UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF GRAM-NEGATIVE BACTERIA ISOLATED FROM LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*) OF THE ISLAND OF MAIO, CAPE VERDE

MATILDE COSTA FERNANDES

ORIENTADORA:
Doutora Maria Manuela Castilho Monteiro de Oliveira

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ANTIBIOTIC RESISTANCE AND VIRULENCE PROFILES OF GRAM-NEGATIVE BACTERIA ISOLATED FROM LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*) OF THE ISLAND OF MAIO, CAPE VERDE

Abstract

Loggerhead sea turtles (*Caretta caretta*) have been suggested as carriers of potential zoonotic pathogens and prime reservoirs of antibiotic-resistant and virulent bacteria. In the present study, the isolation of Gram-negative bacteria of the Cape Verdean loggerhead subpopulation, currently believed to be the largest subpopulation of this species worldwide, is described for the first time.

This study aimed to characterize the aerobic and facultative anaerobic Gram-negative bacteria of the loggerhead colony of the Island of Maio, to evaluate their pathogenic potential and to unveil both the impact on sea turtles' conservation and the underlying public health risks resulting from interactions with these animals and the consumption of turtle-derived products. Cloacal, oral and egg content swab samples from 33 nesting loggerheads (n = 99) were analysed regarding the presence of Gram-negative bacteria and the respective antibiotic resistance and virulence profiles. Conventional bacteriological techniques were applied.

Shewanella putrefaciens (26.32%), *Vibrio alginolyticus* (21.05%) and *Morganella morganii* (21.05%) were the most prevalent species. A low prevalence of antibiotic-resistant bacteria (15.79%) was detected, and no multidrug-resistant isolates were identified. The identified bacterial species revealed the ability to produce numerous virulence factors, including hemolysins (100.0%), DNases (89.47%), lipases (78.95%), biofilms (73.68%), proteases (52.63%), lecithinases (21.05%), and gelatinases (15.79%).

These findings suggest that due to the low anthropogenic impact observed in both their nesting (the Island of Maio) and foraging sites, this loggerhead subpopulation may be less exposed to antimicrobial compounds. Furthermore, Gram-negative bacteria isolated from these turtles may be less susceptible to acquiring resistance genes from environmental bacteria via horizontal gene transfer. Nevertheless, the presence of potentially pathogenic bacteria expressing virulence factors may threat both sea turtles' and human's health.

Key words: *Caretta caretta*, Island of Maio, antibiotic resistance, virulence factors, One Health

PERFIS DE RESISTÊNCIA A ANTIBIÓTICOS E VIRULÊNCIA DE BACTÉRIAS GRAM-NEGATIVAS ISOLADAS DE TARTARUGAS MARINHAS COMUNS (*CARETTA CARETTA*) DA ILHA DO MAIO, CABO VERDE

Resumo

A tartaruga-marinha-comum (*Caretta caretta*) é conhecida por ser portadora de agentes potencialmente patogénicos e zoonóticos, e um relevante reservatório de bactérias virulentas e resistentes aos antibióticos. O presente estudo descreve, pela primeira vez, o isolamento de bactérias Gram-negativas da subpopulação de tartarugas-comuns de Cabo Verde, a qual se estima ser a maior população mundial desta espécie.

Este estudo teve como objectivo a caracterização de bactérias Gram-negativas aeróbias e anaeróbias facultativas da colónia de tartarugas-comuns da Ilha do Maio, a avaliação do seu potencial patogénico e o respectivo impacto na conservação de tartarugas marinhas e do potencial risco para saúde pública, resultante de interações com estes animais e do consumo de productos derivados de tartarugas. Neste trabalho foram analizadas amostras de zaragatoas da cloaca, cavidade oral e ovos de 33 tartarugas comuns fêmeas (n = 99), de modo a isolar bactérias Gram-negativas e os respectivos perfis de resistência a antibióticos e virulência. Para o efeito, foram usados métodos de bacteriologia convencionais.

As espécies isoladas mais prevalentes foram *Shewanella putrefaciens* (26,32%), *Vibrio alginolyticus* (21.05%) e *Morganella morganii* (21.05%). Foi detetada uma baixa prevalência de bactérias resistentes (15.79%), e não foram identificados isolados multirresistentes. As espécies bacterianas identificadas revelaram a capacidade de produzir vários factores de virulência, incluindo hemolisinas (100.0%), DNases (89.47%), lipases (78.95%), biofilmes (73.68%), proteases (52.63%), lecitinases (21.05%), gelatinases (15.79%).

Os resultados deste estudo sugerem que, devido ao reduzido impacto antropogénico observado nos locais de nidificação (Ilha do Maio) e de alimentação da subpopulação em estudo, esta estará menos exposta a compostos antimicrobianos. Além disso, bactérias Gram-negativas isoladas neste estudo, poderão estar menos suscetíveis à aquisição de genes de resistência provenientes de bactérias ambientais, via transferência horizontal de genes. Contudo, a presença de bactérias potencialmente patogénicas e com a capacidade de expressar diversos factores de virulência representa uma ameaça tanto à saúde destes animais como à saúde humana.

Palavras-chave: *Caretta caretta*, Ilha do Maio, resistência a antibióticos, factores de virulência, Uma Só Saúde.

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List of abbreviations, acronyms and symbols

ABR – antibiotic resistance
AMR – antimicrobial resistance
AOO – area of occupancy
API – Analytical Profile Index
ARB – antibiotic-resistant bacteria
ARGs – antibiotic-resistant genes
BF – biofilm
BP – bronchopneumonia
BPW – Buffered Peptone Water
°C – Celsius degrees
CAZ – ceftazidime
CCL – curved carapace length
CFP – cefoperazone
CFU – colony-forming unit
CIP – ciprofloxacin
CITES – Convention on International Trade in Endangered Species
CLSI – Clinical and Laboratory Standards Institute
cm – centimetres
CAT – catalase reaction
COS – Columbia agar supplemented with 5% sheep blood

CPR – Cardiopulmonary Resuscitation

DNA – Deoxyribonucleic acid

DNase – Deoxyribonuclease

E – Enterobacteriaceae

e.g. – *Exempli gratia*

ENR – enrofloxacin

EPS – extracellular polymeric substances

FED – focal erosive dermatitis

FMB – Maio Biodiversity Foundation

FMV – Faculdade de Medicina Veterinária

GEL – gelatinase

GN-B – Gram-negative bacilli

GPS – Global Positioning System

GSP – Glutamate Starch Red Phenol

g/L – gram per litre

h – hour

H – test statistic for the Kruskal Wallis test

HEM – hemolysins

HGT – horizontal gene transfer

I – intermediate

IBM – International Business Machines

IMP – imipenem

IMViC – Indole, Methyl red, Voges-Proskauer, Citrate

IUCN – International Union for Conservation of Nature

k – growth coefficient

km – kilometres

L – mean asymptotic carapace length

L_0 – initial carapace length

L(*t*) – carapace length at age t

LEC – lecithinase

MAC – MacConkey

MAR – multiple antibiotic resistance

MDR – multidrug-resistant

MEM – meropenem

ml – millilitre

N – North

Na – sodium

NE – Non-Enterobacteriaceae

OR – obstructive rhinitis

OX – oxidase test

p – level of significance

PD – papillary dermatitis

PIP – piperacillin

PIT – Passive Integrated Transponder

PPE – Personal protective equipment

PT – protease

r – correlation coefficient

R – resistant

RH – relative humidity

RMU – regional management units

SCUD – septicaemia cutaneous disease

SCW – straight carapace width

sp. – species

T– tetracycline

™ – Trademark

U – test statistic for the Mann-Whitney test

ULisbon – University of Lisbon

US – ulcerative stomatitis

VBGF – Von Bertalanffy (1938) growth function

V. Index – Virulence Index

VSF – Veterinários Sem Fronteiras

W – West

WHO – Word Health Organization

yrs – years

< – less than

/ – division

% – percentage

µg – microgram

® – registered trademark

σ – standard deviation

Chapter 1 – Description of The Traineeship Activities

1.1. Toucan Rescue Ranch (Wildlife facility):

The curricular internship was held in the wildlife facility "Toucan Rescue Ranch", San José, Costa Rica, for six months, 25 weeks (early October 2019 – end of March 2020).

The department assigned was the Headquarters' and Release Site's clinics, which activities consisted of the performance and management of the medical activities. Work was also performed in distinct departments, including the animal husbandry department, the enrichment and education programs and the breeding and release programs.

In the clinic department, the daily tasks included intensive care of the hospitalized animals, specialized care of the two-toed and three-toed sloths (*Choloepus hoffmanni* and *Bradypus variegatus*) under the rehabilitation program, and enrichment training of the infant and juvenile sloths. The following tasks included the monitoring routines, comprising all the animals in the facility, in which the animals' general condition, behaviour, presence of signs of disease, discomfort or stress, as well as diets and the conditions of the enclosures were closely examined. The third set of tasks included treatments, as well as physiotherapy and exercise sessions. Nocturnal animals required specific tasks in different schedules.

I worked with a wide range of different species, including reptiles (iguanas, turtles, lizards), mammals (opossums, sloths, anteaters, wild pigs, squirrels, porcupines, monkeys, tayras, ocelots, oncillas, kinkajous), and birds' species (hummingbirds, parrots, toucans, raptors, including hawks and owls, and several species of Passeriformes).

The clinic work involved the following areas: general clinics (preventive medicine, nutrition and diet formulation, treatments), intensive care, neonatology, surgery, pathology, and physiotherapy and rehabilitation.

In a first approach, the practice involved the patients' intake procedures and a general clinical examination. The protocols differed from different species and animals' condition. Evaluation of weight, body condition score, hydration status, presence of parasites, presence and severity of injuries, level of awareness and responsiveness to stimuli, and auscultation of the heart and lungs were consistently performed. Additionally, in birds, flying tests, grip and strength of the beak and feet, condition of the feathers were evaluated, and, in mammals, the status of the hair coat and mucous membranes were analysed. The presence and level of pain were added to the exam for mammals. The following steps involved the selection of eventually required complementary diagnostic exams and the formulation of differential diagnosis.

During the practice, I performed several procedures regarding complementary diagnostic exams, including blood samples collection (for biochemical and hematologic exams and analysis of hemoparasites) and the analysis of respective results, coprological exams (on-

site) and sample collection for bacteriological examination (external laboratory). Other techniques were performed, such as tube feeding in reptiles and small mammals and tracheal washes in parrots. Regarding the radiographic examination, I assisted in the positioning of the patients and analysed the results. I also performed a complete abdominal ultrasound examination and ultrasonography for pregnancy detection.

The treatments I performed and oversaw included management of wounds, nebulization procedures, burnt protocols, pain management protocols, election and control of fluid therapy, and drug administration.

Emergency and urgency procedures played a prominent role in daily clinical practice and traineeship activities. I oversaw all emergencies during the internship, included the following procedures: cardiopulmonary resuscitation (CPR), the reversal of hypoglycaemic shocks, oxygen therapy, haemorrhage control, and pain control.

Surgeries in which I participated were mostly limbs' amputation procedures in birds and mammals. One orthopaedic surgery was executed by an invited surgeon. We also performed an adult tayra's (*Eira barbara*) orchiectomy. In the surgical team, I held the position of second-hand surgeon and anaesthetist, being responsible for the pre-operative procedures as well as the short-term and long-term post-operative care.

I completed full training in the Neonatology department, in which I provided medical care and hand-raised animals from several species (opossums, squirrels, sloths, porcupines, and owls). The physiotherapy and rehabilitation activities were performed alongside specialists for the recovering of severely traumatized and injured animals. Preventive medicine procedures included the formulation and performance of deworming protocols. I also conducted full necropsies of mammals, reptiles, and birds.

During the practice, I collected swab samples (n = 78) for bacteriological analysis integrated into a research project on antibiotic-resistant Enterobacteriaceae of two-toed and three-toed sloths (*Choloepus hoffmanni* and *Bradypus variegatus*), developed in collaboration with the Laboratory of Bacteriology of FMV/ ULisbon.

Finally, during the internship, I attended different workshops, meetings, and lessons to further my knowledge in wildlife medicine and conservation. During the practice, I had the opportunity to work with specialists in wildlife medicine, wildlife conservation, wildlife neonatology, feline medicine, orthopaedics, ophthalmology, and sloths' rehabilitation and conservation.

1.2. Maio Biodiversity Foundation (FMB) – Cape Verde, Veterinários Sem Fronteiras – Portugal

Considering the present study, a one-month extracurricular internship (August 2019) was held on the Island of Maio, Cape Verde.

The objectives of the internship included the collection of samples from loggerhead sea turtles (*Caretta caretta*) for bacteriological analysis, as well as integrating the scientific team of FMB in the loggerhead sea turtles' conservation program.

The assigned work was a 10-hour night shift, six days a week, which included patrolling several beaches for nesting sea turtle's monitoring and protection. The turtles were evaluated regarding their curved carapace length (CCL), straight carapace width (SCW), GPS coordinates, identification of diseases or anomalies, presence of parasites, risk of predation, clutch size and nest characteristics. Also, the collection of material for parasitological and bacteriological examination for research purposes was performed. If not present, a metallic flipper-tag and a subcutaneous Passive Integrated Transponder (PIT) tag were applied.

If the nests were built in highly threatened areas (high risk of predators, floods due to high tides, litter), we would relocate them to a safe, natural place or a hatchery. The number of eggs, the female identification, and the hatchery characteristics were registered. During the internship, I performed and supervised a loggerhead male necropsy and assisted female turtles' rescues.

Together with FMB and VSF - Portugal, an awareness campaign entitled "Vamos cuidar deles? O meu cão, meu guarda, meu amigo!" was organized and delivered. The campaign included two different activities developed with the young population of the Island, which consisted of short lessons and ludic activities (colouring of informative brochures) on different topics regarding animals' and population's health, including control of populations, the importance of vaccination and deworming, medical care and appropriate diet.

1.3. Laboratory of Bacteriology in FMV-ULisbon

An eight-month internship was held in the Laboratory of Bacteriology to achieve a master's degree in veterinary medicine. Several techniques were performed, including collection, packaging and transportation of biological samples; preparation of culture media, inoculation of plates, observation of bacterial colonies, macro and microscopic characterization, through Gram-staining and metabolic tests; phenotypic identification assays (Analytical Profile Index (API) and Indole, Methyl red, Voges-Proskauer, Citrate test (IMViC)), disk diffusion methods and phenotypic plaque assays. The tasks performed are detailed in Chapter 3 "Materials and Methods".

Chapter 2 – Introduction

2.1. Sea Turtles – characterization of the superfamily Chelonioidea

Sea turtles are marine reptiles that present both the individual adaptations essential for life at sea and the evolutionary pathway of organisms bound to the land and open atmosphere (Witherington 2017).

Life at sea required sea turtles to develop unique morphological and physiological characteristics and adaptations. Their flippers are characterized by paddle-like forelimbs and rudder-shape hind limbs, each with extended phalanges. Sea turtles have a non-retractile head, which, together with the limbs and shell, presents a streamlined, hydrodynamic shape (Pritchard 1997; Witherington 2017). Comparing to other reptiles, sea turtles have a large adult body size, which is indicated as an important feature to prevent predation by fish and promote efficient locomotion in the ocean currents (Williard 2013; Witherington 2017). Sea turtle's body size also allows the retention of body heat in the active tissues (Standora et al. 1982), which is described as gigantothermy (Paladino et al. 1990). The higher body temperature comparing to ambient water temperature may have a beneficial role on several metabolic requirements (Witherington 2017). The shell also has insulator properties (Standora et al. 1982).

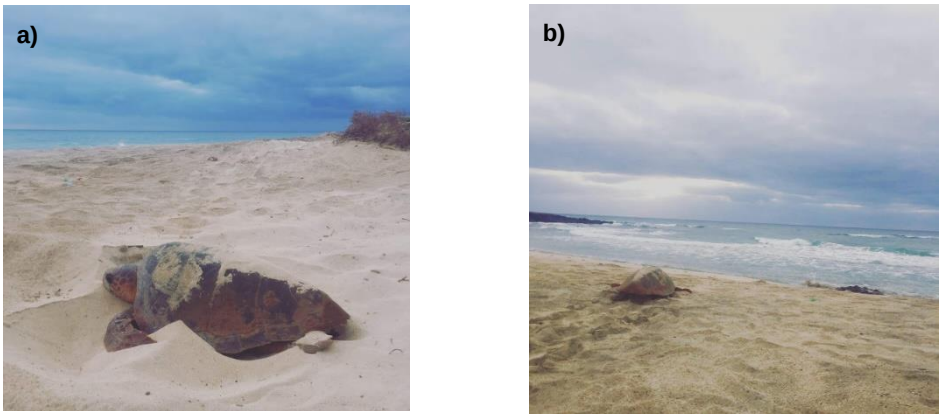
Sea turtles have several adaptations to manage the demands of deep diving, including flexibility of blood flow by cardiac shunting, cerebral resistance to anoxia, and compliant shell walls to prevent "thoracic squeeze" during lung collapse (Berkson 1966; Lutz et al. 1980; Hicks and Wang 1996; Lutcavage and Lutz 1997). Due to life at sea, sea turtles must control water and salt balance. Sea turtles have a large lacrimal (salt) gland dorsal and medial to the eye responsible for excreting the excess of salt. Sea turtles also control salt intake by trapping ingested food in the well-developed sharp and keratinized papillae lining the oesophagus, which allows the turtle to expel water and salt before swallowing (Wyneken 2001; Witherington 2017).

Turtles, and other reptiles, are amniote vertebrates with the embryo developing within four extraembryonic membranes that offer protection, retain energy, provide hydration, and store waste products (Laurin 2005; Witherington 2017). Moreover, the pliable shell of the sea turtles' egg functions as optimal protection for the embryo (Tracy and Snell 1985; Witherington 2017).

2.2. Loggerhead sea turtles

2.2.1. Phenotypic characterization

Loggerhead sea turtles' phenotype is characterized by their large head comparative to their body size (Witherington 2017) (Figure 1). The carapace has an elongated heart profile with a domed-shape anterior portion and an arched posterior portion that extends at a marked sacral hump. A typical loggerhead carapace is composed of non-overlapping scutes, including five pairs of costals, five vertebrals, and a nuchal scute in connection with the first pair of costals. The connection between the plastron and the carapace is made by three pairs of inframarginal scutes (Witherington 2017). The juvenile and adult carapace is characterized by a colour that varies from mahogany to red-brown, and the colour of the plastron extends from cream-white to yellow (Witherington 2017). The carapace may be covered by the growth of commensal organisms, including barnacles, hydroids, and macroalgae. Orange to brown scales with yellowish margins cap the dorsal head and flippers (Witherington 2017). There are two pairs of prefrontal scales between the eyes that often have one or two intervening scales. Each limb has two claws (Witherington 2017). Adult female size varies from 70 to 170 kg and 65 to 113.5 cm curved carapace length (CCL). Mature loggerhead sea turtles from the Mediterranean Sea are smaller than the Pacific populations and, in succession, these loggerheads are smaller than the Atlantic ones (Margaritoulis et al. 2003; Ballell-Valls and López Jurado 2004; Piovano et al. 2011; Marco et al. 2011; Witherington 2017).



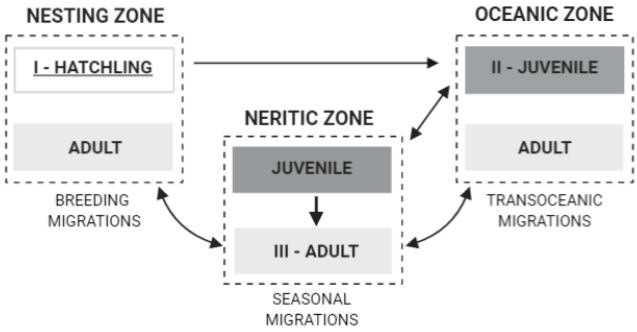
Legend: a) Loggerhead sea turtle from the Island of Maio. Note the large head in comparison with the body size and the orange to brown scales covering the head and flippers; **b)** Female loggerhead turtle returning to the sea after nesting activity.

Figure 1 – **Loggerhead sea turtle (Original).**

2.2.2. Habitat and lifecycle

Loggerheads' habitat extends through temperate and tropical regions of the Mediterranean Sea and the Atlantic, Pacific, and Indian Oceans (Witherington 2017). Insular and mainland sandy beaches represent the nesting sites of loggerhead females, while the foraging sites encompasses coastal, estuarine, and continental shelf waters (Bolten et al. 1998, Witherington 2017).

