Gut bacterial communities in healthy and compromised green turtles (*Chelonia mydas*) and an alternative treatment for gastrointestinal disorders

Thesis submitted by
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Work related to this thesis was completed under the supervision of A/Prof Ellen Ariel, A/Prof Leigh Owens, Dr Jackie Picard, Dr Robert Kinobe and Dr Lisa Elliott. Thanks to James Cook University and the College of Public Health, Medical and Veterinary Sciences for providing the infrastructure to complete this thesis.

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Human (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review (Approval number A2101)

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ABSTRACT

Green turtles (*Chelonia mydas*) are endangered marine herbivores that digest food, primarily sea grasses through microbial fermentation. The gut microbiota and its role in health and disease are largely unexplored although microbial dysbiosis is believed to be associated with the pathogenesis of several intestinal and extra-intestinal disorders. Gut microbial dysbiosis primarily refers to the microbial alteration and or imbalance within the gastrointestinal tract. Debilitated sea turtles are often treated and nursed to health in turtle hospitals and antibiotic therapy may be one of the contributing factors of microbial dysbiosis together with other collateral damages.

The objective of this project was to understand gastrointestinal (GI) diseases of green turtles in rehabilitation by identifying normal and potentially pathogenic microbes, their antimicrobial resistance, and establishing the use of bacteriophages as an alternative to antibiotics. Additionally, the impact of the broad-spectrum antibiotic, enrofloxacin and bacteriophage therapy on the gut bacterial flora of green turtles were investigated.

In this study, both culture dependent and independent techniques were employed to identify the gut bacteria in green turtles. Cloacal swabs were taken from a total of 73 green turtles between 2015 and 2016 for culture dependent identification of Enterobacterales. A total of 16 different bacterial species that represented nine different genera were identified. The predominant isolates were *Citrobacter*, *Edwardsiella*, *Escherichia* and *Klebsiella*.

Antimicrobial resistance against 12 different antibiotics from six different classes was evaluated. The bacterial isolates showed highest resistance to β-lactam antibiotics followed by quinolone and tetracycline classes. Approximately one-third of the isolates identified exhibited multidrug-resistance.

The high-throughput sequencing targeting the V1-V3 regions of the bacterial 16S rRNA gene identified a total of 19 bacterial phyla from a total of 12 samples. The faecal bacterial community of green turtles was largely dominated by Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Firmicutes predominated among wild-captured green turtles while Proteobacteria prevailed in stranded turtles. The predominance of the genus *Bacteroides* in all groups indicates the importance of these bacteria in turtle gut health. Wild-captured green turtles showed the highest microbial diversity and richness compared to

stranded green turtles.

This study also investigated and compared the faecal bacterial communities between pre-hospitalisation (PH) and post-rehabilitation (PR) stranded green turtles. Bacteria within the phylum Proteobacteria dominated in both PH and PR samples without any significant difference. The significant abundance of *Campylobacter fetus*, *Escherichia coli, Clostridium botulinum* and *Vibrio parahaemolyticus* in PH samples indicates pathogenic associations of zoonotic potential within stranded turtles. In this study, all post-rehabilitation green turtles exhibited similar bacterial communities irrespective of their microbial compositions at pre-hospitalisation. The marked differences in the gut microbiota of PH and PR turtles indicate the outcome of dietary, management and environmental shift during rehabilitation.

The mucosa-associated bacterial communities across the GI tract of green turtles were investigated. Bacterial diversity and richness decreased longitudinally along the GI tract from oesophagus to the small intestine while the large intestine showed a higher bacterial diversity and richness compared to the small intestine. The GI tract mucosa-associated microbial community of green turtles was largely dominated by Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria. However, the detailed composition of these phyla was notably distinct for different GI regions.

This study provides a proof-of-concept for the application of bacteriophage (phages) to eliminate targeted bacteria as an alternative to antibiotics. Bacteria-specific phage cocktails were found to significantly reduce the targeted *Acinetobacter* in phage-treated turtles during the therapy. Compared to control turtles, no significant difference was observed in the gut bacterial diversity and compositions in the phage-treated turtles. In contrast, bacterial diversity was significantly reduced in antibiotic-treated turtles during the therapy at day 15 and throughout the trial. The alteration in the bacterial gut communities of antibiotic-treated turtles was largely due to an increase in abundance of Gram-positive Firmicutes and a concurrent decrease in abundance of Gram-negative Bacteroidetes, Proteobacteria and Verrucomicrobia. Additionally, the relative abundance of several bacteria at lower taxonomic levels was much less affected by phages than by antibiotic enrofloxacin.

In conclusion, this is the first detailed characterisation of gut bacterial communities of green turtles in the context of their different health and environmental conditions. The findings offer a helpful reference for further investigations of sea turtle gut microbiome and their metabolic functions to improve their health and nutrition during rehabilitation. The phage

treatment described here provides a targeted alternative to antibiotics, with the possibility to manipulate transient as well as indigenous bacterial flora with a broad application in many gut-related dysbiosis of turtles.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PUBLICATIONS

PUBLISHED

Ahasan, M. S., Picard, J., Elliott, L., Kinobe, R., Owens, L., Ariel, E. (2017). Evidence of antibiotic resistance in Enterobacteriales isolated from green sea turtles, *Chelonia mydas* on the Great Barrier Reef. Marine Pollution Bulletin 120: 18-27.

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LIST OF ABBREVIATIONS

ACE	:	Abundance-based coverage estimator
ANOSIM	:	Analysis of similarity
ANOVA	:	Analysis of variance
BA	:	Blood agar
BGF	:	Bacterial gut flora
BWC	:	Bowen wild-captured
bp	:	Base pair
BLAST	:	Basic local alignment search tool
CCL	:	Curved carapace length
CFU	:	Colony forming unit
cm	:	Centimetre
CLSI	:	Clinical Laboratory Standards Institute
CSS	:	Cumulative sum-scaling
CUP	:	Conditional uncovered probability
DEHP	:	Department of environment and heritage protection
DGGE	:	Gradient gel electrophoresis
DNA	:	Deoxyribonucleic acid
EUCAST	:	European national breakpoint committees
FISH	:	Fluorescent in situ hybridization
FDR	:	False discovery rate
GI tract	:	Gastrointestinal tract
GBR	:	Great Barrier Reef
GBRMPA	:	Great Barrier Reef Marine Parks Authority
Kg	:	kilogram
hr	:	Hour
H&E	:	Haematoxylin and Eosin
H_2S	:	hydrogen sulfide
IC	:	Intra-coelomic
l	:	litre
LI	:	Large intestine
m	:	Metre

MC	:	MacConkey agar
mg	:	Milligram
ml	:	Millilitre
min	:	Minute
MIC	:	Minimum inhibitory concentration
NaCl	:	Sodium chloride
NBGF	:	Normal bacterial gut flora
NCBI	:	National center for biotechnology information
NMDS	:	Non-metric multidimensional scaling
OTU	:	Operational taxonomic unit
Lat.	:	Latitude
Long.	:	Longitude
lda	:	Latent Dirichlet Allocation
PAS	:	Periodic acid Schiff
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PCoA	:	Principle coordinate analysis
PFU	:	Plaque forming unit
PH	:	Pre-hospitalisation
pH	:	Potential of hydrogen
ppt	:	Part per trillion
PR	:	Post-rehabilitation
QIIME	:	The quantitative insights into microbial ecology
QC	:	Quality control
rRNA	:	Ribosomal ribonucleic acid
TGGE	:	Temperature gradient gel electrophoresis
TTGE	:	Temporal temperature gradient gel electrophoresis
T-RFLP	:	Terminal- restriction fragment length polymorphism
TSA	:	Tryptone Soy Agar
TSB	:	Tryptone Soy Broth
TWC	:	Townsville wild-captured
SSCP	:	Single strand conformation polymorphism
Sec	:	Second

SI	:	Small intestine
ST	:	Stranded turtles
UV	:	Ultraviolet
WHO	:	World Health Organisation
WC	:	Wild captured
ZN	:	Ziehl-Neelsen
β	:	Beta
%	:	Percentage
µl	:	Microliter
^{0}C	:	Degree Celsius

CHAPTER 1
GENERAL INTRODUCTION

In both vertebrates and invertebrates, the gastrointestinal (GI) tract harbours a diverse array of microorganisms that play vital roles in maintaining host health (Backhed et al., 2005; Stecher and Hardt, 2008; Costa et al., 2012). The GI microbiota are well-known contributors to maintaining host physiology, host immune homeostasis and several metabolic activities such as energy harvest, fat metabolism and production of short-chain fatty acids (Dethlefsen et al., 2007; Chung and Kasper, 2010). Gut microbiota also prevents the colonisation of pathogenic organisms through a natural barrier referred to as 'colonisation resistance' (Van der Waaij et al., 1971; Adlerberth, 2000; Buffie and Pamer, 2013). Several studies on gut microbiota have confirmed that a balanced gut microbiome is essential for the host's ability to maintain a healthy state. Perturbations in the stability of gut microbial communities dispose the host to pathogenic invasions which may lead to several GI diseases and disorders (MacFarlane and Macfarlane, 2009; Sobhani et al., 2011; Vaarala, 2011; Sartor and Mazmanian, 2012).

The green turtle (*Chelonia mydas*) is one of the largest species of sea turtles and has been listed as endangered globally on the Red List of the International Union for the Conservation of Nature (IUCN) since 1982 (Groombridge and Baillie, 1996; Read et al., 2014). They are primarily herbivorous hind gut fermenters and forage mainly on sea grasses (Bjorndal et al., 1997). The gut bacteria of green turtles are believed to play a crucial role in microbial food degradation and gaining energy from the food sources (Bjorndal, 1979b; Karen et al., 1991). They can also contribute to several other aspects of the health and development of disease, as noted in other animals (Guarner and Malagelada, 2003; Costa et al., 2012; Mao et al., 2015). Previous investigations of the green turtle's gut microbiome have typically involved bacterial identification in faeces by culture-dependent techniques (Aguirre et al., 1994; Santoro et al., 2006a; Al-Bahry et al., 2009; Al-Bahry et al., 2011) that allow only for assessment of a small proportion of bacterial communities as a large proportion of the bacterial communities remains un-cultivable and hence unknown (Daly et al., 2001; Eckburg et al., 2005). Culture-independent molecular approaches are therefore highly suitable for microbial diversity

study of the resident and transient bacterial communities along the GI tract of green turtles (Hugenholtz et al., 1998; Handelsman, 2004). Knowledge of the detailed bacterial gut communities of green turtles and the population dynamics in symbiosis, as well as in dysbiosis can allow the development of strategies to treat gut-associated disorders and restore the host's normal gut microbiome during rehabilitation.

Debilitated green turtles are accommodated in rehabilitation centers and nursed back to health. Broad spectrum antibiotics are often used as part of the general treatment in hospitals and may lead to collateral damages in turtles, as reported in other animals including humans. These include impairment of the indigenous host-associated microbial communities (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011), and alteration of gut metabolism, gene expression and protein activity (Pérez-Cobas et al., 2012; Modi et al., 2014). Although previous investigations have reported the presence of multiple antibiotic resistant bacteria in the GI tract of sea turtles (Foti et al., 2009; Al-Bahry et al., 2011), little information is available on the impacts of broad spectrum antibiotics on green turtle's health. This dissertation therefore aims to address these issues to explore GI disorders of green turtles in rehabilitation by identifying the gut microbiota of healthy and compromised green turtles using both conventional and molecular techniques, determining the impacts of antibiotics on green turtle's gut microbiome and finally, investigating a suitable alternative to antibiotics to treat gut-associated diseases in green turtles.

CHAPTER 2
REVIEW OF LITERATURE

2.1. Background

Microbes evolved over 3.48 billion years, living in a range of environmental conditions and influencing all ecosystems on earth (Noffke et al., 2013). The estimated numbers of bacteria (10^{30}) on the biosphere are 10^9 times higher than the number of stars in the cosmos (Whitman et al., 1998) and scientists believe that 99% of this vast microbial world still remains unexplored. The aquatic ecosystem comprises the largest biome (90%) on earth and it provides a wide range of habitats for different microorganisms including bacteria. These can found in water and sediments or externally and internally of other organisms including turtles (Belkin and Colwell, 2005). In the terrestrial environment, microbes are also highly interactive within and outside their communities, possessing a strong adaptive ability in response to any environmental change. The interactions among microorganisms, constituting synergism or competition, regulate the microbial diversity of an ecosystem (Amin et al., 2012). The success or failure of any bacterial pathogen depends on these interactions. In aquatic and terrestrial ecosystems, the bacterial concentration may reach up to a level of 10^6 cells per millilitre of sea water, 10^8 in marine sediment and 10^9 in soil (Whitman et al., 1998; Schloss and Handelsman, 2006). One bacterial species can colonise different host species resulting in mutualism, commensalism and or parasitism (Estes et al., 2011). In the nineteen century, Anton de Bary described a new type of relationship between the host and the bacteria called “symbiosis” which refers to specific cases where both the host and the microorganism benefit from the association (Walter et al., 2011). Using this term is quite appropriate in the context of gastro-intestinal bacteria in the turtles and tortoises, where there is a lack of valid scientific records on which bacterial species comprises the pathogenic and the mutualistic components within this community.

However, with the advancement of modern technology like that of nucleotide sequencing, there is a dramatic increase in the identification of new species, genera and higher taxa since the 1990s. Moreover, comparative studies on microbial communities reveal the importance of different environmental factors including temperature,

pressure, salinity, hydrogen ion concentration, radiation or biogeography on microbial diversity (Lozupone and Knight, 2007; Desnues et al., 2008; Sullam et al., 2012). The gastro-intestinal systems of all vertebrates, including humans, represent a highly diverse microbial community which is described as "worlds within worlds". For example, human gut bacterial flora is comprised of around 500-1000 species of bacteria belonging from only few described bacterial phyla predominantly Firmicutes and Bacteriodetes (Sommer and Backhed, 2013). The evolution of these gut microbes are equally thought to be true for all other vertebrates including turtles and tortoises.

Turtles and tortoises have existed on earth for around 300 million years, long before the age of dinosaurs. These turtles are now the most endangered group among all major groups of vertebrates (Fund, 2002). There are approximately 335 species of turtles and tortoises of which 8 species have already gone extinct. According to the Red list of International Union for the Conservation of Nature (IUCN) 2013, about 40.3% of all species of turtles are declared as globally "Threatened (Vulnerable, Endangered and Critically endangered)" (Van Dijk et al., 2014). Turtles have evolved with a special body armour that has facilitated their survival over hundreds of years. However, delayed sexual maturity, high juvenile mortality and long life span have increased the vulnerability of turtle species to various threats including human exploitation, climate change and certain diseases (Buhlmann et al., 2009). In regards to marine species, 6 out of 7 are threatened and a sharp fall of turtle populations has been observed over the decades (Van Dijk et al., 2014). Sea turtles have been proposed as the sentinels of marine health and an index of marine pollution (Aguirre and Lutz, 2004); deserving considerable attention. Like other vertebrates, turtles also share the same microbial communities in aquatic and terrestrial environment. They also harbour numerous bacterial floras within their gut. Therefore, this review will attempt to address some rising questions regarding the bacterial communities of the GI tract namely, their diversity and identification; how intricately are they associated with their hosts; what are their common roles; and how distinct are they from the gut bacterial microflora of other vertebrates in similar aquatic environments.

2.2. Green sea turtle at a glance

The green sea turtle (*Chelonia mydas*) is one of the largest species among all sea turtles, distributed circum-globally in the tropical and subtropical oceans (Read et al., 2014). It

is classified within the Cheloniidae which contains an identical hard-shelled carapace. This species is considered to be the closest living relative of the leatherback turtle in the Dermochelyidae although unlike the green sea turtle it lacks keratinised scutes (Gaffney and Meylan, 1988). However, the fossil record suggests that the *Dermochelys* and cheloniid lineages separated over 110 million years ago in the early Cretaceous period (Hirayama, 1997). Green turtles can be distinguished from other hard-shelled turtles by the presence of a single of pair large prefrontal scales in between their eyes, five central scutes flanked by four pairs of lateral scutes and a strongly serrated lower jaw (Witherington et al., 2006). It is a long-lived late-maturing species and catalogued as globally endangered since 1982.

Although the numbers of green turtle populations are largely depleted, their geographic distribution is worldwide with an exception of the Arctic and Antarctic waters (Hirth, 1997). Their highly migratory nature allows them to utilise broadly separated, dispersed, shallow foraging areas and local nesting beaches which represent their regional distribution pattern. Both males and females are involved in the migratory phase that often exceed thousands of kilometres every few years (Witherington et al., 2006). Adult females migrate from their foraging areas to their nesting beach. The nesting predominantly takes place at night in an open sandy beach of mainland shores, barrier islands and atolls. In an undisturbed condition, approximately two hours are required for completing their full nesting process. Approximately 40% of this time is needed for nest preparation, 13% of time for laying eggs and 47% of time for covering and camouflaging the nesting site (Hirth and Samson, 1987). Hatchlings that leave the nests move into convergence zones in the open oceans and it is most likely that post-hatchlings and early juveniles forage at or near the ocean surface for an undetermined period (Carr, 1986). The juveniles leave the pelagic habitat and move into the benthic feeding zone when they achieve a carapace length of 20-25 cm. The young green sea turtles can also be seen in coral reefs, worm reefs, rocky bottoms and/or soft mud in order to familiarise themselves with the foraging areas and to rest themselves without drifting (Witherington et al., 2006). However, with increasing age, immature and adult turtles move into a long-range depending on the availability and type of diet they are feeding on; herbivorous (seagrass) or omnivorous (Godley et al., 2003b).

During the pelagic phase, the post-hatchlings are believed to be omnivorous; feeding at surface waters on both plant and planktonic organisms (Bolten, 2003). Later, when the

juveniles (with a carapace length 20 to 35 cm) move into the neritic habitats like coastal areas and coral reefs, they make a dietary shift to herbivory; feeding principally on seagrasses and algae (Godley et al., 2003a). From this stage to adulthood, all immature and adult turtles feed primarily on seagrasses and macroalgae. Although this feeding habit may vary on the availability of the herbivorous diets, their broad migratory nature allows them to seek food in different oceanic regions (Bugoni et al., 2003; FM et al., 2011). The highly migratory nature of green turtles also exposes them to a diverse range of microbial communities in different marine environments. This can influence the microbial structure of the turtle.

2.3. Threats of green turtles

Several factors drive green turtles to their endangered condition (Donlan et al., 2010). These include natural anthropogenic factors such as climate change, non-human predators, erosion of their nesting habitats, coastal run off etc. (Casale, 2010). However, natural threats are not only the reasons for declining green turtle populations. The biggest threats to turtles by far are human origin such as habitat loss due to development, artificial lighting in their natural habitat, oil spills, poaching and illegal trade, turtle bycatch, and entanglement in marine debris such as plastic bags or fishing nets (Bjorndal, 1995; Casale, 2010; Donlan et al., 2010). Disease is also believed to be another important factor of declining sea turtle population (Flint et al., 2010; Fichi et al., 2016). Green turtles are occasionally recovered as stranded in the shore line or in surrounding shallow waters, either living or dead, exhibits indication of ill health or abnormal behaviour (Hart et al., 2006). Live-stranded animals often need professional assistance or medical attention for their early recovery during rehabilitation and release back to their natural habitat. Knowledge of the microbial community structure of sea turtles will be of importance to provide an appropriate treatment during rehabilitation.

2.4. Interactions between bacteria and turtles

Bacteria can colonise different regions of the turtle including the gut, respiratory tract, plastron or carapace, with the gastrointestinal tract providing a favourable surface for bacterial colonisation (Wyneken et al., 2013). Anatomical studies have reported that the hind gut of the green sea turtle is approximately double the length and volume in comparison to the fore gut (Thompson, 1980). Therefore, the hind gut is able to

accommodate voluminous cellulose diets for a period and where the resident luminal microorganisms ferment the cellulose into volatile fatty acids (Bjorndal, 1979a). However, microbial fermentative enzymes require favourable gut environment to function optimally. The hind gut shows a number of unique environmental features which make it different from other parts of the gastrointestinal system. For example, the essentially isothermal condition of the hindgut that is driven by the host's heterothermic metabolism. A desirable osmotic pressure is always below the isotonic level and a favourable anaerobic or strictly anaerobic condition for anaerobic activities (Savage, 1977). Moreover, a fairly acidic state (average 5.4, range 4-7) was reported in the caecum of a green sea turtle which tends to be more acidic and then subsequently neutral towards the cloaca of the animal. A highly acidic (3.6, range 2-5) and slightly acidic (6.7) pH was revealed subsequently in the stomach and small intestine of the green sea turtle (Thompson, 1980). These different states along the gastrointestinal tract of the host clearly indicate the possible existence of a variety of gut bacterial inhabitants in turtles which include aerobes and predominantly anaerobes. Some bacteria appear to be transient in the gut while others are found as resident flora (Kim et al., 2007). These resident or autochthonous components are often attached to the mucosal wall (Ringo et al., 2001). For intracellular pathogens, adherence is often a prerequisite for bacterial invasion in host cells while in regard to extracellular pathogens, adherence facilitates bacteria to withstand the mechanical clearing mechanism of the host and allows the entry of bacterial products such as toxins into intestinal cells (Boyle and Finlay, 2003). Basically, all gut bacterial communities are believed to be regulated by the host itself and its' environment including diets. Changes in the composition of gut bacterial structure can have detrimental or beneficial effects on growth, maturation and predominantly health of the host which can only be explained in presence of a complete scenario and understanding of gut bacterial communities. Several studies exist on the host-microbial interaction of marine vertebrates and invertebrates (Huchzermeyer et al., 2000; Olafsen, 2001; Horowitz and Horowitz, 2002; Hong et al., 2011; Rungrassamee et al., 2014), however, only limited information exists on the bacterial interaction and microbiology of marine turtles (Belkin and Colwell, 2005; Gibbons and Steffes, 2013; Sarmiento-Ramirez et al., 2014).

2.5. Normal bacterial gut flora (NBGF)

2.5.1. Role of bacterial gut flora

The gastrointestinal system facilitates digestion of food in both humans and animals. This system harbours a diverse range of microbiota, including Archaea, Eukarya, and predominantly Bacteria (Turroni et al., 2008). These gut bacteria can greatly influence the host's gut morphology, biochemistry, physiology, nutrition and non-specific resistance to infection (Hooper et al., 2002; Rawls et al., 2004; Austin, 2006; Li et al., 2008). In herbivorous animals, gut bacteria are believed to be the main component responsible for digestion of complex carbohydrates available from the ingested plant materials (Peter, 1994). These indigenous bacteria ferment the dietary and endogenous carbohydrates into simple short chain fatty acids used for energy production and nutrition (Stevens and Hume, 1998). For example, gut cellulolytic bacteria secrete extracellular cellulase and other enzymes that facilitate the degradation of cellulose and hemicellulose to oligosaccharides. The final products are absorbable glucose, fructose and triosephosphates. However, in birds, these indigenous bacteria are primarily liable for degrading copious amount of mucous secreted by the goblet cells of the intestinal epithelium (Falk et al., 1998).

Several studies have confirmed that the intestinal bacterial flora is involved in a dynamic interaction with the intestinal innate and adaptive immune response (Mead, 2000; Purchiaroni et al., 2013). These bacteria protect the intestines from pathogenic bacterial colonisation through stimulation of the immune response, by competitive exclusion and the production of bacteriocins and toxin neutralisers (Mead, 2000; Purchiaroni et al., 2013). For example, the intestine of germ-free mice showed a low level of secretory IgA followed by marked increase upon intestinal colonisation of the commensal organism, *Bacteroides thetaiotamicron* (Ringo and Birkbeck, 1999).

A comparative study on conventional and germ-free animals (e.g. mice) verified that luminal bacteria influence the development of ultra-structure in the intestinal mucosa at their early life stage (Srikanth and McCormick, 2008). Likewise, gut bacteria in zebra fish have been found to play a vital role in developing their digestive tract at the larval stage (Rawls et al., 2004; Bates et al., 2006). Considering the above functions of gut bacterial flora, Guarner and Malagelada (Guarner and Malagelada, 2003) has

categorized the function for all mammals, including humans into three forms: tropic, metabolic and protective to the host. Tropics effect the gastrointestinal epithelium through the control of epithelial cell proliferation and immune function, metabolic activities result in the salvage of energy as short chain fatty acid and absorbable nutrients, and importantly protectives prevent invasion by exogenous pathogens of the host.

Numerous studies have been undertaken in different animals to determine the factors that influence gut bacterial composition and colonisation. In marine and other aquatic animals, gut bacterial composition and colonisation are greatly influenced by a number of host and environmental determinants including physicochemical aspects of the gut (Huq et al., 1986; Harris et al., 1991), diet (Sochard et al., 1979; Campbell and Buswell, 1983), seasonal and environmental influences (Straub and Dixon, 1993) and even the type of habitats the host inhabits (Harris, 1993). These conditions may lead to the establishment of resident populations of gut flora, which then represent the natural or 'normal' flora of the host animal (Lynch and Hobbie, 1988; van der Waiij, 1992).

2.5.2. Factors influencing the composition of normal bacterial gut flora

The population levels and compositions of gut bacterial communities vary along the gastrointestinal tract of animals which are commonly regulated by multifactorial processes. Some regulatory factors of these processes are exerted by the host itself and some are by the microbes themselves. Environmental factors also have a vital impact on the gut bacterial populations which include diet composition, bacteria from the environment, temperature, antibiotic administration and infection with pathogenic bacteria (Savage, 1977; Ringo and Birkbeck, 1999; Sullam et al., 2012).

2.5.2.1. Host induced factors

Several researchers described bacterial diversities and variations in different life stages of aquatic and terrestrial vertebrates and invertebrates (Lu et al., 2003; Rungrassamee et al., 2013) including humans (O'Toole and Claesson, 2010; Tiihonen et al., 2010). Human bacterial flora are commonly established at birth, predominantly with facultative anaerobes such as *Bifidobacterium*, *Clostridium* and *Bacteroides* (Fanaro et al., 2003). Later, with the introduction of solid food, the bacterial compositions change into a complex and more stable community that continues throughout adulthood. However,

this relative stability is reduced in old age (Power et al., 2014). The age-dependent variations in gut bacterial communities have also been shown in black tiger shrimp (Rungrassamee et al., 2013) and in birds (Lu et al., 2003). Gut bacterial diversity, specifically in a part of the gastrointestinal tract, is also influenced by the forces generated by the host. For example, the peristaltic movement, pH, oxygen availability, oxidation-reduction potential, epithelial turn-over, availability of different vitamins and enzymes influence which type of bacteria can colonise in which part of the gastrointestinal tract (Savage, 1977). Many experimental attempts have also demonstrated the influence of host genetic factors on gut bacterial communities as a result of their historical co-evolution (Rawls et al., 2006; Kovacs et al., 2011). A huge variation in gut microbial communities has been reported in identical twins by Turnbaugh et al. (2009).

2.5.2.2. Microbe induced factors

The bacteria in established microbial communities undoubtedly have an essential role in maintaining their stability inside the microbial communities. For example: some enterobacters produce bacterocins, proteinaceous toxins, which can restrict the invasion of allochthonous (non-indigenous) microbes in the habitat occupied by autochthonous microbes (Tannock and Smith, 1970). Likewise, antimicrobials such as volatile fatty acid and other toxic metabolic end-products produced by strict anaerobes, can also limit the colonisation of gut microbial ecosystem by allochthonous bacteria (Freter and Abrams, 1972). These volatile fatty acids play a vital role in autogenic succession of the gut microbiota from a predominant facultative to strictly anaerobic population at the juvenile stage (Lee and Gemmell, 1972). The anaerobes that are able to produce hydrogen sulphite (H_2S), can repress the population levels of facultative aerobic bacteria including *Escherichia coli* (Savage, 1977). A study also revealed that, the gut bacteria not only influence the gut bacterial compositions directly but also indirectly through altering physiological and immunological responses of the host. For example, gut bacteria deconjugate the bile acids and stimulate the immune response of the host (Drasar and Hill, 1974). They are able to colonise in all areas of the gastrointestinal tract through the stimulation of peristaltic movements. These gastrointestinal bacteria influence several regulatory processes of their host (Savage, 1977). However, little is known about the impact of such factors on gut microbial colonisation, succession and overall compositions of the intestinal microbes in turtles and tortoises.

2.5.2.3. Environmentally induced factors

Certain influences in the environment of the host are able to alter the gut bacterial communities throughout their life. Diet is undoubtedly one of these factors, facilitating nutrients for both the host and bacteria in the intestinal tract (Moschen et al., 2012). The composition of the diets or changes in this composition may influence the intestinal bacterial communities. An approach to define the influences of certain diets in different food habits of people, Zimmer et al. (2012) examined the faecal samples of vegetarians and ordinary omnivorous people where he concluded a significant microbial diversity in between the two groups of people. The above findings were also supported by Muegge et al. (2011) who studied 33 different mammals where the herbivore's microbes were specialised to synthesise amino acids and the carnivore's communities had specialised to degrade protein. However, other environmental factors apart from diet can also shape the gut bacterial communities including hydrogen ion concentration in the oceanic environment (Fierer and Jackson, 2006), temperature (Munro et al., 1994), salinity (Lozupone and Knight, 2007), seasonality (Gilbert et al., 2012) and different ecological interactions (Steele et al., 2011). The bacteria from the marine environment and other eukaryotic organisms can also influence the gut microbial populations and compositions (Sullam et al., 2012). Moreover, certain diseases like gastroenteritis or inflammatory bowel disease owing to either dietary shift or invasion of pathogenic microorganisms can drastically alter the gut microbial compositions (Power et al., 2014). Because of the lack of a complete list of gut microbiota of a healthy intestinal tract, it is difficult to define the specific microbiota responsible for gastrointestinal disorders. Therefore, to overcome these situations, use of indiscriminate antibiotics to control the pathogenic bacteria has also been found to change the entire gut microbiology (Power et al., 2014). Antunes et al. (2011) reported that antibiotic therapy in mice has been shown to abruptly change the luminal metabolome through affecting numerous metabolic pathways of host including carbohydrate, nucleotide and fatty acid metabolisms. In turtles, broad spectrum antibiotics are commonly prescribed in rehabilitation centres to treat different infectious diseases which are thought to be responsible for destroying or altering the gut microbial structure. However, although a variety of factors have been found to influence the composition of intestinal bacterial communities in different vertebrates, limited information is available for aquatic as well as terrestrial tortoises that explore the complex relationship among the host-microbes with their environment.

2.6. Bacterial gut flora (BGF) in gut fermenters

Depending on the anatomical and physiological variation of gastro-intestinal tracts in the host animal, different portions of the tract are adapted to accommodate fermenting microorganisms at various levels. Normally, vertebrate animals are primarily classified into two major groups: foregut and hindgut fermenters (Godoy-Vitorino et al., 2012). In foregut fermenters, the major site of digesta retention, and subsequent microbial digestion, is the fore-stomach such as the rumen or crop. In hindgut fermenters, it is caudal to the stomach (e.g. colon or caecum). Although there is a secondary site of microbial fermentation in the hindgut of foregut fermenters (proximal colon and or caecum), it makes only a minor contribution in terms of energy production (Hume, 2002).

The hindgut fermenters occur in a wide range of aquatic and terrestrial environments and harbour different suites of microbes. Aquatic and amphibian herbivores, are hind gut fermenters and have an enlarged compartment to facilitate fermentation in the caecum and or colon (Stevens and Hume, 1998). This fermentation provides soluble carbohydrate (glycogen) to the host animals. The hindgut fermenters are more efficient in extracting nutrients in comparison with foregut fermenters (Budiansky, 1997).

2.6.1. Aquatic hindgut fermenters

Several authors have documented a wide range of bacterial flora in aquatic hindgut fermenters, including both vertebrates and invertebrates. A study by Rungrassamee et al. (2014) on wild caught black tiger shrimp (*Penaeus monodon*) from the oceanic environment showed clear evidence of the presence of five phyla, including Proteobacteria (*Photobacterium*, *Vibrio*, *Novosphingobium*, *Undibacterium*, *Pseudomonas* and *Sphingomonas*), Actinobacteria, Bacteroidetes (*Cloacibacterium*), Fusobacteria and Firmicutes. These findings concur with those of Chaiyapechara et al. (2012) and Lau et al. (2002) whose studies into the hindgut of black tiger shrimp in wild and farming conditions revealed similar bacterial flora in addition to another seven phyla: Tenericutes, Deinococcus-Thermus, Planctomycetes, Spirochaetes, Synergistetes, Thermotogae, and Verrucomicrobia. A number of studies were also carried out to investigate the intestinal microbial diversity in other crustacean including Norway Lobster (*Nephrops norvegicus*) where the author recorded only two bacteria

phyla Proteobacteria and Tenericutes (Meziti et al., 2012). However, in the mud crab (*Scylla paramamosain*), and copepod (*Eudiaptomus gracilis*), the authors reported the similar findings, with only some variation of intestinal microbes phyla (Li et al., 2007; Homonnay et al., 2012; Li et al., 2012) (Table 2.1)

Moreover, studies on marine herbivorous fishes including parrotfish (*Chlorurus sordidus*) and whitecheek surgeonfish (*Acanthurus nigricans*) exhibited similar phyla where parrotfish had predominantly Proteobacteria and surgeonfish, Bacteroidetes (Sullam et al., 2012) (Table 2.1).

Marine iguanas are herbivorous reptiles that depend on complex gut microbial fermentation to degrade and effectively generate dietary polysaccharides. To explore the microbial communities within the hindgut, a number of studies on the microbial communities within the hindgut of iguanas reported that Firmicutes and Bacteroidetes (at phylum level) dominated (Table 2.1). More specifically, marine iguanas were found to mainly host *Bacteroides* spp., Lachnospiraceae and Clostridiaceae in their gastrointestinal tract, while the land iguanas principally harboured Ruminococcaceae (Hong et al., 2011).

The green sea turtle, another marine reptile that is herbivorous in nature, exhibits hind gut fermentation. Few studies have been carried out with reference to gut microbial communities in green sea turtles. The authors reported only few bacterial genera belonging to the phyla Proteobacteria and Firmicutes (Santoro et al., 2006a; Al-Bahry et al., 2011) whereas the other hind gut fermenters that share the same marine environment harbour a wide range of bacterial phyla (Table 2.1). Moreover, in terms of the fresh water tortoises *Podocnemis expansa* and *P. unifilis*, Proteobacteria is the only phylum described by de Morais et al. (2011). The relatively few studies of gut community compositions in turtles and the use of only culture-based techniques for bacterial identification could be the reasons of oversight the entire intestinal bacterial community except only a small fraction.

The marine mammals, including the dugong, are not exceptions from the other aquatic gut fermenters as they have abundant Firmicutes (83%) along with Bacteriodetes, Actinobacteria, Lentisphaerae, Proteobacteria and Verrucomicrobia in their lower intestinal tract (Tsukinowa et al., 2008) (Table 2.1).

Table 2. 1 Gut bacterial flora in hind gut fermenters

Hind gut fermenters			Gut bacterial phyla															
Groups		**Species**	**Pr**	**Ac**	**Ba**	**Fu**	**Fi**	**Te**	**DT**	**Pl**	**Sp**	**Sy**	**Th**	**Ve**	**Cy**	**Eu**	**Fib**	**Le**	**References**
Aquatic	Crustacean	black tiger shrimp	+	+	+	+	+	+	+	+	+	+	+	+					Rungrassamee et al. (2014), Chaiyapechara et al. (2012) Lau et al. (2002)
		Norway lobster	+					+											(Meziti et al., 2012)
		mud crab	+		+	+	+	+							+				(Li et al., 2007; Li et al., 2012)
	Fish	Parrotfish	+		+	+				+					+				Sullam et al. (2012)
		Surgeonfish	+		+	+	+	+											Sullam et al. (2012)
	Reptile	Iguanas			+		+				+								Hong et al. (2011)
		Sea turtle	+	+			+												Al-Bahry et al. (2011), Santoro et al. (2006a), Santoro et al. (2006b), Santoro et al. (2008b), Foti et al. (2009)
		Fresh water turtle	+																de Morais et al. (2011)
	Mammals	Dugong	+	+	+		+							+				+	Tsukinowa et al. (2008)
Terrestrial	Bird	Chicken	+		+		+				+								Torok et al. (2011)
	Reptile	Terrestrial turtle	+	+			+												Dickinson et al. (2001), Barbour et al. (2007)
	Mammals	Horse	+	+	+		+				+			+		+	+		(Costa et al., 2012; Shepherd et al., 2012; MM et al., 2013)
		White Rhinoceros			+		+												Bian et al. (2013)

Note: +, reported; Pr, Proteobacteria; Ac, Actinobacteria; Ba, Bacteroidetes; Fu, Fusobacteria; Fi, Firmicutes; Te, Tenericutes; DT, Deinococcus-Thermus; Pl, Planctomycetes; Sp, Spirochaetes; Sy, Synergistetes; Th, Thermotogae; Ve, Verrucomicrobia; Cy, Cyanobacteria; Eu, Euryarchaeota; Fib, Fibrobacters; Le, Lentisphaerae

Above all, the findings across a wide range of aquatic hindgut fermenters clearly indicate that the core gut bacterial communities among the hosts are relatively common in a marine environment. However, the host tropic level, habitat and possibly host phylogeny have a potential impact in shaping and colonizing the gut microbiota in different hosts. The salinity of the water has a vital role in structuring the gut microbiota in a free-living marine environment, which was previously described by Lozupone and Knight (2007). Gut bacterial colonisation in different marine herbivores with at least some environmental microbes including *Vibrio, Aeromonas* also indicate the adaptation of different bacterial species with saline environment of their hosts. Although a diverse range of gut bacterial phyla were recorded in different marine herbivores including fish, most were highly united by dominance of bacterial species from the phyla, Proteobacteria (black tiger shrimp, Norway lobster, mud crab, parrotfish, surgeonfish). In contrast, the marine iguanas and the mammalian dugong showed a different scenario, predominant with Firmicutes. The distinction between the predominant phyla among different hosts is more likely driven by the difference in their selectivity of the gut environment, described by Rawls et al. (2006). The variation among the described gut bacterial communities could also be result of differences in techniques used for bacterial identification whether culture independent and or dependent.

2.6.2. Terrestrial hindgut fermenters

In terrestrial animals that utilise hindgut fermentation, the gut bacteria also have a potential impact (Hume, 2002). For instance, the gut bacteria in chicken play a vital role in increasing feed efficiency, which ultimately leads to increased production. Torok et al. (2011) reported twenty-six (26) bacterial species in the chicken. These can be phylogenetically classified into three different phyla including Bacteroidetes, Proteobacteria and predominantly Firmicutes which are associated with improved feed efficiency and production performance (Table 2.1). However, several authors have also reported variation among the gut bacterial flora in different parts of the chicken intestine which may vary with age, diet, health, treatments and environment (Amit-Romach et al., 2004; Wise and Siragusa, 2007; Torok et al., 2008; Torok et al., 2013). These findings were quite similar to the horse, a herbivorous monogastric mammal (Shepherd et al., 2012). In horses, the microbial fermentation mainly takes place in the caecum and colon where an abundance of gut microbiota exists. More than 50% of daily energy in horses comes from microbial fermentation of a fibrous diet, with an end product of

volatile fatty acids. However, little attention has been given to equine hindgut microbiota in comparison with other ruminants' like cattle. A few studies documenting the faecal bacteria in horses show a high diversity of phyla including Firmicutes that predominates while others are Actinobacteria, Bacteroidetes, Euryarchaeota, Spirochaetes, Proteobacteria, Fibrobacters and Verrucomicrobia (Costa et al., 2012; Shepherd et al., 2012; MM et al., 2013) (Table 2.1).

The white rhinoceros, another non-ruminant herbivore possesses a specialised feeding habit; grazing leaves and plants that are somewhat toxic to other animals. This animal shows similar bacterial phyla to the horse where the Firmicutes and Bacteroidetes predominate but are comprised different genera (Bian et al., 2013).

In spite of the variation among the bacterial phyla recorded in aquatic and terrestrial animals, either vertebrates or invertebrates may share a similar function: microbial fermentation. This is evidenced by the presence of short-chain fatty acids as end products of cellulose digestion.

2.6.3. Bacterial gut flora of turtles and tortoises

The luminal bacterial flora reported in turtles and tortoises seem to be dominated by Gammaproteobacteria which include *Salmonella* spp. *Shigella* spp., *Pseudomonas* spp., *Aeromonas* spp., *Escherichia coli*, *Citrobacter* spp. *Photobacterium* spp., *Edwardsiella* spp., *Klebsiella* spp, *Serratia* spp, *Proteus* spp. and *Vibrio* spp. (Table 2.2). *Chromobacterium* sp. in the giant South American tortoise *(Podocnemis expansa)* and yellow-spotted Amazon River tortoise *(P. unifilis)* (de Morais et al., 2011). One study found that *Shigella flexnerii* and *Escherichia coli* were the most frequently isolated Gram-negative bacteria from *Podocnemis expansa* and *P. unifilis* tortoises (de Morais et al., 2011) while in another study, a high prevalence of *Salmonella* was recorded in free living exotic and pet tortoises (Hidalgo-Vila et al., 2007), which is not surprising because Gram-negative bacteria are thought to be common inhabitants of reptiles (Mader, 2006). Gram-positive bacteria have been reported abundantly in terrestrial tortoises. However, other bacteria have also been documented in tortoises, such as Firmicutes (*Staphylococcus spp., Streptococcus spp.*) in the desert tortoise (*Gopherus agassizii*) (Dickinson et al., 2001). A few Gram-positive bacteria like *Staphylococcus, Micrococcus,* and *Bacillus spp*. have also been reported as resident gut flora in sea

turtles including leatherback (Table 2.2). Reviews on different studies revealed that all studies utilising culture-based techniques to identify the luminal bacterial flora of turtles typically fail to spot the major portion of bacterial communities.

Little attention has been given to the characterisation of the gut bacterial flora of the green sea turtle (Santoro et al., 2006a; Al-Bahry et al., 2011) although it is globally in an endangered condition. A detailed gut bacterial profile is absolutely essential for accurate interpretation of the bacteriological culture and to better understand the role of bacterial pathogens in disease events in green sea turtle.

Table 2. 2 Gut bacterial flora isolated from sea water, fresh water and terrestrial turtles & tortoises

Isolated bacteria spp.		**Sea Turtles**				**Fresh water turtle**		**Terrestrial tortoise**	
Genus	**Type**	GS	LH	LB	OR	GSA	YSA	D	ST
Salmonella spp.	gm (-)	+		+		+	+	+	+
Shigella spp.	gm (-)	+				+	+	+	
Aeromonas spp.	gm (-)	+		+	+		+		
Pasteurella spp.	gm (-)	+						+	
Proteus spp.	gm (-)	+	+	+				+	
Pseudomonas spp.	gm (-)	+	+	+		+	+	+	+
Citrobacter spp.	gm (-)	+	+		+	+	+	+	
Enterobacter spp.	gm (-)	+	+	+			+	+	
Escherichia sp.	gm (-)	+	+	+		+	+	+	
Morganella spp.	gm (-)		+						
Providencia spp.	gm (-)		+						
Pantoea spp.	gm (-)		+						
***Shewanella* sp.**	gm (-)		+						
Bordetella sp.	gm (-)	+		+					
Klebsiella sp.	gm (-)	+		+			+	+	
Vibrio sp.	gm (-)	+							
Serratia sp.	gm (-)	+				+			
Chromobacterium sp.	gm (-)					+	+		
Hafnia sp.	gm (-)						+		
Acinetobacter sp.	gm (-)						+		
Campylobacter spp.	gm (-)							+	
Staphylococcussp.	gm (+)	+		+				+	
Bacillus spp.	gm (+)			+				+	
Streptococcus spp.	gm (+)							+	
Lactobacillus spp.	gm (+)							+	
Corynebacterium spp.	gm (+)							+	
Enterococcus sp.	gm (+)			+					
Micrococcus sp.	gm (+)	+							
References		Santoro et al. (2006a) Al-Bahry et al. (2011)	Foti et al. (2009)	Santoro et al. (2008b)	Santoro et al. (2006b)	de Morais et al. (2011)	de Morais et al. (2011)	Dickinson et al. (2001)	Barbour et al. (2007)

Note: +, reported; GS, green sea turtles; LH, loggerhead sea turtles; LB, leatherback turtle; OR, olive Ridley sea turtles; GSA, giant South American tortoise; YSA, yellow-spotted amazon river tortoise; D, desert Tortoise; ST, spur-thighed tortoise

2.6.4. Bacterial flora isolated from eggs of nesting wild sea turtles

Reptilian embryos go through a process of development within a protective environment, inside the egg shell (Soslau et al., 2011b). Introduction of bacterial pathogens into the egg may lead to decreased hatching success. Decreased hatchability and early embryonic mortality will reduce the population at large (Heppell et al., 2003). Therefore, in some species (e.g. python) parental incubation through the entire period of hatching offers the eggs more protection against the environmental pathogen, despite a permeable egg shell (Cook et al., 2005). With respect to sea turtles, eggs are laid in a sandy nest and left unattended over time. This increases the potential for contact with environmental pathogens and may contribute to decreased hatchability and increased mortality (Wyneken et al., 1988). However, factors affecting the hatchability of eggs are not always clear. Some researchers found a strong bacterial association with decreased hatchability of sea turtle eggs (Girondot et al., 1990; Craven et al., 2007). To date, little information is available on the bacterial species harboured in turtle eggs. Therefore, to determine the status of bacterial populations, de Morais et al. (2010) conducted a study on fertile eggs of *Podocnemis expansa* and *P. unifilis* tortoises and concluded that all the bacterial species isolated were potential pathogens. All eggs of both species of tortoise were positive for Enterobacteriaceae. However, *P. expansa* showed positivity only for *Shigella flexneri* whereas *P. unifilis* were positive for *Shigella flexneri, Chromobacterium violaceum,* and less frequently for *Salmonella choleraesuis* subspecies *arizonae* and *S. salmonicid*a subspecies *salmonicida*. Besides these, the other bacterial isolates were *Escherichia coli* and *Aeromonas salmonicida subspecies salmonicida.* Bacteria under the Enterobacteriaceae were also reported in eggs of loggerhead turtle (Wyneken et al., 1988). Their presence among the females and their eggs indicated that they might have a correlation with decreased egg hatchability rate. A similar study was done by Soslau et al. (2011b) in leatherback turtles where nesting adult female turtles and their hatched and unhatched eggs were sampled. These samples were positive for *Bacillus, Pseudomonas* and *Aeromonas* spp. In the olive Ridley turtle, the author hypothesised that the presence of *Vibrio mimicus* in eggs could be due to possible contamination of the eggs from the sand where they were laid (Acuna et al., 1999). To unveil the eggs' microbiome of endangered hawksbill sea turtle, (*Eretmochelys imbricate)* Sarmiento-Ramirez et al. (2014) examined the eggs and confirmed the presence of Proteobacteria as the most predominant phylum along with

Bacteroidetes, Firmicutes, Actinobacteria. From the above discussion, it clearly indicates that all the bacteria identified are present surrounding the turtles and their habitats, transmission may occur vertically and/or horizontally from other animals as well as from the environment.

2.7. Pathogenic bacterial species in turtles and tortoises

Diverse groups of bacteria (Table 2.3 and 2.4) have been identified by many researchers in aquatic and terrestrial turtles and these bacteria seem to play a very important role in their overall health status. Bacteria which are frequently isolated appear to be primary pathogens from localised infections as well as secondary opportunistic organisms in conjunction with other disease. One of the most endangered marine species, green sea turtle *(Chelonia mydas*), was mostly found infected with *Vibrio* spp., *Plesiomonas* spp., *Aeromonas* spp. and *Citrobacter* spp., while other bacteria like *Streptococcus* spp., *Staphylococcus* spp., *Micrococcus* spp., *Corynebacterium* spp. and *Aureobacterium* spp. were also identified in association with other infectious and non-infectious diseases. Such diseases included severe fibropapillomatosis, cardiovascular parasitism and immune-suppressive diseases (Aguirre et al., 1994; Raidal et al., 1998; Work et al., 2003; Chuen-Im et al., 2010b). In leatherback turtle, Soslau et al. (2011a) described a new bacterial pathogen *Acinetobacter* spp. in turtle hatchlings, which is commonly found in soil and water. Later, to determine the extent of other potentially lethal bacteria that may interfere with the hatchling rate of leatherback turtle, they examined and identified *Bacillus* spp., *Pseudomonas* spp. and *Aeromonas* spp. in a cloacal sample (Soslau et al., 2011b). Similar bacterial genera were also reported by Santoro et al. (2008b) in cloacal and nasal samples of nesting leatherback turtle with high prevalence of Gram-negative bacteria under Enterobacteriaceae. Normally enterococci and coliforms, common inhabitants of gastro-intestinal tract, are thought to be primarily responsible for gastro-intestinal disorder in acute and immunosuppressive conditions of animals. Therefore, to explore the reasons behind severe acute gastro-enteritis in leatherback turtle, a necropsy study was carried out in an adult female. This necropsy revealed *Photobacterium damselae* subspecies *piscicida*, an opportunistic agent in conjunction with ingested plastic debris in intestinal tract (Poppi et al., 2012). However, among the other sea turtles, a variety of pathogenic bacteria were also reported. These include *Shewenella* spp. and *Vibrio* spp. in Kemp's Ridley turtle (Williams et al., 2012);

and *Bartonella* spp., *Achromobacter* spp., *Aerococcus* spp., *Bacillus* spp., *Burkholderia* spp., *Citrobacter* spp., *Pasteurella* spp., *Proteus* spp., *Pseudomonas* spp. and *Vibrio* spp. in the loggerhead turtle (Oros et al., 2005; Poppi et al., 2012).

Moreover, in recent decades salmonellosis has become a well-recognised reptilian zoonosis (Raidal et al., 1998; Gonzalez Candela et al., 2005; Hidalgo-Vila et al., 2007; Gaertner et al., 2008; Hidalgo-Vila et al., 2008; Percipalle et al., 2011; Sanchez-Jimenez et al., 2011; Dutton et al., 2013; Lafuente et al., 2013; Silbernagel et al., 2013). Several researchers have also identified many other potential zoonotic pathogens from different aquatic and terrestrial turtles/ tortoises, such as *Mycobacterium* spp. from the Indian flap-shelled turtle (*Lissemys punctate punctata*) (Sakaguchi et al., 2011), Kemp's Ridley sea turtle (*Lepidochelys kempii*) (Greer et al., 2003) and Chinese soft-shelled turtle (*Pelodiscus sinensis*) (Oros et al., 2003); *Helicobacter* spp. from the pancake tortoise (*Malacochersus tornieri*) (Stacy and Wellehan, 2010); *Campylobacter* spp. from the Baur's box tortoise (*Terrapene bauri taylor*) (Harvey and Greenwood, 1985) and other chelonians (Wang et al., 2013); *Listeria* spp. from the eastern box tortoise (*Emys orbicularis*) (Joyner et al., 2006) and *Leptospira* spp. from the spur-thighed tortoise (*Testudo graeca*), Hermann's tortoise (*Testudo hermanni*), European pond tortoise (*Emys orbicularis*) and red-eared slider (*Trachemys scripta elegans*) (Lindtner-Knific et al., 2013). These findings urge a more detailed study on the microbial diversity among the marine and fresh water tortoises with their zoonotic potential.

Table 2. 3 Pathogenic bacteria isolated from sea turtles

Isolated bacteria		**Sea Turtles**			
Genus	**Type**	**GS**	**LH**	**LB**	**KR**
***Salmonella* spp.**	gm (-)	+		+	
***Aeromonas* spp.**	gm (-)	+		+	
***Pseudomonas* spp.**	gm (-)	+		+	
***Citrobacter* spp.**	gm (-)	+		+	
***Escherichia* spp.**	gm (-)	+		+	
***Vibrio* spp.**	gm (-)	+		+	+
***Burkholderia* spp.**	gm (-)	+		+	
***Shewanella* spp.**	gm (-)	+			+
***Pasteurella* spp.**	gm (-)	+			
Edwardsiella spp.	gm (-)	+			
***Moraxella* spp.**	gm (-)	+			
***Enterobacter* spp.**	gm (-)			+	
***Proteus* spp.**	gm (-)			+	
***Acinetobacter* spp.**	gm (-)			+	
***Bordetella* spp.**	gm (-)			+	
***Achromobacter* spp.**	gm (-)			+	
***Chryseobacterium* spp.**	gm (-)			+	
***Bartonella* spp.**	gm (-)		+		
***Aerococcus* spp.**	gm (+)		+	+	
***Staphylococcus* spp.**	gm (+)	+		+	
***Streptococcus* spp.**	gm (+)	+		+	
***Corynebacterium* spp.**	gm (+)	+		+	
Aureobacterium spp.	gm (+)	+			
***Micrococcus* spp.**	gm (+)	+			
***Bacillus* spp.**	gm (+)			+	
***Enterococcus* spp.**	gm (+)			+	
***Arcanobacterium* spp.**	gm (+)			+	
***Mycobacterium* spp.**	gm (+)				+
References		Aguirre et al. (1994) Raidal et al. (1998) Work et al. (2003) Chuen-Im et al. (2010b)	Oros et al. (2005)	Dutton et al. (2013)	Williams et al. (2012)

Note: gm (-), Gram-negative; gm (+), Gram-positive; +, reported; GS, green sea turtles; LH, loggerhead sea turtles; LB, leatherback turtle; OR, Kemp's Ridley sea turtles

Table 2. 4 Pathogenic bacteria isolated from fresh and terrestrial turtles and tortoises

Common name	Scientific name	Isolated bacteria spp.								References
		S	***Le***	***M***	***H***	***P***	***Ca***	***Ci***	***Li***	
Fresh water tortoise										
Western pond tortoise	*Emys marmorata*	+								Silbernagel et al. (2013)
European pond tortoise	*Emys orbicularis*		+							Lindtner-Knific et al. (2013)
Indian flap-Shelled tortoise	*Lissemys punctata punctate*			+						Sakaguchi et al. (2011)
Terrestrial										
Spur-thighed tortoise	*Testudo graeca*	+	+							Lindtner-Knific et al. (2013) Gonzalez Candela et al. (2005) Percipalle et al. (2011)
Hermann's Tortoise	*Testudo hermanni*	+	+							Lindtner-Knific et al. (2013) Percipalle et al. (2011)
Red-eared Slider	*Trachemys scripta elegans*		+							Lindtner-Knific et al. (2013)
Pancake tortoise	*Malacochersus tornier*				+					Stacy and Wellehan (2010)
Eastern box tortoise	*Terrapene carolina carolina*							+	+	Joyner et al. (2006)
Chinese softshell tortoise	*Pelodiscus sinensis*			+						Oros et al. (2003)
Baur's box tortoise	*Terrapene bauri Taylor*	+					+			Harvey and Price (1983)
Desert Tortoise						+				

Note: +, reported; S, *Salmonella* sp.; Le, *Leptospira* spp.; M, *Mycobacterium* sp.; H, *Helicobacter* spp.; P, *Pasteurella* sp.; Ca, *Campylobacter* sp.; Ci, *Citrobacter* sp.; Li, *Listeria* sp.

