EVALUATION OF ANTIMICROBIAL PROPERTIES OF THE CLOACAL FLUID OF LOGGERHEAD SEA TURTLES (*CARETTA CARETTA*)

by

Natalia Libreros Marin

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Biology at Purdue Fort Wayne

Fort Wayne, Indiana

December 2021

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Frank Paladino, Chair
Department of Biology

Dr. Arturo Villalobos
Department of Biology

Dr. Tanya Soule
Department of Biology

Approved by:
Dr. Jordan M. Marshall

To everyone who has encouraged me on this journey

ACKNOWLEDGMENTS

I would first like to thank Dr. Frank Paladino as this wouldn't have been possible without you. Thank you so much for giving me the opportunity to join your lab, for your support during these past two years and for sharing all your advice and knowledge. My sincerest thanks go to my advisors as this thesis would not have come to fruition without your support and your guidance. Dr. Villalobos, you have been of unimaginable help, and I will forever be grateful for all your patience and dedication. Thank you to Dr. Soule, for all the valuable insight and thoughtful mentorship. To all my lab mates, I appreciate the time we spent together and your support during my time at Purdue Fort Wayne. Finally, a special thanks to the Caretta Research project (CRP), particularly Kris Williams, for helping me with my sample collection process, you will have my gratitude forever.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Colonization by bacteria and fungi has been shown to reduce hatching success of sea turtle nests. Presence of microbial pathogens is commonly associated with egg failure, and in some species, it has even been shown to cause up to 90% nest mortality. Developing sea turtle embryos can only rely on non-specific defenses, such as the eggshell and the proteins present in the egg albumen. For a long time, it has been suggested that the fluid in which the eggs are coated during oviposition may contain antimicrobial properties that might protect eggs against potential pathogens that are found in the egg chamber. This study aimed to evaluate the antimicrobial properties of the cloacal fluid of loggerhead sea turtles. Cloacal fluid samples were collected at the Wassaw National Wildlife Refuge, Georgia during the 2021 nesting season (June-July). Protein fractionation of the samples was carried out using commercially available ultracentrifugation devices of 3K, 10K and 30K molecular cut-off weights. A microplate-based turbidimetric growth inhibition assay recorded the antimicrobial activity of the peptide fractions and the crude extract. We found that the cloacal fluid of loggerheads has antimicrobial properties against *Serratia marcescens* and *Morganella morganii*, and some mild action against *Pseudomonas aeruginosa*. Results obtained from the microplate-based turbidimetric assay were confirmed by assessing viability of cells upon 24 hours of exposure to the fluid. Furthermore, this study found that the cloacal fluid contains proteins with molecular weight ranging from approximately 5 to 250 kDa, and that proteins with higher molecular weights (MW>30kDa) are found in greater abundance. Further studies are needed to understand the mechanism of action of the proteins and peptides present in the cloacal fluid to potentially develop antimicrobial compounds that can be used to increase hatching success globally.

CHAPTER 1. INTRODUCTION

1.1 Study species

1.1.1 Taxonomy and external morphology.

The loggerhead sea turtle (*Caretta caretta*) belongs to the superfamily Chelonioidea, which includes the only two extant sea turtle families: Cheloniidae and Dermochelyidae. The Cheloniidae family is comprised by the "hard-shelled" marine turtle species, which include loggerheads (*Caretta caretta*), greens (*Chelonia mydas*), Kemp's Ridley (*Lepidochelys kempii*), hawksbills (*Eretmochelys imbricata*), olive Ridleys (*Lepidochelys olivacea*) and the flatback (*Natator depressus*). The Dermochelyidae family includes only one extant species, the leatherback turtle (*Dermochelys coriacea*) (Bowen *et al*., 1993).

Loggerhead sea turtles (**Figure 1**) are named for their strong jaws and enormous heads (relative to body size). The carapace has a slight heart shape and consists of five pairs of costal scutes that are maroon to dark brown in color, while their plastron is yellowish-brown. Adults tend to measure between 70 and 110 cm in straight carapace length (SCL) and can weight up to 200 kg (Pritchard & Mortimer, 1999). Loggerheads are opportunistic carnivores, and their strong jaws allow them to prey on many hard-shelled invertebrates, however, they also prey on tubeworms, fish, algae, shrimp, and anemones (Paladino & Robinson, 2013).

Figure 1. Adult female loggerhead (*Caretta caretta*).

1.1.2 Distribution

The loggerhead is a marine turtle species that can be found worldwide, primarily in the temperate and tropical regions of the Pacific, Atlantic and Indian Oceans and in the Mediterranean Sea. Loggerheads tend to nest in wide, sandy continental beaches that usually have a low vegetated dune front, and nests are typically laid between the high tide line and the dune front, which decreases the chances of eggs being eroded, as well as the chances of hatchlings being predated (Miller, Limpus & Godfrey., 2001). In the Pacific, there are two distinct loggerhead populations: a southern population, that nests in Australia and New Caledonia; and a northern population, that nests only in Japan. In the South Pacific, nesting is concentrated along the eastern coast of Australia, with most of the nesting occurring in Mon Repos (southeast Queensland) and some minor aggregations on the islands of the Capricon-Bunker Group (mainly Wreck, Tryon and Erskine Islands) and the Swain Reefs (particularly Pryce Island, Frigate, Bylund and Thomas Cays) (Limpus & Limpus, 2003; Limpus, 2009). In New Caledonia, only one nesting population (50 to 80 individuals per year) has been reported to lay eggs in la Roche Percée Beach (Bourail) (Boyle *et al*., 2009). In the North Pacific, loggerhead nesting occurs almost exclusively in Japan, with about 40% of the nesting taking place on Yakushima Island, and the rest on the Atsumi, Minabe and Miyazaki beaches on mainland (Kamezaki *et al*. 2003).

Loggerheads occur throughout the Mediterranean, but nesting tends to be concentrated in the Eastern basin, with the primary sites located along the coasts of Libya, Turkey, Cyprus, and Greece. Low nesting activity levels have been reported in the Western Mediterranean, particularly in Italy, Spain, and France (Casale & Margaritoulis, 2010). In the Indian Ocean, significant loggerhead nesting has been reported on the western part of the basin for both hemispheres: in the north, one of the two primary nesting aggregations is found on Masirah Island (Sultanate of Oman), which hosts over 10,000 females annually, and some minor nesting has also been recorded in the Albalhan Protected Area in Socotra Island (Yemen) (Ross, 1998; Pilcher & Saad, 2000); while in the Southwestern Indian Ocean, loggerhead nesting has been reported in the southern coast of Africa, with important nesting populations (1,000-5,000 nests per year) occurring in South Africa, Mozambique, and some low-density nesting taking place in the Barren Islands and the west coast of Madagascar (Walker & Roberts, 2007; Rakotonirina & Cooke, 1994).

Within the Southwest Atlantic, moderate nesting has been reported from northern Brazil to southern Argentina. In Brazil, the main nesting area ranges from the state of Sergipe (northeast region) to the northern State of Rio de Janeiro (eastern region); while in Argentina, loggerheads tend to occur in the areas of El Rincón and of Samborombón Bay, which are estuarine waters with high levels of productivity (Marcovaldi & Chaloupka, 2007; González Carman *et al.*, 2011). In the Northwest Atlantic, the southeastern coast of the United States hosts one of the largest loggerhead nesting aggregations, with most of the nesting concentrated in North Carolina, Georgia, South Carolina, Florida and along Alabama (**Figure 2**); but there are a few additional nesting beaches found in the Gulf of Mexico and also along the coasts of Central America, Colombia, and Venezuela (Shoop & Dodd., 1989; Moncada G., 2001).

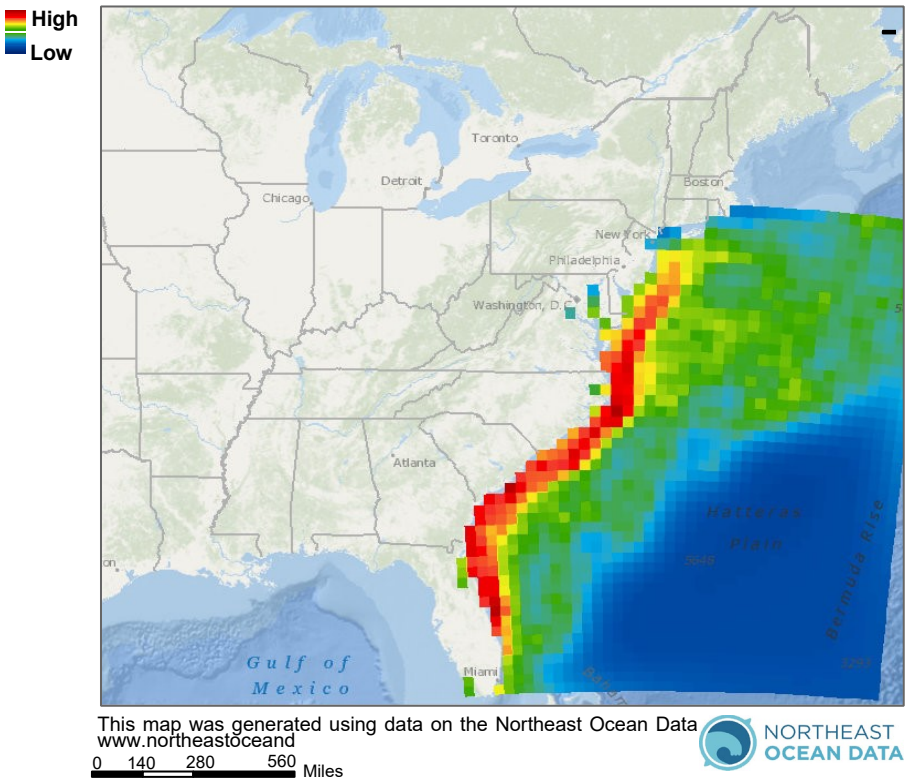


Figure 2. Map of distribution of loggerhead sea turtles (*Caretta caretta*) in the southeastern coast of the United States. Color indicates relative abundance.

In the United States, about 90% of loggerhead nesting occurs in Florida, while the rest occurs on island beaches north of Florida (Moncada G., 2001). There are three subpopulations of loggerhead turtles that nest along the southeastern coast: the *northern subpopulation*, which nests in North Carolina, South Carolina, Georgia, and northeast Florida; the *south Florida population*, which

nests south of Amelia Island; and the *Florida Panhandle subpopulation*, which nests in northwest Florida (Encalada *et al.*, 1998; Plotkin & Spotila., 2002; Williams & Frick., 2008). This study focuses on individuals that belong to the *northern subpopulation* and nest at the Wassaw National Wildlife Refuge, Georgia.

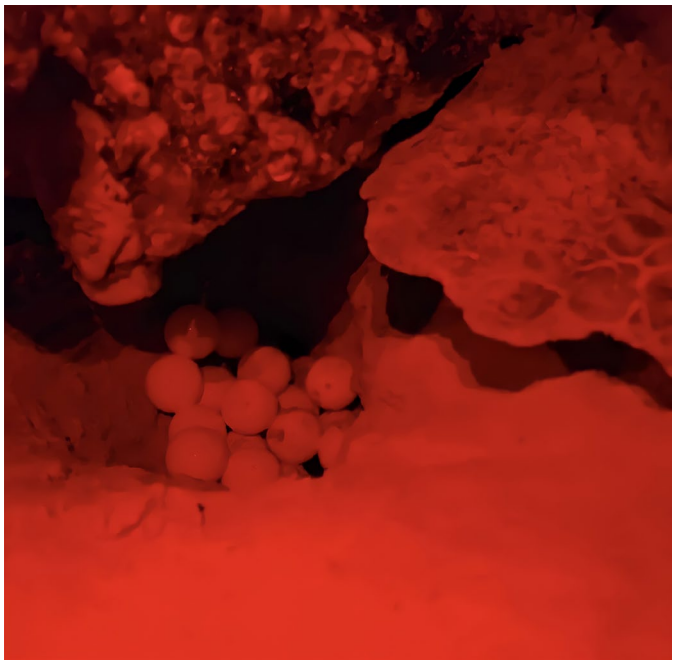
1.1.3 Life history

Loggerheads are long-lived, slow growing reptiles that spend most of their lives in ocean and estuarine waters where they feed, breed, and migrate; and females spend some of that time on beaches where they lay their eggs (Nelson, 1988). The nesting season takes place during the warmer months in the temperate zones, usually from October through March in the Southern Hemisphere, and May through August in the Northern Hemisphere; although nesting in the southeastern U.S., can start as early as mid-March and can continue until late September. In the tropics, nesting tends to occur mostly during the warmer and more productive months but can also occur throughout the year (Miller, Limpus & Godfrey.,2001; McAllister *et al.* 1965; Hughes 1989; Talbert *et al.,* 1980).

Females usually nest every other year or every third year, and the number of clutches per season varies from one to six clutches; with a maximum of 7 clutches reported by Lenarz *et al* (1981). However, the average usually falls somewhere between two to four clutches (Hughes., 1982; Miller, Limpus & Godfrey.,2001). The internesting interval (number of days between consecutively laid nests) of loggerheads is also variable, and it seems to depend on the location, with the shortest interval reported being 9 days in Australia (Limpus & Reed, 1985) and the longest being between 18-28 days in Turkey (Geldiay *et al.*, 1982), however, in most populations the internesting interval tends to be around 14 days (Richardson, 1980).

The number of eggs laid per nest varies among populations, but on average, loggerheads produce between 100-130 eggs per clutch (**Figure 3**), with a hatching success rate (percentage of eggs resulting in emerged hatchlings) that ranges between 60% and 80% (Limpus & Godfrey., 2001; Shoop & Dodd, 1989). In comparison with other species, loggerheads lay medium-sized (~36 g, ~4.0 cm) white, spherical eggs with three eggshell layers: an outer calcareous layer that consists of loose nodular units of calcium carbonate ($CaCO_3$); a middle layer, that has several strata

with rod-shaped crystals made up of 62% aragonite and 38% calcite; and an inner smooth shell membrane with fibers that consist primarily of halite (NaCl) and small amounts of sylvite (KCl) (Al-Bahry *et al.,* 2011).



In loggerheads, the incubation period ranges from 50-70 days and is inversely correlated with temperature (Shoop & Dodd, 1989; McGehee, 1979). Hatchlings of the *northern subpopulation* emerge from nests between July and October and following their emergence they crawl to the shoreline and enter an active swimming period (also known as "swimming frenzy"), in which they vigorously swim through the neritic zone and then, they reach nursery habitats with the help of oceanic currents (Wyneken *et al.*, 2008). Hatchlings from the western North Atlantic are carried away by the Gulf Stream and then enter the North Atlantic Subtropical Gyre (NASG), that ultimately transports them to oceanic developmental habitats in the eastern Atlantic (e.g., Madeira, Cape Verde, or the Azores), that are commonly associated with the presence of floating *Sargassum* communities that offer protection against predators as well as food resources (Mansfield *et al*., 2014). Several years (6.5 – 11.5 y) after this oceanic stage, juveniles shift to neritic waters where they feed on benthic prey, mostly crustaceans and mollusks. Individuals from the *northern subpopulation* use neritic habitats that range from the western Gulf of Mexico to Canada (Bjorndal

et al., 2000; Shoop *et al*., 1981). The age of sexual maturity varies between populations, and it has been estimated to be reached after 22.5-36 years for females and 26-42 years for males of the southeastern U.S, whereupon begins the adult stage (Avens *et al*., 2015). Reproductively mature adults migrate to breeding grounds, where courtship and mating takes place (between late March and early June). Once the mating season ends, males return to foraging areas while the females return to their natal beach area where they will lay their eggs (Miller *et al.,* 1997).

1.2 Microorganisms in sea turtle eggs and cloacal fluid

The presence of microbial pathogens has been shown to be in association with egg failure of sea turtle nests, which ultimately translates in declines in sea turtle populations (Wyneken *et al*., 1988; Zieger *et al.,* 2009). Several studies have suggested that colonization of sea turtle eggs by fungi and bacteria leads to reduced hatching success and in some species, it has even been shown to cause close to 90% nest mortality. For example, in loggerheads high levels of certain gram-negative bacteria genera (e.g., *Aeromonas, Pseudomonas, Enterobacter, Vibrio)* have been shown to be correlated with lower hatching success, and in several sea turtle species (*Chelonia mydas, Caretta caretta, Lepidochelys olivacea*) the presence of the fungal species *Fusarium solani* has been found to increase egg mortality (**Figure 4**) (Craven *et al*., 2007; Phillott & Parmenter, 2001; Wyneken *et al.,* 1988; Sarmiento-Ramírez *et al.,* 2010).

Figure 4. Colonization of loggerhead (*Caretta caretta*) eggs by *Fusarium solani*. (a,b) infected eggs exhibiting colored spots and grayish mycelium covering the shell; (c) healthy eggs; (d) dead embryo (Sarmiento- Ramírez et al., 2010).

Infection of sea turtle eggs also occurs by microbes that are naturally found in the nesting environment (e.g., *Sphingomonas* sp, *Shewanella* sp, *Pseudomonas* sp.) and that might be acting as opportunistic pathogens when eggs lose their natural defenses and/or when the physical environmental factors (e.g., temperature, humidity, etc) of the nesting chamber change (Craven *et al*., 2007; Mo, Caballero & Salas, 1992). Furthermore, it has also been suggested that even when there's no pathogenic action, microbial abundance can negatively affect the development of sea turtle embryos. Several studies have suggested that microbial relative abundance, richness, and diversity might affect success in Olive Ridleys (*Lepidochelys olivacea*). High microbial abundance has been shown to be responsible for the low hatching success observed in *arribada* nesting, due to changes in the oxygen levels and temperature resulting from microbial decomposition (Honarvar *et al*., 2011; Wyneken *et al.,* 1988; Bézy *et al*., 2015).

Besides exposure to bacteria and fungi present in the nest environment, occurrence of pathogens in sea turtle eggs can also be due to other reasons, one of them is eggs being exposed to the mother's bacterial flora during egg formation (Al-Bahry *et al*., 2009). In turtles, the oviduct (reproductive tract) consists of five anatomical regions: infundibulum, uterine tube, isthmus, uterus, and vagina. The uterine tube secretes the albumen after ovulation and the formation of the eggshell layers takes place in the uterus. Eggs are maintained in the reproductive tract for up to two weeks after ovulation, while all the egg components and the eggshell form, which gives enough time for the eggs to become infected if the oviduct of the mother is infected as well (AlKindi *et al.,* 2006; Craven *et al*., 2007). In a study carried out by Al-Bahry *et al*., (2009), 42% of eggs that were collected straight out of the oviduct of green turtles (*Chelonia mydas*) exhibited bacterial contamination, being the yolk as the egg component that was most frequently infected. Another potential source of contamination is exposure of the eggs to the bacterial flora present in the mother's gastrointestinal tract, due to the eggs being mixed with contaminated fecal material at the moment of oviposition, which creates an environment rich in bacteria. Even though many bacteria are naturally present in the gastrointestinal system of turtles, they tend to exhibit opportunistic behavior and become pathogenic in individuals that are immune compromised (Pace *et al*., 2019).

