively less influx of wastewater [40-42]. It is worth mentioning that Greece is one of the major European marine aquaculture producers [43], and this important sector is generally related to a considerable use of antibiotics [44,45]. However, pelicans primarily use freshwater wetlands, and these are not related to any aquaculture activity.

One important pathway for acquiring ARGs is through livestock, which is considered a significant reservoir of ESBL and has a high prevalence of CTX-M clusters [46,47]. Leachates from farms located near wetlands or lakes can end up in water; even the cattle sometimes come to the lakes to graze and drink and could contribute to AMR/ARGs contamination of the aquatic environment [37,38], where it can eventually get into contact with wildlife. Many ESBL-producing *Enterobacteriaceae* have also been reported in a wide diversity of wildlife species [48], although most detections belong to wild birds with family CTX-M as the most prevalent. In Greece, ESBL genes in fecal samples from wild birds were first detected in Eurasian magpies (*Pica pica*) [47]. The same research also analyzed samples from livestock, and results from cattle, pigs, and magpies corroborated the global tendency with all ESBL-Ec harboring *bla*CTX-M-1/15. In a more recent survey including 47 different species of wild birds in the same country [20], the *bla*CTX-M-1 group (along with *bla*TEM) was again isolated in 3.3% of samples. To our knowledge, this is the first report of CTX-M15 *E. coli* in DPs; the presence of these strains of clinical interest in their gut suggests considering their usefulness as sentinels of AMR contamination of aquatic environments to which pelicans are closely related.

Finding an epidemic clone like ST69 in these animals is also important due to their spatial ecology. DPs use numerous wetlands around the year [5,25,49], connecting even five different countries according to previous GPS tracking of the Greek breeding population (Figure 4). In addition, GWP is a long-distance migratory bird that interconnects the European, Asian and African continents [12]. The migratory movements of both species of pelicans, making use of diverse wetlands across several countries and continents, and getting in contact with resident pelicans and other birds highlight the potential capacity of these animals to carry and disperse AMR and ARGs, as described for other wild birds [16, 18, 50].

Finally, ESBL-Ec prevalence found in this study (14.3% of adults sampled) is in accordance with previous reports for other adult wild birds [51-53], although they differ with other studies reporting prevalences higher than 40% [15,54,55]. However, a much lower prevalence was observed in wild birds in Greece [20], and in other European surveys [56,57]. Regional differences in some cases could explain this discrepancy in the results, but another possible explanation suggested by other authors [20] could be the species studied, as some researchers reporting high prevalences have focused on species strongly associated with anthropogenic diet and environments, like seagulls and storks that forage in human wastes or landfills [15,57,58]. A lower prevalence was expected in nestlings from our study, and no ESBL-Ec was found, although some authors have also described it in wild nestlings [17]. Further investigations are needed to look for temporal patterns of ESBL-Ec prevalence and thus persistence of carriage in DP and GWP. Expanding sampling to other breeding colonies (including those where DPs do not come in contact with GWPs) will allow to obtain more data and elucidate different factors involved in the acquisition of these critical bacteria in pelicans.

