

# Antibiotic Resistance and Virulence Profiles of Gram-Negative Bacteria Isolated from Loggerhead Sea Turtles (*Caretta Caretta*) of the Island of Maio, Cape Verde

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## Research article

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## RESEARCH

# Antibiotic resistance and virulence profiles of Gram-negative bacteria isolated from loggerhead sea turtles (*Caretta caretta*) of the Island of Maio, Cape Verde

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## Abstract

**Background:** Several studies detected high levels of antibiotic-resistance in loggerhead sea turtles (*Caretta caretta*) and pointed this species as prime reservoirs of antibiotic-resistant bacteria and carriers of potentially pathogenic bacteria. This study aimed to characterize, for the first time, the Gram-negative aerobic microbiota of the Cape Verdean loggerhead subpopulation. Cloacal, oral and egg content swab samples from 33 nesting loggerheads ( $n = 99$ ) of the Island of Maio were analysed regarding the presence of Gram-negative bacteria and their antibiotic resistance and virulence profiles.

**Results:** *Shewanella putrefaciens* (27.78%), *Morganella morganii* (22.22%) and *Vibrio alginolyticus* (22.22%) were the most prevalent species isolated from the animals under study. A low incidence of antibiotic-resistant bacteria (26%) was detected, and no multidrug-resistant isolates were identified.

Non-Enterobacteriaceae isolates presented the most complex virulence profiles, revealing the ability to produce hemolysins (100%), DNases (89%), lipases (79%), proteases (53%), lecithinases (21%), gelatinases (16%), and also biofilms (74%). Moreover, higher virulence indices were obtained for turtles with high parasite intensities compared with apparently healthy animals, and a positive correlation between antibiotic resistance and virulence was observed.

**Conclusions:** Results suggest that this loggerhead population may be less exposed to antimicrobial compounds, probably due to the low anthropogenic impact observed in both their nesting (the Island of Maio) and foraging sites. Nevertheless, the presence of potentially pathogenic bacteria expressing virulence factors may threaten both sea turtles' and humans' health.

**Keywords:** *Caretta caretta*; Island of Maio; Virulence factors; Antibiotic resistance; One Health

## Background

Loggerhead sea turtles (*Caretta caretta*) are one of the seven species of sea turtles identified worldwide. The International Union for the Conservation of Nature (IUCN) Red List of Threatened Species categorizes this species as vulnerable [1]. However, the *Caretta caretta* subpopulation of the archipelago of Cape Verde (North-East Atlantic subpopulation) is classified as endangered [2]. Recent data shows that this loggerhead subpopulation might be the largest population of this

species worldwide [3, 4]. Furthermore, after the Island of Boavista, the shores of the Island of Maio are, together with the Island of Sal, the most important nesting sites in the archipelago [5, 6].

Previous studies revealed the presence of pathogenic Gram-negative bacteria in loggerhead sea turtles, while the detected isolates were predominantly opportunistic pathogens [7–9]. The bacterial species already described in sea turtles include *Enterobacter* sp., *Citrobacter* sp., *Corynebacterium* sp., *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Salmonella* sp., *Serratia* sp., *Morganella* sp., *Vibrio* sp., *Aeromonas* sp. and *Pseudomonas* sp., which have been associated with disease in loggerhead turtles, being also potential human pathogens [9–15].

According to the World Health Organization (WHO), antimicrobial resistance (AMR) is one of the most serious threats to human health, food safety, and communities' development [16]. Loggerhead sea turtles have specific ecological and physiological characteristics that make them prime reservoirs for antibiotic-resistant bacteria (ARB), including a diverse omnivorous diet, long life-span and high fidelity to coastal feeding habitats [17, 18].

ARB have been studied in live-stranding loggerhead sea turtles, mainly from the Mediterranean subpopulation and high levels of resistance in *Citrobacter* sp., *Pseudomonas aeruginosa*, *Morganella morganii* and *Proteus vulgaris*, have been described, namely to tetracyclines and cephalosporins [8, 17, 19]. To the best of our knowledge, there are no studies available describing the microbial composition of the loggerhead North-East Atlantic subpopulation. However, in distinct loggerhead subpopulations and sea turtles' species, several zoonotic pathogens, including *Salmonella* sp., *Campylobacter* sp., *Vibrio* sp., *Morganella* sp., *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Escherichia coli*, were previously isolated from the turtles' gastrointestinal tract [15, 20–23]. Due to their zoonotic potential, these bacterial species are a concern in the context of One Health [9, 24]. Moreover, the evidence of resistant strains in nesting sea turtles is of great concern, due to the probable transmission to humans, mainly to local populations [25]. Despite illegal, consumption of turtle-derived products in the Island of Maio is a reality, posing a risk to Public Health [15, 25, 26]. Sea turtles, having a high position in the food chain and therefore a higher bacterial load exacerbate the hazards of this practice [26].

Due to the endangered status of the loggerhead colony of the Island of Maio, and the worldwide importance of the Cape Verdean subpopulation, there is an increasing need to further study this subpopulation to reinforce conservation and surveillance strategies. By evaluating the presence of pathogenic Gram-negative bacteria in cloacal, oral and egg samples of loggerhead sea turtles, this study aims to provide the first insight into this loggerhead colony's Gram-negative aerobic microbiota as well as essential data regarding the Cape Verdean *Caretta caretta* nesting subpopulation. Furthermore, the characterization of the resistance and virulence profiles of these bacteria represents key information for this species' conservation strategies, as well as for community sensibilization actions on a public health issue of major importance for the safeguarding of One Health.