Sea turtle eggs can become contaminated by penetration of microorganisms through the eggshell. The outer calcareous layer, which separates the developing embryo from the external environment, is made up of loose nodular units of calcium carbonate ($CaCO_3$) that allow transfer of moisture and gases between the developing embryo and the environment, but under some conditions can also function as a portal for the entry of pathogenic microorganisms present in the nest chamber (Al-Bahry *et al*., 2011). The inner surface of the eggshell is no different, in that it is composed of aragonite crystals that form a loosely organized matrix that hardly prevents embryo colonization by bacteria. In the case of fungi, the spaces present within the inorganic and organic matrices do not seem to facilitate penetration by hyphae, however, species belonging to the *Fusarium* genus secrete enzymes that degrade the components that make up the outer and inner layers (Al-Bahry *et al.,* 2011; Phillott & Parmenter, 2006). Changes in environmental factors such as temperature and humidity might play a role in the colonization process. It has been observed that bacterial growth on the eggshell surface might benefit from high humidity, as water appears to help in the transportation of microorganisms through the eggshell pores; and generally, increasing temperatures tend to lead to increased bacterial growth, as higher temperatures maximize their metabolic activity (Cook *et al*., 2003; Gifari *et al.,* 2018; Ruiz-de-Castañeda *et al.,* 2011).

Marine turtles deposit their eggs in the sand, and these are left unattended during the course of incubation, which makes them more susceptible to attacks by a variety of organisms. In the absence of parental care, developing reptilian embryos must rely on non-specific defenses against microbial invasion (Miller, Limpus & Godfrey., 2001). The eggshell and shell membranes act as physical barriers, but embryos also rely on chemical defenses (e.g., egg albumen). At oviposition, sea turtles and freshwater turtles secrete a clear cloacal fluid that coats the eggs as they are being deposited into the egg chamber (**Figure 5**). The precise purpose of this fluid is not known but it has been suggested that, besides the lubrication it provides during egg deposition, these cloacal secretions might partially protect the eggs against potential pathogens (Phillot, 2002; Hayssen & Blackburn, 1985). For example, in freshwater turtles, eggs that have been washed and are no longer coated in this secretion tend to succumb more easily to infection in comparison with unwashed eggs (Ewert, 1985). In flatback (*Natator depressus*), green (*Chelonia mydas*), hawksbill (*Eretmochelys imbricate*) and loggerhead (*Caretta caretta*) turtles, these cloacal fluids have shown to prevent

fungi (*Fusarium oxysporum*, *Fusarium solani* and *Pseudallescheria boydii*) colonization of the eggs by inhibiting spore germination, but only for a few days (Phillot & Parmenter, 2012).

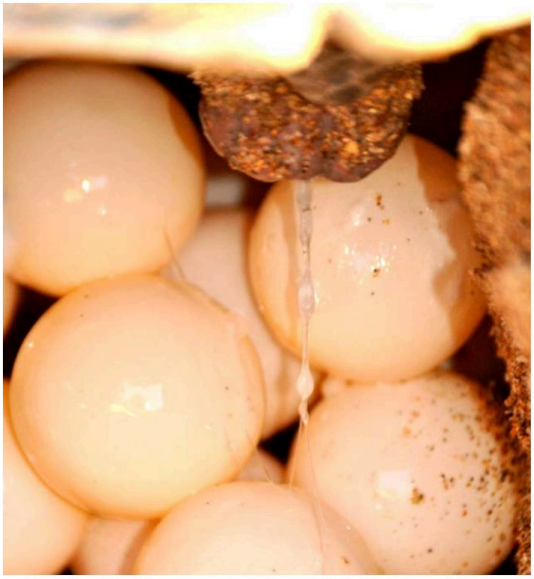


Figure 5. Cloacal fluid coating eggs as they're being deposited into the egg chamber (Keene, 2012).

1.3 Antimicrobial peptides and antimicrobial proteins

Antimicrobial peptides (AMPs) are a multi-functional group of molecules that can be found in a wide variety of organisms, ranging from bacteria to humans, as they are a key component of the innate immune response and act as a first line of defense against potential pathogens. Antimicrobial peptides (AMPs) generally have short amino acid sequences (<100 residues) and tend to be rich in positively charged amino acids (e.g., arginine, lysine), however, there are a few that have a negative net charge (Zhang *et al.,* 2021; Zharkova *et al.,* 2019). In reptiles, AMPs can be found in three of the four orders of reptiles: testudines (turtles and tortoises), crocodilians and the squamata (scaled reptiles) (van Hoek, 2014).

1.3.1 Classification of antimicrobial peptides

1.3.1.1 α-helical peptides.

Peptides that belong to this group adopt an amphipathic α-helical structure once they are in a non-polar environment (e.g., bacterial membrane). The α-helical family is varied, and it includes

cathelicidins, which are synthesized as pre-pro-peptides and contain two domains: a highly conserved amino-terminal cathelin domain and an antimicrobial carboxy-terminal domain that exhibits high variability (Lei *et al*., 2019). Cathelicidins are found exclusively in vertebrates, and even though most research has focused on mammals, several studies have demonstrated that cathelicidins can also be found in different reptile orders. For example, the cathelicidin AcCATH-1 detected in *Anolis carolinensis*, has been proven to negatively influence bacterial growth of *E. coli* and *S. aureus* (Holthaus *et al*., 2016); and in the sea snake *Hydrophis cyanocinctus*, the cathelicidin Hc-CATH has been shown to not only have antimicrobial activity against Gram-negative and Gram-positive bacteria, but also to have anti-inflammatory activity (Wei *et al*., 2015).

In testudines, genome sequencing of the Chinese soft-shell turtle (*Pelodiscus sinensis*), green turtle (*Chelonia mydas*) and the western painted turtle (*Chrysemys picta bellii*) suggests that these species have genes that appear to encode cathelicidin antimicrobial peptides similar to those found in snakes (van Hoek, 2014), and some studies have characterized novel cathelicidins in these species. In the green sea turtle (*Chelonia mydas*), four cathelicidins (Cm-CATH 1-4) have been identified and shown to exhibit antimicrobial activity against Gram-negative and Gram-positive aquatic bacteria, including some antibiotic-resistant strains (Qiao *et al*., 2019). In the soft-shell turtle (*Pelodiscus sinensis*), six cathelicidins (Ps-CATH 1-6) have been characterized and have been shown to not only kill pathogens by altering the structure of the bacterial cell membrane, but to also have immunomodulatory effects (e.g., inhibition of pro-inflammatory responses via MAPKs and NF-κB pathways) (Shi *et al.,* 2019).

1.3.1.2 β-sheets peptides.

This group is comprised by peptides that have a β-sheet structure that is stabilized by cysteine-disulfide bonds, and can be classified as defensins, proline-rich antibacterial peptides, protegrins or tachyplesins (Lei *et al.,* 2019). Defensins are one of the major classes of AMPs found in vertebrates, and similar to cathelicidins, they are synthesized as pro-peptides that are processed by proteases. These small cationic peptides (MW 3-4 kDa) are characterized by the presence of six cysteines arranged in three disulfide bonds and depending on the position of these bonds, they can be classified as α-, β- or θ- defensins (Huan *et al.,* 2020). Within reptiles, only β- defensins seem to be present and these tend to have an additional α-helical region at the amino-terminal that

facilitates anchoring to the bacterial membrane (Zharkova *et al.,* 2019). In the green lizard *Anolis carolinensis*, 32 beta-defensin-like peptides have been identified (Dalla Valle *et al.,* 2012) and in the venom of the South American rattlesnake (*C. durissus terrificus*), Cromatine, a peptide closely related to β-defensins, has been shown to have antibacterial (against *E. coli*) and antifungal (against *Candida* sp) activity (Kerkis *et al.,* 2014).

In testudines, several beta-defensins have been identified and isolated. In the European pond turtle (*Emy orbicularis*), a defensin called TBD-1 (Turtle β-defensin 1) was purified from leukocytes, and it was shown to inhibit growth of Gram-positive and Gram-negative bacteria (Stegemann *et al.*, 2009). Skin of the carapace, neck, digit, and tail of the Soft-shelled turtle (*Apalone spinifera*), expresses four beta-defensin-like peptides (As-BD- 1-4) that have been suggested to form an antimicrobial barrier that prevents colonization by epidermal bacteria (Benato *et al*., 2013). Beta-defensins seem to also play a role as non-specific chemical defenses in developing reptilian embryos. In the Chinese Soft-shelled turtle (*Pelodiscus sinensis*), Pelovaterin (a beta-defensin-like peptide) found in the eggshell matrix, seems to play a role in the stabilization of the nodular units of calcium carbonate ($CaCO_3$) that make up the outer calcareous layer, and it also exhibits strong antimicrobial activity against *Pseudomonas aeruginosa* and *Proteus vulgaris* (Lakshminarayanan *et al.,* 2008). Additionally, the eggwhite of loggerhead turtles (*Caretta caretta*) express a β-defensin-like peptide that exhibits not only antimicrobial activity (against *E. coli* and *S. typhimurium*), but also antiviral activity against Chandipura virus (rhabdovirus) (Chattopadhyay *et al.,* 2006).

1.3.2 Mechanisms of action of antimicrobial peptides

1.3.2.1 Membrane targeting mechanisms

Most antimicrobial peptides (AMPs) act by disrupting membrane integrity (**Figure 6**). The positively charged amino acids present in these peptides (e.g., arginine, lysine) interact directly with the negatively charged bacterial membrane, which results in results in changes of the biophysical properties of the latter (Bals & Wilson, 2003). Currently, there are four models that explain peptide permeation of the target cell membrane. (i) A *toroidal pore* model, in which AMPs bind to phospholipid molecules and accumulate inside the cell membrane, which result in the

formation of a ring hole that allows leakage of intracellular components. (ii) A *barrel stave* model, in which AMPs aggregate with each other and their hydrophobic domains penetrate the cell membrane to form channels. (iii) A *Carpet* model, in which the cell membrane is covered by a lawn of antimicrobial peptides, which leads to changes in the surface tension of the cell membrane and ultimately results in deformation. (iv). An *aggregate* model, in which AMPs bind to the cell membrane and form a peptide-lipid complex, that ultimately leads to outflow of intracellular contents but also facilitates entrance of peptides into the cytoplasm (Lei *et al.,* 2019; Huan et al., 2020; Zhang *et al*., 2021).

1.3.2.2 Non-membrane targeting mechanisms

There are some antimicrobial peptides that can kill microbes without disrupting the cell membrane (**Figure 6**). Recent evidence suggests that AMPs can be intracellularly active, and that there are several other mechanisms that lead to cell death. (i) Inhibition of protein synthesis by affecting transcription, translation, and assembly of ribosomes. (ii) Inhibition of enzyme activity of microbes. (iii) Inhibition of cell division by inhibiting DNA replication and blocking cell cycle. (iv) Inhibiting synthesis of cell wall (Zhang *et al*., 2021).

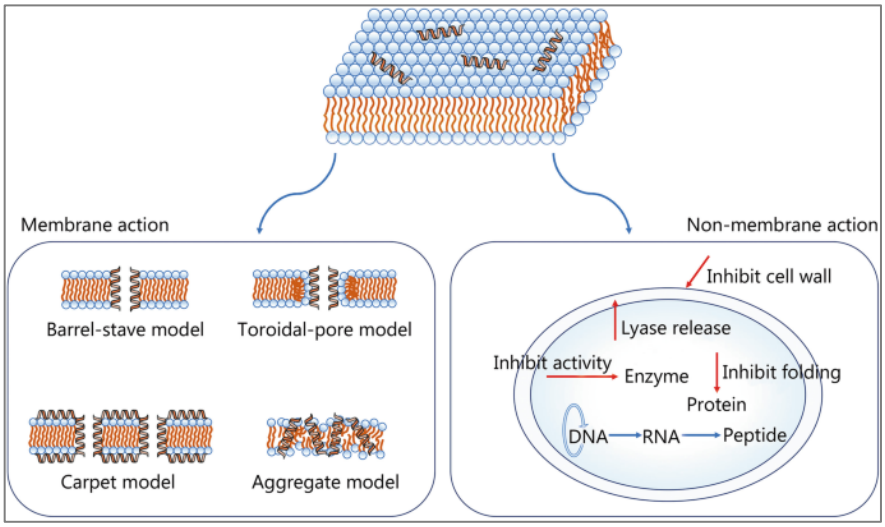


Figure 6. Mechanisms of action of antimicrobial peptides (AMPs) (Zhang et al*.,* 2021).

1.3.3 Antimicrobial proteins

There are some constitutively expressed proteins that exhibit antimicrobial properties and contribute to the host innate immunity response. These antimicrobial proteins tend to be larger (>100 amino acid residues) and some of them are enzymes that exhibit lytic action (Ganz, 2003). In reptiles, some specific transferrins and lysozymes are the most common antimicrobial proteins.

1.3.3.1 Transferrins

Transferrins are a big family of proteins that can be found in vertebrates and invertebrates, and they have been found to play an important role in iron transport and acquisition, and also as defense against potential pathogens (Ciuraszkiewicz *et al.,* 2007). In reptiles, transferrins are glycosylated in the liver and the oviduct, which results in two different types of proteins: a transferrin that mainly binds iron (serum) and an ovotransferrin (egg white) that transfers ferric ions from the oviduct to the embryo but also acts as an antimicrobial agent (Alexander *et al.,* 2017). Ovotransferrin (OTf) has molecular weight of about 80 kDa, and it has been shown to be an important component of the eggs of crocodiles (Chaipayang *et al.,* 2013), snakes (Ciuraszkiewicz *et al*., 2007) and turtles (López-Hurtado *et al*., 2010). It has been suggested, that ovotransferrin inhibits bacterial growth by creating an unfavorable iron-free environment, and by binding copper, which is counterproductive for lysozyme activity (Deeming & Ferguson, 1991).

1.3.3.2 Lysozymes

Lysozymes are small (MW ≈ 15 kDa) antimicrobial enzymes that are involved in the innate immune response of most animals, including reptiles. Lysozymes damage bacterial cells by hydrolyzing the beta-glycosidic linkage between the N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan that makes up cell walls in bacteria (Zhang & Gallo, 2016). A lysozyme-like protein has been isolated from leukocytes of the Siamese crocodile (*Crocodylus siamensis*), and it has been found to inhibit growth Gram-positive and Gram-negative bacteria (Pata *et al.,* 2007). In testudines, lysozyme proteins similar to avian lysozymes have been identified in the egg whites of the Indian soft-shelled turtle (*Trionyx gangeticus*) (Gayen *et al*., 1977), green turtle (*Chelonia mydas*) (Chijiiwa *et al.,* 2006), and the soft-shelled turtle (*Trionyx sinensis*) (Prajanban *et al.,* 2012).

CHAPTER 2. METHODS

2.1 Sample collection

Samples were collected at the Wassaw National Wildlife Refuge, which is one of Georgia's coastal barrier islands (**Figure 7**). Samples were collected from different individuals over the 2021 nesting season, from June to July. Fluid collection took place once the turtle had gone through the normal nesting process of making a body pit and digging an egg chamber, and after approximately 20 eggs had been dropped to allow any possible contaminants to be flushed out (Keene, 2012). Fluid samples were collected by positioning a wide-mouth jar under the cloaca and allowing drips of the fluid into it, then the collected fluid was transferred into a 15 ml sterile Falcon tube. In some cases, a few eggs (2-3) were also collected in the jar and fluid was scrapped off each egg and was then transferred into the sterile tube. Sample volume ranged from 5-8 ml of fluid per individual. Each sample was labeled with the collection dates as well as the individual turtle ID number. To prevent protein degradation, 200 ul of cOmplete, EDTA-free protease inhibitor cocktail working solution (Sigma Aldrich) were added for every 10 ml of fluid. Samples were kept on ice during the rest of the patrol and were then shipped on dry ice to the Department of Biology, Purdue University Fort Wayne. Once they arrived, samples were stored at -80 °C until further processing. All samples were collected under the Scientific Collecting Permit #10000527963 emitted by the Georgia Department of Natural Resources.

Figure 7. Map of loggerhead sea turtle nesting sites in Wassaw Island, Georgia (season 2009). Each dot represents and individual nesting site.

2.2 Protein fractionation

Samples were thawed at room temperature and were kept on ice while they were defrosting. To accelerate the unfreezing process, samples were vortexed at high speed for a few seconds (5-10 seconds) every 30 minutes. Once they were completely defrosted, samples were sonicated using a Fisherbrand™ Model 120 Sonic Dismembrator (Fisher Scientific GSA) at a frequency of 20 KHz. Samples were kept in the 15 ml Falcon tubes, and the tip of the Fisherbrand™ Model 120 Sonic Dismembrator was immersed in the sample to a depth of approximately 2-3 cm. Sonification was carried out in 3 cycles consisting of 20 seconds of sonification followed by 1 minute of cooling period and was followed by centrifugation at 13 000 rpm for 10 minutes. The supernatant was collected, and it was filtered using a polyvinylidene fluoride (PVDF) syringe filter (pore size 0.2 μm) (Thermo Scientific), to remove any remaining particles and microorganisms that could be present in the sample. A PVDF membrane was selected as it allows high protein recovery (Oshima *et al.,* 1996). Three hundred microliters from the filtered supernatant were recovered and stored at -80 °C in a 1.5ml microcentrifuge tube for testing of antimicrobial properties while the remaining volume was subjected to centrifugal ultrafiltration. This filtered supernatant is the *Crude extract.*

Protein fractionation (**Figure 8**) was carried out using commercially available Amicon® Ultra-15 mL centrifugation filters (Merck Millipore) of 3K,10K and 30K molecular cut-off weights. At first, 5-6 mL of the crude extract were placed in the 30K centrifugal filtration device, with no washing, and centrifuged at 5000 x *g* for 20 minutes. The concentrate (~300 μL) retained in the 30K filter device was collected and stored at -80 °C in a 1.5ml microcentrifuge tube. This obtained fraction represents proteins with a molecular weight higher than 30 kDa (*Fraction 30kDa+*). The filtrate (flow-through) of the 30K filter was collected and then centrifuged using the 10K filter under identical conditions. The concentrate (~300 μL) retained in the 10K filter device was collected and stored at -80 °C in a 1.5ml microcentrifuge tube. This obtained fraction represents proteins with a molecular weight between 30 kDa and 10 kDa (*Fraction 10-30kDa*). Finally, the filtrate (flow-through) of the 10K filter device was collected and then centrifuged using the 3K filter at 5000 x *g* for 40 minutes. The concentrate (~300 μL) retained in the 3K filter device was collected and stored at -80 °C in a 1.5ml microcentrifuge tube. This obtained fraction represents proteins with a molecular weight between 10 kDa and 3 kDa (*Fraction 3-10kDa*). All fractions were stored at -80 ° C until testing of antimicrobial properties.