2.8. Bacteria in pelagic water

The oceans harbour a wide range of microorganisms including bacteria. Depending on the oxygen availability, both aerobic and anaerobic bacteria are found in marine water, on the surface of the sediments and within the sediments (Belkin and Colwell, 2005). To determine the extent of bacterial diversity, a number of researches have studied coastal water and marine sediments. In a study on the coastal water of southern Kerala of India, Robin et al. (2012) used a culture-dependent technique and documented six main groups of enteric bacteria including *E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio cholera* and *Vibrio parahaemolyticus* in the costal water. Marine bacteria like *Bacillus*, *Micrococcus*, *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Achromobacter* and *Xanthomonas* with antibacterial activities have also been reported in the oceanic surface and deep see sediments (Jayanth et al., 2002). In the Mediterranean Sea, Gartner et al. (2011) isolated 107 bacterial species from deep sea sediments. These where largely identified as *Bacillus* spp. The other isolated bacterial strains predominantly belonged to two phylogenetic groups: Firmicutes and Actinobacteria. This study was quite similar to the finding of da Silva et al. (2013) who also reported the presence of Firmicutes and Actinobacteria in addition to Gammaproteobacteria in South Atlantic Ocean, indicating aquatic bacterial diversity among the oceans varies on a spatial and temporal scale (Zinger et al., 2011; Jones et al., 2012; Du et al., 2013; Hatosy et al., 2013).

Although several researchers have documented a number of different bacterial species, it only represents a partial picture of the complete aquatic bacterial world. Some bacterial species are indigenous to the oceans like *Vibrio* while *E. coli*, *Shigella* spp. *Salmonella* spp. are allochthonous; usually introduced through waste discharges, rural and urban surface runoff. The presence of the faecal coliforms could be liked with anthropogenic inputs and untreated sewage disposal (Sivri and Seker, 2010; Robin et al., 2012).

2.9. Techniques used for bacterial identification

Successful and accurate identification of any suspected bacterial pathogen depends on the methods and techniques used. There are two distinct methods commonly applied to bacterial identification: phenotyping, or culture-dependent methods, and genotyping. Phenotyping methods are based on identification of phenotypic features including

colony morphology, biochemical tests, pathogenicity test, serology, antibiotic sensitivity and enzymatic tests. On the other hand, genotyping relies on purification, PCR amplification and sequencing of microbial DNA usually the universally conserved molecules 16S ribosomal RNA gene (Houpikian and Raoult, 2002; Spratt, 2004; Spiegelman et al., 2005). Massive parallel sequencing of these genes allows for comparison among the aquatic, terrestrial and host-associated bacterial communities.

The cultural techniques were first used in the 19th century for microbial classification and are still applied in 21th century (Houpikian and Raoult, 2002). The application of phenotypic and biochemical tests has increased in recent years because of the availability of many commercial multi-test systems. These commercial biochemical tests are commonly used for identification of specific gut bacterial pathogens that include Enterobacteriaceae and Vibrionaceae. However, *Vibrio* isolates recovered from marine animals is difficult to differentiate with these systems (Awong-Taylor et al., 2008) although many researchers have used these phenotypic and biochemical techniques for the identification of different bacterial species in fish and aquatic animals including turtles. For example, based on culture-dependent techniques, Santoro et al. (2006a) examined the aerobic cloacal bacterial flora on nesting green turtle in Tortuguero National park, Costa Rica and identified ten Gram-negative and three Gram-positive bacterial genera. Similar techniques aimed at aerobic bacterial isolation and identification were also observed by other researchers (Aguirre et al., 1994; Dickinson et al., 2001; Santoro et al., 2006b; Foti et al., 2009; Chuen-Im et al., 2010b; de Morais et al., 2010; Al-Bahry et al., 2011; de Morais et al., 2011; Liu et al., 2013).

Although culture-dependent techniques have their place, the acceptability of these techniques is declining because of its' drawbacks. These include difficulty in the culture of strict anaerobes, unknown growth requirements of the bacteria, selectivity of media, failure to discriminate closely related strains and it is extremely time consuming (Lauri and Mariani, 2009). Moreover, it is also believed that the phenotypic characteristics are not always stable, which may vary with time depending on the change of environmental conditions like temperature, salinity, pH and growth substrate (Rossello-Mora and Amann, 2001). Furthermore, in regards of the biochemical tests the panel configuration of some tests are rarely changed once they are commercially produced, although reformulation of the tests occasionally occurs (Janda and Abbott, 2002). For example API 20E strip remains as same as it was in 1975, although the newly described taxa

have changed substantially between the 20th and 21st centuries (Euzeby, 1997). The lack of these up-grades has led to less reliable identification of bacterial pathogens. In addition, the traditional culturing technique does not cover the entire microbial diversity of the complex environment (Giraffa and Neviani, 2001; Pogacic et al., 2010).

On the other hand, in terms of gastro-intestinal pathogens, it is also very difficult to maintain an ideal growth environment outside the intestinal tract because of some unexplained complex interactions that occur within the host (Gilmore and Ferretti, 2003). Therefore, a complete survey of the gastrointestinal flora of any animal is quite difficult to perform using only culturing techniques due to an inability to grow many of the gut bacterial pathogens including anaerobes.

To overcome this situation, a molecular based taxonomic approach is mandatory for more accurate identification and characterisation of gut bacterial species (Janda and Abbott, 2002; Pogacic et al., 2010). The promise of genomes without cultivation has made metagenomics a popular culture- independent approach for microbial diversity study (Handelsman, 2004; DeLong, 2005). Researchers working with gut bacterial flora readily use techniques based on sequence diversity including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGE), single strand conformation polymorphism (SSCP), terminal-restriction fragment length polymorphism (T-RFLP), fluorescent *in situ* hybridization (FISH), and diversity microarrays (DNA microarrays) to identify the bacterial species (Zoetendal et al., 2004). For example, Nelson et al. (2013) used 16s rRNA gene sequence data to compare the gut bacterial community of marine and terrestrial mammals. A similar technique has been applied in marine and terrestrial iguanas to evaluate the faecal bacterial communities (Hong et al., 2011). Meziti et al. (2012) used 16s rRNA gene diversity analysis to document the gut bacterial species in the Norway lobster (*Nephrops norvegicus),* a marine crustacean. A high-throughput next generation of pyrosequencing analysis was carried out by Rungrassamee et al. (2014) to characterise the intestinal bacterial flora in wild caught and domestic adult marine black tiger shrimp (*Penaeus monodon*). Aiming to obtain core information on gut bacterial communities, they also compared the result in denatured gradient gel electrophoresis (DGGE). Johnson et al. (2009) used 16s rDNA sequence data to evaluate respiratory microbiome of bottlenose dolphin. Recently, Illumina sequencing which sequences-by-synthesis is based on the same principle as

pyrosequencing is preferred as it generates many more sequence reads at lower costs. Several researchers have applied this technique to identify the microbial community in different vertebrates including humans (Qin et al., 2010), but no application has been reported for aquatic vertebrates including turtles.

It is important to highlight that molecular techniques applied for bacteriological identification and characterisation can either be culture-dependent or independent based on whether the bacterial DNA is extracted from the cultured bacterial colonies on an ideal growth medium or from the original sample (Pogacic et al., 2010). Several studies have applied culture-independent techniques to identify the gut microbiota in different animals (Hong et al., 2011; Chaiyapechara et al., 2012; Rungrassamee et al., 2014), while few studies have also revealed with culture-dependent techniques to describe the intestinal bacterial profile. Sarmiento-Ramirez et al. (2014) used a high density 16S ribosomal DNA oligonucleotide microarray or PhyloChip based on metagenomic analysis to unravel the eggshell microbiome of an endangered species of marine turtle, *Eretmochelys imbricate*. A similar study was done by Soslau et al. (2011b) to identify potentially lethal bacteria in leatherback turtle eggs through isolation of bacterial DNA from the cultured subsamples and amplification of the 16S rRNA gene by polymerase chain reaction (PCR).

All the techniques normally used to identify the bacterial species whether culture-dependent or independent, phenotypic or genotypic, have their limitations because no single technique is able to provide 100% accurate result (Janda and Abbott, 2002). The spectacular development of new molecular techniques makes the traditional culture methods obsolescent. But the undoubted value of culture-independent techniques should not hide the crucial role of culture based identification techniques that have played historically. Moreover, culture-based techniques are still an irreplaceable option for studying emerging bacterial diseases in aquatic and terrestrial animals including turtles (Houpikian and Raoult, 2002). On the contrary, the culture-independent techniques especially the next-generation sequencing provides a reliable and comparatively more convenient platform of examining a large number of samples and determining the taxonomic position of new, still uncultured organisms. The use of culture independent techniques for intestinal microbial identification enables the illumination of a bigger portion of this topic in comparison with culture based techniques (Spiegelman et al., 2005). It helps to profile the intestinal bacterial species and enables researchers to

distinguish among the beneficial and harmful bacteria responsible for certain turtle diseases including gastrointestinal disorders. Moreover, the rapid and reliable detection of fastidious or non-culturable pathogenic organisms applying culture independent molecular techniques facilitates an early and effective antibacterial treatment to recover the animals during rehabilitation.

2.10. Summary

It can be concluded that microflora associated with turtles and tortoises varies among the terrestrial, fresh water and marine environments. These variations reflect the bacterial diversity in their surrounding environment and other vertebrates. Most of the isolated bacterial species are at genus level which restricts the recognition of particular species being resident in turtles apart from the environment. The roles and the relationship among the autochthonous and allochthonous microflora in turtles is not well understood and is hypothesised to vary among the species of turtles and their feeding habits. Although enormous progress has been made in the isolation and identification of gut bacteria in other vertebrates through the application of modern tools such as genomic sequencing, these advancements are yet to be applied to turtles and tortoises. Some potential human pathogens have been recovered from some species of turtles but the relationship is still unclear. The organisms responsible for certain diseases including gastrointestinal disorders in turtles are still not well identified because of the lack of knowledge on intestinal microbial communities. Knowledge of the composition of normal microbiota and in particular bacterial communities will allow for easier identification of pathogenic bacteria present and to select the appropriate antibiotic therapy. Therefore, a more accurate identification, characterisation and pathogenic potential determination of the bacterial species is extremely essential for further advancing our understanding of health, diseases and their treatment in turtles.

CHAPTER 3
GENERAL MATERIALS AND METHODS

The materials and methods described in this chapter were applied in more than one research chapter of the thesis. Those methods used only in one experiment are described in the chapter to which they belong.

3.1. Culture-dependent identification techniques

3.1.1. Phenotypic identification of bacterial isolates

A pilot study was carried out using cloacal swabs collected from ReefHQ rehabilitated turtles before proceeding with the field samples. This study aimed at selecting suitable culture media and incubation temperature for the maximum yield of Enterobacterales from sea turtles. Different media compositions with regard to nutritive ingredients and salt content were tested.

Based on the findings, all samples in this study were plated on two different solid culture media, namely MacConkey agar (Neogen corp., USA) with additional agar (1.5%) and 5% sheep blood agar (Oxoid Ltd., Hampshire, UK) with additional agar (1.5%). Duplicate samples were seeded in EC broth (Oxoid Ltd., Hampshire, UK) for overnight enrichment before plating on the media. Samples were plated within 24 hours of collection at room temperature (25-27 ^{0}C) for overnight incubation. A representative of each colony morphotype was purified by culture and further identified. The isolates that were Gram negative, cytochrome C-oxidase negative and glucose fermentative were suspected as Enterobacterales and were further tested through other biochemical tests that included catalase, spot indole, citrate, urease, hydrogen sulfide (H_2S) production, triple sugar ion and motility test following a standard protocol described by Murray et al. (1999). Presumptive Enterobacterales isolates were fully identified using the API system 20E (BioMerieux, Marcy lEtoile, France). The identification of a bacterial taxon was accompanied by both the percentage of identification accuracy (%id > 98%) and the T index (T> 0.5), an estimate of how closely the profile corresponds to the most typical set of reactions for the stated taxon. API unclassified bacterial isolates

were subsequently sequenced for further identification based on partial sequence of 16S rRNA.

3.1.2. Molecular identification

Colonial purity was ensured by serial culturing of single representative colonies at least 3 times. Genomic DNA of clones of each bacterial isolate was extracted for PCR assay using Isolate II Genomic DNA Kit (Bioline, Australia), in accordance with the manufacturer's instructions with small modifications. The nucleic acid sequence of the 16S rRNA gene was amplified with a set of universal primers, 27F and 1391R (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GACGGGCGGTGTGTRCA-3'; 1350bp) (Lane, 1991). An additional set of primers, RibRNA-454-F and RibRNA-454-R (5'-GCAAGCGTTAATCGGAAT-3' and 5'-ATGTCAAGACCAGGTAAGG-3'; 454bp), was designed in order to obtain a more complete sequence within the same conserved region covered by the universal primer. The PCR reaction mix for 25µl was composed of 12.5µl of 2x My Taq mix (Bioline, Australia), 1µl of 10 pmol forward and reverse primers (Macrogen Inc., Soeul, South Korea), 9µl of ultrapure DNase free water and 1µl of approximately 100 ng DNA template. PCR amplifications were performed using Bio-Rad Thermal Cyclers (Bio-Rad Laboratories Inc., USA) under standard PCR conditions consisting of 95 ^{0}C for 1 min for initial denaturation followed by 30 cycles of amplification of 15 sec at 95 ^{0}C, 15 sec at 55 ^{0}C, 15 sec at 72 ^{0}C and final extension at 72 ^{0}C for 5 min and later, held at 4^{0}C. The presence of a 1,350bp DNA fragment was confirmed on 1% agarose gel electrophoresis and visualized using GelRed under UV illumination. Following confirmation, the PCR products were purified and sequenced by Sanger sequencing (Macrogen Inc., Seoul, South Korea). The nucleotide sequences were analysed and aligned in Geneious (Biomatters Ltd.) followed by identification using NCBI nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The presence of a bacterial taxon was confirmed at the highest nucleotide identity > 98% to the NCBI database.

3.2. Culture-independent identification technique

3.2.1. Nucleic acid extraction

Nucleic acid extraction of the green turtle cloacal swab samples was performed using a PowerLyzer® PowerSoil® DNA Isolation Kit following the manufacturer's standard

protocol (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Once extracted, the quantity of the extracted DNA was determined using GeneQuant Prospectrophotometer (Amersham Pharmacia Biotech) and stored at -20 ^{0}C until used.

3.2.2. PCR amplification and sequencing

Library preparation and sequencing was carried out by the Australian Genome Research Facility, Brisbane, Australia. For MiSeq sequencing analysis, a bacterial 16S rRNA gene library was constructed according to the Illumina 16S metagenomics sequencing library preparation guidelines (Illumina, San Diego, USA). The V1-V3 hypervariable regions of the targeted 16S rRNA gene were PCR amplified using the forward (27F 5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (519R 5'-GWATTACCGCGGCKGCTG-3') primers. The PCR reaction was carried out using an initial denaturation at 95°C for 7 min, followed by 29 cycles of denaturation at 94°C for 45s, primer annealing at 50^{0}C for 1 min and extension at 72°C for 1 min, with a final elongation at 72°C for 7 min. The AmpliTaq Gold 360 mastermix (Life Technologies, Australia) was used for the primary PCR and a secondary PCR to index the amplicons was performed with TaKaRa Taq DNA Polymerase (Clontech). The resulting amplicons were measured by fluorimetry (Invitrogen Picogreen) and normalised. The equimolar pool was then estimated using qPCR (KAPA), followed by sequencing on the Illumina MiSeq (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry.

CHAPTER 4
DETECTION AND ANTIMICROBIAL RESISTANCE OF ENTEROBACTERALES ISOLATES FROM GREEN TURTLES IN THE GREAT BARRIER REEF, AUSTRALIA

4.1. Introduction

Since the World Health Organisation (WHO) first considered the emergence of antimicrobial resistance to be of global concern in the 1980s, the number of types of multi-resistant bacterial strains have continued to increase, outpacing the development of effective antibiotics (Grundmann et al., 2011; Frieri et al., 2016). The Enterobacterales being ubiquitous are easily able to exchange genetic materials including those that code for antimicrobial resistance. These facultative anaerobes usually present as commensal intestinal flora in terrestrial animals, including humans (Bonelli et al., 2014). Several genera of Enterobacterales are responsible for a number of serious, life-threatening infections in humans such as acute gastritis, urinary tract and respiratory tract infections (Wright, 2010; Kresken et al., 2016; Najjuka et al., 2016).

The Great Barrier Reef (GBR) situated off the eastern coast of Queensland is the largest and most diverse ecosystem on the planet. This world heritage site is managed by the Great Barrier Reef Marine Parks Authority (GBRMPA) who have identified risks and vulnerable animals in the Great Barrier Reef Biodiversity Conservation Strategy 2013 (GBRMPA, 2013). In this document, it was indicated that the inshore areas of the GBR were under greatest threat as a result of human-related activities and that they should be better investigated. Furthermore, experts identified a need to evaluate the level of contamination in the GBR of antimicrobial resistance of microbes and the potential role of marine megafauna as their reservoir (Webster and Hill, 2007; Koenig et al., 2011).

Aquatic environments can become contaminated from a variety of sources including urban surface run-off and effluent discharges (Goni-Urriza et al., 2000; Wellington et al., 2013). This suggests that aquatic fish, mammals and reptiles have the potential to harbour antibiotic-resistant bacteria and studies involving several marine reptiles, including sea turtles, support the notion that antibiotic resistant bacteria are present in

the marine environment (Santoro et al., 2006a; Foti et al., 2009; Al-Bahry et al., 2011; Wheeler et al., 2012; Wallace et al., 2013; Stewart et al., 2014).

Green sea turtles, *Chelonia mydas*, have several characteristics that make them a good bio-indicator for environmental health. With a long life-span and high site fidelity to coastal foraging habitats, green sea turtles are exposed long-term to coastal anthropogenic factors and are prime reservoir candidates for antibiotic-resistant bacteria originating from urban run-off. Their reproduction migration inevitably takes them across international borders and exposes them to significant environmental stressors (Foti et al., 2009; Read et al., 2014). These challenges are likely to be extreme in the Pacific Ocean where a wide continental margin harbours significant populations of green turtles in rapidly declining foraging habitats.

This chapter is on an investigation into enteric Enterobacterales and their antimicrobial resistance in wild green sea turtles captured inshore to the central GBR and proximate to urban development, as well as debilitated green sea turtles undergoing rehabilitation in this region.

4.1. Material and methods

4.1.1. Ethics statement

Sample collection from free ranging and rehabilitated green sea turtles was conducted under permissions of James Cook University Animal Ethic Committee (permit no. A2101), Department of Environment and Heritage Protection Authority (permit no. WISP15015914) and Great Barrier Reef Marine Park Authority (permit no. G14/37285.1). Green turtles were restrained without anaesthesia for cloacal swab collection. None of the techniques involved sacrificing turtles and minimal distress was experienced as a result of handing and restraining.

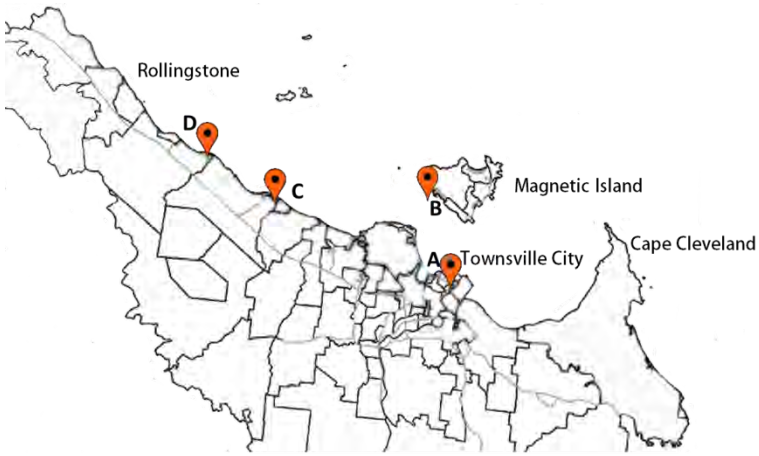

Figure 4. 1 Map of study sites near Townsville. A, ReefHQ turtle hospital; B, Cockle Bay; C, Toolakea Beach and D, Ollera Creek.

4.1.2. Study sites

Green turtles were sampled from three different locations within the inshore areas of the central section of the Great Barrier Reef (GBR) marine park: Cockle Bay, Toolakea and Ollera Creek Beaches (Figure 4.1). Cockle Bay (Lat.: 19°40’34.32” S; Long.: 146°49’51.6” E) is on the south-western shore of Magnetic Island. It is located within the Cleveland Bay and is approximately 5 km away from Townsville city and close to the city port. Toolakea beach (Lat.: 19° 8'41.45"S and Long.: 146°34'53.78"E) is approximately 35 km north-east of Townsville and extends around 8 km from the Blue Water Creek to the mouth of Sleeper Log Creek. Residential habitats can be found in small areas beside the sandy shore of Toolakea. Ollera Creek (Lat.: 18°57'42.23"S and Long.: 146°21'27.19"E) supports significant mangrove and smaller areas of rocky reefs, and has less human exposure. It is approximately 60 km further north-east of Townsville. All three study sites were chosen based on the availability of green turtles, accessibility and frequency of exposure to human activities (frequent: Cockle Bay, less frequent: Toolakea Beach and rare: Ollera Creek, although these sites fall under the category of general use zone that includes recreational boating, fishing, diving and aquaculture. Debilitated and sick green turtles were sampled in this study from Australia’s largest turtle rehabilitation center, ReefHQ; situated in Townsville. Samples

from the stranded turtles were taken immediately after their arrival for rehabilitation and before applying any antimicrobial medication.

4.1.3. Sampling

In this study, a total of 73 live green turtles were sampled between June 2015 and January 2016 (Table 4.1). Free-ranging sea turtles were captured by rodeo (Limpus and Reed, 1985) in shallow water (~5m), and the debilitated turtles accommodated in small water tanks at ReefHQ aquarium turtle hospital were restrained by hand. Depending on size (ranging from juvenile to adult), the turtles were restrained by 1 or 2 volunteers. All turtles were flipper-tagged with Department of Environment and Heritage Protection (DEHP) tags. Morphometric data (weight and curved carapace length, CCL) was recorded according to DEHP standard operating procedures (DEHP, 2013). Repetitive sampling was avoided by excluding turtles that were previously captured and tagged during this period.

Deep cloacal swabs were taken from each healthy and sick turtle for culture dependent identification of the enteric bacteria. For this purpose, a sterile polyester swab was gently inserted and rolled inside the cloaca (10 cm internal depth) immediately after flushing the cloacal opening with 70% ethanol to avoid external contamination. Swabs were then placed in Amies agar gel transport medium (Oxoid, Ltd., Basingstoke, Hampshire, England). All samples were kept in an icebox "Esky™" with ice to maintain a temperature of approximately 4°C and then transported to the laboratory at James Cook University (Townsville, QLD) within 12 hours.

4.1.4. Phenotypic and molecular identification of bacterial isolates

Identification of bacterial isolates was conducted using both phenotypic and molecular techniques. These techniques have been described in Chapter 3.1.

4.1.5. Antibiotic susceptibility study

In order to determine antimicrobial drug resistance, all Enterobacterales isolates were tested for sensitivity to different antibiotic groups using a quantitative method based on broth microdilution and determination of minimal inhibitory concentration (MIC), under defined test conditions (Wiegand et al., 2008). The procedure includes a standard tray containing 96 wells with each well containing a volume of 0.1 ml that allowed 12

antibiotics to be tested at a time in the range of 8 serial two-fold dilutions in a single tray (Jorgensen and Turnidge, 2007; CLSI, 2012). During test performance, a precise volume (100 µl) of pre-weighed and diluted antibiotics was added in the first row of wells containing Mueller-Hinton broth (100 µl) and later, a total of 8 serial two-fold dilutions were achieved in the remaining rows across all the wells. A 100µl volume of a standard suspension (5 x 10^5 CFU/ml) of each bacterial isolate was inoculated into each well of the microdilution tray. Following overnight incubation at 30 ^{0}C, the minimum inhibitory concentration (MIC) was determined by adding 20 µl of a colorless thiazolyl blue tetrazolium bromide dye (Sigma-Aldrich Co.) to each well followed by additional incubation at 37 ^{0}C for 30 minutes, ending with purple changes in those wells containing bacterial growth. The bacterial susceptibility was tested for a range of 12 different antibiotics that represent 6 different classes of antibiotics including, ampicillin, amoxicillin-clavulanic acid, penicillin, cephalexin, ceftiofur, nalidixic acid, enrofloxacin, doxycycline, gentamicin, streptomycin, chloramphenicol and trimethoprim–sulphamethoxazole. These antibiotics were selected based on their frequency of therapeutic application and impact in aquatic and terrestrial animals. The EUCAST epidemiological cut-offs for each antibiotic were considered to determine the susceptibility of tested isolates (http://www.eucast.org/mic_distributions_and_ecoffs/). Clinical break points as recommended by the Clinical Laboratory Standards Institute (CLSI) were used for those antibiotics where cut-offs are not available, which include penicillin and amoxycillin-clavulanic acid. In this study, *Escherichia coli* reference strain ATCC 25922 served as a test control for the MIC test. Isolates that were found to be resistant to three or more antibiotics were categorised as multidrug resistant (Magiorakos et al., 2012).

4.1.6. Statistical analysis

Logistic regression analysis was performed using STATA/IC software for Windows (Version 12.0, Stata Corp, College Station, TX, USA). Univariate association between the binary outcome (resistance, 1 and non-resistance, 0) and independent variables were analysed. Statistical significance of the independent variables was tested using Pearson's Chi-Square test ($p< 0.05$). Odds ratio with 95% confidence intervals was estimated to interpret the results of the categorical variables. Significant differences among the study sites were calculated at 0.05 levels of significance. Types of bacteria

and antibiotic resistance were reported as frequencies, and the percentages of resistance for different antibiotics were calculated in Microsoft Excel 2010.

Table 4. 1 Samples and isolate details from different study sites

Location	Number of turtles	Total Gram-negative isolates	Total Enterobacterales isolates	Ratio between number of turtles & isolates of Enterobacterales
Ollera Creek	12	31	19	1: 1.58
Toolakea Beach	20	110	36	1: 1.80
Cockle Bay	26	109	50	1: 1.92
Rehabilitation Centre	15	91	49	1: 3.27
Total	**73**	**341**	**154**	**1: 2.35**

Table 4. 2 List of isolated Enterobacterales

Sl. no	Bacteria genus	Species	No. of isolates (%)	API accuracy (%)	PCR accuracy (%)
1	*Citrobacter* spp.		**47 (**30.5**)**		
		C. freundii	32 (20.8)	99.9	≥ 99
		C. braaki	6 (3.9)	99.9	≥ 99
		C. youngae	5 (3.3)	99.8[a]	
		C. koseri/amalonaticus	3 (2)	96.7[a]	
		Unclassified	1 (0.7)		≥ 99[b]
2	*Edwardsiella* spp.	*E. tarda*	**33 (21.4)**	99.9	≥ 99
3	*Escherichia* spp.	*E. coli*	**19 (12.4)**	99.8[a]	
4	*Enterobacter* spp.	*E. aerogenes*	**15 (9.7)**	99.7	≥ 99
5	*Klebsiella* spp.		**10 (6.5)**		
		K. oxytoca	7 (4.6)	99.1	≥ 99
		K. pneumoniae ssp. *pneumoniae*	1 (0.7)	99.4[a]	
		K. variicola	2 (1.3)		≥ 99[b]
6	*Morganella* spp.	*M. morganii*	**10 (6.5)**	99.9	≥ 99
7	*Pantoea* spp.	Unclassified	**3 (2)**	99.7[a]	
8	*Providencia* spp.	*P. rettgeri*	**6 (3.9)**	99.9[a]	
9	*Proteus* spp.		**11 (7.1)**		
		P. vulgaris	9 (5.8)	99.8	≥ 99
		P. mirabilis	1 (0.7)	96.7[a]	
		P. penneri	1 (0.7)		≥ 100[b]

[a] indicates isolates were confirmed only by API 20E and [b] indicates isolates were confirmed by 16S rRNA sequencing.

4.2. Results

4.2.1. Bacterial identification

A total of 154 of the 341 Gram-negative bacterial isolates were identified as Enterobacterales using culture-dependent phenotypic, biochemical and molecular techniques (Table 4.1). Sixty-seven samples out of a total of 73 were positive for Enterobacterales and the highest numbers of Enterobacterales were recovered from rehabilitating turtles with a ratio of 3.53 isolates per turtle. A total of 16 different bacterial species were identified that represented 9 different genera of Enterobacterales (Table 4.2).

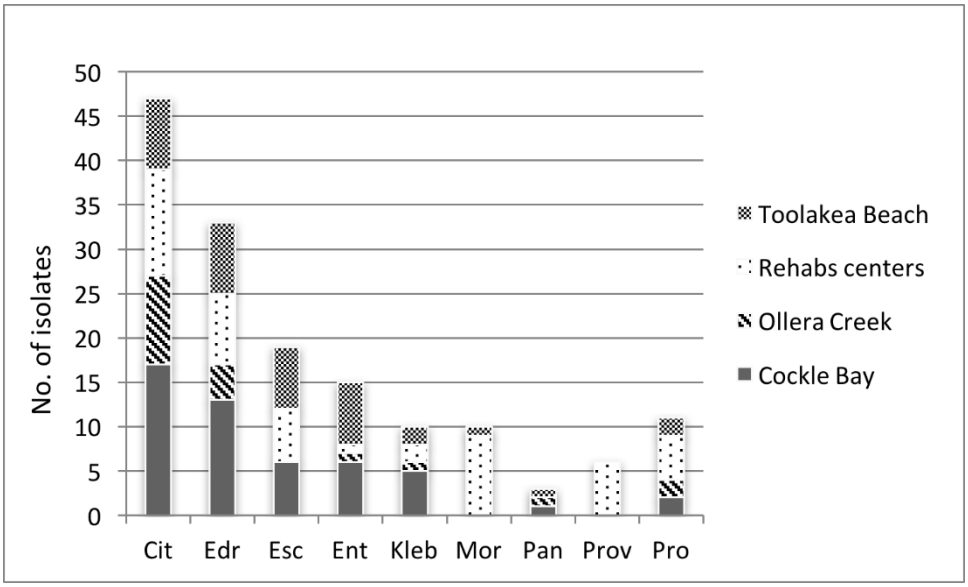

Figure 4. 2 Frequency distribution of Enterobacterales isolated among different sampling sites. Abbreviation: Cit, *Citrobacter* spp.; Edr, *Edwardsiella* spp.; Esc, *Escherichia* sp.; Ent, *Enterobacter* spp.; Kleb, *Klebsiella* spp.; Mor, *Morganella* spp.; Pan, *Pantoea* spp.; Prov, *Providencia* spp.; Pro, *Proteus* spp.

The predominant genera were *Citrobacter* (30.5%), followed by *Edwardsiella* (21.4%) and *Escherichia* (12.4%). The other Enterobacterales identified in this study included *Enterobacter* spp. (9.7%), *Proteus* spp. (71%), *Klebsiella* spp. (6.5%), *Morganella* spp. (6.49%), *Providencia* spp. (3.9%) and *Pantoea* spp. (2%). The species distribution is

summarised in Table 2. Most genera were isolated from both the rehabilitation center and field samples except *Providencia* spp. which was only identified from the rehabilitated turtles while *Pantoea* spp. originated only from field samples. The highest percentages (90%) of *Morganella* spp. were recovered from rehabilitated turtle samples. *Escherichia* spp. was not isolated from the turtles of Ollera Creek (Figure 4.2). In this study, five turtle samples were identified as negative to Enterobacterales and bacteria were not cultured from one sample using the media designed to detect the Enterobacterales.

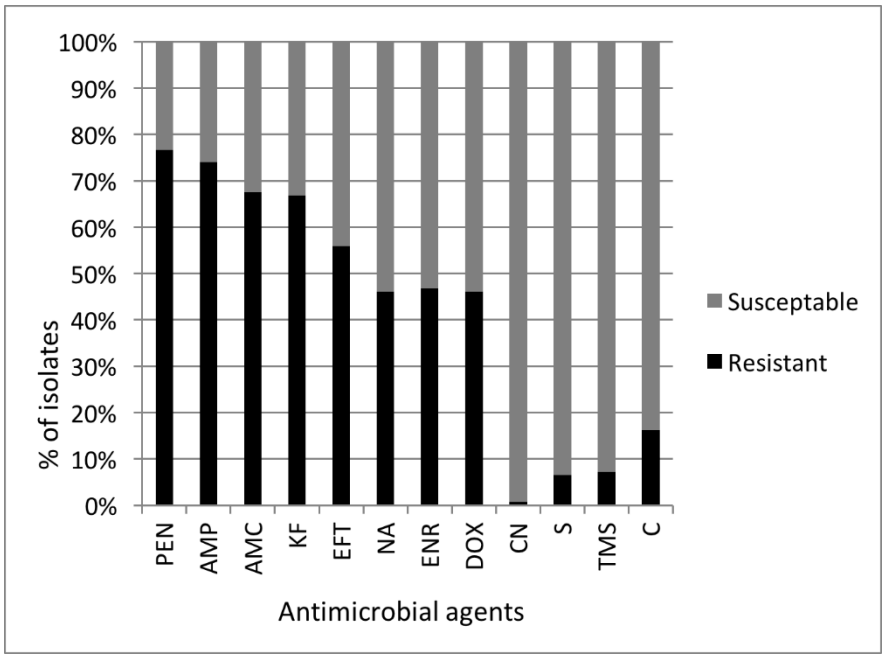

Figure 4. 3 Antimicrobials resistance profile of Enterobacterales to 12 different antimicrobial agents. Abbreviation: PEN, penicillin; AMP, ampicillin; AMC, amoxicillin-clavulanic acid; KF, cephalexin; EFT, ceftiofur; NA, nalidixic acid; ENR, enrofloxacin; DOX, doxycycline; CN, gentamicin; S, streptomycin; TMS, trimethoprim-sulfamethoxazole; C, chloramphenicol.

4.2.2. Antimicrobial resistance

Enterobacterales isolates were found to be resistant to all 12 antimicrobial agents (Figure 4.3). The most frequently displayed resistance was to beta-lactam class of antibiotics that include penicillin (76.6%), ampicillin (74.0%), amoxicillin-clavulanic

acid (67.5%), cephalexin (68.9%) and ceftiofur (55.8). In contrast, the highest antibiotic sensitivity was shown to aminoglycosides (99.4%, gentamicin; 93.5% streptomycin), trimethoprim-sulfamethoxazole (92.9%) and chloramphenicol (83.8%). Enterobacterales isolates exhibited an equal level of antimicrobial resistance to quinolone class (46.1%, nalidixic acid and 46.8%, enrofloxacin), and tetracycline class (46.1%, doxycycline) of antibiotics.

To consolidate the different antimicrobial susceptibilities, an assumption was made that bacteria within the same family or genus would behave similarly. The Enterobacterales isolates were classified into three different families, with the largest family *Enterobacteriaceae*, being divided into 3 genera (Table 4.3). *Edwardsiella*, the only isolated representative of the Hafniaceae showed the lowest resistance (>40%) to all 12 antimicrobial agents where no resistance (0%) to gentamicin, streptomycin, trimethoprim-sulfamethoxazole and chloramphenicol was recorded. In contrast, members of Morganellaceae including *Proteus*, *Morganella* and *Providencia* exhibited the highest resistance (>70%) to most of the antibiotics except gentamicin (0%), streptomycin (7.4%) and trimethoprim-sulfamethoxazole (22.2%). Although antimicrobial activity against Klebsielleae revealed no resistance to gentamicin and trimethoprim-sulfamethoxazole, a lower antimicrobial resistance was also recorded for chloramphenicol (7.1%) and streptomycin (10.7%). Additionally, an equal frequency of resistance was recorded for ceftiofur and doxycycline (39.3%).

Escherichia showed complete resistance (100%) to penicillin and ampicillin while no resistance to gentamicin was noted. Approximately, one-fourth (>21%) of the *Escherichia* isolates were resistant to enrofloxacin, trimethoprim-sulfamethoxazole, nalidixic acid and streptomycin. Resistance to amoxicillin-clavulanic acid and cephalexin were 68.4% and 73.7% respectively.

No antimicrobial agent of the twelve was found to be completely (100%) effective against *Citrobacter*. A low level of antimicrobial resistance was recorded for gentamicin (2.1%), trimethoprim-sulfamethoxazole (2.1%), chloramphenicol (4.2%) and streptomycin (8.5%) but a higher antimicrobial resistance (>75%) was recorded for penicillin, ampicillin, amoxicillin-clavulanic acid, cephalexin and ceftiofur.

Table 4. 3 Antimicrobial resistance profiles of the predominant Enterobacterales isolates. Darkest shades are the upper quantile (75-100% resistant), upper second quantile (50-75% resistant) and lower quartile (25-50% resistant), and lowest quantile (0-25% resistant) is white. The different numbers in the table indicate the number of isolates and in parentheses are the corresponding percentages.

Antibiotics	**Morganellaceae (27)**	***Klebsiella* (28)**	***Escherichia* (19)**	***Citrobacter* (47)**	***Edwardsiella* (33)**
AMP	25 (92.6)	26 (92.9)	19 (100.0)	40 (85.1)	4 (12.1)
AMC	25 (92.6)	26 (92.9)	13 (68.4)	39 (83.0)	1 (3.0)
PEN	25 (92.6)	24 (85.7)	19 (100)	38 (80.9)	12 (36.4)
KF	22 (81.5)	20 (71.4)	14 (73.7)	36 (76.6)	11 (33.3)
EFT	25 (92.6)	11 (39.3)	7 (36.8)	35 (74.5)	8 (24.2)
NA	21 (77.8)	17 (60.7)	5 (26.3)	22 (46.8)	6 (18.2)
ENR	23 (85.2)	16 (57.1)	4 (21.1)	23 (48.9)	6 (18.2)
DOX	23 (85.2)	11 (39.3)	5 (26.3)	19 (40.4)	13 (39.4)
CN	0 (0.0)	0 (0)	0 (0)	1 (2.1)	0 (0)
S	2 (7.4)	3 (10.7)	4 (21.1)	4 (8.5)	0 (0)
C	19 (70.4)	2 (7.1)	2 (10.5)	2 (4.3)	0 (0)
TMS	6 (22.2)	0 (0)	4 (21.1)	1 (2.1)	0 (0)

Table 4. 4 Locality distribution of antimicrobial resistance

Sl no	Antibiotics	Sampling locations	P value	Odds ratio	95% Confidence Interval
1	Ampicillin	Rehab center	0.033	3.64	1.11- 11.90
		Cockle Bay	0.244	1.92	0.64- 5.77
		Toolakea	0.285	1.89	0.59- 6.07
		Ollera	1	1	-
2	Amoxycillin-Clavulinic acid	Rehab center	0.053	3.15	0.99- 10.09
		Cockle Bay	0.600	1.33	0.45- 3.91
		Toolakea	0.975	1.02	0.33- 3.14
		Ollera	1	1	-
3	Penicillin	Rehab center	0.004	6.3	1.82- 21.81
		Cockle Bay	0.055	2.93	0.99- 8.87
		Toolakea	0.097	2.7	0.83- 8.74
		Ollera	1	1	-
4	Cephalexin	Rehab center	0.008	4.62	1.5- 14.35
		Cockle Bay	0.092	2.52	0.86- 7.40
		Toolakea	0.055	3.12	0.99- 9.91
		Ollera	1	1	-
5	Ceftiofur	Rehab center	0.000	10.64	3.09- 36.62
		Cockle Bay	0.014	4.34	1.35- 13.92
		Toolakea	0.593	1.40	0.41-4.81
		Ollera	1	1	-
6	Nalidixic acid	Rehab center	0.053	3.03	0.99- 9.34
		Cockle Bay	0.382	1.64	0.54- 5.01
		Toolakea	0.465	1.55	0.48- 5.00
		Ollera	1	1	-
7	Enrofloxacin	Rehab center	0.020	3.77	1.24- 11.49
		Cockle Bay	0.975	1.01	0.34- 3.03
		Toolakea	0.957	0.97	0.30- 3.07
		Ollera	1	1	-
8	Doxycycline	Rehab center	0.029	3.43	1.13- 10.39
		Cockle Bay	0.783	0.86	0.09- 2.57
		Toolakea	0.729	1.22	0.39- 3.84
		Ollera	1	1	-
10	Streptomycin	Rehab center	0.845	0.78	0.07-9.17
		Cockle Bay	0.921	1.12	0.10- 11.53
		Toolakea	0.483	2.25	0.23- 21.69
		Ollera	1	1	-
11	Trimethoprim-sulpha	Rehab center	model omitted	2.57	0.29- 22.93
		Cockle Bay	0.394	1	-
		Toolakea	0.483	2.25	0.23- 21.69
		Ollera	1	1	-
12	Chloramphenicol	Rehab center	0.026	10.8	1.32- 87.90
		Cockle Bay	0.553	1.96	0.21- 17.92
		Toolakea	0.645	0.51	0.03-8.71
		Ollera	1	1	-

4.2.3. Locality distribution of antimicrobial resistance

In our regression analyses, isolates of Enterobacterales recovered from rehabilitated turtles appeared to be resistant to all beta-lactam antibiotics, including penicillin ($p<0.004$), ampicillin ($p<0.033$), amoxicillin-clavulanic acid ($p<0.053$), cephalexin ($p<0.008$) and ceftiofur ($p<0.000$). Isolates from Cockle Bay were found to be significantly resistant to penicillin ($p<0.054$) and ceftiofur ($p<0.014$). Enterobacterales from other study sites (Toolakea Beach and Ollera Creek) did not exhibit significant association with any beta-lactam antibiotics (Table 4.4).

No significant association was revealed among the field isolates from Toolakea Beach, Ollera Creek and Cockle Bay to quinolone, tetracycline and chloramphenicol classes of antibiotics although isolates from the rehabilitation center were significantly resistant to different tested antibiotics, including nalidixic acid ($p<0.053$), enrofloxacin ($p<0.020$), doxycycline ($p<0.029$) and chloramphenicol ($p<0.026$).

In this study, antimicrobial resistance was recorded least commonly in Ollera Creek isolates (mean: 5.75) and most frequently in rehabilitated green turtles isolates (mean: 26.92) (Figure 4.4).

No significant association was revealed among the field isolates from Toolakea Beach, Ollera Creek and Cockle Bay to quinolone, tetracycline and chloramphenicol classes of antibiotics although isolates from the rehabilitation center were significantly resistant to different tested antibiotics, including nalidixic acid ($p<0.053$), enrofloxacin ($p<0.020$), doxycycline ($p<0.029$) and chloramphenicol ($p<0.026$).

In this study, antimicrobial resistance was recorded least commonly in Ollera Creek isolates (mean: 5.75) and most frequently in rehabilitated green turtles isolates (mean: 26.92) (Figure 4.4).

4.2.4. Antimicrobial multi-resistance

More than 78% of the Enterobacterales isolates were found to be resistant to at least one class and almost half (48.7%) of the isolates were resistant to at least two different classes of antimicrobial agents examined. Fifty-eight (37.7%) of the 154 Enterobacterales isolates were recorded as multidrug resistant as they exhibited resistance to three or more different classes of antimicrobial agents. Isolates recovered

from rehabilitated turtles exhibited significant association ($p<0.009$) with multidrug resistance although no significant association was revealed for the other study locations.

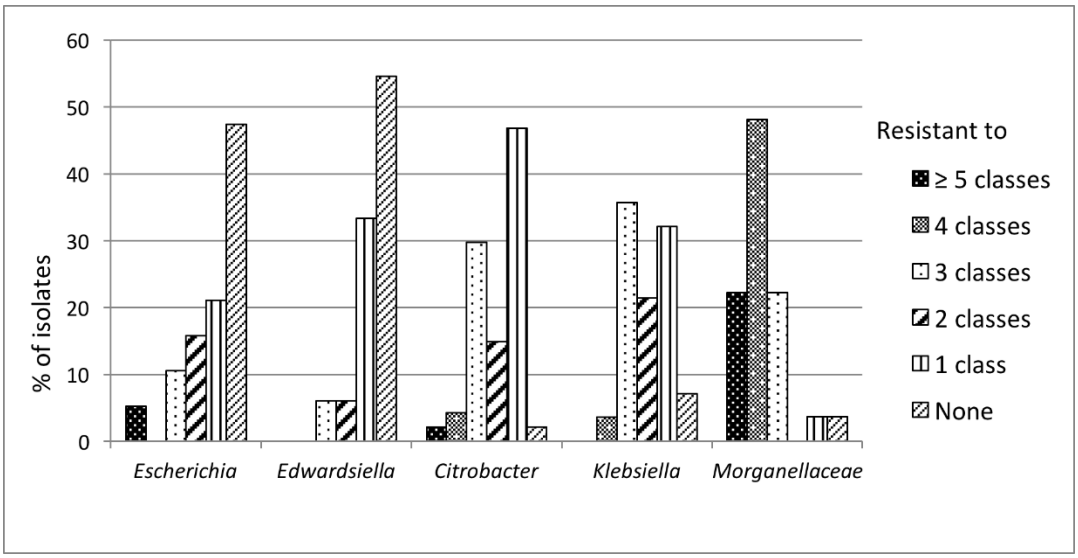

Figure 4. 4 Distribution of antimicrobial multi-resistance of the Enterobacterales. Abbreviation: None, no antibiotic resistance; 1 class, resistant to β-lactam class; 2 classes, resistant to β-lactam and quinolone classes; 3 classes, resistant to β-lactam, quinolone and tetracycline classes; 4 classes, resistant to β-lactam, quinolone, tetracycline and chloramphenicol classes; ≥ 5 classes, resistant to β-lactam, quinolone, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole classes.

The highest multidrug resistance was recorded for the Morganellaceae (92.6%) which included *Proteus* spp. *Morganella* spp. and *Providencia* spp. Isolates from all three genera of Morganellaceae were multidrug resistant (Figure 4.5) where most (80%) were recovered from rehabilitated turtles (Figure 4.6).

Three of the 19 *Escherichia* found to carry multidrug resistance and one isolate showed resistance to ≥ 5 different classes of antibiotics. These multidrug resistant *Escherichia* were isolated from all sampling locations except Cockle Bay. Multidrug resistant *Edwardsiella* were recovered from the samples of Ollera Creek and rehabilitation centre (Figure 4.6).

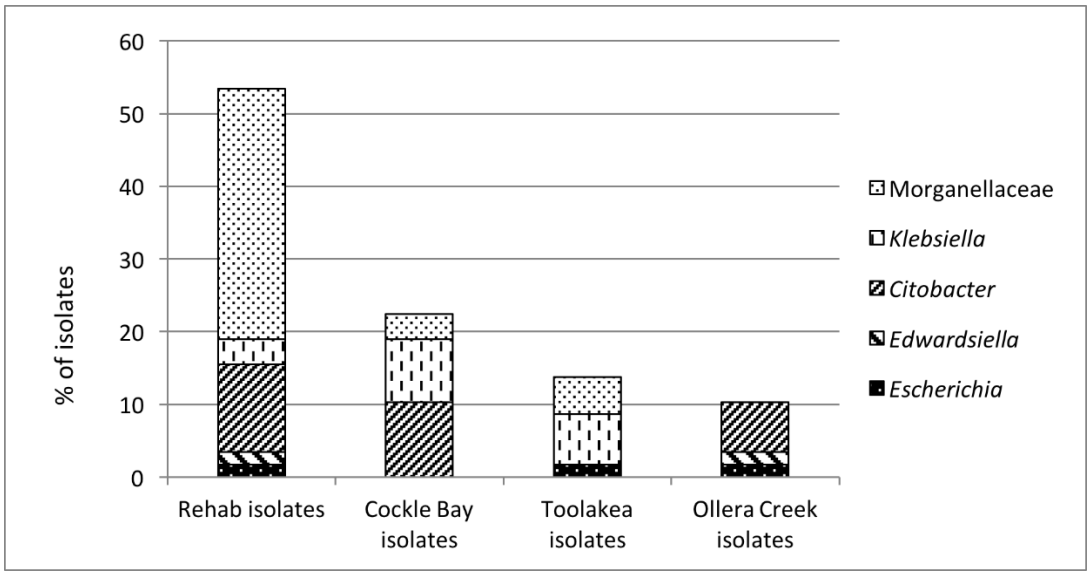

Figure 4. 5 Distribution of multidrug resistant Enterobacterales among different sampling sites

For *Citrobacter*, >39% isolates exhibited multidrug resistance. Two isolates were resistant to ≥ 4 different classes and one isolate was resistant to ≥ 5 different classes of antimicrobial agents. Multidrug resistant *Citrobacter* were identified from both field and rehabilitated turtles. Likewise, a higher percentage of multidrug resistant *Klebsiella* were recovered from field samples (81.8%) and lower in rehabilitated turtles (18.2%) (Figure 4.6).

The frequency distributions of the MIC values for all antimicrobial agents are listed in Table 4.5.

Table 4. 5 Distribution of MICs for Enterobacterales from all study sites. Darkest fields denote range of dilutions tested for each antimicrobial agent. Bold vertical lines indicate epidemiological cut-off values defining resistance

Antimicrobial agents	Distribution (%) of MICs[a] (mg/ml)																
	% resistance	> 32	32	16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	0.02	0.01	< 0.01	total
Ampicillin	74.0	107		7	12	11	8	5	1	2	0					1	154
AMC	67.5	39		50	15	5	8	14	4	7	4					5	154
Penicillin	76.6	86	32	11	11	7	4	0	0	2						1	154
Cephalexin	66.9	103		10	25	10	4	0	0	1	0					1	154
Ceftiofur	55.8	29			10	21	26	25	21	13	5	0				4	154
Nalidixic acid	45.5	70		6	7	8	15	14	14	5	3					12	154
Enrofloxacin	46.1	26						16	11	19	41	21	14	2	0	4	154
Doxycycline	46.1	32		17	22	24	28	17	7	1	0					6	154
Gentamicin	0.7	1					0	2	2	8	31	47	32	19		12	154
Streptomycin	5.8	1	8	12	11	16	17	37	30	9						13	154
Chloramphenicol	16.2	13	12	4	31	41	25	12	12	1						3	154
TMS	7.1	10			0	1	0	2	6	6	11	6				112	154

4.3. Discussion

Most studies carried out on the intestinal microflora of the sea turtle have focused on the most predominant which are the *Vibrionaceae*. However, terrestrial animals, including humans are susceptible to only a few members of this family. This is in contrast to the Enterobacterales which is known to cause opportunistic infections in terrestrial animals and humans. They are well-known culprits of spreading resistant genes from environmental microbes to other members of Enterobacterales and eventually to human microbes, and *vice versa* (Maravic et al., 2015). A number of studies have evaluated the role of sea turtles as a reservoir of pathogenic microbes; this study aimed to specifically identify pathogenic Enterobacterales in green sea turtles (*Chelonia mydas*) on the GBR and the antimicrobial susceptibility of these isolates. The presence of MDR Enterobacterales in the GBR is an important finding that indicates the possible marine microbial pollution in proximity to large urban development.

Our study revealed no notable difference among the Enterobacterales species identified in the different study sites, except the frequency with which they were identified. Among all Enterobacterales, *Citrobacter* spp., *Edwardsiella* spp. and *Escherichia* sp. dominated both wild and rehabilitated green sea turtles (Table 3). The highest prevalence of the *Citrobacter* spp. may be due to their ubiquitous nature, present in a wide range of environmental habitats including soil, water and in the intestines of humans and animals (Chen et al., 2002). Both *C. freundii* and *C. koseri* identified in this study are able to cause human infections (Magiorakos et al., 2012). The results of this study support other findings that claim *Citrobacter* spp. was one of the prevailing Enterobacteriaceae in sea turtles (Foti et al., 2009; Al-Bahry et al., 2011). *Edwardsiella* spp. was rarely reported in high percentages of sea turtles because they are predominantly found in fish and fresh water environments. *Edwardsiella tarda* can cause serious losses in aquaculture and even in humans, although humans are known to be only an occasional host (Stock and Wiedemann, 2001a). Our study indicates *Edwardsiella* sp. is present as a resident flora in green turtles, like other marine reptiles (Sakazaki, 2005). High prevalence of *Escherichia coli* indicates the possibility of widespread terrestrial pollution in marine environments. Generally, *E. coli* is considered to be a commensal inhabitant of the intestinal tract of warm-blooded terrestrial animals, including humans, although a few strains can cause severe illness linked to food

poisoning (Qu et al., 2016) Previous studies also recorded the presence of *E. coli* in sea turtles (Santoro et al., 2008a; Foti et al., 2009) and other marine reptiles (Wheeler et al., 2012). In recent years, the genus *Klebsiella*, including *K. pneumoniae* and *K. oxytoca* identified in this study, have become important pathogens in humans and are believed to cause nosocomial infections in humans.

The high prevalence of *Providencia* spp. (100%) and *Morganella* spp. (90%) in rehabilitated turtles suggests these microbes may act opportunistically in potentially immune-compromised, rehabilitated sea turtles. However, other studies report these species as normal intestinal flora (Foti et al., 2009). The ability of these bacteria to produce biofilm allows them to persist in the tank for longer durations and may be another explanation for their constant presence (Stickler et al., 1993; De et al., 2016). It is difficult to determine dominant bacterial species in rehabilitated turtles, because the composition of bacterial flora shed by turtles is usually linked to diet (Wheeler et al., 2012) and certain host environmental interactions (Liu et al., 2013). Furthermore, bacterial composition can be varied based on different microbiological techniques applied for bacterial isolation. Possibly, the application of culture-independent microbial diversity profiling techniques will be more effective for estimating the predominant bacterial flora in marine reptiles.