Understanding loggerheads' lifecycle is crucial for the comprehension of their ecological role in the marine ecosystem, as well as their feeding behaviour, reproductive ecology and potential interactions with human populations (Figure 2).



Legend: The zones (nesting zone, neritic zone and oceanic zone) and stages (I – hatchling, II – juvenile, III – adult) of loggerhead's lifecycle.

Figure 2 – Loggerhead sea turtle's lifecycle (Original).

As the lifecycle begins, thousands of hatchlings leave the nesting beach and enter an oceanic phase, the juvenile stage (Figure 2). The oceanic stage begins when the turtles enter the oceanic zone. Loggerhead juveniles from specific subpopulations may undertake transoceanic migrations (Witherington 2017). The duration of the oceanic juvenile stage ranges between 6.5 and 11.5 years (Bjorndal et al. 2000; Bolten and Witherington 2003).

The neritic juvenile stage and adult foraging stage occurs in the neritic zone, where both juveniles and adults can complete extensive seasonal migrations (Witherington 2017) (Figure 2). This phase allows loggerheads to forage and grow until maturity (Witherington 2017).

Loggerhead sea turtles, complete maturity with an average age of 36-38 years for females and 37-42 years for males (Avens et al. 2015). Expected adult stage duration (post-maturation longevity) varies from 4 to 46 years, with a mean of 19 years, for both genders (Avens et al. 2015).

After reaching sexual maturity, adults initiate strategic breeding migrations between foraging grounds and nesting sites (Witherington 2017) (Figure 2). Both males and females may navigate through oceanic zones travelling hundreds to thousands of kilometres (Plotkin

2003). The average length of remigration intervals is 2.5-3 years for females (Shroeder et al. 2003), while males complete shorter remigration intervals (Hays et al. 2010). In the non-breeding season, adults locate most of the time in coastal neritic feeding areas that may coincide with juvenile migration paths (Bolten and Witherington 2003; Casale and Marco 2015).

2.2.3. Diet and foraging behaviour

Loggerhead sea turtles are primarily carnivorous marine reptiles (Bjorndal 1997) whose diet varies through different geographic locations and temporal stages of the lifecycle.

In the first few days of active swimming, the post-hatching yolk supports the metabolic requirements of the hatchlings (Kraemer and Bennett 1981). When in the oceanic stage, post-hatchlings and young juveniles feed on sargassum-community associates, primarily marine animals (e.g., hydroids, copepods and pleuston), marine plants (predominantly pelagic Sargassum) and flying insects (Witherington 2002; Witherington et al. 2012).

Surface-pelagic juveniles (1-25 kg) (oceanic stage juveniles) are opportunistic carnivores which diet bases on a variety of oceanic and pelagic organisms, including jellies, fishes, salps, gastropods, crustaceans, barnacles and pelagic coelenterates (Bjorndal 1997; Frick et al. 2009; Witherington 2017).

Larger juvenile and adult loggerheads in the neritic zone feed on benthic invertebrates, including molluscs, crabs, sea pens and jellyfish. They also feed on marine plants and sea horses (Plotkin et al. 1993; Bjorndal 1997; Frick et al. 2009). Neritic loggerhead turtles have been described as larger than the oceanic turtles, possible due to the richness of nutritive benthic fauna in neritic feeding grounds compared to oceanic feeding sites (Hawkes et al. 2006; Eder et al. 2012).

Loggerheads' diet is widely extensive and diverse, which demonstrates the versatile foraging behaviour of this species (Plotkin et al. 1993). Furthermore, loggerhead sea turtles represent an exceptional model of co-evolution between a marine vertebrate and its intestinal bacteria, mainly influenced by their diet and foraging behaviour (Biagi et al. 2018). Biagi et al. (2018) found that loggerhead turtles share more gut microbiota characteristics with marine mammals (e.g., dolphins and seals) then with the herbivorous green turtle (*Chelonia mydas*). This fact suggests that there is an adaptive function of the gut microbiota to diet and the foraging sites' ecosystem.

2.2.4. Loggerhead sea turtles and Environmental Health

Loggerhead turtles have an important role in maintaining the marine ecosystems' health by transporting essential nutrients and energy between marine and coastal areas and maintaining the structure of coral reef ecosystems and seagrass beds (Bouchard and Bjorndal 2000; Bjorndal and Jackson 2003). By adding nutrients into the ecosystems of the nesting sites, loggerhead sea turtles may contribute to maintaining stable dune structures that are crucial to their reproductive output (Bouchard and Bjorndal 2000). Also, loggerhead sea turtles are considered valuable sentinel species among sea turtles. The health status of these animals can provide important information regarding the status of the marine environment. Therefore, *Caretta caretta* is an important model species for the study of both animal and environmental health (Foti et al. 2009).

2.2.5. Conservation status

Loggerhead sea turtles' populations have decreased from their historic abundance. The well-known nesting populations are either declining, stable, or increasing. However, data on many other populations is incomplete (Witherington 2017). Loggerhead sea turtles are protected under the Convention on International Trade in Endangered Species (CITES 2019), Appendix I, throughout their distribution.

The sea turtle's population has been highly threatened by human activities. Therefore, knowing the mechanisms and extent of the anthropogenic impact on sea turtle's population is mandatory for the success and efficacy of conservation projects (Lutcavage et al. 2003; Biagi et al. 2018).

2.2.6. Threats to sea turtles' conservation

Sea turtles suffer from ongoing threats to their survival. Bycatch mortality from trawl, gillnet, longline, and other fisher activities represent the highest threat for loggerhead sea turtles. Habitat loss due to reformation of coastal environments (coastal armouring, beach alteration, mass tourism) represents the second main cause of population decline (Wallace et al. 2011; Casale and Marco 2015; Witherington 2017). Additionally, artificial lighting is responsible for disorientation and hatchling mortality. The third major challenge for the conservation of this species is the direct capture of turtles and eggs for human interest (Witherington 2017). Furthermore, marine pollution can threat loggerheads survival through ingestion of debris, including plastic, or entanglement. Climate change has a significant impact on the rise of sea level, the increase in storm intensity and frequency, and hatchlings sex ratios due to the increase in sand temperature (Wallace et al. 2011; Casale and Marco 2015; Witherington 2017). Although the main mortality causes for loggerhead sea turtles are

associated with direct and indirect anthropogenic activities, sea turtles can also be affected by infectious diseases, particularly fibropapillomatosis, pneumonia, hepatitis, meningitis and septicemia (Óros et al. 2005; Oliveira et al. 2017). Improved efforts to determine and control the effects of these threats to loggerheads should be highly prioritized for future conservation plans.

2.3. The North-East Atlantic subpopulation

All nesting sites of the North-East Atlantic subpopulation are located in the archipelago of Cape Verde (Casale and Marco 2015).

Until recently, it was thought that the Cape Verdean loggerhead nesting subpopulation was the second largest population of this species in the Atlantic and the third worldwide, after Florida, Western North Atlantic subpopulation (83,717 nests per year) and Oman, Northwest Indian subpopulation (70,000 nests per year) (Marco et al. 2011; Casale and Tucker 2017). However, recent data revealed that Cape Verde (North-East Atlantic subpopulation) might be the largest loggerhead nesting subpopulation worldwide, estimating an average of 95,762± 55,038 nests between 2016 and 2017 (Patino-Martinez et al. In Press).

The North-East Atlantic subpopulation has been classified as an isolated genetic pool (Monzón-Argüello et al. 2010). This subpopulation represents a regional management unit (Wallace et al. 2011), genetically distinct from other loggerhead subpopulations and, therefore, requiring management strategies on a regional level (Monzón-Argüello et al. 2010; Wallace et al. 2010; Stiebens et al. 2013; Casale and Marco 2015). Loggerhead sea turtles comprehend ten biologically characterized regional management units (RMUs) (Wallace et al. 2010).

Comparing with the other loggerhead subpopulations, the area of occupancy (AOO) of the Cape Verdean subpopulation (based on nesting sites) is small (<500 km²), and the number of locations limited (<5). In this context, the subpopulation is classified in the category Endangered of the International Union for Conservation of Nature (IUCN) Red List, with the potential to become critically endangered in a short period (Casale and Marco 2015).

2.3.1. Loggerhead sea turtles of the archipelago of Cape Verde and the Island of Maio

Loggerheads are the most important Cape Verdean sea turtle, being the only species nesting in the archipelago (Martins et al. 2013).

Located 500 km west of Senegal, West Coast of Africa, the Cape Verde archipelago (14°48’17°18’N, 22°42’25°18’W) is constituted by ten islands and small islets of volcanic origin. The insular nature of Cape Verde, the separation from the continental coast compared to other East Atlantic islands, the associated sea currents, and the water temperature sustain

the biologic value of this region (López Jurado et al. 2000). Five different species of sea turtles have been detected and studied in the archipelago, including the loggerhead turtle (*Caretta caretta*), hawksbill turtle (*Eretmochelys imbricata*), green turtle (*Chelonia mydas*), olive ridley turtle (*Lepidochelys olivacea*) and leatherback turtle (*Dermochelys coriacea*) (López Jurado et al. 2000).

After the Island of Boavista, the coastal areas of the Island of Maio are, together with the Island of Sal, the second most important nesting sites of the archipelago (Martins et al. 2013). In 2009, the estimated total of nests for the Island of Boavista was 20,500 (Marco et al. 2010). In 2017, 7,771 nests were quantified for the Island of Sal (Laloë et al. 2019), while the number of nests registered in the Island of Maio was 14,364 in 2018 (Patino-Martinez et al. In Press), which has increased to higher than 20,000 nests in 2020 (unpublished data). Considering 3-5 clutches per female per nesting season (Varo-Cruz 2010), a range between 813-4788 nesting females per year was estimated for the Island of Maio (Patino-Martinez et al. In Press).

Being characterized by a pristine environment, Maio is considered a globally important refugee for loggerhead sea turtles' conservation. Its nesting beaches are non-affected by artificial nighttime light (light pollution), vast coastal infrastructure, or unsustainable tourism (Patino-Martinez et al. In Press). Considering that numerous coastal environments worldwide are increasingly disturbed, urbanized and artificially illuminated (Godoy and Stockin 2018; Windle et al. 2018; Patino-Martinez et al. In Press), unique habitats such as the Island of Maio must be prioritized for conservation actions (Antworth et al. 2006; Patino-Martinez et al. In Press).

2.3.2. Habitat, distribution, migration patterns and feeding behaviour

The marine habitats of the North-East Atlantic subpopulation extend through a wide marine area of the northwest African coast, reaching as far as the coastal areas of Sierra Leone and the western part of the Mediterranean (Hawkes et al. 2006; Monzón-Argüello et al. 2010; Casale and Marco 2015).

Genetic studies have connected the Cape Verdean juvenile loggerheads to feeding grounds off the Canary Islands, Madeira, the Azores, and Andalusia (Monzón-Argüello et al. 2009). However, 43% of Cape Verdean juveniles are not linked to known feeding areas, which may suggest that they feed at unknown foraging areas or are eliminated due to poaching or mortality in early ages (Monzón-Argüello et al. 2010).

Adult females feeding grounds extend through the Atlantic coast of Africa, between Mauritania and Sierra Leone (Hawkes et al. 2006). Larger turtles migrate southward to benthic feeding grounds near the coast of Sierra Leone, while small-sized females travel to oceanic

settings off Mauritania, The Gambia and Senegal (Hawkes et al. 2006). The Cape Verdean loggerhead subpopulation is hence characterized by two different feeding strategies (Eder et al. 2012; Cardona et al. 2017), oceanic and neritic. Loggerheads smaller than 90 cm of curved carapace length were identified as oceanic, and turtles larger than 90 cm CCL were observed to have neritic feeding strategies (Eder et al. 2012).

2.3.3. Reproductive biology

The reproductive biology of the Cape Verdean loggerhead turtle is highlighted by the small size of the nesting females. The average CCL is approximately 82 cm (67-107.7) (Ballell-Valls and López Jurado 2004; Varo-Cruz et al. 2007; Marco et al. 2011). These values are larger than the measures collected for the Mediterranean loggerheads, which population have the smallest reproductive sizes of this species (Ballell-Valls and López Jurado 2004; Marco et al. 2011).

Due to the classification of the Cape Verdean loggerhead turtle as a single RMU, this genetic distinctiveness presupposes reproductive isolation, with little or no gene flow from other loggerhead subpopulations (Monzón-Argüello et al. 2010; Marco et al. 2011).

Several female sea turtles typically choose the same nesting beach for one or several seasons, which is referred to as high nesting site fidelity. However, there is great flexibility in nesting fidelity and a considerable flow of nesting females between the Islands of Cape Verde, since some turtles have been detected nesting consecutively at distinct islands (Abella et al. 2010; Monzón-Argüello et al. 2010; Marco et al. 2011).

In the Island of Maio, the nesting activity has been registered along the entire coast, mostly on the eastern side, with “Santo António”, “Praia Gonçalo”, “Pedro Vaz”, “Alcatraz” and “Pilão Cão” being the most important locations (Patino-Martinez et al. In Press).

A high rate of multiple paternity has been detected in this loggerhead population, which may suggest an abundant adult male population, despite the evident threats, mostly associated with the significant number of poachers (Marco et al. 2011).

Between April-May and the beginning of the nesting season (June to mid-October), several adult males and females can be observed mating in coastal waters (Marco et al. 2011).

Nesting success (number of nests laid against the total number of tracks on the beach) varies between 26.0% and 44.2% for the archipelago of Cape Verde (Díaz Merry and López Jurado 2004; Varo-Cruz et al. 2007; Marco et al. 2011).

The average clutch size is 85 eggs (Varo-Cruz et al. 2007; Marco et al. 2011). The incubation temperature interval is approximately 28.1-29.8°C, varying seasonally and with the incubation period, which ranges from 45 to 74 days (Abella et al. 2008).

The hatchlings emerge from the end of August until December (Marco et al. 2011), with the sex ratio (calculated through the average temperature of incubation) biased towards females (84% of females) (Patino-Martinez et al. In Press Tanner et al. 2019). The estimated hatching success for the Island of Maio is 29-38% (Patino-Martinez et al. In Press).

2.3.4. Threats of the North-East Atlantic subpopulation

In the region of the North-East Atlantic, a significant number of females (approximately 5%) are slaughtered for meat consumption (Marco et al. 2012; Casale and Marco 2015), and, besides the existent conservation efforts, the loggerhead colony of the Island of Maio is constantly affected by this threat (Martins et al. 2013).

Despite the nesting activity from continental rookeries being dispersed for thousands of kilometres of coast, the main nesting grounds of the Cape Verde insular population are limited to approximately 40 km of beach. Consequently, these nesting beaches are particularly vulnerable to any eventual disasters (e.g., oil spills, tropical storms, flooding by high tides) and artificial impacts (e.g., urbanization, infrastructures, artificial lighting, unsustainable tourism) (Marco et al. 2011).

The reduction of area and quality requirements of nesting habitats represents a major threat for the North-East Atlantic subpopulation (Marco et al. 2011). The extraction of sand, tourism and fishery bycatch are also important threats (Loureiro 2008; Rocha et al. 2013; Casale and Marco 2015).

In the Island of Maio, the main threats for hatching success vary throughout the Island, including flooding by high tides (1-59%), storms, beach erosion, predation by ghost crabs (*Ocypode cursor* and *Ocypode africana*) (40-42% of clutches), and the high clay content of some incubation substrates (Martins et al. 2013; Marco et al. 2015; Marco et al. 2017; Patino-Martinez et al. In Press). The fungi *Fusarium solani* is also associated with a significant rate of mortality in eggs (Sarmiento-Ramírez et al. 2010). Furthermore, black sand beaches are related to higher nest temperatures, affecting the developing embryos (Marco et al. 2011).

Besides egg poaching being comparatively low (2% of clutches), there is an alarming and emerging threat imposed by domestic animals in the Island of Maio, since 68.4% of all clutches (near the largest human settlement) are affected by dog predation (Patino-Martinez et al. In Press).

2.3.5. Conservation status

In Cape Verde, sea turtles are protected by the Cape Verdean ministry under the international compromise of defence and preservation of these species, including *Chelonia mydas, Dermochelys coriacea, Eretmochelys imbricata, Caretta caretta* and *Lepidochelys olivacea* (Decree-Law nº. 1/2018).

According to the Decree-Law nº 1/2018, the disturbance, capture, traffic, purchase and consumption of sea turtle's meat and eggs are strictly forbidden and a punishable crime. The destroying of nests (even if empty), the capture of hatchlings, the use and installation of artificial lights and the transit with motor vehicles on the beaches constitute additional prohibited practices (Decree-Law nº. 1/2018).

As previously mentioned, the archipelago of Cape Verde represents a single RMU, and all islands have an important role in the life cycle and reproductive ecology of the loggerhead sea turtle. However, most of the conservation efforts are limited to the Island of Boavista due to the high prevalence of nesting activity. It is noteworthy to highlight the significant nesting activity registered in the Island of Maio and the relevance of the implementation of conservation programs for the protection of this loggerhead sea turtle colony. In fact, the increasing number of females nesting on Maio may be crucial to increase the currently limited area of occupancy and therefore improve the conservation status of the species (Martins et al. 2013; Patino-Martinez et al. In Press).

2.4. Bacteria of sea turtles

2.4.1. Bacterial diseases of sea turtles

Infectious diseases are primary factors in sea turtles' mortality, representing a challenge for conservation efforts (Herbst and Jacobson 2003). However, due to the difficulties of sampling wild marine animals in remote areas, disease incidences are usually under-reported (Mashkour et al. 2020). Due to the spatial and temporal scale of sea turtles' lifecycle, the assessment of animals expressing clinical signs is also difficult (Jensen 2010; Mashkour et al. 2020).

Bacterial diseases represent an important threat to sea turtle's health and conservation, being one of the leading causes of morbidity and mortality in reptiles (Rosenthal and Mader 2006). However, the connection between the isolation of pathogenic bacteria and disease manifestation is mostly unknown (Mashkour et al. 2020).

The most common routes for bacterial infection in sea turtles include traumatic injuries and aspiration of contaminated water, after which bacteria can enter the bloodstream and disseminate throughout the host (Work et al. 2003; Pace et al. 2018).

Bacteria can affect turtles' health both as primary and opportunistic pathogens. However, bacterial species of sea turtles are predominantly opportunistic pathogens and have been described as normal microbiota of fish, crustaceans, and other marine organisms (Alfaro et al. 2006; Marshkour et al. 2020). A higher frequency of disease in immunocompromised individuals has been described. The association between immunosuppression and infection by environmental bacteria due to anthropogenic influences has been established (Foti et al. 2009; Pace et al. 2019a, 2019b; Marshkour et al. 2020).

Gram-positive bacteria are rarely described as pathogenic in reptiles (Plowman et al. 1987; Rosenthal and Mader 2006), being common inhabitants of the skin (Rosenthal and Mader 2006). Anaerobic bacteria may also have an important role in reptilian disease (Rosenthal and Mader 2006). However, special attention has been given to Gram-negative bacteria as the most important pathogenic bacteria of sea turtles (Pace et al. 2019a; Marshkour et al. 2020).

Previous studies revealed the presence of Gram-negative bacteria in loggerhead sea turtles (Pace et al. 2019a; Marshkour et al. 2020), including *Enterobacter* sp.*, Citrobacter* sp., *Escherichia coli* (*E. coli*)*, Klebsiella* sp.*, Proteus* sp.*, Salmonella* sp.*, Serratia* sp.*, Morganella* sp.*, Vibrio* sp.*, Aeromonas* sp. and *Pseudomonas* sp., which were described as pathogenic (Glazebrook and Campbell 1990a, 1990b; Work et al. 2003; Óros et al. 2004; Mashkour et al. 2020). Yet, these bacteria may also be common isolates in healthy reptiles (Rosenthal and Mader 2006).

Vibrio sp. (especially *Vibrio alginolyticus*)*, Pseudomonas* sp.*, Aeromonas* sp. (mainly *Aeromonas hydrophila*), *Pasteurella* sp.*,* and *Proteus* sp. are associated with a wide range of diseases in sea turtles, including infectious diseases of the skin and appendages, ulcerative stomatitis, ulcerative esophagitis, gastritis, hepatitis, obstructive-rhinitis, bronchopneumonia and septicaemia-toxaemia (Glazebrook and Campbell 1990a, 1990b; Aguirre et al. 1994; Work et al. 2003; Óros et al. 2004) (Table 1). These bacterial species are also described as a cause of mortality in captive-reared and wild juvenile loggerhead sea turtles (Glazebrook and Campbell 1990a, 1990b; Work et al. 2003; Óros et al. 2004; Mashkour et al. 2020).