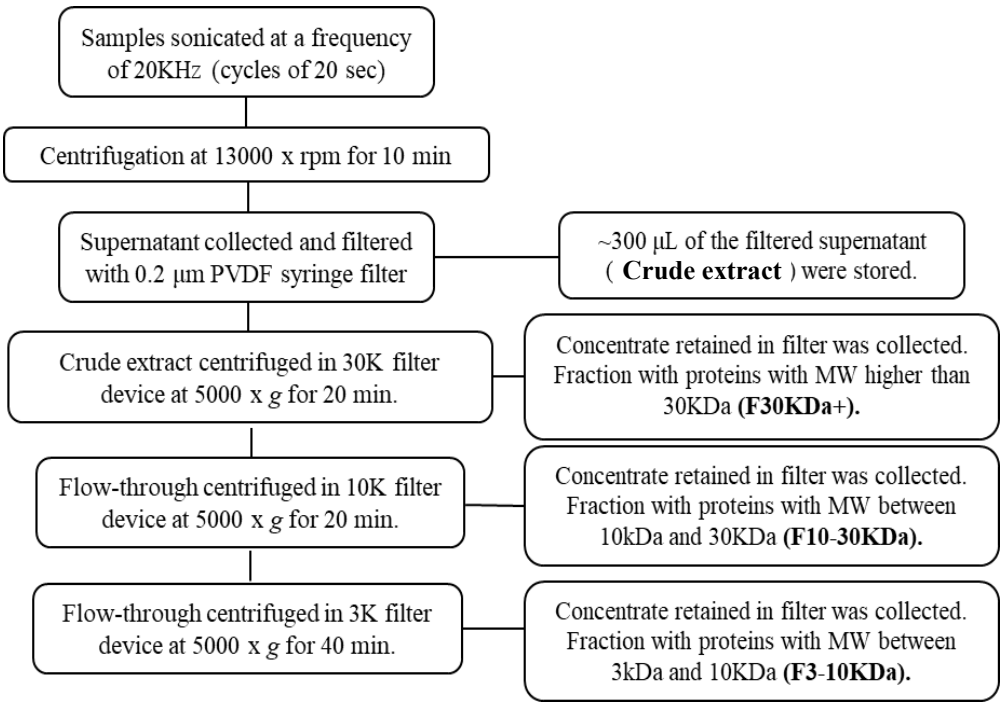


Figure 8. Protocol followed during protein purification.

2.3 Protein quantification

Protein concentration of each sample was measured using the DC Protein Assay (**Figure 9**) (Bio-Rad Laboratories, USA), a sensitive colorimetric assay that is useful in the estimation of low molecular proteins and peptides (Doshi *et al*., 2003). Protein standards containing from 0.03 mg/ml to 1.0 mg/ml of Bovine Serum Albumin (BSA) were prepared using Tris-HCL 50mM buffer pH 8.8. Working reagent A was prepared by mixing 20 µL of reagent S (Sodium dodecyl sulfate) to each ml of reagent A (Sodium hydroxide). Assay was carried out in a Thermo Scientific™ Nunc MicroWell 96-Well Micro-titer plate (Thermo Fisher Scientific Corp). Samples were thawed at room temperature and were kept on ice during the entire time, unfreezing process was sped up by vortexing at high speed for a few seconds (5-10 seconds) every 30 minutes. Once defrosted, samples and standards were denatured at 100°C for 5 minutes. Into each well of the micro-titer plate were pipetted the following: 5 µL of sample or protein standard, 25 µL of working reagent A and 200 µL of reagent B (dilute Folin). Samples and standards were incubated at room temperature for 15 minutes and then absorbances were measure at 750nm using a Thermo Scientific™ Multiskan FC Microplate Reader (Thermo Fisher Scientific Corp). A total of 3 replicates of all samples and standards were run. To determine protein concentration of each

peptide fraction and of the crude extract, a standard curve was created by plotting the net absorbance vs. concentration of the known standards, with net absorbance being the difference of the standard-reagent blank (S-RB). Net absorbance values for each sample and for each fraction were calculated, and these were then interpolated onto the formula of the standard curve to determine concentration (expressed in mg/mL). All replicates values were averaged and a final concentration was recorded.

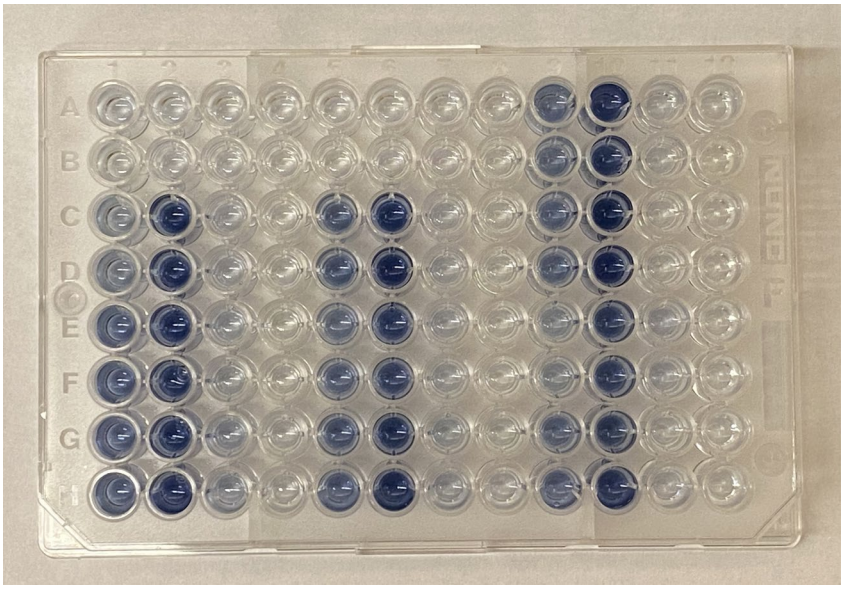


Figure 9. Microplate-based DC Protein Assay.

2.4 Antimicrobial assays

2.4.1 Bacterial strains and growth conditions

Four Gram-negative bacterial strains were used to assess the antimicrobial properties of the different peptide fractions isolated from the cloacal fluid of loggerhead turtles: *Pseudomonas aeruginosa* ATCC 15442 (*P. aeruginosa*), *Enterobacter cloacae* ATCC 25923 (*E. cloacae*), *Serratia marcescens* ATCC 14756 (*S. marcescens*) and *Morganella morganii* ATCC 23585 (*M. morganii*). These bacterial strains were chosen as they have been identified in unhatched eggs of loggerhead turtles (*C. caretta*) that nest in Georgia, USA (Craven *et al*., 2007). All microbial strains were obtained from the Department of Biology of the College of Science at Purdue University Fort Wayne. Bacterial isolates were grown on nutrient agar plates from glycerol stocks stored at -80 °C, isolates were streaked onto the plates using sterile inoculation loops and incubated

for 24 hours at 37 °C. Fresh cultures were prepared from frozen stocks 24 hours before testing of antimicrobial activity.

2.4.2 Inoculum preparation.

Bacterial suspensions were made prior (~20 minutes) to testing using the direct colony suspension method (Balouiri *et al.,* 2016). For each strain, several colonies were suspended in 4 ml of sterile distilled water, and cell suspensions were standardized by comparing the turbidity of each culture to the turbidity of a 0.5 McFarland standard, which represents about 1-2 x 10^8 CFU/ml. This initial inoculum was then further diluted by adding 20 µL of the previously standardized suspension to 1980 µL of nutrient broth, to obtain a final concentration of 5 x 10^5 CFU/ml, which is the recommended final inoculum size for broth dilution. Final inoculum size was verified by making a 1:1000 dilution that was cultured in nutrient agar and incubated at 37°C for 24 hours. Presence of about 50 colonies was expected when the inoculum density was 5 x 10^5 CFU/ml (Wiegand *et al*., 2008).

2.4.3 Microplate-based turbidimetric growth inhibition assay

Antimicrobial activity of the crude extract and the different peptide fractions was evaluated by performing a microplate-based microdilution broth assay. Fifty microliters of the crude extract and the peptide fractions were dispensed into separate wells in the micro-titer plate. Then, 50 µL of the inoculum of each bacterial strain was transferred into each well that contained the cloacal peptide extracts. Three controls were used. First, a positive control (growth control) that contained 50 µL of nutrient broth and 50 µL of the bacterial suspension containing an inoculum size of 5 x 10^5 CFU/ml. Then, a negative control that contained 50 µL of ciprofloxacin (0.1 mg/mL) and 50 µL of the bacterial suspension containing an inoculum size of 5 x 10^5 CFU/ml. The final inoculum size in each well was 2.5 x 10^5 CFU/ml and the final ciprofloxacin concentration in each well was 0.05 mg/ml. Finally, a sterility control that contained 100 µL of culture free nutrient broth. Additionally, a blank for each of the peptide fractions and the crude extract was prepared by mixing 50 µL of culture free nutrient broth and 50 µL of the denatured (at 100°C for 5 minutes) protein extracts. The final assay volume was 100 µL. After inoculation, the micro-titer plates were read immediately using a Thermo Scientific™ Multiskan FC Microplate Reader (Thermo Fisher

Scientific Corp) at 600 nm wavelength. After getting the initial OD, the micro-titer plates were sealed using Parafilm® sealing film and were kept in the 37°C incubator for 24 hours. Absorbance was read every 6 hours. The OD_{600} values obtained for each bacterial strain and for each peptide fraction were used to calculate bacterial growth and these values were then plotted against time to illustrate the inhibitory activity of the peptides found in the cloacal fluid of loggerhead turtles.

2.4.4 Viability assessment

Absorbances obtained from the microdilution broth assays were confirmed by assessing viability of cells upon 24 hours of exposure to the peptide fractions. Cell viability was assessed by the viable plate count (VPC) method, and it was only carried out for the bacterial strains (*S. marcescens* and *M. morganii*) and the peptide fractions for which absorbance at 24 hours in the treated wells (peptide extract + inoculum) was about half of the absorbance in the respective growth control well (inoculum only). VPC was carried out by removing 100 µL from the wells that contained both the peptide fraction and the bacterial inoculum, and then diluting it in 900 µL of nutrient broth to obtain a 1:10 dilution factor. Further dilutions of this suspension (1:10) were made by pipetting 100 µL into 900 µL of nutrient broth until obtaining a 10^{-8} and a 10^{-12} dilution factor for *M. morganii* and *S. marcescens,* respectively. 50 µL microliters of the obtained dilutions were plated in nutrient agar plates and were kept in the 37°C incubator for 24 hours after which colonies were counted. Each sample was analyzed in triplicate.

2.5 SDS-PAGE gel electrophoresis

Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for all peptide fractions and for the crude extract. Samples were prepared by mixing 19.5 µL of peptide extract, 7.5 µL of NuPAGE LDS Sample Buffer (4X) and 3 µL of NuPAGE Reducing Agent (10X) and were then heated at 70°C for 10 minutes. The gel was calibrated with the Precision Plus Protein Dual Color Standard (Bio-Rad Laboratories, USA), which is a protein ladder from the molecular weight of 10 to 250 kDa. Seven microliters of protein marker standard and 13 µL of the denatured peptide solution were loaded onto a 1mm 12 well 12% NuPAGE™ Bis-Tris Precast gel (Invitrogen). The gel was placed in a Xcell Surelock™ gel tank (Invitrogen), and the inner chamber was filled up with NuPAGE™ 1× MES running buffer (Invitrogen).

Electrophoresis was performed at 200 V for 35 minutes until the dye reached the bottom of the gel. After electrophoresis, the gel was taken out and stained using 0.1% (*w/v*) Coomassie Brilliant Blue (CBB). The gel was left incubating at room temperature overnight on an orbital shaker. The CBB-stained gel was then agitated in approximately 100 mL of destaining solution containing 50% (*v/v*) methanol, 10% (*v/v*) acetic acid for 30 minutes on an orbital shaker, and after two replacements of the destaining solution every 15 minutes, the gel was left incubating at room temperature overnight on an orbital shaker. The gel was imaged on a Molecular Imager® Gel Doc™ XR with Image Lab™ Software.

2.6 Statistical analysis

The data collected in this study were analyzed using the statistical software Minitab 17.1.0 (Minitab Inc., n.d.). The two-sample Student's test was used to analyze statistical differences in bacterial growth between the two groups (Control -vs- Treatment), for each peptide fraction and for each bacterial stain. A *p*-value lower than 0.05 was considered statistically significant. A two-sample Student's test was also used to analyze statistical differences in the number on colony forming units (CFUs) between the two groups (Control -vs- Treatment). A one-way analyses of variance (ANOVA) test with Tukey test was used to analyze statistical difference in bacterial growth between the crude extract and the peptide fractions at 24 hours of incubation, to determine which fraction was more efficient at inhibiting bacterial growth. A *p*-value lower than 0.05 was considered statistically significant.

CHAPTER 3. RESULTS

3.1 Protein concentration

Mean protein concentration (mg/ml) was highest for the fraction containing peptides with a molecular weight higher than 30 kDa (Fraction 30kDa+) as the average concentration was 8.52 mg/ml (±1.02). The crude peptide extract (Crude extract) had the second highest concentration being 4.39 mg/ml (±1.01). The two fractions with the smaller peptides (ranging from 3-30 kDa) had the lowest protein concentration, with the fraction containing peptides with a molecular weight between 10-30 kDa (Fraction 10-30kDa) having an average concentration of 1.02 mg/ml (±0.49); and the fraction containing peptides with a molecular weight between 3-10 kDa (Fraction 3-10kDa) having an average concentration of 0.18 mg/ml (±0.10) (**Figure 10-b**).

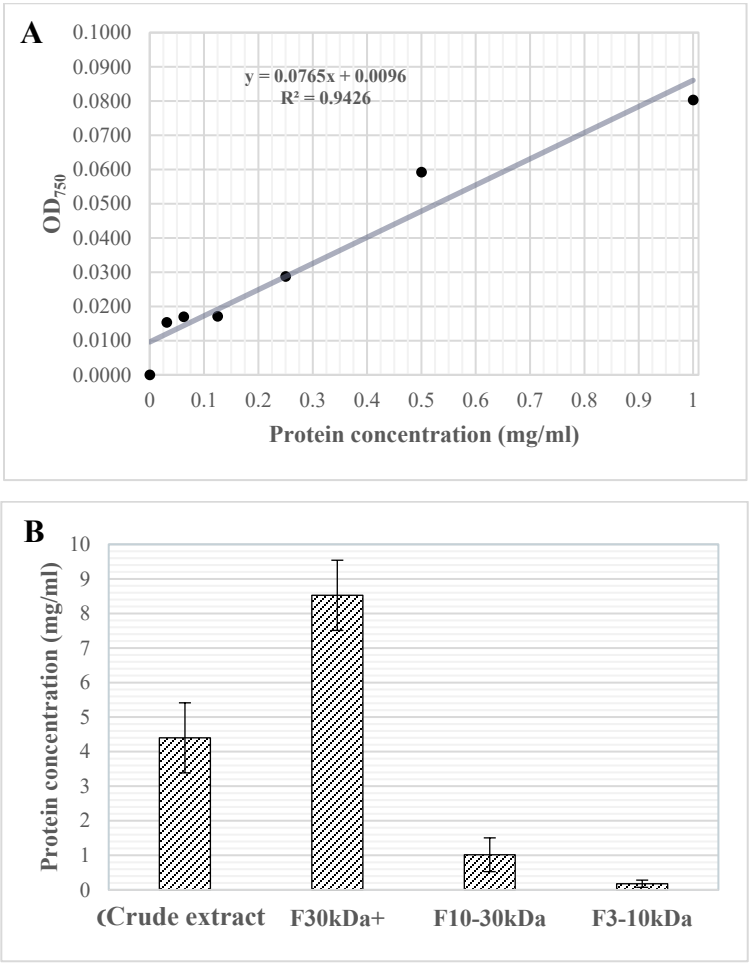


Figure 10. DC protein assay performed on crude extract and peptide fractions from cloacal fluid from loggerheads. (a) Standard curve relating known protein concentration of BSA and absorbance at 750nm. (b). Protein concentration of crude extract and peptide fractions. Error bars indicate standard deviation of 10 different samples analyzed in triplicate.

3.2 Antimicrobial activity of crude extract and peptide fractions

3.2.1 *Serratia marcescens*

The crude extract and the different peptide fractions were observed to have antimicrobial activity against *S. marcescens* (**Figures 11-14**). Bacterial growth was significantly lower ($p<0.01$) in the presence of the crude extract and the different peptide fractions throughout the entire incubation period (6h-24h), except for Fraction 30kDa+ in which bacterial growth was only significantly lower ($p<0.01$) for twelve hours (12h-24h). Bacterial growth inhibition was greatest ($p<0.05$) when incubated with Fraction 30kDa+ (**Figure 15**), as growth (OD_{600}) after 24h of incubation in the treatment was 0.1154 (±0.0089), while in the crude extract, Fraction 10-30kDa+ and Fraction 3-10kDa was 0.1439 (±0.0194), 0.1679 (±0.0375), and 0.1810 (±0.0236) respectively. After 24 hours, there was no significant difference ($p<0.05$) in the bacterial growth between the crude extract and the Fraction 10-30kDa, and between the Fraction 10-30kDa and the Fraction 3-10kDa; however, there was a significant difference ($p<0.05$) between the crude extract and Fraction 3-10kDa, which was the fraction that exhibited lowest levels of antimicrobial activity. Antimicrobial activity of the crude extract and the Fraction 30kDa+ was greatest at 18 and 24 hours of incubation, while the Fraction 10-30kDa and Fraction 3-10kDa had higher levels of antimicrobial activity at 18 hours (**Table 1**).