It is believed that sea turtles can act as a reservoir for different Enterobacterales members with zoonotic potential (Santoro et al., 2008a; Foti et al., 2009; Al-Bahry et al., 2011). The broad migratory nature and longevity of green sea turtles makes them susceptible to hosting and spreading resistant organisms and their associated genes in widespread marine environments. Therefore, our study also aimed to evaluate the microbial resistance to a panel of 12 different antibiotics from 6 different classes using broth microdilution inhibition technique (MIC). In the absence of established clinical breakpoints for wild marine isolates, the epidemiological cut-offs were employed to determine antimicrobial resistance recommended by EUCAST. The epidemiological cut-offs are well-known to distinguish between bacterial populations that are representative of a wild type and those with mutational or acquired resistance to the antimicrobial agent. In contrast, CLSI clinical breakpoints which are based on pharmacokinetic and pharmacodynamic information of the antibiotics in domesticated animal species are not recommended for wild unclassified isolates (Turnidge and Paterson, 2007).

In our study, isolates of Enterobacterales were resistant to all antibiotics except gentamicin. Isolates exhibited lower resistant to streptomycin followed by trimethoprim-sulfamethoxazole and chloramphenicol (Figure 4). A similar finding was reported by Liu et al. (2013) in Red-Eared Slider tortoises (*Trachemys scripta elegans*). However, other studies documented a widespread resistance to gentamicin irrespective of environmental pressure (Heuer et al., 2002). In aquatic animals, especially in reptiles, gentamicin can cause nephrotoxicity, ototoxicity and deposition in body tissue (Fitzgerald and Newquist, 2008). These adverse effects restrict the use of gentamicin, which may contribute to decreasing resistance in comparison to other frequently used antimicrobial agents. Other studies have found relatively low resistance to trimethoprim-sulfamethoxazole in aquaculture and marine environments (Akinbowale et al., 2006; Al-Bahry et al., 2011). Higher resistance rates were also reported outside Australia (Schmidt et al., 2000; Chelossi et al., 2003). The lower rate of chloramphenicol resistance was documented in isolates obtained from green sea turtles in the Arabian Sea (Al-Bahry et al., 2011) and India's coastal waters (Maloo et al., 2014). After 1982, chloramphenicol was introduced, but later prohibited by FDA for food animals and aquaculture practices; however it was still being used in Latin America and Asia (Hernandez-Serrano, 2005). A higher rate of chloramphenicol resistance was reported by Mirand and Zemelman (2002) in Chilean salmon farms. The pattern and incidence of resistance to chloramphenicol and gentamicin in the current study are in keeping with the global trends in exposure of the marine environments to these antibiotics.

Our study revealed that the highest antimicrobial resistance was recorded for antibiotics in the β-lactam class (78.6%), followed by quinolone (50%) and tetracycline (46.1%) classes. The results show that over 78% of all isolates under investigation were resistant to at least one antibiotic in class β-lactam. Enterobacterales resistance to some β-lactam antibiotics is widespread and is recorded frequently (Foti et al., 2009; Wawire et al., 2013). All members of the Enterobacterales can acquire resistance very easily when exposed to any β-lactam antibiotics (Gootz et al., 1984). This is also true for the most common isolate, *Citrobacter* spp., especially *C. freundii* and *C. koseri*, which like *E. coli*, *K. pneumoniae* and *Proteus* spp. produce β-lactamase that is able to hydrolyze the β-lactam ring. Some isolates were resistant to third generation ceftiofur which strongly indicates that these organisms may be able to produce extended-spectrum β-

lactamase (ESBL) (Jacoby, 2009; Al-Bahry et al., 2012). Bacteria producing the chromosomal or plasmid mediated AmpC enzyme are not only resistant to all β-lactam drugs but also to clavulanic acid which is included in potentiated penicillin. The clavulanic acid protects the β-lactam ring from hydrolysis by beta-lactamases. Therefore, future studies should focus on differentiating ESBLs from resistant strains in green sea turtles. The higher rate of β-lactamase resistance was also reported in marine turtles by other researchers even after using CLSI breakpoints (Foti et al., 2009; Al-Bahry et al., 2011).

The resistance patterns of antibiotics revealed in the quinolone class was surprising, especially in comparison to other findings that documented a relatively low frequency of fluoroquinolone resistance (Akinbowale et al., 2006; Al-Bahry et al., 2011). This fluoroquinolone resistance can be due to chromosomal mutation or plasmid-mediated determinant (Partridge, 2015). An overexpression of the efflux pump may also be a possible mechanism to show fluoroquinolone resistance in isolated *Enterobacteriaceae* species, such as *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. (Mazzariol et al., 2002). Additionally, neither nalidixic acid nor enrofloxacin is registered for use in aquaculture and food industry animals in Australia. Resistance to long-acting tetracycline (doxycycline) has been frequently reported by several studies in marine reptiles, including sea turtles, which support findings of the present study. Foti et al. (2009) recorded the presence of high level of doxycycline resistant Gram-negative bacteria in loggerhead turtles (*Carreta carreta*) in the central Mediterranean Sea. In addition, tetracycline is one of the most common globally used antibiotics in aquaculture and livestock, including Australia (Akinbowale et al., 2006). This antibiotic is more likely to be excreted slowly from the body over a longer duration; their low degradative nature causes an increase in selective pressure that may lead to acquiring microbial resistance typically encoded by plasmids and/or transposable elements (Romero et al., 2012).

Microbial resistance to multiple antimicrobial agents in aquatic and terrestrial environment have previously been reported and support our present study (Akinbowale et al., 2006; Janatova et al., 2014). Our results indicate that all the genera of isolated Enterobacterales were resistant to more than one class of antibiotics. More than 36% of isolates were resistant to multiple drugs dominated by Morganellaceae (43%), *Citrobacter* (29%), *Klebsiella* (18%) and *Escherichia* (5%). These isolates were found

to be resistant to between three and five different classes of antibiotics and would be very difficult to treat.

Multidrug resistant Morganellaceae was significantly associated with rehabilitated turtles compared with isolates from other study sites. Morganellaceae primarily includes *Morganella* spp., *Providencia* spp. and *Proteus* spp. The multidrug resistance of Morganellaceae is more likely to be associated with their intrinsic resistant properties. *Morganella* spp. are naturally resistant to a wide range of antibiotics such as penicillin, amoxicillin, sulfamethoxazole and macrolides (Stock and Wiedemann, 1998a). *Providencia* spp. are naturally resistant to tetracycline, some penicillin, first generation cephalosporin and sulfamethoxazole have also been reported (Stock and Wiedemann, 1998b).

The members of genus *Klebsiella* including *K. pneumoniae* and *K. oxytoca* were found to be resistant to multiple antibiotics that include all the β-lactam antibiotics, quinolone and tetracycline classes of antibiotics. In this study, wild strains of *Klebsiella* were resistant to antibiotics in quinolone and tetracycline classes, although ESBL producing *Klebsiella* has become resistant to many more antibiotics (Stock and Wiedemann, 2001b). *Escherichia coli* and *Edwardsiella* sp. recovered from wild and rehabilitated turtles exhibited multidrug resistance (Figure 4.6) and contradicted other findings with other wild sea turtles outside of Australian waters (Foti et al., 2009).

The present study revealed that Enterobacterales isolates from rehabilitation center were significantly resistant to most antibiotics, except aminoglycosides and trimethoprim-sulfamethoxazole (Table 4.4). These isolates were also significantly multidrug resistant ($p<0.009$) compared to the isolates from free-ranging green turtles of other study sites. Resistance of these isolates to multiple antibiotics is less likely due to therapeutic application of antibiotics in rehabilitation centers because all specimens were collected immediately after the turtles were admitted to rehabilitation centers and before exposure to antibiotics. Sea turtles are usually released close to the habitat where they were found after recovering and it is rare they return to rehabilitation a second time. However, rehabilitated sea turtles that were given antibiotics or were exposed to antibiotic resistant bacteria while undergoing treatment, may play a vital role in spreading these and bacterial genes in their natural environment.

The results suggest there is limited significant difference in the level of antimicrobial resistance between free ranging turtle study sites, except isolates collected from Cockle Bay were significantly resistant to ceftiofur. A higher percentage (22.4%) of isolates from Cockle Bay also found multi-drug resistant with no significant difference between study sites. These may be due to Cockle Bay's close proximity to the city and city port. These may be an indication that the area is contaminated by urban runoff and/or effluents because ceftiofur is one antibiotic recommended by veterinarians that is used frequently in livestock (Hornish and Kotarski, 2002). Wheeler et al. (2012) also found a higher level of resistant bacteria in the wild terrestrial populations proximate to human and livestock activities. In the absence of a statistically significant difference, all free-ranging turtle sampling sites demonstrated a minimal level of antimicrobial resistance for all classes of antibiotics examined (Table 4.4). This indicates resistance to certain antibiotics may either be inherent or an acquirement in response to stressors in that environment. Generally, statistical analysis can only show the comparative differences in resistance patterns, but does not reliably estimate the absolute likelihood of antibiotic resistance and stressors present in that environment. Moreover, simply evaluating the microbial resistance to certain antibiotics in a defined population is not enough to identify the possible source of stressors and to infer conclusions on transmission dynamics (Wheeler et al., 2012). This may be especially true for green sea turtles that are migratory in the marine environment and more likely exposed to a range of potential stressors (Read et al., 2014).

This study has raised the question of the extent of antimicrobial resistance throughout the GBR because a number of bacterial isolates with antimicrobial resistance were observed in all study sites in the Townsville region. Microbial resistance to antibiotics found in this study was lower in the study locations furthest away from the urban areas. However, it is difficult to claim that pristine areas in the GBR are free from antimicrobial resistance because antimicrobial resistant bacteria and associated resistant genes can be spread in the marine environment via hosts, such as the green sea turtles. Moreover, marine bacteria are known to be capable of producing antibiotics and it is not impossible to obtain a baseline of intrinsic resistance to certain antibiotics (Rosenfeld and Zobell, 1947). Further investigation is suggested to determine whether the resistance is inherent in origin or due to selective environmental pressure of antibiotics, including pollution. Determination of the resistant gene is important to understand their

transmission dynamics and potential effect on human health. The finding of our study can provide guidance when choosing appropriate antibiotics for debilitated marine reptiles, including sea turtles, in rehabilitation. The results can also serve as baseline information on antimicrobial resistance while revealing gaps in need of further research to evaluate the level of contamination in the Great Barrier Reef**.**

CHAPTER 5
FAECAL BACTERIAL COMMUNITIES OF WILD-CAPTURED AND STRANDED GREEN TURTLES (*CHELONIA MYDAS)* ON THE GREAT BARRIER REEF

5.1. Introduction

In both vertebrate and invertebrates, it is now well-recognised that the gut microbiome plays a vital role in maintaining the host health as well as in a wide range of diseases (Backhed et al., 2005; Stecher and Hardt, 2008; Costa et al., 2012). The microbiome is a well-established contributor to the digestion and utilisation of complex food particles, and proliferation of intestinal epithelium within the gastrointestinal (GI) tract of the host (Karen et al., 1991; Sommer and Backhed, 2013; Yang et al., 2016). Studies in different animal models also recognised their role in the modulation of the immune system and host physiology (Mao et al., 2015; Yang et al., 2016). Gut microbes can prevent the overgrowth of pathogenic organisms through a natural barrier referred to as 'colonisation resistance' (Van der Waaij et al., 1971; Adlerberth, 2000; Buffie and Pamer, 2013). Recent investigations in terrestrial vertebrates including humans have noticed that gut microbial community composition can be influenced by several factors such as gut structure and physiology (Sommer and Backhed, 2013), feeding strategy (Ravussin et al., 2012; Wang et al., 2016b), genotype of host (Zhang et al., 2010; Carmody et al., 2015), host developmental stage (Sommer and Backhed, 2013), antimicrobial exposure (Jernberg et al., 2010; Dethlefsen and Relman, 2011) and certain environmental conditions such as temperature and salinity (Yoshimizu and Kimura, 1976; MacFariane et al., 1986). Moreover, several studies in terrestrial herbivores elucidated the distinct roles of complex gut microbiota in detoxification of certain compounds and synthesis of different metabolites such as vitamins and minerals required for normal host development (O'Mahony et al., 2015; Yang et al., 2016).

To date, little is known about the gut microbial ecology of marine herbivores that forage primarily in sea grass meadows (Hong et al., 2011; Eigeland et al., 2012; Nelson et al., 2013; Merson et al., 2014). The microbiome in sea turtles is believed to be distinct from

other marine mammals because they exhibit a long life-span and high site fidelity to coastal foraging habitats, increasing the chance of long-term exposure to coastal anthropogenic factors (Bjorndal et al., 1997; Lutz et al., 2002). Moreover, their reproduction migration inevitably takes them across long distances from their normal home range, increasing the risk of exposure to significant environmental stressors (Read et al., 2014). In addition, green turtles exhibit different digestive and reproduction physiologies compared to other marine mammals. They rely on hind-gut fermentation to digest their herbivorous diet and are therefore especially vulnerable to stressors that may influence their gut microbial community. In contrast to mammals, the maternal microbiota in turtles cannot be passed on to their offspring through birth and post-parental care (Funkhouser and Bordenstein, 2013). Conversely, sea turtle hatchlings are more likely to share the microbiota of the nesting beaches where the females lay eggs due to their oviparous nature (Hirth, 1980). A detailed understanding of the core bacterial community is of importance given that gastro-intestinal disorders are a major factor in declining sea turtle populations (Flint et al., 2010) and other health problems (George, 1996). The link between altered gut microbes and disease risk is now a well-established concept in both vertebrates and invertebrates (Costa et al., 2012). Moreover, several studies have shown that the gut microbiota harbour opportunistic pathogens, which can colonise the GI tract of immuno-compromised animals (Owens Jr et al., 2008; Li et al., 2015). Determination of the core microbiota present within the GI tract of healthy animals would allow for a better understanding of their health state and promote gut health. To date, studies on the gut microbial communities in sea turtles were limited to culture dependent phenotypic and biochemical techniques (Aguirre et al., 1994; Al-Bahry et al., 2009; Chuen-Im et al., 2010a; Ahasan et al., 2017a), which do not represent the whole biome including the non-cultivable bacteria.

Our study first characterised in detail the faecal bacterial communities of wild-captured green turtles from different geographical locations that include Bowen and Townsville and compared these bacterial communities to those encountered in stranded green turtles with unknown illness.

5.2. Materials and Methods

Sample collections from free-ranging and stranded green turtles were done under permit from Department of Environment and Heritage Protection Authority (DEHP) (permit

no. WISP15015914), Great Barrier Reef Marine Park Authority (Permit no. G14/37285.1) and James Cook University Animal Ethic Committee (Permit no. A2101).

Figure 5. 1 Map showing the sampling locations (highlighted) in the central Great Barrier Reef Marine Park, Queensland, Australia.

5.2.1. Study site and sample collection

Samples were collected from clinically healthy green turtles that were captured while foraging and from stranded turtles that were in a poor state of health and admitted for rehabilitation. Deep cloacal swab samples from eight green turtles were obtained from two different locations of the central section of the Great Barrier Reef (GBR) Marine Park (Figure 5.1). The wild free-ranging green turtles (n=8) were captured from Edgecumbe Bay, Bowen (Lat: 20° 07' 55" S and Long: 148° 23' 14" E) and Cockle Bay, Townsville (Lat: 19°40’34.32” S; Long: 146°49’51.6” E). Edgecumbe Bay is a coastal embayment near the township of Bowen, which supports large patches of seagrass foraging areas along the fringe of the Bay and an approximately 200m^2 coral reef habitat (Jensen et al., 2016). Cockle Bay is located on the south-western shore of

Magnetic Island, approximately 5 km from the city of Townsville and facing the city port. In addition to seagrass beds, the bay supports rocky reefs associated with rocky outcrops. Cloacal swabs from stranded green turtles (n=4) were collected from turtles admitted to ReefHQ Turtle Hospital, situated in Townsville. Samples from the stranded turtles were taken immediately after their arrival for rehabilitation and before treatment with any antimicrobial medication.

All samples from Edgecumbe Bay and Cockle Bay were collected by conducting two separate rodeos during September 2015. Samples from stranded turtles were collected between October 2015 and March 2016 as turtles were admitted to hospital. Free-ranging wild green turtles were captured by hand (Limpus and Reed, 1985) in shallow water (~5m) and the stranded turtles were accommodated at ReefHQ Turtle Hospital. All wild turtles were flipper-tagged with Department of Environment and Heritage Protection (DEHP) tags. Morphometric data including weight and curved carapace length (CCL) were recorded according to DEHP standard operating procedures (DEHP, 2013) and are presented in Table 5.1.

For sample collection, a sterile cotton swab was gently inserted and rolled inside the cloaca (10 cm internal depth) immediately after flushing the cloacal opening with 70% ethanol to avoid external contamination. Swabs were then immediately placed in 1.5 ml microcentrifuge tubes and transported on ice within 4-7 hours directly to the laboratory at James Cook University (Townsville, QLD), where they were stored at -80 ^{0}C until processed.

Table 5. 1 Site location, sample size and morphometric data of wild captured and stranded green sea turtles

Sl. No.	Group Name	Type	Location	Sample size	Curved carapace length (CCL) range	Body weight (Kg) range
1	Bowen wild-captured (BWC)	Wild captured	Edgecumbe, Bowen	4	42.7-46.4	8.1-12.4
2	Townsville wild-captured (TWC)		Cockle Bay, Townville	4	42.6-55.8	8.3-18.35
3	Townsville stranded (ST)	Stranded	Various, Townsville	4	41.3-87.6	6.25-55

5.2.2. DNA extraction, PCR amplification and sequencing

The extraction of bacterial nucleic acid from the cloacal swab samples, PCR amplification and sequencing of the bacterial 16S rRNA gene were conducted using the techniques described in Chapter 3.2.

5.2.3. Bioinformatics and statistical analyses

To remove primer sequences, fastx_trimmer from the FASTX toolkit (Gordon and Hannon, 2010) was used to trim 20 bp from the forward and 18 bp from the reverse reads. This was followed by quality trimming (q=20) using Sickle (version 1.33) (https://github.com/najoshi/sickle). The paired-ends reads were merged using PANDAseq, discarding orphaned reads (Masella et al., 2012). The quality filtered and merged reads were further processed using the Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010). First, identify_chimeric_seqs.py was used to identify chimeric sequences, which were subsequently filtered using filter_fasta.py. Then USEARCH (version 8.0.1623) (Edgar et al., 2011) was used to cluster operational taxonomic units (OTUs) at 97% similarity applying the UCLUST algorithm (Edgar, 2010). Using QIIME, taxonomy was assigned using Silva database (version 128, Sep 2016) (https://www.mothur.org/wiki/Silva_reference_files#Release_128).

For statistical analyses, the QIIME OTUs table was filtered based on their relative abundance to remove low abundance OTUs (< 1%) and samples that showed <1000 sequence reads. Alpha diversity matrices (ACE, Chao1, Shannon diversity index and Simpson index) were estimated in R to determine the host specific microbial richness and diversity (McMurdie and Holmes, 2013). Strip charts were generated through the Calypso web server (Zakrzewski et al., 2017). Good's coverage was estimated in QIIME to evaluate the completeness of sampling. To evaluate the variation among different groups of samples the rarefied dataset was analysed using Bray-Curtis (Bray and Curtis, 1957) and Chao1 (Chao, 1984) distance matrices, which were later visualised by Principle Coordinate Analysis (PCoA). Clustering of the samples was also evaluated applying non-metric multidimensional scaling (NMDS) with 2 dimensions. Analysis of similarity (ANOSIM) was performed by applying Bray-Curtis distance matrix to evaluate the association between wild-captured and stranded turtle groups. ANOSIM tested the null hypothesis that faecal bacterial compositions are significantly

different between wild-captured and stranded turtles. Venn diagrams were constructed in Calypso to visualise the amount of unique and mutually exclusive bacterial communities among different groups of samples. The positive and negative correlations among the bacterial communities were analysed, and visualised by Calypso using Spearman's rho correlation (Zakrzewski et al., 2017). Finally, the abundance of microbial communities among different groups at different taxonomic level was estimated and compared using parametric ANOVA and paired *t*-test. Linear discriminant analysis was performed to identify the specific biomarker candidates of gut bacterial communities at different taxonomic levels (Segata et al., 2011).

5.3. Results

5.3.1. Summary of sequencing data and depth

Illumina sequencing of 12 cloacal swab samples resulted in a total of 729,689 raw merged reads. Merged reads with a quality score >33 and 350-600 bp in size were included for further analysis. A total of 483,443 high quality reads were identified after filtering (QC) of low quality sequences and potential chimeras (~17% of the total reads) (Table S1). Operational taxonomic units (OTUs) clustering resulted in a total of 669 unique OTUs that were successfully identified and classified to at least a domain level using 97% sequence similarity threshold against the Silva database. The top 20 most abundant OTUs in green turtle samples are shown in Figure S 5.1.

5.3.2. Host specific bacterial richness and diversity estimation

Good's coverage was estimated to evaluate the completeness of sampling. The coverage ranged from 99.5 to 99.9%, indicating that the majority of bacterial phylotypes was present in each sample (Table S 5.2). Bacterial diversity and richness of each sample were estimated at OTU level using ACE, Chao1, Shannon index and Simpson index (Figure 2). The number of OTUs identified in the samples covered 59.62-93.47% and 62.86-92.44% of the richness, as estimated by the ACE and Chao1 respectively. Both richness indices revealed significant difference ($p < 0.05$) between the groups of green turtles (Figure 5.2). Bacterial diversity of the faecal communities was found to be significantly different ($p < 0.05$) between wild-captured and stranded green turtles, as evaluated with the Shannon and Simpson index (Figure 5.2). The diversity was significantly higher in wild-captured green sea turtles from both the Bowen and

Townsville regions, compared to stranded green turtles in the Townsville region (Figure 5.2).

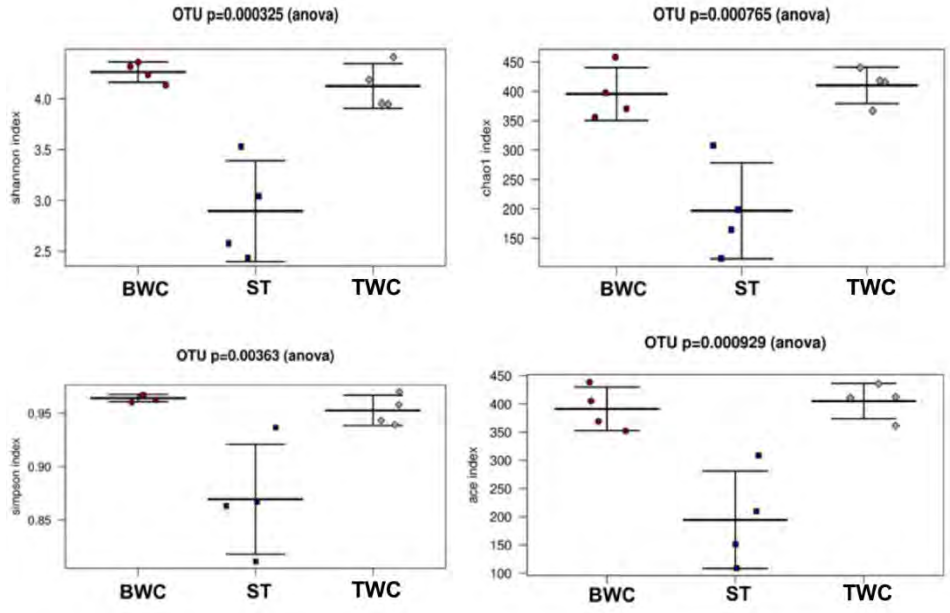

Figure 5. 2 Estimated operational taxonomy unit (OTU) richness (ACE, Chao1) and diversity indexes (Shannon, Simpson) using different methods. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

5.3.3. Taxonomic composition of the faecal bacterial communities in green turtles

The operational taxonomic units identified in the samples were assigned to 20 different bacterial phyla (Figure 5.3a). OTUs that were unable to be assigned were categorised as "Unclassified". In the Bowen-wild-captured (BWC) green turtles group, the most predominant phylum was Firmicutes, with an average relative abundance of 62.6%, followed by Bacteroidetes (27.6%) and Proteobacteria (8.8%). In the Townsville-wild-captured (TWC) group, Firmicutes (60.5%) and Bacteroidetes (31.9%) in addition to Verrucomicrobia (3.6%) and Lentisphaerae (2.7%) were the highly abundant phyla. In the stranded green turtles of Townsville (ST), the bacterial communities were dominated by the phylum Proteobacteria (47.6%), followed by Bacteroidetes (19.0%),

Firmicutes (18.7%) and Fusobacteria (13.6%) (Table 5.2). The estimated cumulative abundance of these six most predominant phyla was ≥98% of the identified OTUs across all the samples.

Bacterial diversity at the lower taxonomic level was assessed and a total of 44 classes and 89 orders were recovered from the complete dataset. At a family level, 167 families were identified and the most abundant 20 families in each sample are presented in Figure 5.3b. The BWC group of samples was dominated by Clostridiaceae (28.3%), Lachnospiraceae (22.2%) and Bacteroidaceae (19.3%), whereas the TWC group was predominantly Lachnospiraceae (28.2%), Bacteroidaceae (22.2%) and Peptostreptococcaceae (13.8%). The ST group was dominated by Enterobacteriaceae (19.4%), Bacteroidaceae (11.6%) and Moraxellaceae (10.3%) (Table S 5.3).

At genus level (Figure 5.3c), a total of 416 genera were identified. *Bacteroides* was the most predominant genus in both wild-captured and stranded green turtles. The proportion of unclassified genera ranged from 6.57% to 47.71% among the samples. The top five most abundant genera in each turtle group are shown in Table 5.3.

5.3.4. Variation in beta diversity

PCoA analysis was performed to visualize the dissimilarities in the faecal bacterial communities among different groups of green turtles. PCoA plots and hierarchical dendrograms based on Bray-Curtis distance matrices showed that the samples from the same environmental location clustered together except the samples from TS group (Figure 5.4a). The results indicated that wild-captured green turtles harboured bacterial microbial communities that were different from the stranded green turtles. ANOSIM confirmed that the difference was significant (R= 0.502, *P*= 0.001, Figure S 5.2) between wild-captured and stranded turtles while no significant difference was noticed between BWC and TWC groups of green turtles. PCoA plot constructed applying Chao1 distance matrices (Figure S 5.3) and non-metric multidimensional scaling (NMDS) (Figure 5.4b) yield similar results.

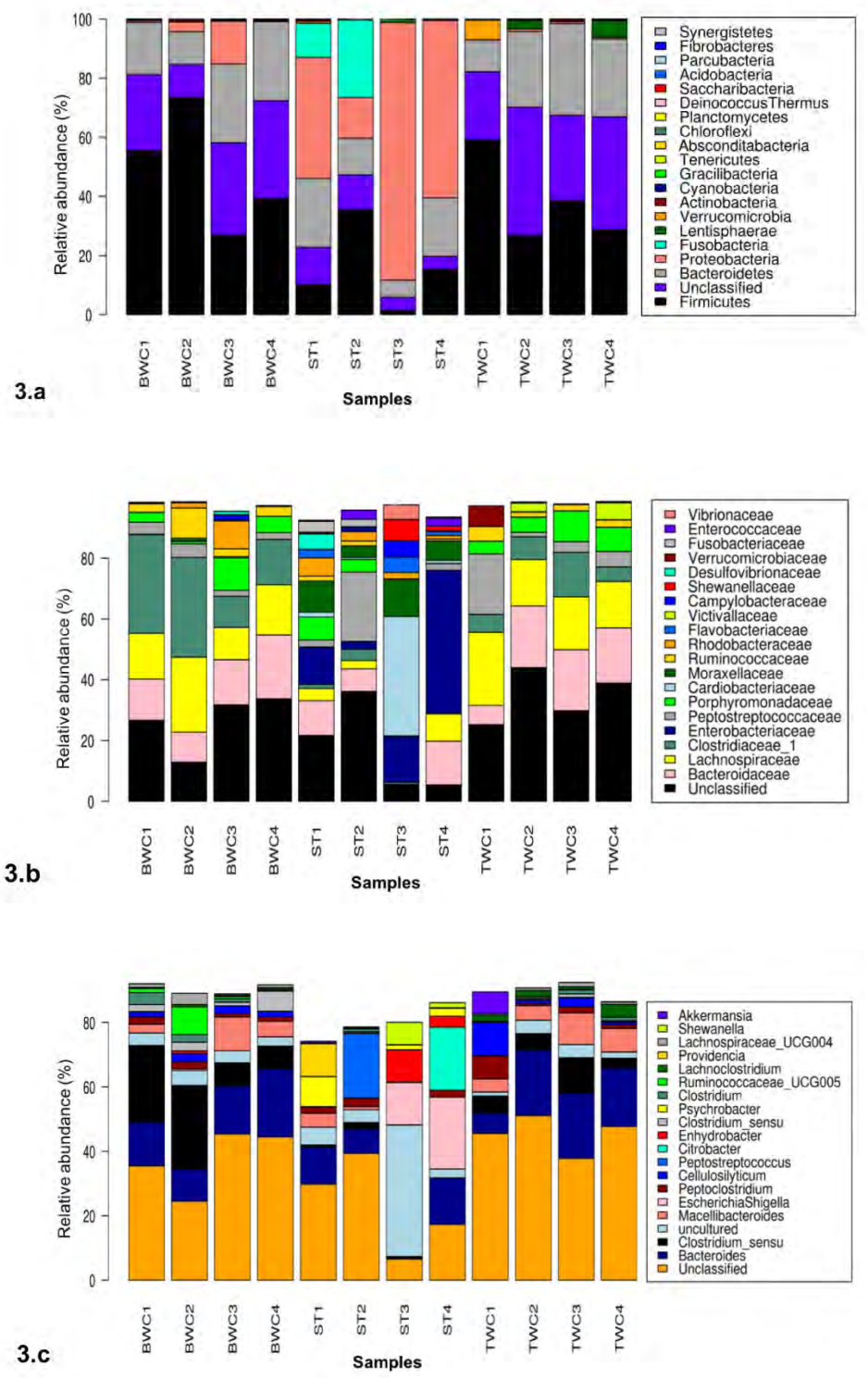

Figure 5. 3 Distribution and the relative abundance (%) of different bacterial communities in the samples from green turtles at different taxonomic level (a) phylum, (b) family, and (c) genus. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

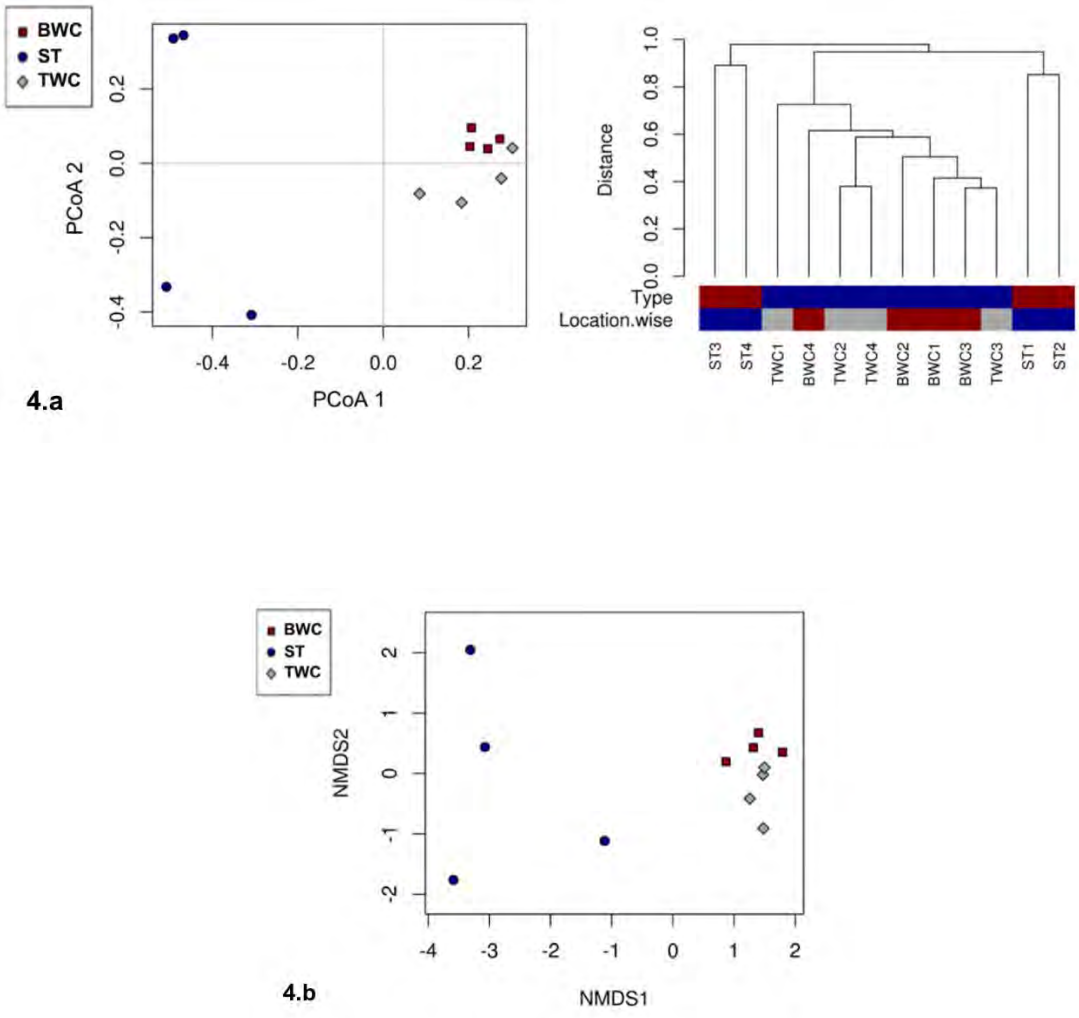

Figure 5. 4 Differential gut bacterial communities across all samples at OTU level. Principle coordinate analysis plot and hierarchical dendrogram of Bray-Curtis distances (a). Nonmetric multidimensional scaling (NMDS) plot, (b) comparing the gut bacterial communities of all samples from different study groups. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

Table 5. 2 The abundance of predominant phyla in different groups of green turtles. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

Phylum	Overall (%)	Location wise (%)			Type (%)	
		BWC	TWC	ST	Wild-captured	Stranded
Firmicutes	47.59	62.62	60.49	18.72	61.63	18.72
Bacteroidetes	26.15	27.64	31.85	19.03	29.6	19.03
Proteobacteria	18.97	8.79	0.69	47.64	5.02	47.64
Fusobacteria	4.47	0.00	0.04	13.62	0.02	13.62
Verrucomicrobia	1.24	0.02	3.64	0.28	1.7	0.28
Lentisphaerae	0.88	0.05	2.73	0.02	1.3	0.02
Actinobacteria	0.42	0.58	0.28	0.37	0.44	0.37
Cyanobacteria	0.17	0.23	0.19	0.08	0.21	0.08

Table 5. 3 The most abundant genera (% of the relative abundance of OTUs) in wild-captured and stranded groups of green turtles

Wild-captured group		Stranded group	
Phylum	**Genus (%)**	**Phylum**	**Genus (%)**
Bacteroidetes	*Bacteroides* (24.9%)	Bacteroidetes	*Bacteroides* (12.6%)
Firmicutes	*Clostridium* (18.3%)	Firmicutes	*Peptostreptococcus* (7.7%)
Bacteroidetes	*Macellibacteroides* (9.8%)	Proteobacteria	*Escherichia-Shigella* (7.5%)
Firmicutes	*Cellulosilyticum* (5.9%)	Proteobacteria	*Psychrobacter* (6.2%)
Firmicutes	*Peptoclostridium* (4.2%)	Proteobacteria	*Providencia* (5.9%)

5.3.5. Analysis of group-specific bacterial communities

Among the 527 OTUs (>1% relative abundance) identified in the study, 380 (72.1%) OTUs were strictly associated with the wild-captured turtles and 67 (12.7%) OTUs were associated with the stranded turtles (Figure 5.5a). Only 50 (9.5%) OTUs were shared among the BWC, TWC and ST groups of green turtles. Conversely, 256 (48.7%) of the total were shared between only BWC and TWC groups of green turtles (Figure 5.5a). Among the identified bacteria, a total of 25 (48. 1%) families were shared among the three groups and 14 (26.42%) families were strictly associated with the stranded green turtles group (Figure 5.5b).

ANOVA showed that several intestinal bacteria were significantly associated with specific group of green turtles. At phylum level, Proteobacteria and Chlorofexi were significantly associated with stranded turtles ($P<0.05$) and the "unclassified" OTUs were found to be significantly associated with both BWC and TWC groups of turtles. In the ST turtles, gut microbiota belonging to several families were significantly different from the BWC and TWC groups of turtles (Figure 5.6). These families are Alteromonadaceae, Enterobacteriaceae, Flavobacteriaceae, Moraxellaceae, Rhodospirillaceae, Vibrionaceae, Pseudomonadaceae and Shewallaceae ($P<0.05$, figure 5.6). At genus level, *Escherichia/Shigella*, *Psychrobacter*, *Tanacibaculum*, *Pseudomonas* and *Vibrio* predominantly showed significant association ($P < 0.05$) with the ST turtles group (Table S 5.4). Several bacteria that were found to be similar both at family and genus level in the BWC and TWC groups, were significantly different from the ST group of turtles.

A major research objective of our study was to identify whether specific faecal bacterial communities could be determined for wild-captured and stranded green turtles. To identify these bacteria, linear discriminant analysis was used to identify specific biomarker candidates belonging to different taxonomic groups (Figure 5.7). Latent Dirichlet Allocation (lda) score ≥ 4 was used as a threshold for this analysis. The results showed that the most abundant phylum in the wild-captured group was Firmicutes (class: Clostridia), while Proteobacteria (class: Gammaproteobacteria) was the most common phylum in the stranded group of turtles. In addition, several candidates from different phyla were identified in lower taxonomic level especially in stranded turtles. At family level, Flavobacteriaceae (Phylum: Bacteroidetes) and Enterococcaceae

(Phylum: Firmicutes) were identified together with the other candidates from Proteobacteria that includes Enterobacteriaceae, Cardiobacteriaceae, Moraxellaceae, Vibrionaceae, Shewallaceae, and Pseudomonadaceae. The microbial candidates for the wild-captured groups (BWC and TBC) were consistently classified with the phylum Firmicutes that include Lachnospiraceae, Clostridiaceae and Ruminococcaceae. No common group-specific phylum, family or genus was identified between the wild-captured and the stranded turtles in this analysis.

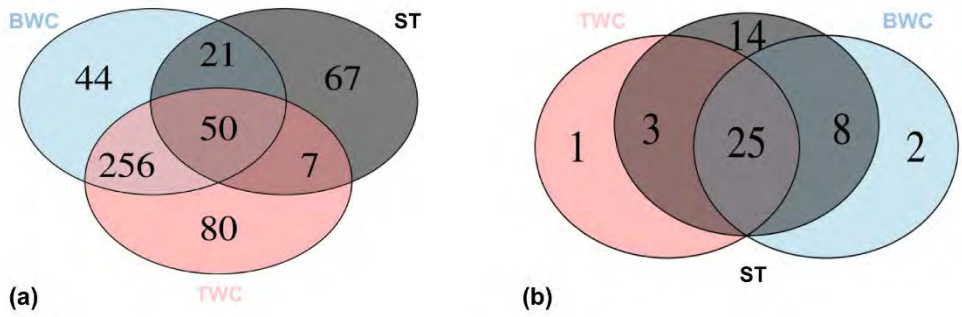

Figure 5. 5 Venn diagrams showing the number of shared and exclusive OTUs (a) and families (b) in different group of turtles. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

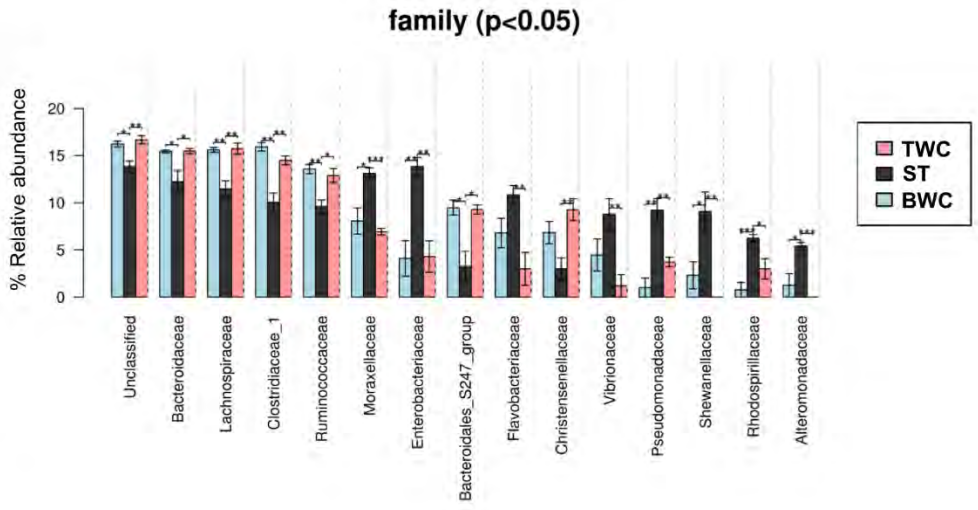

Figure 5. 6 The top 15 most significantly different families of bacterial communities in different groups of green turtles based on ANOVA. Significantly different taxa are shown as bar chart (P<0.05, ANOVA). Standard error is depicted by error bars. Pair-wise comparisons are done by t-test and annotated as *, p<0.05; **, p<0.01; ***, p<0.001. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

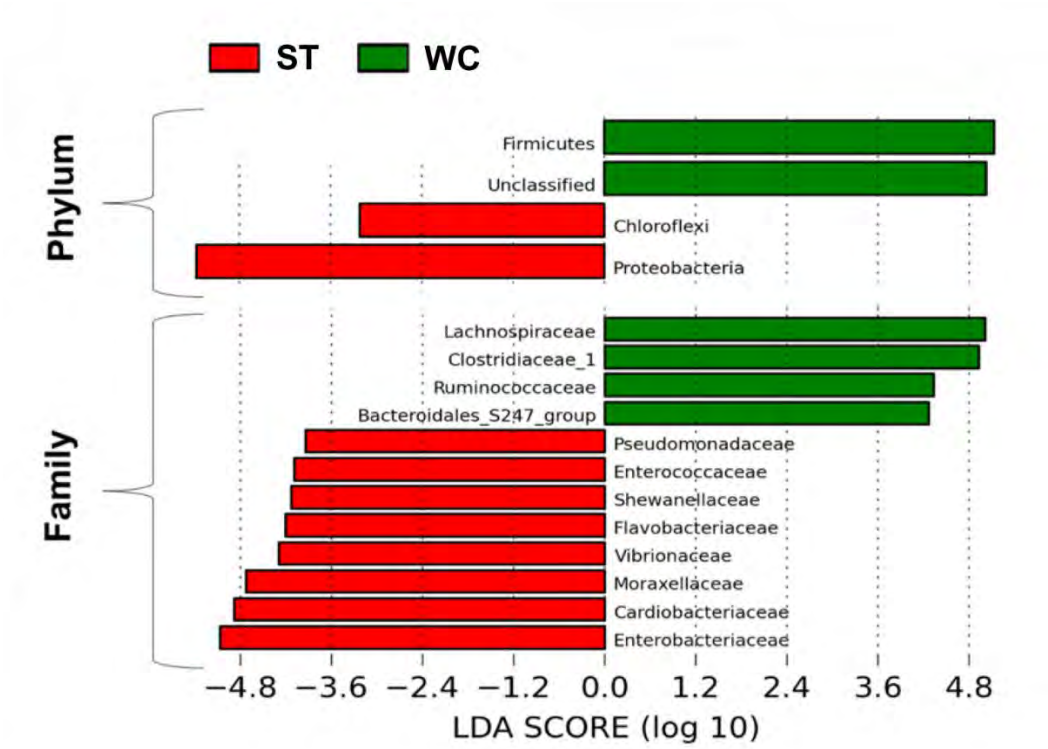

Figure 5. 7 Biomarker candidates of gut microbial communities at phylum and family levels for wild-caught (WC) and stranded (ST) groups of green turtles.

5.4. Discussion

This study presents the first detailed analyses of faecal bacterial communities of sea turtles by high-throughput sequencing technology, and provide evidence that faecal bacterial communities differ between wild-captured and stranded green turtles.

Our results suggest that wild-captured green turtles have the higher bacterial diversity and richness compared to stranded turtles. The possible explanation could be the diverse microbial requirements of herbivores to access complex carbohydrates in plant materials, they consume as a primary food source. In contrast, stranded turtles are often restricted to natural diet (seagrass) and their debilitated health state allows the opportunistic bacteria to colonise and overgrow within the GI tract. Despite slightly different phylogenetic trees, PCoA plots and NMDS plot constructed by the different statistical tests, all results clearly suggest the presence of significant differences in microbial gut compositions among the wild-captured and stranded turtles. The analysis of samples in this study indicates that the free-living green turtles of these sampling locations share similar bacterial communities, while the stranded turtles from the same environment showed distinctly different bacterial communities with higher intra-group variation. One reason for this could be due to their diseased or debilitated health conditions.

The 16S rRNA-based Illumina sequencing revealed that green turtles appear to harbour highly diverse bacterial communities largely within the four phyla Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. Other phyla including Cyanobacteria, Fusobacteria, and Verrucomicrobiota were present at variable levels. All cloacal swab samples from wild-captured green turtles were dominated by Firmicutes. In contrast, Proteobacteria was the most abundant phylum in stranded green turtles and Firmicutes was one of the three most predominant phyla. The higher abundance of Firmicutes in wild-captured turtles is consistent with the findings in other marine herbivorous reptiles (Hong et al., 2011), and mammals (Tsukinowa et al., 2008; Nelson et al., 2013; Merson et al., 2014). Preliminary studies on stranded loggerhead sea turtles (*Caretta caretta*) also showed the presence of elevated abundance of Proteobacteria (Abdelrhman et al., 2016). Firmicutes includes bacteria that were found to be the most ubiquitous and common phylum in all vertebrates due to their ability to harvest energy and absorb nutrients from ingested feed materials (Wang et al., 2016b). The predominance of the

Firmicutes present in wild-captured green turtles may reflect the natural state of turtles in good health. In addition to health status, the age, habitat, body temperature and diet has been found to influence the gut microbiome in other marine (André et al., 2005; Nelson et al., 2015) and terrestrial vertebrates and invertebrates (Guan et al., 2016; Kohl and Yahn, 2016), and might also shape the gut bacterial composition of green turtles. The presence of higher abundance of *Clostridium*, *Peptoclostridium* and *Cellulosilyticum* in wild-captured green turtles could be due to their ability to breakdown and utilise various complex plant-derived polysaccharides, such as cellulose, hemicellulose and xylan, which constitute the major part of seagrass and other plant fibers (Uffen, 1997; Uz and Ogram, 2006). The higher abundance of *Peptostreptococcus* within the GI tract of stranded green turtles may indicate possible opportunistic infection as many *Peptostreptococcus* species are recorded to cause for clinical infections in marine mammals and humans (Murphy and Frick, 2013; Nielsen et al., 2013) or alternatively it is a commensal which is an important component of the microbial communities within the GI tract (Murdoch, 1998).

The phylum Proteobacter is known member of the microbial communities within the gut of many vertebrates including sea turtles (Abdelrhman et al., 2016). Our study revealed that the faecal bacterial community of stranded turtles was significantly dominated by Proteobacteria compared to wild-captured green turtles, where a lower abundance of Proteobacteria was recorded in both BWC and TWC groups. A low frequency of Proteobacteria (0.6%) was also reported by Hong et al. (2011) within the gut of wild marine herbivorous iguanas (*Amblyrhynchus cristatus*). Tsukinowa et al. (2008) also recorded a lower percentage of Proteobacteria in wild-captured herbivorous dugongs (*Dugong dugong*). Proteobacteria is one of the most physiologically and metabolically diverse groups that has been observed to be ubiquitous in habitats such as soil (Lauber et al., 2009), water (Teeling et al., 2012; Pascault et al., 2014), plant (Redford and Fierer, 2009) and atmosphere (Després et al., 2012). Members within the phylum Proteobacteria are well-known to establish pathogenic as well as symbiotic relationships with their hosts (López-García and Moreira, 1999; Bergey and Garrity, 2004; Shin et al., 2015). Our study demonstrated that the high abundance of Proteobacteria in stranded turtles were largely due to a high proportion of Gammaproteobacteria particularly Enterobacteriaceae, Moraxellaceae, Shewanellaceae, Pseudomonadaceae and Vibrionaceae. These families were significantly associated with

ST turtles compared to other two groups BWC and TWC. Although previous studies based on culture dependent techniques reported that these microbes can be present in free-ranging wild sea turtles as a part of normal gut inhabitants (Foti et al., 2009; Al-Bahry et al., 2011), the high abundance of *Escherichia-Shigella* in this study may indicate overgrowth within the GI tract of stranded turtles which is often recorded in clinical infections in immunosuppressed animals including sea turtles (Raidal et al., 1998; Shin et al., 2015). The genus *Pseudomonas* is one of the top three causes of opportunistic infections in humans and were in higher abundance in stranded green turtles (Stover et al., 2000). Furthermore, potential pathogens within the genera *Vibrio*, *Moraxella* and *Shewanella* identified in this study can cause opportunistic infections in freshwater and marine vertebrates (Reed and Francis-Floyd, 1996; Austin and Zhang, 2006). In stranded turtles, these microbes might exacerbate underlying health conditions. However, their role within the GI tract of green turtle is unknown and further study is necessary to understand the functions of these microbes.

Bacteroidetes was one of the three most abundant phyla detected in both wild-captured and stranded green turtles. At a family level, Bacteroidaceae and Porphyromonadaceae were in significantly higher abundances in wild-captured green turtles while stranded turtles showed predominantly Flavobacteriaceae. At genus level, *Bacteroides* was the most predominant genus among all genera from different phyla identified in all turtles (Table 6). Generally, the members of *Bacteroidetes* and *Macellibacteroides* are common gut associated microbes reported in several aquatic and terrestrial vertebrates including herbivorous mammals such as the dugong (Tsukinowa et al., 2008; Hong et al., 2011; Nelson et al., 2013). In addition to *Bacteroides*, *Tenacibaculum* was another genus detected significantly within the GI tract of stranded green turtles. Little is known about their roles within the GI tract of the host including green turtle.

A lower abundance of bacteria from the phyla Actinobacteria, Verrucomicrobia and Lentisphaerae was observed in the microbial communities of both wild-captured and stranded turtles. This result is consistent with the preliminary findings in the faecal and intestinal samples of stranded loggerhead sea turtles (Abdelrhman et al., 2016). The low number of sequences of Actinobacteria and Verrucomicrobia were also reported in other marine iguanas (*Amblyrhynchus cristatus*) and mammals (*Dugong dugong*; seal, *Phoca vitulina*) (Tsukinowa et al., 2008; Hong et al., 2011; Nelson et al., 2013; Numberger et al., 2016).

Our study demonstrated that the phylum Fusobacteria was in higher abundance in stranded turtles compared to wild-captured green turtles. A higher abundance of Fusobacteria was also observed in captive (harbour) seals that were mainly fed with fish (Nelson et al., 2013; Numberger et al., 2016). While the members of the phylum Fusobacteria have been identified from both aquatic and terrestrial vertebrates including humans, the phylum is poorly-studied and comprises of approximately 32 species with an overall undefined phylogenetic position (Keenan et al., 2013; Nelson et al., 2013). The members of Fusobacteria are reported to be associated with diseased corals (Vega Thurber et al., 2009). It is hypothesised that the presence of Fusobacteria in stranded turtles could be due to a dietary shift. Stranded turtles are often experienced with unusual diets such as fish, small invertebrates or sponges which are reported to carry a higher abundance of Fusobacteria (David, 2001; Vega Thurber et al., 2009).

Most importantly, our study demonstrated that a large proportion of the gut microbes in all samples were “unclassified bacteria”. Although a proportion of unclassified reads can result from PCR or sequencing errors, such an abundance of unclassified sequences argues for a significant presence of novel/undescribed bacteria. Our finding of numerous unclassified microbes within the GI tract is consistent with the findings of others in vertebrates from different marine environments (Hong et al., 2011; Nelson et al., 2013). Future research is needed to identify these undescribed microbes within the GI tract of green turtles.

The present study investigated the core bacterial communities of green turtles, which have not previously been investigated in any sea turtle species. The core gut microbiota represents the set of most abundant microbial lineages that are mutually shared by all members from the same species. Our data indicate that Firmicutes were the main phylum represented in the specific microbial cores associated with the gut contents of wild-captured green turtles. At the family level, a total of 25 families were identified that were mutually shared by all green turtles. The four representative dominant core families within the wild-captured green turtles include Lachnospiraceae, Clostridaceae, Ruminococcaceae and Bacteroidaceae. The present study also investigated the potential biomarkers to access the health status of stranded turtles. The class Gammaproteobacteria under the phylum Proteobacteria dominated all samples from stranded turtles. In addition, the high dominance of Enterobacteriaceae, Cardiobacteriaceae, Moraxellaceae, Vibrionaceae, Flavobacteriaceae, Shewanellaceae,

Enterococcaceae and Pseudomonadaeae clearly indicates the opportunistic behaviour (i.e. overgrowth potential) of these bacteria in debilitated animals.

In conclusion, the present study aimed to investigate the faecal bacterial communities of green turtles (*Chelonia mydas*) by high-throughput sequencing analysis. The diverse bacteria inhabiting the GI tract of green turtles identified in this study may provide several important functions which are still unknown, but based on the findings in other marine and terrestrial herbivorous hindgut fermenters, they may play an important role in microbial food assimilation as well as utilisation. The predominance of Firmicutes and Bacteroidetes demonstrate the importance of these bacteria in healthy gut microbiome. The high abundance of Gammaproteobacteria in stranded turtles deserves special attention and further investigations, as the role of this bacteria within the GI tract of green turtles are still unknown. The OTUs richness reported in this study revealed the complexity of green turtle gut bacterial communities and the study provides the most comprehensive indication of this important and complex gut bacterial microbiome to date.