The link between the presence of pathogenic Gram-negative bacteria species and unhatched loggerhead sea turtles' eggs has been studied, as its association with *Aeromonas hydrophila, Alcaligenes* sp.*, Pseudomonas aeruginosa* (*P. aeruginosa*) and *Shewanella putrefaciens,* was identified (Wyneken et al. 1998; Awong-Taylor et al. 2008; Mashkour et al. 2020).

2.4.1.1. Bacterial infections of the skin and appendages

Infectious diseases of the skin and appendages are often described in sea turtles, mainly in captive animals. Dermal bacterial infections include dermatitis, ulcerative dermatitis, and ulcerative shell disease. *Pseudomonas* sp., *Vibrio alginolyticus* (*V. alginolyticus*), *Aeromonas hydrophila and Flavobacterium* sp. have been related to these diseases (Glazebrook and Campbell 1990a; Mashkour et al. 2020). Ulcerative dermatitis is associated with the isolation of both *Pseudomonas* sp. and *Vibrio alginolyticus* (Marshkour et al. 2020). Also, Gram-positive cocci have been isolated from dermatitis lesions (Óros et al. 2020) (Table 1, Part I).

Previous studies described bacterial dermatitis syndromes (Wiles et al. 1987; Glazebrook and Campbell 1990a; George 1997), including focal erosive dermatitis (FED), septicaemia, ulcerative cutaneous disease (SCUD), and papillary dermatitis (PD). These syndromes were previously associated with the isolation of *Aeromonas* sp., *V. alginolyticus, Pseudomonas* sp., *Proteus* sp., and *Citrobacter* sp. (Wiles et al. 1987; Glazebrook and Campbell 1990a; George 1997). Frequently observed lesions include ulceration and discolouration of the dermis (FED, SCUD) and superficial and proliferative papilla-like lesions (PD). Dermatitis with profound ulcerative lesions (SCUD) may lead to septicaemia (George 1997).

Miller et al. (2009) found several bacterial species associated with skin lesions in hatchlings and post hatchling leatherback sea turtles (*Dermochelys coriacea*) and suggested that these lesions may act as possible entries for opportunistic invaders and a consequent cause of systemic infection.

2.4.1.2. Ulcerative stomatitis (US), Obstructive rhinitis (OR) and Bronchopneumonia (BP) complex

A group of bacterial diseases known as ulcerative stomatitis (US), obstructive rhinitis (OR), and bronchopneumonia (BP) occurs mainly in captive sea turtles. The US-OR-BP complex has been described as a cause of high mortality in hatchlings and juvenile loggerhead sea turtles (Glazebrook and Campbell 1990a; Glazebrook et al. 1993; George 1997). The different diseases can be expressed separately or in combination. The common first clinical sign is the presence of caseous material in one of the nares. Anorexia is also a common clinical sign in sick turtles. If BP occurs, there may be a loss of equilibrium and the ability to maintain an evenly distributed neutral buoyancy. The mortality rate can be as high as 70% in untreated animals (Glazebrook et al. 1993; George 1997).

The most frequently isolated bacterial species associated with the US-OR-BP complex are *Pseudomonas* sp., *Vibrio alginolyticus, Aeromonas hydrophila* and *Flavobacterium* sp..

Other bacterial species have been isolated from US lesions, including *Achromobacter* sp.*, Burkholderia cepacia, Proteus* sp.*,* and *Staphylococcus* sp.. *Escherichia coli* was also associated with BP lesions (Glazebrook and Campbell 1990; Óros et al. 2004; Mashkour et al. 2020) (Table 1, Part II).

2.4.1.3. Osteoarticular bacterial infections

Osteomyelitis in sea turtles has been described in cases of cold-stunning, penetrating injuries and pneumonia (Pace et al. 2018). Bacterial infections by *Mycobacterium cheloneae, Vibrio alginolyticus, Nocardia* sp.*, Aeromonas* sp.*, Pseudomonas* sp.*, Flavobacterium* sp.*, Enterococcus faecalis* and coagulase-positive staphylococci were previously associated with this condition (Glazebrook and Campbell 1990a; Harms et al. 2002; Greer et al. 2003; Solano et al. 2008; Innis et al. 2014; Pace et al. 2018) (Table 1, Part II). *Aeromonas hydrophila* was isolated from a case of osteomyelitis, which was taken as secondary to an *A. hydrophila* septicaemia (Pace et al. 2018).

1 **Table 1** – **Sea turtles' bacterial diseases. Part I.**

SYSTEMS	DISEASE	GRAM-NEGATIVE SPECIES	GRAM-POSITIVE SPECIES	ANIMAL SPECIES	REFERENCE
Integumentary System and appendages	Dermatitis	-	Gram-positive cocci	*Caretta caretta*	Óros et al. 2020
	Ulcerative dermatitis	*Vibrio* sp., *Pseudomonas* sp. + *Vibrio alginolyticus, Aeromonas hydrophila, Cytophaga-Flavobacterium* sp.	-	*Caretta caretta, Chelonia mydas, Eretmochelis imbricata*	Glazebrook and Campbell 1990a, 1990b; Mashkour et al. 2020
	Ulcerative shell disease	*Vibrio alginolyticus, Pseudomonas* sp.	-		Glazebrook and Campbell 1990a
Eye	Keratoconjunctivitis-ulcerative blepharitis	*Pseudomonas* sp., *Cytophaga-Flavobacterium* sp.	-	*Caretta caretta, Chelonia mydas*	Glazebrook and Campbell 1990a, 1990b;
	Adenitis (salt-secreting gland infection)	*Pseudomonas* sp.	-		Glazebrook and Campbell 1990a, 1990b
Digestive Oral cavity	Fibrinonecrotizing stomatitis	Gram-negative bacilli	-	*Caretta caretta*	Óros et al. 2020
	Ulcerative stomatitis	*Pseudomonas* sp. + *Vibrio alginolyticus, Vibrio alginolyticus, Aeromonas hydrophila, Achromobacter* sp., *Burkholderia cepacia, Proteus* sp., *Cytophaga-Flavobacterium* sp.	*Staphylococcus* sp.	*Caretta caretta, Chelonia mydas*	Glazebrook and Campbell 1990a, 1990b; Óros et al. 2004; Mashkour et al. 2020
Oesophagus	Ulcerative esophagitis	*Aeromonas hydrophila, Pseudomonas* sp., *Citrobacter* sp., *Escherichia coli, Vibrio alginolyticus*	*Aerococcus viridans, Staphylococcus* sp.	*Caretta caretta, Chelonia mydas*	Aguirre et al. 1994; Óros et al. 2004
Stomach	Gastritis	*Pasteurella* sp., *Escherichia coli; Proteus, Vibrio alginolyticus*	*Bacillus* sp., *Staphylococcus* sp., *Streptococcus* sp.	*Caretta caretta, Chelonia mydas*	Aguirre et al. 1994; Óros et al. 2004
	Ulcerative gastritis	*Vibrio alginolyticus*	-	*Caretta caretta*	Óros et al. 2004
Intestine	Solitary Large Intestinal Diverticulitis	*Morganella morganii*		*Dermochelys coriacea*	Stacy et al. 2015

Table 1 – Sea turtles' bacterial diseases. Part II.

SYSTEMS	DISEASE	GRAM-NEGATIVE SPECIES	GRAM-POSITIVE SPECIES	ANIMAL SPECIES	REFERENCE
Hepatic **Liver**	Hepatitis	*Citrobacter* sp., *Escherichia coli, Proteus* sp. *Serratia marcescens, Vibrio alginolytcus, Salmonella* Enteritidis	*Staphylococcus* sp., *Streptococcus* sp.	*Caretta caretta, Chelonia mydas*	Glazebrook and Campbell 1990a; Aguirre et al. 1994; Óros et al. 2004
Cardiovascular **Heart**	Necrotizing myocarditis	Chlamydiae	-	*Chelonia mydas*	Homer et al. 1994
Respiratory **Lungs**	Pneumonia	*Arizona hinshairi, Flavobacterium* sp., *Mycobacterium* sp.	-	*Chelonia mydas*	Glazebrook and Campbell 1990a, 1990b
	Bronchopneumonia	*Vibrio alginolyticus, Aeromonas hydrophila, Pseudomonas* sp., *Cytophaga-Flavobacterium* sp., *Escherichia coli*	-	*Chelonia mydas*	Glazebrook and Campbell 1990a, 1990b
Osteoarticular	Osteomyelitis	*Aeromonas* sp., *Nocardia* sp., *Vibrio alginolyticus, Pseudomonas* sp., *Cytophaga-Flavobacterium* sp.	-	*Caretta caretta, Lepidochelys kempi*, *Chelonia mydas*	Glazebrook and Campbell 1990a; Harms et al. 2002; Pace et al. 2018
	Osteoarthritis	*Mycobacterium chelonae*	-	*Lepidochelys kempi*	Greer et al. 2003
Other	Complex ulcerative stomatitis−obstructive rhinitis−bronchopneumonia	*Vibrio alginolyticus, Aeromonas* sp., *Aeromonas hydrophila, Pseudomonas* sp., *Cytophaga-Flavobacterium* sp.,	-	*Chelonia mydas*	Glazebrook and Campbell 1990a, 1990b
	Peritonitis	*Pseudomonas* sp.	-	*Chelonia mydas, Eretmochelys imbricata*	Glazebrook and Campbell 1990a, 1990b

2.5. Antimicrobial resistance and One Health

Antimicrobial resistance (AMR) is described by the World Health Organization (WHO 2018a) as one of the most serious threats to human health, food safety, and communities' development (WHO 2018a). Antimicrobial resistance is an increasingly alarming problem in human and veterinary medicine worldwide (Foti et al. 2009; Grundmann et al. 2011), primarily due to the wide misuse of antimicrobial drugs, promoting the selection and dissemination of resistant strains. As such, antimicrobial drugs have become less effective or even ineffective, which culminates in an expansive global health security emergency, quickly outpacing available treatment alternatives (Amann et al. 2019).

AMR is now visualized within the conceptional framework of the One Health initiative (Queenan et al. 2016; Le Quesne et al. 2018), which recognizes the benefit for human, animal and environmental health as well as financial and social stability, from improved cooperation of professionals in these different but interconnected areas (Zinsstag et al. 2012).

2.5.1. AMR in marine wildlife and marine environments

Wildlife species represent valuable reservoirs of antibiotic-resistant bacteria (ARB) in natural environments, as they actively aid in the dissemination of resistant bacteria and resistance determinants throughout different environments (Hu et al. 2017; Grilo et al. 2020).

Continuing exposure to wastes with antimicrobial drugs' residues promotes the development of resistance by wildlife enteric bacteria by selecting pre-existing natural antimicrobial-resistance genes or through the acquisition of AMR genes via horizontal gene transfer (HGT) (Arnold et al. 2016). Therefore, wildlife species can be an active element of the environmental cycles of AMR dissemination (Vittecoq et al. 2016; Hu et al. 2017).

Agriculture and aquaculture's contaminated wastes and human sewage products are often consumed by wild fish and other marine wildlife, which can travel vast distances, and, afterwards, integrate the human food chain (Cabello et al. 2013). However, there are not enough studies available identifying AMR contamination sources. Special concern is increasing on the prevalence and mechanisms of the transmission of AMR from wildlife to humans and domestic animals (Huijbers et al. 2015; Arnold et al. 2016). It is currently known that wildlife bacteria can exchange resistance genes with human pathogens via HGT (Arnold et al. 2016).

It is critical to ponder AMR in wildlife as a significant threat to human health, particularly considering that nearly 42% of human emergent diseases have their origin in wildlife (Jones et al. 2008; Arnold et al. 2016).

Recent research found important antibiotic-resistant genes (ARGs) in bacteria of several wild marine organisms, including fish, sponges, sea birds, penguins, whales, dolphins

and sea turtles (Foti et al. 2009; Barros et al. 2011; Laport et al. 2016; Prichula et al. 2016; Liu et al. 2018). Marine animals are, hence, valuable ecological indicators (Foti et al. 2009; Prichula et al. 2016; Ahasan et al. 2017), supporting the fact that antibiotic resistance bacteria (ARB) are widely present in the marine ecosystems (Santoro et al. 2008; Foti et al. 2009; Al-Bahry et al. 2011; Wheeler et al. 2012; Wallace et al. 2013; Stewart et al. 2014; Ahasan et al. 2017).

Besides the fact that ARB and ARGs are considered less relevant per se for wildlife, change in host-microbiota interactivity can affect the animals' digestion, increasing the risk of intestinal disease, as well as the animal's immune system and behaviour (Trevelline et al. 2019).

2.5.2. Sea turtles' antibiotic-resistant bacteria

ARB have been studied in live-stranding and wild-living loggerhead sea turtles, mainly from the Mediterranean subpopulation. High levels of resistance were revealed for Gram-negative bacteria, including *Citrobacter* sp., *Pseudomonas aeruginosa*, *Morganella morganii* and *Proteus vulgaris,* namely to tetracyclines and cephalosporins (Kelly et al. 2006; Foti et al. 2009; Pace et al. 2019a).

Studies in other species, including green sea turtles (*Chelonia mydas*), olive ridley sea turtles (*Lepidochelys olivacea*) and hawksbill sea turtles (*Eretmochelys imbricata*), also identified opportunistic Gram-negative bacteria, such as *Vibrio* sp.*, Escherichia coli, Pseudomonas* sp. and *Salmonella* sp.*,* and high percentages of resistance to ampicillin, sulfamethoxazole and streptomycin (Al-Bahry et al. 2009; Al-Bahry et al. 2011; Zavala-Norzagaray et al. 2015; Ahasan et al. 2017; Oliveira et al. 2017).

Furthermore, bacteria revealing high levels of multidrug-resistance have been isolated from clinically healthy and wild-living sea turtles near urbanised regions, suggesting an increase of ARGs in marine bacteria due to the exposure to anthropogenic factors (Ahasan et al. 2017; Blasi et al. 2020).

2.5.3. Characteristics that make loggerhead sea turtles optimal sentinel species

Studies support that sea turtles are important environmental health indicators for coastal marine habitats (Aguirre and Lutz 2004; Owens et al. 2006; Foti et al. 2009). Ecological and physiological characteristics of sea turtles that make them reliable bio-indicators include long lifespan, long period to reach sexual maturity, high site fidelity to coastal feeding habitats and complex feeding behaviours (Foti et al. 2009).

The long life-span and high site fidelity to coastal feeding habitats expose loggerhead sea turtles to constant anthropogenic impacts, which makes them prime reservoirs for antibiotic-resistant bacteria derived from urban run-off. Due to the extensive migratory nature, sea turtles may cross international borders and be exposed to significant environmental stressors (Foti et al. 2009; Read et al. 2014; Ahasan et al. 2017). Also, the longevity and intense migratory behaviour predispose sea turtles to host and spread pathogenic and antibiotic-resistant bacteria in innumerable marine environments (Foti et al. 2009; Ahasan et al. 2017; Oliveira et al. 2017; Blasi et al. 2020).

Due to the long lifespan of sea turtles, it is predictable that the exposure to environmental bacteria and parasites is comparatively higher. Also, it is hypothesized that their immune response and coping mechanisms are proportionally more efficient (Warwick et al. 2013). However, sea turtles are exceptionally vulnerable to chemical and organic pollution (Foti et al. 2009; Oliveira et al. 2017; Biagi et al. 2018), which enhances these species as good sentinels for environmental health monitoring (Barbour et al. 2007; Al-Bahry et al. 2009, 2012; Foti et al. 2009; Oliveira et al. 2017).

Furthermore, sea turtles can interact with other animals, also known reservoirs of resistant bacteria (fish, wild birds, sea mammals), and promote an additional pathway for the dissemination of resistant strains (Delport et al. 2015; Prichula et al. 2016; Liu et al. 2018).

2.6. Virulence profile of bacteria isolated from marine wildlife

The ability of an organism to infect a host and initiate disease is defined as virulence. Virulence factors are described as inherent elements of a pathogen with the ability to cause damage to host cells and tissues, as well as molecules or structures (e.g., capsule, biofilm) that enable the pathogen to evade or modulate host defence mechanisms to its replicative advantage (Johnson 2018).

In extracellular pathogens, the secretory virulence factors may act in concert to damage the host cells (Sharma et al. 2016). Therefore, the entire virulence profile should be analysed regarding the potential relationships between different virulence factors. For example, phospholipases and lipases may act synergistically, modulating host responses beneficially for the microorganism (König et al. 1996; Stehr et al. 2003). Gelatinase activities can contribute to the formation of biofilm, as it supports cell aggregation in microcolonies to create a three-dimensional structure, being one of the first steps in the process of biofilm establishment (Hancock and Perego 2004; Thurlow et al. 2010; Hashem et al. 2017).

The virulence phenotype of bacteria isolated from different wild fish species was previously described, especially of the species *Aeromonas* sp., *Vibrio* sp., and *Shewanella* sp.

(Lee et al. 1996). However, to the best of our knowledge, there are no studies describing the virulence phenotype of bacteria isolated from sea turtles.

2.6.1. Hemolysins

Hemolysins are toxic extracellular proteins produced by several Gram-negative (e.g., *E. coli, Vibrio* sp., *Pasteurella* sp., *Pseudomonas aeruginosa*) and Gram-positive bacteria (e.g., *Streptococcus* sp., *Staphylococcus* aureus) (Goebel et al. 1988). Most hemolysins cause lysis of erythrocytes by forming pores in their membrane (Goebel et al. 1988). These cytolytic polypeptides can disrupt host cell membranes, causing defects in ion homeostasis that ultimately lead to the influx of water (H_2O), cell bulging, and lysis (Johnson 2018). The ability to produce hemolysins (α and β) was observed in *Shewanella putrefaciens* isolates from freshwater fish, and *Vibrio alginolyticus* isolates from diseased tiger prawns (*Penaeus monodon*) (Lee et al. 1996; Paździor et al. 2019).

2.6.2. DNases

DNases represent virulence factors by promoting the use of extracellular DNA as a nutrient source for bacterial growth (Mulcahy et al. 2010), as well as a matrix component of biofilms (Mann et al. 2009). DNases can also be a vital tool to develop resistance against the immune system by degrading neutrophil extracellular traps (NETs), important effectors of the host's innate immune response (Berends et al. 2010; Haas et al. 2014). DNase activity was identified in marine *Vibrio* species (Castillo et al. 2018).

2.6.3. Lipases

Lipases act by digesting lipids for nutrient acquisition (Stehr et al. 2003). These enzymes might also help pathogens grow in a carbohydrate-restricted environment or conditions where lipids are the sole carbon source (Stehr et al. 2003). Lipase activity was detected in marine *Vibrio* species and *Aeromonas* species associated with fish diseases (Beaz-Hidalgo and Figueras 2013; Castillo et al. 2018).

2.6.4. Biofilm

Biofilm is considered an essential factor in the pathogenesis of numerous bacteria (Hancock and Perego 2004; Thomas et al. 2008). Biofilm bacteria live in a self-created matrix of hydrated extracellular polymeric substances (EPS), including polysaccharides, proteins, nucleic acids and lipids. These substances confer the mechanical stability of biofilms and facilitate their adhesion to surfaces, developing a consistent, three-dimensional polymer network (Flemming and Wingender 2010). Polysaccharides and proteins in biofilms can also

act as a protective barrier, being involved in the development of resistance to host defences during infection and the tolerance to antimicrobial agents (Flemming and Wingender 2010). The ability to produce biofilms was previously detected in *Aeromonas* species associated with fish diseases (Beaz-Hidalgo and Figueras 2013).

2.6.5. Proteases

Proteases represent important mechanisms of virulence throughout the infection cycle of the bacterium. Secreted proteases can contribute to the degradation of the host's physical barriers, enabling penetration and efficient dissemination of the bacterium (Hueck 1998). Protease activity was previously detected in *Aeromonas* species associated with fish diseases (Beaz-Hidalgo and Figueras 2013). Protease activity was also related to the pathogenesis of marine fish vibriosis, a deadly hemorrhagic septicemic disease caused by *Vibrio anguillarum* (Frans et al. 2011).