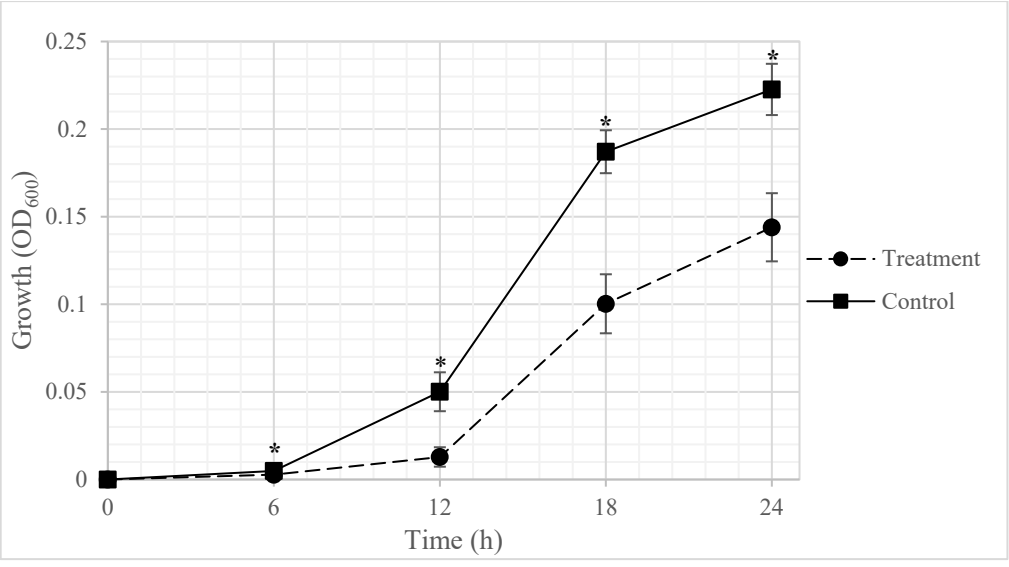


Figure 11. Growth curve of *S. marcescens* in the presence of the crude extract under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

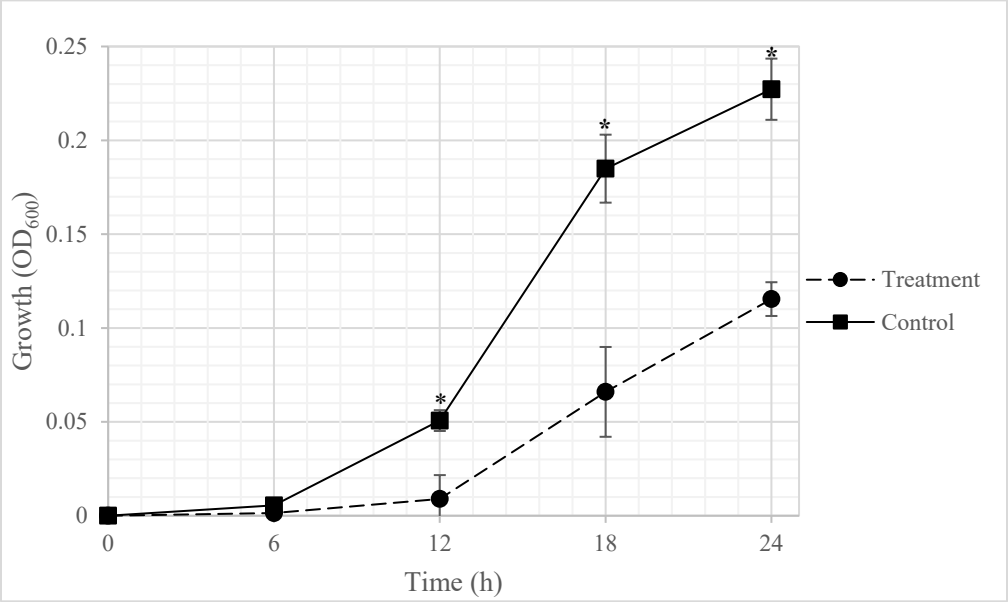


Figure 12.Growth curve of *S. marcescens* in the presence of the fraction containing peptides with a MW>30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

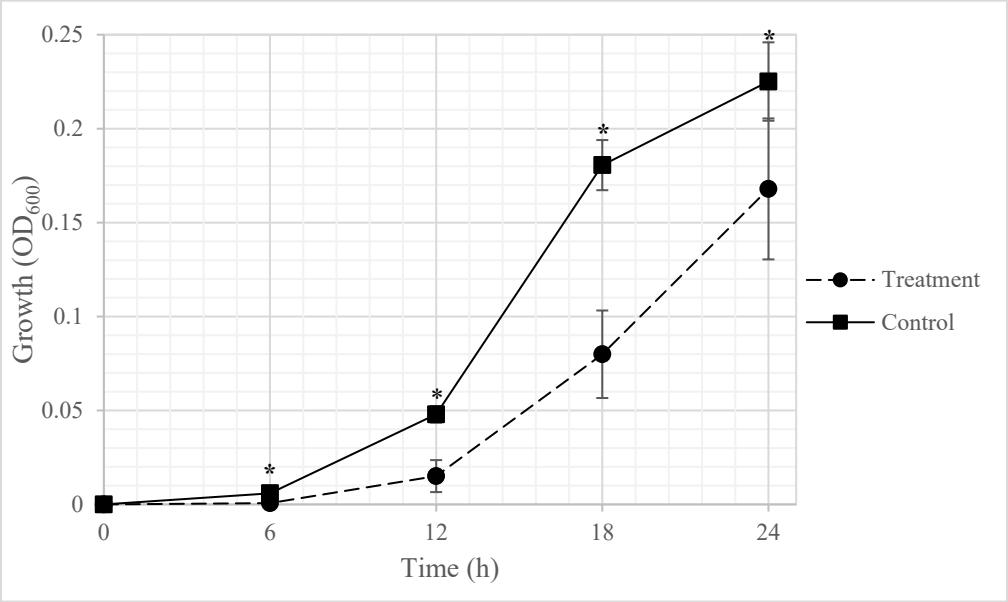


Figure 13.Growth curve of *S. marcescens* in the presence of the fraction containing peptides with a MW between 10-30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

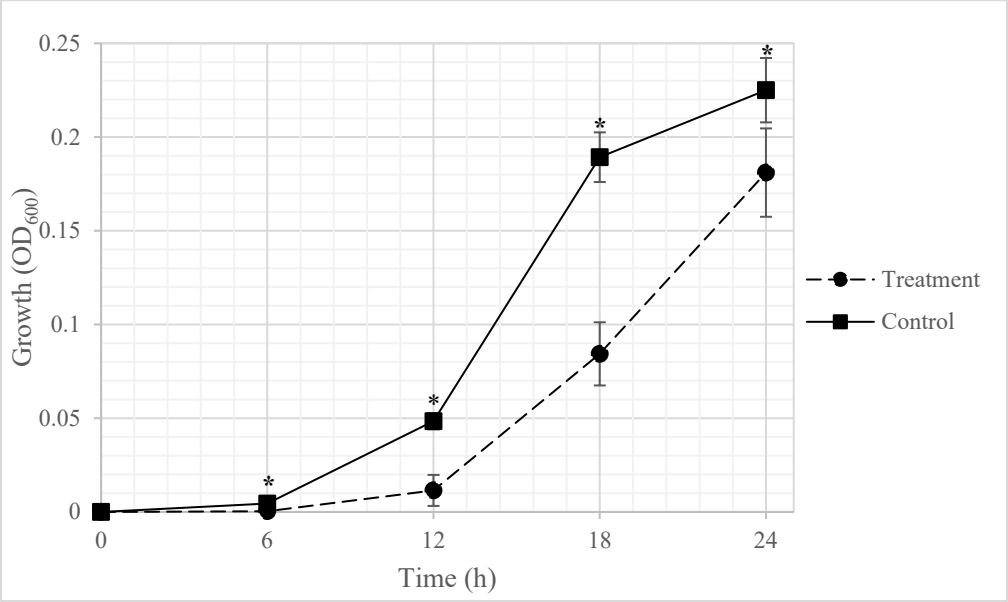


Figure 14. Growth curve of *S. marcescens* in the presence of the fraction containing peptides with a MW between 3-10kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

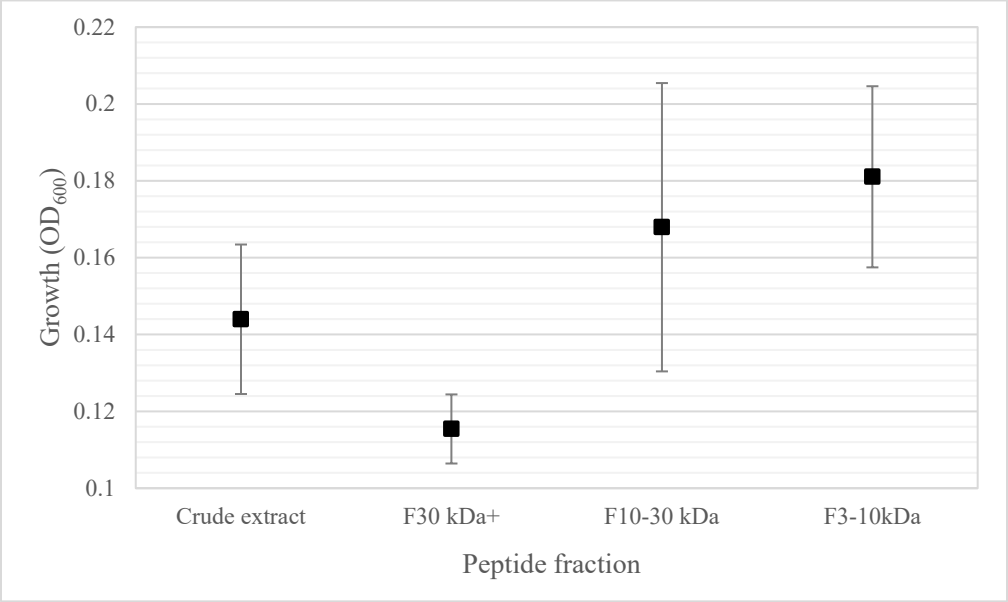


Figure 15. Growth of *S. marcescens* in the presence of the crude extract and peptide fractions after 24 h. All values represent a mean of ten replicate tests. Error bars indicate standard deviation.

3.2.2 *Morganella morganii*

The crude extract and the different peptide fractions were observed to have antimicrobial activity against *M. morganii* (**Figures 16-19**). Bacterial growth was significantly (p<0.01) lower in the presence of the crude extract and the different peptide fractions throughout the entire incubation period (6h-24h). After 24 hours of incubation, bacterial growth inhibition was greater (p<0.05) in the presence of the cloacal peptide fractions in comparison with the crude extract (**Figure 20**), as bacterial (OD_{600}) when incubated with Fraction 30kDa+, Fraction 10-30kDa and Fraction 3-10kDa was 0.0667 (±0.009), 0.0691 (±0.0077), 0.0700 (±0.0063), respectively, while in the crude extract was 0.0962 (±0.0094). Antimicrobial activity also varied with time, and it was higher at 18 and 24 hours of incubation in presence of the different peptide fractions. Levels of antimicrobial activity of the crude extract remained somewhat constant from 12 to 24 hours of incubation but were lower at 6 hours of incubation (**Table 1**).

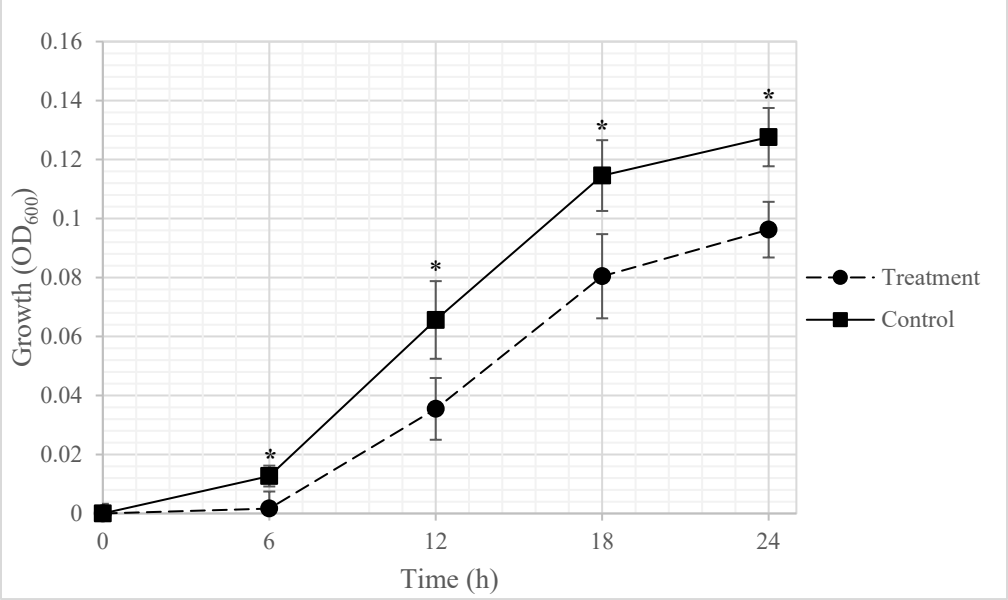


Figure 16. Growth curve of *M. morganii* in the presence of the crude extract under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) p<0.01. Error bars indicate standard deviation.

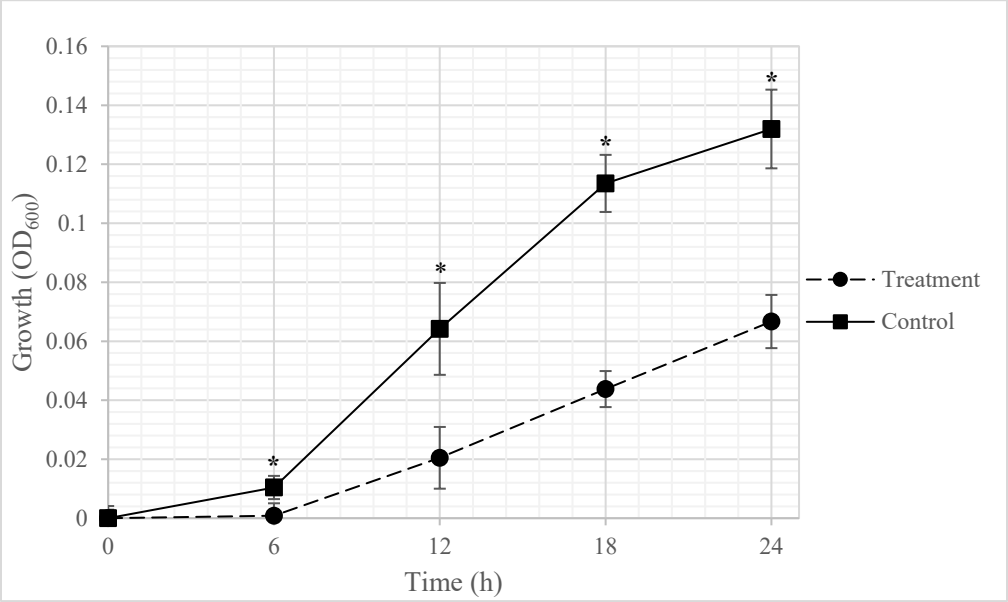


Figure 17. Growth curve of *M. morganii* in the presence of the fraction containing peptides with a MW>30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

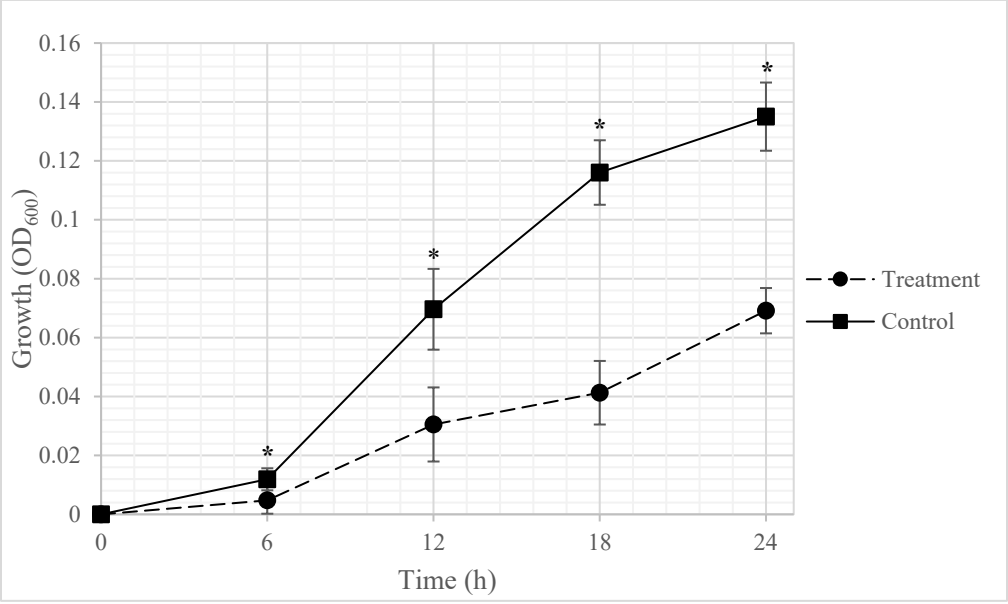


Figure 18. Growth curve of *M. morganii* in the presence of the fraction containing peptides with a MW between 10-30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

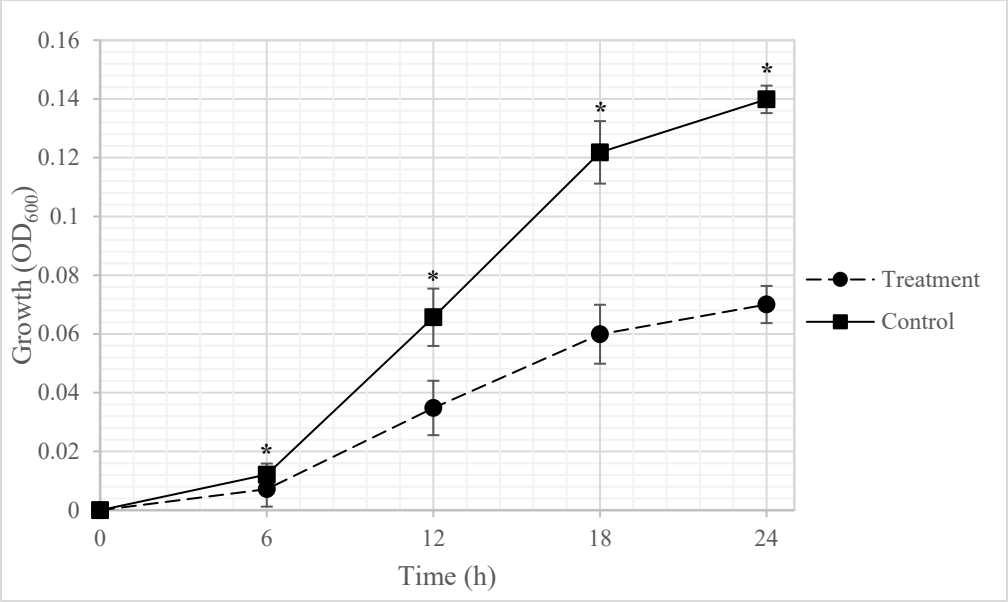


Figure 19. Growth curve of *M. morganii* in the presence of the fraction containing peptides with a MW between 3-10kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

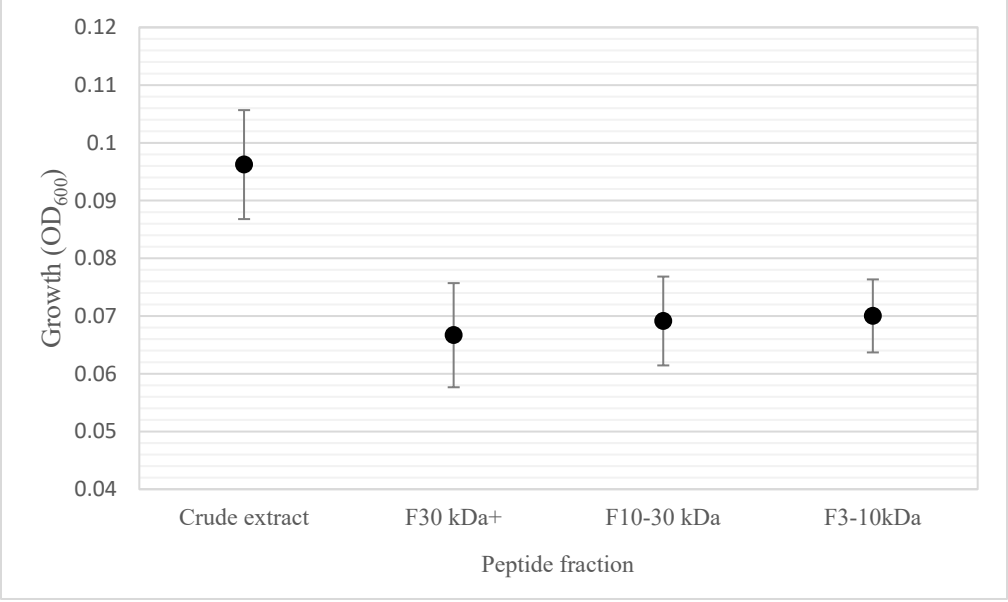


Figure 20. Growth of *M. morganii* in the presence of the crude extract and peptide fractions after 24 h. All values represent a mean of ten replicate tests. Error bars indicate standard deviation.