CHAPTER 6
COMPARATIVE ANALYSIS OF GUT BACTERIA OF GREEN TURTLES PRE-HOSPITALISATION AND POST-REHABILITATION

2.11. Introduction

Recent advances in sequencing technologies and biological computational tools have enabled the scientific communities to explore the enormous influence of host gut microbiome and their effect on health and disease states (Flint et al., 2012b). In a healthy state, gut microbes expand the host's metabolic potential by harvesting energy and nutrients from complex food particles, such as plant materials (Sommer and Backhed, 2013). For example, herbivores can maintain a diet based on plant materials by acquiring the gut microbiota capable of digesting the cellulose compounds present in plant fibers (Choat and Clements, 1998). Moreover, several studies have showed that gut microbial commensalisms and mutualisms contribute to the host development (Sommer and Backhed, 2013), vitamin synthesis (LeBlanc et al., 2013), colonisation resistance and immune homeostasis in the host (Hooper et al., 2012). Negative consequences, however, can include infection with opportunistic pathogens (Shin et al., 2015), involvement in gastrointestinal (GI) disorders (Mukhopadhya et al., 2012) and possible contributions to allergies (Bisgaard et al., 2011), obesity (Ley et al., 2006), diabetes (Vaarala, 2011), and cancer (Sobhani et al., 2011). It is therefore crucial to have a balanced gut microbiome for maintenance of normal health of the host.

Studies in vertebrates and invertebrates identified several exogenous and endogenous factors that regulate the establishment and maintenance of host gut microbiome. For example, diet (Flint et al., 2012b; Ravussin et al., 2012), host phylogeny (Benson et al., 2010), gut anatomy (Sommer and Backhed, 2013), physiology (Sommer and Backhed, 2013), reproduction (Funkhouser and Bordenstein, 2013), and antimicrobial exposures (Dethlefsen and Relman, 2011; Pérez-Cobas et al., 2012) have been identified to have effects on host microbial composition. Several environmental factors such as season (Carey et al., 2013), temperature and salinity (MacFariane et al., 1986; Sullam et al., 2012) can also influence the intestinal bacterial composition of the host.

To date, the knowledge base on the gut microbial ecology of sea turtles, including endangered green turtles (*Chelonia mydas*), is very limited. Green turtles, as a hind-gut fermenter, primarily rely on the microbial fermentation of ingested plant particles as their energy source (Bjorndal, 1985). Although recent studies have provided baseline information on the gut bacterial communities of sea turtles (Abdelrhman et al., 2016; Ahasan et al., 2017b; Price et al., 2017), their role in the event of health and disease are yet to be explored. Moreover, in the marine environment, green turtles are exposed to several infectious agents and the susceptibility of turtles to these infectious agents is likely affected by several anthropogenic stressors such as depletion of food resources, habitat degradation and pollution of the marine environment (Orth et al., 2006; Casale, 2010). Succeeding events occurring after an initial infection may lead to microbial dysbiosis, followed by gastrointestinal disorder and even death of the animal (Myers, 2004). A detailed knowledge of the host's normal gut microbiome, together with possible alterations, is crucial for the restoration of a host's gut microbiome.

Stranded sea turtles with unknown illnesses are often cared for in rehabilitation centers until they recover. Despite the substantial financial costs, sea turtle rehabilitation centers offer unique support to the sick and injured sea turtles (Baker et al., 2015). Knowledge of gut microbial communities in sea turtles would allow rehabilitation centers to choose better dietary, treatment and management procedures to limit microbiota associated diseases with early recovery (Reid et al., 2011; Costa et al., 2012). Furthermore, it will allow these centers to establish proper animal handling guidelines for hospital personnel to avoid accidental infections with potential pathogens. The objective of the present study was to characterise and compare the gut bacterial communities between pre-hospitalisation and post-rehabilitation stranded green turtles. Additionally, this study investigated the presence of any bacterial pathogens that have the potential to infect human during rehabilitation. Furthermore, the role of recovered and released turtles in spreading pathogenic microbes into their natural habitat was investigated.

2.12. Materials and Methods

2.12.1. Target population

This study was conducted under permissions of James Cook University Animal Ethic Committee (Permit no. A2101), Department of Environment and Heritage Protection

Authority (permit no. WISP15015914) and Great Barrier Reef Marine Park Authority (Permit no. G14/37285.1). In this study, samples taken from a total of four green turtles rehabilitated in the Australia's largest Turtle Hospital, ReefHQ, situated in Townsville, Qld. Two successive samples were taken from each green turtle as follows: (1) at the time of their arrival at hospital (Pre-hospitalisation [PH] samples) and, (2) before being released back to their natural habitat (Post-rehabilitation [PR] samples). The green turtles were brought from different regions of Townsville including Magnetic island, Ollera Creek, Lucinda and Toolakea beach. Immediately after their arrival at hospital, all turtles were flipper-tagged with Department of Environment and Heritage Protection (DEHP) tags. Morphometric data such as body weight and curved carapace length (CCL) were recorded in accordance to DEHP standard operating procedures (DEHP, 2013) and are presented in Table 6.1.

Table 6. 1 Identification number, age-class, length of rehabilitation and morphometric data of each green turtle at arrival and release. T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

Details	**T 1**	**T 2**	**T 3**	**T 4**
Identification number	QA42562	QA33971	QA47482	QA47535
Age-class	Sub-adult	Juvenile	Juvenile	Juvenile
Total duration at hospital (months)	~ 10	~ 4	~ 4	~ 1
Weight at arrival (kg)	38.8	8.1	9.5	6.0
Weight at release (kg)	49.3	13.2	16.0	6.2
CCL at arrival (cm)	78.9	45.5	47.6	40.8
CCL at release (cm)	79.0	47.2	48.8	41.4

The health status of the stranded turtles was assessed by an expert veterinary technician as follows: Turtle 1 was diagnosed with Floating Syndrome and turtle 2, 3 and 4 were severely dehydrated, underweight and anaemic. Heavy barnacle infestation was also recorded for turtle 3. No visible traumatic lesions were observed for any of the turtles. In hospital, all green turtles were housed separately in individual plastic tanks (~1.5m diameter and ~1.1m depth) with recirculated sea water. Water quality factors including temperature (25-27 °C), pH and salinity (30 ppt) were routinely tested. The turtles were fed human grade seafood such as squid (*Loligo opalescens)* and were rehabilitated for anywhere between less than 1 month up to 10 months. All green turtles were dewormed by praziquantal using standard oral dose @ 25-50mg/kg body weight. None of the green turtles were provided any antibiotic therapy during rehabilitation.

2.12.2. Sample collection and nucleic acid extraction

Green turtles were restrained by hand for specimen collections. A sterile cotton swab was gently inserted and rolled inside the cloaca immediately after flushing the cloacal opening with 70% ethanol to avoid external contamination. Swabs were then placed in microfuge tubes and transported on ice, directly to the laboratory at James Cook University (Townsville, QLD), where they were frozen at -80^{0}C until processing.

Nucleic acid extraction of the green turtle cloacal swab samples was performed according to the techniques described in Chapter 3.2.

2.12.3. PCR amplification and sequencing

Library preparation and sequencing was carried out by the Australian Genome Research Facility, Brisbane, Australia following the methods described in Chapter 3.2.

2.12.4. Illumina sequencing data analysis

The Quantitative Insights Into Microbial Ecology (QIIME) pipeline (version 1.9.1) was used to process and filter the de-multiplexed sequence reads (Caporaso et al., 2010). Primers were removed from both forward and reverse reads of the paired fastq files using Fastx_trimmer from the Fastx toolkit (Gordon and Hannon, 2010). The raw paired-ends fastq files were quality trimmed (q< 20) to remove low-quality and single end reads using Sickle (Joshi and Fass, 2011). The paired-ends reads were then merged using PANDAseq (Masella et al., 2012). Using USEARCH (version 8.0.1623),

sequences were quality filtered to remove the chimeric reads and full length duplicate sequences (Edgar et al., 2011). Sequences were clustered in to operational taxonomic units (OTUs) at 97% sequence similarity applying UCLUST algorithm in QIIME (Edgar, 2010). Taxonomy was assigned using Silva database (version 128, Sep 2016) (https://www.mothur.org/wiki/Silva_reference_files#Release_128). Species level identification of the selected sequences was performed by blasting against NCBI database (Pruitt et al., 2005). Species were confirmed that showed sequence similarity at least 99%.

2.12.5. Statistical analyses

Based on the relative abundance of OTUs, a new OTU table was constructed with the following criteria: (1) relative abundance of OTUs > 0.01 and, (2) samples that showed ≥ 1000 sequence reads. Microbial richness and diversity of the samples were determined by abundance based coverage estimator (ACE), Chao1, Shannon diversity index and Simpson index using R (version 3.3.0) (McMurdie and Holmes, 2013). Individual based rarefaction curves were constructed to determine whether read coverage was sufficient to delineate the gut bacterial composition of each turtle. Furthermore, conditional uncovered probability was calculated for each sample in QIIME with default parameter (r= 25). Good's coverage was estimated to evaluate the completeness of sampling. Distances between microbial communities in different samples were calculated using Bray-Curtis (Bray and Curtis, 1957) and Chao1 (Chao, 1984) distance matrices, and further visualised by Principle Coordinate Analysis (PCoA) using Calypso (Zakrzewski et al., 2017). Using Ordination based on Bray-Curtis dissimilarity matrix, we performed a non-metric multidimensional scaling (NMDS) to illustrate the relationships between PH and PR samples. An analysis of similarity (ANOSIM) was performed by applying the Bray-Curtis distance matrix to evaluate the association between PH and PR samples. A significant p-value indicated if the microbial communities were significantly different between the sample groups. Venn diagrams were constructed considering the OTUs with relative abundance >1% to visualise the amount of unique and mutually exclusive gut microbial communities in PH and PR samples. In this study, the gut microbial communities of two sample groups were compared using DESeq2 function in R software (Love et al., 2014).

2.13. Results

2.13.1. Data acquisition of 16S rRNA and analysis

Illumina sequencing of 16S rRNA gene was used to determine and compare the faecal bacterial populations of stranded green turtles between pre-hospitalisation (PH) and post-rehabilitation (PR). After filtering the low-quality reads, chimeric reads, singletons and quality trimming (quality score >33) a total of 299,523 high-quality reads were obtained from a total of eight samples. The mean read length ranged from 463 to 489 bp (Table S 6.1). In this study, all sequences were delineated into OTUs with 97% nucleotide sequence identity threshold against Silva reference database, where a total of 495 OTUs were retained after filtering the low abundant OTUs (relative abundance <1%). The top 20 most abundant OTUs are presented in Figure 6.1. Individually based rarefaction curves were generated which revealed the saturation plateau in most samples (Figure S 6.1). The estimates of the conditional uncovered probability for each sample indicated that the OTU coverage was sufficient for all samples (Table 6.2). Furthermore, the Good's coverage was > 99% for all samples, suggesting that the majority of the bacterial phylotypes are present in each sample and sufficient to describe the gut bacterial composition in each turtle (Table 6.2).

2.13.2. Bacterial diversity and richness estimation

Bacterial diversity estimated by the Shannon index varied from 2.57 to 3.94 in PH samples, and 2.95 to 3.99 in PR samples, which indicates a similar range of diversity between the two sample groups (Table 6.2). The Simpson indices were also estimated, but revealed no significant difference between the sample groups. Furthermore, bacterial richness estimators such as Chao1 and ACE analyses estimated a higher number of phylotypes, ranging between 181 to 285 and 164 to 285 respectively, than the actual observed in each library (Table 6.2). Under our sequencing depth, both richness indices revealed no significant difference between PH and PR samples of green turtles.

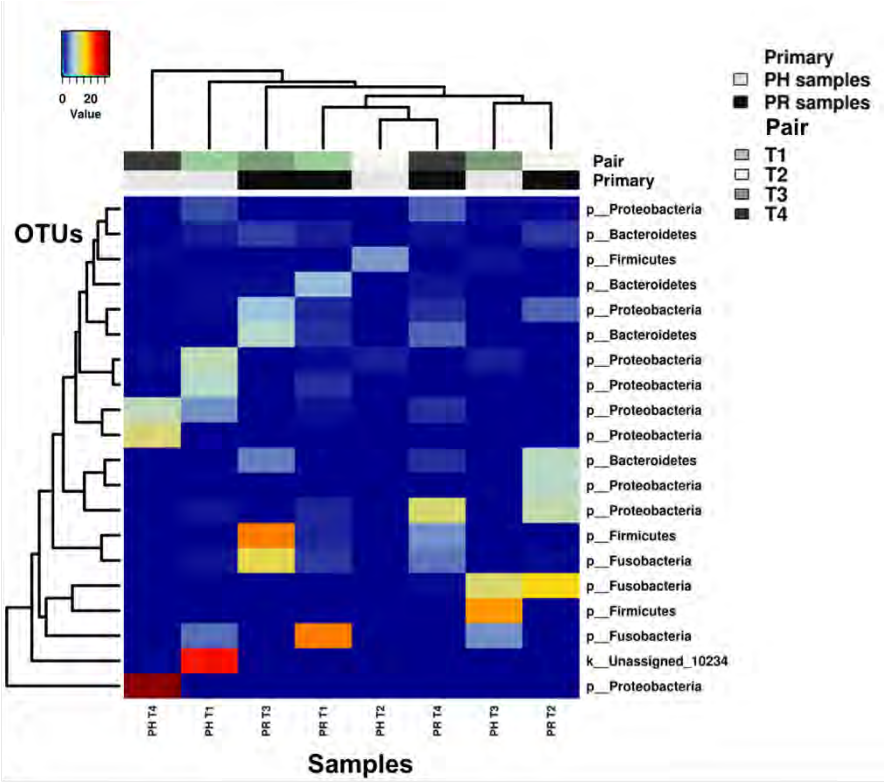

Figure 6. 1 The top 20 most abundant operational taxonomic units (OTUs) in each turtle sample. The prefix PH indicates sample collected pre-hospitalization of green turtle and PR indicates sample collected post-rehabilitation of green turtle. T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

2.13.3. Variation in bacterial gut communities between PH and PR groups

PCoA plots and hierarchical dendrograms were constructed to determine the relationships of the bacterial gut communities between PH and PR samples. The PCoA plots and hierarchical dendrograms based on Bray-Curtis (Figure 6.2) and Chao1 (Figure S 6.2) distance matrices showed that PR samples are likely to cluster together. In contrast, a higher variation was noticed in PH samples. Furthermore, an NMDS plot constructed by applying the non-metric multidimensional scaling (NMDS) technique yielded similar results (Figure 6.3). Analysis of similarity (ANOSIM) confirmed that the gut microbial communities were significantly different ($R< 0.47$, $P< 0.023$, Figure S 6.3) between PH and PR samples.

Table 6. 2 Alpha diversity metrics for the gut bacterial communities of green turtle samples. The prefix PH indicates sample collected pre-hospitalization of green turtle and PR indicates sample collected post-rehabilitation of green turtle. T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

Indices	**PH samples**				**PR samples**			
	T1	**T2**	**T 3**	**T 4**	**T 1**	**T 2**	**T 3**	**T 4**
Number of OTUs	193	158	226	124	250	199	119	240
Shannon index	3.13	3.94	3.39	2.57	3.81	3.40	2.95	3.99
Chao1index	262.87	189.13	272.57	205.00	285.79	272.58	181.00	255.04
ACE index	250.29	187.95	270.84	201.05	285.59	244.71	164.74	257.35
Simpson index	0.09	0.02	0.06	0.14	0.05	0.06	0.08	0.03
Good's Coverage (%)	99.8	99.9	99.8	99.7	99.9	99.8	99.9	99.9
Conditional Uncovered Probability (CUP)								
PE	0.003	0.003	0.003	0.005	0.002	0.002	0.003	0.002
Lower bound	0.001	0.000	0.000	0.001	0.000	0.001	0.000	0.000
Upper bound	0.006	0.002	0.004	0.008	0.004	0.005	0.003	0.003

2.13.4. Gut bacterial community structure of green turtles

The taxonomic classification of the nucleotide sequences from the study samples resulted in 26 different, mostly rare, phyla. The sequences unable to be assigned were categorized as “Unclassified”. In PH samples, the most predominant phylum was Proteobacteria (33.6%), followed by Firmicutes (25.5%), Bacteroidetes (14.4%) and Fusobacteria (9.1%) (Figure 4a). Proteobacteria (36.9%) was also the most abundant phylum in PR samples, however, the second most abundant was Bacteroidetes (25.4%), followed by Fusobacteria (16.1%) and Firmicutes (14.2%) (Figure 6.4a). The estimated cumulative abundance of these four predominant phyla was above 98% of the identified OTUs. Moreover, the intra-phylum variation of the four predominant phyla was higher in PH samples compared to PR samples (Figure 6.4b).

In our study, a total of 57 bacterial classes were identified, and both groups of samples which were dominated by Bacteroidia, Gammaproteobacteria, Fusobacteria, Alphaproteobacteria and Clostridia. A total of 174 families were identified from the complete dataset and the top 20 most abundant families in each sample are presented in figure 6.5. *Bacteroides* was the most prevalent genus in both PH and PR samples (Table 6.3). It is worth noting that *Escherichia coli*, *Vibrio harveyi, V. owensii* and *V. parahaemolyticus* were predominantly identified in the PH turtle 4 sample. The higher abundance of *Campylobacter fetus* was detected in the PH turtle 2 sample. *Clostridium botulinum* was present only in the PH turtle 3 sample. More interestingly, *Salmonella enterica* was only identified in PR turtle 1 sample.

2.13.5. Comparison of the bacterial population between PH and PR groups of samples

A Venn diagram showed that only 73 (21.0%) of the 347 OTUs were common between PH and PR samples, while 126 (36.3%) and 148 (42.7%) OTUs were strictly associated with PH and PR samples, respectively (Figure 6.6). 52 (12.5%) OTUs were mutually shared in all PR samples while only 10 (2.1%) were common in PH samples (Figure S4). At genus level, almost half (46.7%) of all genera were unique to either PH or PR samples (Figure 6.6).

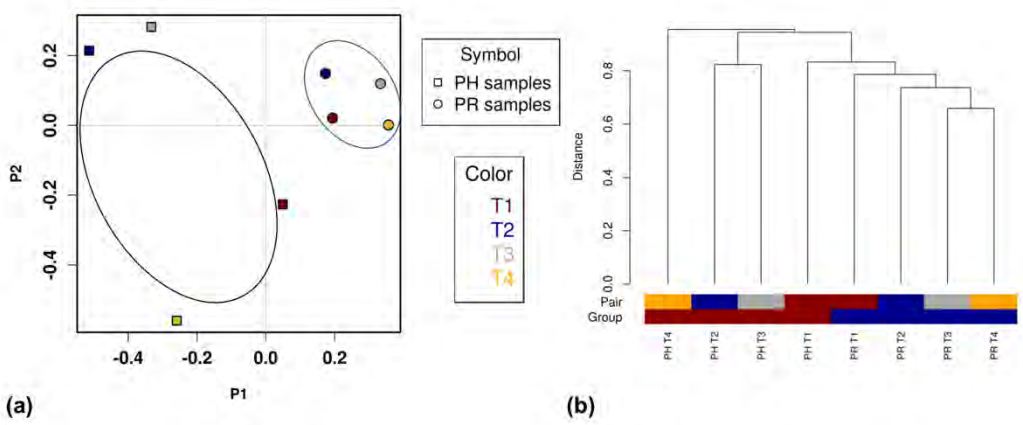

Figure 6. 2 (a) Principle coordinates analysis (PCoA) and (b) hierarchical dendrogram between pre-hospitalization (PH) and post-rehabilitation (PR) samples of green turtles using Bray-Curtis distance matrix. T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

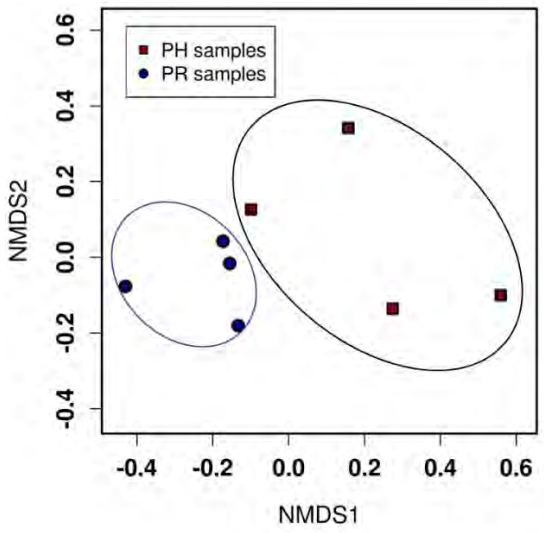

Figure 6. 3 Non-metric multidimensional scaling (NMDS) analysis of the dissimilarity in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR).

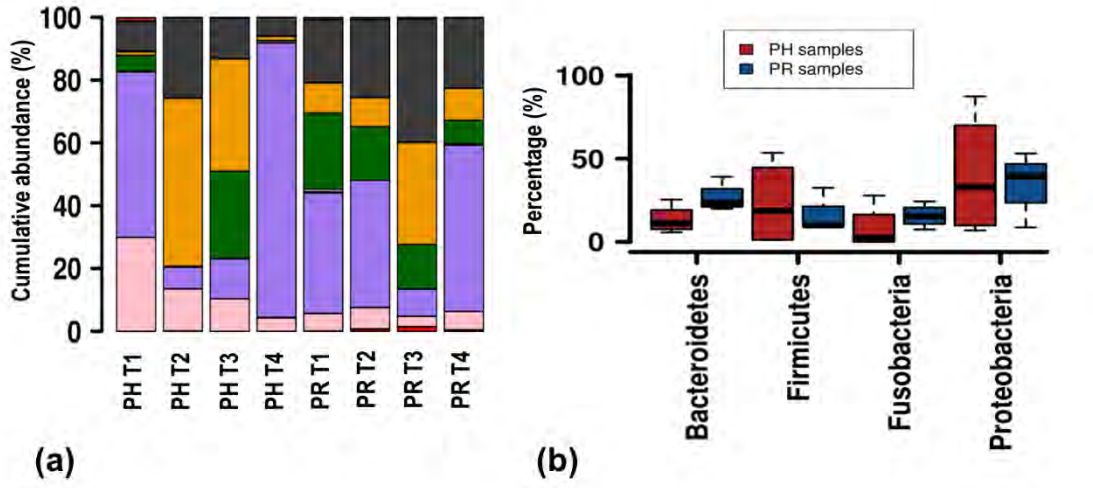

Figure 6. 4 Composition of green turtle gut microbiomes. (a) Cumulative abundance (%) of different phyla present in samples from green turtle (b) Intra-phylum variation of the most abundant phyla present in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR). T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

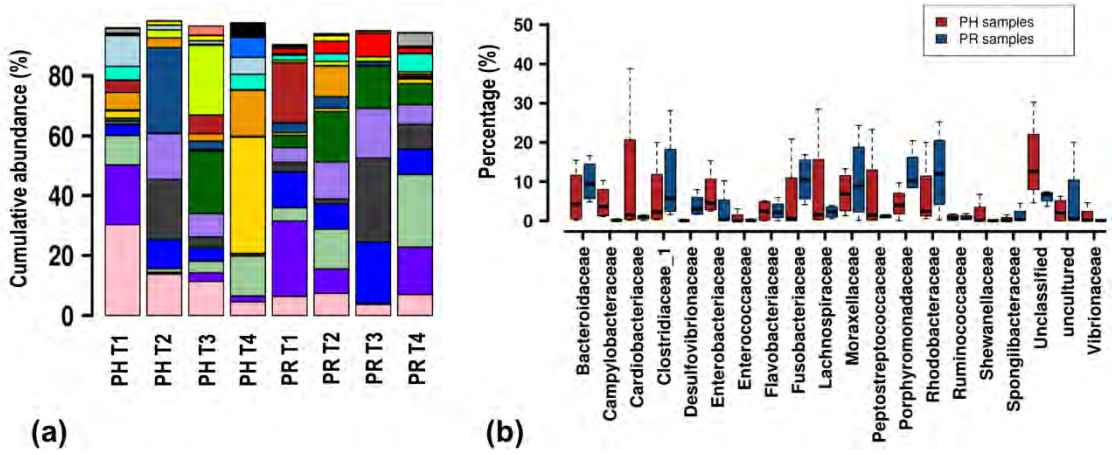

Figure 6. 5 Compositions and intra-family variations of the top 20 most abundant families present in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR). T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

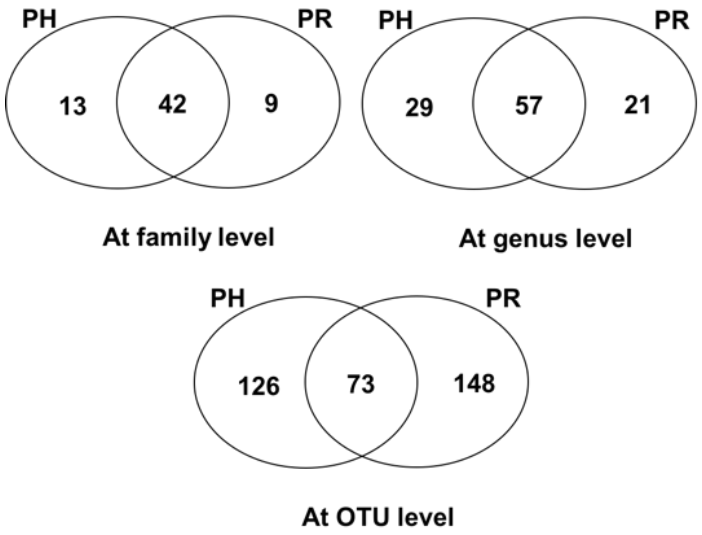

Figure 6. 6 Venn diagrams represent the number of shared and exclusive families, genera and OTUs between pre-hospitalization (PH) and post-rehabilitation (PR) samples of green turtles.

Our analysis showed that none of the predominant bacterial phyla that include Proteobacteria, Firmicutes, Bacteroidetes and Fusobacteria were significantly ($P > 0.05$) different between PH and PR samples. In contrast, lower abundance phyla were significantly associated with one of the treatments. For example, Lentisphaerae was significantly ($P < 0.026$) higher in the PH samples and Parcubacteria predominantly showed significant ($P < 0.05$) association with the PR samples. However, it is worth noting that the significant abundance of Parcubacteria in the PR group may be skewed by Turtle 1. At a class level, the relative abundance of Epsilonproteobacteria was significantly ($P< 3.1E-06$) higher in the PH samples and Deltaproteobacteria exhibited significant ($P< 6.9E-06$) association with PR samples. Although the abundance of Gammaproteobacteria was not statistically different between the two groups of samples, families within the class Gammaproteobacteria such as Shewanellaceae, Cardiobacteriaceae and Vibrionaceae were significantly ($P< 0.05$) higher in PH samples compared to PR samples (Table S 6.2). Moreover, the *Campylobacteraceae* was also significantly ($P< 0.05$) associated with the PH samples. In contrast, bacteria within the Verrucomicrobiaceae, Desulfovibrionaceae, Clostridiales_vadinBB60 group, Rhodocyclaceae, Rikenellaceae and Erysipelotrichaceae exhibited significant ($P< 0.05$) association with PR samples (Table S 6.2). Furthermore, several bacterial genera were found to be significantly associated among the sample groups. For example, *Campylobacter*, *Citrobacter*, *Escherichia*, *Edwardsiella*, *Mobilitalea*, *Shewanella*, *Peptostreptococcus*, *Eubacterium*, *Arcobacter*, *Fonticella* and *Vibrio* showed significant ($P < 0.05$) association with the PH samples (Table 6.4). In contrast, the relative abundance of *Sarcina*, *Akkermansia* and *Coprococcus* were significantly higher in the PR samples (Table 6.4).

Table 6. 3 The abundance of predominant genera (% of OTU) at pre-hospitalization (PH) and post-hospitalization (PR) of green turtles

PH samples		PR samples	
Genus (%)	**Phylum**	**Genus (%)**	**Phylum**
Bacteroides (6.8)	Bacteroidetes	*Bacteroides* (9.2)	Bacteroidetes
Peptostreptococcus (5.6)	Firmicutes	*Parabacteroides* (7.9)	Bacteroidetes
Cetobacterium (5.3)	Fusobacteria	*Psychrobacter* (7.7)	Proteobacteria
Clostridium sensu stricto *1* (4.4)	Firmicutes	*Sarcina* (6.0)	Firmicutes
Campylobacter (4.0)	Proteobacteria	*Fusobacterium* (5.0)	Fusobacteria

Table 6. 4 The top 15 most significantly different genera in pre-hospitalization (PH) and post-rehabilitation (PR) samples of green turtles

Genus	P value	P Bonferroni	FDR value	PH samples mean	PR samples mean
Sarcina	1.40E-06	1.80E-04	1.80E-04	3.5	1530.75
Mobilitalea	2.80E-05	3.60E-03	1.10E-03	571.75	0
Edwardsiella	2.80E-05	3.60E-03	1.10E-03	253.25	0
Akkermansia	3.50E-05	4.50E-03	1.10E-03	0.75	151.5
Coprococcus_1	2.80E-04	3.60E-02	7.20E-03	1.5	127.25
EscherichiaShigella	5.70E-04	7.30E-02	1.20E-02	470.5	11.5
Citrobacter	6.90E-04	8.90E-02	1.30E-02	395.75	4
Cellulosilyticum	1.10E-03	1.40E-01	1.80E-02	67.25	0
Shewanella	1.20E-03	1.60E-01	1.80E-02	246.75	17.5
Campylobacter	1.50E-03	2.00E-01	2.00E-02	963.75	38.25
Peptostreptococcus	1.90E-03	2.50E-01	2.20E-02	1353.5	1.25
Eubacterium	2.80E-03	3.60E-01	2.70E-02	31	0
Arcobacter	3.00E-03	3.90E-01	2.70E-02	172	15.75
Fonticella	3.30E-03	4.30E-01	2.70E-02	40.25	0
Vibrio	3.40E-03	4.30E-01	2.70E-02	168.75	16

2.14. Discussion

Our results demonstrated that bacteria within the phylum Proteobacteria were predominant in both pre-hospitalisation (PH) and post-rehabilitation (PR) samples of green turtles. The presence of Proteobacteria in the samples is concordant with the findings of other studies in stranded sea turtles using culture dependent techniques (Foti et al., 2009; Ahasan et al., 2017a). However, the high-throughput sequencing data on wild free-ranging green turtles revealed that Firmicutes was the most predominant phylum, followed by Bacteroidetes and Proteobacteria (Ahasan et al., 2017b). Abdelrhman et al. (2016) reported Proteobacteria was the second most abundant phylum in stranded loggerhead (*Caretta caretta*) turtles. The high prevalence of Proteobacteria within the gastrointestinal (GI) tract is one of the recognised signature of dysbiosis as well as indication of disease in animals, including humans (Shin et al., 2015). Several researchers have identified an unusual expansion of different members of Proteobacteria in various disease conditions such as immune (Carvalho et al., 2012), metabolic (Zhang et al., 2012) and GI disorders (Dworkin et al., 2006; Liou et al., 2013). However Proteobacteria, as a physiologically and metabolically diverse group, can play a vital role in preparing the juvenile gut through consuming oxygen, altering the gut pH, and producing carbon dioxide and nutrients for successive colonisation by strict anaerobes (Wilson, 2005; Chow and Lee, 2006). In addition, they are also well-known for utilisation of a wide variety of carbon compounds (Bergey and Garrity, 2004).

The high abundance of Proteobacteria in the study samples was largely due to the higher proportion of Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria. Noteworthy, Epsilonproteobacteria was significantly associated with the PH samples while Deltaproteobacteria were predominantly found in PR samples. Epsilonproteobacteria is a diverse bacterial group that has been primarily recognised as a group of pathogenic organisms (Nakagawa and Takaki, 2009; Röling, 2015). Several members of this group are well-known pathogens which mainly colonise the GI tract of many animals, including humans (Cornelius et al., 2012; Röling, 2015). The most prominent representatives of human and animal pathogens belong to the order Campylobacterales and include *Campylobacter*, *Helicobacter*, *Wolinella* and *Arcobacter* (Bocian-Ostrzycka et al., 2015). Among these genera, *Campylobacter* and

Arcobacter were significantly associated with PH samples of stranded turtles. The higher abundance of *Campylobacter fetus* in turtle 2 may indicate the possible overgrowth of the bacteria because they are frequently reported in enteritis, bacteremia, abortion, endocarditis or meningitis in both humans and animals (Simor et al., 1986; Monno et al., 2004; Ausselet et al., 2009). Studies in humans demonstrated that GI infections with the major pathogenic species such as *Campylobacter jejuni* and *C. coli* may lead to severe autoimmune disorder in immunocompromised patients (Cornelius et al., 2012). Although no information is available on *C. fetus* in sea turtles, Wang et al. (2013) reported the presence of *C. fetus* in pet reptiles where most of the *C. fetus* strains were genetically distinct from classically mammalian *C. fetus*.

In the present study, the high abundance of Deltaproteobacteria in PR samples was mainly due to a larger proportion (98%) of the genus *Bilophila*. Bacteria within the genus *Bilophila* are strict anaerobes and contribute less than 0.01% of the normal gut microbiota in human (Baron, 1997). *Bilophila wadsworthia* was the only pathogenic species recovered from the clinical specimens of patients with perforated gangrenous appendicitis (Baron, 1997), however, little is known about their composition and role in terrestrial and aquatic animals including green turtles but they do produce 2-sulfoacetaldehyde at sites of inflammation. More detailed investigation is required to judge the role of *Bilophila* spp. in health and nutrition of green turtles.

Our results demonstrated that the relative abundance of several genera within the class Gammaproteobacteria differed significantly between PH and PR samples. Bacteria within the genera *Escherichia, Edwardsiella, Citrobacte*r, *Shewanella* and *Vibrio* were significantly higher in abundance in PH samples. The presence of *Escherichia coli, Vibrio harveyi, V. owensii* and *V. parahaemolyticus* in PH turtle 4 might indicate their overgrowth, followed by opportunistic infections in potentially immune-compromised, stranded turtles. This finding is congruent with the findings of Work et al. (2003) who identified several species of *Vibrio* from free-ranging green turtles that had been diagnosed with bacteraemia and fibropapillomatosis. Zavala-Norzagaray et al. (2015) reported the presence of different antibiotic resistant *Vibrio* species that have zoonotic potential in sea turtles. The presence of *Escherichia coli* in sea turtle is also reported in other studies (Foti et al., 2009; Ahasan et al., 2017a). Furthermore, our results revealed the presence of *Salmonella* spp. in PR turtle 1 sample, although it was low in abundance. The presence of *S. enterica* in only PR sample might indicate the possibility

of acquiring this bacterium via food or from the environment during rehabilitation. This finding deserves considerable attention because it raises the question about what role rehabilitated turtles play in spreading infectious agents after their release.

The phylum Firmicutes was the second most abundant microbiota in PH turtles while a lower frequency was noticed in PR turtles. The predominance of Firmicutes present in PH turtles may be associated with their wild feeding habits, foraging mainly on sea grasses. Among the identified Firmicutes, the genera *Clostridium*, *Peptostreptococcus*, *Cellulosilyticum*, *Mobilitalea* and *Eubacterium* predominated in PH samples. In contrast, the genera *Sarcina* and *Coprococcus* significantly were prevalent in PR samples. The members of Firmicutes are well-known to harvest energy and assimilation nutrients from the fibrous food particles. These genera possess the ability to break down and utilise complex carbohydrates, such as cellulose, hemicellulose and xylan present in the seagrasses and other plant fibers (Uffen, 1997; Uz and Ogram, 2006). The phylum Firmicutes has been reported as one of the most predominant gut inhabitants of many terrestrial and aquatic vertebrates, including sea turtles (Nelson et al., 2013; Merson et al., 2014; Abdelrhman et al., 2016); however, several genera within the phylum Firmicutes were also reported in clinical infections. For example, *Peptostreptococcus* and *Eubacterium* identified in the PH samples are frequently reported in clinical infections in immune-compromised hosts (Fouad et al., 2003; Murphy and Frick, 2013). Noteworthy, a significant proportion of *Peptostreptococcus* was identified from turtle 3 only, in addition to another pathogen *Clostridium botulinum*, which is well-known for the production of botulinum, the neurotoxin responsible for botulism in animals and humans (Cherington, 2004). A higher abundance of *Coprococus* spp. identified in PR samples is rarely reported in clinical infections (Murphy and Frick, 2013). The genus *Sarcina spp.* was highly prevalent in PR samples of stranded turtles. Although the members of this genus are considered to be normal GI inhabitants (Crowther, 1971), *Sarcina ventriculi* was reported in a clinical infection of emphysematous gastritis in a human (Laass et al., 2010). Therefore, it can be suggested that future research should be focused on species level identification of this bacterium to determine their exact role within the GI tract of green turtles.

In our study, a marked shift in the abundance of the phylum Bacteroidetes was clearly observed between PH and PR samples. Bacteria within the phylum Bacteroidetes are known to be common gut associated microbiota in many vertebrates, including sea

turtles (Nelson et al., 2013; Abdelrhman et al., 2016). The higher abundance of Bacteroidetes in PR turtles was largely due to the outcome of improved health and dietary shift during rehabilitation. At the ReefHQ turtle hospital, sea turtles are mostly fed on a high protein diet such as squid (*Loligo opalescens*). It has been reported in humans that a high protein diet can increase the abundance of Bacteroidetes within the GI tract (Wu et al., 2011). A similar finding was also reported in bar-headed geese where Bacteroidetes abundance was significantly higher in artificially reared geese compared to the wild population (Wang et al., 2016c). Despite the differences in abundance of Bacteroidetes within the GI tract of green turtles, the genus *Bacteroides* was present in equally high numbers in both PH and PR samples. The members of Bacteroidetes possess diverse gene-encoding carbohydrate active compounds, which allow them to switch easily between different energy sources using complex regulatory mechanisms within the GI tract depending on availability (Xu et al., 2007; Thomas et al., 2011). It is hypothesised that the emergence of an increased abundance of Bacteroidetes in PR turtles may contribute to the green turtle adaption to the digestion and utilisation of available food resources. Further study is warranted to explore the exact association between a high protein diet and the abundance of Bacteroidetes within the gut of green turtles.

Fusobacteria was another bacterial phylum detected in high abundance in both PH and PR turtles without any significant difference between two groups. It is noteworthy that the genus *Cetobacterium* showed a similar abundance in both PH and PR samples while *Fusobacterium* predominated in PR samples. Both *Cetobacterium* and *Fusobacterium* have been reported as common gut inhabitants of several aquatic mammals and fishes (Roeselers et al., 2011; Nelson et al., 2013; Larsen, 2014). *Cetobacterium* can produce vitamin B12 at high efficiency which may be of particular relevance to turtle nutrition. Moreover, bacteria within both genera are able to produce butyrate as an end-product of carbohydrate fermentation (Bennett and Eley, 1993). In mammals, it has been reported that butyrate provides the majority of energy supply to the gastrointestinal cells and acts as an anti-inflammatory and anti-carcinogenic compound (McBain et al., 1997; Von Engelhardt et al., 1998; Andoh et al., 1999). Ortin et al. (2012) demonstrated the ability of butyric acid to inhibit the potential freshwater fish pathogens; however, several studies also reported that a number of Fusobacteria species can act as potential pathogens to animals as well as humans (Langworth, 1977; Roberts, 2000).

A low abundance (<0.01%) of bacteria from the phyla Actinobacteria, Lentisphaerae and Parcubacteria was noticed in both PH and PR turtles. The presence of Actinobacteria and Lentisphaerae was also reported by earlier studies in stranded loggerhead turtles (Abdelrhman et al., 2016). The significant association of the phylum Parcubacteria with PR samples was due to the higher abundance of the bacteria in Turtle 1 for unknown reasons. Parcubacteria have been identified in a wide range of anoxic environments (Nelson and Stegen, 2015). Bacteria within this phylum lack biosynthetic and DNA repair capabilities (Kantor et al., 2013). The presence of potential attachment and adhesion proteins suggest that these bacteria can be ectosymbionts or parasites of other living organisms (Nelson and Stegen, 2015).

Principle coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) analysis clearly revealed that there was a marked difference between PH and PR samples of green turtles. All PR samples were clustered together tightly, irrespective of their bacterial compositions at pre-hospitalisation. Furthermore, our OTU based bacterial diversity analyses revealed that only 21% OTUs were common between PH and PR samples while there were still a vast number of unique OTUs present in both PH and PR samples. This indicates a marked shift in the gut bacterial communities in rehabilitated turtles. Green turtles reared in individual water tank during rehabilitation were less likely to experience diverse bacterial communities compared to wild turtles. Moreover, transient bacteria, as well as bacteria associated with their food (e.g. squid), could also influence the observed individual bacterial variation. It is hypothesised that bacterial structure within the gut of green turtles can be influenced by several determinants such as selective pressure within the gut, immune-response, water quality and feeding habits, as shown in other animals (Huertas and Michán, 2014; Wang et al., 2016b).

In conclusion, despite the small sample size and high variation among the individuals, our high-throughput sequencing data revealed important information that increases the understanding of the underlying factors responsible for low success rates of sea turtle rehabilitation. Since rehabilitation of stranded turtles frequently requires extensive investment of resources and personnel, these findings will help the rehabilitators to develop cost-effective treatment and management procedures for early recovery of stranded sea turtles during rehabilitation. Our present findings, combined with our previous study (Ahasan et al., 2017b), revealed that bacterial gut compositions differ

markedly among free-ranging wild populations, PH stranded turtles and PR stranded turtles. The marked differences in gut bacterial compositions may be of dietary (high protein diet), environmental or management origin. It is therefore suggested to offer a herbivorous diet, such as green vegetables, to the stranded green turtles to support the restoration of normal gut microbiota during rehabilitation. The predominance of Proteobacteria in PH stranded turtles indicates microbial dysbiosis, followed by a deteriorating health condition of the green turtles. The higher abundance of Fusobacteria in PR turtles deserves future study to understand the role of these bacteria within the GI tract of green turtles. The presence of human pathogenic bacterial species in stranded turtles warrants considerable attentions and further investigation to explore the role of the stranded turtles spreading these bacteria to humans, or on the other hand, immune-compromised turtles may get human pathogens from the environment. It is also important to understand the capacity of restoring normal gut microbiota of recovered green turtles which are released back to their natural habitat.

CHAPTER 7
CHARACTERISATION AND COMPARISON OF THE MUCOSA-ASSOCIATED BACTERIAL COMMUNITIES ACROSS THE GASTROINTESTINAL TRACT OF STRANDED GREEN TURTLES

7.1. Introduction

Microbial communities inhabiting the gastrointestinal (GI) tract comprise a complex ecosystem and play a vital role in maintaining host physiology, ranging from metabolic activity to host immune homeostasis (Dethlefsen et al., 2007; Chung and Kasper, 2010). They are also involved in many functions that are not encoded in the host‘s DNA (Gill et al., 2006). In recent decades, several studies on gut microbiota have confirmed that a balanced gut microbiome is essential for the host's ability to maintain a healthy state. Perturbations in the stability of gut microbial communities dispose the host to pathogenic invasions which may lead to several GI diseases and disorders (MacFarlane and Macfarlane, 2009; Sobhani et al., 2011; Vaarala, 2011; Sartor and Mazmanian, 2012). Knowledge of microbial community and population dynamics in symbiosis, as well as in dysbiosis, are essential for developing management strategies for treating GI associated diseases and disorders.

Recent advancement in the molecular biology and computational techniques have empowered the scientific community to explore several influencing factors on the establishment and maintenance of host gut microbiome. Studies revealed that diet is one of the primary drivers of functional capacity within the GI tract, resulting in a convergence of bacterial communities and the phylogenetically un-related host (MM et al., 2013; Power et al., 2014). Besides diet, several host factors such as physiology, gut structure and genetics can also shape gut microbial communities (Benson et al., 2010; Sommer and Backhed, 2013). Studies in terrestrial and aquatic animals revealed that several environmental factors such as habitat, temperature and salinity can influence the microbial community compositions within the GI tract of the host (MacFariane et al., 1986; Sullam et al., 2012; Carey et al., 2013).

To date, large scale research on host gut microbiome has been conducted on several aquatic and terrestrial mammals (MM et al., 2013; Nelson et al., 2013; Mao et al., 2015), fishes (Li et al., 2015), birds (Wang et al., 2016b; Wilkinson et al., 2016; Yang et al., 2016) and to a lesser extent invertebrates (Li et al., 2007; Li et al., 2016a; Souza et al., 2016). Very few studies have investigated marine reptiles, including the endangered green turtles (Eigeland et al., 2012; Price et al., 2017). Green turtles are long-distance migrators, hind gut fermenters that forages primarily on sea grasses (Bjorndal et al., 1997). The gut microbiota of green turtles are believed to play a crucial role in the turtles gaining energy from their food sources (Bjorndal, 1979b; Karen et al., 1991). Moreover, they can also contribute to several other aspects of their health and development of disease, as noted in other animals (Guarner and Malagelada, 2003; Costa et al., 2012; Mao et al., 2015). Therefore, detailed understanding of resident gut bacterial communities along the digestive tract of green turtles is very important to develop strategies to treat gut associated disorders and restore the host's normal gut microbiome during rehabilitation.

Most investigations of the green turtle's gut microbiome have typically involved bacterial identification in faeces either by culture-dependent (Santoro et al., 2006a; Ahasan et al., 2017a) or independent techniques (Ahasan et al., 2017b; Price et al., 2017). Faecal samples only represent the overall gut microbiota rather than bacterial communities of specific anatomical regions of the GI tract (Li et al., 2017). Furthermore, it is hypothesised that faecal microbiota represents a pool of both resident and transient microbial population in the GI tract (Eckburg et al., 2005). The mucosal microbiota is more likely to be resident and will have a higher potential to influence the host than transient microbial populations and is therefore of more interest to intestinal health (Kelly et al., 2016). In terms of bacterial identification, culture based approaches allow only for assessment of a small proportion of bacterial communities where a large proportion of the bacterial microbiome remains un-culturable and hence unknown (Daly et al., 2001; Eckburg et al., 2005). Culture-independent molecular approaches are therefore highly suitable for research on microbial diversity, this combined with selection of appropriate samples within the GI tract allow a detailed understanding of the gut microbiome. The aim of this study was to characterise and compare the mucosa-associated bacterial communities across different regions of the GI tract of green turtles using high-throughput sequencing analysis.

7.2. Materials and Methods

7.2.1. Target population and sample collection

Stranded green turtles are occasionally cared for in rehabilitation centers until they recover. In this study, samples were taken from a total of four stranded green turtles that were collected from two different beaches of the central Great Barrier Reef that include Airlie Beach and Whitsunday Beach (Table 7.1). These turtles died shortly (1 to 7 days) after arriving for rehabilitation. During rehabilitation, turtles were fed human grade seafood such as squid (*Loligo opalescens)*. The freshly dead turtles were kept frozen (below -20^0 C) for further post-mortem examination. The health status, curved carapace length (CCL) and body weight were recorded in accordance to DEHP (Department of Environment and Heritage Protection) standard operating procedures upon the turtle's arrival to the rehabilitation center (DEHP, 2013) (Table 1). Turtle 1 was underweight and emaciated. Turtle 2 was determined to have a healed boat-strike scar on the carapace. No visible traumatic injuries were recorded for turtle 3, however, it was emaciated and heavily infested by barnacles. Turtle 4 had a swollen right front flipper with a respiratory problem.

During the necropsies, samples were collected from four different regions of the GI tract, including the oesophagus, stomach, small intestine (duodenum) and large intestine (caecum) of the animals. Using a sterile scalpel blade, the luminal cavity of each GI region was opened and the gut contents were separated from the mucosal wall. A sterile cotton swab was thoroughly rubbed against the outer mucosal layer of the gut lumen with minimum contamination by gut contents. Swabs were placed in microfuge tubes, transported on ice within 4-6 hrs directly to the laboratory at James Cook University (Townsville, QLD), and frozen at -80 ^{0}C until processing. This study was conducted under permit from James Cook University Animal Ethic Committee (Permit no. A2101), Department of Environment and Heritage Protection Authority (permit no. WISP15015914) and Great Barrier Reef Marine Park Authority (Permit no. G14/37285.1).

7.2.2. Extraction of bacterial DNA, PCR amplification and sequencing

Bacterial DNA extraction from the specimens, PCR amplification and sequencing of the bacterial 16S rRNA gene were done following the procedure described in Chapter 3.2.

7.2.3. Bioinformatic analysis

All bioinformatics analyses were performed using the software package "The Quantitative Insights Into Microbial Ecology (QIIME v1.9.1)" (Caporaso et al., 2010). A unique barcode was used to label each sample and Illumina sequencing resulted in 1,722,641 paired-end reads from a total of 16 samples. The raw paired-ends Fastq files were quality trimmed (q< 20) to remove low-quality and single end reads using Sickle (version 1.33) (https://github.com/najoshi/sickle). The forward and reverse reads were assembled using PANDAseq Assembler (Masella et al., 2012). Using USEARCH (version 8.0.1623), sequences were quality filtered to remove the chimeric reads and full length duplicate sequences (Edgar et al., 2011). Sequences were then clustered into operational taxonomic units (OTUs) using UCLUST taxonomy assigner in QIIME. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. The reads that failed to hit the reference database were subsequently clustered as *de novo*. Using QIIME, taxonomy was assigned using Silva database (version 128, Sep 2016) (https://www.mothur.org/wiki/Silva_reference_files#Release_128). The sequences that were unable to assign to any known taxa were categorized as "Unclassified".

7.2.4. Statistical analyses

A new OTU table was constructed considering OTUs that showed a relative abundance > 1% and samples that retained > 1000 sequence reads. Bacterial count data were then normalised by cumulative sum-scaling (CSS) and $\log_2$ transformation. The abundance based coverage estimator (ACE), Chao1 and Shannon diversity indices were used to determine bacterial richness and diversity of samples using R software (version 3.3.0) (McMurdie and Holmes, 2013). One-way ANOVA was used to test for differences in the bacterial diversity/richness among different GI regions. Individually based rarefaction curves were constructed to determine whether sampling yielded sufficient OTU coverage to delineate the gut bacterial composition of each turtle. Furthermore, conditional uncovered probability (CUP) and Good's coverage were estimated for each sample in QIIME. Distances between bacterial communities of different samples of different GI regions were calculated using weighted UniFrac distance matric (Lozupone and Knight, 2005) and further visualised by Principle Coordinate Analysis (PCoA) in Calypso (Zakrzewski et al., 2017). The analysis of similarity (ANOSIM) was performed

by applying Bray-Curtis (Bray and Curtis, 1957) and weighted UniFrac (Lozupone and Knight, 2005) distance matrices to evaluate the association between bacterial communities of different GI regions. The taxa level differences among bacterial communities of different GI regions were assessed by ANOVA and Wilcoxon signed-rank test. A significant p-value ($P< 0.05$) indicated if the taxa were significantly different between GI regions. P-values were also adjusted for multiple testing by the false discovery rate (FDR) using Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Venn diagrams were constructed to visualise the amount of unique and mutually shared gut bacterial communities in different GI regions.

Table 7. 1 Identification number, age-class and morphometric data of each green turtle at arrival and release. T1: turtle 1, T2: turtle 2, T3: turtle 3 and T4: turtle 4

Details	T 1	T 2	T 3	T 4
Identification number	QA54944	QA56724	QA56888	QA56344
Location	Airlie Beach, QLD	Whitsunday Beach, QLD	Whitsunday Beach, QLD	Whitsunday Beach, QLD
Age-class	Juvenile	Juvenile	Juvenile	Adult
Body weight (kg)	6.1	8.2	12	100
CCL (cm)	48	48.2	52.3	102

Table 7. 2 Alpha diversity metrics for the bacterial communities across different regions of the gastrointestinal (GI) tract at an evolutionary distance D= 0.03. Values indicates means and standard errors derived from total samples for each GI regions. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Indices	ES	ST	SI	LI
Sequences	48535.50 ± 10864.05	37154.00 ± 2571.05	24939.25 ± 3098.35	41552.50 ± 10551.54
OTUs	109.25 ± 19.01	114.00 ± 9.14	64.25 ± 6.49	123.00 ± 29.16
Chao 1 index	144.80 ± 22.71	139.86 ± 12.77	87.07 ± 11.33[a]	157.41 ± 31.99
ACE index	148.61 ± 25.40	138.05 ± 13.10	90.62 ± 11.93[b]	157.28 ± 28.99
Shannon index	4.91 ± 0.47	4.33 ± 0.40	2.82 ± 0.63[c]	4.24 ± 1.01
Good's coverage	0.97 ± 0.01	0.97 ± 0.01	0.97 ± 0.01	0.96 ± 0.01
Conditional Uncovered Probability (CUP)				
PE	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.04 ± 0.01
Lower bound	0.004 ± 0.002	0.003 ± 0.001	0.004 ± 0.001	0.006 ± 0.002
Upper bound	0.042 ± 0.016	0.028 ± 0.006	0.040 ± 0.014	0.056 ± 0.017

Note: Superscript '[a, b]' indicate Chao 1 and ACE index of SI were significantly different from ST indices (ANOVA, $P < 0.05$). '[c]' indicates Shannon index of SI was significantly different (ANOVA, $P< 0.05$) from ES and ST.

7.3. Results

A total of 703,799 high-quality sequences obtained after assembly and quality filtering were used for downstream analyses. The mean sequence length ranged from 434 to 500 bp (avg. 468.43 bp). Individual samples were covered by an average 43,987 reads (ranging from 15,125 to 75, 952). The highest number of reads was recorded for the oesophageal samples. All sequences were delineated into OTUs with 97% nucleotide sequence identity threshold, where 23,396 unique OTUs were identified in total and 407 OTUs were retained after filtering the low abundant OTUs (relative abundance < 1%). A total of 62 OTUs were identified that retained above 1,000 sequences (up to 57,928 sequences). The highest number of OTUs were recorded for large intestinal (LI) samples (123.00 ± 29.16) while a lower number of OTUs (64.25 ± 6.49) were in the small intestinal (SI) samples of green turtles (Table 7.2). The individually based rarefaction curves were constructed that tended to approach saturation plateau in our current sequencing depth (Figure S 7.1). The mean conditional uncovered probability (PE) for different GI region samples ranged from 0.02 to 0.04 (Table 7.2) and the Good's coverage was > 92% (ranging from 92 to 99%) for all samples, suggesting the presence of sufficient phylotypes for the current analysis although more phylotypes may be recovered by increasing the sequencing depth (Table 7.2).