2.6.6. Gelatinases

Gelatinase is thought to allow the degradation of a wide range of host substrates, such as fibrinogen and fibrin (Waters et al. 2003; Thurlow et al. 2010). Gelatinase activity was previously detected in *V. alginolyticus* isolates from diseased tiger prawns (*Penaeus monodon*) (Lee et al. 1996).

2.6.7. Lecithinases

Lecithinases promote the disruption of bacterial membranes through hydrolysis of phospholipids, which may result in lysis of the bacterial membranes (Songer 1997; Johnson 2018). Lecithinase activity was revealed in *V. alginolyticus* isolates from tiger prawns (*Penaeus monodon*) and *Aeromonas* species associated with fish diseases (Lee et al. 1996; Beaz-Hidalgo and Figueras 2013).

2.7. Loggerhead sea turtles and Public Health

Historically, sea turtles were an important food source to several human populations worldwide, particularly in the Cape Verde islands (Loureiro 2008). Nowadays, the consumption of sea turtle-related products (e.g., meat, organs, blood, adipose tissue, and eggs) is strictly prohibited in most sea turtle habitats worldwide. However, and disregarding the established regulations, this practice is still a reality in several communities worldwide, including the Island of Maio (Aguirre et al. 2006; Zavala-Norzagaray et al. 2015; Pace et al. 2019a, Patino-Martinez et al. In Press).

The consumption of sea turtle-related products represents a risk to Public Health, supported by the absence of safety protocols to eliminate these potentially unsafe products from distribution and the limited information on disease associated with sea turtle consumption (Aguirre et al. 2006; Warwick et al. 2013).

2.7.1. Hazards of the consumption of sea turtles and their products

Hazards of contact with sea turtles and sea turtle-related products are mostly associated with exposure to microbiologic (bacteria, viruses, parasites, and fungi) and macrobiologic (macroparasites) agents, as well as environmental contaminants (biotoxins, organochlorines and heavy metals) (Aguirre et al. 2006; Warwick et al. 2013). The risks of sea turtle consumption are aggravated by the fact that these animals have a high position in the food chain and, therefore, a consequent higher contaminant and bacterial load (Aguirre et al. 2006).

Environmental contaminants stored in sea turtles' edible tissues impose a risk to Public Health, supported by the detection of high levels of these compounds, often exceeding the international food safety standards (Aguirre et al. 2006). These contaminants can have toxic effects, including neurotoxicity, kidney disease, liver neoplasia, and developmental impacts in fetuses and infants (Aguirre et al. 2006). Also, the sea turtle associated toxin, chelonitoxin, is a primary health concern. Chelonitoxism can affect healthy people, which symptoms include nausea, vomiting, pain, polyarthralgia, flu-like symptoms, coma, and ultimately death (Fussy et al. 2007; Warwick et al. 2013).

Potential zoonotic parasites and bacteria have been identified in sea turtles, including coccidia, *Corynebacterium* sp.*, Campylobacter* sp.*, Escherichia coli, Klebsiella* sp.*, Proteus* sp.*, Salmonella* sp.*, Serratia* sp.*, Morganella* sp.*, Vibrio* sp.*, Aeromonas* sp., and *Pseudomonas* sp. (Santoro et al. 2008; Magnino et al. 2009; Dutton et al. 2013; Warwick et al. 2013; Zavala-Norzagaray et al. 2015; Ives et al. 2016). Bacteria belonging to the Enterobacteriaceae family are of special concern for the One Health view (Zavala-Norzagaray et al. 2015; Mashkour et al. 2020). These bacteria represent a hazard for Human Health due

to their pathogenic ability and resistance profile (Santoro et al. 2008; Foti et al. 2009; Warwick et al. 2013; Pace et al. 2019a; Mashkour et al. 2020).

2.7.1.1. Bacterial Infections

Salmonella, Mycobacterium, Vibrio sp. and *E. coli*, identified in sea turtles, are also important humans' pathogens (Raidal et al. 1998; Aguirre et al. 2006; Dutton et al. 2013; Prichula et al. 2016). Infection routes include the shedding of pathogenic bacteria into water surfaces, beaches or directly through close interactions with sea turtles (Ives et al. 2016).

E. coli infections are associated with abdominal cramps, diarrhoea, fever, vomiting, acute renal failure (haemolytic uraemic syndrome), and even death, especially in children (WHO 2018b). Symptoms of *Vibrio* sp. infections may include diarrhoea, vomiting, and severe dehydration (Campos et al. 1996). *Salmonella* sp. infections symptoms include headache, nausea, vomiting, abdominal pain, and diarrhoea (Lamm et al. 1972).

Salmonellosis, mainly caused by *Salmonella enterica*, stands out as the most notorious zoonotic disease of reptiles. This disease has been transmitted from pet turtles to infants, revealing a high incidence of infection (Lamm et al. 1972; Rosenthal and Mader 2006). Turtles may become reservoirs of *Salmonella* sp. without showing clinical signs. The rate of infection in these animals is significantly high (Rosenthal and Mader 2006).

Mycobacterium species have been described in reptiles, including *M. marinum, M. avium*, and *M. tuberculosis,* all described as zoonotic pathogens (Aguirre et al. 2006). *M. marinum* causes cutaneous and subcutaneous nodular disease in humans. The potential routes of transmission of this pathogen include inhalation, solutions of continuity or tissue breakage, and contact with the oral mucosa (Aguirre et al. 2006; Rosenthal and Mader 2006).

Furthermore, *Leptospira* sp. serotypes have been detected in sea turtles, suggesting that sea turtles may represent reservoirs of these potential zoonotic pathogens (Cordero-Tapia 2005; Aguirre et al. 2006). The presentation of this zoonosis is characterized by nonspecific symptoms, including fever, myalgia, and headache (Katz et al. 2001).

It has been suggested that the impact of sea turtles' consumption on human health should be further investigated. In fact, both Public Health and sea turtles' conservation could be improved by reducing the consumption of these endangered species (Aguirre et al. 2006).

Chapter 3. Materials and Methods

3.1. Objectives of the study

This study aimed to characterize the Gram-negative bacterial species present in the cloaca, oral cavity and eggs of loggerhead sea turtles from the Island of Maio; to evaluate their pathogenic potential; and to unveil both the impact of these species on sea turtles' conservation and the underlying public health risk resulting from interactions with these animals and the consumption of turtle-derived products. This work also aimed to examine the antibiotic resistance and virulence profiles of the isolated and identified bacterial species. The study included the following tasks:

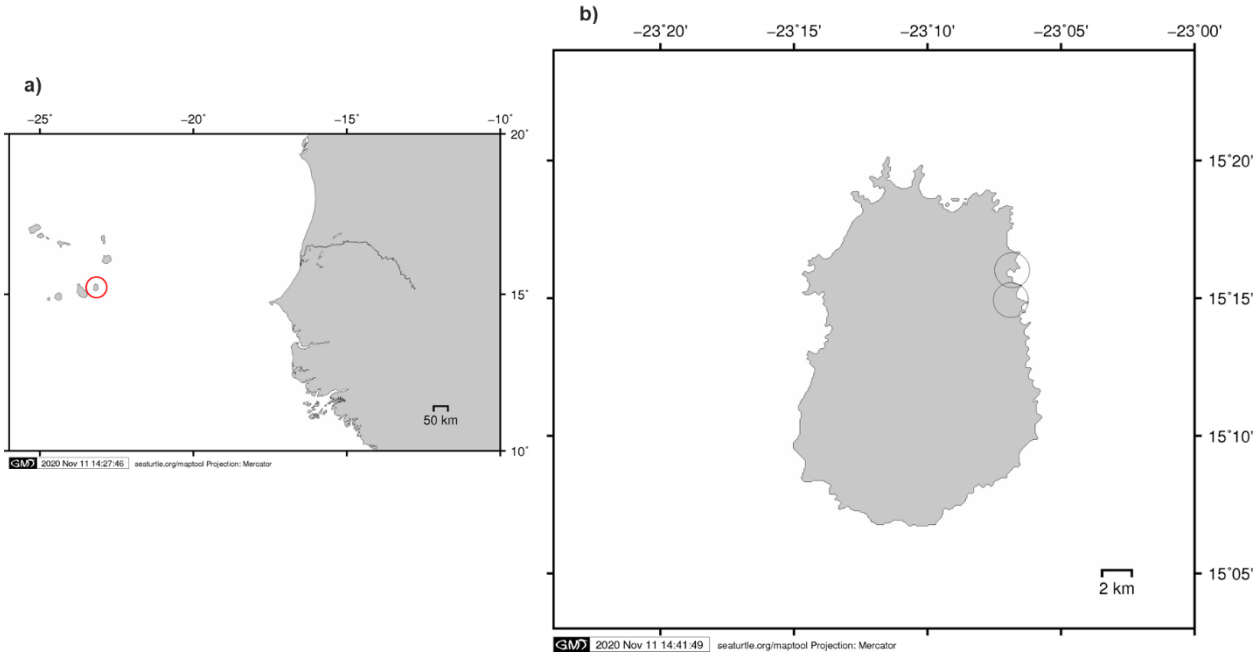
1. The collection of oral, cloacal and egg samples (n = 99) from nesting loggerhead sea turtles (*Caretta caretta*) during a one-month internship integrated into the loggerhead conservation program of Maio Biodiversity Foundation in the Island of Maio, Cape Verde;
2. The isolation of potential pathogenic Gram-negative bacteria from the collected samples using Glutamate Starch Red Phenol (GSP) Agar (Merck) and MacConkey Agar (Oxoid), incubated at 37°C for 24h, performed in the Microbiology and Immunology Laboratory from FMV/ ULisbon;
3. The identification of the isolated aerobic and facultative anaerobic Gram-negative bacilli through the biochemical identification galleries API 20E and API 20NE (bioMérieux), according to the manufacturer's instructions;
4. The assessment of the identified isolates' antibiotic resistance profile, regarding twelve different antibiotics commonly used in veterinary and human medicine and belonging to six different classes by the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2013);
5. The evaluation of the isolates' phenotypic virulence profile by the analysis of the production of enzymes associated with bacteria pathogenic potential, including DNases, lipases, lecithinases, gelatinases, hemolysins, proteases and biofilm production;
6. The evaluation of the relationship between the antibiotic resistance profile of the identified isolates and the type of sample (oral cavity, cloaca, egg) (Kruskal-Wallis test analysis);
7. The evaluation of the relationship between the virulence profile of the isolated bacteria and the type of sample and health status classes of the respective turtle (Kruskal-Wallis test analysis);
8. The assessment of the correlation between the antibiotic resistance profile and the virulence profile for the tested isolates (Spearman correlation test).

3.2. Sampling procedure

3.2.1. Area of study

Samples were collected from loggerhead sea turtles from the Island of Maio (15°13′50″N 23°09′22″W), the archipelago of Cape Verde (14°48’17°18’N, 22°42’25°18’W), West Africa (Figure 3). The Island of Maio comprehends an area of 269 km^2 (maximum 24 km long x 16 km wide) and hosts loggerhead nesting activity along 38 km of sandy beaches throughout 110 km of coastline (Patino-Martinez et al. In Press). The insular sandy beaches of the Island of Maio are characterized by a great variety of sand colour and grain size (Patino-Martinez et al. In Press).

The area of study (Figure 3) 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.



Legend: a) The archipelago of Cape Verde (14°48’17°18’N, 22°42’25°18’W) and **b)** the Island of Maio (15°13′50″N 23°09′22″W) (red circle in **a**). The location of “Praia Gonçalo” (15°15′25.9″N 23°06′34.5″W) is highlighted in the top circle and the location 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 (SEATURTLE.ORG 2002).

Figure 3 – The Area of Study.

3.2.2. Period of study

Samples were collected during August 2019, which coincides with the peak of loggerhead nesting activity (July and August) in the Island of Maio. The nesting season extends from June to mid-October (Marco et al. 2011). Sampling was performed during night shifts completing a total of fifteen days. For each shift, an average of 5 nesting females was selected for sampling.

3.2.3. Selected animals

The sea turtles selected for the present study constituted adult nesting loggerhead sea turtles' females from the North-East Atlantic subpopulation, which represents a single regional management unit (Monzón-Argüello et al. 2010, Wallace et al. 2011). A total of 33 nesting turtles (*Caretta caretta*) were selected for sampling. Turtles were sampled sequentially after being observed from a safe distance as they arrived at the beach and started building the nest. Inclusion criteria included loggerhead adult females in the beginning of oviposition, free from any potential external sources of stress, e.g., another female nesting in proximity. Exclusion criteria included non-nesting animals, for example, rescued turtles or females that have completed oviposition.

3.2.4. Sampling technique

Oral, cloacal, and egg content AMIES (without carbon) swab (1814-002) (VWR^{TM}) samples (n = 99) of loggerhead nesting sea turtles (*Caretta caretta*) were collected during August 2019 in the coastal areas of the Island of Maio (Cape Verde).

The following information was collected regarding each sample: date and time of sampling; local of sample collection; flipper tag identification number and PIT identification (when available); curved carapace length (CCL); clutch size (nº. eggs) (when available); type of sample (oral cavity, cloaca and egg); turtle health status (class I – good (good apparent body condition, no evident lesions or disease), class II – external superficial lesions (e.g., superficial erosions or deformation of the carapace), class III – high number of parasites present in the cloaca or abnormal oviposition. The estimated age was calculated through the Von Bertalanffy (1938) growth function (VBGF) (Equation. 1) with the obtained curved carapace length (CCL) values (Casale et al. 2011).

$$L(t) = L_{\infty} - (L_{\infty} - L_0)e^{(-kt)}$$

Legend: *L*(*t*) – carapace length at age t; ***L*∞** – mean asymptotic carapace length; ***L*0** – initial carapace length; ***k*** – growth coefficient (*k*= 0.667; *L*∞=99 cm; *L*0=30 cm) (Casale et al. 2011).

Equation 1 - Von Bertalanffy (1938) growth function.

To minimize the contact with the studied turtles, actions were coordinated before each procedure. Only red-filtered lights were used.

The three samples were collected sequentially: cloacal, oral, and egg samples. A sterile swab was gently inserted approximately 4 cm into the cloaca (dilated during oviposition), and with a rotative movement, the oviductal fluid was collected encompassing the entire internal surface of the cloaca (Figure 4). Samples were taken after the laying of 5 to 10 eggs.

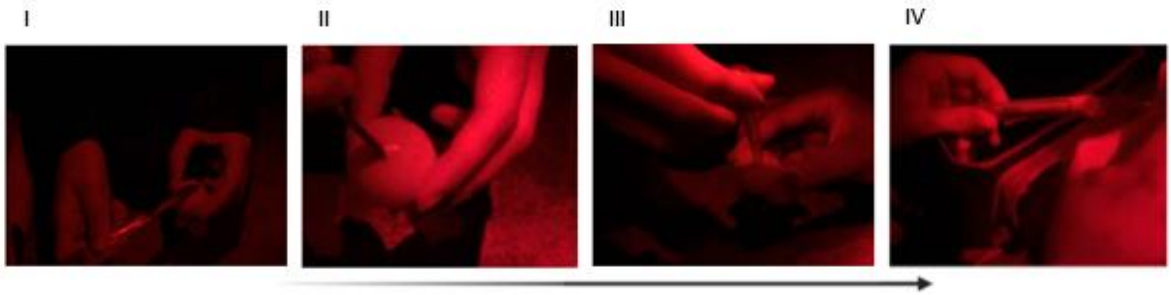
Legend: A swab is gently inserted into the cloaca, dilated at oviposition. The carapace, cloaca and swab are labeled.

Figure 4 - Cloacal sampling procedure (Original).

Oral sampling was performed by opening the rhamphotheca with a previously disinfected (ethylic alcohol 70%) wooden pry bar. The soft tissue portion of the mouth (tongue and palate) was gently swabbed for approximately 5 seconds. The sample was taken without contacting with the mouth opener or any external surface of the rhamphotheca.

After the laying of a minimum of five eggs, an egg was collected directly from the cloaca without contacting the surrounding environment. For the egg sample, a small surface of the eggshell was sterilized with a fire-heated bistoury and a circle shape window was opened. A sterile Pasteur pipette was used to collect approximately 1.5 ml of the egg content, including

both the egg yolk and the albumen. The egg content was introduced in the transport medium (Figure 5).



Legend: I) The top surface of the eggshell was sterilized with a fire-heated bistoury; **II)** A circle shape window was opened in the eggshell; **III)** A sterile Pasteur pipette was used to collect the egg content; **IV)** The egg content was introduced in the transport medium.

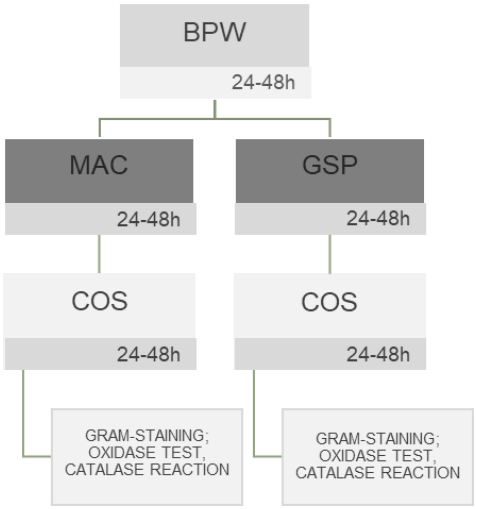
Figure 5 - Egg content sampling procedure (Original).

The samples were identified with date, time, type of sample, and flipper tag ID number and then, safely placed in a thermal bag, at 4°C. After the sampling period, the collected samples were transported to the Microbiology and Immunology Laboratory from the Veterinary Faculty in Lisbon, Portugal, by plane for further processing in a one-month period.

A maximum of 5 minutes was required for each sampling procedure, after which oviposition was normally concluded and the turtle naturally returned to the sea.

3.3. Isolation and identification of Gram-negative bacteria

Samples were processed in Microbiology and Immunology Laboratory from the Veterinary Faculty in Lisbon, Portugal, after a maximum period of three weeks. After pre-enrichment in Buffered Peptone Water at 37°C for 24h, Gram-negative aerobic bacteria were isolated from the collected samples using Glutamate Starch Red Phenol (GSP) Agar plates supplemented with 100,000 IU sodium penicillin g/L (Merck) and MacConkey Agar (Oxoid), incubated at 37°C for 24-48 h (Foti et al. 2009; Igbinosa et al. 2020). The bacterial colonies obtained were isolated in Columbia agar supplemented with 5% sheep blood (COS) (bioMérieux) (Figure 6). The isolates were characterized regarding their macro and microscopic morphology, Gram-staining, catalase test and oxidase reaction (Figure 6). Gram-negative, oxidase-positive, and oxidase-negative bacilli were identified through the biochemical identification galleries API 20E and API 20NE (bioMérieux), according to the manufacturer's instructions.



Legend: Buffered Peptone Water (**BPW**); Glutamate Starch Red Phenol (**GSP**); Columbia agar supplemented with 5% sheep blood (**COS**); MacConkey agar (**MAC**), hours (h).

Figure 6 - Protocol for the isolation of Gram-negative bacteria (Original).

3.4. Evaluation of isolates' antibiotic resistance profile.

Isolates' susceptibility profile regarding 12 different antibiotics commonly used in veterinary and human medicine and belonging to six different classes was determined using the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2013). Briefly, isolates were cultured in Columbia agar supplemented with 5% sheep blood (bioMérieux) and incubated at 37 ºC for 24 h. A 10^8 CFU/mL bacterial suspension, with a 0.5 turbidity in the McFarland scale, was prepared by collecting the colonies with a sterile swab and suspended it in a sterile saline solution. The bacterial suspension was evenly spread over a Mueller-Hinton agar (VWR™) plate and antibiotic-impregnated disks were placed over the surface of the agar plates, which were incubated at 37°C for 18 h. A 10% replica was performed.

The tested antibiotics (Oxoid and MASTDISCS) included aminoglycosides (amikacin 30 µg, gentamicin 120 µg, tobramycin 10 µg), third generation cephalosporins (cefoperazone 75 µg, ceftazidime 30 µg), fluoroquinolones (ciprofloxacin 5 µg, enrofloxacin 5 µg, ofloxacin 5 µg), carbapenems (imipenem 10 µg, meropenem 10 µg), ureidopenicillin (piperacillin 100 µg) and tetracycline (tetracycline 30 µg), as described elsewhere (Foti et al. 2009; Serrano et al. 2016). The reference strain *Escherichia coli* ATCC®25922™ was used for quality control. The inhibition zones were measured and scored as susceptible, intermediate, or resistant, according to the CLSI guidelines (CLSI 2013).