3.2.3 *Pseudomonas aeruginosa*

The crude extract and the different peptide fractions were observed to have mild levels of antimicrobial activity against *P. aeruginosa* (**Figures 21-24**). Bacterial growth was significantly (p<0.01) lower in the presence of the crude extract and the different peptide fractions throughout the entire incubation period (6h-24h). There was no significant difference in the levels of antimicrobial activity after 24 hours of incubation between the crude extract and the different peptide fractions. Peak levels of antimicrobial activity were observed at 12 hours of incubation for both the crude extract and the peptide fractions, and activity seemed to decrease after that.

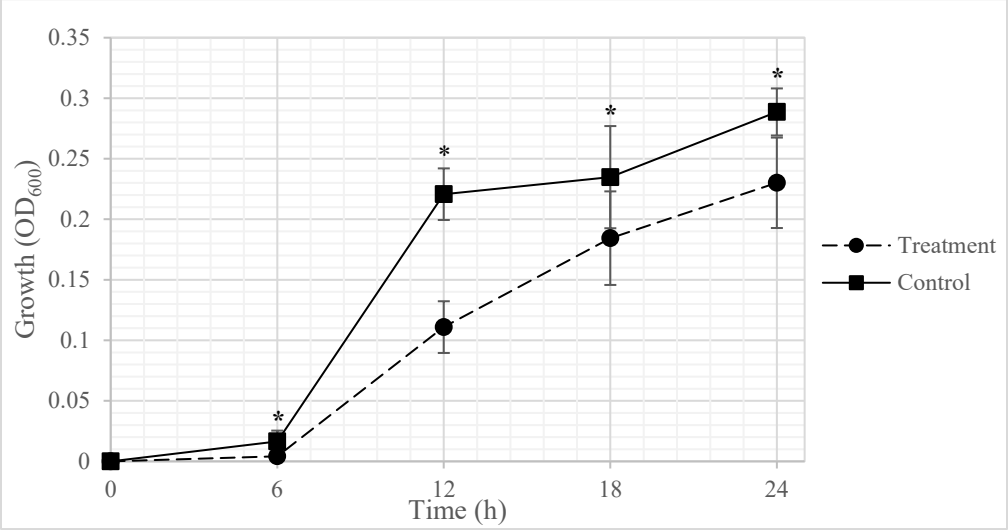


Figure 21. Growth curve of *P. aeruginosa* in the presence of the crude extract under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) p<0.01. Error bars indicate standard deviation.

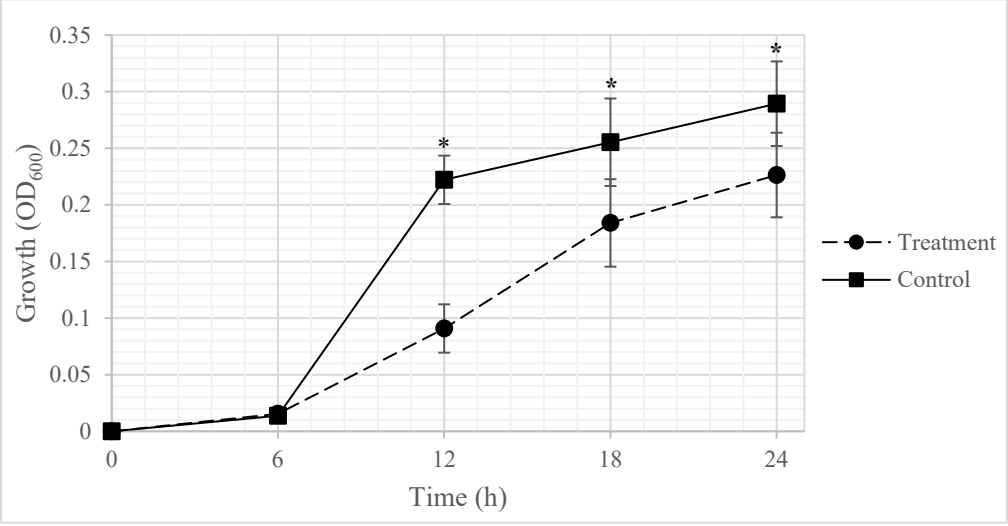


Figure 22. Growth curve of *P. aeruginosa* in the presence of the fraction containing peptides with a MW>30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) p<0.01. Error bars indicate standard deviation.

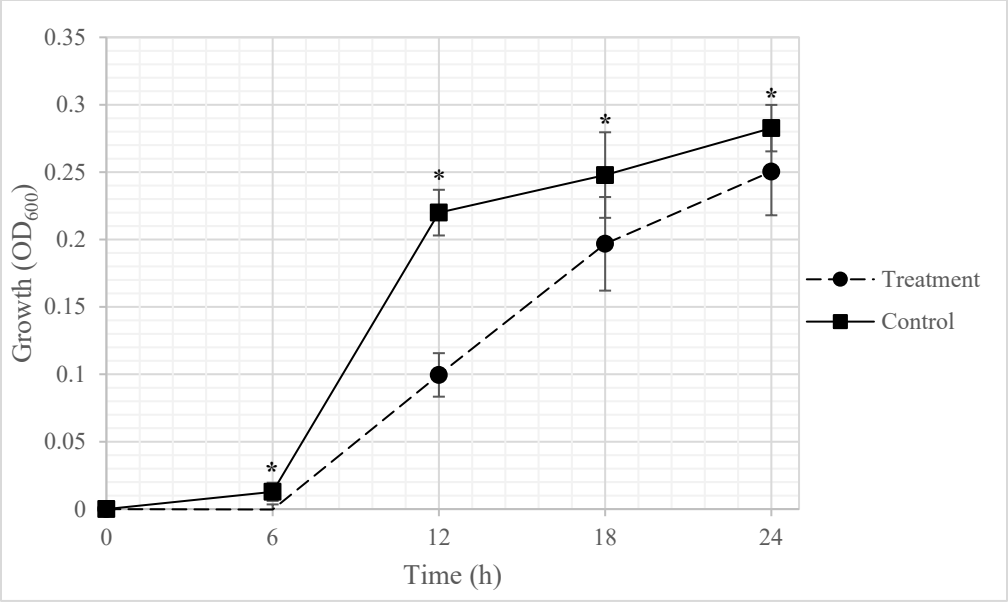


Figure 23. Growth curve of *P. aeruginosa* in the presence of the fraction containing peptides with a MW between 10-30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

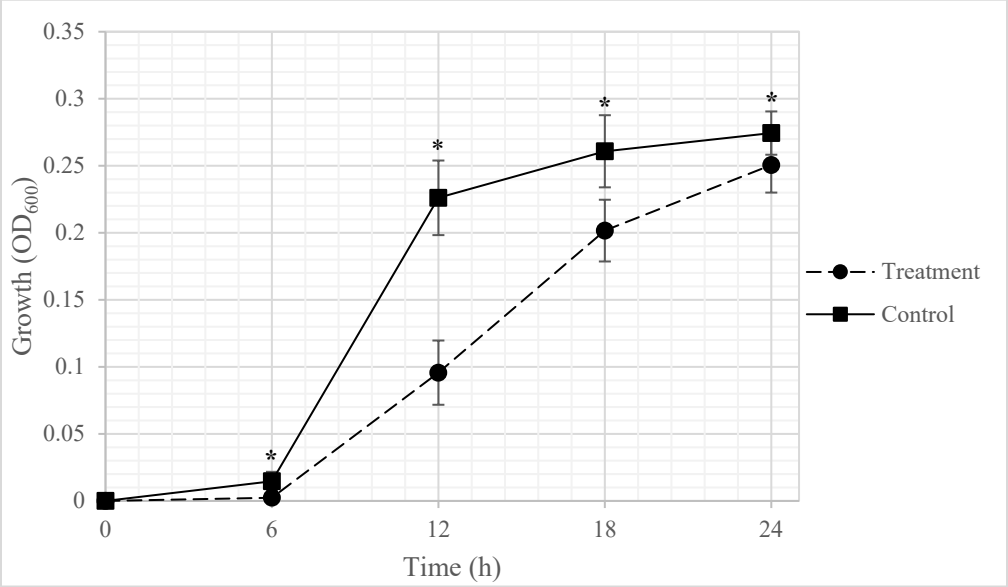


Figure 24. Growth curve of *P. aeruginosa* in the presence of the fraction containing peptides with a MW between 3-10kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

3.2.4 *Enterobacter cloacae*

The crude extract and the different peptide fractions were observed to have little to almost no antimicrobial activity against *E. cloacae (***Figures 25-28**). Even though statistical analysis points

at a significant difference (p<0.05) between treatment and control group, it is evident from the growth curves that bacterial growth in the presence of both the crude extract and the peptide fractions, was very similar and in some cases greater than the control.

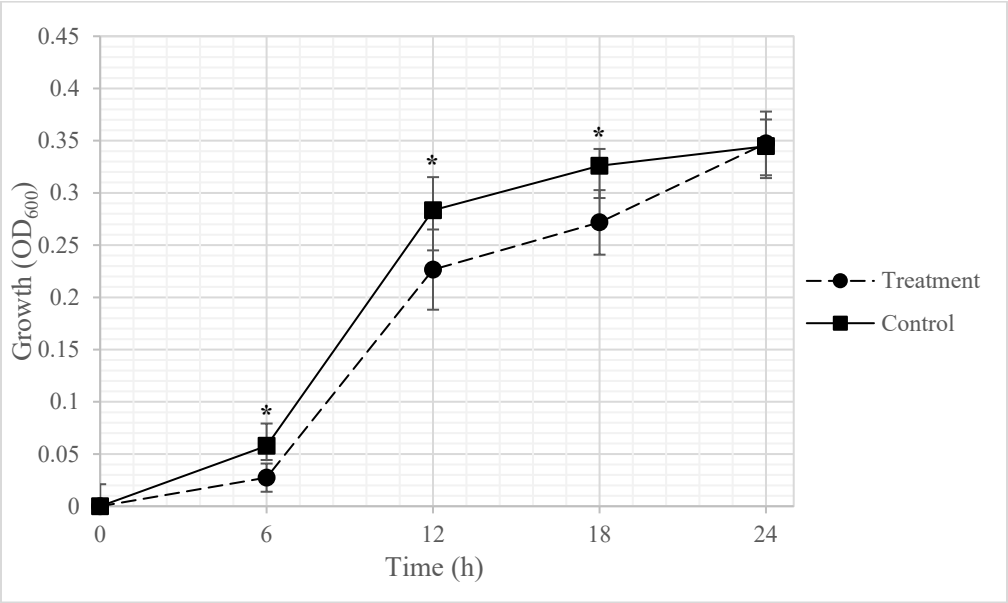


Figure 25. Growth curve of *E. cloacae* in the presence of the crude extract under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) p<0.01. Error bars indicate standard deviation.

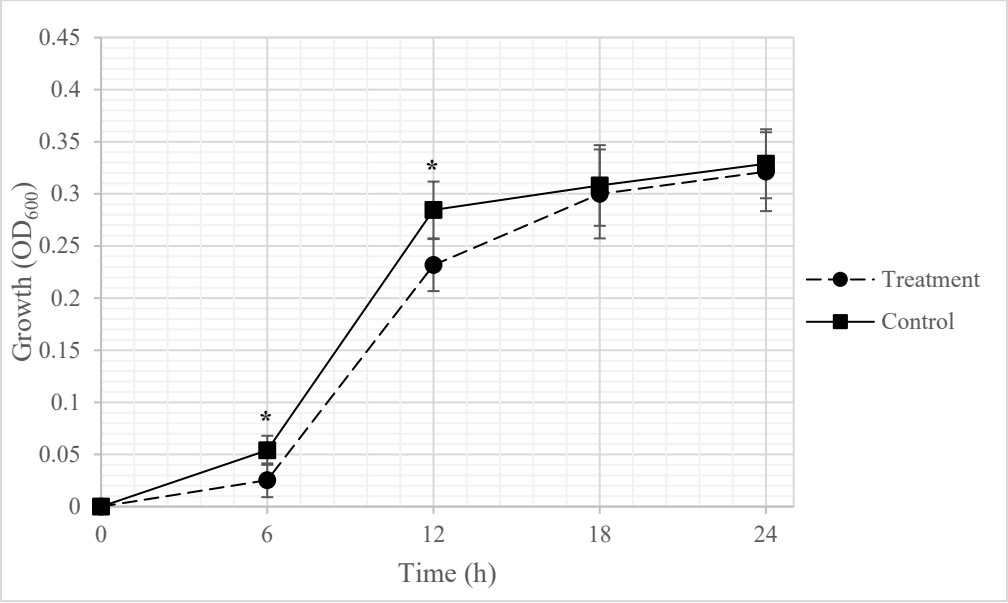


Figure 26. Growth curve of *E. cloacae* in the presence of the fraction containing peptides with a MW>30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) p<0.01. Error bars indicate standard deviation.

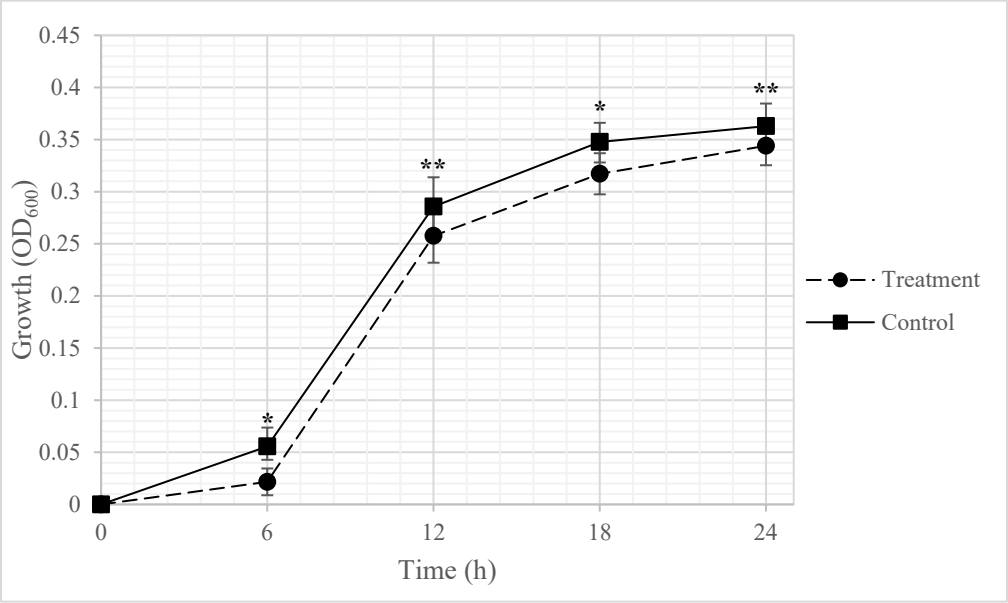


Figure 27. Growth curve of *E. cloacae* in the presence of the fraction containing peptides with a MW between 10-30kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. (**) $p<0.05$. Error bars indicate standard deviation.

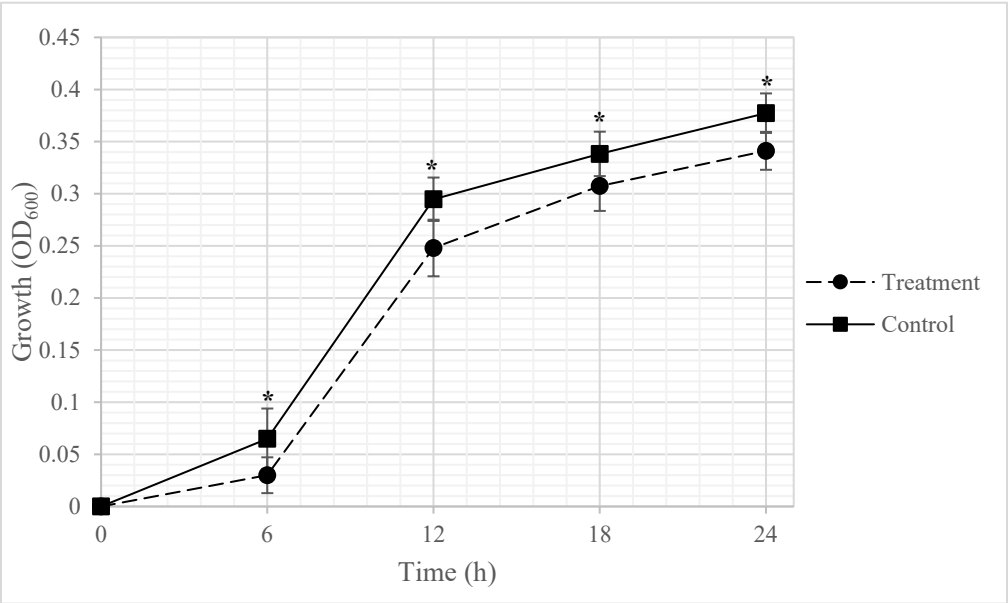


Figure 28. Growth curve of *E. cloacae* in the presence of the fraction containing peptides with a MW between 3-10kDa under standard conditions, 37°C and 24 h incubation. All values represent a mean of ten replicate tests. (*) $p<0.01$. Error bars indicate standard deviation.