7.3.1. Assessment of bacterial richness and diversity

The Chao 1 and abundance-based coverage estimators (ACE) were used to evaluate the community richness of each sample from different regions of GI tract (Table 7.2). Bacterial richness was lower in the small intestinal samples compared to oesophageal, gastric and large intestinal samples. However, no significant difference (P> 0.2) was found between observed OTUs and actual OTUs, estimated by Chao 1 and ACE estimators for any GI region. Bacterial diversity as estimated by the Shannon index (SI) revealed significant difference between oesophageal and small intestinal samples (t test, P= 0.037). Additionally, the gastric and large intestinal mucosa also harboured a relatively higher level of diverse bacteria than the small intestinal mucosa. However, at this sequencing depth, oesophageal samples showed the highest bacterial diversity (mean SI 4.91 ± 0.47) compared to other samples from different GI regions.

7.3.2. Variation in bacterial microbiomes across the GI tract

Although several bacterial communities exhibited a consistent presence across the length of the green turtles' GI tract, the overall composition of mucosa-associated bacterial microbiome was found to be significantly associated with GI regions (ANOSIM R: 0.28, $P < 0.05$, Bray-Curtis, figure S 7.2). The strongest association between bacterial microbiomes and GI regions was recorded for the small and large intestinal mucosa-associated bacterial communities (ANOSIM R: 0.469, $P < 0.05$, Bray-Curtis). PCoA plot and hierarchical dendrograms were constructed based on weighted UniFrac distance metric, clearly demonstrated that the majority of large intestinal samples were clustered together and distinctly separated from small intestinal samples although a higher variation was noticed for small intestinal samples (Figure 7.1). The oesophageal and gastric samples were clustered closely to one another and clearly separated from small intestinal samples.

7.3.3. Comparison of mucosa-associated bacterial communities of different regions of the GI tract

Phylum-level analysis of the mucosa-associated bacterial communities of green turtle GI tract revealed a total of 30, mostly rare, bacterial phyla. The majority of the bacterial community present within the GI tract belonged to Firmicutes (57.8%) and Proteobacteria (21.3%), while the rest were distributed amongst Actinobacteria (6.4%), Bacteroidetes (3.6%), Fusobacteria (2.4%), Spirochaetae (0.19%), Saccharibacteria (0.16%), and unclassified bacteria (7.9%) (Figure 7.2a). Other phyla, such as Cyanobacteria, Thermotogae, Tenericutes, Synergistetes, Gracilibacteria, Chloroflexi, comprised <0.25% of total composition. Oesophageal and gastric mucosa harboured the majority of the identified phyla (25 and 23 of the 30), while the lowest number of bacterial phyla were observed in the small intestinal mucosa. Among the phyla identified in this study, only Firmicutes, Proteobacteria and Actinobacteria were present in samples of all GI regions. These phyla were ubiquitously distributed in the GI tract whilst the relative abundance varied between the gut sites. The phylum Firmicutes was significantly abundant in oesophageal (30.3%), gastric (85.1%) and large intestinal (86.5%) mucosa-associated microbial communities, while a drastically lower abundance was observed for the small intestinal bacterial population (Figure 7.2b). The relative abundance of Proteobacteria was significantly higher ($P < 0.05$) in small intestinal

microbial communities than other GI regions (Figure 7.2b). In summation, Proteobacteria showed an inverse profile to Firmicutes and was the second most abundant phylum across the GI tract. The relative abundance of Actinobacteria, Bacteroidetes and Fusobacteria was higher in oesophageal mucosa-associated bacterial communities compared to other GI regions (Figure 7.2a).

A total of 192 families were identified from the complete data set. The 20 most abundant families (Figure 7.3) belonged to nine different classes that include Clostridia, Gammaproteobacteria, Bacilli, Actinobacteria, Bacteroidia, Alphaproteobacteria, Coriobacteria and Erysipelotrichia (Figure 7.4). Clostridia was one of the most abundant bacterial classes in all anatomic regions, except the small intestine, where a significantly lower ($P< 0.05$) abundance of Clostridia was observed. Clostridia was mainly comprised of seven families, Peptostreptococcaceae, Lachnospiraceae, Clostridiaceae 1, Ruminococcaceae, Eubacteriaceae, Family XIII and Family XI that accounted for an average 45.6% of the total identified families. Bacteria within the Peptostreptococcaceae were significantly abundant ($P< 0.007$) in gastric and large intestinal samples compared to oesophageal and small intestinal samples (Figure 7.5). A high abundance ($P= 0.02$) of Lachnospiraceae was observed only in large intestinal samples compared to oesophageal and small intestinal samples. Bacteria within the Ruminococcaceae was significantly lower ($P= 0.02$) in the oesophageal mucosa compared to other GI regions. The relative abundance of Gammaproteobacteria was higher in small intestinal samples without any significant difference ($P= 0.064$) to other GI regions. Gammaproteobacteria includes Aeromonadaceae, Vibrionaceae, Enterobacteriaceae and Xanthomonadaceae, all of which were predominant in small intestinal samples except Xanthomonadacea*e*, found only in oesophageal samples. The prevalence of Bacilli was lower in the large intestinal mucosa compared to other GI regions. A higher abundance of Bacteroidia (Porphyromonadaceae and Marinilabiliaceae) was observed within the oesophageal and gastric mucosa-associated bacterial communities (Figure 7.5). Likewise, Alphaproteobacteria (Rhodobacteraceae) and Actinobacteria (Dietziaceae and Propionibacteriaceae) were absent in large intestinal samples, but were higher in abundance in oesophageal samples. The Campylobacteraceae, Helicobacteraceae, Acidaminococcaceae, Desulfobulbaceae, Flavobacteriaceae and Marinilabiaceae significantly prevailed in the oesophageal mucosa-associated bacterial communities. Our analysis revealed that one or two

families represented the majority of OTUs and were highly abundant in the turtles. For example, Vibrionaceae 17162, *Aeromonas* 19906, uncultured *Clostridium* sp., Peptostreptococcaceae 17846 and Clostridiaceae 1 5902 comprised of 5.3%, 4.6%, 4.5%, 4.2%, 3.3%, and 2.0% of the total sequences respectively. At the genus level, 452 taxa were identified across the GI tract of green turtles while more than 35.8% of the total sequences were not identified at the genus level.

Venn diagrams were constructed to determine the unique and mutually shared bacterial taxa present across the GI tract of green turtles. Our results showed that only 12 (6%) of the total OTUs (relative abundance > 1%) were common in all gut regions, while 38 (19.1%), 6 (3%), 23 (11.6%) and 57 (28.6%) OTUs were strictly associated with oesophageal, gastric, small and large intestinal bacterial communities respectively (Figure 7.6a). Moreover, eight of the 12 common OTUs were within the phylum Firmicutes and four OTUs represented Proteobacteria, Bacteroidetes, Fusobacteria and Cyanobacteria respectively (Table S 7.7). Seventeen (8.5%) of the total OTUs were common between small intestinal and large intestinal bacterial communities. At the family level, 19 (38%) families were shared among all GI regions and 32 (64%) families were exclusively shared between oesophageal and gastric mucosa-associated microbiomes (Figure 7.6a). Seven of the 11 phyla (relative abundance >1%) were distributed across the GI tract of green turtles while three phyla were exclusively present in oesophageal and gastric mucosa-associated bacterial communities (Figure 7.6a). It is worth noting that inter-turtle variations were also observed from the phylum to OTU levels of the bacterial communities of green turtles (Figure 7.6b). Only six of the total (407) OTUs were found in all green turtles, where five represented the phylum Firmicutes.

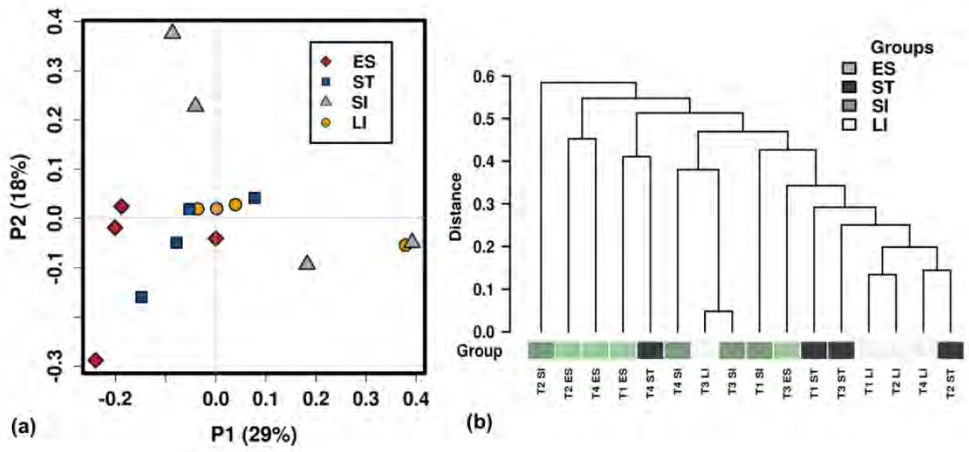

Figure 7. 1 Principle coordinates analysis (PCoA) of the dissimilarity among samples from different regions of gastrointestinal tract of green turtles. (a) PCoA plots and (b) hierarchical dendrograms were constructed using weighted UniFrac distance matric. T1: turtle 1, T2: turtle 2, T3: turtle 3, T4: turtle 4, ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine.

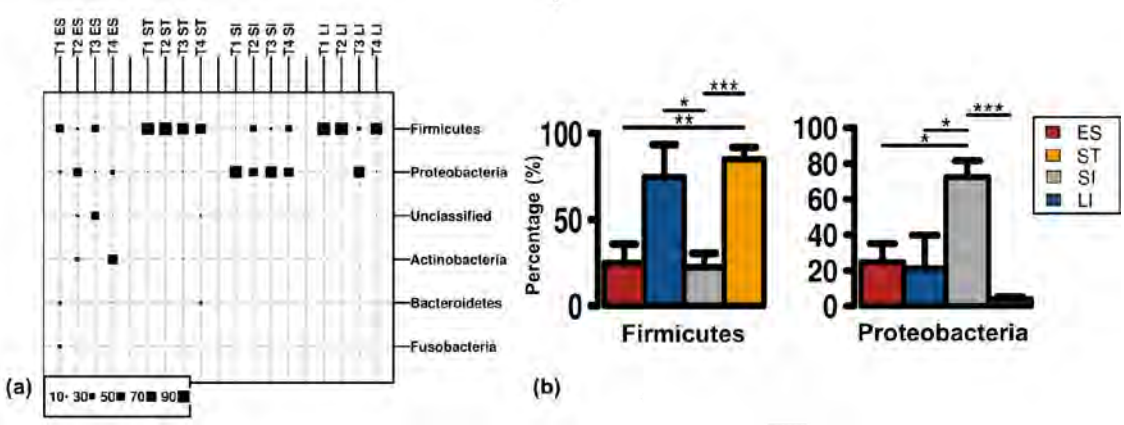

Figure 7. 2 Composition of green turtle gut microbiomes. (a) cumulative abundance (%) of the bacterial communities at phylum level across different regions of the gastrointestinal (GI) tract. (b) Relative abundance of two most predominant phyla across the GI tract. T1: turtle 1, T2: turtle 2, T3: turtle 3, T4: turtle 4, ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine. Significantly different regions are shown in bar chart using Wilcoxon signed- rank test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Standard error is depicted by error bars.

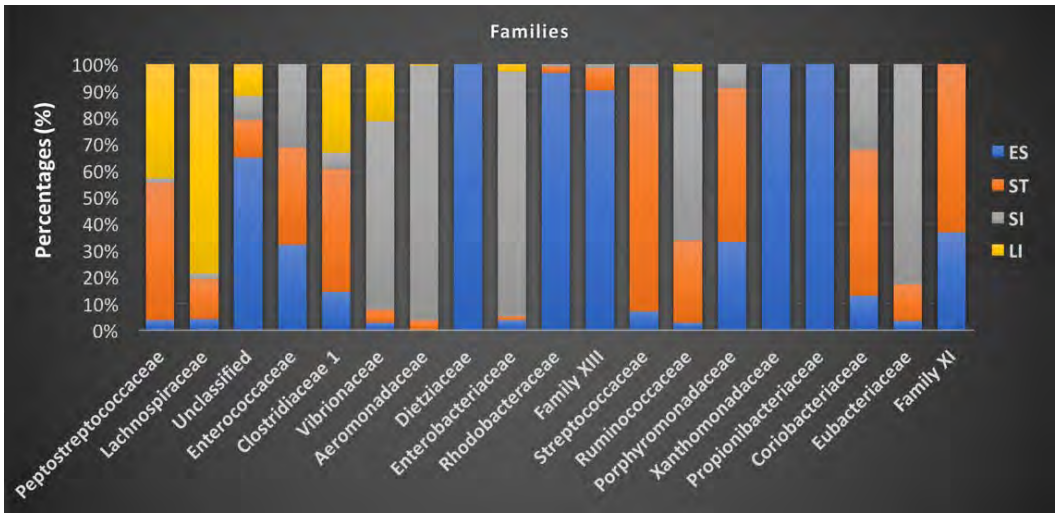

Figure 7. 3 The top 20 most abundant families across different regions of the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

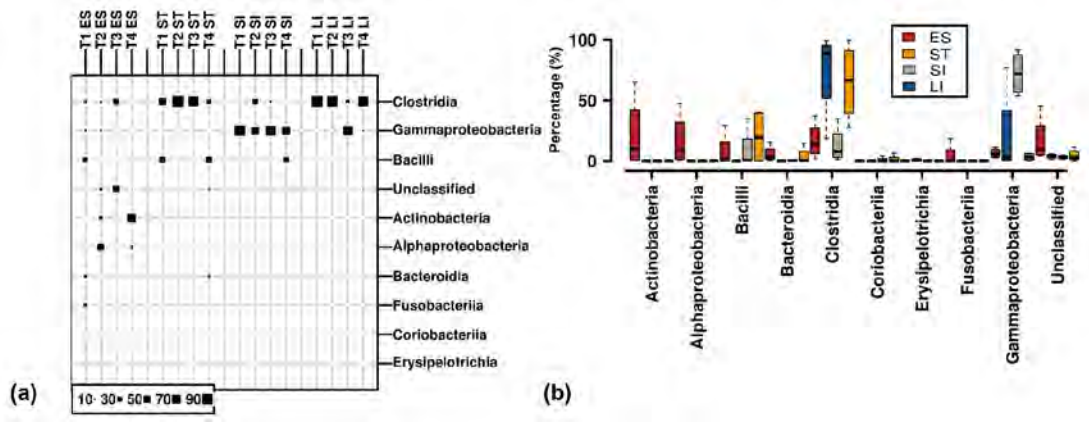

Figure 7. 4 Composition (a) and intra-class variations (b) of top 10 most abundant classes across different regions of the gastrointestinal tract of green turtles. T1: turtle 1, T2: turtle 2, T3: turtle 3, T4: turtle 4, ES: Esophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

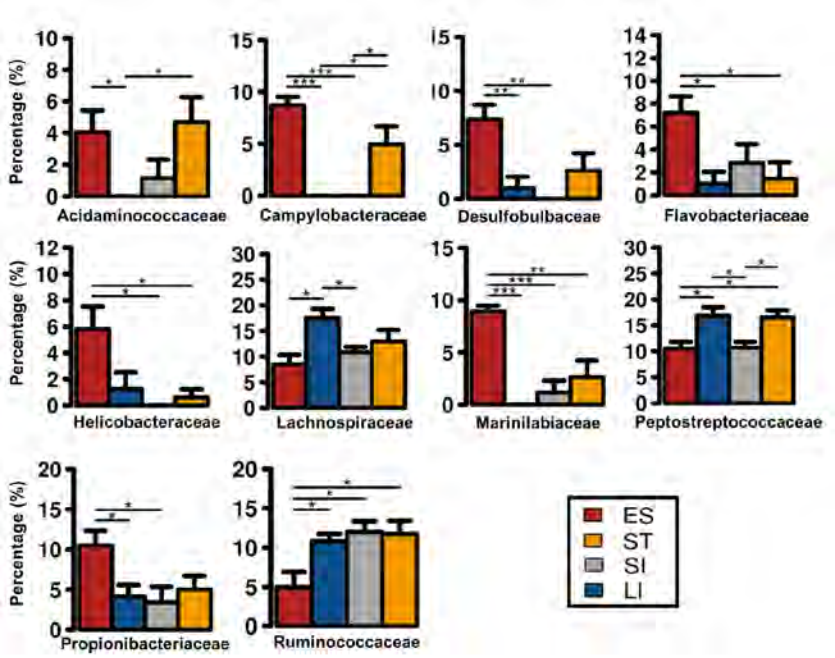

Figure 7. 5 The top 10 most significant families across the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine. Significantly different regions are shown in bar chart using Wilcoxon signed-rank test (*, p<0.05; **, p<0.01; ***, p<0.001). Standard error is depicted by error bars.

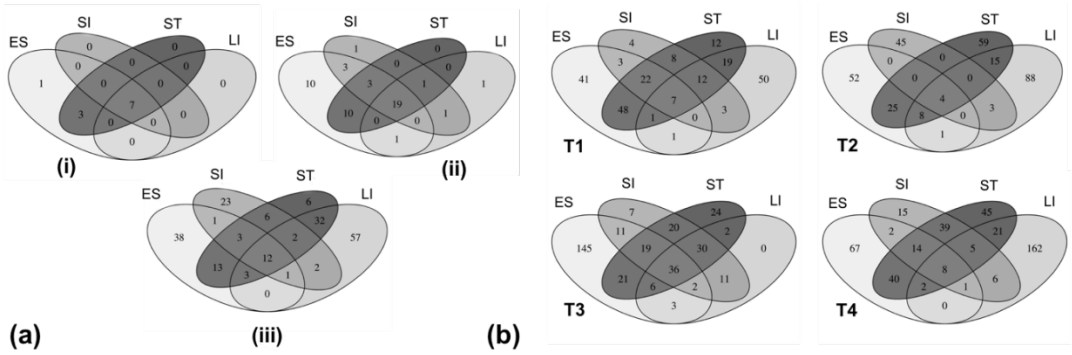

Figure 7. 6 Venn diagrams represent the numbers of total shared and exclusive taxa. (a) Taxa at phylum (i), family (ii), and OTU (iii) level within the different gut regions of green turtles. (b) Taxa at OTU level in each green turtle. T1: turtle 1, T2: turtle 2, T3: turtle 3, T4: turtle 4, ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

7.4. Discussion

To date, little is known about the diverse gut microbiota of endangered green turtles in contrast to the extensive knowledge of their ecology, as well as distribution (Bjorndal, 1995; Lutz et al., 2002). Knowledge of the microbial community is imperative to understand the host-microbial interaction in both healthy and diseased animals. The present study explored the community composition and structure of the mucosa-associated bacterial microbiomes across the length of gastrointestinal (GI) tract of stranded green turtles using high-throughput sequencing and identified an enormous diversity of microbes.

As turtles are endangered species, it is close to impossible to obtain permits to kill healthy turtles for research purposes in Australia. The samples for this study were therefore collected from turtles that died within one week of entering rehabilitation and without having consumed the food offered. Although the underlying causes of the strandings may have varied between the 4 turtles and thereby influenced the intestinal microbiota in different ways, there was little variation between samples collected in the four regions of the intestinal tract and it was therefore decided to proceed to make general conclusions based on these samples although the bacterial flora described in these animals may not entirely reflect the flora of clinically healthy animals.

Gut bacterial diversity decreased across the longitudinal axis of the green turtle GI tract from oesophagus to small intestine while the large intestine showed a higher bacterial diversity compared to the small intestine but less than the stomach. This finding is in accordance with findings in terrestrial herbivores, such as cattle, where bacterial diversity was higher in the stomach compared to small intestinal mucosa (Mao et al., 2015). The presence of highly diverse bacterial communities in the oesophageal and gastric mucosa could be due to the existence of transient microbiota. Highly diverse bacterial communities in sea water might influence the diversity of bacterial microbiomes in the oesophageal mucosa of green turtles because turtles are continuously ingesting bacteria associated with their food and water (Pedrós-Alió, 2006; Zinger et al., 2011). These findings support the theory speculating that the mouth is an access point for gut microbes (highest diversity) with selection for a subgroup of the cumulative diversity appearing as these microbes pass through the GI tract. However, studies in laboratory mice revealed that the microbial diversity in gastric and small

intestinal mucosa were similar to the large intestinal mucosa (Gu et al., 2013). The presence of a diverse bacterial community in the large intestinal mucosa of green turtles indicates the occurrence of a more complex micro-ecosystem that might be associated with nutrient absorption and assimilation (Flint et al., 2012b) and possibly the availability of oxygen (Byndloss et al., 2017). A similar finding was also observed in the colonic mucosal samples of rat and humans (Li et al., 2017).

The GI mucosa-associated microbiota of green turtles were largely dominated by Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Fusobacteria and a considerable number of unclassified bacteria. However, the detailed composition of these phyla at a lower taxonomic level differed notably in different GI regions. Our results demonstrated that Firmicutes were the predominant phylum across different regions of the GI tract mucosa except in the small intestine, where Proteobacteria prevailed. The higher prevalence of Firmicutes was supported by findings in other studies on green turtle using cloacal swab samples (Ahasan et al., 2017b; Price et al., 2017) as faecal swab represents the overall gut microbial population of green turtles. Members of Firmicutes have been found to be associated with harvesting energy and absorption of nutrients through microbial fermentation of food components (Turnbaugh et al., 2008; Power et al., 2014). Studies suggested that the caecum is the initial site for this fermentation activity while little digestion and absorption may take place in the stomach and small intestine (Bjorndal, 1979b; Karen et al., 1991). In green turtles, examination of the GI ingesta demonstrated that many of the plant cell materials remained undigested until it reached the caecum where microbial fermentation occurs (Bjorndal, 1979b). Our current study revealed that several families within the phyla Firmicutes, such as Ruminococcaceae, Lachnospiraceae, and Peptostreptococcaceae, was significantly elevated in the large intestinal mucosa while Peptostreptococcaceae and Ruminococcaceae also dominated the gastric mucosa. This finding is concordant with findings in terrestrial herbivores, such as cattle (Mao et al., 2015), and pigs (Kelly et al., 2016). Bacteria within these families are known to proliferate by hydrolyzing dietary fibre and complex carbohydrates (Kim et al., 2011) with Ruminococcaceae and Lachnospiraceae being major butyrate produces that reduce leeky gut syndrome, reduce available oxygen in the gut and prevent pathogenic Proteobacteria from dominating the gut (Byndloss et al., 2017). Peptostreptococcaceae can also play a vital role in feed degradation and digestion (Mao et al., 2012; Kim et al., 2014). However, bacterial

community structure and membership within the GI tract can be influenced by factors such as, physiochemical conditions along the GI tract including GI motility, pH and GI secretions. A recent study using humanized mice (germ-free mice colonised with human faecal microbiota) clearly revealed that GI motility can alter the gut microbial composition of the host although it is dependent on diet (Kashyap et al., 2013). Studies in human colonic microbiota demonstrated that a lowering pH from substrate fermentation in the large intestine may increase butyrate production and populations of butyrate-producing bacteria while at the same time inhibiting the *Bacteroides* spp. (Walker et al., 2005). The availability of different substrates and nutrients along the GI tract may also promote distinct bacterial populations in different GI regions of the host (Bartram et al., 2011). Moreover, the availability of oxygen in different GI regions can also play a vital role in gut bacterial compositions (Savage, 1977). Oxygen availability in the oesophagus and stomach was higher, therefore, the aerobic and facultative bacteria including Propionibacteriaceae, Desulfobulbaceae, Marinilabiaceae, Campylobacteriaceae, Helicobacteriaceae, and Flavobacteriaceae were significantly enriched in oesophageal and gastric mucosa. In contrast, in absence of oxygen, anaerobes or oxygen sensitive bacterial communities such as Lachnospiraceae, Clostridiaceae, and Ruminococcaceae were enriched in the large intestinal mucosa of GI tract.

Our results demonstrate that bacteria within the phylum Proteobacteria were significantly less abundant in oesophageal, gastric and large intestinal mucosa while its prevalence was higher in small intestinal mucosa of the GI tract. The abundance of Proteobacteria in the small intestinal mucosa was largely due to a higher prevalence of the bacteria belonging to the class Gammaproteobacteria, which includes the Vibrionaceae, Enterobacteriaceae and Aeromonadaceae. The majority of these bacteria are known to be facultative anaerobes and multiply at neutral to slightly basic pH (~ 7.4) (Bergey and Garrity, 2004). However, previous studies in green turtles revealed a fairly acidic pH (5.5- 6) environ with the large intestinal contents (different from the other reptiles) and a very acidic pH (3.85- 4) conditions in the gastric contents (Bjorndal, 1979b). In contrast, the small intestine exhibited a fairly neutral pH (7.0) which provides a suitable environment for the occurrence of Gammaproteobacteria (Bjorndal, 1979b; Bergey and Garrity, 2004). Gammaproteobacteria, a physiologically and metabolically diverse class, can play an important role in preparing the gut for

successive colonisation by strict anaerobes through utilizing oxygen, changing the gut pH, producing CO_2 and nutrients (Wilson, 2005; Chow and Lee, 2006). However, an over colonisation of these opportunistic bacteria (Vibrionaceae and Enterobacteriaceae) common markers of dysbiosis (Byndloss et al., 2017) within the small intestinal mucosa may competitively exclude the other normal flora and result in decreased bacterial diversity in the small intestinal mucosa (Brenner and Farmer, 1984; Amaro and Biosca, 1996). Further investigation is suggested to explore these phenomena.

Actinobacteria was the third most abundant phylum in the oesophageal mucosa. However, they were very low in other GI regions. The higher prevalence of Actinobacteria in the oesophageal mucosa was mainly due to higher abundance of bacteria in the Dietziaceae and Propionibacteriaceae. The members of Dietziaceae are characterised by the presence of mycolic acids (Dworkin et al., 2006). They are mainly chemoorganotrophic and exhibit an oxidation type of metabolism. However, the diversity of the metabolic activities of different members of this family are still unknown which demand better genetic tools to characterise and exploit their diverse functions within the GI tract of animals (Dworkin et al., 2006). Bacteria within the Propionibacteriaceae are well recognised, normal inhabitants of the GI tract for both humans and animals (Schaal et al., 1980; Stackebrandt et al., 2006). The pathogenic potential for many propionibacteria, such as *Propionibacterium* or *Eubacterium*, have been claimed for many years and later on, recognised as opportunistic rather than obligate pathogens (Schaal et al., 1980). These bacteria are often encountered in multiple infection processes where it is difficult to determine which component of the mixed microbial community is a true pathogen and which is only a “hanger-on” (Schaal et al., 1980). Furthermore, in this study, bacteria within the phylum Bacteroidetes were less abundant in the small and large intestinal mucosa while it was prominent in oesophageal and gastric mucosa and composed of mainly Porphyromonadaceae known members of the buccal cavity associated with the salivary biome (Wang et al., 2016a). Bacteria within the Porphyromonadaceae were also observed in previous studies on cloacal swabs of green turtles (Ahasan et al., 2017b). However, in this study, the reason for lower abundance of Bacteroidetes in the small and large intestinal mucosa is still unclear because the majority of food fermentation of green turtles usually takes place in the caecum (Bjorndal, 1979b). Bacteroidetes species are well known to degrade a variety of plant polysaccharides including pectin, xylan, galactomannan,

arabinogalactan, alginate and glucomannan (Salyers et al., 1977; Martens et al., 2011; Flint et al., 2012a) and are major butyrate producers. However, the turtles examined herein were ones that died and there may have been a bacterial shift in composition before death. Therefore, further research is recommended to explore the exact reasons of the low level of Bacteroidetes prevalence in small and large intestinal mucosa.

Furthermore, it is worth noting that the predominance of different phyla in study samples was largely due to the dominance of a small number of distinct OTUs. The top 10 most abundant OTUs, within each GI region, comprised 70.2%, 87.1% and 77.9% of the total resident microbiomes in gastric, small intestinal and large intestinal mucosa respectively. Additionally, four of the top 10 most abundant OTUs of gastric and large intestinal mucosa were common and accounted for 38.4% and 50.4% of the total OTUs. This finding indicates the possibility of similar mucosa-associated microbiota between the stomach and large intestine of green turtles. However, further investigation is suggested to determine the core mucosa-associated gut bacterial community of healthy green turtles.

In conclusion, this study has generated the first comprehensive landscape of green turtle bacterial microbiomes across the GI tract. This result will improve our understanding of how mucosa-associated bacterial microbiomes changes along the digestive tract. Our results reveal that bacterial communities of various compositions occupied different regions of the GI tract. The oesophagus and large intestine exhibit the highest bacterial diversity compared to stomach and small intestine of green turtles. This finding supports the recognised notion that different anatomic regions of the GI tract have their own physiochemical conditions such pH and oxygen, which exert selective pressures on the bacterial communities and play an important role in shaping the microbiota of GI tract. Interestingly, our study identified several members of opportunistic bacteria across the GI tract which might be overrepresented in stranded turtles. Our results also indicate that faecal samples can only represent the overall bacterial communities rather than microbiota of specific regions of the GI tract. It is therefore suggested to be attentive when selecting proper samples to investigate gut-associated disease. Our study provides a helpful reference for further detailed investigation of sea turtle gut microbiomes and their metabolic functions to improve their health and nutrition during rehabilitation.

CHAPTER 8
BACTERIOPHAGE VERSUS ANTIBIOTIC THERAPY ON GUT BACTERIAL COMMUNITIES OF JUVENILE GREEN TURTLE

8.1. Introduction

Since the industrialisation of antibiotics in the 1940s the contribution of antibiotics in the fields of public health, agriculture and medicine has been remarkable. However, in recent decades, our control over microbial diseases is diminishing because of the emergence of antibiotic-resistance and this is increasingly threatening our ability to use clinical antibiotics against resistant pathogens (O'Neill, 2016). Approximately, 70% of bacteria that are responsible for infectious diseases in human and animal health are resistant to at least one of the antibacterial agents commonly used for treatment (Bisht et al., 2009). Moreover, the incidence of antibiotic resistant bacterial infections is rising while the discovery of new antimicrobial agents is not advancing (Kinch et al., 2014). Additionally, the development of cheap and robust tools to screen for antibiotic resistant microbes has proven to be elusive (WHO, 2014).

Recent investigations on the impact of antibiotics have shed new light on the collateral damage they impart on the indigenous host-associated microbes (Langford et al., 2003; Pérez-Cobas et al., 2012; Looft et al., 2014; Panda et al., 2014; Langdon et al., 2016). Broad spectrum antibiotics can affect the abundance of up to 25% of the bacteria within the gut microbial community, causing a significant drop in taxonomic diversity and richness (Dethlefsen et al., 2008; Dethlefsen and Relman, 2011). Beyond altering the microbial community structure, antibiotics have also been found to alter gene expression, protein activity and overall gut metabolism, with effects that are rapid and sometimes persistent (Pérez-Cobas et al., 2012; Modi et al., 2014). Additionally, the uses of broad spectrum antibiotics to treat infectious diseases can induce indigenous microbiota to serve as reservoirs of resistance genes and contribute towards the development and spread of antibiotic resistance (Courvalin, 1994; Aarestrup, 1999; Sullivan et al., 2001). Broad spectrum antibiotics pave the way for intestinal overgrowth by antibiotic resistant and potentially pathogenic bacteria and can predispose animals to

gastrointestinal disorders that are difficult to treat (Quigley and Quera, 2006). The scientific community has surmised therefore, that without the necessary vigilance and stewardship, we might be approaching the post-antibiotic era.

Bacteriophages (phages) are promising supplements to antibiotics that can be used for medicinal or biological control purposes (Abedon et al., 2017; Doss et al., 2017). Phages are believed to be highly specific because they only invade the bacterial cells that possess specific cell surface receptors; this makes phages species and even strain specific (Wittebole et al., 2014). Moreover, phages are less likely to affect the non-targeted host-associated microbial communities (Kutter and Sulakvelidze, 2005). Additionally, unlike chemotherapeutic compounds or molecules, phages have the ability to evolve with their host, are ubiquitous in nature and possess less deleterious environmental effects (Kutter et al., 2010). The concept of utilising phage therapy against bacterial infections came out about a century ago but it was overshadowed by the success of antibiotics. The renewed interest in the antimicrobial potential of phages in the scientific community took shape predominantly due to the increase in multiple antibiotic-resistant bacterial infections in humans (Housby and Mann, 2009). Phage therapy is now being extensively investigated for application in agriculture (Susianto et al., 2014; Wittmann et al., 2016), and other food industries to eliminate harmful and zoonotic bacteria (Goodridge and Abedon, 2003; Endersen et al., 2014). Research has also focused on the therapeutic potential of phages to treat animal pathogens in both aquatic and terrestrial environments (Wagenaar et al., 2005; Richards, 2014; Doss et al., 2017; Wang et al., 2017). However, to date, the application of phage therapy in marine turtles is not known.

Green sea turtles (*Chelonia mydas*) are endangered marine herbivorous hindgut fermenters that rely on the microbial fermentation of ingested plants as their energy source (Bjorndal, 1985). Debilitated sea turtles are often treated with broad spectrum antibiotics as a general treatment in turtle hospitals. Treatment may alter the intestinal ecosystem allowing the overgrowth of pathogenic and antibiotic-resistant bacteria thus promoting intestinal disease and even death of the animals. A few studies have also reported the presence of multiple antibiotic-resistant bacteria in the gastrointestinal tract of free-ranging and stranded sea turtles (Foti et al., 2009; Al-Bahry et al., 2011; Ahasan et al., 2017a). Under these circumstances, phage therapy could be instituted as a suitable alternative to antibiotics in treating bacterial diseases in sea turtles. Therefore, the

objectives of this study were to provide a proof of concept for the therapeutic use of phages to eliminate targeted bacteria in captive green turtles and, investigate as well as compare effects of a broad-spectrum enrofloxacin and phage therapy on the gut bacterial communities of green turtles.

8.2. Materials and Methods

8.2.1. Ethics statement

This study was conducted under permits issued by James Cook University Animal Ethic Committee (Permit no. A2101 and A2309). Animals were collected from Heron island, QLD with the permission from the Department of Environment and Heritage Protection, QLD (permit no. WITK15765815) and Great Barrier Reef Marine Park Authority (Permit no. G14/36593.1).

8.2.2. Experimental setup

One day-old juvenile green sea turtles were collected from Heron Island, QLD and accommodated in individual tanks inside the animal facility at James Cook University. These turtles were raised for 11 months at a controlled environmental temperature (25-27 ^{o}C), pH (7.8-8.5), salinity (27-35 ppt) and recirculated water sterilised by UV. All turtles were fed daily with an equal amount of floating pelleted diet that comprised of mainly freshly blended human grade sea food and vegetables. Routine husbandry followed the protocol established by James Cook University Turtle Health Research Facility. Before starting the experiment, all turtles were morphologically examined for any visible signs of infections including inflammation, wounds and inappetence. A total of 36 green turtles were selected for the experiment where individual weight ranged from 93 to 208 g (mean: 164 g) (Table 8.1).

Table 8. 1 Experimental animals, treatments and sampling details of the trial

Treatment status	Sample collection	Samples	Trial animals (N=36)		
Pre-treatment (Day 0)	Day 0	Faecal samples	**Group A** Antibiotic (N=12)	**Group P** Phages (N=12)	**Group C** Control (N=12)
		Organ samples	0	0	Sub-group 1 (n=3)
Treatment (Day 01-14)	Day 06	Faecal and organ samples	Sub-group 1 (n=4)	Sub-group 1 (n=4)	Sub-group 2 (n=3)
	Day 15		Sub-group 2 (n=4)	Sub-group 2 (n=4)	Sub-group 3 (n=3)
Post-treatment (Day 15-28)	Day 28		Sub-group 3 (n=4)	Sub-group 3 (n=4)	Sub-group 4 (n=3)

Turtles included in this study were divided randomly into three equal groups (N=12): treatment groups (A, antibiotic and P, phages) and negative group (C, control). Subsequently, the treatment and control groups were subdivided into 3 and 4 equal subgroups respectively. At day 0, deep cloacal swab samples were collected from all turtles from the three groups. Additionally, turtles from one subgroup (n=3) of the control group, C were euthanized to collect organ samples that included liver, kidney, heart and lung.

Turtles within group A received an oral dose of enrofloxacin (10 mg/kg body weight) according to a procedure outlined by (Jacobson et al., 2005; Giorgi et al., 2013), and Group P turtles received an oral dose of *Acinetobacter venetianus* specific phage cocktail (10^9 plague forming units) that was prepared in normal saline solution (0.9% NaCl) according to the procedure outlined by (Mai et al., 2015) respectively. Group C turtles received an oral dose of pure distilled water. In all three groups, treatments continued from day 1 to day 14 followed by post-treatment observation until day 28. Body weights were recorded for all turtles at day 0, 6, 15 and 28. One sub-group (n = 4, group A and P, and n=3, group C) of each group was euthanized at day 6, 15 and 28 for the collection of organ samples and faecal swabs. Euthanasia was by intra-coelomic (IC) injection of 350 mg/kg pH-neutralised 1% MS-222, followed by 1 ml 50% unbuffered MS-222 as outlined by Conroy et al. (2009). All samples were collected into sterile microfuge tubes with minimum contamination. In this study, all turtles were

monitored at least twice daily and examined for feed intake, physiological changes and abnormal behaviours.

8.2.3. Bacterial identification and determination of antimicrobial susceptibility

To identify the representative Gram-negative bacteria for bacteriophage development, deep cloacal swabs were taken from a total of 9 randomly selected turtles before the beginning of the trial. Samples were plated immediately on MacConkey agar (Neogen corp., USA), 5% sheep blood agar (Oxoid Ltd., Hampshire, UK) and EC broth (Oxoid Ltd., Hampshire, UK) for overnight incubation at room temperature (25-27 $^{\circ}$C). A representative of each colony morphotype was purified by culture and further identified according to the procedure described in Chapter 3.1. However, in this study, the nucleic acid sequence of the bacterial 16S rRNA gene was amplified using only one set of universal primers, 27F and 1391R (5$^{'}$-AGAGTTTGATCMTGGCTCAG-3$^{'}$ and 5$^{'}$-GACGGGCGGTGTGTRCA-3$^{'}$; 1350bp).

The antimicrobial susceptibility of the identified bacterial isolates was tested for a range of 10 different antibiotics including penicillin, oxacillin, ampicillin, amoxicillin-clavulanic acid, polymyxin, ceftiofur, cefoxitin, oxytetracycline, enrofloxacin and chloramphenicol applying the disk diffusion techniques followed by CLSI guidelines (Schwalbe et al., 2007; CLSI, 2012).

8.2.4. Bacteriophage identification, purification and titration

Based on the findings of the study mentioned in chapter 8.2.3 the genus *Acinetobacter* has been chosen as a representative taxon to identify bacteria specific phages. In this study, a total of eight *Acinetobacter* isolates were selected as host bacteria to recover lytic phages from sewage sludge, which are assumed to be a rich source of bacteriophages within the environment (Jurczak-Kurek et al., 2016). An adequate volume (2 *l*) of raw sewage sludge was obtained from Cleveland Bay Waste Treatment Plant in Townsville, Queensland, Australia. Immediately after collection, sludge was taken into the laboratory and centrifuged at 10,000 *g* for 20 minutes at 4°C to remove the course particles from the sludge. A viral suspension was prepared by aseptically filtering the supernatant through 0.45 µm membrane filter (Whatman^{TM}) to remove bacterial and other cellular debris. For amplification of the bacteria-specific phage, the filtrate (50 ml) was mixed with an equal volume of 2x tryptone soy broth (TSB) and 1

ml of log-phase bacterial culture, incubated overnight at 30°C on a shaker at 75 rpm. The phage suspension was centrifuged at 2,655 *g*, for 30 mins and then filtered with a 0.45 µm membrane filter. The phage filtrate (10 µl) was then inoculated into a log-phase bacterial lawn on a tryptone soy agar (TSA) plate and incubated overnight for plaque examination.

For purification of phages, all isolates were serially diluted and plated on TSA following a modified threefold successive single-plaque isolation method described by Sambrook (2001). The high titre phage stocks were also prepared by serial dilution of the purified phage filtrates following drop wise (10 µl) inoculation into a log-phase bacterial lawn on TSA plate to yield a concentration that would provide confluent lysis (80-90% clear zone) of the host bacteria after overnight incubation. The phage filtrate of the selected concentration was added to an equal volume (500 µl) of log-phase bacterial culture. The mixture was left at room temperature for 30 minutes before plating on to a TSA plate and incubated overnight at 30 °C. Following incubation, phages were recovered by adding 10 ml of PBS in the TSA plate and left for 6 hours at room temperature. The PBS suspension was collected, centrifuged at 2,655 *g*, for 30 min and then filtered through 0.45 µm membrane filters.

The phage titre was determined by a drop count method (Luo et al., 2016). Ten-fold serial dilutions of the phage suspension was prepared in PBS buffer. Ten microliters (10 µl) of each suspension of phage dilution was spotted onto a TSA plate that overlayed a log-phase bacterial culture. The plate was allowed to dry and incubated overnight for plaque formation. The plaques in each drop were counted and the titres of the phage suspension were expressed as plaque forming unit (PFU)/ml of suspension. All phage lysates were stored at 4 °C.

8.2.5. Histopathological examination

Tissue samples from liver, lung, kidney and spleen were collected during necropsy of turtles and preserved in 10% neutral buffered formaldehyde. Tissues were processed routinely, and embedded in paraffin wax. Tissue sections were cut at a thickness of 5 µm and stained routinely with Haematoxylin and Eosin (H&E), Gram-Twort, Ziehl-Neelsen (ZN) and Periodic acid Schiff (PAS) (Bancroft and Gamble, 2008). Slides were mounted routinely and examined under light microscopy.

8.2.6. DNA extraction, PCR amplification and sequencing of faecal specimens

The nucleic acid extraction of the faecal specimens, PCR amplification and sequencing of the bacterial 16S rRNA gene were carried out according to the protocol described in Chapter 3.2.

8.2.7. Bioinformatics and statistical analyses

Fastx_trimmer from the FASTX toolkit was used to remove the primer sequences from the forward and reverse reads of paired Fastq files as described previously (Gordon and Hannon, 2010). This was followed by quality trimming (q=20) using Sickle (Joshi and Fass, 2011). Multiple_join_paired_ends.py script was used in the Quantitative Insights into Microbial Ecology (QIIME 1.8) to merge the pair-ends reads (Caporaso et al., 2010). Chimeric reads were identified and filtered using identify_chimeric_seqs.py and filter_fasta.py module in QIIME. Sequences were then clustered into Operational Taxonomic Units (OTUs) at 97% similarity threshold applying UCLAST algorithm (Edgar et al., 2011).Taxonomy was assigned in QIIME using Silva database (version 128, Sep 2016) (https://www.mothur.org/wiki/Silva_reference_files#Release_128). OTUs that were unable to be assigned were categorized as “Unclassified”.

For statistical analyses, the QIIME and OTUs table was filtered based on their relative abundance to remove low abundance OTUs (< 0.01%) and samples that showed <1000 sequence reads. Alpha diversity metrics including ACE (abundance-based coverage estimator), Shannon diversity index and Simpson index were estimated to determine the host specific microbial richness and diversity using Calypso web server (Zakrzewski et al., 2017). The conditional uncovered probability and Good’s coverage were estimated in QIIME to evaluate the completeness of sampling (Esty, 1986; Lladser et al., 2011). To evaluate the variation among different groups of samples, we analyzed the rarefied dataset using Bray-Curtis distance matrix (Bray and Curtis, 1957), and further visualized by principle coordinate analysis (PCoA). Clustering of samples was also evaluated using a non-metric multidimensional scaling (NMDS) technique (Kruskal, 1964). Analysis of similarity (ANOSIM) was performed by applying Bray-Curtis distance matrix to evaluate the association between different treatments and control groups. The abundance of microbial communities among different groups of samples at different taxonomic level was estimated and compared using ANOVA and Wilcoxon

signed-rank test. Taxa that exhibited a relative abundance < 0.01 were excluded from the analysis.

8.3. Results

8.3.1. Bacterial identification and determination of antimicrobial susceptibility

A total of 61 Gram-negative bacterial isolates were recovered from 9 cloacal swab samples of green turtles. Sixteen of the 61 isolates exhibited similar results in all biochemical tests. Moreover, these 16 isolates were found to be common and represented in a maximum of 8 of the 9 turtle samples. These isolates were further confirmed to be members of the genus *Acinetobacter* using API 20E system and bacterial 16S rRNA gene sequencing.

Antimicrobial susceptibility of the *Acinetobacter* isolates against selected antimicrobial agents including penicillin, ampicillin, amoxicillin-clavulanic acid, ceftiofur, cefoxitin, chloramphenicol, enrofloxacin, oxytetracycline and polymyxin were determined. None of the isolates were found resistant to any of these antimicrobial agents.

8.3.2. Phage identification and phage titres

Screening of sewage sludge samples yield a total of 9 phages effective against the eight selected *Acinetobacter* isolates. The plaques caused by the lytic phages varied in size and were characterised by the presence of clear and/or transparent zones (Figure 8.1). Eight phage filtrates were active against seven *Acinetobacter* isolates while one phage filtrate was effective against three *Acinetobacter* isolates. Titres of the isolated phages ranged from 10^{10} to 10^{11} PFU/ml following 24 hours incubation with the host bacteria (Table 8.2). Phage cocktail was prepared by adding 9 different phage lysates at 10^{9} PFU/ml up to a total volume of 70 ml of PBS.

Table 8. 2 Plaque morphology of the isolated bacteriophages from sewage sludge samples

Phage ID	Plaque morphology	
	Number (PFU/ml)	Zone
PH06	2.6 X 10^{11}	Clear
PH13	9.5 X 10^{10}	Clear
PH241	2.1 X 10^{11}	Clear
PH242	1.2 X 10^{11}	Clear
PH35	4.5 X 10^{11}	Clear
PH41	6.6 X 10^{11}	Clear
PH481	5.0 X 10^{10}	Clear
PH482	1.6 X 10^{11}	Clear
PH50	1.3 X 10^{11}	Clear

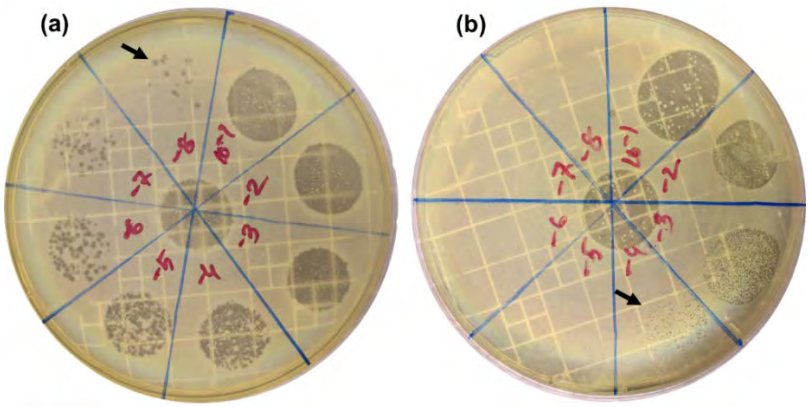

Figure 8. 1 Plaque morphology of the isolated bacteriophages (a) PH481 and (b) PH41 on *Acinetobacter* lawns.

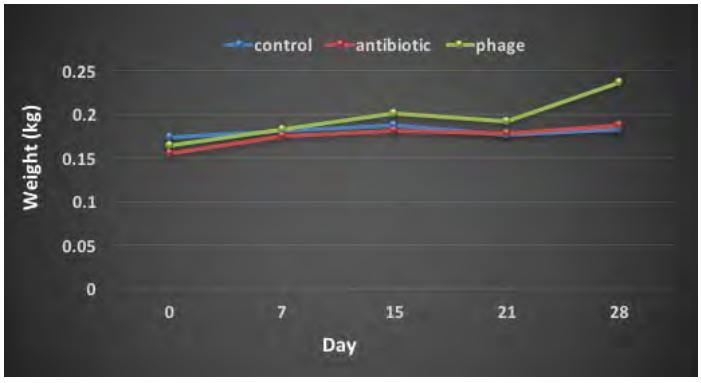

Figure 8. 2 Growth performance of green turtles, *Chelonia mydas* in different treatments (antibiotics and phages) and control groups.

8.3.3. Morphometric and clinical examination

Body weights were measured to determine effects of the different treatments on the turtle's appetites, weight and growth trajectory. No significant difference ($P>0.05$) was recorded in weight gain of the turtles between groups while an apparent higher weight gain was observed for phage treatment group (Group P) of turtles compared to control (Group C) and antibiotic (Group A) treatment groups (Figure 8.2). All turtles remained healthy and no visible clinical abnormalities were recorded for any turtle during the experiment.

8.3.4. Histopathological examination

The livers from 11/36 turtles (from all groups) showed focal and/or mid-zonal to central lobular hepatocyte vacuolation and degeneration, which was attributed to artifact from the anesthetic agent, MS-222 used in this study (Figure 8.3b). In lung tissues, single or multiple granulomas containing multinucleated giant cells, macrophages and lymphocytes, were identified in both control C (3/12) and treatment groups (A, antibiotic [4/12] and P, phages [5/12]) turtles (Figure 8.3a). Periodic acid-Schiff (PAS) stain confirmed the presence of pigmented fungal hyphae within lung granulomas of group A (1/12) and group C (1/12) turtles (Figure 8.3c). Ziehl-Neelsen (ZN) stain identified the presence of acid-fast bacteria within lung granulomas of group A (1/12) and group P (1/12) turtles (Figure 8.3). These bacteria were presumptive *Mycobacterium* spp. (Figure 8.3e, f). Mild congestion was present in the heart and kidneys of turtles from all three groups, but no other abnormalities were detected.

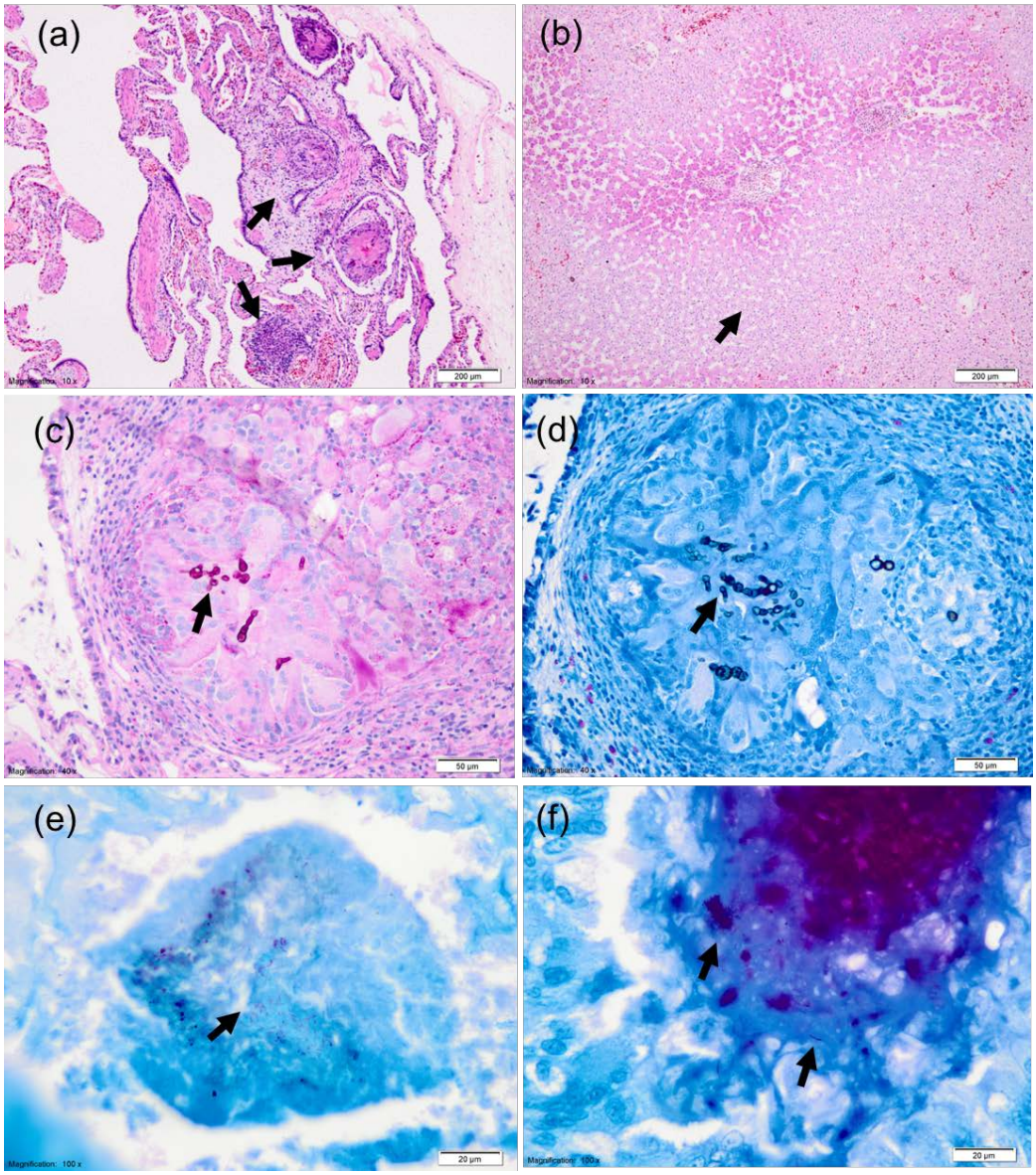

Figure 8. 3 Examples of photomicrographs from the histopathological examination of different organ samples collected from experimental green turtles (*Chelonia mydas*) (a) a multiple granuloma surrounded multinucleated giant cells, macrophage and neutrophils in a lung section (H&E stain), (b) a mid-zonal to central lobular hepatocyte vacuolation in a liver section (H&E stain), (c) and (d) sections of fungal hyphae in a lung granuloma (PAS stain and ZN stain), (e) and (f) presence of *Mycobacteria* spp. within a lung granuloma (ZN stain).