## Results

Cloacal (oviductal fluid), oral and egg content swab samples were collected from 33 animals, making a total of 99 samples. The average curved carapace length (CCL) of the studied loggerhead females was 76.7 cm and the average clutch size 69 eggs.

From the 99 samples under study, it was possible to obtain a total of 49 isolates (49%), from 24 samples. Considering the animal and the type of sample, 19 Gram-negative bacilli were selected for further characterization, including Non-Enterobacteriaceae ( $n = 12$ ; 63%) and Enterobacteriaceae isolates ( $n = 7$ ; 37%).

The isolates were identified using the API 20NE and 20E galleries, obtaining the following results: *Shewanella putrefaciens* ( $n=5$ ; 27.78%), *Vibrio alginolyticus* ( $n = 4$ ; 22.22%), *Morganella morganii* ( $n = 4$ ; 22.22%), *Enterobacter cloacae* ( $n = 2$ ; 11.11%), *Aeromonas hydrophila/caviae* ( $n = 1$ ; 5.56%), *Brevundimonas vesicularis* ( $n = 1$ ; 5.56%), *Burkholderia cepacia* ( $n = 1$ ; 5.56%), and *Citrobacter* sp. ( $n = 1$ ; 5.56%). *Shewanella putrefaciens* was the most prevalent species (27.78%), followed by *Vibrio alginolyticus* (22.22%) and *Morganella morganii* (22.22%).

The turtles under study were categorized in three different classes regarding their health status: class I – good body condition, no evident lesions or disease ( $n = 19$ ), class II – external superficial lesions (i.e. superficial erosions or flaking of the carapace) ( $n = 7$ ) and class III – animals with a high number of parasites in the cloaca or abnormal oviposition ( $n = 7$ ). *Vibrio alginolyticus* isolates had a higher prevalence in samples from class III loggerhead females compared with apparently healthy individuals (class I and II) ( $\chi^2=7,031$ ,  $d.f. = 2$ ,  $p < 0,05$ ). No significant differences were observed regarding the prevalence of the identified bacterial species and the type of sample (cloacal, oral and egg).

A considerable percentage of isolates under study (70%) were resistant or intermediately resistant to at least one of the twelve antibiotics tested, with 26% of isolates showing resistance to two or more antibiotics. Higher frequencies of resistance were detected regarding tetracyclines (26%), and none of the isolates presented resistance or intermediate resistance to aminoglycosides (amikacin, gentamicin, and tobramycin) and fluoroquinolones (ofloxacin) (Table 1).

The bacterial species that showed higher MAR indices were *Aeromonas hydrophila/caviae* (MAR index mean value = 0.33), *Burkholderia cepacia* (MAR index mean value = 0.17), and *Enterobacter cloacae* isolate (MAR index value = 0.25) (Table 1). According to Magiorakos *et al.* [27] classification, no multidrug-resistant (MDR) isolates were detected, as none was non-susceptible to at least three antimicrobial agents of different categories. A significant difference between the MAR index values of isolates from different sample types was observed, with the values of isolates from cloacal samples (mean rank = 59.49) being significantly higher when compared with the index values of isolates collected from oral (mean rank = 49.56) or egg content samples (mean rank = 48.09) ( $H(2) = 9.747$ ,  $p < 0.01$ ).

Regarding virulence characterization, all isolates were able to produce hemolysins (100%) (Table 2). Most isolates were able to produce DNases (89%), lipases (79%) and biofilm (74%). Protease production was revealed in 53% of isolates. Lecithinase (21%) and gelatinase (16%) activities were less observed among the tested isolates. Higher virulence profile index (V. Index) values were obtained for *Aeromonas hydrophila/caviae* (V. Index value = 0.86), *Brevundimonas vesicularis* (V. Index

Table 1: Selected isolates' Resistance Profile

ISOLATE CODE	SAMPLE TYPE	SPECIES ID	RESISTANCE PROFILE		MAR INDEX
			I	R	
276/030-2	C	<i>Aeromonas hydrophila/ caviae</i>	IMP; ENR	CFP	0.33
786/785	C	<i>Brevundimonas vesicularis</i>	IMP	-	0.08
329/328	E	<i>Burkholderia cepacia</i>	ENR; CFP	-	0.17
49/50-2	C	<i>Shewanella putrefaciens</i>	-	-	0.00
46/45-2	C	<i>Shewanella putrefaciens</i>	-	T	0.08
46/45-1.2b	E	<i>Shewanella putrefaciens</i>	-	-	0.00
72/73-1	C	<i>Shewanella putrefaciens</i>	IMP	-	0.08
276/030-1	C	<i>Shewanella putrefaciens</i>	IMP	-	0.08
276/030-1	E	<i>Vibrio alginolyticus</i>	-	-	0.00
276/030-1	E	<i>Vibrio alginolyticus</i>	-	-	0.00
60/61-1	C	<i>Vibrio alginolyticus</i>	-	-	0.00
504/503-2	O	<i>Vibrio alginolyticus</i>	-	T	0.08
330/331	C	<i>Enterobacter cloacae</i>	-	-	0.00
49/50-1	C	<i>Enterobacter cloacae</i>	CIP	ENR; PIP	0.25
46/45-2.1a	C	<i>Morganella morganii</i>	MEM	T	0.08
46/45-2.2	E	<i>Morganella morganii</i>	-	T	0.00
276/030-1	O	<i>Morganella morganii</i>	IMP	T	0.08
504/503	C	<i>Morganella morganii</i>	IMP	T	0.08
229/228	C	<i>Citrobacter sp.</i>	CAZ	-	0.08