3.5. Evaluation of isolates' virulence profile

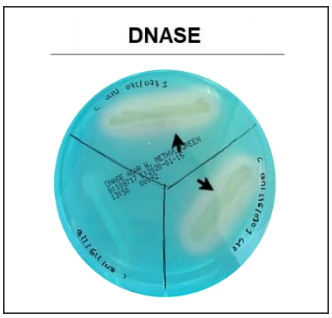
Isolates were characterized regarding their phenotypic virulence profile by the evaluation of the production of enzymes associated with bacteria pathogenic potential.

3.5.1. Haemolysins

Hemolysins production was evaluated using Columbia Agar with 5% sheep blood (bioMérieux). After incubation for 24 h at 25°C, alpha-hemolytic bacteria partially lysed erythrocytes, producing a yellowish-green colour of the area surrounding the colonies. Beta-hemolytic bacteria completely lysed erythrocytes, which results in a clear zone surrounding the colonies (Balashova et al. 2006).

3.5.2. DNase

DNase activity was evaluated using DNase Agar supplemented with 0,005% methyl green (VWR™), followed by incubation for 24 h at 25°C. *Aeromonas hydrophila* ATCC®7966™ and *Escherichia coli* ATCC®25922™ were used as positive and negative controls, respectively. Positive DNase activity was identified by the change of the medium around the tested organism from pale green to colourless (Forbes et al. 2007; Seixas et al. 2014) (Figure 7).

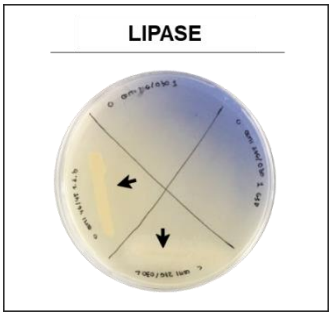


Legend: DNase test (DNase Agar) – colonies surrounded by a clear halo (arrows) are classified as positive.

Figure 7 – DNase plaque assay (Original).

3.5.3. Lipase

Lipase activity was tested using Spirit Blue agar (Difco™) added with Tween® 80 and olive oil. *Pseudomonas aeruginosa* ATCC®27853™ and *Staphylococcus aureus* ATCC® 29213™ were used as positive and negative controls, respectively. The lipolytic activity was identified by the presence of a clear zone around the colony, after incubation for 24 h at 25°C (Pogaku et al. 2010) (Figure 8).

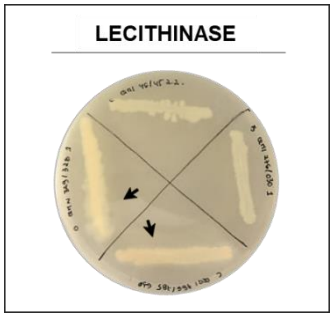


Legend: Lipase test (Spirit Blue Agar) – positive results are determined by a clear area around the colonies (arrow).

Figure 8 – Lipase plaque assay (Original).

3.5.4. Lecithinase

Lecithinase activity was determined using Tryptic Soy Agar (VWR™) supplemented with 10% egg yolk emulsion (VWR™). *Pseudomonas aeruginosa* ATCC®27853™ and *Escherichia coli* ATCC®25922™ were used as positive and negative controls. Positive isolates for lecithinase production were identified by the development of a white, opaque, diffuse precipitation area around the colonies, following incubation for 24 h at 25°C (Chrisope et al. 1976; Chapin and Lauder 2007; Gonçalves et al. 2007) (Figure 9).

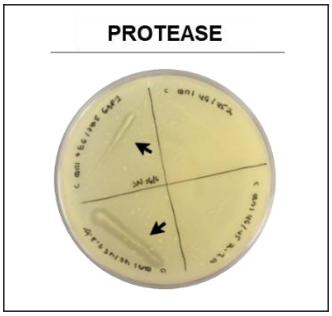


Legend: Lecithinase test (Tryptic Soy Agar) – positive results are identified by a precipitate around the colonies (arrows).

Figure 9 – Lecithinase plaque assay (Original).

3.5.5. Protease

Protease activity was analysed resorting to Skim Milk powder (Oxoid) added with bacteriological agar (VWR™). *Pseudomonas aeruginosa* ATCC®27853™ and *Staphylococcus aureus* ATCC®29213™ were used as positive and negative controls, respectively. After incubation for 24 h at 25°C, isolates showing a clear halo around the colonies were classified as positive protease producers (Brown and Foster 1970; Sokol et al. 1979; Burke et al. 1991; Gonçalves et al. 2007) (Figure 10).



Legend: Protease test (Skim Milk Medium) – the clear halo around the tested isolates (arrows) is identified as a positive result.

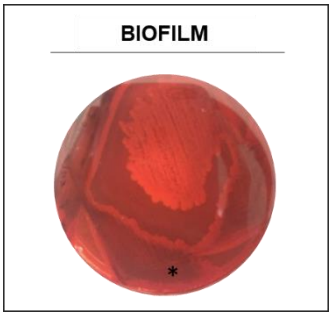
Figure 10 – Protease plaque assay (Original).

3.5.6. Gelatinase

Gelatinase activity was detected using Nutrient Gelatin Agar (Oxoid). This test is applied to verify the ability of an isolate to produce proteolytic enzymes (gelatinases) that liquefy gelatin (Forbes et al. 2007). Positive gelatinase activity was detected by partial or total liquefaction of the inoculated tube after storage at 4°C for 30 min, following incubation for 24 h at 25°C. *Pseudomonas aeruginosa* ATCC®27853™ and *Escherichia coli* ATCC®25922™ were used as positive and negative controls, respectively (Semedo et al. 2003; Forbes et al. 2007).

3.5.7. Biofilm production

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). *Enterococcus faecium* ATCC®35667™ and *Escherichia coli* ATCC®25922™ were used as positive and negative controls, respectively. After incubation for 24 h at 25°C, dark red or black colonies, with crystalline or dry consistency, were identified as positive, indicating biofilm production (Freeman et al. 1989; Rewatkar and Wadher 2013; Lima et al. 2017) (Figure 11).



Legend: Biofilm production test (Congo Red Agar) – positive results are determined by the blackening of the tested isolate (*).

Figure 11 – Biofilm production plaque assay (Original).

3.6. Statistical analysis

Results relative to the isolation and characterization of Gram-negative bacteria were analyzed to 1) assess differences between the frequency of different bacterial species in oral, cloacal, and egg content swab samples among bacterial species, 2) test possible tendencies between antibiotic resistance and virulence profiles of bacterial species isolated from different samples.

The MAR (multiple antibiotic resistance) index (nº. antibiotics for which resistance was revealed / nº. antibiotics tested) and the Virulence index (nº. positive virulence factors / nº. virulence factors tested) were calculated for the bacterial isolates obtained (Krumperman 1983; Singh et al. 2017).

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

A Pearson's Chi-square Test was performed to test the differences between the number of positive and negative results regarding the bacterial species identified for different types of sample (cloaca, oral cavity and egg content) and the turtle's health status (class I, II, III).

The variables MAR index and Virulence index did not follow a normal distribution (evaluated with the Skewness and Kurtosis coefficients, the Shapiro-Wilk test and Histogram). Therefore, for evaluating the differences between the MAR index and Virulence index of the isolated bacteria and the respective sample (sample type and turtles' health status classes), a nonparametric Kruskal-Wallis test was applied.

The differences in MAR index and Virulence index values among distinct bacterial families (Enterobacteriaceae and Non-Enterobacteriaceae) of the tested bacteria were analysed with the nonparametric Mann-Whitney U-test. This test was also applied to evaluate the differences in MAR index values between biofilm-producer and non-biofilm-producer 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.

Chapter 4. Results

4.1. Sampled animals' data

According to the available data, the studied females had an average curved carapace length (CCL) of 77.1 cm (σ = 2.34) and an average clutch size of 72 eggs (σ = 7.47). The average age calculated through the Von Bertalanffy (1938) growth function was 17 years (σ = 1.65) (Annexe 1 – Table 5, Part I-VI).

4.2. Isolates' Identification

Cloacal (oviductal fluid), oral and egg content swab samples were collected from 33 animals, making a total of 99 samples. From this total, it was possible to obtain 26 and 8 bacterial isolates (Gram-negative bacilli) from Glutamate Starch Red Phenol (GSP) Agar (Merck) and McConkey Agar (Oxoid), respectively (Annexe 2 – Table 6). 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 19 isolates were identified through the API 20NE and API 20E galleries, as follows: *Shewanella putrefaciens* (n = 5; 26.32%), *Vibrio alginolyticus* (n = 4; 21.05%), *Morganella morganii* (n = 4; 21.05%), *Enterobacter cloacae* (n = 2; 10.53%), *Aeromonas hydrophila/caviae* (n = 1; 5.26%), *Brevundimonas vesicularis* (n = 1; 5.26%), *Burkholderia cepacia* (n = 1; 5.26%), and *Citrobacter* sp. (n = 1; 5.26%).

Shewanella putrefaciens was the most prevalent species (26.32%), followed by *Vibrio alginolyticus* (21.05%) and *Morganella morganii* (21.05%) (Table 2) (Graphic 1).

As mentioned before, the turtles under study were categorized in three different classes regarding their health status: class I - good apparent body condition, no evident lesions or disease (n = 19), class II - external superficial lesions (e.g., superficial erosions or flacking of the carapace) (n = 7), and class III – animals with a high number of parasites observed in the cloaca or abnormal oviposition (n = 7).

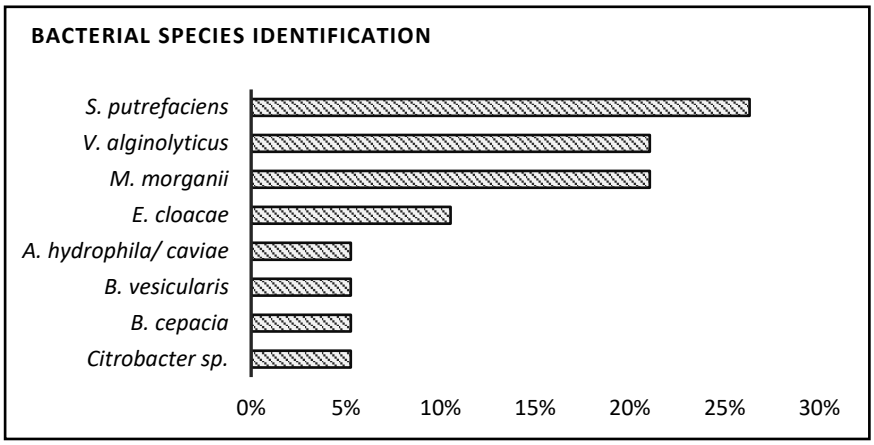
A higher prevalence of *Vibrio alginolyticus* isolates from samples from class III loggerhead females, compared with apparently healthy individuals (classes I and II), was observed ($\chi 2 = 7.031$; d.f. = 2; $p < 0.05$). No significant differences were observed regarding the prevalence of the identified bacterial species and the type of sample (cloacal, oral and egg) ($p > 0.05$).

Table 2 - Isolates' identification by the API 20E and NE galleries.

ISOLATE	SAMPLE CODE	SAMPLE TYPE	ISOLATE ID	%ID
1	276/030-2	C	*Aeromonas hydrophila/caviae*	95.1
2	786/785	C	*Brevundimonas vesicularis*	93.7
3	329/328	E	*Burkholderia cepacia* complex	91.8
4	276/030-1	C	*Shewanella putrefaciens*	99.1
5	49/50-2	C	*Shewanella putrefaciens*	99.9
6	46/45-2	C	*Shewanella putrefaciens*	99.9
7	46/45-1.2B	E	*Shewanella putrefaciens*	99.9
8	72/73-1	C	*Shewanella putrefaciens*	99.9
9	276/030-1	E	*Vibrio alginolyticus*	99.8
10	276/030-1G	E	*Vibrio alginolyticus*	99.3
11	60/61-1	C	*Vibrio alginolyticus*	99.6
12	504/503	O	*Vibrio alginolyticus*	99.8
13	330/331	C	*Enterobacter cloacae*	44.1*
14	49/50-1	C	*Enterobacter cloacae*	99.2
15	46/45-2.1A	C	*Morganella morganii*	99.9
16	46/45-2.2	E	*Morganella morganii*	99.9
17	276/030-1	O	*Morganella morganii*	99.9
18	504/503	C	*Morganella morganii*	99.9
19	229/228	C	*Citrobacter* sp.	68.1

Legend: Cloaca (C), egg content (E), oral cavity (O), species' identification (ID), probability of species identification (identification percentage) (%ID). * Confirmed by the non-formation of yellow colonies.

Graphic 1 – Percentage frequency of bacterial species identified by the API 20NE and API 20E galleries.



Legend: *Shewanella putrefaciens* (*S. putrefaciens*)*; Vibrio alginolyticus* (*V. alginolyticus*); *Morganella morganii* (*M. morganii*)*; Enterobacter cloacae* (*E. cloacae*)*; Aeromonas hydrophila/caviae* (*A. hydrophila/caviae*); *Brevundimonas vesicularis* (*B. vesicularis*)*; Burkholderia cepacia* (*B. cepacia*)*.*

4.3. Characterization of isolates' antibiotic resistance profile

A considerable percentage of isolates under study (68.42%) were resistant or intermediately resistant to at least one of the twelve antibiotics tested, with 15.79% of isolates showing resistance to two or more antibiotics. According to Magiorakos et al. (2012) classification, no multidrug-resistant (MDR) isolates were detected, as non was non-susceptible to at least three antimicrobial agents of different categories.

Higher frequency of resistance was detected to tetracyclines (26%), and none of the isolates presented resistance or intermediate resistance to aminoglycosides (amikacin, gentamicin, and tobramycin) and fluoroquinolones (ofloxacin) (Table 3).

Table 3 – Antimicrobial susceptibility, intermediate resistance, and resistance of the isolates under study, regarding twelve antibiotics of six different classes.

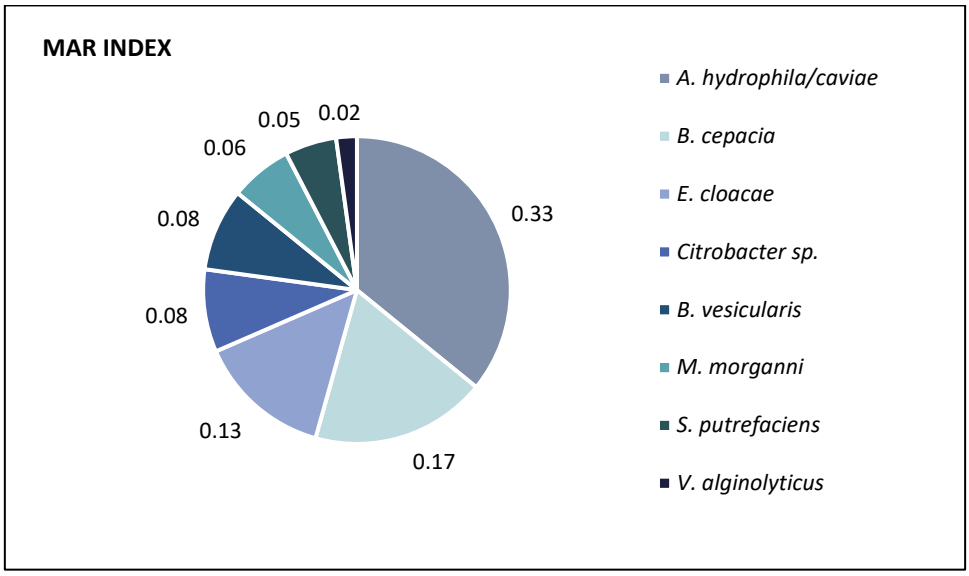
ANTIMICROBIAL CLASS	ANTIMICROBIAL COMPOUND	BACTERIAL ISOLATES (%)		
		SUSCEPTIBLE	INTERMEDIATE	RESISTANT
Aminoglycosides	Amikacin (30 µg)	19 (100)	0 (0)	0 (0)
	Gentamicin (120 µg)	19 (100)	0 (0)	0 (0)
	Tobramycin (10 µg)	19 (100)	0 (0)	0 (0)
Carbapenems	Meropenem (10 µg)	18 (95)	1 (5)	0 (0)
	Imipenem (10 µg)	12 (63)	6 (32)	2 (11)
Cephalosporins	Cefoperazone (75 µg)	16 (84)	2 (11)	1 (5)
	Ceftazidime (30 µg)	18 (95)	1 (5)	0 (0)
Fluoroquinolones	Ciprofloxacin (5 µg)	18 (95)	1 (5)	0 (0)
	Enrofloxacin (5 µg)	16 (84)	2 (11)	1 (5)
	Ofloxacin (5 µg)	19 (100)	0 (0)	0 (0)
Tetracyclines	Tetracycline (30 µg)	13 (68)	1 (5)	5 (26)
Ureidopenicillins	Piperacillin (100 µg)	18 (95)	0 (0)	1(5)

Legend: Percentage (%).

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 an *Enterobacter cloacae* isolate (MAR index value = 0.25) (Table 4) (Graphic 2).

No significant differences were observed for the MAR index values of isolates from different sample types: cloacal – MAR index mean value = 0.10; oral – MAR index mean value = 0.08; and egg content – MAR index mean value = 0.03; despite the ones from cloacal samples being higher ($p > 0.05$).

Graphic 2 – MAR index mean values for the tested bacterial species.



Legend: *Vibrio alginolyticus* (*V. alginolyticus*)*; Morganella morganii* (*M. morganii*)*; Brevundimonas vesicularis* (*B. vesicularis*)*; Enterobacter cloacae* (*E. cloacae*)*; Burkholderia cepacia* (*B. cepacia*)*; Shewanella putrefaciens* (*S. putrefaciens*)*, Aeromonas hydrophila/caviae* (*Aeromonas* sp.). The highest MAR index value was registered for *Aeromonas hydrophila/caviae*.

4.4. Characterization of isolates' virulence profile

Regarding the virulence characterization, all isolates were able to produce hemolysins (Table 4). Most isolates were able to produce DNases (89.47%), lipases (78.95%) and biofilm (73.68%). Protease production was revealed in 52.63% of isolates. Lecithinase (21.05%) and gelatinase (15.79%) activities were less observed among the tested isolates (Table 4).

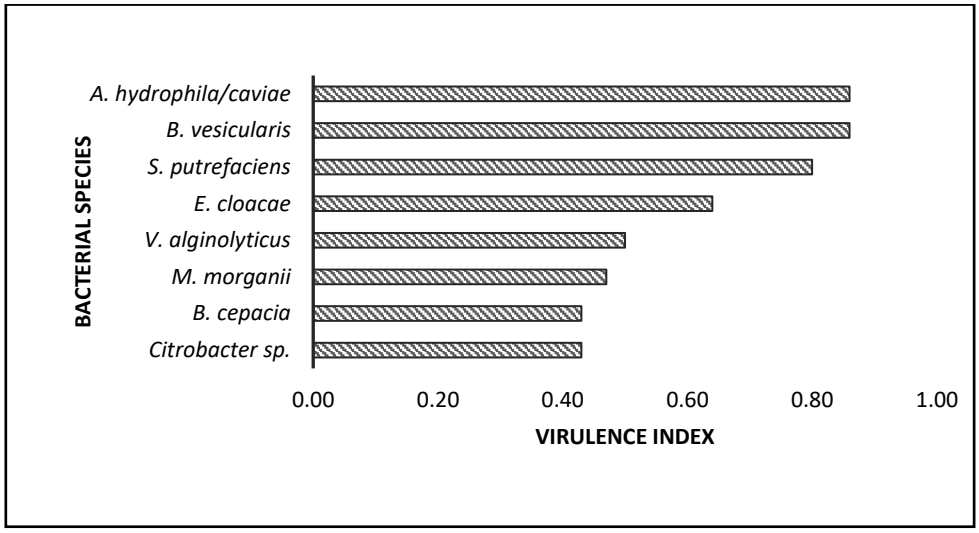
Higher virulence profile index (V. Index) values were determined for *Aeromonas hydrophila/caviae* (V. Index value = 0.86), *Brevundimonas vesicularis* (V. Index value = 0.86) and *Shewanella putrefaciens* (V. Index mean value = 0.80). Bacterial species revealing the lowest values included *Vibrio alginolyticus* (V. Index mean value = 0.50), *Morganella morganii* (V. Index mean value = 0.47), *Burkholderia cepaciae* (V. Index value = 0.43) and *Citrobacter* sp. (V. index value = 0.43) (Graphic 3) (Table 4).