Table 1. Bacterial growth (OD_{600}) of *S. marcescens*, *M. morganii* and *P. aeruginosa* when incubated with the crude extract and the different peptide fractions. All values represent a mean of ten replicate tests ($p<0.01$).

Bacteria	Treatment	Time (h)	OD_{600} (Treatment)	OD_{600} (Control)	$(OD_{600}^{Control} - OD_{600}^{Treatment})$
S. marcescens	Crude extract	6	0.0028 (±0.0035)	0.0049 (±0.0028)	0.0022
S. marcescens	Crude extract	12	0.0129 (±0.0056)	0.0501 (±0.0111)	0.0372
S. marcescens	Crude extract	18	0.1003 (±0.0168)	0.1871 (±0.0123)	0.0868
S. marcescens	Crude extract	24	0.1440 (±0.0195)	0.2227 (±0.0146)	0.0787
S. marcescens	F30kDa+	6	0.0014 (±0.0059)	0.0055 (±0.0028)	0.0041
S. marcescens	F30kDa+	12	0.0089 (±0.0127)	0.0507 (±0.0055)	0.0417
S. marcescens	F30kDa+	18	0.0660 (±0.0239)	0.1849 (±0.0181)	0.1189
S. marcescens	F30kDa+	24	0.1154 (±0.0090)	0.2272 (±0.0163)	0.1118
S. marcescens	F10-30kDa	6	0.0007 (±0.0045)	0.0059 (±0.0039)	0.0053
S. marcescens	F10-30kDa	12	0.0151 (±0.0085)	0.0479 (±0.0044)	0.0329
S. marcescens	F10-30kDa	18	0.0799 (±0.0233)	0.1806 (±0.0133)	0.1007
S. marcescens	F10-30kDa	24	0.1679 (±0.0375)	0.2251 (±0.0209)	0.0572
S. marcescens	F3-10kDa	6	0.0004 (±0.0023)	0.0045 (±0.0027)	0.0040
S. marcescens	F3-10kDa	12	0.0115 (±0.0083)	0.0484 (±0.0043)	0.0369
S. marcescens	F3-10kDa	18	0.0843 (±0.0169)	0.1893 (±0.0132)	0.1049
S. marcescens	F3-10kDa	24	0.1810 (±0.0236)	0.2250 (±0.0172)	0.0440
M. morganii	Crude extract	6	0.0017 (±0.0058)	0.0127 (±0.0035)	0.0110
M. morganii	Crude extract	12	0.0355 (±0.0105)	0.0656 (±0.0132)	0.0301
M. morganii	Crude extract	18	0.0804 (±0.0143)	0.1146 (±0.0120)	0.0341
M. morganii	Crude extract	24	0.0962 (±0.0094)	0.1276 (±0.0099)	0.0314
M. morganii	F30kDa+	6	0.0008 (±0.0042)	0.0104 (±0.0039)	0.0096
M. morganii	F30kDa+	12	0.0205 (±0.0105)	0.0642 (±0.0156)	0.0437
M. morganii	F30kDa+	18	0.0438 (±0.0061)	0.1135 (±0.0097)	0.0697
M. morganii	F30kDa+	24	0.0667 (±0.0090)	0.1320 (±0.0133)	0.0653
M. morganii	F10-30kDa	6	0.0048 (±0.0045)	0.0119 (±0.0037)	0.0071
M. morganii	F10-30kDa	12	0.0305 (±0.0126)	0.0696 (±0.0137)	0.0391
M. morganii	F10-30kDa	18	0.0413 (±0.0108)	0.1161 (±0.0109)	0.0748
M. morganii	F10-30kDa	24	0.0691 (±0.0077)	0.1350 (±0.0116)	0.0659
M. morganii	F3-10kDa	6	0.0072 (±0.0060)	0.0121 (±0.0038)	0.0050
M. morganii	F3-10kDa	12	0.0348 (±0.0093)	0.0657 (±0.0098)	0.0309
M. morganii	F3-10kDa	18	0.0599 (±0.0100)	0.1218 (±0.0106)	0.0619
M. morganii	F3-10kDa	24	0.0700 (±0.0063)	0.1399 (±0.0047)	0.0698
P. aeruginosa	Crude extract	12	0.1109 (±0.0214)	0.2207 (±0.0213)	0.1098
P. aeruginosa	Crude extract	18	0.1844 (±0.0386)	0.2348 (±0.0422)	0.0504
P. aeruginosa	Crude extract	24	0.2301 (±0.0374)	0.2887 (±0.0194)	0.0585
P. aeruginosa	F30kDa+	12	0.0908 (±0.0228)	0.2221 (±0.0189)	0.1313
P. aeruginosa	F30kDa+	18	0.1841 (±0.0349)	0.2553 (±0.0242)	0.0713
P. aeruginosa	F30kDa+	24	0.2264 (±0.0283)	0.2894 (±0.0288)	0.0630
P. aeruginosa	F10-30kDa	12	0.0995 (±0.0161)	0.2199 (±0.0169)	0.1204
P. aeruginosa	F10-30kDa	18	0.1968 (±0.0347)	0.2478 (±0.0317)	0.0510
P. aeruginosa	F10-30kDa	24	0.2504 (±0.0324)	0.2826 (±0.0172)	0.0322
P. aeruginosa	F3-10kDa	12	0.0957 (±0.0239)	0.2261 (±0.0278)	0.1304
P. aeruginosa	F3-10kDa	18	0.2016 (±0.0230)	0.2608 (±0.0269)	0.0592
P. aeruginosa	F3-10kDa	24	0.2506 (±0.0205)	0.2744 (0.01614)	0.0238

3.3 Cell viability assay

Both *M. morganii* and *S. marcescens* showed significant viability losses after 24 hours. As shown in **Figure 29** and **Figure 30,** the number of colony forming units (CFU/ml) was significantly lower (p<0.01) in the treated samples in comparison with the growth control for both bacterial strains. In *M. morganii* (**Figure 29**) incubated with the fraction containing peptides with a MW>30kDa (Fraction 30kDa+) the number of CFU/ml was 2.11E+11 (±3.77E+10), while in the control was 4.03E+11(±6.76E+10). When incubated with the fraction containing peptides with a MW between 10 and 30kDa (Fraction 10-30kDa) the number of CFU/ml was 1.52E+11 (±4.50E+10), while in the control was 3.56E+11 (±1.03E+11). Finally, when incubated with the fraction containing peptides with a MW between 3 and 10kDa (Fraction 3-10kDa) the number of CFU/ml was 1.61E+11 (±2.27E+10), while in the control was 3.63E+11 (±4.59E+10). In *S. marcescens* (**Figure 30**), the number of CFU/ml when incubated with the Fraction 30kDa+ was 2.44E+15 (±2.91E+14), while in the control was 4.28E+15 (±3.26E+14).

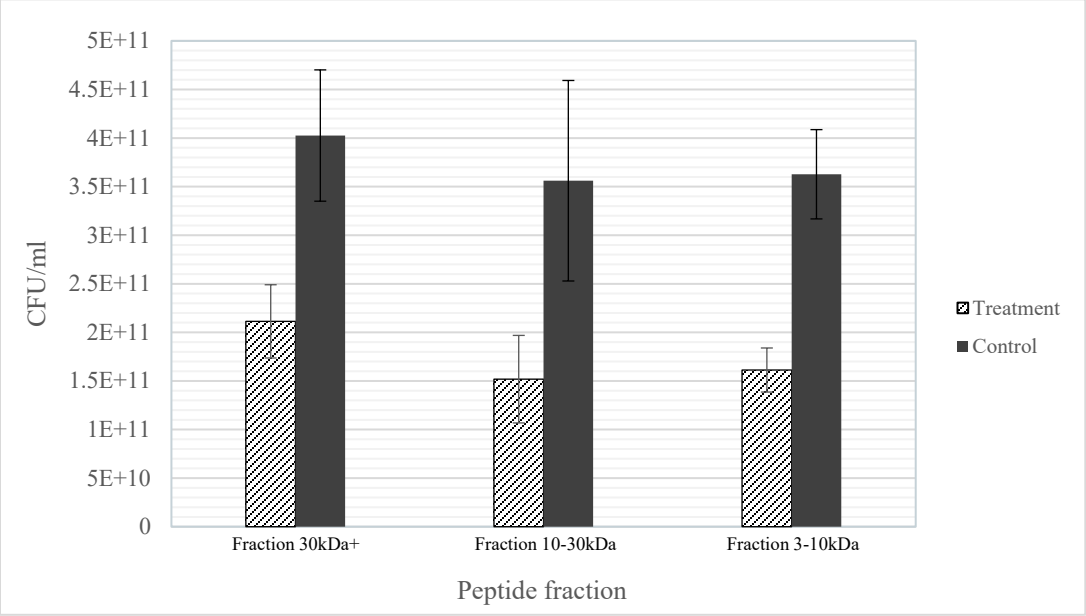


Figure 29. Cell viability assay assessed by the viable plate count (VPC) method for *M. morganii* under standard conditions, 37°C and 24 hours of incubation. Incubation with the different peptide fractions significantly reduced (p<0.01) cell viability upon 24 hours of exposure to the different peptide fractions. All values represent a mean of nine individual samples analyzed in triplicates. Error bars indicate standard deviation.

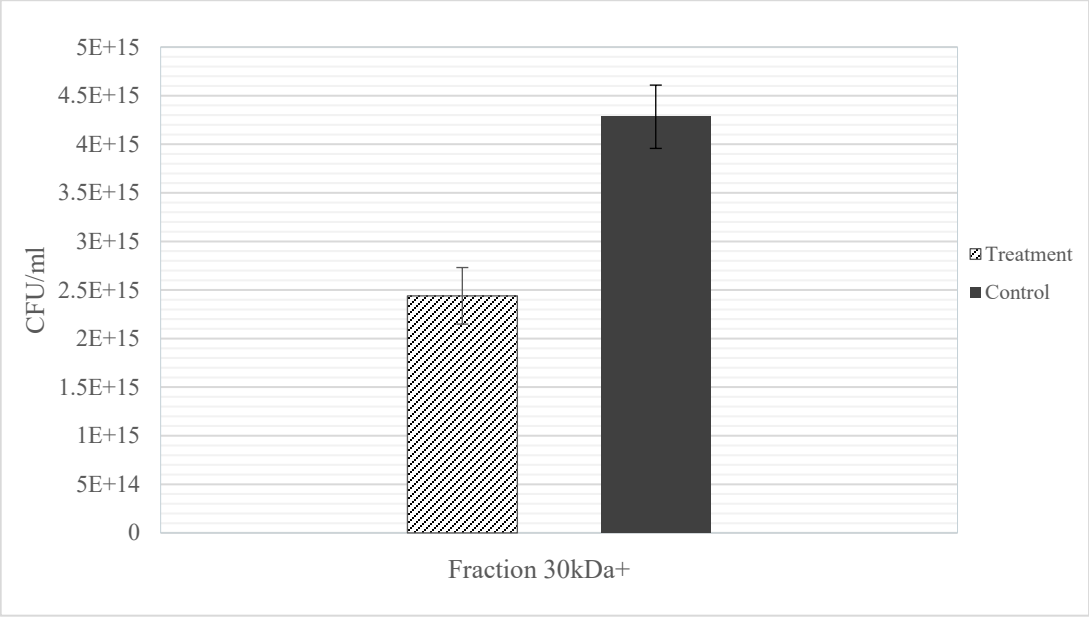


Figure 30. Cell viability assay assessed by the viable plate count (VPC) method for *S. marcescens* under standard conditions, 37°C and 24 hours of incubation. Incubation with the Fraction 30kDa+ significantly reduced ($p<0.01$) cell viability upon 24 hours of exposure. All values represent a mean of nine individual samples analyzed in triplicates. Error bars indicate standard deviation

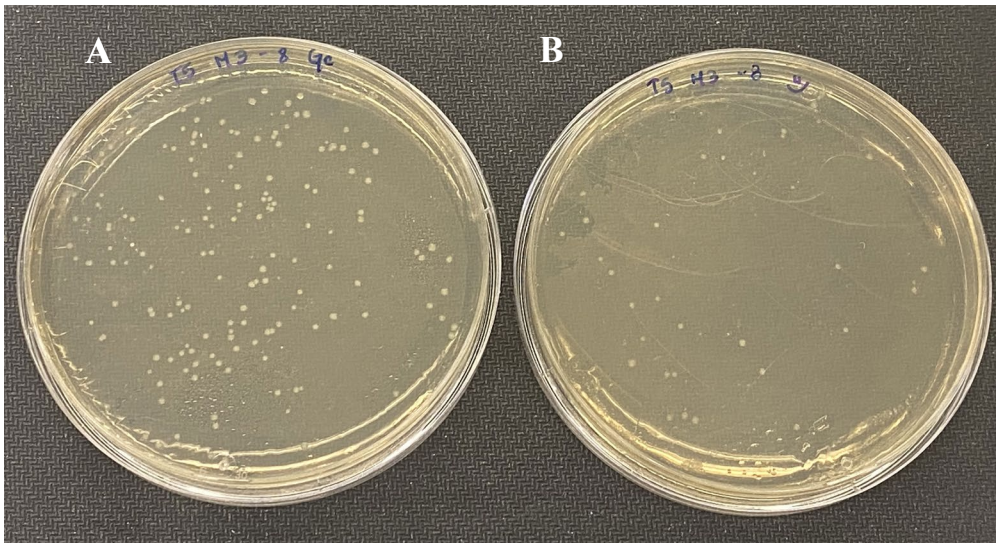


Figure 31. Cell viability assay in nutrient broth agar for *M. morganii* after 24 hours of incubation. (a) Control growth (6.87E+08 CFU/ml); (b) Treatment with Fraction 3-10kDa (3.05E+08 CFU/ml).

3.4 SDS-PAGE gel electrophoresis

Protein patterns for the crude extract and the different fractions were studied using SDS-PAGE analysis. As observed in **Figure 32**, cloacal fluid from loggerheads showed several protein bands with molecular weights ranging from approximately 5 – 250 kDa. It was expected that protein fractionation carried out with centrifugal filters would separate proteins according to their size, however, as observed in **Figure 32 (lanes F30kDa+, F1030kDa)**, this was not the case. Fraction 30kDa+ was supposed to contain proteins with a MW>30kDa, however, this fraction has bands with molecular weights of 14 and ~5 kDa. Similarly, fraction 10-30kDa was supposed to contain proteins with a MW between 10 and 30 kDa, but a strong band with a molecular weight of approximately 5kDa can be observed.

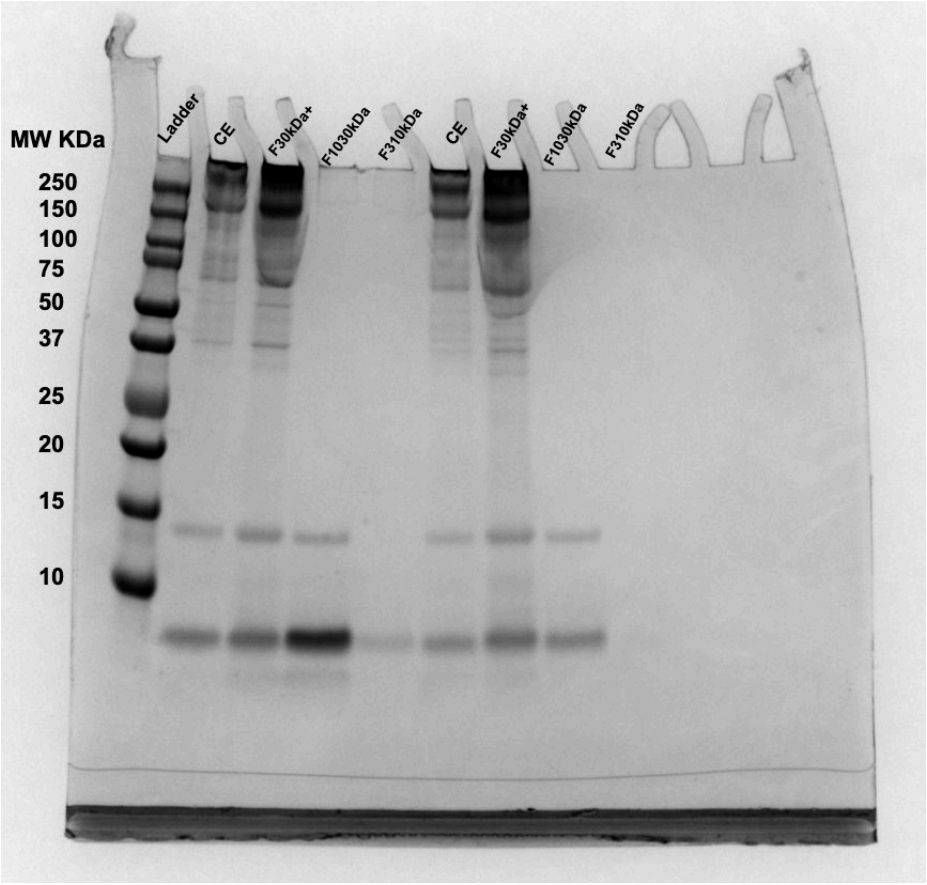


Figure 32. SDS-PAGE analysis of proteins present in the cloacal fluid of loggerhead turtles. Proteins were resolved on a 12% NuPAGE™ Bis-Tris Precast gel and then stained with Coomassie Brilliant Blue. Lane ladder: molecular weight markers; Lanes CE: Crude extract; Lanes F30kDa+: fraction of peptides with MW>30kDa; Lanes F1030kDa: fraction of peptides with MW between 10 and 30 kDa; Lanes F310kDa: fraction of peptides with a MW between 3 and 10kDa.