Multiple antibiotic resistance (MAR), cloaca (C), oral cavity (O), egg (E), intermediately resistant (I), resistant (R), imipenem (IMP), enrofloxacin (ENR), cefoperazone (CFP), tetracycline (T), ciprofloxacin (CIP), piperacillin (PIP), meropenem (MEM), ceftazidime (CAZ).

value = 0.86) and *Shewanella putrefaciens* (V. Index mean value = 0.80). Bacterial species revealing the lowest virulence index values included *Vibrio alginolyticus* (V. Index mean value = 0.50), *Morganella morganii* (V. Index mean value = 0.47), *Burkholderia cepacia* (V. Index mean value = 0.43) and *Citrobacter sp.* (V. Index mean value = 0.43) (Table 2). There was a significant difference between the V. Index values of isolates obtained from cloacal (mean rank = 60.67), oral (mean rank = 45.82) and egg content (mean rank = 50.40) swab samples, with the index values of the ones from cloacal samples being significantly higher ( $H(2) = 9.763$ ,  $p < 0.01$ ). Concerning the animal health status classes, the virulence profile index results were significantly higher in isolates from class III turtles (mean rank = 62.91), when comparing with class I (mean rank = 49.28) or class II animals (mean rank = 47.71) ( $H(2) = 9.663$ ,  $p < 0.01$ ). No significant differences were observed for the MAR and virulence indices of isolates from the Enterobacteriaceae and the Non-Enterobacteriaceae families.

A positive correlation was observed between MAR index and V. Index ( $r = 0.751$ ,  $p < 0.01$ ). Also, a significant difference was observed for the MAR index results between biofilm-producer (mean rank = 78.30) and non-biofilm-producer (mean rank = 48.83) isolates, with the first registering higher MAR index values ( $U(2) = 340.500$ ,  $p < 0.001$ ).

## Discussion

The present study provides initial insight data on the Gram-negative aerobic microbiota of a firstly examined loggerhead turtle subpopulation, addressing the antimicrobial resistance issue on a wider scale. This study also represents the first characterization of the virulence phenotypic profile of sea turtles' bacteria, underlining the role of loggerhead sea turtles as carriers of potentially pathogenic and zoonotic bacteria.

For the collection of oral and cloacal swabs, AMIES swabs were used, being described as a reliable, effective, non-traumatic technique for the characterization of

Table 2: Selected isolates' Virulence Profile

ISOLATE CODE	SPECIES ID	VIRULENCE PROFILE							V. INDEX
		HEM	DNase	Lipase	LEC	PT	GEL	BF (h)	
276/030-2	<i>Aeromonas hydrophila/ caviae</i>	$\beta$	+	+	+	+	-	24	0.86
786/785	<i>Brevundimonas vesicularis</i>	$\alpha$	+	+	-	+	+	24	0.86
329/328	<i>Burkholderia cepacia</i>	$\alpha$	-	+	-	+	-	-	0.43
49/50-2	<i>Shewanella putrefaciens</i>	$\alpha$	+	+	-	+	+	24	0.86
46/45-2	<i>Shewanella putrefaciens</i>	$\alpha$	+	+	-	-	-	-	0.43
46/45-1.2b	<i>Shewanella putrefaciens</i>	$\beta$	+	+	+	+	+	24	1.00
72/73-1	<i>Shewanella putrefaciens</i>	$\alpha$	+	+	+	+	-	24	0.86
276/030-1	<i>Shewanella putrefaciens</i>	$\alpha$	+	+	+	+	-	24	0.86
276/030-1	<i>Vibrio alginolyticus</i>	$\alpha$	+	-	INC	+	-	48	0.57
276/030-1	<i>Vibrio alginolyticus</i>	$\alpha$	+	-	INC	+	-	24	0.57
60/61-1	<i>Vibrio alginolyticus</i>	$\alpha$	+	-	INC	-	-	24	0.43
504/503-2	<i>Vibrio alginolyticus</i>	$\alpha$	+	-	-	-	-	24	0.43
330/331	<i>Enterobacter cloacae</i>	$\alpha$	+	+	-	-	-	48	0.57
49/50-1	<i>Enterobacter cloacae</i>	$\alpha$	+	+	-	+	-	48	0.71
46/45-2.1a	<i>Morganella morganii</i>	$\beta$	+	+	-	-	-	-	0.43
46/45-2.2	<i>Morganella morganii</i>	$\alpha$	+	+	-	-	-	-	0.43
276/030-1	<i>Morganella morganii</i>	$\alpha$	+	+	-	-	-	-	0.43
504/503	<i>Morganella morganii</i>	$\alpha$	+	+	-	-	-	24	0.57
229/228	<i>Citrobacter sp.</i>	$\alpha$	-	+	-	-	-	72	0.43

alpha-hemolysis ( $\alpha$ ), beta-hemolysis ( $\beta$ ), positive (+); negative (-), inconclusive (INC), hemolysins (HEM), lecithinase (LEC), protease (PT); gelatinase (GEL); biofilm (BF), hours (h), V. Index (Virulence Index).

loggerhead sea turtle's microbiota and the assessment of populations' conservation status [8, 28]. Also, Oliveira *et al.* [29] showed that similar collection and transport methods permit the isolation and characterization of bacteria, even when requiring large distances and processing periods.