No significant differences were observed between the V. Index values of isolates from cloacal (V. Index mean value = 0.66), oral (V. Index mean value = 0.60) and egg content (V. Index mean value = 0.43) samples ($p > 0.05$), despite the values for isolates from samples from class III animals being higher.

Concerning the animal health status classes, no significant differences were observed between the V. Index results of isolates from class I (V. Index mean value = 0.57), class II (V. Index mean value = 0.50), and class III turtles (V. Index mean value = 0.67) ($p > 0.05$), despite the values for isolates from samples from class III animals being higher.

No significant differences were observed for the MAR and Virulence indices of isolates from the Enterobacteriaceae family – MAR index mean value = 0.08; V. Index mean value = 0.51; and the Non-Enterobacteriaceae family – MAR index mean value = 0.08; V. Index mean value = 0.68 ($p > 0.05$).

Graphic 3 – Virulence index mean values for the tested bacterial species.



Legend: *Aeromonas hydrophila/caviae* (*A. hydrophila/caviae*)*; Brevundimonas vesicularis* (*B. vesicularis*)*; Shewanella putrefaciens* (*S. putrefaciens*)*; Enterobacter cloacae* (*E. cloacae*)*; Vibrio alginolyticus* (*V. alginolyticus*); *Morganella morganii (M. morganii); Burkholderia cepacia* (*B. cepacia*)*;* The highest virulence index value was observed for *Aeromonas hydrophila/caviae.*

No significant correlation was observed between MAR index and V. Index ($p > 0.05$). Also, no significant differences were observed for the MAR index mean values between biofilm-producer (MAR index mean value = 0.68) and non-biofilm-producer isolates (MAR index mean value = 0.43), despite the first group registering higher MAR index values ($p > 0.05$).

Table 4 - Isolates' antibiotic resistance and virulence profiles.

ISOLATE CODE	SAMPLE CODE	SAMPLE TYPE	SPECIES ID	RESISTANCE PROFILE		MAR INDEX	VIRULENCE PROFILE							V. INDEX
				I	R		HEM	DNase	LIP	LEC	PT	GEL	BF (h)	
1	276/030-2	C	*Aeromonas hydrophila/caviae*	IMP; ENR	CFP	0.33	β	+	+	+	+	-	24	0.86
2	786/785	C	*Brevundimonas vesicularis*	IMP	-	0.08	α	+	+	-	+	+	24	0.86
3	329/328	E	*Burkholderia cepaciae*	ENR; CFP	-	0.17	α	-	+	-	+	-	-	0.43
4	49/50-2	C	*Shewanella putrefaciens*	-	-	0.00	α	+	+	-	+	+	24	0.86
5	46/45-2	C	*Shewanella putrefaciens*	-	T	0.08	α	+	+	-	-	-	-	0.43
6	46/45-1.2b	E	*Shewanella putrefaciens*	-	-	0.00	β	+	+	+	+	+	24	1.00
7	72/73-1	C	*Shewanella putrefaciens*	IMP	-	0.08	α	+	+	+	+	-	24	0.86
8	276/030-1	C	*Shewanella putrefaciens*	IMP	-	0.08	α	+	+	+	+	-	24	0.86
9	276/030-1	E	*Vibrio alginolyticus*	-	-	0.00	α	+	-	INC	+	-	48	0.57
10	276/030-1	E	*Vibrio alginolyticus*	-	-	0.00	α	+	-	INC	+	-	24	0.57
11	60/61-1	C	*Vibrio alginolyticus*	-	-	0.00	α	+	-	INC	-	-	24	0.43
12	504/503-2	O	*Vibrio alginolyticus*	-	T	0.08	α	+	-	-	-	-	24	0.43
13	330/331	C	*Enterobacter cloacae*	-	-	0.00	α	+	+	-	-	-	48	0.57
14	49/50-1	C	*Enterobacter cloacae*	CIP	ENR; PIP	0.25	α	+	+	-	+	-	48	0.71
15	46/45-2.1a	C	*Morganella morganii*	MEM	T	0.08	β	+	+	-	-	-	-	0.43
16	46/45-2.2	E	*Morganella morganii*	-	T	0.00	α	+	+	-	-	-	-	0.43
17	276/030-1	O	*Morganella morganii*	IMP	T	0.08	α	+	+	-	-	-	-	0.43
18	504/503	C	*Morganella morganii*	IMP	T	0.08	α	+	+	-	-	-	24	0.57
19	229/228	C	*Citrobacter* sp.	CAZ	-	0.08	α	-	+	-	-	-	72	0.43

Legend: Multiple antibiotic resistance (MAR), V. Index (Virulence index), cloaca (C), oral cavity (O), egg (E), hours (h), intermediate (I), resistant (R), imipenem (IMP), enrofloxacin (ENR), cefoperazone (CFP), tetracycline (T), ciprofloxacin (CIP), piperacillin (PIP), meropenem (MEM), ceftazidime (CAZ), positive (+), negative (-), hemolysins (HEM), alpha (α), beta (β), lipase (LIP), lecithinase (LEC), protease (PT), gelatinase (GEL), biofilm (BF), inconclusive (INC) (due to swarming ability of *Vibrio alginolyticus*).

Chapter 5. Discussion

The present study provides initial insight data on the Gram-negative aerobic and facultative anaerobic microbiota of a firstly examined loggerhead sea turtle subpopulation, addressing the antimicrobial resistance issue on a broader scale. It 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 bacteria.

Isolates were obtained from oral and cloacal swabs, which are described as reliable, effective, non-traumatic techniques for the characterization of loggerhead sea turtle's microbiota and the assessment of turtle populations' conservation status (Lanci et al. 2012; Pace et al. 2019a). Also, Oliveira et al. (2010) proved that similar collection and transport methods permit the isolation of bacteria, even when requiring long distances and processing periods.

In the present study, *Shewanella putrefaciens* was the most prevalent species found in *Caretta caretta*, as previously reported by Blasi et al. (2020). Unexpectedly, no *Pseudomonas* sp. isolates were detected, despite *P. aeruginosa* be one of the most prevalent species isolated from sea turtles in previous studies (Al-Bahry et al. 2009, 2011; Oliveira et al. 2017). Also, a low selectivity of the GSP medium for the isolation of *Pseudomonas* sp. and *Aeromonas* sp., for the tested samples was noted, as it allowed the growth of isolates identified as *Burkholderia cepacia, Brevundimonas vesicularis, Shewanella putrefaciens* and *Vibrio alginolyticus*.

All identified bacterial species have been previously isolated from both injured and stranded sea turtles, as well as healthy wild animals (Óros et al. 2005; Rodgers et al. 2018; Alduina et al. 2020; Blasi et al. 2020), except 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 (Glazebrook and Campbell 1990a; Di Ianni et al. 2015; Ahasan et al. 2017; Pace et al. 2018).

Aeromonas hydrophila and *Vibrio alginolyticus* have been associated with ulcerative stomatitis, ulcerative esophagitis, granulomatous hepatitis, granulomatous nephritis, and bronchopneumonia (Aguirre et al. 1994; Óros et al. 2004, 2005; Mashkour et al. 2020). *Aeromonas hydrophila* is a straight, rigid, oxidase and catalase-positive, non-spore-forming, facultative anaerobic bacilli of the Aeromonodaceae family. It occurs singly, in pairs or short chains and is motile by polar flagella (Martin-Carnahan and Joseph 2005). *Aeromonas* species are primarily aquatic, most frequently isolated from fresh and estuarine waters and in association with aquatic animals; they are also found on sewage, surface waters, sediments, and biofilms (Martin-Carnahan and Joseph 2005).

The genus *Vibrio* belongs to the Vibrionaceae family and is characterized by small, straight, slightly curved, or comma-shaped, facultative anaerobic bacilli; motile by polar flagella, with the ability to synthesize numerous lateral flagella on solid media, and capable of both fermentative and respiratory metabolism (Farmer et al. 2005). These bacteria are primarily aquatic, and species distribution is usually dependent on salt (Na) content, nutrient availability, and water temperature. *Vibrio* species are common in marine and estuarine environments and the intestinal contents of marine animals (Farmer et al. 2005).

Burkholderia cepacia complex is a group of non-spore-forming, facultative anaerobic bacilli belonging to the Burkholderiaceae family and has been associated with lesions of the oral cavity (e.g., ulcerative stomatitis) and traumatic skin lesions of sea turtles (Óros et al. 2004, 2005). *Burkholderia cepacia* complex is widely spread in the environment and is known to have both beneficial and detrimental effects on plants (Coenye et al. 2001; Mahenthiralingam et al. 2005).

Shewanella putrefaciens has been regarded as a severe opportunistic bacterium (Craven et al. 2007; Paździor 2016; Ahasan et al. 2017; Yousfi et al. 2017; Lloyd et al. 2018) and was described in one case of septicemia in green sea turtles (*Chelonia mydas*) with underlying fibropapillomatosis (Work et al. 2003). Members of the *Shewanella* genus (Alteromonadaceae family) are straight or curved, motile by a single, polar flagellum, oxidase, and catalase-positive, non-endospore-forming and facultative anaerobic bacilli (Bowman 2005). These bacteria are commonly isolated from clinical samples, freshwater, freshwater sediment, estuarine environments, marine algae, seawater, marine sediment, fish, marine invertebrates, sea ice, marine snow, and pelagic ocean waters (Bowman 2005).

Citrobacter sp. has been implicated in cases of ulcerative esophagitis and hepatitis in sea turtles, and *Morganella morganii* was identified as a cause of conjunctivitis in turtles (Glazebrook and Campbell 1990a; Aguirre et al. 1994; Óros et al. 2004; Di Ianni et al. 2015). *Citrobacter* sp. and *Morganella morganii* belong to the Enterobacteriaceae family (Davies J and Davies D 2010; Di Ianni et al. 2015). The genus *Citrobacter* is characterized by facultative anaerobic straight bacilli, occurring single or in pairs, and motile by peritrichous flagella. Citrate can be utilized as a sole carbon source by most strains (Frederiksen 2005). This genus occurs in the faeces of humans and some animals as normal intestinal inhabitants or isolated from clinical specimens as opportunistic pathogens. These bacteria can also be found in soil, water, sewage, and food (Frederiksen 2005). *Morganella morganii* isolates are facultative anaerobic straight bacilli, motile through peritrichous flagella, occurring in the faeces of humans, dogs and other mammals, and reptiles (Janda and Abbot 2005).

In this study, *Burkholderia cepacia, Shewanella putrefaciens* and *Vibrio alginolyticus,* isolated from egg samples, were previously associated with the occurrence of unhatched eggs (Wyneken et al. 1988; Craven et al. 2007; Awong-Taylor et al. 2008). Craven et al. 2007,

suggested that environmental bacteria found in adult females can be potential opportunistic pathogens and a cause of embryonic mortality. Also, Candan O and Candan E (2020) found a higher number of unhatched *Chelonia mydas'* eggs in bacteria-infected nests compared to nests free of bacterial infection, significantly contributing to low hatching success.

Morganella morganii was previously isolated from the outside eggshell of unhatched loggerhead eggs (Craven et al. 2007). In this study, it was possible to obtain a *Morganella morganii* isolate from the internal fluid of one egg, representing, to the best of our knowledge, the first finding of this bacterial species associated with the sea turtle egg content microbiota.

In the present study, eggs were collected directly from the cloaca during oviposition. Therefore, bacterial species isolated from the egg's content should have their origin from the nesting female (Al-Bahry et al. 2009). *Shewanella putrefaciens* and *Morganella morganii* were found in both the oviductal fluid and the egg content of one loggerhead female. This finding is in line with the study by Wyneken et al. (1988), in which the association between the bacterial species found in *Caretta caretta* females and the respective clutch was described. Wyneken et al. (1988) also suggested that bacteria shedding from females to eggs may be more common than described, possible due to a periodically shedding of the pathogen, nonequivalent to the period of sampling of the oviductal fluid.

The time between ovulation and nesting (the nesting cycle), during which the egg components and eggshell are formed, is approximately 9 to 15 days (Owens and Morris 1985). In this period, the egg can be in contact with bacteria present in the turtle's reproductive apparatus (Al-Bahry et al. 2009). In fact, the ovaries and oviducts, if infected, can be a source of egg contamination before oviposition. This vertical transmission has been studied in chickens, mainly infected with *Salmonella* sp., in which infection of the reproductive organs acted as the source of contamination of the egg yolk, albumen and eggshell membranes (Okamura et al. 2001; Gantois et al. 2009).

Compared to previous studies, the prevalence of resistant isolates was low. The percentage of bacteria resistant or intermediately resistant to two or more antibiotics was 15.79%, while other studies identified significantly higher values (Foti et al. 2009; Oliveira et al. 2017; Pace et al. 2019a). No MDR bacteria were detected, which is in line with a previous study conducted in juvenile sea turtles hawksbill *Eretmochelys imbricata* and *Chelonia mydas* from potential coincident feeding grounds (Oliveira et al. 2017), but discordant to prior studies focusing on other *Caretta caretta* and *Chelonia mydas'* populations (Al-Bahry et al. 2009, 2011; Foti et al. 2009; Ahasan et al. 2017; Pace et al. 2019a). Furthermore, and despite not being statistically significant, the higher level of resistance in isolates 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.

Following previous results, higher resistance levels were observed for tetracyclines and the lower ones for the aminoglycoside class (Al-Bahry et al. 2009; Foti et al. 2009; Ahasan et al. 2017; Pace et al. 2019a; Blasi et al. 2020). To the best of our knowledge, no resistance to imipenem was previously described for loggerhead sea turtles' bacteria. Here, *Aeromonas hydrophila/caviae*, *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 prevalence of resistance for the carbapenem class, this finding should be further assessed due to the categorization of this antibiotic as a last resort option for the treatment of serious Gram-negative infections, being of major importance for Human Medicine (Thomson and Bonomo 2005; Wellington et al. 2013).

Being characterised mainly by a pristine environment, the Island of Maio is less affected by human impacts, such as the discharge of wastewater carrying high levels of antibiotics, associated with aquaculture, intensive farms and medical settings (Shah et al. 2014; Le Quesne et al. 2018; Vitale et al. 2018; Blasi et al. 2020). Effluent and runoff contamination of marine ecosystems are critical contact points between marine wildlife and antibiotic-resistant bacteria (Arnold et al. 2016). The aquaculture industry is also a significant source of ARG, ARB and antimicrobials to the marine environments (Arnold et al. 2016).

Sea turtles dwelling in ecosystems affected by anthropogenic activities are at higher risk of being exposed to antibiotic environmental pressure (Ahasan et al. 2017). Comparing with other loggerhead colonies, both feeding and nesting sites of the colony of the Island of Maio are less exposed to anthropogenic pressures (Foti et al. 2009; Ahasan et al. 2017; Pace et al. 2019a; Alduina et al. 2020), which suggests a low contact between the studied loggerheads and the tested antimicrobial compounds, consequently explaining the low prevalence of antibiotic-resistant isolates in this loggerhead subpopulation.

Furthermore, the studied females had an average CCL of 77.1 cm, which, according to Eder et al. (2012), shows that these turtles have expected oceanic feeding strategies, travelling to the oceanic settings of Mauritania, The Gambia and Senegal (Hawkes et al. 2006). This separation from the coast also supports a little exposure to human impacts and sustains our findings.

Isolates' phenotypic virulence characterization showed that the isolates from this study could express several virulence traits that may contribute to the evasion of the host's immune system as well as the host's tissue colonization and damage (Seixas et al. 2014). The expression of a high number of virulence factors may play an essential role in the pathogenesis of infections (Seixas et al. 2014).

Higher virulence indices were detected for the isolates from the Non-Enterobacteriaceae family (*Aeromonas hydrophila/caviae, Brevundimonas vesicularis,*

Shewanella putrefaciens), suggesting the pathogenic potential of these species. Although lower virulence index values were revealed for *Vibrio alginolyticus*, this species has been associated with numerous serious diseases in sea turtles (Aguirre et al. 1994; Óros et al. 2004, 2005; Mashkour et al. 2020). Contrarily to our results, *V. alginolyticus* isolates obtained from sea fishes along the Tunisian coast were previously described as being able to produce gelatinases, lipases, and β-hemolysins, also presenting high levels of antimicrobial resistance (Sadok et al. 2013). Moreover, Zavala-Norzagaray et al. (2015) identified *Vibrio cholerae* isolates carrying virulence genes in olive ridley sea turtles (*Lepidochelys olivacea*), also referring to the epidemic potential of these bacterial species and the consequent negative impact of sea turtle consumption.

Despite not being statistically significant, the virulence indices of the isolates from cloacal samples were higher compared to the ones for isolates from other sample types (oral cavity and egg content), which may suggest that the cloacal and gastrointestinal environments host bacteria conserving a more complex phenotypic virulence profile. These bacteria are potentially more pathogenic and with higher survival fitness.

The virulence profile index of isolates cultured from animals showing high parasitic loads (class III) was higher when compared to the ones of isolates from individuals without evident cloacal parasites (class I and class II). Parasites may exert selective pressures to modulate the genome of a bacterium or microbial population and consequently select for the production and expression of specific virulence determinants (Toft and Andersson 2010; Bliven and Maurelli 2016). Additionally, the high burden of gastrointestinal parasites can affect sea turtles' health and predispose them to secondary infections by depressing the immune system. 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 in these animals and a compromised immune response. These factors may include high parasite loads (observed in class III animals), which can cause gastrointestinal and cloacal injuries, inflammatory reaction, and nutrient depletion (Óros et al. 2004, 2005; Pace et al. 2019b; Santoro et al. 2019); and superficial lesions (e.g., erosions of the carapace) (observed in class II animals), probably indicating a previous traumatic event (Óros et al. 2005). However, the long co-evolution between parasites and their sea turtle host, usually characterized by their low pathogenicity, may contribute to the stable functioning of the immune system and even protect against the colonization by less adapted pathogenic species (Greiner et al. 2013, Pace et al. 2019b).

Although no significant positive correlation was observed between MAR and virulence indices, the association between virulence and resistance has been studied. Beceiro et al. (2013) 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 the development of infection mainly in environments where selective antibiotic pressure predominates.

However, not statistically significant, MAR index values were higher in biofilm-producer isolates compared with non-biofilm-producers. In fact, biofilms can be responsible for antibiotic resistance up to 1,000-fold higher than their free-swimming, planktonic counterparts (Hoyle and Costerton 1991; Mah et al. 2003). Biofilms can resist the effect of antibiotic compounds by multiple mechanisms, requiring a set of recognizable genetic determinants (Mah et al. 2003). These mechanisms may include the impairment of antibiotic diffusion by the matrix components, decreased metabolic activity and nutrient limitation, expression of chromosomal beta-lactamases, upregulated efflux pumps, horizontal gene transfer, quorum-sensing, and mutations in antibiotic target molecules (Mah et al. 2003; Driffield et al. 2008; Høiby et al. 2010). The increased antibiotic-resistance in biofilm bacteria makes these microbial communities extremely difficult to control in medical settings (Costerton et al. 1999; Mah et al. 2003).

Bacteria identified in the present study may act as agents of zoonotic disease and represent an important threat to Public Health (Di Ianni et al. 2015; Mashkour et al. 2020).

In humans, *Aeromonas* sp. can cause gastroenteritis, septicemia, skin and soft tissue infections, peritonitis, and empyema (Janda and Abbott 2010). *Burkholderia* sp. and *Enterobacter* sp. can be implicated in nosocomial infections, with *Burkholderia cepacia* being also described as a cause of necrotizing pneumonia in humans (Saini et al. 1999; Óros et al. 2005; Davies J and Davies D 2010). *Vibrio alginolyticus* has been associated with ear and wound infections (Stewart et al. 2014). *Morganella morganii* (*M. morganii*) has been described as a cause of neonatal sepsis, urinary tract infections, wound infections, and as one of the most frequent pathogens isolated from wounds' secondary infections following snakebites (Chang et al. 2011; Chen et al. 2011). The presence of resistant *M. morganii* isolates in sea turtles should not be disregarded since, even uncommon, this bacterium can be an agent of zoonotic disease, especially in immunocompromised humans (Di Ianni et al. 2015). *Brevundimonas vesicularis* has been described as a cause of bacteriemia, and despite this bacterial species being considered of minor clinical importance, it has been the main *Brevundimonas* species associated with human infection (Ryan and Pembroke 2018). Finally, *Shewanella putrefaciens* has been implicated in ears, skin, and soft tissue infections, with or without associated bacteriemia (Holt et al. 2005).