8.3.5. Gut microbiota

8.3.5.1. Summary of sequencing data and depth

Illumina sequencing of bacterial 16S rRNA gene targeting V1-V3 hypervariable regions generated a total of 3,534,759 high quality reads from a total of 44 samples (Table S 8.1). Individual samples were covered in depth by an average of 80,335 reads where the median read length ranged from 460- 486 bp. At 97% sequence identity threshold, all sequences were delineated into OTUs where 25,690 OTUs were identified in total and 338 OTUs were retained after removing the low abundant OTUs (relative abundance < 0.01). A total of 151 OTUs were identified as they retained above 1,000 sequences with a maximum of 959,802 sequences. Samples from the antibiotic treatment (group A) represented the highest number of OTUs (242.62 ± 6.41) followed by group P phages (221.25 ± 22.45) and group C controls (219.08 ± 24.28) (Table S 8.2). Constructed rarefaction curves for individual samples revealed the saturation plateau was reached in most samples (Figure S 8.1). The conditional uncovered probability of each sample was also estimated to evaluate the completeness of samples. Results indicated there was sufficient OTU coverage in all samples (Table S 8.2). Furthermore, our results of Good's coverage revealed > 95% phylotypes in all samples, which suggested the presence of sufficient phylotypes to describe the gut bacterial community in each green turtle (Table S 8.2).

8.3.5.2. Gut bacterial diversity and richness estimation

Bacterial diversity of each sample estimated at OTU level using Shannon and Simpson indices revealed significant differences in inter-day subgroups of the antibiotic treatment group (Figure 8.4). Bacterial diversity was significantly lower ($P< 0.05$) in day 15 and day 28 samples compared to day 0 samples. No significant difference was present between subgroups of group P and group C (Figure 8.4). Bacterial richness was also estimated for each group of samples applying abundance-based coverage estimators (ACE). The estimates revealed that bacterial richness was significantly lower in day 0 samples compared to day 6 and day 15 samples of group A (Figure 8.4). However, in group P, a significantly higher microbial richness was observed at day 6 samples compared to day 28 samples. No significant difference was observed between subgroups of group C samples.

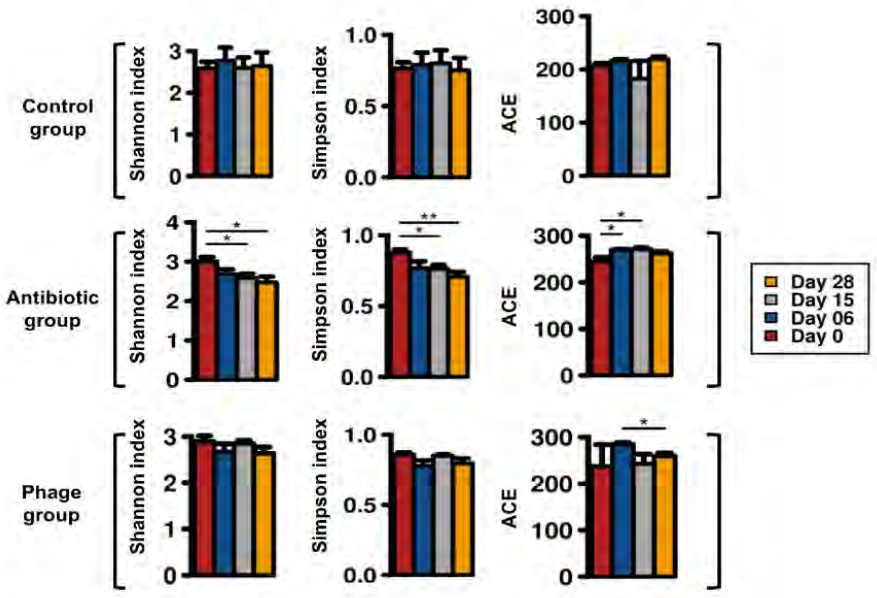

Figure 8. 4 Alpha diversity metrics for the gut bacterial communities from samples of green turtles, *Chelonia mydas* with different treatments and control groups.

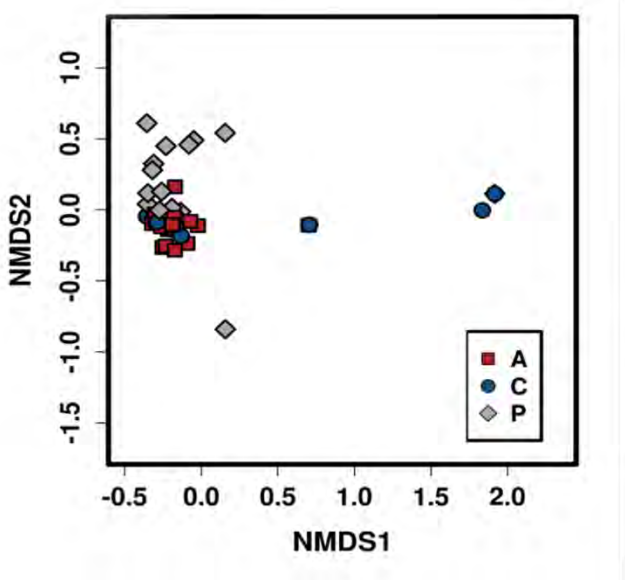

Figure 8. 5 Non-metric multidimensional scaling (NMDS) analysis of the dissimilarity in samples of different treatment and control groups. A, antibiotics group; P, phages group and C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

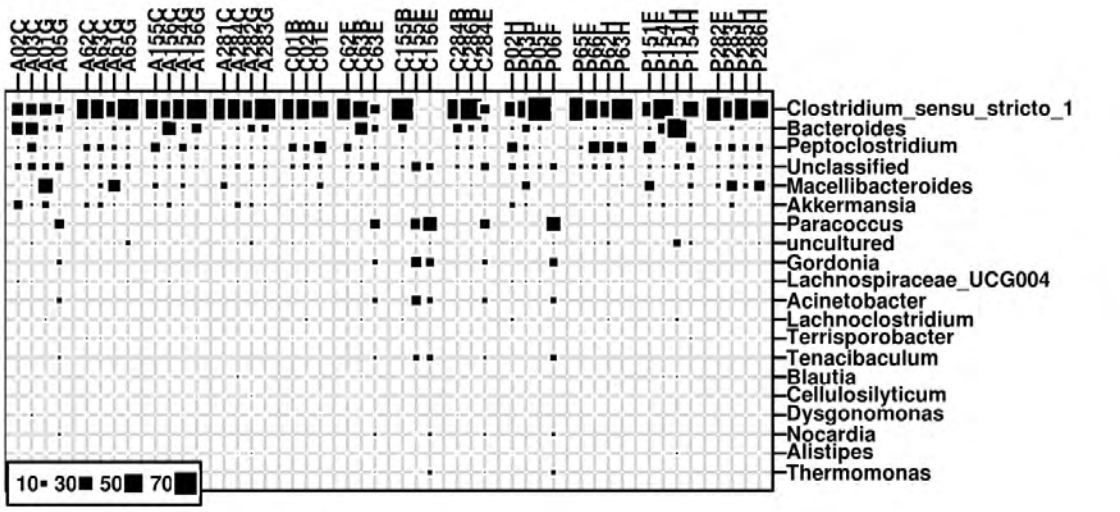

Figure 8. 6 Distribution and the relative abundance (%) as identified by QIIME of the 20 most abundant bacterial genera in samples of different treatment and control groups. The prefix A0, A6, A15 and A28 indicate data obtained from samples in the antibiotics group at day 0, 6, 15 and 28 respectively. The prefix P0, P6, P15 and P28 indicate data obtained from samples in the phages group at day 0, 6, 15 and 28 respectively. The prefix C0, C6, C15 and C28 indicate data obtained from samples in the control group at day 0, 6, 15 and 28 respectively.

8.3.5.3. Variation in bacterial communities between treatments and control groups

Principle coordinate analysis (PCoA) plots applying Bray-Curtis distance matrix constructed to visualize the dissimilarities in the bacterial communities of different treatments and control group of samples (Figure S 8.2). The PCoA plot revealed that samples from the same day subgroup from the different treatment groups mostly clustered together but sometimes loosely. The non-metric multidimensional scaling (NMDS) plots also revealed the variation in the microbial communities in samples of group A, P and C (Figure 8.5). The control group had the widest variation on NMDS1 axis whilst the Antibiotic group was the tightest group. Our analysis of similarities (ANOSIM) applying Bray-Curtis distance matrix confirmed that the microbial gut communities were significant different in samples of group A, P and C, collected at day 15 and day 28 (Figure S 8.3). All groups lost diversity as the trial continued with the control group keeping its diversity longest. However, no significant difference was observed in the microbial gut communities of group A, P and C samples, collected at day 0 and day 6 (Figure S 8.3).

8.3.5.4. Gut microbial community composition

The taxonomic assignment of the operational taxonomic units (OTUs) performed by QIIME revealed a total of 19 phyla, 48 classes, 93 orders, 193 families and 402 bacterial genera. The most predominant phyla were Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia and Actinobacteria which contributed to more than 99% of the bacterial communities in all three treatment groups (Table 8.3). At class level, Clostridia was the most dominant class in both group A and P with a relative abundance >71%, followed by Bacteroidia, Verrucomicrobiae, Alphaproteobacteria and Erysipelotrichia (Table 8.4). In group C, the most abundant class was also Clostridia at a slightly lower level (~60%) followed by Alphaproteobacteria, Bacteroidia, Actinobacteria and Gammaproteobacteria (Table 8.4). At the genus level, *Clostridium_sensu_stricto*_1 prevailed in all three groups with a relative abundance >41% (Figure 8.6). The higher abundance of *Acinetobacter* was observed in group C, followed by group A and P. Again, diversity seemed to drop with the increasing number of days in the treatment trial.

8.3.5.5. Analysis of group specific microbiota

In this study, bacterial communities that were found to be significantly different between treatments and control groups have been summarised in Table 8.5.

Day 0

None of the bacterial phyla were significantly different between treatments and control groups of samples except Bacteroidetes. A significantly higher abundance ($P< 0.02$) of Bacteroidetes was present in group A samples compared to group P and C. At the genus level, members only within the phylum Firmicutes that include *Anaerofilum*, *Ruminococcus*_1, *Terrisporobacter* and *Epulopiscium* prevailed significantly ($P< 0.05$) in group C samples (Table 8.6).

Day 6

The genera *Hydrogenoanaerobacterium, Parabacteroides* and *Terrisporobacter* were significantly ($P< 0.05$) associated with group A samples (Table 8.7). A non-statistical significance higher abundance of Firmicutes in group P and Verrucomicrobia in group A was observed compared to group C (Table 8.7). At the genus level, the relative abundance of *Acinetobacter* was lower in group A and P samples compared to group C but not significantly different.

Day 0 and day 6

We also compared between day 0 and day 6 samples of both treatment groups. In antibiotics group (A), our analysis revealed the occurrence of significantly higher abundance of Firmicutes at day 6 samples compared to day 0 (Table 8.8). At genus level, *Bacteroides* and *Parabacteroides* were significantly ($P< 0.05$) lower in the antibiotic treatment samples at day 6 (Table 8.8). In contrast, other genera including *Anaerotruncus*, *Anaerofilum*, *Lachnoclostridium*_10, *Clostridium_sensu_stricto*_1 and *Ruminiclostridium*_5 were significantly higher ($P< 0.05$) at day 6 compared to day 0 (Table 8.8).

In phages group (P), bacteria phylum characterised as Bacteroidetes and the genera *Actinobacter* and *Paracoccus* were significantly lower ($P< 0.05$) at day 6 compared to day 0 (Table 8.9). However, the members within the genera *Eubacterium_eligens*_group and Ruminococcaceae_UCG002 were significantly predominant in samples at day 6.

Day 15

The relative abundances of Proteobacteria and Actinobacteria were significantly lower ($P< 0.05$) in group A and P samples compared to group C samples (Table S 8.3) whilst Verrucomicrobia were significantly higher. Bacteria within the genus *Acinetobacter* were significantly lower in both group A and P samples while *Tenacibaculum*, *Vibrio*, *Paracoccus* and *Gordonia* were significantly associated with group C samples (Figure 8.7). The genera including *Butyricicoccus* and *Akkermansia* were significantly abundant in group P samples followed by group A. *Eubacterium_nodatum*_group and Lachnospiraceae_NC2004_group significantly dominated group A samples compared to other groups (Figure 8.7).

Day 28

None of the bacterial phyla differed significantly between treatment and control groups although a relatively higher abundance of Firmicutes was observed in group A and P samples (Table 8.10). Proteobacteria prevailed in group C samples compared to group A and P samples. At the genus level, *Peptoclostridium* and *Macellibacteroides* were significantly abundant in group P samples and, *Bilophila*, *Eubacterium*, Ruminococcaceae_UCG013, *Oscillibacter* and *Erysipelatoclostridium* were significantly more prevalent in group A samples (Table 8.10). *Bacteroides* was significantly higher in C samples whilst *Lachnoclostridium* was significantly lower in the P group. No significant difference was observed for the genus *Acinetobacter*.

Table 8. 3 Composition and the relative abundance (%) as identified by QIIME of the five most abundant bacterial phyla in samples of different treatment and control groups. A, antibiotics group; P, phages group and C, control group.

Phylum	Group A (%)	Group P (%)	Group C (%)
Firmicutes	72.6	77.6	60.9
Bacteroidetes	17.5	13.2	13.8
Proteobacteria	2.4	3.5	16.8
Verrucomicrobia	4.8	2.9	1.5
Actinobacteria	1.2	1.1	5.8

Table 8. 4 Composition and the relative abundance (%) as identified by QIIME of the 10 most abundant bacterial classes in samples of different treatment and control groups. A, antibiotics group; P, phages group and C, control group.

Class	Group A (%)	Group P (%)	Group C (%)
Clostridia	71.5	76.4	59.9
Bacteroidia	17.2	12.6	10.8
Alphaproteobacteria	1.5	2.3	11.0
Verrucomicrobiae	4.8	2.9	1.5
Actinobacteria	0.9	1.0	5.7
Gammaproteobacteria	0.8	1.0	5.5
Unclassified	1.5	1.6	1.3
Flavobacteriia	0.3	0.6	2.8
Erysipelotrichia	1.0	1.1	0.9
Coriobacteriia	0.2	0.2	0.1

Table 8. 5 The taxonomic classification of the bacteria that were significantly associated in different treatments (A) antibiotics; (P) phages and control (C) groups of turtles during the trial for easy reference.

Phylum Firmicutes					
Class	Clostridia				
	Family	Lachnoshpiraceae			
		Genus	*Anaerofilum*	Genus	*Lachnoclostridium*_10
		Genus	*Lachnoclostridium*	Genus	*Lachnospiraceae*_NC2004_group
	Family	*Clostridiaceae*			
		Genus	*Anaerotruncus*	Genus	*Butyricicoccus*
	Family	Ruminococcaceae			
		Genus	*Anaerofilum*	Genus	*Hydrogenoanaerobacterium*
		Genus	*Ruminococcus*_1	Genus	*Ruminococcaceae*_UCG002
		Genus	*Ruminiclostridium*_5	Genus	*Ruminococcaceae*_UCG013
	Family	Eubacteriaceae		Genus	*Eubacterium_eligens*_group
	Family	Peptostreptococcaceae			
		Genus	*Terrisporobacter*	Genus	*Peptoclostridium*
	Family	*Epulopiscium*		Genus	*Epulopiscium*
	Family	Clostridiaceae 1		Genus	*Clostridium_sensu_stricto*_1
	Family	Oscillospiraceae		Genus	*Oscillibacter*
Class	Erysipelotrichia				
	Family	Erysipelotrichaceae		Genus	*Erysipelatoclostridium*
Phylum Bacteroidetes					
Class	Bacteroidia				
	Family	Bacteroidaceae		Genus	*Bacteroides*
	Family	Porphyromonadaceae			
		Genus	*Parabacteroides*	Genus	*Macellibacteroides*
Class	Flavobacteriia				
	Family	Flavobacteriaceae		Genus	*Tenacibaculum*
Phylum Proteobacteria					
Class	Gammaproteobacteria				
	Family	Moraxellaceae			
		Genus	*Pseudomonas*	Genus	*Acinetobacter*
	Family	Vibrionaceae		Genus	*Vibrio*
Class	Alphaproteobacteria				
	Family	Rhodobacteriaceae		Genus	*Paracoccus*
Class	Deltaproteobacteria				
	Family	Desulfovibrionaceae		Genus	*Bilophila*
Phylum Actinobacteria					
Class	Actinobacteria				
	Family	Gordoniaceae		Genus	*Gordonia*
	Family	Mycobacteriaceae		Genus	*Mycobacterium*
Phylum Verrucomicrobia					
Class	Verrucomicrobia				
	Family	Verrucomicrobiaceae		Genus	*Akkermansia*

Table 8. 6 Comparison of bacterial taxa between different experimental groups at day 0. Group A, antibiotics group; group P, phages group; group C, control group. Asterisk (*) indicates taxa are significantly different at P<0.05 level. For easier visualisation, greyed-out values were significant.

Taxa	P value	Group A mean	Group P mean	Group C mean
Phylum				
Bacteroidetes	0.019*	30.83	13.45	8.9
Firmicutes	0.16	44.54	59.67	87.04
Verrucomicrobia	0.25	7.69	3.24	2.47
Proteobacteria	0.66	9.63	16.69	0.14
Actinobacteria	0.68	4.08	4.95	0.16
Genus				
Anaerofilum	0.002*	0.005	0.038	0.12
Ruminococcus_1	0.008*	0	0	0.047
Terrisporobacter	0.014*	0.66	0.49	1.39
Epulopiscium	0.015*	0.11	0.13	0.61
Bacteroides	0.052*	17.39	5.65	3.11
Anaerosporobacter	0.069	0.0025	0.057	0.0067

Table 8. 7 Comparison of bacterial taxa between different experimental groups at day 6. Group A, antibiotics group; group P, phages group; group C, control group. Asterisk (*) indicates taxa are significantly different at P<0.05 level. For easier visualisation, greyed-out values were significant.

Taxa	P value	Group A mean	Group P mean	Group C mean
Phylum				
Verrucomicrobia	0.11	4.82	1.89	1.86
Firmicutes	0.16	79.52	91.31	61.56
Bacteroidetes	0.25	13.68	4.88	17.4
Proteobacteria	0.29	0.24	0.1	12.49
Actinobacteria	0.3	0.38	0.24	5.26
Genus				
Hydrogenoanaerobacterium	0.001*	0.02	0.0025	0.0067
Parabacteroides	0.002*	0.03	0.015	0.077
Terrisporobacter	0.027*	1.74	0.88	0.69

Table 8. 8 Comparison of bacterial taxa between day 0 and day 6 for samples in the antibiotics group (A). Asterisk (*) indicates taxa are significantly different at P<0.05 level. For easier visualisation, greyed-out values were significant.

Taxa	P (rank test)	Day 0 mean	Day 6 mean
Phylum			
Firmicutes	0.029*	44.54	79.52
Bacteroidetes	0.11	30.83	13.68
Proteobacteria	0.15	9.63	0.24
Verrucomicrobia	0.49	7.69	4.82
Actinobacteria	0.69	4.08	0.38
Genus			
Anaerotruncus	0.028*	0.062	0.1
Anaerofilum	0.028*	0.005	0.07
Parabacteroides	0.029*	0.12	0.03
Lachnoclostridium_10	0.029*	0.048	0.18
Clostridium_sensu_stricto_1	0.029*	22.51	51.44
Bacteroides	0.029*	17.39	4.17
Ruminiclostridium_5	0.041*	0.1	0.2
Pseudomonas	0.19	0.31	0
Mycobacterium	0.19	0.035	0
Acinetobacter	0.41	1.82	0.002
Vibrio	0.45	0.065	0

Table 8. 9 Comparison of bacterial taxa between day 0 and day 6 for samples in the phages group (P). Asterisk (*) indicates taxa are significantly different at P<0.05 level. For easier visualisation, greyed-out values were significant.

Taxa	P (rank test)	Day 0 mean	Day 6 mean
Phylum			
Bacteroidetes	0.029*	13.45	4.88
Proteobacteria	0.064	16.69	0.1
Firmicutes	0.11	59.67	91.31
Actinobacteria	0.2	4.95	0.24
Verrucomicrobia	1	3.24	1.89
Unclassified	1	1.97	1.57
Genus			
Acinetobacter	0.02*	2.15	0
Paracoccus	0.027*	7.9	0.0025
*Eubacterium_eligens*_group	0.029*	0.042	0.38
Ruminococcaceae_UCG002	0.047*	0.005	0.018

Table 8. 10 Comparison of bacterial taxa between samples from different treatment groups at day 28. Group A, antibiotics group; group P, phages group; group C, control group. Asterisk (*) indicates taxa are significantly different at P<0.05 level.

Taxa	P value	Group A mean	Group P mean	Group C mean
Phylum				
Verrucomicrobia	0.29	4.77	2.5	1.7
Proteobacteria	0.3	0.14	0.33	12.42
Actinobacteria	0.3	0.22	0.075	5.2
Firmicutes	0.41	80.35	79.31	62.42
Bacteroidetes	0.79	13.35	16.15	16.83
Genus				
Peptoclostridium	0.0002*	4.62	10.2	4.69
Bilophila	0.011*	0.03	0.01	0.0033
Eubacterium	0.013*	0.16	0.032	0.09
Ruminococcaceae_*UCG013*	0.027*	0.34	0.09	0.15
Oscillibacter	0.033*	0.082	0.012	0.0067
Erysipelatoclostridium	0.033*	0.15	0.035	0.1
Macellibacteroides	0.039*	3.92	13.07	1.42
Bacteroides	0.041*	6.95	2.28	11.43
Lachnoclostridium	0.048*	0.9	0.28	0.82
Acinetobacter	0.29	0.001	2.42	0.003

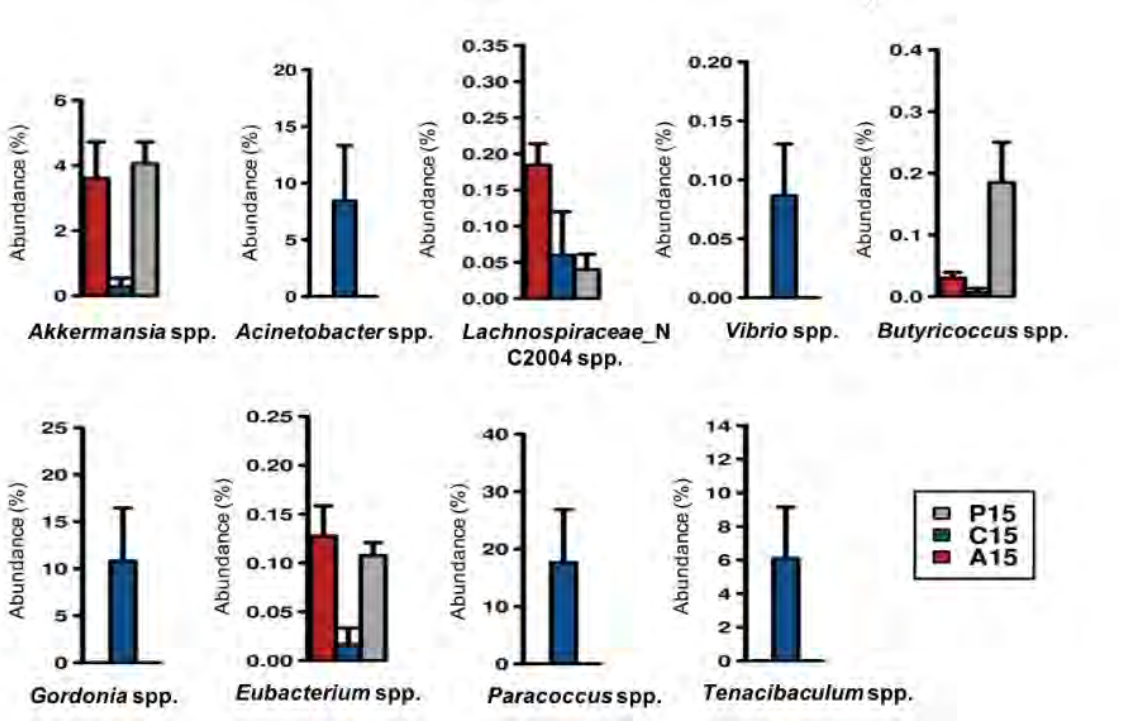

Figure 8. 7 Qualitative and quantitative differences in bacteria genera in the treatment and control groups at day 15. A, antibiotics group; P, phages group and C, control group. 15, day 15

8.4. Discussion

Currently, there is substantial interest in the development of phage-based antibacterials and to date, several studies have proven the efficacy of phage therapy in treating various pathogenic bacterial infections in both humans and animals (Merril et al., 2003; Sulakvelidze and Kutter, 2005; Sarker et al., 2012; Doss et al., 2017). The present study provides the first proof of concept for the therapeutic use of phages *in vivo* to eliminate selective bacteria in captive juvenile green turtles. In addition, our study also demonstrated that phages exert minimal effects on the community composition and structure of indigenous host-associated gut bacterial communities of green turtles.

Bacteria specific phage cocktail administered orally to the turtles significantly reduced the abundance of targeted *Acinetobacte*r during treatment (at day 6 and 15) while a slow recovery occurred following cessation of the phage challenge. Phage therapy was also found to be effective in treating targeted bacterial pathogens in juvenile sea cucumbers (Li et al., 2016b) and shrimp (*Penaeus monodon*) (Alagappan et al., 2016) and marine corals (Atad et al., 2012). In addition, the effective elimination of the targeted pathogenic bacteria from the gastrointestinal tract have also been demonstrated in several phage therapy experiments in both humans (Sulakvelidze and Kutter, 2005) and animals (Sulakvelidze and Barrow, 2005). However, Xu et al. (2016) reported that the oral administration of *Burkholderia*-specific phages was unable to eliminate the crypt-associated *Burkholderia* in the southern chinch bug (*Blissus insularis*). Additionally, the observed inactivity of these phages was attributed to the blockage of the connection between the anterior and posterior mid-gut regions. As the anatomical limitations in the southern chinch bug are less apparent in the sea turtle, the present study sought to provide a first proof of concept for the effective use of phage in turtles. In our study, oral administration of phage cocktails at a higher dose ($1x10^9$ PFU/ml) did not negatively affect the physical condition or survival of turtles without any significant differences in weight between phage treated animals and controls. It has been hypothesised that phages may facilitate changes in the microenvironment in colonising the microbial communities that have higher potential in nutrient assimilation and energy release (Letarov and Kulikov, 2009). However, the evaluations of specific effects of phage treatment on nutrient intake, absorption and assimilation were beyond the scope

of the current study but do warrant further studies, with the application of extensive proteomic and genomic approaches.

To date, the impact of phage therapy on the indigenous gut-associated microbial communities has rarely been examined although it is known that the diversity of microbial communities play important roles in maintaining the health of gut microbiome (Ventura et al., 2011). Moreover, studies have shown that the host gut microbial populations can interact and compete with each other, and phages are expected to play an important role in driving the microbial ecology of the complex ecosystem (Ventura et al., 2011; Duerkop and Hooper, 2013). Our data revealed that oral administration of phage cocktails resulted in an initial increase in bacterial richness after a relatively short period (day 6) while a significant reduction was observed two weeks after cessation of the treatment (day 28). However, no significant difference was observed in the gut bacterial diversity of green turtles during the trial. These data are consistent with findings from earlier studies where bacterial diversity was much less affected by phages than by antibiotics (Galtier et al., 2016; Hong et al., 2016). Additionally, our finding support the host density-dependent "Kill-the-winner" model which suggests that lytic phages are highly specific to their target host cells, at least at the species level (Thingstad, 2000).

In this study, the gut bacterial composition of green turtles, regardless of treatments, resembled the typical green turtle gut microbiome shown in earlier studies (Ahasan et al., 2017b; Price et al., 2017). The bacterial communities of green turtles in this study were predominated by the bacteria belonging to the phylum Firmicutes, Bacteroidetes and Proteobacteria while *Clostridium* and *Bacteroides* were the most predominant genera. Our data revealed that the oral administration of *Acinetobacter* spp. specific phage cocktail did not permanently alter the overall composition of the gut bacterial communities of green turtles and compared to the control, no consistent changes were observed in the relative abundance of bacteria in phage treated turtles. This finding is in agreement with the finding of Hong et al. (2016) who also revealed no consistent difference in the faecal bacterial communities of pigs after oral challenge of *E. coli* O157:H7 phages at low (10^6 PFU/mL) and high (10^8 PFU/mL) doses. A similar result was also reported in healthy adult human volunteers who received an oral administration of a high dose (3 X 10^9 PFU) of *E. coli* specific T-4 like phage cocktail (Sarker et al., 2012). However, this study showed that bacteria within the phylum

Bacteroidetes was significantly lower in abundance at day 6 (Table 8.7) while a relatively higher abundance was observed at day 15 compared to the control. Additionally, some bacterial taxa at the genus level were shown to be significantly changed in the phage-treated turtles. For example, bacteria within the genera *Butyricicoccus*, *Akkermansia* and *Eubacterium* were significantly ($P< 0.05$) higher at day 15 (Figure 8.7) while *Peptoclostridium* prevailed at day 28 (Table 8.10). Members of *Butyricicoccus* are well-known for their ability to degrade plant fibre and produce butyrate which is an important energy source for the intestinal epithelial cells (Hamer et al., 2008). Likewise, *Peptoclostridium* species have the ability to breakdown and utilise various complex plant-derived polysaccharides, such as cellulose, hemicellulose and xylan, which constitute the major part of plant fibers (Uffen, 1997; Uz and Ogram, 2006). Bacteria within the genus *Akkermansia* are universally distributed in the intestinal tracts all over the animal kingdom (Belzer and De Vos, 2012). The genus *Akkermansia* has been identified as one of the most abundant mucus degrading true symbiont in the healthy gut microbiome of humans (Derrien et al., 2008). Collectively, the results from our study support the general hypothesis that phages can act in concert with favourable, physiologically relevant bacteria to re-establish normal microflora (Galtier et al., 2016). Likewise, data in the current study as well as those reported in other studies (Harcombe and Bull, 2005; Brockhurst et al., 2006), show that phage can alter competitive interactions between bacterial population within a microbial ecosystem. Previous studies have also reported that phage can act to regulate bacterial density and alter the relative abundance of bacterial species within a microbial community (Weinbauer and Höfle, 1998; Wommack and Colwell, 2000; Weinbauer and Rassoulzadegan, 2004). However, detailed mechanisms by phages impact the establishment and maintenance of indigenous host-associated microbial communities are poorly understood. Detailed investigations on whether phage-induced changes in the abundance of certain bacterial community do have deleterious effects on the health of the animal were beyond the scope of the current study, but do warrant further research before applying the phage therapy to manipulate the transient pathogenic or indigenous host-associated bacterial flora.

In marked contrast, our investigation on the impact of broad-spectrum antibiotic on the gut bacterial communities revealed that oral administration of enrofloxacin significantly altered the gut bacterial communities of green turtles. The oral antibiotic challenge

effectively eliminated the targeted *Acinetobacter*, however, the gut bacterial diversity of green turtles decreased gradually during and after antibiotic therapy without any recovery. Previous studies have also shown a significant collapse in the gut bacterial diversity during antibiotic therapy in both humans (Jernberg et al., 2010; Pérez-Cobas et al., 2012) and animals (Rettedal et al., 2009; Lin, 2011) while the impact of antibiotic therapy might differ between short-term and long term antibiotic challenge (Jakobsson et al., 2010). In this study, the alteration in the bacterial gut communities of antibiotic-treated turtles was mainly driven by an increase abundance of Gram-positive bacteria, Firmicutes (Class: Clostridia) and a concurrent decrease in the Gram-negative bacteria, Bacteroidetes (Class Bacteroidia), Proteobacteria (Class: Gammaproteobacteria) and Verrucomicrobia (Class: Verrucomicrobia). Gram-positive bacteria within the phylum Actinobacteria were also relatively low in abundance at day15. Our results are consistent and supported by the general notion that broad-spectrum antibiotics such as enrofloxacin are bactericicdal to both Gram-positive and Gram-negative bacterial communities (Brown, 1996; Schaumann and Rodloff, 2007). Similar results were also reported in earlier studies in animals and humans (Lu et al., 2006; Looft et al., 2014). Pérez-Cobas et al. (2012) reported a significant reduction in the Gram-negative Bacteroidetes and a concurrent increase in Gram-positive Firmicutes following broad-spectrum ß-lactam therapy in human. In addition, the work of Pérez-Cobas et al. (2012) also revealed that there was a general tendency towards restoration of the microbial community towards its original status at day 40. However, in this study, the gut bacterial community structure of antibiotic-treated turtles was restored largely 2 weeks after discontinuation of the antibiotic therapy. No significant difference was observed at the phylum level while few bacterial genera such as *Bilophila, Eubacterium,* Ruminococcaceae_UCG013, *Oscillibacter* and *Erysipelatoclostridium* within the phylum Firmicutes were significantly dominant in antibiotic-treated turtles compared to the control turtles. Although several members of these genera have been previously reported in the faecal samples of green turtles (Ahasan et al., 2017b), studies have not yet explored the exact roles of these bacteria within the gut microbiome of green turtles. Bacteria within the genera *Bilophila, Eubacterium* and *Ruminococus* are well-recognised as bile-tolerant microbes. They have been reported in higher abundance in the individuals who feed on animal-based diet compared plant-based diet (David et al., 2014). In the current study however, the cause of higher abundance of this select group of bacteria following cessation of antibiotic therapy is unclear since all turtles (control

and treatment groups) were fed the same mixed diet as described in our methods section. Although studies have suggested that the ability of the bacterial gut community to recover to its original state following cessation of antimicrobial therapy depends on the antimicrobials regimen administered to the individual (Antonopoulos et al., 2009), further investigation is required to explore the factors that may govern the abundance of such bacteria and their contribution on green turtle health.

Generally, gut bacterial communities and the host maintain a commensal relationship with each other (Kostic et al., 2013; Sommer and Backhed, 2013). However, it is also reported that bacterial microbiota can cause deleterious effects to the host when the microbiome undergoes any abnormal changes (Boulangé et al., 2016). Several members within the phylum Firmicutes and Bacteroidetes play crucial roles in modulating physiological, immune and metabolic processes within the gastrointestinal tract of the host; however, the imbalance in the ratio between these two predominant phyla has been reported to be associated with several diseases and disorders (Kostic et al., 2013; Zhang et al., 2015; Komaroff, 2017). The prolonged antibiotic therapy has been recognised as one of the possible causes of such long-lasting alterations in the gut bacterial communities of the host (Antonopoulos et al., 2009; Pérez-Cobas et al., 2012). It is suggested therefore, to have a better understanding of the impact of such changes on the health of sea turtles before consideration of any antibiotic regimen.

In conclusion, the implications of applying broad-spectrum antibiotics are very extensive. Significant alterations in the bacterial diversity as well as abundance were noticed in green turtles following antibiotic challenge. Although gut bacterial community structure of antibiotic-treated turtles recovered largely at phylum level after cessation of antibiotic therapy, it remained altered at lower taxonomic level. This may indicate that severe reduction of bacterial communities that are sensitive to the administered antibiotic provide space for resistant communities to overgrow and dominate the niche. Further studies are suggested to investigate whether such changes in the abundance of certain bacterial community are deleterious to the health of patient. Since different antimicrobial agents can influence the host indigenous microbial community in different ways, future studies involving other classes of antibiotics could help better understanding if the findings of this study could be generalised to other findings. The present study is the first to provide a proof of concept for the therapeutic use of phages in captive sea turtles. Phages were found to be safe and effective in

elimination of the targeted bacteria from the gut of sea turtles without any deleterious effects to their health. This study offers a new possibility of applying phages to manipulate transient as well as indigenous bacterial flora with a broad application in many gut-related diseases/disorders in sea turtles.

CHAPTER 9
GENERAL DISCUSSION

Gastrointestinal (GI) diseases have been identified as one of the major causes of mortality in stranded green turtles (Flint et al., 2010; Rousselet et al., 2014). Knowledge of the gut bacterial communities of healthy and compromised green turtles is likely the key to elucidate the complex association between host and its microbiota, and to identify the possible causal agent(s) of disease. The major goal of this project was to characterise the bacterial gut flora of healthy and compromised green turtles using both culture dependent and independent techniques. To our knowledge, this research is the first to use both conventional and molecular techniques to characterise the gut bacterial communities of sea turtles. Our study identified a diverse array of bacterial communities inhabiting the gastrointestinal tract of green turtles that contribute to the health and diseases of the host.

Our culture-dependent identification revealed the presence of several members of Enterobacterales that are known to be gut commensal and have the ability to cause opportunistic infections in both aquatic and terrestrial animals including sea turtles (Aguirre et al., 1994; Bergey and Garrity, 2004; Santoro et al., 2008a). The predominant Enterobacterales belonged to the genus *Citrobacter*, *Edwardsiella*, *Escherichia* and *Klebsiella*. Bacteria within these genera such as *C. freundii*, *C. koseri*, *E. coli*, *K. pneumoniae* and *K. oxytoca* have the potential to infect humans although their pathogenicity is unknown (Podschun and Ullmann, 1998; Magiorakos et al., 2012; Qu et al., 2016). In this study, the highest number of Enterobacterales were recovered from stranded (rehabilitating) turtles (3.53 isolates per turtle) compared to wild-captured turtles (<2 isolates per turtle). This finding indicates the possible overgrowth of Enterobacterales in the gut of debilitated green turtles. Furthermore, the present study investigated antimicrobial resistance of these Enterobacterales against different groups of antibiotics. The highest antimicrobial resistance was recorded for the antibiotics in the β-lactam class followed by quinolone, tetracycline and aminoglycoside classes. A lower antimicrobial resistance was recorded for streptomycin, trimethoprim-sulfamethoxazole and chloramphenicol classes. The presence of antibiotic-resistant

Enterobacterales with potential to infect humans and other animals is an important finding that indicates marine microbial pollution in proximity to large urban development. Microbial resistance to multiple antibiotics revealed that isolates recovered from rehabilitating turtles exhibited higher multidrug resistant compared to wild-captured turtles' isolates. This finding indicates that these multidrug resistant bacteria might be associated with the compromised health condition of stranded turtles. The antimicrobial resistance of the Enterobacterales to certain antibiotics determined in this study may either be inherent or acquired in response to stressors present in the environments. Further investigation is suggested to determine the resistant gene in the bacterial isolates to understand the transmission dynamics and potential effect on human health.

The high-throughput sequencing analysis targeting the hypervariable V1- V3 regions of the bacterial 16S rRNA gene revealed that the faecal bacterial communities of green turtles were largely dominated by bacteria within the phyla Firmicutes, Bacteroidetes and Proteobacteria while a lower abundance of Verrucomicrobia, Actinobacteria and Fusobacteria were also recorded. This finding is in agreement with the findings in other marine animals including both reptiles and marine mammals (Tsukinowa et al., 2008; Hong et al., 2011; Nelson et al., 2013; Numberger et al., 2016). In this study, Firmicutes dominated among the wild-captured green turtles and Proteobacteria (Gammaproteobacteria) prevailed in stranded turtles. The presence of a higher abundance of Gammaproteobacteria (order Enterobacterales) in stranded turtles support the finding of our culture-dependent identification study where the majority of Enterobacterales were recovered from stranded turtles before rehabilitation. Preliminary studies on stranded loggerhead sea turtles (*Caretta caretta*) also showed the presence of an elevated abundance of Proteobacteria (Abdelrhman et al., 2016). In contrast, the higher abundance of Firmicutes was reported in other marine herbivorous reptiles (Hong et al., 2011), and mammals (Tsukinowa et al., 2008; Nelson et al., 2013; Merson et al., 2014). Our results also revealed that the faecal bacteria of wild-captured green turtles were highly diverse and rich compared to stranded turtles. The diverse microorganisms present in the GI tract of wild-captured green turtles may be associated with various functions in their gut microbiome. However, to date, little information is available on the functional role of gut microbiota in sea turtles and therefore, information on the role of these bacteria must be elucidated from previous studies in

other terrestrial and aquatic vertebrates. The bacterial communities of green turtles appear to be associated with microbes that are responsible for harvesting energy, absorption of nutrients, butyrate production, maintain intestinal barrier functions, host-immune function, intestinal permeability, strengthen epithelial cell barrier properties and protection against stress-induced injuries (Backhed et al., 2005; Turnbaugh et al., 2008; Mao et al., 2012; Serino et al., 2012; Power et al., 2014). These microbes have also been implicated in gastrointestinal pathologies, brain-gut axis and neurological conditions (Guarner and Malagelada, 2003; Keszthelyi et al., 2009). However, it is worth investigating the actual role of these microbes in health and diseases of green turtles.

Debilitated sea turtles with unknown illness are often cared for in rehabilitation centers until they recover. Alteration in the gut bacterial community and its activities are believed to be one of the contributing factors to their illness (Myers, 2004). Therefore, the present study investigated and compared the faecal bacterial communities between pre-hospitalisation and post-rehabilitation stranded green turtles. Additionally, we investigated whether recovered green turtles are able to restore their normal gut flora during rehabilitation. Our study identified significant difference in the faecal bacterial communities between pre-hospitalisation and post-rehabilitation at lower taxonomic level while no significant difference was present at phylum level. Bacteria within the phylum Proteobacteria dominated in both pre-hospitalisation and post-rehabilitation samples. In addition to Proteobacteria, Firmicutes also prevailed in pre-hospitalisation samples and a higher abundance of Bacteroidetes was observed in post-rehabilitation samples. Proteobacteria is one of the most physiologically and metabolically diverse groups. Members within this phylum are well-known to establish mutualistic as well as pathogenic relationships with their hosts (López-García and Moreira, 1999; Bergey and Garrity, 2004; Lauber et al., 2009; Pascault et al., 2014; Shin et al., 2015). Bacteria such as *Campylobacter*, *Arcobacter*, *Escherichia*, *Edwardsiella*, *Citrobacte*r, *Shewanella* and *Vibrio* were significantly abundant in pre-hospitalisation samples. This indicates these bacteria might be overrepresented in the gut of stranded turtles. Importantly, they can cause opportunistic infections in several terrestrial and aquatic vertebrates including sea turtles (Daniels et al., 2000; Imhoff, 2005; Chuen-Im et al., 2010a; Bocian-Ostrzycka et al., 2015). The higher abundance of Bacteroidetes in post-rehabilitation turtles may be associated with the high protein diets (squid) that were offered during rehabilitation.

Although there was a marked shift in the bacterial communities between pre-hospitalisation and post-rehabilitation turtles, no significant correlation was found in the bacterial communities between post-rehabilitation and wild-captured green turtles of earlier study. It is therefore, interesting to investigate the capacity to restore normal gut microbiota of recovered green turtles which are released back to their natural habitat.

Furthermore, reading of the literature revealed that most investigations of sea turtle's gut microbiome have typically involved bacterial identification in faeces (Aguirre et al., 1994; Santoro et al., 2006a; Foti et al., 2009; Al-Bahry et al., 2011; Price et al., 2017) rather than along the GI tract. Faecal microbiota represents a pool of both resident and transient microbial population in the GI tract (Eckburg et al., 2005) while samples from the GI mucosal wall are more likely to represent the resident bacterial communities that have a higher potential to influence several host functions compared to transient microbial population (Kelly et al., 2016). Therefore, this study characterised and compared the mucosa-associated bacterial communities across different regions of the GI tract of freshly dead green turtles. The results revealed that bacterial communities of various compositions occupied different regions of the GI tract. Gut bacterial diversity and richness decreased longitudinally along the GI tract from oesophagus to small intestine while the large intestine showed a higher bacterial diversity and richness compared to the small intestine but less than the stomach. This finding supports the recognised notion that different anatomic regions of the GI tract have their own physiochemical conditions such as pH and oxygen, which exert selective pressures on the bacterial communities and play an important role in shaping the microbiota of the GI tract. The occurrence of highly diverse bacterial communities in the oesophageal and gastric mucosa of green turtles could be due to the existence of transient bacteria that is continuously swallowed with the food and sea water (Pedrós-Alió, 2006; Zinger et al., 2011). The presence of diverse bacterial community in the large intestinal mucosa as found in the caecum, indicates the occurrence of a more complex microbial ecosystem because the caecum is identified as the initial site for the microbial fermentation activity in green turtles (Bjorndal, 1979b; Karen et al., 1991). Furthermore, the GI mucosa-associated bacterial communities of green turtles were largely dominated by Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes and Fusobacteria which are likely to be similar to the faecal bacterial communities identified previously in wild-captured green turtles in this study. However, in this study, the gut bacterial composition at different

anatomical regions of the GI tract of green turtles differed notably. Bacteria within the phylum Firmicutes were most abundant in all GI regions except in the small intestine, where Proteobacteria prevailed. The predominance of Proteobacteria in the small intestinal mucosa was largely due to a higher prevalence of the bacteria belonging to the class Gammaproteobacteria. Bacteria within this class are known to be facultative anaerobes and multiply at neutral to slightly basic pH (~ 7.4) (Bergey and Garrity, 2004). Previous studies in green turtles revealed a fairly neutral pH (7.0) in the small intestine which provides a suitable environment for the occurrence of these bacteria (Bjorndal, 1979b; Bergey and Garrity, 2004). However, an over colonisation of these opportunistic bacteria (Gammaproteobacteria: Vibrionaceae and Enterobacteriaceae) are a common marker of dysbiosis (Byndloss et al., 2017) within the small intestinal mucosa and these bacteria may competitively exclude the other normal flora and result in decreased bacterial diversity in the small intestinal mucosa (Brenner and Farmer, 1984; Amaro and Biosca, 1996).

Dysbiosis of the gut microbial communities is often associated with the pathogenesis of several intestinal and extra-intestinal disorders (Carding et al., 2015). Antibiotic therapy is believed to be one of the major contributing factors of microbial dysbiosis together with other collateral damage in debilitated patients (Sullivan et al., 2001; Myers, 2004; Pérez-Cobas et al., 2012; Modi et al., 2014). Therefore, this study established a proof of concept for the use of bacteriophage (phage) therapy as a suitable alternative to antibiotics in order to eliminate targeted bacteria from the GI tract of green turtles. Additionally, this study identified minimal effects of phages on the gut bacterial community of green turtles compared to antibiotic therapy. Our study revealed that the oral challenge with a bacterial specific phage cocktail significantly reduced targeted *Acinetobacte*r abundance during treatment while a slow recovery was seen to occur following cessation of the challenge. This finding is in accordance with the finding of other studies in both humans and animals where phage therapy was found to be effective in treating targeted bacterial pathogens (Sulakvelidze and Barrow, 2005; Sulakvelidze and Kutter, 2005; Atad et al., 2012; Alagappan et al., 2016; Li et al., 2016b). However, to date, the impact of phage therapy on the indigenous gut microbes has rarely been examined although the diverse microbial communities play important roles in maintaining the healthy gut microbiome (Ventura et al., 2011). Our results revealed an initial increase in bacterial richness following the phage challenge at early

treatment period (day 6) while a significant reduction was observed two weeks after cessation of the treatment (day 28). No significant difference was observed in the gut bacterial diversity of green turtles during the trial. This result indicates that bacteria-specific phage cocktail had minimal effects on the gut microbial diversity of green turtles. Furthermore, compared to the control, no consistent changes were observed in the relative abundance of bacteria in phage treated turtles. This supports the hypothesis that phages can act in concert with "favourable" bacteria for re-establishing the normal microflora (Galtier et al., 2016).

In this study, the oral enrofloxacin challenge was also effective in eliminating the targeted *Acinetobacter*, however, the side-effects on the gut bacterial communities were extensive. Significant alterations in the bacterial diversity as well as in abundance were observed in green turtles following antibiotic challenge. Previous studies have also shown a substantial collapse in the gut bacterial diversity during antibiotic therapy in both humans (Jernberg et al., 2010; Pérez-Cobas et al., 2012) and animals (Rettedal et al., 2009; Lin, 2011) while it might differ between short-term and long term antibiotic challenge (Jakobsson et al., 2010). In this study, the alteration in the bacterial gut communities of antibiotic-treated turtles was mainly driven by an increased abundance of Gram-positive bacteria, Firmicutes and a concurrent decrease in the Gram-negative bacteria, Bacteroidetes, Proteobacteria and Verrucomicrobia. Gut bacterial community structure of antibiotic-treated turtles recovered largely at phylum level after cessation of antibiotic therapy, however, it remained altered at lower taxonomic level. Multiple bacterial populations did not recover after a two weeks withdrawal from antibiotics. This may indicate that severe reductions of bacterial communities' that are sensitive to administered antibiotic provide space for resistant communities to overgrow and dominate the niche. Further studies are suggested to investigate whether such changes in the abundance of certain bacterial community are deleterious to the health of the patient. Since different antimicrobial agents can influence the host indigenous microbial community in different ways, future studies involving other classes of antibiotics could help to better understanding if the findings of this study can be extrapolated to other findings.

In conclusion, a complex community of microbes that inhabit the GI tract of green turtles and contribute to various aspects of their health and diseases have been identified in this study. The impacts of broad spectrum antibiotics on the gut bacterial

communities identified in this study highlights the need for careful consideration in the selection and application of antibiotics to treat diseases of sea turtles. This study has also established a proof-of-concept for the use of bacteriophages to effectively eliminate the target bacteria from the GI tract of sea turtles. This offers a new possibility of applying phages to manipulate transient as well as indigenous bacterial flora with a broad application in many gut-related dysbiosis of turtles.