In the present study, *Shewanella* sp. was the most prevalent species found in *Caretta caretta*, as previously reported by Blasi *et al.* [30]. Unexpectedly, no *Pseudomonas* sp. isolates were detected, despite *P. aeruginosa* being one of the most prevalent species isolated from sea turtles in previous studies [25, 31, 32].

All identified bacterial species have been previously isolated from both injured and stranded sea turtles, as well as healthy wild animals [30, 33–35], with the exception for *Brevundimonas vesicularis*, which was isolated for the first time from the oviductal fluid of sea turtles in this study.

*Aeromonas* sp., *Burkholderia* sp., *Vibrio* sp., *Citrobacter* sp. and *Morganella morganii* have been described as pathogens of loggerheads and other sea turtle's species [10, 18, 36, 37]. *Aeromonas hydrophila* and *Vibrio alginolyticus* have been associated with ulcerative stomatitis, ulcerative esophagitis, granulomatous hepatitis, granulomatous nephritis and bronchopneumonia [9, 13, 33, 38]. *Burkholderia cepacia* has been associated with lesions of the oral cavity of these animals including ulcerative stomatitis, and traumatic skin lesions. *Shewanella putrefaciens* has been regarded as a severe opportunistic bacterium [18, 39–42] and was described in one case of septicemia in green sea turtles (*Chelonia mydas*) with underlying fibropapillomatosis [12]. *Citrobacter* sp. has been implicated in cases of ulcerative oesophagitis and hepatitis and *Morganella morganii* was identified as a cause of conjunctivitis in tortoises [10, 13, 36, 38].

*Burkholderia cepacia*, *Shewanella putrefaciens* and *Vibrio alginolyticus*, isolated from the egg samples under study, were previously associated with unhatched eggs [39, 43, 44]. Craven *et al.* [39], suggested that environmental bacteria found on adult females can be potential opportunistic pathogens and a cause of embryonic mortality.

*Shewanella putrefaciens* and *Morganella morganii* were found in both the oviductal fluid and respective egg of one loggerhead female. This finding is in line with

the study by Wyneken *et al.* [43], in which the association between the bacterial species found in *Caretta caretta* females and the respective clutch was described. In the present study, eggs were collected directly from the cloaca during oviposition. Therefore, bacteria isolated from the egg's content should have their origin in the nesting female [31].

Compared to previous studies, the incidence of resistant isolates was low. The prevalence of bacteria resistant or intermediately resistant to one or more antibiotics was 68%, while other studies identified significantly higher values [8, 17, 25, 32]. No MDR bacteria were detected, which is in line with a previous study conducted in juvenile sea turtles' hawksbill (*Eretmochelys imbricata*) and green turtle (*Chelonia mydas*) from potential coincident feeding grounds [25], but discordant with previous studies in other *Caretta caretta* and *Chelonia mydas* populations [8, 17, 18, 31, 32]. Following previous results, higher resistance levels were observed for tetracyclines, and the lower ones for the aminoglycoside class [8, 17, 18, 30, 31]. To the best of our knowledge, no resistance to imipenem was previously described for loggerhead sea turtles' bacteria. Here, *Aeromonas* sp., *Brevundimonas vesicularis*, *Shewanella putrefaciens* and *Morganella morganii* presented intermediate resistance to imipenem, with *Morganella morganii* also showing intermediate resistance to meropenem. Regardless of the low incidence of resistance detected for the carbapenem class, this finding should be further assessed, due to the categorization of this antibiotic class as a last resort option for the treatment of serious Gram-negative infections, being of major importance for Human Medicine [45, 46].

The higher prevalence of resistance in isolates obtained from cloacal samples comparing with the ones from oral and egg samples could indicate that the cloaca can act as a favourable environment for the colonization of resistant bacteria.

Being mostly characterized by a pristine environment, the Island of Maio, is less affected by anthropogenic impacts, such as the discharge of wastewater carrying high levels of antibiotics, associated with aquaculture, intensive farms, and human and veterinary clinics and hospitals [30, 47–49]. Also, according to Eder *et al.* [50], the average CCL (76.7 cm) of the studied loggerhead females shows that these animals have expected oceanic feeding strategies, travelling to the oceanic settings off Mauritania, The Gambia and Senegal [51]. Sea turtles living in ecosystems affected by humans' activities are in higher risk of being exposed to antibiotic environmental pressure [18, 52]. Comparing with the studies performed in other loggerhead subpopulations, both feeding and nesting sites of the colony of the Island of Maio are less exposed to anthropogenic pressures [8, 17, 18, 25, 35], which suggests a low contact between the studied loggerheads and the tested antimicrobial compounds and explains the comparatively low prevalence of resistant isolates in this loggerhead subpopulation.

Virulence characterization showed that the isolates from this study can express virulence traits that may contribute for the evasion of the host immune system as well as host tissue colonization and damage [53]. The expression of a high number of virulence factors may play an important role in the pathogenesis of infections [53].