Biosecurity measures are, hence, required, particularly concerning volunteers and biologists interacting with sea turtles, working on nesting beaches, and handling eggs from natural nests (Santoro et al. 2008). Personal protective equipment (PPE) and hygiene practices have been proposed as effective and accessible options to decrease the risk of

pathogen transmission (Mashkour et al. 2020). Moreover, Mashkour et al. (2020) revealed that sea turtles' health experts encourage preventive solutions for the reduction of the risk of Enterobacteriaceae and multi-resistant bacterial infections by promoting education and awareness initiatives.

Besides the existent conservation efforts, there is a severe ongoing slaughter of females on the beaches of the Island of Maio (Martins et al. 2013; Patino-Martinez et al. In Press). The consumption of turtle-related products represents a risk behaviour, aggravated by the fact that there are no safety controls for turtle related-products, which may pose a high-risk of food-borne disease (Zavala-Norzagaray et al. 2015). The risk to local populations is also worsened by the limited access to health assistance and therapeutic options.

Furthermore, on this Island, there is an increasing number of nest destruction by dog predation, including nests in both natural sites and hatcheries (Patino-Martinez et al. In Press). Predation of sea turtles' eggs by these animals can represent an additional factor of pathogenic bacteria and ARB dissemination, increasing the Public Health risk. Nest protection and monitoring were described as crucial to prevent nest loss and justify the implementation of a hatchery program (Mashkour et al. 2020). In truth, Patino-Martinez et al. (In Press) showed that hatcheries do not represent the best solution to increase hatching success in the Island of Maio and that, alternatively, efforts should be made in controlling the ongoing threats.

The individual ecological features, as the migratory lifestyle and feeding behaviour characterizing each loggerhead subpopulation, have a significant influence on the microbial composition and parasitic communities of these animals (Valente et al. 2009; Santoro et al. 2010; Pace et al. 2019b).

The low levels of antimicrobial resistance in the studied group represent positive and encouraging results regarding the evaluation of the conservation status of this loggerhead colony. The low prevalence of antimicrobial resistance revealed by loggerhead sea turtles of Cape Verde suggests that the North-East Atlantic subpopulation and respective environments (migration routes, feeding and nesting sites) may not select for AMR dissemination.

Nonetheless, the evidence of potential pathogenic Gram-negative bacteria expressing virulence traits may represent a risk to sea turtles' health and consequently affect the conservation of this endangered species.

The revealed findings, as well as the increasing numbers of nesting females registered in the Island of Maio (approximately 20000 nests in 2020), endorse the safe animal interactions practised and supervised by the Maio Biodiversity Foundation to prevent human disturbance and reduce the anthropogenic impacts in this protected species. However, the continuous monitoring of the presence of pathogenic and antibiotic-resistant bacteria in this endangered *Caretta caretta* subpopulation is of major importance for the success of future conservation programs (Al-Bahry et al. 2009; Oliveira et al. 2017).

A thorough understanding of both the microbiota of loggerhead sea turtles and the role of these animals as reservoirs of antibiotic-resistant and virulent bacteria is far from being accomplished. Nevertheless, the baseline information will undoubtedly increase as more bacterial species from distinct loggerhead subpopulations, and sample sources are discovered, disease manifestations are described, and diagnostic methodologies are proposed. For example, Alduina et al. (2020) focused on metagenome analysis to evaluate the presence of antibiotic resistance genes in loggerhead sea turtles, sustaining the importance of these techniques to further understanding the spread and persistence of antimicrobial resistance in the marine environment.

Chapter 6. Conclusion

Over the past ten years, studies revealed a surprisingly high level of antimicrobial resistance in sea turtles' bacteria, mainly on the Mediterranean loggerhead subpopulation. Also, loggerhead sea turtles have been suggested as carriers of potentially pathogenic bacteria and zoonotic agents.

No information on these topics was available for the Cape Verdean nesting subpopulation (North-East Atlantic subpopulation), which is currently believed to be the largest subpopulation of this species worldwide. As the archipelago of Cape Verde, and especially the Island of Maio, are characterized by a lower anthropogenic impact compared to other coastal environments, a lower prevalence of antibiotic-resistant bacteria in the loggerhead colony of the Island of Maio was expected.

We quantified and evaluated the antibiotic resistance and virulence profiles of the aerobic and facultative anaerobic Gram-negative bacteria present in the cloaca, oral cavity and eggs of loggerhead females from the Island of Maio. We also assessed the impact of these species on sea turtles' conservation and the underlying public health risk resulting from interactions with these animals, and the consumption of turtle-derived products.

Our findings revealed a low prevalence of antibiotic-resistant bacteria in the loggerhead colony of the Island of Maio, which may be explained by the low anthropogenic pressure observed on this island.

Furthermore, the bacterial species analysed in the present study were able to produce several virulence factors, including biofilms, which raises concern about their pathogenic potential. Therefore, the consumption of turtle-related products by both humans and domestic animals represents a risk behaviour for Public Health. Nevertheless, this risk can be mitigated through awareness campaigns and the sharing of scientific-based knowledge.

Given the association between sea turtle's health and human health, our findings were assessed in the context of One Health, which promotes the cooperation between professionals from the areas of human's, animal's and environmental health.

This study also highlights the importance to perform bacteriological analysis on non-captive loggerhead sea turtles from distinct subpopulations with individual ecological features to assess the AMR issue in a global context.

Finally, further investigation of the microbiota of the Cape Verdean loggerhead subpopulation is encouraged to englobe different nesting groups and temporal scales as well as to monitor the evolution of the antibiotic resistance and virulence profiles of the Gram-negative bacteria of this endangered loggerhead subpopulation.

Chapter 7. References

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Annexe 1 – Sampling data.

Table 5 – Sampling data. Part I.

Sample nº.	Date	Time	Beach/ local	Flipper Tag ID	PIT	CCL	Estimated age (yrs)	Clutch size	Sample type	Animal general condition
1	8/1/2019	21h51	"Praiona", "Pedro Vaz"	QMN326/27	9.81098(...)258	79.5	18.95	59	cloaca	good
2	8/1/2019	21h51	"Praiona", "Pedro Vaz"	QMN326/27	9.81098(...)258	79.5	18.95	59	oral cavity	good
3	8/1/2019	21h51	"Praiona", "Pedro Vaz"	QMN326/27	9.81098(...)258	79.5	18.95	59	egg	good
4	8/1/2019	23h00	"Praiona", "Pedro Vaz"	QMN329/328	-	75.5	16.15	72	cloaca	good
5	8/1/2019	23h00	"Praiona", "Pedro Vaz"	QMN329/328	-	75.5	16.15	72	oral cavity	good
6	8/1/2019	23h00	"Praiona", "Pedro Vaz"	QMN329/328	-	75.5	16.15	72	egg	good
7	8/1/2019	23h21	"Praiona", "Pedro Vaz"	QMN330/331	-	78.3	18.05	62	cloaca	superficial erosions of the carapace
8	8/1/2019	23h21	"Praiona", "Pedro Vaz"	QMN330/331	-	78.3	18.05	62	oral cavity	superficial erosions of the carapace
9	8/1/2019	23h21	"Praiona", "Pedro Vaz"	QMN330/331	-	78.3	18.05	62	egg	superficial erosions of the carapace
10	8/1/2019	6h10	"Praiona", "Pedro Vaz"	QMN335/334	9.81098(..)250	76.4	16.73	-	cloaca	good
11	8/1/2019	6h10	"Praiona", "Pedro Vaz"	QMN335/334	9.81098(...)250	76.4	16.73	-	oral cavity	good
12	8/1/2019	6h10	"Praiona", "Pedro Vaz"	QMN335/334	9.81098(...)250	76.4	16.73	-	egg	good
13	8/2/2019	22h30	“Areia Preta” “Praia Gonçalo”	QMJ104/QMI098	9.81098(...)598	-	-	83	cloaca	superficial erosions of the carapace
14	8/2/2019	22h30	“Areia Preta” “Praia Gonçalo”	QMJ104/QMI098	9.81098(...)598	-	-	83	oral cavity	superficial erosions of the carapace
15	8/2/2019	22h30	“Areia Preta” “Praia Gonçalo”	QMJ104/QMI098	9.81098(...)598	-	-	83	egg	superficial erosions of the carapace
16	8/2/2019	23h32	“Cozinha fácil” “Praia Gonçalo”	QMI 24/25	9.81098(...)803	-	-	-	cloaca	good
17	8/2/2019	23h32	“Cozinha fácil” “Praia Gonçalo”	QMI 24/25	9.81098(...)803	-	-	-	oral cavity	good
18	8/2/2019	23h32	“Cozinha fácil” “Praia Gonçalo”	QMI 24/25	9.81098(...)803	-	-	-	egg	good

Legend: number (nº), hour (h), Passive Integrated Transponder (PIT)**,** curved carapace length (CCL), years (yrs).

Table 5 – Sampling data. Part II.

Sample nº.	Date	Time	Beach/ local	Flipper Tag ID	PIT	CCL	Estimated age (yrs)	Clutch size	Sample type	Animal general condition
19	8/2/2019	23h55	"Cozinha fácil" "Praia Gonçalo"	QMI 64/65	9.81098(...)690	-	-	-	cloaca	superficial erosions of the carapace
20	8/2/2019	23h55	"Cozinha fácil" "Praia Gonçalo"	QMI 64/65	9.81098(...)690	-	-	-	oral cavity	superficial erosions of the carapace
21	8/2/2019	23h55	"Cozinha fácil" "Praia Gonçalo"	QMI 64/65	9.81098(...)690	-	-	-	egg	superficial erosions of the carapace
22	8/2/2019	01h26	"Areia Preta" "Praia Gonçalo"	QMI 99/100	9.81098(...)752	-	-	-	cloaca	superficial erosions of the carapace, external parasites
23	8/2/2019	01h26	"Areia Preta" "Praia Gonçalo"	QMI 99/100	9.81098(...)752	-	-	-	oral cavity	superficial erosions of the carapace, external parasites
24	8/2/2019	01h26	"Areia Preta" "Praia Gonçalo"	QMI 99/100	9.81098(...)752	-	-	-	egg	superficial erosions of the carapace, external parasites
25	8/2/2019	02h10	"Areia Preta" "Praia Gonçalo"	QMI 784/783	9.81098(...)606	75.2	15.96	78	cloaca	good
26	8/2/2019	02h10	"Areia Preta" "Praia Gonçalo"	QMI 784/783	9.81098(...)606	75.2	15.96	78	oral cavity	good
27	8/2/2019	02h10	"Areia Preta" "Praia Gonçalo"	QMI 784/783	9.81098(...)606	75.2	15.96	78	egg	good
28	8/2/2019	02h40	"Areia Preta" "Praia Gonçalo"	QMI 786/785	9.81098(...)330	72.5	14.35	78	cloaca	good
29	8/2/2019	02h40	"Areia Preta" "Praia Gonçalo"	QMI 786/785	9.81098(...)330	72.5	14.35	78	oral cavity	good
30	8/2/2019	02h40	"Areia Preta" "Praia Gonçalo"	QMI 786/785	9.81098(...)330	72.5	14.35	78	egg	good
31	8/4/2019	21h50	"Areia Preta" "Praia Gonçalo"	QMI 039/038	-	-	-	82	cloaca	good
32	8/4/2019	21h50	"Areia Preta" "Praia Gonçalo"	QMI 039/038	-	-	-	82	oral cavity	good
33	8/4/2019	21h50	"Areia Preta" "Praia Gonçalo"	QMI 039/038	-	-	-	82	egg	good
34	8/5/2019	00h34	"Areia Preta" "Praia Gonçalo"	QMI 051/052	9.81098(...)652	74.6	15.59	-	cloaca	low body condition, deformation - carapace
35	8/5/2019	00h34	"Areia Preta" "Praia Gonçalo"	QMI 051/052	9.81098(...)652	74.6	15.59	-	oral cavity	low body condition, deformation - carapace
36	8/5/2019	00h34	"Areia Preta" "Praia Gonçalo"	QMI 051/052	9.81098(...)652	74.6	15.59	-	egg	low body condition, deformation - carapace

Legend: number (nº), hour (h), Passive Integrated Transponder (PIT)**,** curved carapace length (CCL), years (yrs).

Table 5 – Sampling data. Part III.

Sample nº.	Date	Time	Beach/ local	Flipper Tag ID	PIT	CCL	Estimated age (yrs)	Clutch size	Sample type	Animal general condition
37	8/5/2019	01h20	"Areia Preta" "Praia Gonçalo"	QMI 028/029	9.81098(...)018	79.2	18.72	65	cloaca	good
38	8/5/2019	01h20	"Areia Preta" "Praia Gonçalo"	QMI 028/029	9.81098(...)018	79.2	18.72	65	oral cavity	good
39	8/5/2019	01h20	"Areia Preta" "Praia Gonçalo"	QMI 028/029	9.81098(...)018	79.2	18.72	65	egg	good
40	8/6/2019	22h13	"Cozinha fácil" "Praia Gonçalo"	QMI 062/063	-	-	-	-	cloaca	good
41	8/6/2019	22h13	"Cozinha fácil" "Praia Gonçalo"	QMI 062/063	-	-	-	-	oral cavity	good
42	8/6/2019	22h13	"Cozinha fácil" "Praia Gonçalo"	QMI 062/063	-	-	-	-	egg	good
43	8/6/2019	22h30	"Cozinha fácil" "Praia Gonçalo"	QMI 060/061	-	76	16.47	-	cloaca	flacking of the carapace
44	8/6/2019	22h30	"Cozinha fácil" "Praia Gonçalo"	QMI 060/061	-	76	16.47	-	oral cavity	flacking of the carapace
45	8/6/2019	22h30	"Cozinha fácil" "Praia Gonçalo"	QMI 060/061	-	76	16.47	-	egg	flacking of the carapace
46	8/6/2019	22h47	"Cozinha fácil" "Praia Gonçalo"	QMI 064/065	-	75.8	16.34	-	cloaca	good
47	8/6/2019	22h47	"Cozinha fácil" "Praia Gonçalo"	QMI 064/065	-	75.8	16.34	-	oral cavity	good
48	8/6/2019	22h47	"Cozinha fácil" "Praia Gonçalo"	QMI 064/065	-	75.8	16.34	-	egg	good
49	8/6/2019	23h20	"Cozinha fácil" "Praia Gonçalo"	QMI 066/067	-	-	-	-	cloaca	good
50	8/6/2019	23h20	"Cozinha fácil" "Praia Gonçalo"	QMI 066/067	-	-	-	-	oral cavity	good
51	8/6/2019	23h20	"Cozinha fácil" "Praia Gonçalo"	QMI 066/067	-	-	-	-	egg	good
52	8/6/2019	00h00	"Cozinha fácil" "Praia Gonçalo"	QMK 067/068	-	-	-	-	cloaca	good
53	8/6/2019	00h00	"Cozinha fácil" "Praia Gonçalo"	QMK 067/068	-	-	-	-	oral cavity	good
54	8/6/2019	00h00	"Cozinha fácil" "Praia Gonçalo"	QMK 067/068	-	-	-	-	egg	good

Legend: number (nº), hour (h), Passive Integrated Transponder (PIT)**,** curved carapace length (CCL), years (yrs).

Table 5 – Sampling data. Part IV.

Sample nº.	Date	Time	Beach/ local	Flipper Tag ID	PIT	CCL	Estimated age (yrs)	Clutch size	Sample type	Animal general condition
55	8/6/2019	01h40	"Cozinha fácil" "Praia Gonçalo"	KMJ 504/503	-	75.5	16.15	78	cloaca	difficult dilatation of the cloaca
56	8/6/2019	01h40	"Cozinha fácil" "Praia Gonçalo"	KMJ 504/503	-	75.5	16.15	78	oral cavity	difficult dilatation of the cloaca
57	8/6/2019	01h40	"Cozinha fácil" "Praia Gonçalo"	KMJ 504/503	-	75.5	16.15	78	egg	difficult dilatation of the cloaca
58	8/8/2019	21h53	"Areia Preta" "Praia Gonçalo"	QMI 073/072	9.81098(...)147	78	17.83	73	cloaca	parasites cloaca
59	8/8/2019	21h53	"Areia Preta" "Praia Gonçalo"	QMI 073/072	9.81098(...)147	78	17.83	73	oral cavity	parasites cloaca
60	8/8/2019	21h53	"Areia Preta" "Praia Gonçalo"	QMI 073/072	9.81098(...)147	78	17.83	73	egg	parasites cloaca
61	8/8/2019	22h30	"Areia Preta" "Praia Gonçalo"	QMI 770/771	9.81098(...)885	74	15.22	-	cloaca	parasites cloaca, deformation of the carapace
62	8/8/2019	22h30	"Areia Preta" "Praia Gonçalo"	QMI 770/771	9.81098(...)885	74	15.22	-	oral cavity	parasites cloaca, deformation of the carapace
63	8/8/2019	22h30	"Areia Preta" "Praia Gonçalo"	QMI 770/771	9.81098(...)885	74	15.22	-	egg	parasites cloaca, deformation of the carapace
64	8/8/2019	23h16	"Areia Preta" "Praia Gonçalo"	QMI 773/772	9.81098(...)591	-	-	-	cloaca	good
65	8/8/2019	23h16	"Areia Preta" "Praia Gonçalo"	QMI 773/772	9.81098(...)591	-	-	-	oral cavity	good
66	8/8/2019	23h16	"Areia Preta" "Praia Gonçalo"	QMI 773/772	9.81098(...)591	-	-	-	egg	good
67	8/8/2019	01h40	"Areia Preta" "Praia Gonçalo"	QMM 229/228	-	79	18.57	72	cloaca	good
68	8/8/2019	01h40	"Areia Preta" "Praia Gonçalo"	QMM 229/228	-	79	18.57	72	oral cavity	good
69	8/8/2019	01h40	"Areia Preta" "Praia Gonçalo"	QMM 229/228	-	79	18.57	72	egg	good
70	8/8/2019	02h10	"Areia Preta" "Praia Gonçalo"	QME 50/258	-	75.2	15.96	-	cloaca	good
71	8/8/2019	02h10	"Areia Preta" "Praia Gonçalo"	QME 50/258	-	75.2	15.96	-	oral cavity	good
72	8/8/2019	02h10	"Areia Preta" "Praia Gonçalo"	QME 50/258	-	75.2	15.96	-	egg	good

Legend: number (nº), hour (h), Passive Integrated Transponder (PIT)**,** curved carapace length (CCL), years (yrs).

Table 5 – Sampling data. Part V.