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APPENDIX 1

SAMPLE INFORMATION

Serial no	Sample id	Collection site	Sampling site	Animal description		
				Animal id	CCL (cm)	Weight (kg)
1	sae01	reefhq	cloaca			11.2
2	sae02	reefhq	cloaca		87.6	55
3	sae03p	QWPS	cloaca			
4	sae04p	QWPS	cloaca			
5	sae05p	QWPS	cloaca			
6	sae06p	QWPS	cloaca			
7	sae07f	ollera beach	cloaca	QA33971	45.3	8.1
8	sae08f	ollera beach	cloaca	QA39281	47.7	11.1
9	sae09f	ollera beach	cloaca	QA33974	40.8	6.4
10	sae10f	ollera beach	cloaca	QA36642	47.8	11.5
11	sae11f	ollera beach	cloaca	QA33965	44.1	8.6
12	sae12f	ollera beach	cloaca	QA33948	46	9.3
13	sae13f	ollera beach	cloaca	QA33937	46.6	11
14	sae14f	ollera beach	cloaca	QA33952	51.1	14.5
15	sae15r	reefhq	cloaca		45.3	8.1
16	sae16r	reefhq	cloaca		45.5	9.5
17	sae17f	toolakea	cloaca	QA42921	40.7	7.5
18	sae18f	toolakea	cloaca	QA42918	48.5	12.2
19	sae19f	toolakea	cloaca	QA20337	46.7	10.8
20	sae20f	toolakea	cloaca	QA19725	48.8	9.4
21	sae21f	toolakea	cloaca	QA7323	45.8	10.6
22	sae22f	toolakea	cloaca	QA89294	46.8	11.2
23	sae23h	reefhq	Gut content			
24	sae24h	reefhq	Gut content			
25	sae25h	reefhq	Gut content			
26	sae26f	toolakea	cloaca	K89295	49.1	13.4
27	sae27f	toolakea	cloaca	QA20327	47.2	10.73
28	sae28f	toolakea	cloaca	QA20326	46.9	10.62
29	sae29f	toolakea	cloaca	QA42921	40.6	7.6
30	sae30f	toolakea	cloaca	QA51073	40.8	7.01
31	sae31f	toolakea	cloaca	QA7343	40.8	7.02
32	sae32f	toolakea	cloaca	QA47531	43.5	9.19
33	sae33r	reefhq	cloaca			
34	sae34r	reefhq	cloaca			
35	sae35r	reefhq	cloaca			
36	sae36f	toolakea	cloaca	QA47542	51.2	15.17
37	sae37f	toolakea	cloaca	QA47543	42.5	9.5
38	sae38f	toolakea	cloaca	QA47545	41.4	8.45
39	sae39f	toolakea	cloaca	QA47544	46.1	11.35
40	sae40f	toolakea	cloaca	QA47535	41.3	6.25

41	sae42f	Bowen	cloaca	QA42274		
42	sae43f	Bowen	cloaca	QA42271	42.7	8.1
43	sae44f	Bowen	cloaca	QA42254	55.6	19.4
44	sae45f	Bowen	cloaca	QA42286	51.8	13.5
45	sae46f	Bowen	cloaca	QA47546	42.8	8.4
46	sae47f	Bowen	cloaca	QA47547	46.4	12.4
47	sae48f	Bowen	cloaca	QA47548	42.1	9.15
48	sae49f	toolakea	cloaca			
49	sae50f	toolakea	cloaca			
50	sae51r	reefhq	cloaca	QA47535	41.3	6.25
51	sae52r	reefhq	cloaca			18.2
52	sae53r	Bowen	cloaca		48.4	9.5
53	sae54f	cockle bay	cloaca	QA7394	106.5	
54	sae55f	cockle bay	cloaca	QA47530	48.1	12.7
55	sae56f	cockle bay	cloaca	QA7414	53.7	18.2
56	sae57f	cockle bay	cloaca	QA7454	42.6	8.3
57	sae58f	cockle bay	cloaca	QA7406	43.5	9.7
58	sae59f	cockle bay	cloaca	QA7403	64.5	25.5
59	sae60f	cockle bay	cloaca	K59363	53.3	16.2
60	sae61f	cockle bay	cloaca	QA7404	47.7	12.4
61	sae62f	cockle bay	cloaca	QA7392	50.2	12.9
62	sae63f	cockle bay	cloaca	QA7401	49.5	13.4
63	sae64f	cockle bay	cloaca	QA47550	43.5	8.2
64	sae65f	cockle bay	cloaca	QA42248	44.2	8.78
65	sae66f	cockle bay	cloaca	QA32746	44.5	9.95
66	sae67f	cockle bay	cloaca	QA42272	46.8	10.7
67	sae68f	cockle bay	cloaca	QA42201	49.4	12.85
68	sae69f	cockle bay	cloaca	QA7418	46.7	10.36
69	sae70f	cockle bay	cloaca	QA42218	55.8	18.35
70	sae71f	cockle bay	cloaca	QA7397	44.5	10.25
71	sae72f	cockle bay	cloaca	QA7433	44.9	8.82
72	sae73f	cockle bay	cloaca	QA7388	49.5	13.1
73	sae74f	cockle bay	cloaca	QA7381	53	14.9
74	sae75f	cockle bay	cloaca	QA42923	43.6	6.5
75	sae76f	cockle bay	cloaca	QA7382	45.5	9.2
76	sae77f	cockle bay	cloaca	QA42922	47.3	11.1
77	sae78f	cockle bay	cloaca	QA7400	46.3	10.4
78	sae79f	cockle bay	cloaca	QA7398	46.4	9.4
79	sae80r	reefhq	cloaca		67.5	26.6
80	sae81	reefhq	cloaca	QA47641	70.6	28.4

APPENDIX 2

BEAGENTS AND BACTERIOLOGICAL MEDIA

5% Sheep blood agar

For 1 litre preparation

Blood agar base no 2 (Acumedia)	:	39.5 g
NaCl	:	10 g
Bacteriological agar	:	15 g
Distilled water	:	1000 ml
Sheep blood	:	50 ml

MacConkey agar with crystal violet

For 1 litre preparation

MacConkey agar w CV (Acumedia)	:	50 g
Bacteriological agar	:	10 g
Distilled water	:	1000 ml

Bismuth sulfite agar

For 1 litre preparation

Bismuth sulfite agar (Acumedia)	:	40 g
Distilled water	:	1000 ml

Endo agar

For 1 litre preparation

Endo agar base (Oxoid)	:	36 g
10% w/v alcoholic solution of basic fuchsin BR 50 (95% ethylalcohol)	:	4 ml
Distilled water	:	1000 ml

EC broth

For 1 litre preparation

EC broth with mug (Oxoid)	:	37 g
Distilled water	:	1000 ml

Brilliant green bile broth (2%)

For 1 litre preparation

Brilliant green bile 2% (Oxoid)	:	40 g
Distilled water	:	1000 ml

Urease media

For 1 litre preparation

Urease agar base	:	25.6 g
Distilled water	:	948 ml
Autoclave at 115 ^{0}C for 20 min		
40% sterile urea solution	:	52 ml

Oxidative fermentative media

For 1 litre preparation

Oxidative fermentative media (Oxoid)	:	1 g
Distilled water	:	900 ml
10% glucose D anhydrous (+) [add after autoclave]	:	100 ml

Simmon's citrate agar

For 1 litre preparation

Simmon's citrate agar (Oxoid)	:	23 g
Distilled water	:	1000 ml

Motility broth

For 1 litre preparation

Nutrient broth	:	13 g
Gelatin	:	50 g
Bacteriological agar	:	4 g
10% glucose D anhydrous (+) [add after autoclave]	:	2 g
Distilled water	:	1000 ml

Normal saline solution

For 1 litre preparation

NaCl	:	9 g
Distilled water	:	1000 ml
Tween 80		0.50 ml

APPENDIX 3

BACTERIAL IDENTIFICATION

1. Bacterial identification using morphological examination and conventional biochemical tests

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
1	163	r	sae35r	1.5	brown round convex	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1110
2	165	r	sae35r	1.5	brown round convex	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1110
3	206	t	sae42f	0.5	whitish brown	n	n	cb	0	f	ng	m	1	0	0	1	1	fa	ngp	1110
4	207	b	sae43f	0.5	brown	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1111
5	208	b	sae43f	0.5	brown	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1111
6	219	b	sae42f	1	brown	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1110
7	273	c	sae56f	1	rough ash brown	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1110
8	314	c	sae64f	1	ash brown convex	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1011
9	316	c	sae64f	1	ash brown convex	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	ngp	1011
10	325	c	sae66f	1-1.5	ash brown convex	n	n	cb	0	f	NLF	m	1	0	0	1	1	fa	gp	1010
11	149	r	sae33r	1.5	brown	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1110
12	205	t	sae42f	0.5	whitish brown	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1111
13	232	t	sae50f	1	brown convex	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1110
14	233	t	sae50f	1	brown convex	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1110
15	234	t	sae50f	1	brown convex	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1110

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
16	274	c	sae56f	1	rough ash brown	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1110
17	313	c	sae64f	1	ash brown convex	n	n	cb	0	f	NLF	m	0	0	0	1	1	fa	ngp	1011
18	241	t	sae50f	1-1.5	brown convex	n	n	cb	0	f	LF	m	0	0	0	1	1	fa	ngp	1110
19	249	r	sae52r	1	brown convex	n	n	cb	0	f	LF	m	0	0	0	1	1	fa	ngp	1110
20	240	t	sae50f	1-1.5	brown convex	n	n	cb	0	f	LF	m	0	1	0	1	1	fa	ngp	1110
21	116	r	sae23j			n	n	cocci	0	f	LF	nm	1	1	0	1	1	fa	ngp	1110
22	43	o	sae11f	1.5	transparent grey	n	n	cb	0	f	LF	m	0	1	0	1	0	fa	ngp	1110
23	44	o	sae11f	1.5	grey	n	n	cb	0	f	LF	m	0	1	0	1	0	fa	gp	1110
24	55	o	sae13f	1	transparent	n	n	cb	0	f	LF	m	0	1	0	1	0	fa	gp	1111
25	217	b	sae48f	1	brown	n	n	cb	0	f	LF	m	1	1	1	1	1	fa	ngp	1110
26	226	c	sae47f	1-1.5	brown	n	n	cb	0	f	LF	m	1	1	1	1	1	fa	ngp	1110
27	308	c	sae63f	0.5	white convex	n	n	cb	0	f	LF	m	1	1	1	1	1	fa	gp	1110
28	49	o	sae12f	1	flat grey	n	n	cb	0	f	LF	m	1	1	1	1	0	fa	ngp	1111
29	27	o	sae07f	1.5	grey, connvex	n	n	cb	0	f	LF	m!!!	0	0	1	1	0	fa	ngp	1110
30	45	o	sae11f	1.5	grey	n	n		0	f	LF	m	1	0	0	1	0	fa	ngp	1111
31	50	o	sae12f	1.5	grey, connvex	n	n	cb	0	f	LF	m	1	0	0	1	0	fa	ngp	1110
178	221	b	sae44f	0.5-1	brown	n	n	cocci	0	nr	NLF	nm	0	0	0	0	1	a	gp	0000
181	315	c	sae64f	1	white brown	b hem	n	cocci	0	nr	NLF	nm	0	0	0	0	1	fa	gp	0000
32	153	r	sae34r	1.5	brown	n	n		0	f	NLF	m	1	0	1	1	1	fa	ngp	1110
33	368	c	sae79f	1-1.5	brown convex	n	n	cb	0	f	NLF	m	1	0	1	1	1	fa	ngp	1110
34	369	c	sae79f	1-1.5	brown convex	n	n	cb	0	f	NLF	m	1	0	1	1	1	fa	ngp	1110
35	370	c	sae79f	1-1.5	brown convex	n	n	cb	0	f	NLF	m	1	0	1	1	1	fa	ngp	1110
36	41	o	sae10f	1.5-2	grey	n	n	cb	0	f	LF	m	1	0	1	1	p	fa	ngp	1110
37	287	c	sae59f	0.5	whitish	n	n	cb	0	f	LF	m	1	0	1	1	1	fa	ngp	1010

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
38	309	c	sae63f	1	ash brown convex	n	n	cb	0	f	LF	m	1	0	1	1	1	fa	ngp	1010
39	29	o	sae07f	2	grey, connvex	n	n	curved cocci	0	f	LF	m	1	0	1	1	0	fa	ngp	1110
40	64	r	sae15r	1-1.5	creamy light brown	n	n	cb	0	f	LF	nm	1	0	1	1	1	fa	ngp	1110
41	66	r	sae15r	1.5	light brown	n	n	cb	0	f	LF	nm	1	0	1	1	1	fa	gp	1110
42	51	o	sae12f	1	grey, connvex	n	n	cb	0	f	LF	m	0	0	0	1	0	fa	ngp	1110
43	54	o	sae13f	1	grey	n	n	cb	0	f	LF	m	0	0	0	1	0	fa	gp	1111
44	52	o	sae12f	1.5	grey, connvex	n	n	cb	0	f	LF	m	1	1	0	1	0	fa	ngp	1111
45	53	o	sae13f	1	grey	n	n	cb	0	f	LF	m	1	1	0	1	0	fa	ngp	1111
46	56	o	sae13f	0.5-1	transparent	n	n	cb	0	f	LF	nm	0	1	0	1	0	fa	ngp	1111
47	3E		sae01e	1	Creamy convex	N	n	Cocci		F	NLF	M		0	1	0	1	FA	NG	
48	4E		sae02e	1.5	Dark creamy	N	n	cb		F	NLF	M		0	1	0	1	FA	NG	
49	6E		sae03e	1	Dark brown convex	N	n	cb		F	NLF	M		0	1	0	1	FA	NG	
50	157	r	sae34r	1.5	brown	n	n	cb	0	f	NLF	m	1	1	1	0	1	fa	gp	1100
51	256	r	sae52r	0.5-1	brown convex	n	n	bacillus	0	f	ng	m	0	0	0	1	1	fa	ngp	1110
52	145	r	sae33r	1.5	brown	n	n	bacillus	0	f	NLF	m	0	1	0	1	1	fa	ngp	1110
53	146	r	sae33r	1.5	brown	n	n	cb	0	f	NLF	m	0	1	0	1	1	fa	ngp	1110
54	231	t	sae50f	1	brown convex	n			0	f	NLF	m	0	1	0	1	1	fa	ngp	1110
55	184	t	sae39f	1	brown	n	n	cocci	0	f	dead isolate	m	0	0	1	1	1	fa	ngp	1110
56	191	t	sae40f	0.5	brown	n	n	cocci or cb	0	f	dead isolate	m	0	0	1	1	1	fa	ngp	1110
57	192	t	sae40f	0.5	brown	n	n	cocci or cb	0	f	dead isolate	m	0	0	1	1	1	fa	ngp	1010

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
58	298	c	sae60f	<0.5	white transparent	n	n	cocci	0	f	NLF	m	0	0	1	0	1	fa	ngp	1100
59	32	o	sae08f	1-1.5	transparent	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1010
60	62	r	sae15r	0.5-1	dark brown	b hem	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1111
61	63	r	sae15r	1	dark brown	b hem	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1111
62	67	r	sae15r	1	dark brown	b hem	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1111
63	78	t	sae17f	0.5-1	white transparent	b hem	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1111
64	80	t	sae17f	1	white transparent	b hem	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1011
65	88	t	sae19f	1-1.5	dark brown	n	n	cb	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
66	91	t	sae19f	0.5-1	dark brown convex	n	n	cb	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
67	98	t	sae21f	1-1.5	dark brown convex	b hem	n	cb	0	f	NLF	m	0	0	1	1	1	fa	ngp	1011
68	166	t	sae36f	1-1.5	brown convex	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
69	167	t	sae36f	1-1.5	brown convex	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1111
70	168	t	sae36f	1-1.5	brown convex	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
71	169	t	sae36f	1-1.5	brown convex	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
72	170	t	sae36f	0.5	brown convex	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
73	239	t	sae50f	0.5-1	whitish brown	n	n	cb	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
74	276	c	sae56f	1	rough ash brown	n	n	cb	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
75	277	c	sae56f	<0.5	ash brown	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
76	278	c	sae56f	<0.5	ash brown	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1110
77	294	c	sae60f	<0.5	white transparent	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	ngp	1010
78	295	c	sae60f	<0.5	white transparent	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	gp	1110

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
79	322	c	sae65f	<0.5	white transparent	n	n	cocci	0	f	NLF	m	0	0	1	1	1	fa	gp	1111
80	328	c	sae67f	<0.5	white transparent	n	n	cb	0	f	NLF	m	0	0	1	1	1	fa	gp	1110
81	81	t	sae17f	1	white transparent	b hem	n		0	f	NLF	nm	0	0	1	1	1	fa	ngp	1111
82	100	t	sae21f	1-1.5	dark brown convex	b hem	n		0	f	NLF	nm	0	0	1	1	1	fa	ngp	1011
83	101	t	sae21f	1-1.5	dark brown convex	b hem	n		0	f	NLF	nm	0	0	1	1	1	fa	ngp	1011
84	350	c	sae74f	<0.5	white transparent	n	n	bacilli or cb	0	f	NLF	nm	0	0	1	1	1	fa	gp	1110
85	352	c	sae74f	<0.5	white transparent	n	n	cocci	0	f	NLF	nm	0	0	1	1	1	fa	gp	1010
86	359	c	sae76f	<0.5	white transparent	n	n	cb	0	f	NLF	nm	0	0	0	1	1	fa	gp	1110
87	319	c	sae65f	<0.5	white transparent	n	n	cocci	0	f	ng	m	0	0	1	1	1	fa	gp	1110
182	317	c	sae65f	<0.5	white transparent	n	n	cocci	0	nr	NLF	nm	0	0	0	0	1	fa	gp	0001
88	204	t	sae39f	1	brown flat	n	n	cb	0	f	dead isolate	nm	0	0	0	1	1	ng	ngp	1011
89	243	r	sae51r	1-1.5	whitish brown	n	n	bacilli or CB	0	f	LF	m	0	0	0	0	1	fa	gp	1101
90	244	r	sae51r	1-1.5	whitish brown	n	n	bacilli or !!!	0	f	LF	m	0	0	0	0	1	fa	gp	1101
91	160	r	sae34r	1.5	brown	n	n	bacillus	0	f	NLF	m	0	0	0	0	slow p	fa	ngp	1100
92	245	r	sae51r	1-1.5	whitish brown	n	n	bacilli or !!!	0	f	NLF	m	0	0	0	0	1	fa	gp	1101
93	246	r	sae51r	1-1.5	whitish brown	n	n	bacilli or !!!	0	f	NLF	m	0	0	0	0	1	fa	gp	1101
94	292	c	sae60f	0.5	whitish brown	n	n	cb	0	f	NLF	m	0	0	0	0	1	fa	gp	1100
179	293	c	sae60f	0.5-1	whitish brown	b hem	n	cocci	0	nr	NLF	nm	0	0	0	0	1	fa	ngp	0000

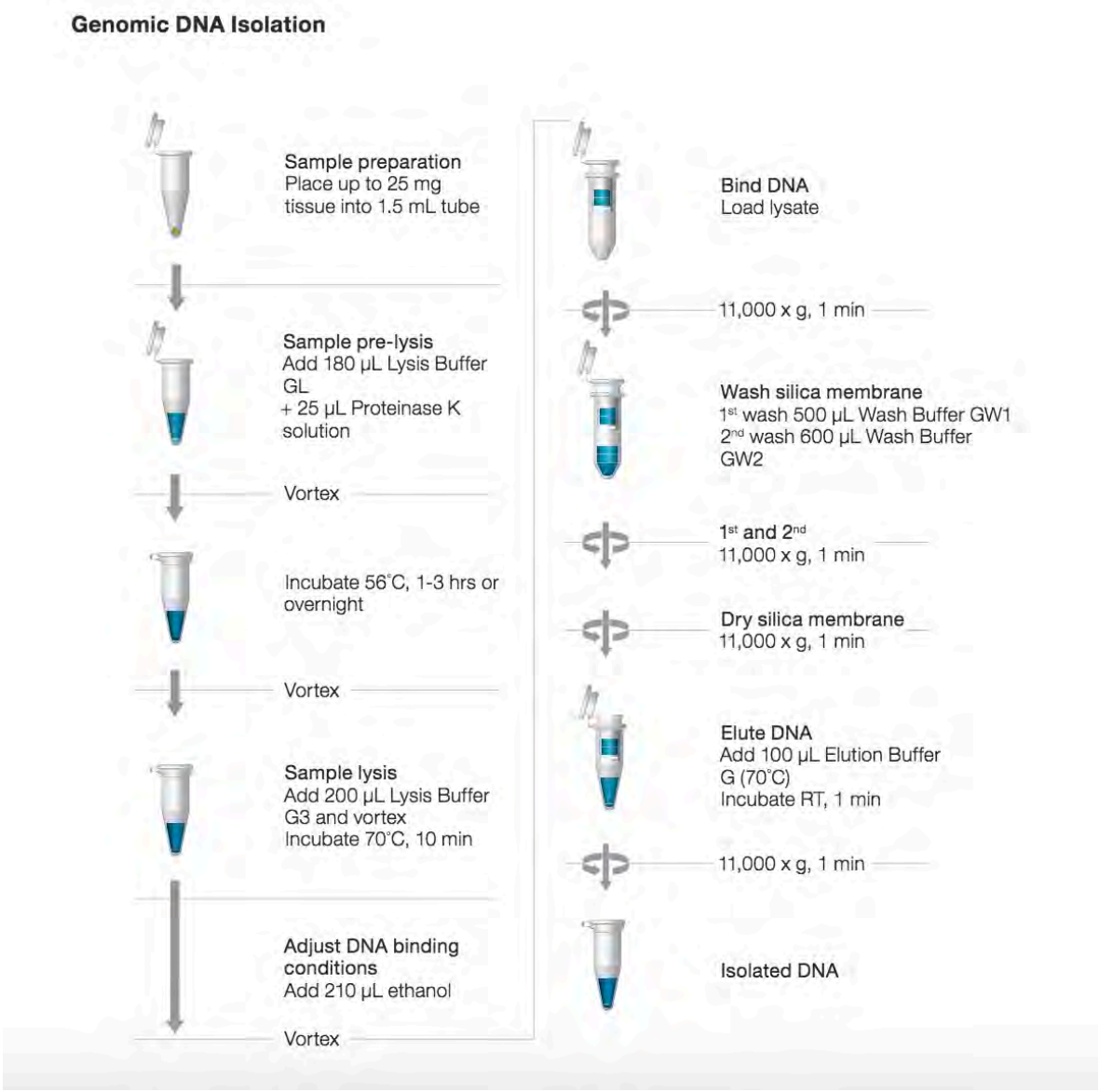
Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
95	306	c	sae62f	0.5	brown chocolotae	n	n	cocci	0	f	NLF	m	0	0	0	0	1	fa	gp	1100
96	188	t	sae40f	1-1.5	dark brown	n	n	cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
97	193	t	sae40f	1-1.5	brown	n	n	cocci or cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
98	194	t	sae40f	1-1.5	brown	n	n	cocci or cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
99	195	t	sae40f	1-1.5	ash brown	n	n	cocci or cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
100	196	t	sae40f	1-1.5	ash brown	n	n	cocci or cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1101
101	197	t	sae40f	1-1.5	ash brown	n	n	cocci or cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
102	198	t	sae40f	1-1.5	dark brown	n	n	cocci or cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
103	255	r	sae52r	0.5	brown	n	n	CB* or bacillus	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
104	327	c	sae67f	1-1.5	ash brown convex	n	n	cb	0	f	LF	m	0	0	1	0	1	fa	gp	1101
105	329	c	sae67f	1-1.5	ash brown convex	n	n	cb	0	f	LF	m	0	0	1	0	1	fa	ngp	1100
106	355	c	sae75f	1-1.5	ash brown convex	n	n	cb	0	f	NLF	nm	0	0	1	0	1	fa	ngp	1100
107	58	o	sae14f	1-1.5	grey	n	n	cb	0	f	LF	m	1	1	0	0	1	fa	ngp	1100
108	227	t	sae49f	1	brown convex	n			0	f	LF	m	1	1	0	0	1	fa	ngp	1100
109	228	t	sae49f	1	brown convex	n			0	f	LF	m	1	1	0	0	1	fa	ngp	1100
110	229	t	sae49f	1	brown convex	n	n	cocci	0	f	LF	m	1	1	0	0	1	fa	ngp	1100
111	230	t	sae49f	1	brown convex	n			0	f	LF	m	1	1	0	0	1	fa	ngp	1100
112	235	t	sae49f	1-1.5	brown convex	n			0	f	LF	m	1	1	0	0	1	fa	ngp	1100
113	236	t	sae49f	1-1.5	brown convex	n			0	f	LF	m	1	1	0	0	1	fa	gp	1100
114	237	t	sae49f	1-1.5	brown convex	n	n	cocci* or cb	0	f	LF	m	1	1	0	0	1	fa	ngp	1100
115	303	c	sae62f	0.5-1	chocolate brown	n	n	cb	0	f	NLF	nm	1	0	0	0	1	fa	gp	1100
116	331	c	sae68f	1-1.5	brown convex	n	n	cb	0	f	NLF	nm	1	0	0	0	1	fa	ngp	1100

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
117	156	r	sae34r	1.5	brown	n	n	cb	0	f	NLF	m	1	0	0	0	1	fa	gp	1100
118	296	c	sae60f	1.5-2	brown mucoid	n	n	cb	0	f	NLF	m	1	0	0	0	1	a	gp	1100
119	330	c	sae68f	1-1.5	brown convex	n	n	cb	0	f	NLF	m	1	0	0	0	1	fa	ngp	1100
120	332	c	sae68f	1-1.5	brown convex	n	n	cb	0	f	NLF	m	1	0	0	0	1	fa	ngp	1000
180	302	c	sae62f	<0.5	chocolate brown	n	n	CB* or bacillus	0	nr	NLF	nm	1	1	0	0	1	fa	gp	0000
121	310	c	sae63f	0.5	white convex	n	n	cocci	0	f	NLF	nm	1	1	1	0	1	fa	gp	1101
122	311	c	sae63f	0.5	white convex	n	n	cocci	0	f	NLF	nm	1	1	1	0	1	fa	ngp	1000
123	312	c	sae63f	0.5	white convex	n	n	cocci	0	f	NLF	nm	1	1	1	0	1	fa	ngp	1000
124	28	o	sae07f	1.5	grey, connvex	n	n	cb	0	f	NLF	nm	0	1	1	0	0	fa	ngp	1100
125	75	r	sae16r	1	transparent convex	n	n	rod	0	f	LF	m	0	1	0	0	1	fa	ngp	1101
126	74	r	sae16r	2	white convex	n	n	rod	0	f	LF	m	0	1	1	0	1	fa	1	1101
127	141	t	sae32f	1-1.5	whitish	n	n	bacillus	0	f	LF	m	1	1	1	0	1	fa	ngp	1100
128	238	t	sae49f	1-1.5	brown convex	n			0	f	LF	nm	0	1	0	0	1	fa	ngp	1100
129	335	c	sae70f	1-1.5	white brown convex	n	n	cb	0	f	NLF	nm	1	1	0	0	1	fa	ngp	1100
130	336	c	sae70f	1-1.5	white brown convex	n	n	cb	0	f	NLF	nm	1	1	0	0	1	fa	ngp	1100
131	5a	r	sae01h		whitish mucoid	a hem	n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1000
132	5b	r	sae01h		whitish mucoid	a hem	n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1100
133	5c	r	sae01h		whitish mucoid	a hem	n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1100
134	8a	r	sae02h	1	whitish creamy	b hem	n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1100
135	13	r	sae01h	3	creamy		n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1100
136	9b	r	sae02h	2	creamy		n	rod	0	f	NLF	m	1	1	1	1	1	fa	ngp	1110
137	9a	r	sae 02h	1	whitish creamy	n	n	rod	0	f	NLF	m	0	1	0	1	1	fa	gp	1010
138	253	r	sae51r	0.5	brown	n	n	cb or bacillus	0	f	ng	m	0	1	0	0	1	fa	gp	1101

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
139	140	t	sae32f	1-1.5	whitish brown	b hem	n	cb	0	nr	NLF	m	0	1	1	0	1	fa	ngp	1000
174	8b	r	sae 02h	1	whitish	n	n	cocci	0	nr	NLF	nm	0	0	0	0	1	a	ngp	0000
140	31	o	sae08f	1.5	white greyish	n	n	cocci	0	f	LF	m	1	1	1	0	1	fa	ngp	1100
141	39	o	sae10f	1-1.5	grey, connvex	n	n	cb	0	f	LF	m	1	1	1	0	1	fa	ngp	1100
142	97	t	sae20f	1-1.5	white convex	n	n	cb	0	f	LF	m	1	1	1	0	1	fa	ngp	1101
143	102-1	t	sae21f	1-1.5	white	n	n	cb	0	f	LF	m	1	1	1	0	1	fa	ngp	1100
144	102-2	t	sae21f	1-1.5	white convex	n	n	cb	0	f	LF	m	1	1	1	0	1	fa	ngp	1100
145	147/1	r	sae33r	1-1.5	brown	b hem	n	cocci	0	f	NLF	m	1	1	0	0	1	fa	gp	1000
146	150	r	sae33r	1.5	brown round convex	b hem	n	bacillus	0	f	NLF	m	1	1	0	0	1	fa	ngp	1100
147	151	r	sae33r	1.5	brown round convex	b hem	n		0	f	NLF	m	1	1	0	0	1	fa	gp	1000
149	164	r	sae35r	1.5	brown	n	n	cb	0	f	NLF	m	0	1	0	0	1	fa	ngp	1100
150	252	r	sae52r	1	brown convex	n	n	cb	0	f	NLF	m	0	1	0	0	1	fa	gp	1101
151	12	r	sae 02h		whitish swarming		n	cocci	0	f	NLF	m	0	1	0	0	1	fa	ngp	1100
152	34	o	sae08f	1.5	white greyish	n	n	cocci	0	f	LF	nm	1	1	1	0	1	fa	ngp	1100
153	103	t	sae21f	1-1.5	white convex	n	n	cb	0	f	LF	nm	1	1	1	0	1	fa	ngp	1100
154	307	c	sae63f	0.5	white convex	n	n	cb	0	f	LF	nm	1	1	1	0	1	fa	gp	1100
155	99	t	sae21f	1-1.5	white convex	n	n		0	f	LF		1	1	1	0	1	fa	ngp	1101
156	257-1	r	sae52r	0.5-1	brown convex	n	n	cb or bacillus	0	f	ng	m	1	1	0	0	1	fa	gp	1101
157	15	r	sae02h	3	creamy		n	rod	0	f	NLF	m	0	1	1	1	1	fa	ngp	1110
158	290	c	sae59f	0.5	whitish brown	n	n	cb	0	f	LF	m	1	0	1		1	fa	ngp	
159	155	r	sae34r	1.5	brown	b hem	n	rod	0	f	NLF	m	0	1	0	1	1	fa	ngp	1111
160	162	r	sae35r	1.5-2	brown	b hem	n	rod	0	f	NLF	m	0	1	0	1	1	fa	ngp	1110
161	158	r	sae34r	1.5	brown	n	n	bacillus	0	f	NLF	m	1	1	0	1	1	fa	ngp	1110

Sl no	Culture no	Locations	Sample no.	c. size (mm)	Colony colour	haemolysis	Gram's stain	Col. in micros	Oxidase	OF	Lactose fermt.	motility	Citrate	Urease	spot indole	H_2S	catalase	type	Gas P.	TSI
162	61	o	sae14f	0.5-1	white greyish	b hem	n	cocci	0	nr		nm	0	0	0	0	1	a	gp	1000
163	265	c	sae54f	0.5-1	chocolate brown	n	n	cb	0	f	NLF	nm	0	0	0	0	1	fa	gp	1100
164	343	c	sae72f	1	white brown convex	b hem	n	cocci	0	f	NLF	nm	0	0	0	0	1	fa	gp	0000
165	345	c	sae72f	1-1.5	white brown convex	b hem	n	cocci	0	f	NLF	nm	0	0	0	0	1	fa	gp	0000
166	17	r	Sae 02h	1	whitish creamy fimbriated		n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1100
167	18	r	sae 02h	1	whitish creamy fimbriated		n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1000
168	20	r	sae 02h	1	creamy		n	rod	0	f	NLF	m	0	1	1	0	1	fa	ngp	1100
175	37	o	sae09f	2-2.5	dark brown	n	n	rod	0	nr	NG	nm	0	0	1	0	1	ng	gp	0000
169	5E		sae02e	3	Dark brown concave rough	Beta slightly	n	Rod shape		O	NLF	M		0	0	0	0	A	ng	
176	40	o	sae10f	1	dark grey	n	n	rod or cb	0	nr	NLF	nm	1	0	0	0	1	fa	gp	0000
170	200	t	sae36f	0.5-1	dark brown	n	n	rod	0	f	NLF	nm	0	0	1	1	1	fa	ngp	1110
177	203	t	sae39f	0.5	ash brown	n	n	cocci or cb	0	nr	dead isolate	m	0	0	1	1	1	fa	ngp	1110
172	60	o	sae14f	2-2.5	white greyish	n	n	rod	0	nr		nm	1	0	0	1	1	fa	gp	1111
173	1E		sae 01E	0.5	creamy	N	n	cb		F	NLF	nm		0	1	0	0	fa	ng	

1. Genomic DNA extraction from pure bacterial culture



9.2 BACTERIA

Before you start:

- Make sure Wash Buffer GW2 and Proteinase K are prepared (see section 7.1).
- For hard-to-lyse bacteria make up the following lysis buffer (20 mM Tris/HCl; 2 mM EDTA; 1% Triton X-100; pH 8, supplemented with 20 mg/mL lysozyme or 0.2 mg/mL lysostaphin) (not supplied).
- Set an incubator or water bath to 56°C.
- Preheat Elution Buffer G to 70°C.

1 **Sample preparation**

Up to 1 mL of bacterial culture can be used for the preparation depending on density, culture medium, bacterial strain etc.

Centrifuge up to 1 mL culture for 5 min at 8,000 x g. Remove supernatant.

2 **Pre-lysis**

Resuspend pellet in 180 µL Lysis Buffer GL and 25 µL Proteinase K solution and vortex vigorously.

Incubate at 56°C for 1-3 hours (until completely lysed), shake or vortex occasionally.

Note: Samples can be incubated overnight. If RNA-free DNA is needed for downstream applications, an RNase digest may be performed (RNase not included).

Note: For hard-to-lyse bacteria such as Gram-positive bacteria, a preincubation is necessary: Resuspend the pelleted cells in a lysis buffer (instead of Lysis Buffer GL, see above) supplemented with lysozyme or lysostaphin and incubate for 30-60 min at 37°C. Add 25 µL Proteinase K, incubate at 56°C until complete lysis is obtained.

Proceed with step 3 of the standard protocol (see section 8.1).

1 **Sample preparation**

1.1 **Human or animal tissue**

Cut 25 mg of tissue into small pieces. Place the sample in a 1.5 mL microcentrifuge tube (proceed to step 2).

Note: Samples that are difficult to lyse can be ground under liquid nitrogen or may be treated in a mechanical homogenizer: Add 25 mg of tissue to a 1.5 mL microcentrifuge tube (not supplied), add 50-75 µL PBS (not supplied) and homogenize.

1.2 **Cultured cells**

Resuspend up to 10^7 cells in a final volume of 200 µL Lysis Buffer GL. Add 25 µL Proteinase K solution and 200 µL Lysis Buffer G3. Incubate the sample at 70°C for 10-15 min (proceed to step 4).

2 **Pre-lysis**

Add 180 µL Lysis Buffer GL and 25 µL Proteinase K solution, completely cover sample with solution and vortex.

Note: If processing several samples, Proteinase K and Lysis Buffer GL may be premixed directly before use (no more than 10-15 min before addition to the sample, as Proteinase K will self-digest in Lysis Buffer GL without substrate).

Incubate at 56°C for 1-3 hours (until completely lysed), shake or vortex occasionally.

Note: Samples can be incubated overnight. If RNA-free DNA is needed for downstream applications, an RNase digest may be performed (RNase not included).

3 **Lyse sample**

Vortex sample briefly and add 200 µL Lysis Buffer G3. Vortex vigorously and incubate at 70°C for 10 min.

Note: If insoluble particles are visible, centrifuge for 5 min at high speed and transfer the supernatant to a new microcentrifuge tube.

4 **Adjust DNA binding conditions**

Vortex briefly and add 210 µL ethanol (96-100%) to the sample. Vortex vigorously.

Note: After addition of ethanol a stringy precipitate may become visible. This will not affect the DNA isolation.

5 **Bind DNA**

For each sample, place an ISOLATE II Genomic DNA Spin Column into a Collection Tube. Add all of the sample to the column and centrifuge for 1 min at 11,000 x g. Discard the flow-through and reuse Collection Tube. Repeat at a higher g force if samples are not completely filtered through matrix.

6 **Wash silica membrane**

- Add 500 µL Wash Buffer GW1. Centrifuge for 1 min at 11,000 x g. Discard flow-through and reuse Collection Tube.
- Add 600 µL Wash Buffer GW2 to the column and centrifuge for 1 min at 11,000 x g. Discard flow-through and reuse Collection Tube.

7 **Dry silica membrane**

Centrifuge 1 min at 11,000 x g, to remove residual ethanol. Place the ISOLATE II Genomic DNA Spin Column in a 1.5 mL microcentrifuge tube (not supplied).

8 **Elute DNA**

Add 100 µL preheated Elution Buffer G (70°C) directly onto the silica membrane. Incubate at room temperature for 1 min. Centrifuge 1 min at 11,000 x g.

Note: For alternative elution procedures see section 7.1.

APPENDIX 4

ANTIMICROBIALS SENSITIVITY DATA

The following **locations** (b, Bowen; c, Cockle Bay; o, Ollera Beach; r, ReefHQ; t, Toolakea Beach) and **antibiotics** (AMP, Ampicillin; AMC, Amoxycillin-clavulanic acid; PEN, Penicillin; KF, Ceftiofur; NA, Nalidixic acid; ENR, Enrofloxacin; CN, Gentamicin; S, Streptomycin; SXT, Sulphamethoxazole-trimethoprim; C, Chloramphenicol) were considered in this study.

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
1	r	1	5a	>16	>16/08'	>32	>16	4	>16	>1	>16	0.06	32	>8	>32
2	r	1	5b	>16	>16/08'	>32	>16	4	>16	>1	>16	0.125	8	>8	>32
3	r	1	5c	>16	>16/08'	>32	>16	4	>16	>1	>16	0.125	16	>8	>32
4	r	2	8a	>16	16/08'	>32	8	4	0.5	0.25	16	0.03	2	<0.06	32
5	r	2	8b	0.25	<0.13/0.06	0.25	4	4	>16	1	<0.125	<0.02	<0.25	<0.06	32
6	r	2	9a	>16	>16/08'	>32	>16	8	>16	1	>16	0.125	8	<0.06	>32
7	r	2	9b	>16	16/08'	>32	>16	8	>16	0.5	>16	0.06	16	<0.06	>32
8	r	2	12	>16	16/08'	>32	>16	2	4	0.25	8	0.06	2	<0.06	>32
9	r	1	13	>16	>16/08'	>32	>16	>8	>16	>1	>16	0.06	32	>8	>32
10	r	2	15	>16	16/08'	>32	>16	4	>16	1	<0.125	0.25	16	<0.06	>32
11	r	2	17	>16	16/08'	>32	>16	2	>16	1	>16	0.06	4	<0.06	>32
12	r	2	18	>16	16/08'	>32	4	2	1	0.125	16	0.03	1	<0.06	>32
13	r	2	20	>16	>16/08'	>32	>16	4	>16	>1	>16	0.06	1	<0.06	>32
14	r	2	24	>16	16/08'	>32	>16	4	>16	>1	16	0.06	1	<0.06	>32

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
15	o	7	27	<0.125	<0.13/0.06	<0.25	<0.25	0.5	>16	1	2	0.5	16	<0.06	2
16	o	7	28	0.25	<0.13/0.06	0.25	0.25	0.125	0.5	0.03	0.25	0.06	1	<0.06	0.5
17	o	7	29	>16	16/08'	16	>16	8	>16	1	>16	0.125	8	<0.06	8
18	o	8	31	>16	8/4'	16	8	1	0.5	0.125	1	0.03	1	<0.06	2
19	o	8	32	0.5	0.5/0.25	2	8	<0.06	<0.125	<0.01	0.5	0.125	2	<0.06	0.5
20	o	8	34	>16	16/08'	32	16	0.5	1	0.03	0.5	0.125	0.5	<0.06	1
21	o	9	37	<0.125	<0.13/0.06	<0.25	0.25	8	0.25	0.06	<0.25	<0.02	<0.25	<0.06	1
22	c	10	39	>16	16/08'	32	>16	>8	>16	>1	>16	0.03	0.5	<0.06	4
23	c	10	40	>16	16/08'	32	16	>8	>16	1	2	0.03	1	2	8
24	o	10	41	4	1/0.5	32	16	0.5	2	0.03	2	0.06	4	1	4
25	o	11	43	16	8/4'	32	>16	<0.06	8	0.06	1	0.06	4	<0.06	2
26	o	11	44	16	8/4'	32	>16	1	1	0.06	1	0.06	4	<0.06	4
27	o	11	45	>16	>16/08'	32	>16	>8	>16	1	16	0.03	1	4	32
28	o	12	49	>16	16/08'	>32	>16	>8	16	1	>16	0.06	8	0.06	4
29	o	12	50	>16	>16/08'	32	>16	>8	>16	>1	16	0.06	2	<0.06	4
30	o	12	51	>16	16/08'	16	4	0.25	4	<0.01	<0.125	0.02	1	<0.06	<0.25
31	o	12	52	>16	16/08'	32	>16	4	>16	1	4	>2	16	0.125	2
32	c	13	53	>16	>16/08'	16	>16	>8	>16	>1	16	0.125	8	<0.06	8
33	c	13	54	>16	>16/08'	32	>16	>8	>16	>1	>16	0.06	2	<0.06	4
34	c	13	55	>16	>16/08'	16	2	2	0.25	<0.01	<0.25	0.03	1	<0.06	0.5
35	c	13	56	>16	>16/08'	>32	>16	>8	>16	>1	>16	0.125	4	0.06	8
36	o	14	58	>16	>16/08'	32	2	0.25	<0.125	<0.01	<0.125	0.02	1	<0.06	<0.25
37	o	14	60	2	0.125/0.06	4	>16	>8	>16	0.125	<0.125	0.02	<0.25	<0.06	16
38	o	14	61	1	0.5/0.25	2	2	1	8	0.125	1	1	8	0.5	2
39	o	17	78	2	1/0.5	4	>16	1	0.5	0.06	8	0.125	1	0.5	1
40	o	17	80	8	2/1'	32	4	0.25	>16	>1	8	0.06	1	0.125	2

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
41	o	17	81	4	<0.13/0.06	4	4	0.25	1	0.125	8	0.125	0.5	0.125	0.5
42	t	19	88	4	1/0.5	8	8	0.25	0.125	0.06	8	0.125	<0.25	<0.06	<0.25
43	t	19	91	2	0.5/0.25	8	8	0.5	0.25	0.06	8	0.125	0.5	<0.06	0.5
44	t	21	99	>16	>16/08'	>32	>16	>8	>16	0.125	8	0.125	0.25	0.06	2
45	t	21	100	1	0.5/0.25	2	8	0.25	<0.125	0.03	8	0.125	0.5	<0.06	0.5
46	t	21	101	2	0.125/0.06	4	8	0.25	<0.125	0.02	8	0.25	0.5	0.06	0.25
47	t	21	102	>16	16/08'	>32	>16	>8	>16	0.25	16	0.125	1	0.06	8
48	t	21	103	>16	16/08'	>32	4	>8	2	0.125	8	0.02	<0.25	0.125	1
49	t	22	112	<0.125	<0.13/0.06	<0.25	1	0.25	0.25	0.06	2	<0.02	0.25	2	1
50	r	23	116	>16	16/08'	32	>16	8	>16	1	16	0.03	0.5	<0.06	4
51	t	32	140	>16	>16/08'	>32	>16	>8	>16	0.5	>16	0.125	4	0.5	32
52	t	32	141	>16	>16/08'	>32	>16	>8	>16	0.25	>16	0.06	1	0.5	8
53	r	15	62	2	0.25/0.13	16	16	1	0.125	0.125	8	0.06	1	<0.06	2
54	r	15	63	8	2/1'	32	>16	8	>16	0.5	16	0.125	2	<0.06	2
55	r	15	64	>16	16/08'	>32	>16	>8	>16	0.5	>16	0.03	16	0.25	8
56	r	15	66	>16	16/08'	>32	>16	>8	>16	0.25	>16	0.06	16	<0.06	8
57	r	15	67	2	0.25/0.13	32	>16	2	0.25	0.125	4	0.03	0.5	<0.06	1
58	r	16	74	>16	8/4'	>32	>16	>8	>16	0.5	16	0.06	1	0.125	4
59	r	16	75	>16	16/08'	>32	>16	>8	>16	0.5	8	0.125	4	1	4
60	r	33	145	>16	>16/08'	>32	>16	2	0.125	0.125	4	0.03	2	<0.06	2
61	r	33	146	>16	>16/08'	>32	>16	2	2	0.125	4	0.03	1	<0.06	4
62	r	33	147	>16	>16/08'	>32	>16	2	>16	>1	>16	0.06	1	0.125	32
63	r	33	149	>16	>16/08'	>32	8	2	2	0.125	2	0.02	1	<0.06	4
64	r	33	150	>16	8/4'	>32	>16	4	>16	>1	>16	0.03	4	0.25	32
65	r	33	151	>16	8/4'	>32	>16	4	>16	>1	>16	0.03	4	0.25	32
66	r	34	153	>16	>16/08'	>32	>16	4	8	0.5	16	0.03	8	<0.06	8

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
67	r	34	155	>16	16/08'	>32	>16	2	>16	>1	8	0.03	4	0.25	4
68	r	34	156	>16	16/08'	>32	8	1	0.25	0.125	4	0.02	1	<0.06	4
69	r	34	157	>16	16/08'	>32	8	1	1	0.125	4	0.02	1	<0.06	4
70	r	34	158	>16	>16/08'	>32	>16	2	>16	>1	>16	0.125	8	<0.06	32
71	r	34	160	>16	>16/08'	>32	>16	1	2	0.125	4	0.06	1	<0.06	2
72	r	35	162	>16	>16/08'	>32	>16	2	>16	>1	>16	0.125	1	<0.06	16
73	r	35	163	>16	16/08'	>32	>16	2	>16	>1	2	0.06	2	<0.06	8
74	r	35	164	>16	16/08'	>32	>16	>8	>16	0.5	>16	0.06	1	<0.06	2
75	r	35	165	>16	>16/08'	>32	>16	>8	>16	>1	16	0.03	4	<0.06	4
76	r	36	166	2	0.25/0.13	4	8	0.5	1	0.125	4	0.02	0.25	<0.06	0.5
77	r	36	167	>16	2/1'	32	>16	2	16	0.125	4	0.03	0.5	<0.06	0.5
78	r	36	168	4	0.25/0.13	8	8	0.125	16	0.125	8	0.06	1	<0.06	0.5
79	r	36	169	1	0.125/0.06	8	>16	0.5	<0.125	0.06	4	0.03	0.5	<0.06	1
80	r	36	170	2	0.25/0.13	4	8	0.5	<0.125	0.06	1	<0.02	0.5	<0.06	2
81	t	39	184	1	<0.13/0.06	2	16	0.25	<0.125	0.06	2	<0.02	<0.25	<0.06	1
82	t	40	188	>16	4/2'	>32	>16	8	>16	0.25	16	0.25	32	>8	4
83	t	40	191	1	0.125/0.06	4	>16	0.5	1	0.125	4	<0.02	<0.25	<0.06	0.5
84	t	40	192	4	1/0.5	16	>16	0.5	1	0.125	8	0.03	0.5	<0.06	1
85	t	40	193	>16	4/2'	>32	>16	1	2	0.03	4	0.06	1	<0.06	8
86	t	40	194	>16	16/08'	>32	>16	1	>16	0.125	2	0.06	1	<0.06	16
87	t	40	195	>16	4/2'	>32	>16	1	0.5	0.06	2	0.125	>32	>8	8
88	t	40	196	>16	4/2'	>32	8	0.5	0.5	0.03	1	0.25	32	>8	8
89	t	40	197	>16	8/4'	>32	>16	1	1	0.03	2	0.25	32	>8	8
90	t	40	198	>16	4/2'	>32	8	1	0.5	0.06	2	0.125	2	<0.06	8
91	r	36	200	>16	16/08'	>32	>16	8	>16	1	8	0.125	0.5	0.125	8
92	t	39	203	2	<0.13/0.06	8	4	<0.06	0.125	0.03	2	0.06	2	<0.06	1

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
93	t	39	204	4	0.25/0.13	4	4	0.125	1	0.125	4	0.02	2	<0.06	16
94	t	42	205	4	0.25/0.13	16	>16	8	1	0.125	16	0.03	2	<0.06	2
95	t	42	206	4	0.25/0.13	8	>16	8	0.25	0.125	>16	0.03	2	<0.06	1
96	b	43	207	4	0.5/0.25	32	>16	8	1	0.125	16	0.03	2	<0.06	2
97	b	43	208	8	0.5/0.25	8	>16	>8	>16	0.125	16	0.125	2	<0.06	2
98	b	48	217	8	1/0.5	8	4	2	1	0.06	4	0.02	2	<0.06	4
99	b	42	219	8	0.5/0.25	16	>16	1	>16	0.125	4	0.5	8	<0.06	4
100	b	44	221	>16	>16/08'	>32	>16	4	4	0.125	<0.125	<0.02	<0.25	<0.06	>32
101	b	47	226	>16	16/08'	>32	>16	2	>16	>1	>16	0.06	4	<0.06	2
102	t	49	227	>16	16/08'	>32	>16	0.5	1	0.125	4	<0.02	<0.25	<0.06	4
103	t	49	228	>16	16/08'	>32	>16	1	>16	1	8	<0.02	<0.25	<0.06	4
104	t	49	229	>16	16/08'	>32	>16	1	>16	1	8	0.02	0.25	<0.06	8
105	t	49	230	>16	>16/08'	>32	16	0.5	>16	1	2	0.125	<0.25	<0.06	4
106	t	50	231	>16	>16/08'	>32	16	0.5	1	0.06	4	0.02	0.25	<0.06	2
107	t	50	232	>16	16/08'	>32	>16	2	>16	0.25	2	0.03	1	<0.06	4
108	t	50	233	>16	16/08'	>32	>16	1	2	0.125	2	0.03	0.5	<0.06	4
109	t	50	234	>16	>16/08'	>32	>16	1	2	0.125	2	0.02	0.25	<0.06	4
110	t	49	235	>16	>16/08'	>32	>16	1	>16	0.25	2	0.02	<0.25	<0.06	4
111	t	49	236	>16	16/08'	>32	>16	1	>16	0.5	2	0.03	0.5	<0.06	4
112	t	49	237	>16	16/08'	>32	>16	2	>16	0.25	2	0.02	<0.25	<0.06	4
113	t	49	238	>16	16/08'	>32	>16	8	>16	0.25	4	0.03	0.25	<0.06	4
114	t	50	239	8	1/0.5	32	2	0.25	<0.125	0.125	0.5	0.06	0.5	<0.06	0.5
115	t	50	240	>16	>16/08'	>32	>16	1	2	0.125	4	0.03	0.5	<0.06	4
116	t	50	241	>16	>16/08'	>32	>16	4	>16	0.25	1	0.03	0.5	0.06	1
117	r	51	243	>16	>16/08'	>32	>16	0.5	2	0.125	16	0.06	1	<0.06	32
118	r	51	244	>16	8/4'	>32	8	0.25	4	0.125	1	0.02	1	<0.06	4

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
119	r	51	245	>16	8/4'	>32	8	2	>16	>1	>16	0.125	0.5	<0.06	4
120	r	51	246	>16	>16/08'	>32	>16	2	4	0.25	4	0.06	2	<0.06	8
121	r	52	252	>16	>16/08'	>32	>16	2	>16	0.25	>16	0.06	16	>8	>32
122	r	51	253	>16	16/08'	>32	>16	4	>16	0.5	>16	0.06	16	0.125	>32
123	r	52	255	>16	16/08'	>32	>16	2	>16	0.25	8	0.06	0.5	<0.06	8
124	r	52	256	>16	16/08'	>32	>16	2	16	0.25	8	0.03	1	<0.06	8
125	r	52	257	>16	16/08'	>32	>16	2	>16	>1	>16	0.125	4	>8	32
126	c	54	265	>16	2/1'	32	8	1	1	0.06	1	0.02	<0.25	<0.06	8
127	c	56	273	8	1/0.5	8	4	2	8	0.125	4	0.02	0.25	<0.06	2
128	c	56	274	8	1/0.5	32	4	4	8	0.125	4	0.02	1	<0.06	2
129	c	56	276	8	1/0.5	8	4	4	8	0.125	16	0.02	<0.25	<0.06	8
130	c	56	277	8	2/1'	32	8	1	0.5	0.03	16	0.125	1	<0.06	2
131	c	56	278	8	1/0.5	32	>16	4	>16	>1	1	0.03	0.5	<0.06	2
132	c	59	287	>16	>16/08'	>32	>16	4	>16	1	>16	0.06	32	<0.06	4
133	c	59	290	>16	16/08'	>32	>16	2	8	0.25	16	0.06	16	<0.06	8
134	c	60	292	>16	16/08'	>32	>16	0.5	0.5	0.06	0.5	<0.02	4	<0.06	4
135	c	60	293	>16	16/08'	>32	>16	0.5	2	0.125	1	0.125	1	0.125	>32
136	c	60	294	8	1/0.5	8	8	0.5	>16	1	1	0.02	0.25	<0.06	2
137	c	60	295	16	2/1'	32	>16	2	>16	1	2	0.03	0.5	<0.06	2
138	c	60	296	>16	8/4'	>32	>16	1	>16	1	16	0.03	0.5	<0.06	>32
139	c	60	298	8	1/0.5	16	8	0.5	<0.125	0.06	2	0.02	0.25	<0.06	4
140	c	62	302	>16	8/4'	16	>16	4	4	0.125	1	<0.02	<0.25	0.5	16
141	c	62	303	4	1/0.5	4	8	0.5	<0.125	0.06	2	<0.02	<0.25	<0.06	8
142	c	62	306	>16	>16/08'	>32	>16	1	>16	0.06	1	<0.02	0.5	<0.06	2
143	c	63	307	>16	16/08'	>32	>16	4	>16	0.25	>16	0.06	1	<0.06	>32
144	c	63	308	>16	16/08'	>32	>16	4	>16	0.25	8	0.06	32	<0.06	8

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
145	c	63	309	>16	>16/08'	>32	16	2	0.5	0.03	8	0.06	8	<0.06	8
146	c	63	310	>16	8/4'	>32	>16	0.25	4	0.125	2	1	1	0.25	2
147	c	63	311	>16	>16/08'	>32	>16	1	4	0.125	2	0.06	0.5	<0.06	4
148	c	63	312	>16	8/4'	>32	16	0.25	0.5	0.06	2	0.02	8	<0.06	8
149	c	64	313	>16	8/4'	32	16	1	2	0.125	2	0.25	2	<0.06	4
150	c	64	314	8	1/0.5	16	>16	4	1	0.125	4	0.125	2	<0.06	8
151	c	64	315	>16	16/08'	32	>16	>8	2	0.03	1	0.25	8	0.125	32
152	c	64	316	>16	16/08'	32	>16	>8	4	0.125	4	<0.02	2	<0.06	8
153	c	65	317	16	2/1'	32	8	4	1	0.06	<0.125	<0.02	0.5	<0.06	16
154	c	65	319	4	0.5/0.25	8	>16	0.25	<0.125	0.03	0.5	0.06	1	<0.06	1
155	c	65	322	2	0.125/0.06	8	4	<0.06	0.5	0.06	1	0.06	1	<0.06	1
156	c	65	325	16	2/1'	32	>16	0.5	>16	0.125	4	0.03	2	<0.06	8
157	c	67	327	16	2/1'	32	>16	>8	1	0.06	2	0.25	4	<0.06	8
158	c	67	328	4	1/0.5	8	8	0.5	2	0.03	0.5	0.06	0.5	<0.06	0.5
159	c	67	329	16	8/4'	32	16	>8	2	0.125	8	0.125	4	<0.06	8
160	c	68	330	>16	>16/08'	>32	>16	>8	>16	>1	>16	0.03	0.5	<0.06	4
161	c	68	331	>16	16/08'	>32	>16	>8	>16	>1	>16	0.06	0.5	<0.06	4
162	c	68	332	>16	16/08'	>32	>16	>8	>16	>1	>16	0.06	1	0.125	4
163	c	70	335	>16	16/08'	>32	8	0.125	0.5	0.03	2	0.06	1	<0.06	4
164	c	70	336	>16	16/08'	>32	>16	0.125	16	0.06	4	0.06	0.5	0.25	32
165	c	72	343	>16	>16/08'	32	>16	>8	2	0.06	2	0.5	8	<0.06	32
166	c	72	345	>16	>16/08'	>32	>16	>8	>16	0.06	2	0.25	8	0.125	32
167	c	74	350	4	0.5/0.25	>32	4	<0.06	<0.125	0.03	1	0.06	1	<0.06	1
168	c	74	352	8	0.5/0.25	32	8	0.125	16	0.02	0.5	0.125	0.5	<0.06	2
169	c	75	355	>16	>16/08'	32	>16	2	0.5	0.06	4	0.125	2	0.125	8
170	c	76	359	>16	16/08'	32	>16	8	>16	>1	1	0.06	4	0.5	4

Sl no	locations	Sample no	Isolates no	Minimum Inhibitory Concentration of Antibiotics (µg/ml)											
				AMP	AMC	PEN	KF	EFT	NA	ENR	DOX	CN	S	SXT	C
171	c	79	368	>16	16/08'	32	>16	>8	>16	0.25	2	0.06	16	<0.06	4
172	c	79	369	>16	16/08'	32	>16	>8	>16	0.125	2	0.06	16	<0.06	4
173	c	79	370	>16	16/08'	>32	>16	>8	>16	0.5	2	0.5	32	<0.06	8

APPENDIX 5

SUPPLEMENTARY FILES OF CHAPTER 5

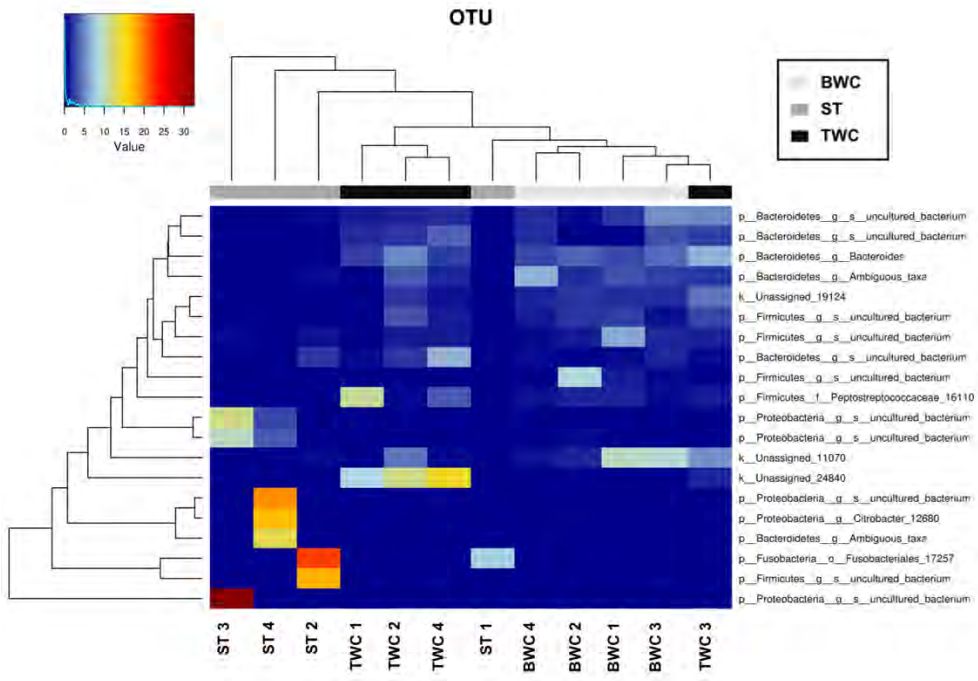


Figure S 5.1. The top 20 most abundant operational taxonomic units (OTUs) in cloacal samples collected from wild-captured and stranded sea turtles. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

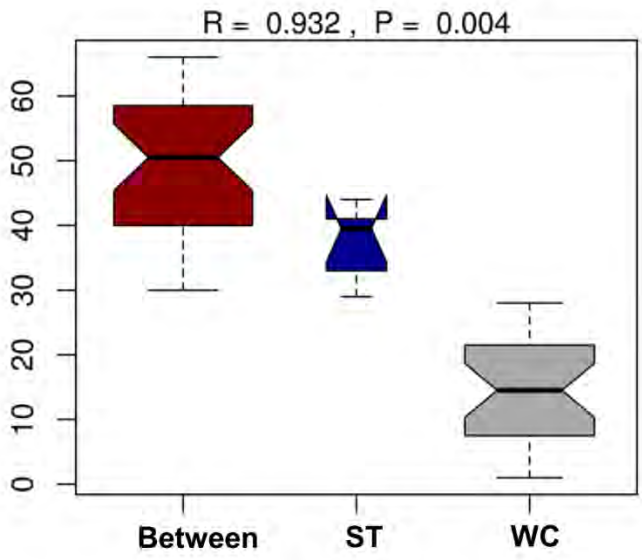


Figure S 5.2. Analysis of similarity (ANOSIM) between wild-captured (WC) and stranded (ST) green turtles. Note: ST represents stranded green turtles of Townsville and WC represents wild-captured green turtles.