On one hand, higher virulence indices were detected for the Non-Enterobacteriaceae family (*Aeromonas* sp., *Brevundimonas vesicularis*, *Shewanella putrefaciens*), sug-



gesting the pathogenic potential of these species. On the other hand, *Vibrio alginolyticus* isolates showed the lowest virulence indices. Contrarily to our results, *V. alginolyticus* isolates from sea fishes along the Tunisian coast were previously described as being able to produce gelatinases, lipases, and  $\beta$ -hemolysins, also presenting high levels of antibiotic resistance [54]. Also, Zavala-Norzagaray *et al.* [24], identified virulent *Vibrio cholerae* strains in olive ridley sea turtles (*Lepidochelys olivacea*), referring to the epidemic potential of these bacteria. As mentioned before, *Vibrio alginolyticus* has been associated with numerous serious diseases in sea turtles [9, 13, 33, 38]. In this case, the low virulence index obtained for this bacterial species could indicate that these isolates developed a stable symbiosis with their turtle-host.

Virulence indices of isolates from cloacal samples were significantly higher compared to isolates from other samples, which may suggest that the cloacal and gastrointestinal environments host bacteria conserving a more complex virulence profile. These bacteria are potentially more pathogenic and with a higher survival fitness. Moreover, the virulence profile of isolates obtained from animals showing high parasitic loads (class III) was significantly higher when compared to isolates from individuals without evident cloacal parasites (class I and II). Parasites may exert selective pressures to modulate the genome of a bacterium or microbial population, and consequently prompt the expression of specific virulence determinants [55, 56]. Additionally, the high burden of gastrointestinal parasites can predispose to secondary infections by depressing the immune system, and consequently affecting sea turtles' health [23]. Therefore, in class III animals, there might be a higher risk of these bacterial species becoming opportunistic pathogens and induce disease.

In conclusion, it is important to assess potential factors that may suggest a deteriorated health status or underlying disease, and a consequent decreased immune response. These factors may include high parasite intensities (observed in class III animals), which can cause gastrointestinal and cloacal injuries, inflammatory reaction, and nutrient depletion [13, 23, 57]; and superficial lesions (i.e. erosions of the carapace) (observed in class II animals), probably indicating a previous traumatic event [33]. Also, different biological and ecological factors, including the migratory lifestyle and feeding behaviour characterizing different loggerhead subpopulations have a significant influence in both the microbial composition and endoparasitic communities of these animals [23, 58, 59].

A significant positive correlation was observed between MAR and virulence indices, which suggests an association between virulence and resistance. Beceiro *et al.* [60] described the relationship between mechanisms of antimicrobial resistance and virulence, indicating that antimicrobial resistance may increase the virulence or fitness of certain species by facilitating the colonization of new ecological niches and infection development. Furthermore, MAR index values were higher in biofilm-producer isolates, compared with non-biofilm-producers. In fact, biofilms can develop antibiotic resistance up to 1,000-fold greater than their free-swimming, planktonic counterparts [61, 62]. Biofilms can resist the effect of antibiotics compounds by multiple mechanisms, requiring a set of recognizable genetic determinants [62]. The increased antibiotic-resistance in biofilm bacteria makes these microbial communities extremely difficult to control [62, 63].

Bacteria identified in the present study may act as agents of zoonotic disease and represent an important threat to Public Health. In humans, *Aeromonas hydrophila/caviae* can cause gastroenteritis, septicemia, and skin and soft tissue infections [64]. *Burkholderia* sp. and *Enterobacter* sp. can be implicated in nosocomial infections [65]. *Vibrio alginolyticus* has been associated with ear and wound infections [66]. *Morganella morgani* has been described as a cause of urinary tract and wound infections, and neonatal sepsis [67]. *Brevundimonas* species have been identified in cases of bacteriemia [68]. Finally, *Shewanella putrefaciens* has been implicated in ears, skin, and soft tissue infections, with or without bacteriemia [69]. Biosecurity measures are, hence, required, particularly concerning volunteers and biologists interacting with sea turtles, working on nesting beaches, and handling eggs from natural nests [14]. Personal protective equipment (PPE) and hygiene practices have been proposed as effective and accessible options to decrease the risk of pathogen transmission [9]. Moreover, Mashkour *et al.* [9] revealed that sea turtles' health experts encourage preventive solutions for the reduction of the risk of Enterobacteriaceae and multi-resistant bacterial infections based on education and awareness initiatives.

Besides the existent conservation efforts, slaughter of females on the beaches of the Island of Maio and illegal harvesting of eggs are frequent [5]. Furthermore, in the Island of Maio, the increased number of stray dogs and cats contributes for a higher destruction of both nests in natural settings and protected hatcheries [4]. Sea turtles' eggs consumption by these animals can represent an additional factor for pathogenic and ARB dissemination, increasing the public health risk.

## Conclusion

The low incidence of potentially pathogenic bacteria and the low levels of antibiotic resistance in the studied loggerhead colony represent positive and encouraging results regarding the conservation status of this subpopulation. The low incidence of antibiotic resistance revealed by loggerhead sea turtles of Cape Verde, seems to indicate that the North-East Atlantic subpopulation and respective environment (migration routes, feeding and nesting areas) do not select for resistance dissemination. Nonetheless, the presence of potentially pathogenic Gram-negative bacteria expressing virulence factors may represent a risk to sea turtles' health and consequently affect the conservation of this endangered species. Furthermore, due to their virulence profile and zoonotic potential, aggravated by the unsafe and uncontrolled consumption of turtle-related products, the identified bacteria may represent a major risk for Public Health.