Sample nº.	Date	Time	Beach/ local	Flipper Tag ID	PIT	CCL	Estimated age (yrs)	Clutch size	Sample type	Animal general condition
73	13/8/19	01h04	"Cozinha fácil" "Praia Gonçalo"	QMI 094/095	-	-	-	-	cloaca	good
74	13/8/19	01h04	"Cozinha fácil" "Praia Gonçalo"	QMI 094/095	-	-	-	-	oral cavity	good
75	13/8/19	01h04	"Cozinha fácil" "Praia Gonçalo"	QMI 094/095	-	-	-	-	egg	good
76	13/8/19	02h38	"Cozinha fácil" "Praia Gonçalo"	QMI 049/050	-	76	16.47	78	cloaca	parasites cloaca
77	13/8/19	02h38	"Cozinha fácil" "Praia Gonçalo"	QMI 049/050	-	76	16.47	78	oral cavity	parasites cloaca
78	13/8/19	02h38	"Cozinha fácil" "Praia Gonçalo"	QMI 049/050	-	76	16.47	78	egg	parasites cloaca
79	13/8/19	03h10	"Cozinha fácil" "Praia Gonçalo"	QMI 046/045	-	74.5	15.52	63	cloaca	good
80	13/8/19	03h10	"Cozinha fácil" "Praia Gonçalo"	QMI 046/045	-	74.5	15.52	63	oral cavity	good
81	13/8/19	03h10	"Cozinha fácil" "Praia Gonçalo"	QMI 046/045	-	74.5	15.52	63	egg	good
82	13/8/19	03h20	"Cozinha fácil" "Praia Gonçalo"	QMI 276/035	-	79.5	18.95	64	cloaca	parasites cloaca
83	13/8/19	03h20	"Cozinha fácil" "Praia Gonçalo"	QMI 276/035	-	79.5	18.95	64	oral cavity	parasites cloaca
84	13/8/19	03h20	"Cozinha fácil" "Praia Gonçalo"	QMI 276/035	-	79.5	18.95	64	egg	parasites cloaca
85	13/8/19	03h44	"Cozinha fácil" "Praia Gonçalo"	QMI 277/278	-	78	17.83	75	cloaca	good
86	13/8/19	03h44	"Cozinha fácil" "Praia Gonçalo"	QMI 277/278	-	78	17.83	75	oral cavity	good
87	13/8/19	03h44	"Cozinha fácil" "Praia Gonçalo"	QMI 277/278	-	78	17.83	75	egg	good
88	16/8/19	22h43	"Cozinha fácil" "Praia Gonçalo"	QMM 298/505	-	81	20.15	-	cloaca	external parasites, parasites cloaca
89	16/8/19	22h43	"Cozinha fácil" "Praia Gonçalo"	QMM 298/505	-	81	20.15	-	oral cavity	external parasites, parasites cloaca
90	16/8/19	22h43	"Cozinha fácil" "Praia Gonçalo"	QMM 298/505	-	81	20.15	-	egg	external parasites, parasites cloaca

Legend: number (nº), hour (h), Passive Integrated Transponder (PIT), curved carapace length (CCL), years (yrs).

Table 5 – Sampling data. Part VI.

Sample nº.	Date	Time	Beach/ local	Flipper Tag ID	PIT	CCL	Estimated age (yrs)	Clutch size	Sample type	Animal general condition
91	16/8/19	00h36	"Cozinha fácil" "Praia Gonçalo"	QMM 284/283	-	80.5	19.74	-	Cloaca	Parasites cloaca
92	16/8/19	00h36	"Cozinha fácil" "Praia Gonçalo"	QMM 284/283	-	80.5	19.74	-	Oral cavity	Parasites cloaca
93	16/8/19	00h36	"Cozinha fácil" "Praia Gonçalo"	QMM 284/283	-	80.5	19.74	-	Egg	Parasites cloaca
94	16/8/19	01h36	"Cozinha fácil" "Praia Gonçalo"	QMM 285/286	-	78	17.83	-	Cloaca	good
95	16/8/19	01h36	"Cozinha fácil" "Praia Gonçalo"	QMM 285/286	-	78	17.83	-	Oral cavity	Good
96	16/8/19	01h36	"Cozinha fácil" "Praia Gonçalo"	QMM 285/286	-	78	17.83	-	Egg	Good
97	16/8/19	02h37	"Areia preta" "Praia Gonçalo"	QMI 288/287	-	81	20.15	-	Cloaca	Flipper atrophy, mark of fishhook
98	16/8/19	02h37	"Areia preta" "Praia Gonçalo"	QMI 288/287	-	81	20.15	-	Oral cavity	Flipper atrophy, mark of fishhook
99	16/8/19	02h37	"Areia preta" "Praia Gonçalo"	QMI 288/287	-	81	20.15	-	Egg	Flipper atrophy, mark of fishhook

Legend: number (nº), hour (h), Passive Integrated Transponder (PIT), curved carapace length (CCL), years (yrs).

Annexe 2 – Results of the protocol for the isolation of Gram-negative bacteria.

Table 6 – Results of the protocol for the isolation of Gram-negative bacteria. Part I.

Sample ID	BPW	GSP (24h)	GSP (48h)	MAC	GRAM	OX	CAT	Sample ID	BPW	GSP (24h)	GSP (48h)	MAC	GRAM	OX	CAT
C 298/505	N	N	N	N	-	-	-	C 065/064	N	N	N	N	-	-	-
O 298/505	N	N	N	N	-	-	-	O 065/064	N	N	N	N	-	-	-
E 298/505	P	N	N	N	-	-	-	E 065/064	P	N	N	N	-	-	-
C 066/067	N	N	N	N	-	-	-	C P258	N	N	N	N	-	-	-
O 066/067	N	N	N	N	-	-	-	O P258	N	N	N	N	-	-	-
E 066/067	P	N	N	N	-	-	-	E P258	P	N	N	N	-	-	-
C 038/039	P	N	N	N	-	-	-	C 329/328	N	N	N	N	-	-	-
O 038/039	N	N	N	N	-	-	-	O 329/328	N	N	N	N	-	-	-
E 038/039	P	N	N	N	-	-	-	E 329/328	P	P	P	P	GN-B	P	P
C 330/331	N	N	P	P	GN-B	N	P	C 770/771	N	N	N	N	-	-	-
O 330/331	P	N	N	N	-	-	-	O 770/771	P	N	N	N	-	-	-
E 330/331	P	N	N	N	-	-	-	E 770/771	P	N	N	N	-	-	-
C 67/68	N	N	N	N	-	-	-	C 025/024	N	N	N	N	-	-	-
O 67/68	N	N	N	N	-	-	-	O 025/024	N	N	N	N	-	-	-
E 67/68	P	N	N	N	-	-	-	E 025/024	P	N	N	N	-	-	-
C 051/052	N	N	N	N	-	-	-	C 64/65	N	N	N	N	-	-	-
O O51/052	N	N	N	P	yeasts	-	-	O 64/65	N	N	N	N	-	-	-
E 051/052	P	P	P	N	-	-	-	E 64/65	P	N	N	N	-	-	-
C 772/773	N	N	N	N	-	-	-	C 104/505	N	N	N	N	-	-	-
O 772/773	N	N	N	N	-	-	-	O 104/505	P	N	N	N	-	-	-
E 772/773	P	N	N	N	-	-	-	E 104/505	P	N	N	N	-	-	-
C 335/334	N	N	N	N	-	-	-	C 229/228	P	N	N	P	-	N	P
O 335/334	N	N	N	N	-	-	-	O 229/228	P	N	N	N	-	-	-
E 335/334	P	N	N	N	-	-	-	E 229/228	P	N	N	N	-	-	-

Legend: cloaca (C), oral cavity (O), egg (E), positive (P), negative (N), Gram-negative bacilli (GN-B), Buffered Peptone Water (BPW), Glutamate Starch Red Phenol (GSP), MacConkey (MAC), oxidase test (OX), catalase reaction (CAT), hours (h).

Table 6 – Results of the protocol for the isolation of Gram-negative bacteria. Part II.

Sample ID	BPW	GSP (24h)	GSP (48h)	MAC	GRAM	OX	CAT	Sample ID	BPW	GSP (24h)	GSP (48h)	MAC	GRAM	OX	CAT
C 062/063	N	N	N	N	-	-	-	C 029/028	P	N	N	N	-	-	-
O 062/063	N	N	N	N	-	-	-	O 029/029	P	N	N	N	-	-	-
E 062/063	P	N	N	N	-	-	-	E 029/028	P	N	N	N	-	-	-
C 99/100	N	N	N	N	-	-	-	C 94/95	N	N	N	N	-	-	-
O 99/100	N	N	N	N	-	-	-	O 94/95	P	N	N	N	-	-	-
E 99/100	P	N	N	N	-	-	-	E 94/95	P	N	N	N	-	-	-
C 784/783	N	N	N	N	-	-	-	C 288/287	N	N	N	N	-	-	-
O 784/783	P	N	N	N	-	-	-	O 288/287	N	N	N	N	-	-	-
E 784/783	P	N	N	N	-	-	-	E 288/287	P	N	N	N	-	-	-
C 050/258	P	N	N	N	-	-	-	C 276/030	P	P	P	P	-	N	P
O 050/258	P	N	N	N	-	-	-	O 276/030	P	P	P	P	-	N	P
E 050/258	P	N	N	N	-	-	-	E 276/030	P	P	P	P	-	P	P
C 284/283	N	N	N	N	-	-	-	C 060/061	P	P	P	P	-	P	P
O 284/283	N	N	N	N	-	-	-	O 060/061	P	N	N	N	-	-	-
E 284/283	P	N	N	N	-	-	-	E 060/061	P	N	N	N	-	-	-
C 785/786	N	P	P	P	GN-B	N	P	C 286/285	N	N	N	N	-	-	-
O 785/786	N	N	N	N	-	-	-	O 286/285	N	N	N	N	-	-	-
E 785/786	P	N	N	N	-	-	-	E 286/285	P	N	N	N	-	-	-
C 277/278	N	N	N	N	-	-	-	C 072/073	N	N	N	P	-	-	-
O 277/278	N	N	N	N	-	-	-	O 072/073	N	N	N	N	-	-	-
E 277/278	P	N	N	N	-	-	-	E 072/073	P	N	N	P	-	-	-
C 049/050	P	P	P	P	GN-B	1-N; 2-P	P	C 504/503	P	N	N	P	-	-	-
O 049/050	N	N	N	N	-	-	-	O 504/503	N	P	P	N	-	-	-
E 049/050	P	N	N	N	-	-	-	E 504/503	P	N	N	P	-	-	-
C 046/045	P	P	P	P	GN-B	1/2-P	P								
O 046/045	N	N	N	P	-	-	-								
E 046/045	P	P	P	P	-	1-P; 2-N	P								

Legend: cloaca (C), oral cavity (O), egg (E), positive (P), negative (N), Gram-negative bacilli (GN-B), BPW (Buffered Peptone Water), Glutamate Starch Red Phenol (GSP), MacConkey (MAC), oxidase test (OX), catalase reaction (CAT), hours (h).

Annexe 3 – Chi-Square, Kruskal-Wallis, Mann-Whitney and Spearman correlation tests

Table 7 - Chi-Square test

Bacterial species		Value	Asymptotic significance (2-sided)		Value	Asymptotic significance (2-sided)
Aeromonas hydrophila/caviae	**Sample type (cloaca, oral, egg content)**	1.907	0.385	**Animal's health status classes (I, II, III)**	2.880	0.237
Brevundimonas vesicularis		1.907	0.385		0.865	0.649
Burkholderia cepacia		1.991	0.370		0.865	0.649
Citrobacter sp.		1.907	0.385		0.865	0.649
Enterobacter cloacae		3.852	0.146		2.449	0.294
Morganella morganii		0.436	0.804		1.777	0.411
Shewanella putrefaciens		5.083	0.079		3.592	0.166
Vibrio alginolyticus		0.501	0.778		6.141	0.046*

Legend: *significant value.

Table 8 - Mann-Whitney test

Variables tested	Mann-Whitney U	Wilcoxon W	Asymptotic significance	z
MAR Index and Biofilm production	340.500	4345.500	0.000*	-5.459
MAR Index and bacterial family (E/NE)	38.000	116.000	0.713	-0.368
V. Index and bacterial family (E/NE)	24.000	52.000	0.109	-0.109

Legend: multiple antibiotic resistance index (MAR index), Virulence index (V. Index), Enterobacteriaceae (E), Non-Enterobacteriaceae (NE), z-score (z), *significant value.

Table 9 - Kruskal-Wallis test

Variables tested	Kruskal-Wallis H	Asymptotic significance
MAR index and sample type	9.747	0.008*
V. Index and sample type	9.763	0.008*
V. Index and health status classes	9.663	0.008*

Legend: multiple antibiotic resistance index (MAR index), Virulence index (V. Index), *significant value.

Table 10 - Spearman correlation test

Correlation coefficient (MAR index and V. Index)	0.751
Sig. (2-sided)	0.000*

Legend: multiple antibiotic resistance index (MAR index), Virulence index (V. Index), significance level (sig.), *significant value.

Annexe 4 – Ethics statement

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, approved by the 27th Meeting of IUCN Council, Gland Switzerland, 14 June 1989, and the Sea Turtle Research (IUCN 1989) Techniques Manual (Stokes et al. 2008).

Annexe 5 – Declaration of use and purpose for samples' transportation – Faculty of Veterinary Medicine, University of Lisbon

Universidade de Lisboa

Faculdade de Medicina Veterinária

Lisboa, 20 de Julho de 2019

DECLARAÇÃO DE USO E FINALIDADE

Declaro para os devidos fins que serei responsável pelo processamento das amostras de esfregaços ambientais em meio de cultura em recipiente estanque (swabs de transporte – VWR 1814-002), transportadas pelo Doutora Matilde Costa Fernandes.

Estas amostras serão processadas para fins de pesquisa científica (Projecto UIDB/00276/2020 (Funded by the Foundation of 4 Science and Technology (FCT)), a título de serviço de terceiros no exterior, pelo Centro de Investigação Interdisciplinar em Sanidade Animal – CIISA, Universidade de Lisboa, Lisboa (Portugal).

Não constituem produto farmacológico para análise clínica ou médica, e não representam qualquer risco químico ou biológico, sendo absolutamente seguro seu manuseio no caso de derramamento.

Maria Manuela Castilho Monteiro de Oliveira

Maria Manuela Castilho Monteiro de Oliveira, DVM, MSc, PhD
Professora Auxiliar
Departamento de Sanidade Animal
Faculdade de Medicina Veterinária da Universidade de Lisboa
Telefone +351213652800, extensão 431263
moliveira@fmv.ulisboa.pt

Annexe 6 – Declaration of samples' transportation – Maio Biodiversity Foundation, Cape Verde

Fundação Maio Biodiversidade – Maio Biodiversity Foundation
Cidade Porto Ingles, 6110 Ilha do Maio, Cabo Verde. NIF: 561424390

Dr Juan Patiño Martínez

Scientific Coordinator FMB

Tel: + 238 9706912

Email: juan.patino@fmb-maio.org

26-08-2019

Autorização para transportar amostras de estudo

Fundação Maio Biodiversidade (FMB), com sede na Cidade do Porto Inglês, Ilha do Maio, NIF 561424390 é uma ONG fundada em 2010 no Maio, Cabo Verde. A sua missão é preservar a fauna e flora da ilha e criar oportunidades e benefícios duradouros para os seus habitantes. O projecto de conservação das tartarugas marinhas é um dos principais programas da FMB, dado o importante papel das tartarugas marinhas, não somente no ecossistema marinho, mas também na cultura nacional e como património natural.

Juan Patiño Martínez, coordenador científico da Fundação Maio Biodiversidade (FMB), informa que as amostras transportadas pela aluna Matilde Costa Fernandes do Mestrado Integrado em Medicina Veterinária da Faculdade de Veterinária da Universidade de Lisboa, são necessárias para realizar o estudo intitulado: “microbiota fecal de animais selvagens em meio tropical”, em colaboração científica com a FMB.

As referidas amostras são fluidos externos (Saliva e Muco) de tartarugas marinhas.

Incluo a actual autorização da Direcção Nacional do Ambiente (DNA) de Cabo Verde para realizar os estudos científicos relevantes para a conservação das espécies.

Muito obrigado por facilitar o transporte a frio.

Eu permaneço atento para responder a quaisquer perguntas.

Att.

Juan Patiño Martínez

Annexe 7 – Authorization of the sea turtles' conservation project of Maio Biodiversity Foundation

Ministério da Agricultura e Ambiente
Direcção Nacional do Ambiente
Direcção Serviço de Conservação da Natureza

AUTORIZAÇÃO Nº 067/2019

A Direção Nacional do Ambiente (DNA) vem por este meio, autorizar a **ONG Fundação Maio Biodiversidade**, a implementar o projeto de conservação das tartarugas marinhas durante o ano de 2019, conforme o Artigo 12º do Decreto Legislativo nº 1/2019 e mediante a ficha de projeto submetida a DNA, nas seguintes áreas na ilha do Maio:

- **Todo o litoral da ilha do Maio;**

Condicionantes:

1. O relatório da campanha deve ser enviado à DNA até o dia 31 de Janeiro de 2020.
2. A presente autorização não é válida para as atividades de ecoturismo ou turtle watching.
3. A translocação dos ovos para o viveiro, é permitido somente em casos especiais, em que os ovos se encontram em zonas de risco de sobrevivência.

Esta autorização tem uma validade até 31 de Janeiro de 2020.

O Director Nacional

Alexandre Nevsky Rodrigues

MINISTÉRIO DA AGRICULTURA E AMBIENTE
DIREÇÃO NACIONAL DO AMBIENTE

Annexe 8 – Abstract accepted for poster presentation at the 2nd International Conference of the European College of Veterinary Microbiology

Virulence of Gram-Negative bacteria of Loggerhead sea turtles (*Caretta caretta*) of Maio Island, Cape Verde

Matilde Fernandes[1, 2,*], Miguel Grilo[1], Carla Carneiro[1], Eva Cunha[1], Luís Tavares[1], Juan Patino-Martinez[3], Manuela Oliveira[1]

[1]CIISA-Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal

[2]Veterinários Sem Fronteiras, Portugal, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal

[3]Maio Biodiversity Foundation (FMB), Cidade Porto Ingles, Ilha do Maio, Cabo Verde

*cfernandes.matilde@gmail.com

Loggerhead sea turtles (*Caretta caretta*) have been suggested as carriers of potential zoonotic pathogens and prime reservoirs of antibiotic-resistant and virulent bacteria. To date, investigations have mainly focused on the Mediterranean's population. In the present study, the isolation of Gram-negative bacteria of the Cape Verdean loggerhead population is first described.

We aimed to characterize the aerobic Gram-negative bacteria of the endangered loggerhead population of Maio Island, evaluate their pathogenic potential and unveil the impact on sea turtles' conservation and the underlying public health risks resulting from turtle-derived products consumption.

Cloacal, oral and egg content swab samples from 33 nesting loggerheads (n=99) were analyzed regarding the presence of Gram-negative bacteria and their antibiotic resistance and virulence profiles by conventional bacteriological techniques.

Shewanella putrefaciens (27.78%), *Vibrio alginolyticus* (22.22%) and *Morganella morganii* (22.22%) were the most prevalent species. A low incidence of bacteria resistant to more than two antibiotics (26%) was detected, and no multidrug-resistant isolates were identified. Isolates were able to produce numerous virulence factors, including hemolysins, DNases, lipases, lecithinases, proteases, gelatinases, and biofilm.

These findings suggest that due to the low anthropogenic impact observed in Maio Island, this loggerhead population may be less exposed to antimicrobial compounds selecting for resistant bacteria. Nevertheless, the presence of potentially pathogenic bacteria expressing virulence factors may threat both sea turtles' and human's health.

In conclusion, virulence characterization of Gram-negative 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.

Acknowledgements: This work was supported by CIISA–Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa (Project UIDP/CVT/00276/2020). We acknowledge the Foundation for Science and Technology (Eva Cunha PhD fellowship SFRH/BD/131384/2017) and the University of Lisbon (Miguel Grilo PhD fellowship C10571K).

Annexe 9 – Abstract of the Manuscript submitted for publication in Environmental Microbiology

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

Matilde Fernandes[1,2*], Miguel L. Grilo[1] , Carla Carneiro[1] , Eva Cunha[1] , Luís Tavares[1], Juan Patino-Martinez[3] and Manuela Oliveira[1]

[1]CIISA-Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal

[2]Veterinários Sem Fronteiras, Portugal, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal

[3]Maio Biodiversity Foundation (FMB), Cidade Porto Inglês, Ilha do Maio, Cabo Verde

*Correspondence: cfernandes.matilde@gmail.com

Summary

The antibiotic resistance and virulence profiles of Gram-negative bacteria were analysed, for the first time, in 33 nesting loggerhead sea turtles (C*aretta caretta*) from the Island of Maio, Cape Verde. For this evaluation, conventional bacteriological techniques were applied. *Shewanella putrefaciens* (26.32%) was the most prevalent species isolated in this study. Lower levels of antibiotic resistance were detected for the isolates obtained from this loggerhead subpopulation (North-East Atlantic) compared with previous studies performed in other subpopulations (e.g., Mediterranean subpopulation). However, the detection of multiple antibiotic resistance (MAR) indices equal to or higher to 0.20 and the evidence of resistance to carbapenems for the studied isolates raises concern about the potential exposure of these loggerheads to points of high antimicrobial contamination. Furthermore, virulence phenotypic characterisation revealed that the isolates presented complex virulence profiles, with 57.90% of isolates classified as high or moderate threat as potential pathogens. Finally, due to their pathogenic potential, added to the evidence of unsafe and illegal consumption of turtle-related products on the Island of Maio, the identified bacteria may represent a significant threat to Public Health.