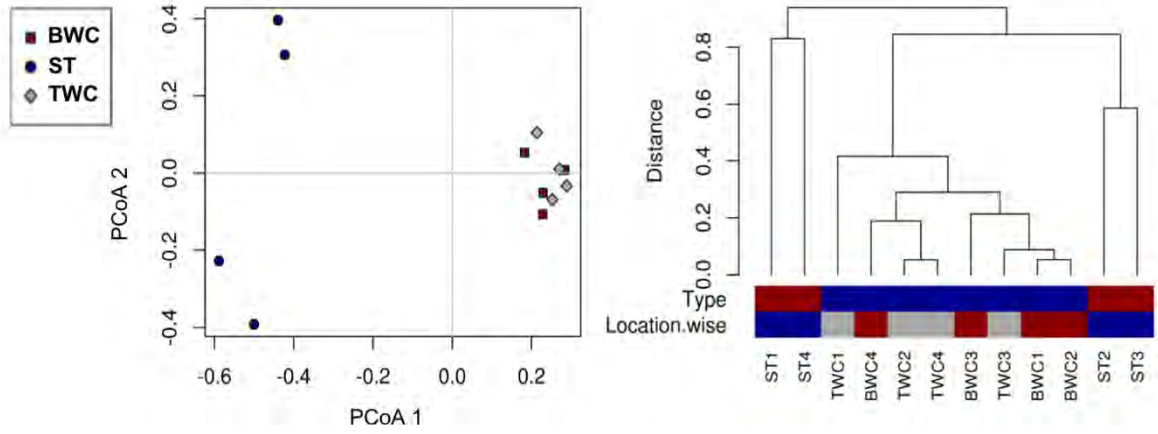


Figure S 5.3. Differential gut bacterial communities across all samples at OTU level. Principle coordinate analysis plot and hierarchical dendrogram of Chao1 distances for different groups of samples. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

Table S 5.1. The total number of reads in each sample before and after standard quality control (QC) filters. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

Sample ID	Raw reads	Post QC reads	Post QC Mean Length
BWC 1	72,381	27,326	465.00
BWC 2	104,722	54,088	465.00
BWC 3	121,993	65,002	467.00
BWC 4	64,493	33,119	475.00
ST 1	71,735	49,332	452.00
ST 2	73,190	38,023	466.00
ST 3	59,601	21,817	489.00
ST 4	65,011	24,041	489.00
TWC 1	95,114	58,302	459.00
TWC 2	69,352	35,171	473.00
TWC 3	78,643	38,223	470.00
TWC 4	80,169	38,999	470.00

Table S 5.2. Total number of operational taxonomic units (OTUs), estimated OTU richness (ACE, Chao1), diversity index (Shannon, Simpson) and estimated samples coverage (Good's coverage) for 16S rRNA sequences for each sample. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

Sample ID	OTUs	Shannon	Chao1	ACE	Simpson	coverage (%)
BWC 1	329	4.24	355.90	351.98	0.04	99.7
BWC 2	312	4.32	370.50	368.95	0.03	99.9
BWC 3	365	4.36	458.00	438.60	0.03	99.9
BWC 4	297	4.13	397.59	405.06	0.04	99.5
ST 1	125	3.53	198.86	209.65	0.06	99.9
ST 2	200	3.04	308.04	308.36	0.14	99.7
ST 3	112	2.43	164.80	151.14	0.19	99.7
ST 4	93	2.58	115.75	108.79	0.13	99.8
TWC 1	284	3.96	366.87	361.19	0.06	99.9
TWC 2	346	4.19	440.08	435.59	0.04	99.5
TWC 3	364	4.41	415.86	412.50	0.03	99.8
TWC 4	322	3.95	418.25	410.75	0.06	99.6

Table S 5.3. The most abundant family (% of OTU) in each group of green turtles. BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

Family	**All samples**	**Type (%)**		**Location-wise (%)**		
		Wild-captured	Stranded	BWC	TWC	ST
Lachnospiraceae	18.91	24.98	4.93	22.18	28.19	4.93
Bacteroidaceae	17.92	20.66	11.62	19.32	22.19	11.62
Clostridiaceae 1	15.05	20.62	2.21	28.32	11.75	2.21
Peptostreptococcaceae	9.00	8.63	9.86	4.10	13.84	9.86
Porphyromonadaceae	7.68	8.64	5.47	7.75	9.67	5.47
Ruminococcaceae	4.37	5.58	1.58	6.56	4.44	1.58
Rhodobacteraceae	3.50	2.91	4.86	5.33	0.12	4.86
Moraxellaceae	3.30	0.28	10.27	0.46	0.07	10.27
Enterobacteriaceae	5.90	0.03	19.41	0.04	0.03	19.41
Cardiobacteriaceae	2.59	0.09	8.35	0.16	0.01	8.35

Table S 5.4. The top 25 most significant genera of the bacterial communities in different groups of green turtles. ST represents stranded green turtles of Townsville and WC represents wild-captured green turtles.

Genera	P value	P Bonferroni	FDR value	ST mean	WC mean
Pseudomonas	0.00E+00	0.00E+00	0.00E+00	324	0.88
EscherichiaShigella	6.60E-13	6.50E-11	3.20E-11	1348	3
Shewanella	5.10E-10	5.10E-08	1.30E-08	314.5	1.12
Citrobacter	5.30E-10	5.30E-08	1.30E-08	776.25	0.38
Tenacibaculum	2.10E-09	2.10E-07	4.10E-08	268	4.62
Vibrio	4.90E-07	4.80E-05	8.00E-06	192.75	5.12
Lachnospira	7.90E-07	7.90E-05	1.10E-05	0	159.12
uncultured_marine_bacterium	1.10E-06	1.00E-04	1.30E-05	248.25	1
Peptostreptococcus	2.00E-06	2.00E-04	2.20E-05	1400.75	0
Cellulosilyticum	2.20E-06	2.20E-04	2.20E-05	12.25	1105.62
Lachnospiraceae_UCG004	2.90E-06	2.90E-04	2.60E-05	17.5	371.25
Psychrobacter	6.70E-06	6.70E-04	5.50E-05	1115.25	9.5
Bilophila	8.10E-06	8.10E-04	5.80E-05	526.75	0.5
uncultured	8.60E-06	8.50E-04	5.80E-05	2405.25	1044.75
Providencia	9.50E-06	9.40E-04	5.80E-05	1059.25	0
Arcobacter	9.70E-06	9.60E-04	5.80E-05	171.75	97.88
Cetobacterium	9.90E-06	9.80E-04	5.80E-05	337.75	0.12
Faecalibacterium	1.30E-05	1.30E-03	7.10E-05	1.5	76.62
Parabacteroides	1.60E-05	1.60E-03	8.40E-05	481.75	0.62
BD17_clade	1.80E-05	1.80E-03	9.10E-05	41.5	0.12
Edwardsiella	2.60E-05	2.50E-03	1.20E-04	38.25	0
Fusobacterium	2.70E-05	2.60E-03	1.20E-04	197.75	0.12
Klebsiella	4.50E-05	4.50E-03	1.90E-04	41	0
Lachnospiraceae_UCG001	4.60E-05	4.60E-03	1.90E-04	0.25	179.38
Tyzzerella	5.30E-05	5.20E-03	2.10E-04	29.75	0

Table S5: The most abundant genera (% of the relative abundance of OTUs) in wild-captured and stranded groups of green turtles.

BWC, Bowen wild-captured green turtles; TWC, Townsville wild-captured green turtles and ST, stranded green turtles of Townsville.

BWC		TWC		ST	
Phylum	**Genus (%)**	**Phylum**	**Genus (%)**	**Phylum**	**Genus (%)**
Bacteroidetes	*Bacteroides* (22.98)	Bacteroidetes	*Bacteroides* (27.22)	Bacteroidetes	*Bacteroides* (12.64)
Firmicutes	*Clostridium* (24.41)	Firmicutes	*Clostridium* (11.05)	Firmicutes	*Peptostreptococcus* (7.74)
Bacteroidetes	*Macellibacteroides* (8.61)	Bacteroidetes	*Macellibacteroides* (11.24)	Proteobacteria	*Escherichia-Shigella* (7.45)
Firmicutes	*Clostridium sensu stricto* 13 (4.25)	Firmicutes	*Cellulosilyticum* (8.69)	Proteobacteria	*Psychrobacter* (6.16)
Firmicutes	*Ruminococcaceae* UCG-005 (4.77)	Firmicutes	*Peptoclostridium* (6.10)	Proteobacteria	*Providencia* (5.85)

APPENDIX 6

SUPPLEMENTARY FILES OF CHAPTER 6

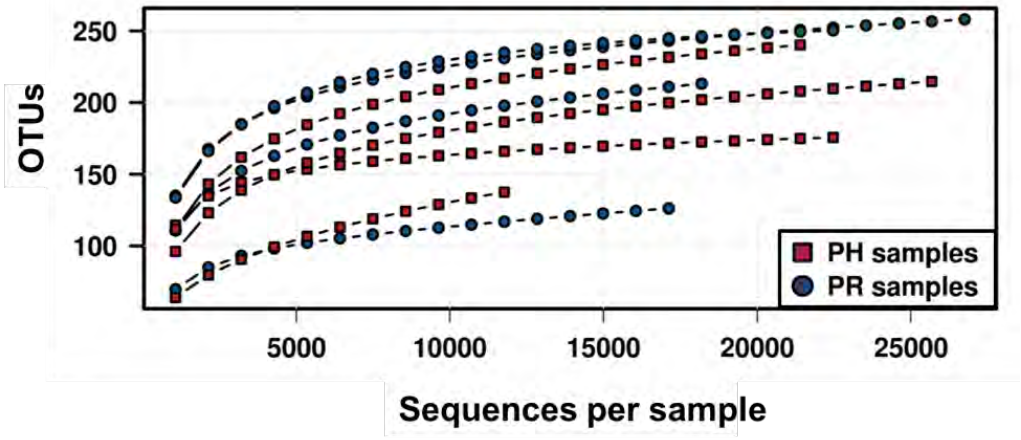


Figure S 6.1. Rarefaction analysis based on operational taxonomic unit (OTUs) (3% divergence) in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR).

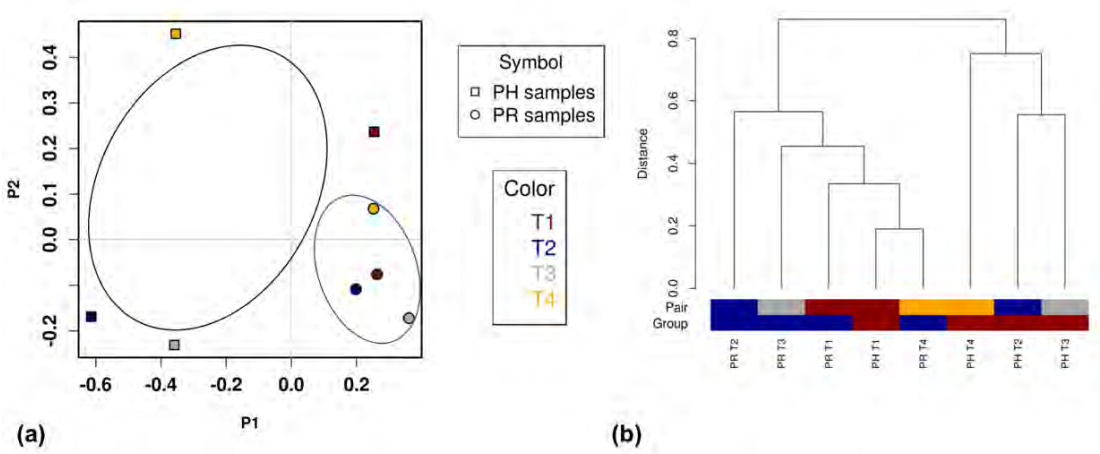


Figure S 6.2. (a) Principle coordinates analysis (PCoA) and (b) hierarchical dendrogram between pre-hospitalization (PH) and post-rehabilitation (PR) samples of green turtles using Chao1 distance matrix. T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

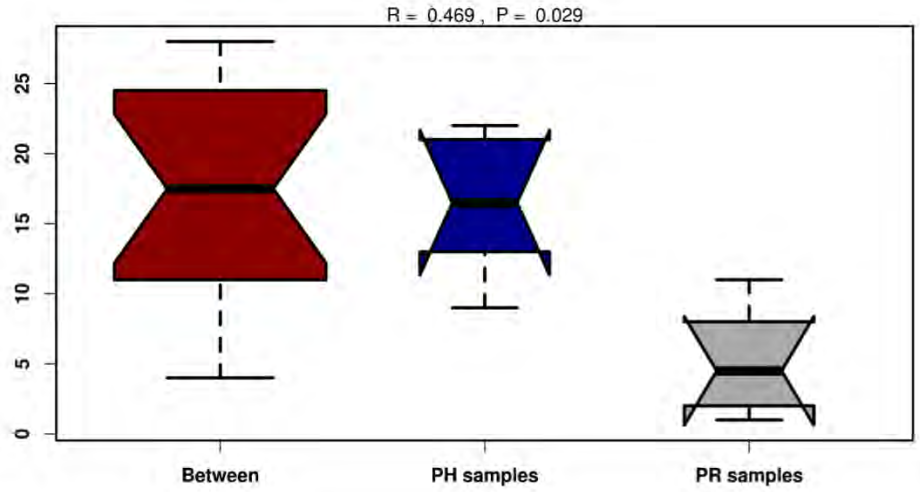


Figure S 6.3. Analysis of Similarity (ANOSIM) in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR).

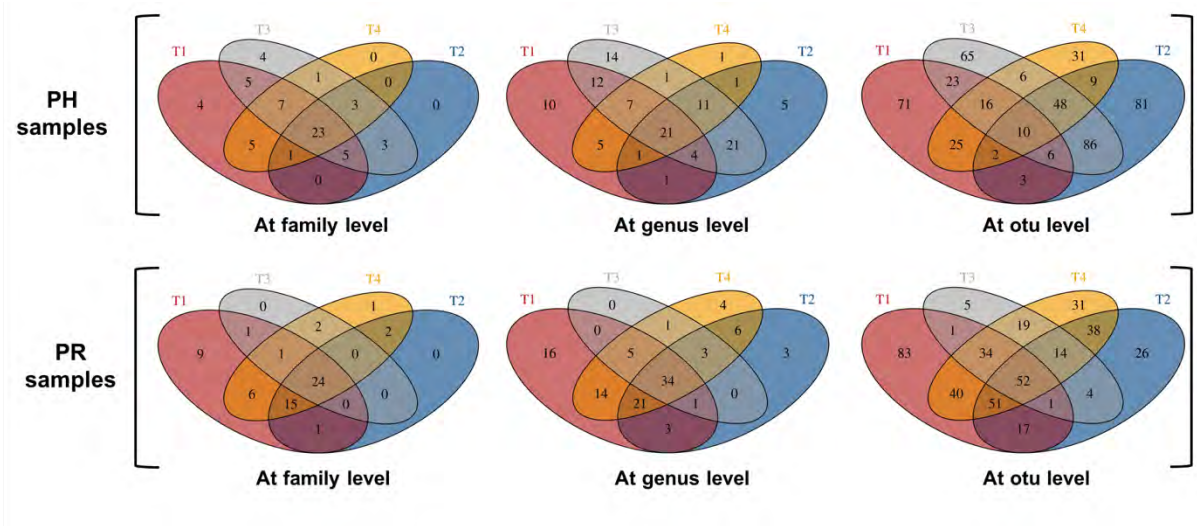


Figure S 6.4. Venn diagrams represent the number of shared and exclusive families, genera and OTUs among pre-hospitalization (PH) and post-rehabilitation (PR) samples of green turtles. T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

Table S 6.1. Number of reads before and after standard quality control (QC) filters and post QC mean read length. PH, pre-hospitalization; PR, post-rehabilitation; T1, turtle 1; T2, turtle 2; T3, turtle 3 and T4, turtle 4

Sample ID	Raw reads	Post QC reads	Post QC Mean Length
PH T1	71,735	32,519	467
PR T1	107,715	54,269	463
PH T2	87,585	40,155	469
PR T2	87,665	40,662	464
PH T3	73,190	38,036	466
PR T3	56,660	27,326	465
PH T4	59,601	21,143	489
PR T4	97,644	45,413	468

Table S2: The top 10 most significant families of the bacterial communities in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR).

Family	P value	P Bonferroni	FDR value	PH samples mean	PR samples mean
Verrucomicrobiaceae	3.80E-06	2.60E-04	2.60E-04	1.25	153.25
Desulfovibrionaceae	1.50E-04	1.00E-02	5.10E-03	33.75	878
Campylobacteraceae	5.10E-04	3.50E-02	9.80E-03	1136.25	54.25
Shewanellaceae	5.70E-04	3.90E-02	9.80E-03	246.75	17.75
Rhodocyclaceae	9.40E-04	6.50E-02	1.30E-02	0.5	222
Clostridiales_vadinBB60_group	1.30E-03	8.80E-02	1.50E-02	39.5	0
Rikenellaceae	1.80E-03	1.20E-01	1.70E-02	5.5	168.25
Cardiobacteriaceae	2.20E-03	1.50E-01	1.90E-02	1545.75	240.75
Vibrionaceae	4.20E-03	2.90E-01	3.30E-02	173	23.75
Erysipelotrichaceae	1.30E-02	9.20E-01	8.30E-02	11.75	73.75

Table S3: The abundance of predominant phyla in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR).

Phylum	PH samples (%)	PR samples (%)
Proteobacteria	33.7	36.9
Bacteroidetes	14.4	25.4
Firmicutes	25.5	14.2
Fusobacteria	9.1	16.1
Actinobacteria	0.4	0.5

Table S4: The abundance of predominant families (% of OTU) in the samples from green turtles collected at pre-hospitalization (PH) and post-rehabilitation (PR).

Family	PH samples (%)	PR samples (%)
Rhodobacteraceae	7.3	14.1
Moraxellaceae	6.3	10.9
Porphyromonadaceae	5.	11.9
Bacteroidaceae	6.8	9.2
Clostridiaceae 1	6.9	9.1
Fusobacteriaceae	6.0	9.6
Lachnospiraceae	9.0	2.3
Peptostreptococcaceae	7.2	1.1
Enterobacteriaceae	5.5	2.4
Cardiobacteriaceae	6.3	1.0

APPENDIX 7

SUPLLEMENTARY FILES OF CHAPTER 7

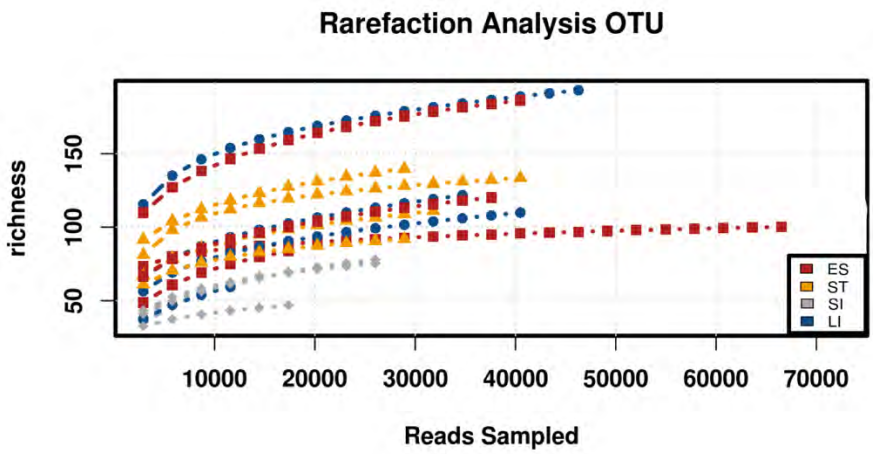


Figure S 7.1. Rarefaction analysis based on operational taxonomic unit (OTUs) (3% divergence) in the samples from different regions of the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

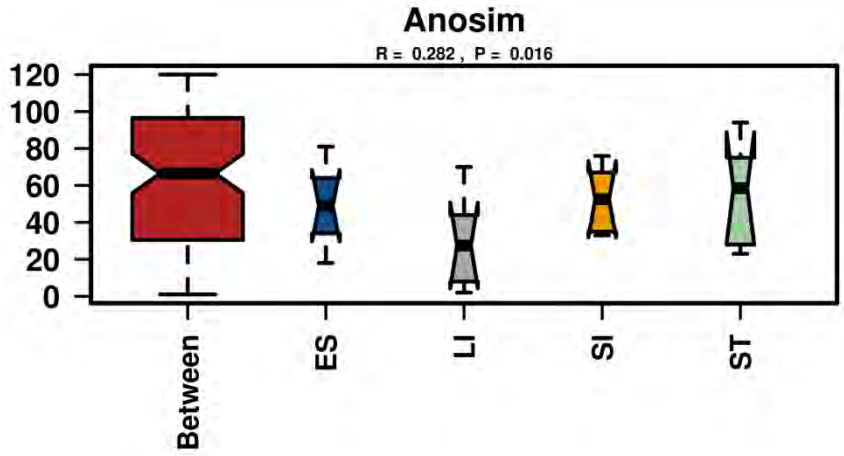


Figure S 7.2. Analysis of Similarity (ANOSIM) in the samples from different regions of the gastrointestinal tract of green turtles using weighted UniFrac distance metric at OTU level.

Table S 7.1. Raw data before and after standard quality control (QC) filters.

Sample ID	Raw reads	Post QC reads	Post QC Mean Length
T1 ES	2,28,578	75,840	465.00
T2 ES	66,397	35,334	434.00
T3 ES	1,22,224	57,615	469.00
T4 ES	1,24,195	58,503	464.00
T1 ST	1,03,949	45,186	453.00
T2 ST	63,353	38,608	453.00
T3 ST	1,22,396	43,934	460.00
T4 ST	1,07,307	39,110	463.00
T1 SI	1,28,147	30,106	493.00
T2 SI	86,010	29,894	483.00
T3 SI	90,390	19,258	500.00
T4 SI	1,46,916	35,806	491.00
T1 LI	91,370	50,906	453.00
T2 LI	60,603	52,612	460.00
T3 LI	88,492	15,135	501.00
T4 LI	92,314	75,952	453.00

Table S 7.2. Alpha diversity metrics for the bacterial communities across different regions of the gastrointestinal (GI) tract at an evolutionary distance D= 0.03. Values indicates means and standard errors derived from total samples for each GI regions.

ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Sample	Reads	OTUs	Chao 1 index	ACE index	Shannon index	Good's coverage	Conditional uncovered probability (CUP)		
							PE	Lower bound	Upper bound
T1 ES	75491	93	116.00	112.72	4.58	0.99	0.012	0.001	0.009
T2 ES	22542	75	109.10	106.77	5.08	0.96	0.035	0.005	0.054
T3 ES	50663	163	208.71	216.49	6.09	0.95	0.055	0.008	0.080
T4 ES	45446	106	145.40	158.47	3.88	0.98	0.022	0.003	0.026
T1 ST	44252	130	147.33	145.68	3.84	0.98	0.017	0.002	0.021
T2 ST	32438	94	105.00	102.81	4.13	0.97	0.024	0.004	0.036
T3 ST	37353	103	141.11	138.10	3.84	0.95	0.014	0.002	0.015
T4 ST	34573	129	166.00	165.62	5.53	0.97	0.026	0.004	0.042
T1 SI	29339	77	99.09	96.54	1.04	0.99	0.008	0.001	0.011
T2 SI	21853	48	53.14	57.55	3.72	0.97	0.020	0.003	0.030
T3 SI	17746	60	99.63	114.45	2.85	0.95	0.046	0.008	0.078
T4 SI	30819	72	96.43	93.92	3.68	0.97	0.022	0.004	0.042
T1 LI	40873	126	165.33	166.75	4.59	0.98	0.025	0.002	0.024
T2 LI	48158	109	140.55	141.83	3.52	0.96	0.035	0.007	0.069
T3 LI	13347	58	84.75	90.48	2.04	0.98	0.019	0.003	0.035
T4 LI	63832	199	239.00	230.05	6.83	0.92	0.063	0.010	0.096

Table S 7.3. The top 5 most abundant bacterial phyla across different regions of the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Phylum	Total (%)	ES (%)	ST (%)	SI (%)	LI (%)
Firmicutes	57.8	30.3	85.1	22.9	86.5
Proteobacteria	21.3	18.8	4.0	72.3	9.1
Unclassified	7.9	16.3	4.3	2.9	4.2
Actinobacteria	6.4	18.2	1.8	1.1	0.1
Bacteroidetes	3.6	8.1	3.7	0.6	0.1
Fusobacteria	2.4	7.2	0.2	0.1	0.1

Table S 7.4. The top 10 most abundant bacterial classes across different regions of the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Class	Overall	ES (%)	ST (%)	SI (%)	LI (%)
Clostridia	46.4	17.8	64.0	11.3	85.0
Gammaproteobacteria	17.3	6.9	3.6	72.1	8.9
Bacilli	10.9	12.1	20.9	11.3	0.2
Unclassified	7.9	16.4	4.4	2.9	4.2
Actinobacteria	5.7	17.8	0.1	0.1	0.0
Bacteroidia	3.5	7.7	3.7	0.6	0.1
Alphaproteobacteria	3.3	10.1	0.3	0.1	0.0
Fusobacteriia	2.4	7.2	0.2	0.1	0.1
Coriobacteriia	0.7	0.4	1.7	1.0	0.0
Erysipelotrichia	0.5	0.3	0.1	0.2	1.3

Table S 7.5. The top 20 most abundant bacterial families across different regions of the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Family	Total (%)	ES (%)	ST (%)	SI (%)	LI (%)
Peptostreptococcaceae	18.7	2.7	37.4	1.3	31.1
Lachnospiraceae	14.7	2.3	8.2	1.1	42.9
Unclassified	12.7	28.5	6.2	3.9	5.3
Enterococcaceae	8.6	11.4	13.0	11.2	0.0
Clostridiaceae 1	7.6	4.2	13.6	1.8	9.8
Vibrionaceae	6.6	0.9	1.7	23.8	7.2
Aeromonadaceae	4.6	0.0	1.0	26.5	0.1
Dietziaceae	4.5	14.0	0.0	0.0	0.0
Enterobacteriaceae	3.8	0.8	0.3	20.1	0.6
Rhodobacteraceae	3.0	9.1	0.2	0.1	0.0
Family XIII	2.2	6.4	0.6	0.1	0.0
Streptococcaceae	2.1	0.6	7.8	0.1	0.0
Ruminococcaceae	1.4	0.2	2.2	4.5	0.2
Porphyromonadaceae	1.2	1.5	2.6	0.4	0.0
Xanthomonadaceae	1.1	3.5	0.0	0.0	0.0
Propionibacteriaceae	1.0	2.9	0.0	0.0	0.0
Coriobacteriaceae	0.7	0.4	1.7	1.0	0.0
Eubacteriaceae	0.5	0.1	0.4	2.4	0.0
Family XI	0.5	0.7	1.2	0.0	0.0

Table S 7.6. The top 20 most abundant bacterial genera across different regions of the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Genus	Total (%)	ES (%)	ST (%)	SI (%)	LI (%)
Unclassified	35.8	43.5	33.0	27.4	34.4
Coprococcus 1	9.7	1.3	6.7	0.0	28.1
Peptoclostridium	9.5	1.2	13.3	0.5	21.1
Aeromonas	4.6	0.0	1.0	26.5	0.1
Vagococcus	4.6	11.3	3.7	0.2	0.0
Dietzia	4.5	14.0	0.0	0.0	0.0
Enterococcus	4.1	0.0	9.2	11.0	0.0
Intestinibacter	3.5	0.1	9.6	0.1	3.9
uncultured	2.3	3.7	1.7	4.0	0.2
Streptococcus	2.1	0.6	7.8	0.1	0.0
Escherichia-Shigella	1.9	0.0	0.0	11.5	0.1
Stenotrophomonas	1.1	3.5	0.0	0.0	0.0
Serratia	1.1	0.0	0.1	6.5	0.0
Vibrio	0.9	0.1	0.1	4.5	0.2
Clostridium sensu stricto 1	0.9	0.2	2.1	0.2	0.9
Luteococcus	0.8	2.6	0.0	0.0	0.0
Cellulosilyticum	0.6	0.0	0.1	0.0	2.2
Paracoccus	0.6	1.9	0.1	0.1	0.0
Lachnoclostridium	0.6	0.1	0.2	0.3	1.9
Eubacterium brachy group	0.6	1.7	0.2	0.0	0.0

Table S 7.7. The abundance of 12 shared OTUs across the gastrointestinal tract of green turtles. ES: Oesophagus, ST: Stomach, SI: Small intestine and LI: Large intestine

Operational taxonomic units (OTU)	Cumulative abundance (%)			
	ES	ST	SI	LI
Proteobacteria__f__Vibrionaceae_17162	0.67	1.515	23.023	18.348
Fusobacteria__o__Fusobacteriales_19186	4.04	0.085	0.07	0.01
Firmicutes__g__Terrisporobacter_6106	0.05	0.775	0.008	0.09
Firmicutes__g__s__uncultured_Clostridium_sp.__s___9532	0.775	7.345	0.298	6.17
Firmicutes__g__s__uncultured_bacterium__s___808	1.165	6.487	0.055	23.76
Firmicutes__g__s__uncultured_bacterium__s___20735	0.202	10.502	0.058	3.367
Firmicutes__g__Clostridium_sensu_stricto_1_14141	0.08	0.178	0.082	0.168
Firmicutes__g__Ambiguous_taxa__s___8680	5.46	2.282	0.158	0.012
Firmicutes__f__Peptostreptococcaceae_17846	0.922	10.912	0.62	2.203
Firmicutes__f__Clostridiaceae_1_7975	0.842	0.64	0.155	3.635
Cyanobacteria__c__Chloroplast_22627	0.015	0.022	0.055	0.045
Bacteroidetes__g__s__uncultured_bacterium__s___13932	0.022	0.028	0.188	0.04

APPENDIX 8
SUPPLEMENTARY FILES OF CHAPTER 8

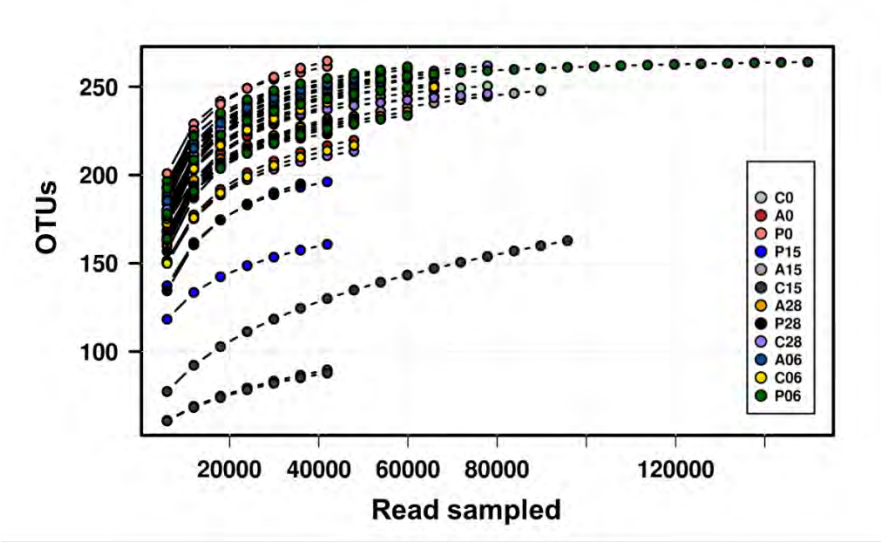


Figure S 8.1. Rarefaction analysis based on operational taxonomic unit (OTUs) (3% divergence) in the samples of different treatments and control groups. A, antibiotics group; P, phages group and C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

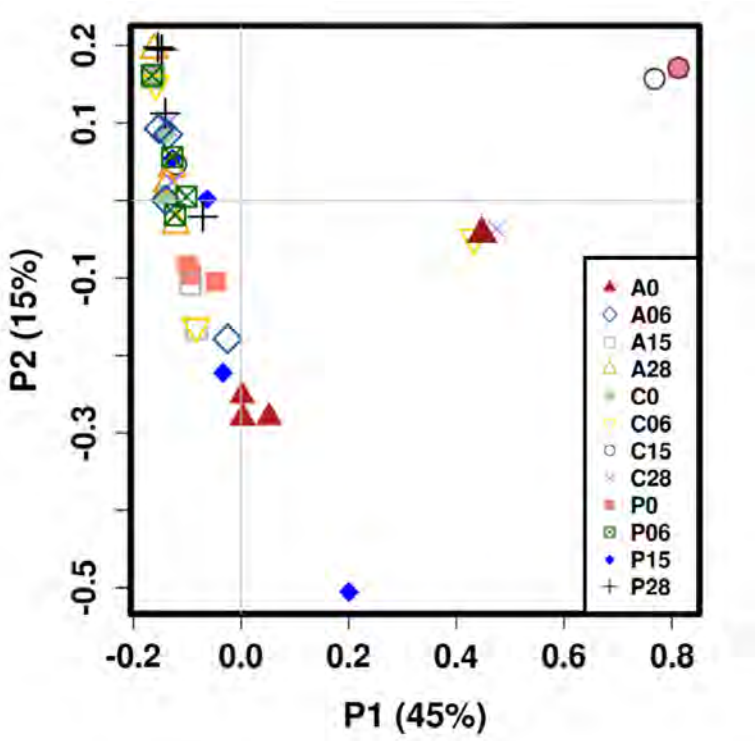


Figure S 8.2: Principle coordinate analysis (PCoA) analysis of the dissimilarity in the samples of different treatments and control groups. A, antibiotics group; P, phages group and C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

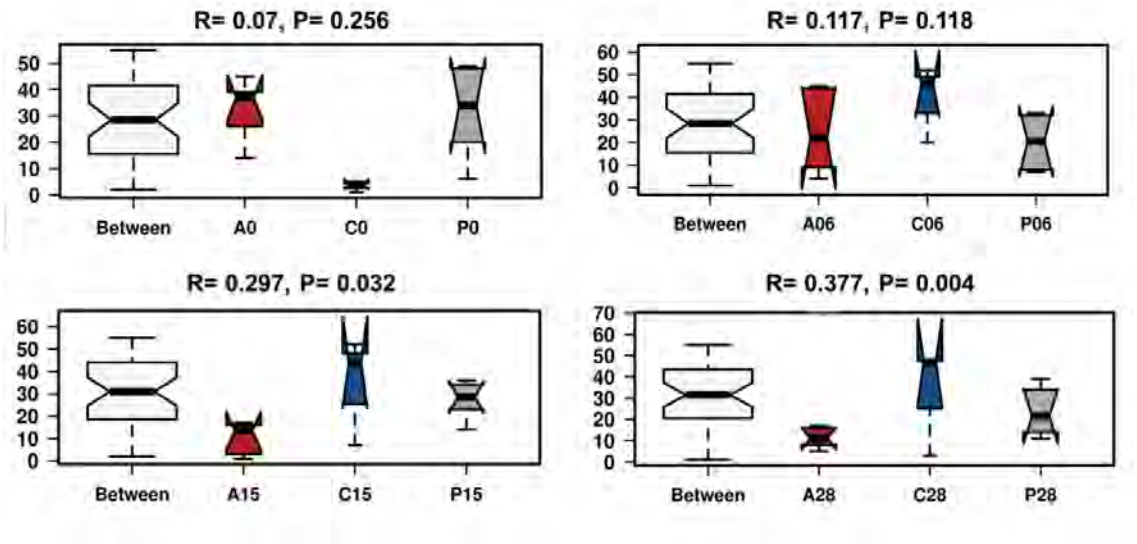


Figure S 8.3: Analysis of similarity (ANOSIM) using the Bray-Curtis distance matrix between different treatments and control groups. The prefix A0, A6, A15 and A28 indicate antibiotics group of samples at day 0, 6, 15 and 28 respectively. The prefix P0, P6, P15 and P28 indicate phages group of samples at day 0, 6, 15 and 28 respectively. The prefix C0, C6, C15 and C28 indicate control group of samples at day 0, 6, 15 and 28 respectively.

Table S 8.1. Number of reads and mean read length before and after standard quality control (QC) filters. A, antibiotics group; P, phages group and C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

Sl. No	Group	Sample ID	Total raw sequences	Raw sequence length (mean)	Post QC sequences	Post QC sequence length (median)
1	A0	01G-16S	61331	474.4	60570	472
2	A0	02C-16S	38740	472.3	38343	467
3	A0	03C-16S	55616	473.5	55059	473
4	A0	05G-16S	77591	464.4	76575	463
5	A06	61G-16S	34667	472.9	34147	467
6	A06	62C-16S	94373	465.2	93505	463
7	A06	63C-16S	74508	466.2	73818	463
8	A06	65G-16S	71320	468.7	70483	463
9	A15	154G-16S	86261	466.3	85456	463
10	A15	155C-16S	73993	465.7	73297	463
11	A15	156C-16S	125434	471.8	124026	463
12	A15	156G-16S	99747	468.9	98666	463
13	A28	281C-16S	92233	467.6	91338	463
14	A28	282G-16S	74051	470.9	73063	463
15	A28	283G-16S	68629	468.9	67973	463
16	A28	284C-16S	55565	467.6	54784	463
17	P0	02H-16S	75822	467.9	75059	463
18	P0	03H-16S	69899	472.1	69141	466
19	P0	05F-16S	61499	466.7	60895	463
20	P0	06F-16S	67441	458.1	66581	460
21	P06	62H-16S	230732	465.7	228487	463
22	P06	63H-16S	85388	464.5	84677	463
23	P06	65F-16S	91740	465.7	90912	463
24	P06	66F-16S	88702	464.8	87876	463
25	P15	151F-16S	56859	467	56193	463
26	P15	151H-16S	72362	481.9	71197	486
27	P15	154F-16S	59664	471	58945	463
28	P15	154H-16S	106936	466.6	105881	463
29	P15	282F-16S	49889	464.6	49424	463
30	P28	283F-16S	50583	469.6	49992	463
31	P28	285H-16S	73658	466.7	72953	465
32	P28	286H-16S	67125	470.2	66374	465

Sl. No	Group	Sample ID	Total raw sequences	Raw sequence length (mean)	Post QC sequences	Post QC sequence length (median)
33	C0	01B-16S	116365	465.7	115313	463
34	C0	01E-16S	71797	465.3	71198	463
35	C0	02B-16S	86757	466.5	85950	463
36	C06	62E-16S	90361	465.5	89535	463
37	C06	63B-16S	64881	473.4	64056	469
38	C06	63E-16S	77463	464.4	76443	463
39	C15	155B-16S	93903	469	92936	463
40	C15	155E-16S	119805	459.1	118201	465
41	C15	156E-16S	67179	458.1	66279	460
42	C28	284B-16S	104963	469.7	103835	463
43	C28	284E-16S	76366	464.4	75366	463
44	C28	286B-16S	111011	467.8	109957	463
		Total	**3573209**		**3534759**	

Table S 8.2. Total operational taxonomic units (OTU) and coverage of OTUs in each sample of different treatments and control groups. A, antibiotics group; P, phages group and C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

Sl. No	Group	Sample ID	Total OTUs	Good's coverage	Conditional Uncovered Probability (CUP)		
					lladser_pe	*Lower_bound*	*Upper_bound*
1	A0	01G-16S	230	0.98	0.03	0.003	0.03
2	A0	02C-16S	228	0.95	0.04	0.009	0.089
3	A0	03C-16S	237	0.96	0.03	0.008	0.083
4	A0	05G-16S	218	0.99	0.01	0.001	0.011
5	A06	61G-16S	231	0.98	0.02	0.003	0.026
6	A06	62C-16S	252	0.99	0.02	0.002	0.016
7	A06	63C-16S	255	0.99	0.01	0.002	0.017
8	A06	65G-16S	254	0.98	0.02	0.002	0.02
9	A15	154G-16S	256	0.99	0.01	0.001	0.014
10	A15	155C-16S	251	0.99	0.02	0.002	0.024
11	A15	156C-16S	247	0.98	0.02	0.002	0.02
12	A15	156G-16S	257	0.98	0.02	0.002	0.024
13	A28	281C-16S	255	0.99	0.01	0.001	0.011
14	A28	282G-16S	233	0.99	0.01	0.001	0.011
15	A28	283G-16S	249	0.98	0.01	0.002	0.023
16	A28	284C-16S	229	0.98	0.01	0.002	0.02
17	P0	02H-16S	260	0.97	0.03	0.003	0.034
18	P0	03H-16S	264	0.98	0.02	0.002	0.023
19	P0	05F-16S	227	0.98	0.02	0.004	0.044
20	P0	06F-16S	91	0.99	0.01	0.001	0.009
21	P06	62H-16S	263	0.99	0.01	0.002	0.025
22	P06	63H-16S	248	0.99	0.01	0.001	0.014
23	P06	65F-16S	232	0.99	0.02	0.001	0.013
24	P06	66F-16S	258	0.98	0.02	0.002	0.021
25	P15	151F-16S	233	0.98	0.02	0.003	0.031
26	P15	151H-16S	161	0.97	0.03	0.005	0.048
27	P15	154F-16S	192	0.98	0.02	0.003	0.029
28	P15	154H-16S	242	0.99	0.01	0.002	0.016
29	P28	282F-16S	193	0.98	0.01	0.002	0.019
30	P28	283F-16S	222	0.98	0.03	0.002	0.023
31	P28	285H-16S	233	0.98	0.01	0.002	0.018
32	P28	286H-16S	221	0.98	0.02	0.002	0.023
33	C0	01B-16S	249	0.99	0.01	0.001	0.012
34	C0	01E-16S	228	0.99	0.01	0.002	0.017
35	C0	02B-16S	243	0.98	0.02	0.003	0.032

36	C15	155B-16S	251	0.99	0.02	0.002	0.02
37	C15	155E-16S	163	1	0	0.001	0.005
38	C15	156E-16S	89	0.99	0.01	0.001	0.007
39	C28	284B-16S	243	0.99	0.01	0.003	0.027
40	C28	284E-16S	211	0.99	0.01	0.001	0.01
41	C28	286B-16S	261	0.99	0.01	0.002	0.021
42	C06	62E-16S	248	0.99	0.01	0.001	0.014
43	C06	63B-16S	243	0.98	0.02	0.003	0.026
44	C06	63E-16S	215	0.99	0	0.001	0.014

Table S 8.3: Comparison of bacterial taxa between samples from different treatment groups at day 15. Group A, antibiotics group; group P, phages group; group C, control group. Asterisk (*) indicates taxa are significantly different at P<0.05 level.

Taxa	P value	Group A mean	Group P mean	Group C mean
Phylum				
Proteobacteria	0.028*	0.1	0.12	42.92
Actinobacteria	0.029*	0.18	0.2	13.25
Verrucomicrobia	0.034*	3.6	4.05	0.28
Firmicutes	0.1	77.3	67.52	28.84
Bacteroidetes	0.43	17.86	27.03	13.62
Genus				
Eubacterium_ndatum_group	0.025*	0.13	0.11	0.017
Tenacibaculum	0.028*	0	0	6.1
Vibrio	0.029*	0	0	0.087
Paracoccus	0.033*	0.0025	0	17.66
Butyricicoccus	0.033*	0.03	0.18	0.0067
Akkermansia	0.033*	3.6	4.05	0.28
Gordonia	0.034*	0.005	0.005	10.81
Lachnospiraceae_NC2004_group	0.036*	0.18	0.04	0.06
Acinetobacter	0.047*	0.0025	0	8.44

Table S4: The relative abundance (%) of top 5 most abundant phyla in different groups at day 0, 6, 15 and 28 of *Chelonia mydas*. Group A, antibiotics group; group P, phages group; group C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

Genus	Overall (%)	A0	A6	A15	A28	P0	P6	P15	P28	C0	C6	C15	C28
Firmicutes	70.8	87.5	64.0	29.1	67.5	41.7	82.2	75.5	80.2	59.6	90.9	69.1	80.2
Bacteroidetes	14.8	8.7	16.0	13.5	16.6	29.3	10.6	19.4	13.8	13.6	4.7	25.4	15.6
Proteobacteria	7.2	0.1	11.8	42.6	9.1	13.6	0.3	0.1	0.1	16.6	0.1	0.1	0.3
Verrucomicrobia	3.1	2.3	1.8	0.3	1.7	6.6	5.2	3.9	4.4	3.3	2.4	4.0	2.2
Actinobacteria	2.6	0.1	5.0	13.5	3.8	5.7	0.4	0.2	0.2	4.9	0.2	0.2	0.1
Unclassified	1.4	1.2	1.4	1.0	1.3	3.1	1.3	0.9	1.2	2.0	1.6	1.1	1.6

Table S5: The relative abundance (%) of top 20 most abundant genera in different groups at day 0, 6, 15 and 28 of *Chelonia mydas*. Group A, antibiotics group; group P, phages group; group C, control group. 0, day 0; 06, day 6; 15, day 15 and 28, day 28

Genus	Overall (%)	A0	A6	A15	A28	P0	P6	P15	P28	C0	C6	C15	C28
Clostridium sensu stricto 1	44.84	21.5	53.7	51.2	56.1	37.2	51.2	32.2	56.5	53.1	41.6	22.5	48.8
Peptoclostridium	9.20	5.9	8.6	8.4	4.7	7.4	19.1	11.5	10.1	15.1	7.1	1.4	5.0
Bacteroides	8.28	15.5	3.9	15.4	6.8	5.7	2.9	18.1	2.0	3.3	11.6	4.8	11.5
Unclassified	7.85	12.0	7.2	5.6	6.4	9.0	7.5	7.6	6.1	7.3	8.6	11.6	7.6
Macellibacteroides	3.98	9.2	5.4	3.1	4.5	3.6	0.9	6.1	12.7	4.2	1.2	0.2	1.6
Akkermansia	3.12	6.6	5.2	3.9	4.4	3.3	2.4	4.0	2.2	2.3	1.8	0.3	1.7
Paracoccus	3.03	6.3	0.0	0.0	0.0	7.8	0.0	0.0	0.0	0.0	5.4	16.6	4.1
Uncultured	2.29	1.7	2.7	1.5	2.7	1.9	2.8	4.9	1.8	2.1	1.6	1.7	1.5
Gordonia	1.66	2.6	0.0	0.0	0.0	3.3	0.0	0.0	0.0	0.0	2.3	11.5	1.8
Lachnospiraceae UCG-004	1.51	1.2	1.9	1.8	1.6	2.1	1.9	1.1	0.7	1.4	1.6	0.9	1.6
Acinetobacter	1.40	2.6	0.0	0.0	0.0	2.1	0.0	0.0	0.0	0.0	2.3	9.5	1.8
Lachnoclostridium	1.15	1.2	1.0	1.1	0.9	1.7	1.5	1.9	0.3	1.3	1.2	0.8	0.8
Terrisporobacter	0.96	0.6	1.9	1.3	0.8	0.5	0.9	1.2	1.0	1.4	0.7	0.4	0.6
Tenacibaculum	0.93	1.5	0.0	0.0	0.0	2.3	0.0	0.0	0.0	0.0	1.3	6.0	1.0
Blautia	0.66	0.5	0.9	0.7	0.9	0.8	0.6	1.3	0.1	0.3	1.1	0.2	0.4
Cellulosilyticum	0.59	0.7	0.8	0.6	1.0	0.3	0.6	0.4	0.4	0.8	1.0	0.1	0.5
Dysgonomonas	0.55	1.1	0.7	0.5	0.7	0.2	0.2	0.2	0.4	1.0	0.7	0.2	1.0
Turicibacter	0.46	0.0	0.1	0.3	0.8	0.1	0.3	1.0	0.4	0.8	0.4	0.0	1.1
Eubacterium eligens group	0.26	0.0	0.3	0.1	0.1	0.0	0.4	0.3	0.3	1.0	0.1	0.0	0.4
Pseudomonas	0.21	0.4	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.4	1.0	0.3

APPENDIX 9

PUBLICATIONS

Publication 1

ARTICLE IN PRESS

Marine Pollution Bulletin xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Evidence of antibiotic resistance in Enterobacteriales isolated from green sea turtles, *Chelonia mydas* on the Great Barrier Reef

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ARTICLE INFO

Keywords:
Antibiotic resistance
Enterobacteriales
Enteric bacteria
Green sea turtle
Great Barrier Reef

ABSTRACT

This study investigated Enterobacteriales and their antimicrobial resistance in green sea turtles captured adjacent to the central Great Barrier Reef (GBR) and proximate to urban development. Cloacal swabs were taken from 73 green turtles between 2015 and 2016. A total of 154 out of 341 Gram-negative bacterial isolates were identified as Enterobacteriales that represent 16 different species from 9 different genera. The dominant isolates were *Citrobacter* (30.52%), *Edwardsiella* (21.43%) and *Escherichia* (12.34%). The resistance against 12 antibiotics belonging to 6 different classes was determined. The isolates showed highest resistance to β-lactam antibiotics (78.57%) followed by quinolone (50%) and tetracycline classes (46.1%). Approximately one-third (37.7%) of the isolates identified exhibited multidrug-resistance. Isolates recovered from rehabilitated turtles were significantly multidrug resistant ($p < 0.009$) compared to isolates from other study sites. These results provide baseline information on antimicrobial resistance while revealing gaps for further research to evaluate the level of pollution in the GBR.

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Publication 2

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Publication 3

ological Research 207 (2018) 91–99

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Comparative analysis of gut bacterial communities of green turtles (*Chelonia mydas*) pre-hospitalization and post-rehabilitation by high-throughput sequencing of bacterial 16S rRNA gene

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Keywords:
Green sea turtle
Chelonia mydas
Gut microbiota
Dysbiosis
Bacterial diversity
Rehabilitation

ABSTRACT

Stranded green turtles (*Chelonia mydas*) are often cared for in rehabilitation centers until they recover. Although the specific causal agents of diseases in stranded turtles are difficult to diagnose, we know that gut microbiota of green turtles play a vital role in health as well as a wide range of diseases. The objective of this study was to characterize and compare the gut bacterial communities between pre-hospitalization (PH) and post-rehabilitation (PR) stranded green turtles using high-throughput sequencing analysis targeting V1–V3 regions of the bacterial 16S rRNA gene. A total of eight cloacal swab samples were collected from four green turtles undergoing rehabilitation. Proteobacteria dominating in both PH and PR samples without any significant difference. Firmicutes was the second and Bacteroidetes was the third most abundant phylum in PH samples, while Bacteroidetes prevailed in PR samples, followed by Firmicutes. The predominance of the genus *Bacteroides* in both PH and PR samples indicates the importance of this genus in turtle gut health. At a class level, Epsilonproteobacteria was significantly ($P < 0.05$) associated with PH samples and Deltaproteobacteria predominated ($P < 0.05$) in PR samples. The significant abundance of *Campylobacter fetus*, *Escherichia coli*, *Clostridium botulinum* and *Vibrio parahaemolyticus* in PH samples indicate pathogenic associations with stranded green turtles with zoonotic potential. The presence of *Salmonella enterica* in only PR samples suggest possible acquisition of this bacteria during rehabilitation. In this study, all post-rehabilitation green turtles exhibited similar bacterial communities, irrespective of their microbial compositions at pre-hospitalization. The marked differences in the gut bacterial communities of PH and PR turtles indicate the outcome of dietary, management and environmental shift during rehabilitation. Therefore, it is important to address the process of restoring normal gut microbiota of recovered turtles prior to release back to their natural habitat.

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