## Methods

This study aimed to characterize the cloacal, oral and egg content of Gram-negative aerobic microbiota of loggerhead sea turtles from the Island of Maio, to evaluate their antibiotic resistance and virulence profiles and to assess both the impact on sea turtles' conservation and the underlying public health risk resulting from interactions with these animals and the consumption of turtle-derived products.

### Area of study

Samples were collected from loggerhead sea turtles in the Island of Maio (15°13' 50''N 23°09' 22''W), of the archipelago of Cape Verde (14°48' 17''N, 22°42' 25''W), West Africa (1). The Island of Maio comprehends an area of 269 km<sup>2</sup>, and hosts loggerhead nesting activity along 38 km of sandy beaches throughout 110 km of coastline [4]. The area of study (1) included the coastal areas of “Pedro Vaz” (15°14' 52.2''N 23°06' 54.5''W) and “Praia Gonçalo” (15°15'25.9''N 23°06'34.5''W), namely “Praiona”, “Cozinha fácil” and “Areia Preta” beaches.

Figure 1: **Area of study.** **a** The archipelago of Cape Verde (14°48' 17''N, 22°42' 25''W) and **b** the Island of Maio (15°13' 50''N 23°09' 22''W) (red circle in **a**). The locality of “Praia Gonçalo” (15°15'25.9''N 23°06'34.5''W) is highlighted on the top circle and the locality of “Pedro Vaz” (15°14' 52.2''N 23°06' 54.5''W) is marked with the circle underneath. The maps were created with SEATURTLE.ORG Maptool. 2002 [70].

### Sample collection

A total of 33 nesting loggerhead sea turtles (*Caretta caretta*) was sampled, during August 2019. Oral, cloacal, and egg content samples were obtained from each female, using AMIES (without carbon) swabs 1814-002 (VWR<sup>TM</sup>). The three samples were collected sequentially, in the following order: cloacal, oral, and egg. For the cloacal sample, the swab was gently inserted approximately 5 cm into the cloaca and with a rotative movement, the material from the internal surface was collected. Oral sampling was performed by opening the rhamphotheca with a previously disinfected (ethylic alcohol 70%) wooden pry bar. The soft tissue of the mouth (tongue and palate) was gently swabbed for approximately 5 seconds. After the laying of a minimum of five eggs, an egg was collected directly from the cloaca without contacting with the surrounding environment. For the egg sample, a small surface of the shell was sterilized with a fire-heated bistoury and a circle shape window was cut. A sterilized Pasteur pipette was used to collect approximately 1.5 ml of the egg content, which was introduced directly in the transport medium of the AMIES swabs. The samples were identified with date, time, type of sample, and flipper tag number and then, safely placed in a thermic bag, at 4°C. After the sampling period, the collected samples were transported to the Microbiology and Immunology Laboratory of the Veterinary Faculty, University of Lisbon, Portugal, for further processing.

The following information was collected regarding each sample from each animal: date and time of sampling; local of sample collection; flipper tag identification number; curved carapace length (CCL); clutch size (n°. eggs laid); type of sample (cloaca, oral cavity and egg content). The animals were categorized regarding their health status as class I - good body condition, no evident lesions or disease, class II - external superficial lesions (i.e. superficial erosions of the carapace), and class III animals - high number of parasites in the cloaca or abnormal oviposition.

### Isolation and Identification of Gram-negative bacteria

After pre-enrichment in Buffered Peptone Water at 37°C for 24h, Gram-negative aerobic bacteria were isolated from collected samples using Glutamate Starch Red Phenol (GSP) Agar plates supplemented with 100,000 UI penicillin g/L (Merck) and MacConkey Agar (Oxoid), incubated at 37°C for 24–48 h [17, 71]. Positive bacterial colonies were isolated in Columbia agar supplemented with 5% sheep blood (Oxoid). Isolates were characterized regarding their macro and microscopic morphology, Gram staining, catalase test and oxidase reaction. Gram-negative bacilli were selected for further characterization, including identification through the biochemical identification galleries API 20E and 20NE (bioMérieux), according to the manufacturer's instructions.

### Evaluation of isolates' antibiotic resistance profile

Isolates' susceptibility profile regarding 12 different antibiotics commonly used in veterinary and human medicine and belonging to different classes was determined using the disk diffusion method, in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [72]. The tested antibiotics (Oxoid and MASTDISCS) were: amikacin (30 µg), cefoperazone (75 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), enrofloxacin (5 µg), gentamicin (120 µg), imipenem (10 µg), meropenem (10 µg), ofloxacin (5 µg), piperacillin (100 µg), tetracycline (30 µg) and tobramycin (10 µg), as described elsewhere [17, 73]. The reference strain *Escherichia coli* (ATCC®25922™) was used as quality control. The inhibition zones were measured and isolates were scored as susceptible, intermediate, and resistant, according to the CLSI guidelines [72].

### Evaluation of isolates' virulence profile

Isolates were characterized regarding their phenotypic virulence profile, by assessing the production of enzymes associated with bacteria pathogenic potential.