CHAPTER 4. DISCUSSION

Microorganisms have been shown to be in association with failure of sea turtle nests as presence of bacteria and fungi is often correlated with reduced hatching success (Wyneken *et al*., 1988; Zieger *et al*., 2009; Craven *et al*., 2007; Phillott & Parmenter, 2001; Sarmiento-Ramírez *et al*., 2010). Developing reptilian embryos rely on a combination of physical (e.g., eggshell) and chemical (e.g., egg albumen) non-specific defenses against microbial invasion (Miller, Limpus & Godfrey.,2001), which is why it has been suggested that the fluid secreted at oviposition might also provide some protection against potential pathogens. The purpose of this study was to evaluate the antimicrobial properties of the cloacal fluid of loggerhead sea turtles against bacterial strains that are often associated with failed eggs in this species.

The cloacal fluid produced by the shell-forming section of the oviduct is a clear liquid that consists mainly of glycoproteins (Phillot & Parmenter, 2012). Total protein concentration was evaluated for each sample and for each peptide fraction. Results indicated that proteins with higher molecular weights (>30kDa) are found in greater abundance in the cloacal fluid, as the fraction containing peptides with a MW>30kDa exhibited the highest protein concentration (8.52 mg/ml ± 1.01). Smaller peptides (MW<30kDa) were found to be a smaller component of the cloacal fluid, as fractions 10-30kDa and 3-10kDa had a concentration of 1.02mg/ml ±0.49 and 0.18mg/ml ±0.10, respectively. Currently there are no studies in which protein concentration of cloacal fluid has been determined, so the obtained results cannot be compared for either loggerhead turtles, or for any other turtle species. Likewise, even though the DC protein assay is a commonly used method for protein quantification due its simplicity and sensitivity, it is important to note that is highly nonspecific and many substances (e.g., potassium ions) that are present in the biological samples, particularly one as complex as the cloacal fluid of sea turtles, might interfere with the absorbance readings (Bensadoun & Weinstein, 1976). Additionally, the mass of the protein can impact absorbance readings as the dye reagents don't bind as efficiently to low molecular weight proteins (Rodríguez-Díaz *et al*., 2005).

Protein patterns for the crude extract and the different peptide fractions showed that the cloacal fluid from loggerheads is composed by proteins with a molecular weight ranging from

approximately 5 to 250 kDa. As observed in **Figure 32**, it seems as if proteins and peptides were not accurately separated according to their molecular weight as it was expected. In **lanes F30kDa+ and F1030kDa (Figure 32),** protein bands with molecular weights outside of the expected size range were observed, which means that proteins with a molecular weight lower than the established molecular weight cut-off of the filtering device remained in the separation membrane, instead of ending up in the flow-through. This result can be due to several reasons: (i) oftentimes there are some limitations in the processing of complex biological samples with centrifugal filter devices, like the ones used in this study, which end up altering performance and ultimately results in lowered fractioning selectivity (Johnsen *et al.,* 2016); (ii) in the case of cloacal fluid from sea turtles, the time recommended by the manufacturer might not have been enough to allow proper separation. It is important to note that due to the low initial volume of the samples, the recommended centrifugation time was followed to allow recovery of at least 300 µL of retentate. Since protein fractionation was not as accurate as initially thought, it means that the protein concentration of fractions 30kDa+ and 10-30kDa might actually be lower than the obtained values, while the concentration of fraction 3-10kDa might actually be higher as the strongest bands observed in the gel have a molecular weight of approximately 5 kDa.

The results from the microplate-based turbidimetric growth inhibition assay suggest that the proteins and peptides present in the cloacal fluid of loggerheads exhibit antimicrobial activity. The crude extract and the different peptide fractions were found to be active against *M. morganii* and *S. marcescens.* Species belonging to the genus *Serratia* and *Morganella*, are widespread opportunistic pathogens that cause severe infections in humans and animals, including turtles. *S. marcescens* has been found to be present in the interior of failed loggerhead eggs, and in adults it's been associated with ulcerative shell disease, integumental lesions, bronchopneumonia, and traumatic ulcerative dermatitis (Craven *et al*., 2007; Santoro *et al*., 2006). *M. morganii* is also a common bacterium in failed eggs of loggerheads and it's been associated with development of aural abscess in free-ranging and captive turtles (Joyner *et al*., 2006). In this study, both *S. marcescens* and *M. morganii* growth was significantly inhibited when incubated with the crude extract and the different peptide fractions. In the case of *S. marcescens,* fraction 30kDa+ displays the highest antimicrobial activity, particularly at 24 hours of incubation. Protein patterns obtained by SDS-PAGE electrophoresis, indicate that this peptide fraction might contain ovotransferrin,

lysozyme and probably some type of antimicrobial peptide as there are three visible bands with molecular weights of ~75, 14 and 5 kDa (**Figure 32, lane F30kDa+**). Ovotransferrin (OTf) is a glycoprotein that has a molecular weight of approximately 77.7kDa, and that has been shown to be present in the egg white of several marine and freshwater turtle species (Thammasirira *et al.,* 2007). OTf has been shown to exhibit antimicrobial activity due to its iron-binding capacity that ultimately prevents iron utilization by microorganisms, which is deleterious for growth. However, bacteria possess iron-acquisition systems, like siderophores, that are high-affinity iron-chelating molecules that allow bacteria to counteract to some degree OTf activity (Legros *et al*., 2021) and this might explain why bacteria continued to grow even when incubated with fraction 30kDa+. Lysozymes are small enzymes that cleave the glycosidic linkage of the peptidoglycan present in the cell wall, ultimately leading to cell death. Due to the presence of an outer membrane, Gram-negative bacteria tend to be lysozyme resistant, however, synergistic effects can occur between OTf and lysozyme, as OTf also binds divalent ions (e.g., Mg^{2+}) that are required for the integrity of the outer membrane, and this can potentially allow peptidoglycan lysis (Zhang & Gallo, 2016; Legros *et al*., 2021). Lysozyme also seem to be present in fraction 10-30 kDa as a band with a MW ~14 kDa was observed in the gel. Since only Gram-negative bacteria were used in this study, in the absence of OTf, there should be no negative effect of lysozyme on bacterial growth. This was not the case, as bacterial growth was significantly lower when incubated with the fraction 10-30 kDa, and this could potentially be due to the presence of other protein components. A strong band of approximately 5 kDa was observed in all fractions, which suggests that either cathelicidins or defensin-like peptides might be present in the cloacal fluid of loggerheads. The antimicrobial activity of AMPs is synergistic with other defense molecules, such as lysozyme, which might explain why growth was inhibited when incubated with fraction 10-30kDa (Bals & Wilson, 2002). It is worth noting that results suggest that the small peptides (band of ~5 kDa) found in the cloacal fluid seem to exhibit high levels of antimicrobial activity, as bacterial growth was inhibited even when incubated with Fraction 3-10kDa which only seemed to contain these peptides and also had the lowest total protein concentration.In the case of *M. morganii* there was no significant difference when incubated with the different peptide fractions as growth (OD_{600}) was very similar at 24 hours, and this suggests that *M. morganii* might be equally susceptible to the different antimicrobial proteins and peptides that seem to be present in the cloacal fluid of loggerheads. The absorbance results obtained in the turbidimetric assay are likely to be accurate because both bacterial strains

showed significant viability losses (**Figure 29, Figure 30**), as the number of CFU/ml in all treatments was about half of the number of CFU of the respective growth controls.

Incubation with the crude extract and the different peptide fractions seem to have mild antimicrobial effects against *P. aeruginosa* and little to no effect against *E. cloacae.* *P. aeruginosa* is a pathogen that has been isolated from the interior of failed loggerhead eggs and is also frequently attributed to nosocomial infections (Craven *et al*., 2007). In this study, growth was mildly inhibited as peak levels of antimicrobial activity were observed at 12 hours of incubation in all treatments, however, it was also observed that growth inhibition decreased with time after that. It is possible that a resistance mechanism to the cloacal fluid may have built up for these species and that's why there seems to be almost no effect on their growth. Several studies have described loggerheads as a reservoir of antimicrobial-resistant bacteria and high levels of resistance have been described in *Pseudomonas* sp and *Enterobacter* sp, which might explain the obtained results (Fernandes *et al*., 2021; Blasi *et al*., 2020). There are several mechanisms present in bacteria that confer resistance to antimicrobial peptides: (i) secretion of extracellular proteins, particularly proteases, that lead to proteolytic degradation; (ii) formation of a bacterial biofilm that decreases penetration of AMPs due to the exopolymers present (e.g., alginate in *Pseudomonas* sp.); (iii) alteration of the net charge and permeability of the outer membrane (e.g., addition of positively charged molecules) (Joo *et al.,* 2016). Additionally, bacteria also exhibit mechanisms that counteract the activity of ovotransferrin and lysozyme. High-affinity iron-acquisition systems (e.g., siderophores) are present in Gram-negative bacteria, and these function by chelating iron and making it accessible to the bacterial cell. In *P. aeruginosa* and *E. cloacae* there are two different siderophores: pyoverdine and pyochelin in *Pseudomonas*; and aerobactin and enterochelin in *Enterobacter* (Legros *et al.,* 2021).

In conclusion, this study found that the cloacal fluid of loggerhead sea turtles from Georgia, US, does have antimicrobial properties against some Gram-negative bacterial strains. Results from this study can potentially be used to develop antimicrobial compounds that mimic those found naturally to prevent microorganism contamination in the nesting chamber, particularly in hatcheries where there's a higher risk of microbial contamination due to high nest density. Development of these compounds can help potentially increase hatching success globally and help with sea turtle

conservation efforts Further studies should aim to isolate and purify each specific protein and peptide for characterization purposes and to determine its individual effect on bacterial growth. Purification of proteins and peptides will allow to determine the MIC (minimum inhibitory concentration), since in this study a standard protein concentration was not used. In addition, future research should be conducted using other bacteria, including Gram-positive, and fungi commonly associate with failed eggs.

REFERENCES

Alexander, D. B., Vogel, H. J., & Tsuda, H. (2017). Lactoferrin researchers descend on Nagoya Castle. *Biochemistry and Cell Biology*, *95*(1), 1–4. https://doi.org/10.1139/bcb-2017-0009

Al-Bahry, S., Mahmoud, I., Elshafie, A., Al-Harthy, A., Al-Ghafri, S., Al-Amri, I., & Alkindi, A. (2009). Bacterial flora and antibiotic resistance from eggs of green turtles Chelonia mydas: An indication of polluted effluents. *Marine Pollution Bulletin*, *58*(5), 720–725. https://doi.org/10.1016/j.marpolbul.2008.12.018

Al-Bahry, S. N., Mahmoud, I. Y., Melghit, K., & Al-Amri, I. (2011). Analysis of Elemental Composition of the Eggshell before and after Incubation in the Loggerhead Turtle (Caretta caretta) in Oman. *Microscopy and Microanalysis*, *17*(3), 452–460. https://doi.org/10.1017/s1431927611000298

AlKindi, A. Y. A., Mahmoud, I. Y., Woller, M. J., & Plude, J. L. (2006). Oviductal morphology in relation to hormonal levels in the snapping turtle, Chelydra serpentina. *Tissue and Cell*, *38*(1), 19–33. https://doi.org/10.1016/j.tice.2005.10.001

Avens, L., Goshe, L. R., Coggins, L., Snover, M. L., Pajuelo, M., Bjorndal, K. A., & Bolten, A. B. (2015). Age and size at maturation- and adult-stage duration for loggerhead sea turtles in the western North Atlantic. *Marine Biology*, *162*(9), 1749–1767. https://doi.org/10.1007/s00227-015-2705-x

Baldwin, R., Hughes, G.R. & Prince, R.I.T. (2003). Loggerhead turtles in the Indian Ocean. *In: Loggerhead Sea turtles*, Bolten, A. B. and Witherington, B. E.14. Smithsonian Books, Washington.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Bals, R., & Wilson, J. M. (2003). Cathelicidins - a family of multifunctional antimicrobial peptides. *Cellular and Molecular Life Sciences (CMLS)*, *60*(4), 711–720. https://doi.org/10.1007/s00018-003-2186-9

Blasi, M. F., Migliore, L., Mattei, D., Rotini, A., Thaller, M. C., & Alduina, R. (2020). Antibiotic Resistance of Gram-Negative Bacteria from Wild Captured Loggerhead Sea Turtles. *Antibiotics*, *9*(4), 162. https://doi.org/10.3390/antibiotics9040162

Benato, F., Dalla Valle, L., Skobo, T., & Alibardi, L. (2013). Biomolecular Identification of Beta-Defensin-Like Peptides from the Skin of the Soft-Shelled Turtle Apalone spinifera. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *320*(4), 210–217. https://doi.org/10.1002/jez.b.22495

Bensadoun, A., & Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. *Analytical Biochemistry*, *70*(1), 241–250. https://doi.org/10.1016/s0003-2697(76)80064-4

Bézy, V. S., Valverde, R. A., & Plante, C. J. (2015). Olive Ridley Sea Turtle Hatching Success as a Function of the Microbial Abundance in Nest Sand at Ostional, Costa Rica. *PLOS ONE*, *10*(2), e0118579. https://doi.org/10.1371/journal.pone.0118579

Bjorndal, K., Bolten, A., & Martins, H. (2000). Somatic growth model of juvenile loggerhead sea turtles Caretta caretta: duration of pelagic stage. *Marine Ecology Progress Series*, *202*, 265–272. https://doi.org/10.3354/meps202265

Bowen, B. W., Nelson, W. S., & Avise, J. C. (1993). A molecular phylogeny for marine turtles: trait mapping, rate assessment, and conservation relevance. *Proceedings of the National Academy of Sciences*, *90*(12), 5574–5577. https://doi.org/10.1073/pnas.90.12.5574

Boyle, M. C., FitzSimmons, N. N., Limpus, C. J., Kelez, S., Velez-Zuazo, X., & Waycott, M. (2009). Evidence for transoceanic migrations by loggerhead sea turtles in the southern Pacific Ocean. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1664), 1993–1999. https://doi.org/10.1098/rspb.2008.1931

Casale, P. & Dimitris, M. (2010). *Sea turtles in the Mediterranean: Distribution, Threats and Conservation Priorities*. IUCN, Cop.

Chaipayang, S., Heamatorn, N., Keha, L., Daduang, S., Songsiriritthigul, C., Swatsitang, P., Dhiravisit, A., & Thammasirirak, S. (2013). Purification and Characterization of Ovotransferrin from Crocodylus siamensis. *The Protein Journal*, *32*(2), 89–96. https://doi.org/10.1007/s10930-012-9461-2

Chattopadhyay, S., Sinha, N. K., Banerjee, S., Roy, D., Chattopadhyay, D., & Roy, S. (2006). Small cationic protein from a marine turtle has β-defensin-like fold and antibacterial and antiviral activity. *Proteins: Structure, Function, and Bioinformatics*, *64*(2), 524–531. https://doi.org/10.1002/prot.20963

Chijiiwa, Y., Kawamura, S., Torikata, T., & Araki, T. (2006). Amino Acid Sequence and Activity of Green Turtle (Chelonia mydas) Lysozyme. *The Protein Journal*, *25*(5), 336–344. https://doi.org/10.1007/s10930-006-9019-2

Ciuraszkiewicz, J., Biczycki, M., Maluta, A., Martin, S., Wątorek, W., & Olczak, M. (2007). Reptilian transferrins: Evolution of disulphide bridges and conservation of iron-binding center. *Gene*, *396*(1), 28–38. https://doi.org/10.1016/j.gene.2007.02.018

Ciuraszkiewicz, J., Olczak, M., & Watorek, W. (2007). Isolation and characterisation of crocodile and python ovotransferrins. *Acta Biochimica Polonica*, *54*(1), 175–182. https://doi.org/10.18388/abp.2007_3284

Cook, M. I., Beissinger, S. R., Toranzos, G. A., Rodriguez, R. A., & Arendt, W. J. (2003). Trans–shell infection by pathogenic microorganisms reduces the shelf life of non–incubated bird's eggs: a constraint on the onset of incubation? *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1530), 2233–2240. https://doi.org/10.1098/rspb.2003.2508

Craven, K. S., Awong-Taylor, J., Griffiths, L., Bass, C., and M. Muscarella. (2007). Identification of bacterial isolates from unhatched loggerhead (Caretta caretta) sea turtles' eggs in Georgia, USA. *Marine Turtle Newsletter*, *115*, 9-7.

Dalla Valle, L., Benato, F., Maistro, S., Quinzani, S., & Alibardi, L. (2012). Bioinformatic and molecular characterization of beta-defensins-like peptides isolated from the green lizard Anolis carolinensis. *Developmental & Comparative Immunology*, *36*(1), 222–229. https://doi.org/10.1016/j.dci.2011.05.004

Deeming, D. C; Ferguson, M. W. J. (1991). Egg incubation (Its effects on embryonic development in birds and reptiles) || Oviductal proteins and their influence on embryonic development in birds and reptiles., 10.1017/CBO9780511585739(3), 29–46. doi:10.1017/cbo9780511585739.004

Doshi, J. B., Ravetkar, S. D., Ghole, V. S., & Rehani, K. (2003). Comparative quantitation for the protein content of diphtheria and tetanus toxoids by DC protein assay and Kjeldahl method. *Biologicals*, *31*(3), 187–189. https://doi.org/10.1016/s1045-1056(03)00038-1

Encalada, S. E., Bjorndal, K. A., Bolten, A. B., Zurita, J. C., Schroeder, B., Possardt, E., Sears, C. J., & Bowen, B. W. (1998). Population structure of loggerhead turtle (Caretta caretta) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA

control region sequences. *Marine Biology*, *130*(4), 567–575. https://doi.org/10.1007/s002270050278

Ewert, M.A. (1985). In: Biology of Reptilia Vol. 14. Gans, C., Billett, F., and Maderson, P.F.A. (eds). John Wiley and Sons, New York. Pp. 76-267.

Fernandes, M., Grilo, M. L., Carneiro, C., Cunha, E., Tavares, L., Patino-Martinez, J., & Oliveira, M. (2021). Antibiotic Resistance and Virulence Profiles of Gram-Negative Bacteria Isolated from Loggerhead Sea Turtles (Caretta caretta) of the Island of Maio, Cape Verde. *Antibiotics*, *10*(7), 771. https://doi.org/10.3390/antibiotics10070771

Ganz, T. (2003). The Role of Antimicrobial Peptides in Innate Immunity. *Integrative and Comparative Biology*, *43*(2), 300–304. https://doi.org/10.1093/icb/43.2.300

Garcia, L. S., & Isenberg, H. D. (2010). Clinical microbiology procedures handbook. Asm Press.