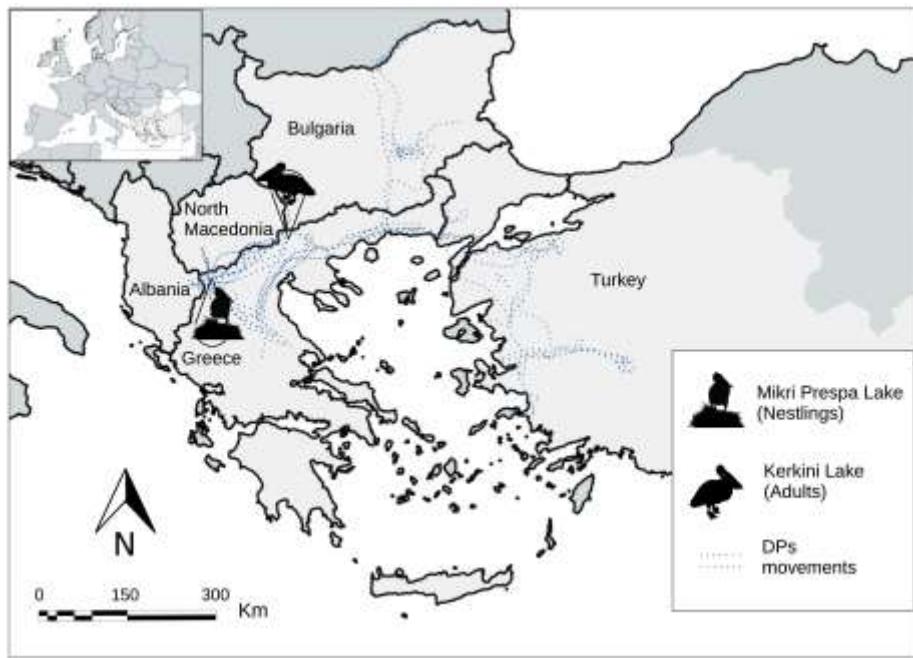


Figure 4. Map showing sampling sites, and movements of GPS tagged DP from Greek eastern DP population (n=53; 2012-2024). Tracking data provided by Society for the Protection of Prespa. Available online: <https://www.movebank.org/cms/movebank-main>

4. Material and Methods

4.1. Study Sites

Samples of pelicans from two different locations in Greece [Mikri Prespa Lake (N40°45', E21°06') and Kerkini Lake (N41°12', E23°09')] were collected (Figure 4), and both locations are quite connected through frequent movements of adult pelicans [49]. Mikri Prespa Lake is the largest breeding colony of DP in the world with 1,226-1,585 pairs until 2022 [59] and is also the nesting site for the majority of GWP in Greece, with 500-780 pairs [60]. Both species nest side by side, forming large colonies that allow them to strengthen their sense of safety and consequently increase their breeding success [8]. In 2022, after the massive mortality caused by the HPAI outbreak, around 100 pairs of DPs were able to complete the reproductive process and raise 90 chicks [13], while GWP were not affected. A total of 31 cloacal samples (20 DP/11 GWP) were obtained in July 2022 from nestlings belonging to these remaining breeding pairs.

The second location was Kerkini Lake, that supports the greatest diversity of waterbird species in freshwater in Greece, besides being an important wintering and stop-over site for many species, including many of the DPs that breed at Prespa [4,5,49]. The HPAI outbreak also affected the DP population in Kerkini, but to a lesser extent than in Mikri Prespa Lake [13]. In January 2023, a total of 21 DPs were sampled at Kerkini Lake, mostly adults but also some second and third-year immatures.

4.2. Sampling

Adults were captured using leg-hold traps set on the shore, or by hand from a boat, after being attracted very close to the boat with fish as bait, while nestlings were approached in their nests and gently restrained by manual handling or trapped from a boat using a fishing net pole. Cloacal samples were taken and were stored in Amies transport medium (Aptaca, Caneli, Italy), kept refrigerated in the field and frozen at -20°C for sending to the laboratory, where they were frozen at -80°C until further processing. Blood samples were taken from the medial metatarsal vein and kept in sterile tubes with heparin lithium as anticoagulant. Tubes were spun at 10000 rpm for two minutes

to separate plasma from bottom pellet and both were kept separated and frozen at -20°C for further analysis.

4.3. Bacterial Isolation And Identification

Cloacal swabs were inoculated in 3ml of brain heart infusion (BHI) broth for enrichment and the resulting suspension was incubated at 37°C for 24 hours. For enterobacteria and enterococci isolation, MCA and S-B agar plates were used, adding 50 µL of enriched suspension in each one for streaking over the plates; from these plates *E. coli* and enterococci were recovered in media not supplemented with antibiotics to obtain a collection of non-selected isolates. After incubation at 37°C for 24 hours, one or two isolated colonies morphologically compatible with *E. coli* (MCA) or enterococci (S-B) were selected and identified by MALDI-TOF mass spectrometry (MALDI Biotyper®, Bruker).

Moreover, 50-100 µL of suspension was also spread over chromogenic selective media for detection of ESBL producing bacteria (Brilliance™ ESBL Agar; Oxoid) and carbapenem resistant (CP) bacteria (Brilliance™ CRE Medium; Oxoid), to analyze the prevalence of samples with *E. coli* carrying these specific resistance mechanisms. Plates were incubated at 37°C for 24h and colonies with the compatible morphology were identified by MALDI-TOF, as previously indicated.

4.4. Antimicrobial Susceptibility Testing

All identified isolates as *E. coli* or enterococci were tested for antimicrobial susceptibility by the disc-diffusion method, following methodology and breakpoints of the Clinical Laboratory Standards Institute (CLSI) [61]. Twelve antibiotics were tested for *E. coli*: ampicillin, amoxicillin/clavulanate, cefoxitin, cefotaxime, ceftazidime, imipenem, gentamicin, tobramycin, trimethoprim/sulfamethoxazole, chloramphenicol, ciprofloxacin, and tetracycline. The double disc synergy test, using cefotaxime, ceftazidime, and amoxicillin/clavulanate discs, was used for the detection of phenotypic ESBL production [61]. In the case of enterococci, eleven different antibiotics were tested: vancomycin, teicoplanin, penicillin, ampicillin, tetracycline, erythromycin, gentamicin, streptomycin, linezolid, chloramphenicol and ciprofloxacin. In the collection of isolates recovered in MCA and S-B plates, non-repetitive isolates were considered for further analysis, and they represented one isolate of each species per sample, or more than one if they presented different antibiotic resistance phenotypes. For ESBL-Ec isolates recovered in chromogenic Brilliance™ ESBL Agar media, one isolate per sample was considered and further characterized.