DNase activity was evaluated using DNase Agar supplemented with 0,005% methyl green (VWR™). Isolates expressing extracellular nuclease activity, produced a clear zone on the DNase test agar, following incubation for 24h at 25°C [53, 74]. *Aeromonas hydrophila* (ATCC® 7966™) and *Escherichia coli* (ATCC®25922™) were used as positive and negative controls, respectively.

Hemolysins production was evaluated using Columbia Agar with 5% sheep blood (bioMérieux). After incubation for 24h at 25°C,  $\alpha$ -hemolytic bacteria partially lysed erythrocytes, producing a yellowish-green colour of the area surrounding the colonies, while  $\beta$ -hemolytic bacteria completely lysed erythrocytes, which resulted in a clear zone surrounding the colonies [75].

Lecithinase activity was determined using Tryptic Soy Agar (VWR™) supplemented with 10% egg yolk emulsion (VWR™). Positive and negative controls, *Pseudomonas aeruginosa* (ATCC®27853™) and *Escherichia coli* (ATCC®25922™) respectively were used. Positive isolates for lecithinase production were identified through the presence of a white, opaque, diffuse precipitation area around the colonies, following incubation for 24h at 25°C [76, 77].

Gelatinase activity was detected using Nutrient Gelatin Agar (Oxoid). This test is applied to evaluate the ability of an isolate to produce proteolytic enzymes (gelatinases) that liquefy gelatin [74]. Positive gelatinase activity was detected by partial

or total liquefaction of the inoculated medium after incubation at 4°C for 30 min, following incubation for 24h at 25°C. *Pseudomonas aeruginosa* (ATCC®27853™) and *Escherichia coli* (ATCC®25922™) were used as positive and negative controls, respectively [74, 78].

Biofilm production ability was assessed using Congo Red Agar Plates, composed by Brain Heart Infusion broth (VWR™), Bacteriological Agar (VWR™) and Red Congo reagent (Sigma-Aldrich). Positive and negative controls, *Enterococcus faecium* (ATCC®35667™) and *Escherichia coli* (ATCC®25922™) respectively were used. After incubation for 24h at 25°C, isolates producing dark red or black colonies, with crystalline or dry consistency, were identified as positive biofilm producers [79–81].

Protease activity was analysed resorting to Skim Milk agar (Oxoid), using *Pseudomonas aeruginosa* (ATCC®27853™) and *Staphylococcus aureus* (ATCC®29213™) as positive and negative controls, respectively. After incubation for 24h at 25°C, isolates showing a clear halo around the colonies were classified as positive protease producers [77, 82–84].

Lipase activity was tested using Spirit Blue agar (Difco™) added with Tween® 80 (Sigma-Aldrich) and olive oil, using *Pseudomonas aeruginosa* (ATCC®27853™) and *Staphylococcus aureus* (ATCC® 29213™) as positive and negative controls, respectively. The lipolytic activity was identified by the presence of a clear zone around the colony, after incubation for 24h at 25°C [85].

### Statistical analysis

Isolation and characterization results were analyzed 1) to assess differences between bacterial diversity and sample types (oral, cloacal, and egg content); 2) to test possible tendencies between antibiotic resistance and virulence profiles of bacterial species isolated from different sample types.

The MAR (multiple antibiotic resistance) indices (n°. antibiotics to which isolates were resistant/ n°. antibiotics tested) and the virulence indices (n°. positive virulence factors/ n°. virulence factors tested) were calculated for the isolates obtained [86, 87].

All statistical analyses were performed using IBM® SPSS® Statistics v.25.0 for Windows.

A Chi-square test was performed to test the differences between the number of positive samples and the sample type (cloaca, oral cavity and egg content) and the animal's health status (class I, II, III). As the variables health status, place of sampling, bacterial family, MAR index and virulence index did not follow a normal distribution, for evaluating the differences between the MAR and virulence indices of isolates and the sample type and sampled animal (health status classes), a nonparametric Kruskal-Wallis test was applied. The differences in MAR index and virulence index values among isolates from distinct bacterial families (Enterobacteriaceae and Non-Enterobacteriaceae) were analysed using the nonparametric Mann-Whitney U-test. This test was also applied to evaluate the differences in MAR index values between biofilm-producers and non-biofilm-producers isolates. The correlation between MAR index and virulence index was analysed with the Spearman correlation test. Significant differences were calculated at 0.05 (two-tailed) levels of significance.

### Abbreviations

ARB: antibiotic-resistant bacteria; AMR: antimicrobial resistance; API: Analytical Profile Index; BF: biofilm; CAZ: ceftazidime; CCL: curved carapace length; CFP: cefoperazone; CIP: ciprofloxacin; CLSI: Clinical and Laboratory Standards Institute; E: Enterobacteriaceae; ENR: Enrofloxacin; GEL: gelatinase; GSP: Glutamate Starch Red Phenol; HEM: hemolysins; IMP: Imipenem; IUCN: International Union for Conservation of Nature; LEC: lecithinase; MAR: multiple antibiotic resistance; MDR: multidrug-resistance; MEM: meropenem; NE: Non-Enterobacteriaceae; PIP: piperacillin; PPE: personal protective equipment; PT: protease; T: tetracycline; V. Index: Virulence Index; WHO: World Health Organization.