Gayen, S. K., Som, S., Sinha, N. K., & Sen, A. (1977). Lysozyme in egg whites of tortoises and turtle. *Archives of Biochemistry and Biophysics*, *183*(2), 432–442. https://doi.org/10.1016/0003-9861(77)90378-2

Gifari, T., Elfidasar, D., & Sugoro, I. (2018). The effects of contaminant microorganism towards Chelonia mydas eggs hatchery results in Pangumbahan Green Sea Turtles Conservation, Sukabumi, Indonesia. *Biodiversitas Journal of Biological Diversity*, *19*(4), 1207–1212. https://doi.org/10.13057/biodiv/d190404

Geldiay, R., T. Koray, and S. Balik. (1982). Status of sea turtle populations (Caretta c. caretta and Chelonia m. mydas) in the northern Mediterranean Sea, Turkey. Pages 425-434 in K. Bjorndal (ed.), Biology and conservation of sea turtles. Smithsonian Institution Press, Washington, DC.

González Carman, V., Álvarez, K. C., Prosdocimi, L., Inchaurraga, M. C., Dellacasa, R. F., Faiella, A., Echenique, C., González, R., Andrejuk, J., Mianzan, H. W., Campagna, C., & Albareda, D. A. (2011). Argentinian coastal waters: A temperate habitat for three species of threatened sea turtles. *Marine Biology Research*, *7*(5), 500–508. https://doi.org/10.1080/17451000.2010.528772

Hayssen, V., & Blackburn, D. G. (1985). α-Lactalbumin and the Origins of Lactation. *Evolution*, *39*(5), 1147. https://doi.org/10.2307/2408741

Holthaus, K., Spisni, E., & Alibardi, L. (2016). Microbicide Activity of Two Reptilian Antimicrobial Peptides on Gram Positive and Gram-Negative Bacteria. *Journal of Immunobiology*, *1*(1).

Honarvar, S., Spotila, J. R., & O'Connor, M. P. (2011). Microbial community structure in sand on two olive ridley arribada nesting beaches, Playa La Flor, Nicaragua and Playa Nancite, Costa Rica. *Journal of Experimental Marine Biology and Ecology*, *409*(1-2), 339–344. https://doi.org/10.1016/j.jembe.2011.09.015

Huan, Y., Kong, Q., Mou, H., & Yi, H. (2020). Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.582779

Hughes, G.R (1982). Nesting cycles in sea turtles—typical or atypical? In K. A. Bjorndal (ed.). Biology and conservation of sea turtles. Washington, D.C.: Smithsonian Institution Press

Johnsen, E., Brandtzaeg, O. K., Vehus, T., Roberg-Larsen, H., Bogoeva, V., Ademi, O., Hildahl, J., Lundanes, E., & Wilson, S. R. (2016). A critical evaluation of Amicon Ultra centrifugal filters for separating proteins, drugs and nanoparticles in biosamples. *Journal of Pharmaceutical and Biomedical Analysis*, *120*, 106–111. https://doi.org/10.1016/j.jpba.2015.12.010

Joo, H.-S., Fu, C.-I., & Otto, M. (2016). Bacterial strategies of resistance to antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *371*(1695). https://doi.org/10.1098/rstb.2015.0292

Kamezaki, N., Y. Matsuzawa, O. Abe, H. Asakawa, T. Fujii, K. Goto, S. Hagino, M. Hayami, M. Ishii, T. Iwamoto, T. Kamata, H. Kato, J. Kodama, Y. Kondo, I. Miyawaki, K. Mizobuchi, Y. Nakamura, Y. Nakashima, H. Naruse, K. Omuta, M. Samejima, H. Suganuma, H. Takeshita, T. Tanaka, T. Toji, M. Uematsu, A. Yamamoto, T. Yamato, and I. Wakabayashi. (2003). Loggerhead turtles nesting in Japan. Pages 210-217 in Bolten, A.B. and B.E. Witherington (editors). Loggerhead Sea Turtles. Smithsonian Books, Washington D.C

Keene, Erin (2012). Microorganisms from Sand, Cloacal Fluid, and Eggs of Lepidochelys Olivacea and Standard Testing of Cloacal Fluid Antimicrobial Properties. *Master's thesis*. Indiana University - Purdue University Fort Wayne.

Kerkis, I., Hayashi, M. A. F., Prieto da Silva, A. R. B., Pereira, A., De Sá Júnior, P. L., Zaharenko, A. J., Rádis-Baptista, G., Kerkis, A., & Yamane, T. (2014). State of the Art in the Studies

on Crotamine, a Cell Penetrating Peptide from South American Rattlesnake. *BioMed Research International*, *2014*, 1–9. https://doi.org/10.1155/2014/675985

Kobayashi, S., Wada, M., Fujimoto, R., Kumazawa, Y., Arai, K., Watanabe, G., & Saito, T. (2017). The effects of nest incubation temperature on embryos and hatchlings of the loggerhead sea turtle: Implications of sex difference for survival rates during early life stages. *Journal of Experimental Marine Biology and Ecology*, *486*, 274–281. https://doi.org/10.1016/j.jembe.2016.10.020

Lakshminarayanan, R., Vivekanandan, S., Samy, R. P., Banerjee, Y., Chi-Jin, E. O., Teo, K. W., Jois, S. D. S., Kini, R. M., & Valiyaveettil, S. (2008). Structure, Self-Assembly, and Dual Role of a β-Defensin-like Peptide from the Chinese Soft-Shelled Turtle Eggshell Matrix. *Journal of the American Chemical Society*, *130*(14), 4660–4668. https://doi.org/10.1021/ja075659k

Legros, J., Jan, S., Bonnassie, S., Gautier, M., Croguennec, T., Pezennec, S., Cochet, M.-F., Nau, F., Andrews, S. C., & Baron, F. (2021). The Role of Ovotransferrin in Egg-White Antimicrobial Activity: A Review. *Foods*, *10*(4), 823. https://doi.org/10.3390/foods10040823

Lei, J., Sun, L., Huang, S., Zhu, C., Li, P., He, J., Mackey, V., Coy, D. H., & He, Q. (2019). The antimicrobial peptides and their potential clinical applications. *American Journal of Translational Research*, *11*(7), 3919–3931.

Lenarz, M. S., N. B. Frazer, M. S. Ralston and R. B. Mast. (1981). Seven nests recorded for loggerhead turtle (Caretta caretta) in one season. *Herpetological Review, 12*, 9.

Limpus, C. J., and P.C. Reed. (1985). The loggerhead turtle, Caretta caretta, in Queensland: observations on internesting behaviour. *Australian Wildlife Research, 12*, 535-540.

Limpus C.J., Limpus D.J (2003). Loggerhead turtles in the equatorial and southern Pacific Ocean: a species in decline. In: Bolten A.B., Witherington B.E., editors. *Loggerhead sea turtles.* Smithsonian Books; Washington, DC 199–209.

Limpus C.J., (2009). *A biological review of Australian marine turtles.* Queensland Environmental Protection Agency. Qld Government.

Mansfield, K. L., Wyneken, J., Porter, W. P., & Luo, J. (2014). First satellite tracks of neonate sea turtles redefine the “lost years” oceanic niche. *Proceedings of the Royal Society B: Biological Sciences*, *281*(1781), 20133039. https://doi.org/10.1098/rspb.2013.3039

Marcovaldi, M., & Chaloupka, M. (2007). Conservation status of the loggerhead sea turtle in Brazil: an encouraging outlook. *Endangered Species Research*, *3*, 133–143. https://doi.org/10.3354/esr003133

McAllister, H.J., Bass, A.J. & Van Schoor, H.J. (1965). Marine turtles on the coast of Tongaland, Natal. *The Lammergeyer* 3: 11-40

McGehee, M. Angela. (1979). "Factors Affecting the Hatching Success of Loggerhead Sea Turtle Eggs (Caretta caretta caretta)" Retrospective Theses and Dissertations. 437.

Miller, J. D., Lutz, P. L. & Musick, J. A (1997). Reproduction in sea turtles. Biol. Sea Turt. I, 51–82.

Miller, J.D., Limpus, C.J. & Godfrey, M.H. (2001). Nest site selection, oviposition, eggs, development, hatching and emergence of loggerhead turtles. *In: Biology and conservation of loggerhead sea turtle*, Bolten, A. B. and Witherington, B. E.University of Florida Press.

Mo CL, Caballero M, Salas I. 1992. Microorganism infection of olive ridley eggs. In: Richardson JI, Richarson TH compilers, editors. Proceedings of the Twelfth Annual Workshop on Sea Turtle Biology and Conservation; February 25–29. Jeckyll

Island, Georgia, NOAA Technical Memorandum NMFSSEFSC-361: 274. p. 81–84. Moncada G., F. (2001). Status and Distribution of the Loggerhead Turtle, Caretta caretta, in the Wider Caribbean Region. *In Marine Turtle Conservation in the Wider Caribbean Region - a Dialogue for Effective Regional Management*, 36–40.

Nelson, D. A. (1988). *Life history and environmental requirements of loggerhead turtles*. Fish and Wildlife Service, U.S. Department of The Interior.

Oshima, K. H., Evans-Strickfaden, T. T., Highsmith, A. K., & Ades, E. W. (1996). The Use of a Microporous Polyvinylidene Fluoride (PVDF) Membrane Filter to Separate Contaminating Viral Particles from Biologically Important Proteins. *Biologicals*, *24*(2), 137–145. https://doi.org/10.1006/biol.1996.0018

Pace, A., Rinaldi, L., Ianniello, D., Borrelli, L., Cringoli, G., Fioretti, A., Hochscheid, S., & Dipineto, L. (2019). Gastrointestinal investigation of parasites and Enterobacteriaceae in loggerhead sea turtles from Italian coasts. *BMC Veterinary Research*, *15*(1). https://doi.org/10.1186/s12917-019-2113-4

Paladino, F. V., & Robinson, N. J. (2013). *Earth Systems and Environmental Sciences* (2013th ed., pp. 1–11). El Sevier.

Pata, S., Daduang, S., Svasti, J., & Thammasirirak, S. (2007). Isolation of lysozyme like protein from crocodile leukocyte extract (Crocodylus siamensis). *KMITL Science and Technology Journal*, *7*(S1).

Pilcher, N.J., Saad, M.A. (2000). Sea turtles of Socotra. Report to the Senckenberg Research Institute, Frankfurt, Germany. 15 pages.

Phillot, A. D. (2002). Fungal colonization of sea turtle nests in Eastern Australia. PhD Dissertation. Central Queensland University.

Phillott, A. D., & Parmenter, C. J. (2006). The ultrastructure of sea turtle eggshell does not contribute to interspecies variation in fungal invasion of the egg. *Canadian Journal of Zoology*, *84*(9), 1339–1344. https://doi.org/10.1139/z06-125

Phillot, A. D., & Parmenter, C. J. (2012). Anti-fungal Properties of Sea Turtle Cloacal Mucus and Egg Albumen. *Marine Turtle Newsletter*, 134, 17–21.

Phillott, A. D., & Parmenter, C. J. (2001). The distribution of failed eggs and the appearance of fungi in artificial nests of green (Chelonia mydas) and loggerhead (Caretta caretta) sea turtles. *Australian Journal of Zoology*, *49*(6), 713. https://doi.org/10.1071/zo00051

Plotkin, P. T., & Spotila, J. R. (2002). Post-nesting migrations of loggerhead turtles Caretta caretta from Georgia, USA: Conservation Implications for a Genetically Distinct Subpopulation. *Oryx*, *36*(04).https://doi.org/10.1017/s0030605302000753

Prajanban, B., Shawsuan, L., Daduang, S., Kommanee, J., Roytrakul, S., Dhiravisit, A., & Thammasirirak, S. (2012). Identification of five reptile egg whites protein using MALDI-TOF mass spectrometry and LC/MS-MS analysis. *Journal of Proteomics*, *75*(6), 1940–1959. https://doi.org/10.1016/j.jprot.2012.01.004

Pritchard, P.C.H. and Mortimer, J.A. (1999) Taxonomy, External Morphology and Species Identification. In: Eckert, K.L., Bjorndal, K.A., Abreu-Grobois, F.A. and Donnelly, M., Eds., Research and Management Techniques for the Conservation of Sea Turtles, No. 4, IUCN/Species Survival Commission Marine Turtles Specialist Group Publication, Washington DC, 31-48.

Qiao, X., Yang, H., Gao, J., Zhang, F., Chu, P., Yang, Y., Zhang, M., Wang, Y., & Yu, H. (2019). Diversity, immunoregulatory action and structure-activity relationship of green sea turtle cathelicidins. *Developmental & Comparative Immunology*, *98*, 189–204. https://doi.org/10.1016/j.dci.2019.05.005

Rakotonirina, B., Cooke, A., (1994). Sea turtles of Madagascar - their status, exploitation and conservation. *Oryx, 28*, 51-61.

Richardson, J. I. (1980). A population model for adult female loggerhead sea turtles (Caretta caretta) nesting in Georgia. Dissertation. University of Georgia, Athens, Georgia, USA

Rodríguez-DíazR., Wehr, T., & Tuck, S. (2005). Analytical techniques for biopharmaceutical development. M. Dekker.

Ross, J. (1998). Estimations of the nesting population size of loggerhead sea turtles, Caretta caretta, Masirah Island, Sultanate of Oman. in S. Epperly and J. Braun, editors. Proceedings of the Seventeenth Annual Symposium on Sea Turtle Biology and Conservation. NOAA Techinical Memorandum NMFS-SEFSC-415.

Ruiz-de-Castañeda, R., Vela, A. I., Lobato, E., Briones, V., & Moreno, J. (2011). Bacterial Loads on Eggshells of the Pied Flycatcher: Environmental and Maternal Factors. *The Condor*, *113*(1), 200–208. https://doi.org/10.1525/cond.2011.100035

Sarmiento-Ramírez, J. M., Abella, E., Martín, M. P., Tellería, M. T., López-Jurado, L. F., Marco, A., & Diéguez-Uribeondo, J. (2010). Fusarium solani is responsible for mass mortalities in nests of loggerhead sea turtle, Caretta caretta, in Boavista, Cape Verde. *FEMS Microbiology Letters*, *312*(2), 192–200. https://doi.org/10.1111/j.1574-6968.2010.02116.x

Shi, N., Cai, S., Gao, J., Qiao, X., Yang, H., Wang, Y., & Yu, H. (2019). Roles of polymorphic cathelicidins in innate immunity of soft-shell turtle, Pelodiscus sinensis. *Developmental & Comparative Immunology*, *92*, 179–192. https://doi.org/10.1016/j.dci.2018.11.010

Shoop, C. R., & Dodd, C. K. (1989). Synopsis of the Biological Data on the Loggerhead Sea Turtle Caretta caretta (Linnaeus 1758). *Copeia*, *1989*(2), 534. https://doi.org/10.2307/1445468

Stegemann, C., Kolobov, A., Leonova, Y. F., Knappe, D., Shamova, O., Ovchinnikova, T. V., Kokryakov, V. N., & Hoffmann, R. (2009). Isolation, purification and de novo sequencing of TBD-1, the first beta-defensin from leukocytes of reptiles. *PROTEOMICS*, *9*(5), 1364–1373. https://doi.org/10.1002/pmic.200800569

Talbert, O.R., Jr., S.E. Stancyk, J.M. Dean, and J.M. Will. (1980). Nesting activity of the loggerhead turtle Caretta caretta in South Carolina I: a rookery in transition. *Copeia, 1980,* 709-718.

Thammasirira, S., Daduang, S., & Shawsuan, L. (2007). Comparative study of reptile egg white proteins by 2D-PAGE. *Current Applied Science and Technology*, *7*(2-1), 127–136.

van Hoek, M. (2014). Antimicrobial Peptides in Reptiles. *Pharmaceuticals*, *7*(6), 723–753. https://doi.org/10.3390/ph7060723

Walker, R. C., & Roberts, E. (2007). Notes on the status and incidental capture of marine turtles by the subsistence fishing communities of Southwest Madagascar. *Western Indian Ocean Journal of Marine Science*, *4*(2). https://doi.org/10.4314/wiojms.v4i2.28491

Wei, L., Gao, J., Zhang, S., Wu, S., Xie, Z., Ling, G., Kuang, Y.-Q., Yang, Y., Yu, H., & Wang, Y. (2015). Identification and Characterization of the First Cathelicidin from Sea Snakes with Potent Antimicrobial and Anti-inflammatory Activity and Special Mechanism. *The Journal of Biological Chemistry*, *290*(27), 16633–16652. https://doi.org/10.1074/jbc.M115.642645

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*(2), 163–175. https://doi.org/10.1038/nprot.2007.521

Williams, K. L., & Frick, M. G. (2008). Tag Returns from Loggerhead Turtles from Wassaw Island, GA. *Southeastern Naturalist*, *7*(1), 165–172. https://doi.org/10.1656/1528-7092(2008)7[165:trfltf]2.0.co;2

Wyneken, J., Burke, T. J., Salmon, M., & Pedersen, D. K. (1988). Egg Failure in Natural and Relocated Sea Turtle Nests. *Journal of Herpetology*, *22*(1), 88. https://doi.org/10.2307/1564360

Wyneken, J., Madrak, S. V., Salmon, M., & Foote, J. (2008). Migratory activity by hatchling loggerhead sea turtles (Caretta caretta L.): evidence for divergence between nesting groups. *Marine Biology*, *156*(2), 171–178. https://doi.org/10.1007/s00227-008-1074-0

Zhang, L., & Gallo, R. L. (2016). Antimicrobial peptides. *Current Biology*, *26*(1), R14–R19. https://doi.org/10.1016/j.cub.2015.11.017

Zhang, Q.-Y., Yan, Z.-B., Meng, Y.-M., Hong, X.-Y., Shao, G., Ma, J.-J., Cheng, X.-R., Liu, J., Kang, J., & Fu, C.-Y. (2021). Antimicrobial peptides: mechanism of action, activity and

clinical potential. *Military Medical Research*, *8*, 48. https://doi.org/10.1186/s40779-021-00343-2

Zharkova, M. S., Orlov, D. S., Golubeva, O. Yu., Chakchir, O. B., Eliseev, I. E., Grinchuk, T. M., & Shamova, O. V. (2019). Application of Antimicrobial Peptides of the Innate Immune System in Combination With Conventional Antibiotics—A Novel Way to Combat Antibiotic Resistance? *Frontiers in Cellular and Infection Microbiology*, *9*. https://doi.org/10.3389/fcimb.2019.00128

Zieger, U., Trelease, H., Winkler, N., Mathew, V., & Sharma, R. (2009). Bacterial Contamination of Leatherback Turtle (Dermochelys coriacea) eggs and sand in nesting chambers at Levera Beach, Grenada, West Indies -a preliminary Study. *West Indian Veterinary Journal*, *9*(2).