4.5. *E. coli* and Enterococci DNA Extraction

The DNA extraction of *E. coli* isolates was performed suspending fresh isolated colonies in 1 ml of sterile Milli-Q water and incubating in boiled water at 100°C for 8 minutes. After that, suspension was thoroughly mixed by vortex and centrifuged at 12000 revolutions per minute (rpm) for 2 min. The supernatant with DNA was stored at -20 °C. For DNA extraction of enterococci isolates, fresh colonies were suspended in 1 ml of sterile Milli-Q water, mixed by vortex and centrifuged at 12000 rpm for 3 min. After removing the supernatant, 20 µL of Insta-Gene matrix (Bio-Rad Laboratories, Hercules, CA, USA) was added to the sediment, mixed by vortex and incubated in bath at 56°C for 20 min. Another incubation was repeated at 100°C for 8 min and centrifuged at 12000 rpm for 3 min. The resultant supernatant with DNA was stored at -20°C.

4.6. Molecular Characterization Of Antibiotic-Resistant Genes

Based on the resistant phenotype, genes conferring resistance to tetracycline (*tet(A)/tet(B)* for *E. coli* and *tet(K)/tet(M)/tet(L)* for enterococci), aminoglycosides (*aac(3')-II*), vancomycin (*vanA/vanB*) and streptomycin (*str*) were studied by PCR. In addition, all phenotypic ESBL-Ec isolates were selected for *blasHV*, *blaCTX-M*, *blaTEM* and *blaOXA* screening, and the resultant PCR products were further analyzed using sanger sequencing and confirmed by mapping against the NCBI database. The clonality of ESBL-Ec isolates were characterized by MultiLocus Sequence Typing (MLST) based on the Achtman

scheme, amplifying and sequencing the seven housekeeping genes (*adk*, *fumC*, *icd*, *gyrB*, *mdh*, *purA* and *recA*) and comparing them with the MLST database to assign sequenced types (ST) (<https://pubmlst.org/organisms/escherichia-spp>). Conditions of PCRs and primers for genes tested, as well as the molecular typing, are described in Supplementary Tables S1 and S2.

4.7. Sex Determination

DNA was extracted from centrifuged blood, taking 5 µL of bottom pellet diluted in 1 ml of Milli-Q water, and boiled at 100°C for 10 minutes. The resultant DNA was used in a modified version of the PCR described by Fridolfsson and Ellegren [62], using the commercial kit Phusion blood direct PCR (ThermoFisher, Madrid, Spain). Horizontal electrophoresis in a 1.5% agarose gel was carried out to allow for separation and posterior visualization of DNA bands.

4.8. Statistical Analysis

Data analysis was run in the Statistical Package for Social Sciences (SPSS) Version 28 (IBM, California, USA). The association between the carriage rate of different bacteria and the bird species/age/sex groups was compared using the chi-square test at a 95% confidence interval. All analyses were considered statistically significant with probability values less than 0.05.

5. Conclusions

The present study describes the prevalence of enterococcal cloacal microbiota and *E. coli* in pelican nestlings from two different species and DP adults. Specifically, this study focused on phenotypic and genotypic characteristics of isolates with clinical interest, with clonality characterization of ESBL-producing *E. coli* due to its importance for Public Health.

We can outline that the high rate of susceptibility of most of the *E. coli* and enterococci recovered from the tested pelicans is in line with the habitat they live in. Nevertheless, at the same time, the detection of ESBL-producing *E. coli* isolates of the epidemic clone ST69 carrying the gene encoding CTX-M-15 in species like DPs, feeding quite restricted diets and under a moderate influence of anthropogenic activities is of relevance. This fact makes further investigation mandatory to clarify the main sources and face the AMR threat from a 'One Health' approach.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Primers and protocols employed in the PCR analyses to detect genes encoding for resistance to different antibiotics selected based on resistant phenotype.; Table S2: Primers and protocols employed in the PCR analyses to detect MLST genes. References [63-71] are cited in the Supplementary Materials.

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