### Declarations

Ethics approval and consent to participate

This study did not involve human subjects, animal experiments or collection of specimens, and none of the procedures implicated the disturbance of the animal natural behaviour. The handling time did not exceed 5 minutes, before and after which the animals were observed from a safe distance to ensure that oviposition proceeded normally. Collection of samples was conducted under Maio Biodiversity Foundation guidelines and by the permits of the Environmental National Authority DNA (Direção Nacional do Ambiente). Research protocols were performed per the IUCN Policy Statement on Research Involving Species at Risk of Extinction [88], approved by the 27th Meeting of IUCN Council, Gland Switzerland, 14 June 1989, and the Sea Turtle Research Techniques Manual [89].

Consent for publication

Not Applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

MF, JPM, MO designed the study and respective protocols. MF performed the sampling procedures and laboratory techniques. JPM supervised the sampling processes, and MG, EC, CC and MO assisted in the laboratory practices. MF analysed and interpreted the data obtained in the present study, guided by MO. All authors read and approved the final manuscript.

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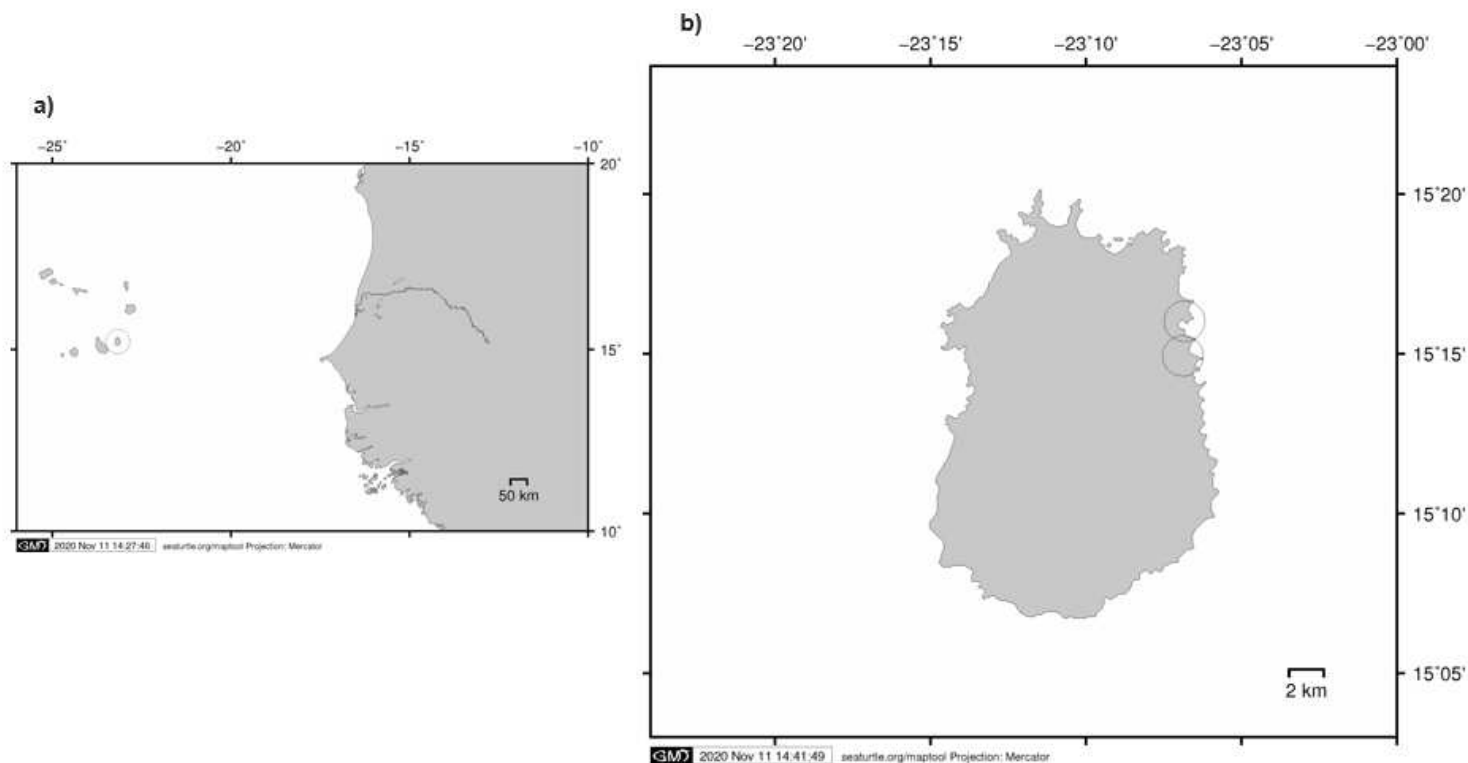
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# Figures



**Figure 1**

Area of study. a The archipelago of Cape Verde ( $14^{\circ}48' 17''18''N$ ,  $22^{\circ}42' 25''8''W$ ) and b the Island of Maio ( $15^{\circ}13' 50''N$   $23^{\circ}09' 22''W$ ) (red circle in a). The locality of “Praia Gonçalo” ( $15^{\circ}15'25.9''N$   $23^{\circ}06' 34.5''W$ ) is highlighted on the top circle and the locality of “Pedro Vaz” ( $15^{\circ}14' 52.2''N$   $23^{\circ}06' 54.5''W$ ) is marked with the circle underneath. The maps were created with SEATURTLE.ORG Maptool. 2002 [70]. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.