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Detection, Activity Measurement and Phylogeny of Ureolytic Bacteria Isolated from Elasmobranch Tissue

Yimu Yang
University of Southern Mississippi

Detection, Activity Measurement and Phylogeny of Ureolytic Bacteria Isolated from Elasmobranch Tissue

by

Yimu Yang

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Approved by:

Dr. Darrell Jay Grimes, Committee Chair
Dr. Wei Wu, Major Professor
Dr. Robert J. Griffitt, Committee Member

Dr. Darrell Jay Grimes Committee Chair	Dr. Robert J. Griffitt Department Chair	Dr. Karen S. Coats Dean of the Graduate School

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ABSTRACT

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Free-ranging marine elasmobranch tissue-associated micro-organisms were cultured from free-ranging Atlantic stingray (*Dasyatis sabina*) and Atlantic sharpnose sharks (*Rhizoprionodon terraenovae*). 16S rRNA gene phylogeny indicated bacteria community structure in both elasmobranchs were under phylum Proteobacteria, Firmicutes and Actinobacteria. By conducting split-plot ANOVA, we found the microbial richness is significantly different (P=0.0814) between two superorders of elasmobranch, which may largely due to their preferred habitats and feeding habits. Urease presentence and activity was detected in phylogenetically diverse bacterial strains. Species with high urea-hydrolyzing ability, such as *Micrococcus luteus* (shark blood isolate: 46.84 mU/mg protein; stingray blood isolate: 24.36 mU/mg protein) and *Staphylococcus saprophyticus* (could also be *xylosus*) (66.46 mU/mg protein) were both isolated from blood samples. This study suggests the examination of urease activity to promote the better profile of the virulence of some novel bacteria species. The phylogeny of bacterial 16S rRNA genes and urease-coding ureC genes were analyzed and compared, combined with the examination of urease activity of ureolytic bacteria, we found ureC gene as a potential functional marker. The study of enzymatic (urease) activity and ureC gene-based phylogeny provides a better understanding of ureolytic bacteria for their urea-utilizing

potential, enables the further study of urease-positive strains on bioengineering and bioremediating of marine urea eutrophication in a larger scale.

To our knowledge, this should be the first study to unveil the urea-hydrolyzing ability of marine elasmobranch tissue-associated ureolytic microbes, and the potential of the ureC gene to be a functional marker.

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LIST OF ABBREVIATIONS

USM	The University of Southern Mississippi
AIC	Akaike information criterion
AMP	Adenosine monophosphate
ARG1	Arginase 1
ASL	Argininosuccinate lyse
ASS	Argininosuccinate synthetase
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CNS	Coagulase Negative Staphylococci
CPS1	Carbamoyl phosphate synthetase 1
HGT	Horizontal Gene Transfer
HVR	Hypervariable region
LB	Lysogeny broth
MRSA	Methicillin-resistant Staphylococcus aureus
MS-222	Tricaine methanesulfonate
NAGS	N-acetyl glutamate synthetase
OTC	Ornithine transcarbamoylase
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
R2A	Reasoner's 2A Agar

CHAPTER I – INTRODUCTION

The study of bacterial urease is of vital importance because urease is not only a microbial enzyme that is responsible for the hydrolysis of nitrogenous waste – urea, but also known as a general microbial virulent factor. By hydrolyzing urea, urease derives highly toxic ammonia, which would be fatal when accumulated in the body. Accumulation of ammonia, increase the concentration of NH_4^+, cause the depolarization of neurons and activation of glutamate receptor, which furtherly damage the central nervous system (Randall et al., 2002; Konieczna et al., 2012). Additionally, pH changes mediated by urease (ammonia generated through urea hydrolyzation) is responsible for the promotion of many bacterial infections, so urease has the potential to be a therapeutic target (Rutherford et al., 2014). Ureolytic bacteria are capable of producing urease, and impressively, pathogenic bacteria are frequently observed with ureolytic bacteria (Konieczna et al., 2012). In this study, we screened for ureolytic isolates from the kidney, liver and blood samples from Atlantic stingray (*Dasyatis sabina*) and Atlantic sharpnose shark (*Rhizoprionodon terraenovae*), we found over half of ureolytic isolates in both stingray and shark are opportunistic pathogens. We examined the urease-positive bacteria for their urease activity and we compared the urea-hydrolyzing ability of pathogenic (opportunistic) isolates against non-pathogenic isolates. Additionally, we explored the microbial community structure in the tissue samples of elasmobranchs, and we also determined the utility of ureC gene-based phylogeny as a potential functional marker to classify ureolytic bacteria according to their urease activity performance.

A. Urea and Ornithine-Urea Cycle

Urea ($CO(NH_2)_2$), a small organic compound, has two amino ($-NH_2$) groups and a carbonyl (C=O) functional group. The molecular composition of urea, make it known as carbamide. Urea is a colorless and odorless compound, with high solubility in water; it creates neither acidic nor alkaline environment once dissolved in water (Fisher et al., 2017). As an organic nitrogen compound, urea is a widely used fertilizer and feed additive in the agricultural industry.

Urea, together with ammonia, uric acid, and creatinine, are normally considered as nitrogenous waste and they are all produced from protein metabolism; for many animals, urine is the primary and main route to excrete such wastes. In ureotelic organisms, urea is produced from ornithine-urea cycle which mainly takes place in the liver, and then in the kidneys, to a lesser degree (Timberlake, 2015). The cycle is composed of biochemical reactions that convert ammonia (NH_3) to urea, amino acids produced through metabolism of muscle protein, or ingested food that is not used for the protein synthesis but utilized by the body through oxidation as an alternate source of energy (Sakami et al., 1963). The oxidation pathway begins with transaminase removing the amino group, the amino acid from protein into metabolic waste which results in ammonia. Ammonia is a byproduct of nitrogenous compounds metabolism, the pH value in cells will raise when ammonia is accumulated, which is harmful and poisonous to cells (Ghalehkandi et al., 2012). Because the elevation of ammonium ion (NH_4^+) displaces potassium ion (K+) and depolarizes neurons, activating glutamate receptor (synaptic receptors located mainly on the membranes of neuronal cells, plays a crucial role in mediating the transmission of excitatory synaptic), which leads to an influx of excessive

calcium ion (Ca^{2+}) and cell death in the central nervous system subsequently; in that case, ammonia is believed to be poisonous to all vertebrates, which can cause convulsions, coma and even death (Randall et al., 2002). Most aquatic organisms excrete ammonia without converting it. For bony fishes (teleost), the excretion of ammonia requires huge volumes of water to pass over their gills; however, elasmobranchs (cartilaginous fishes) undergo a complex ornithine-urea cycle to convert highly toxic substance (ammonia) to less toxic substance (urea) (Nelson et al., 2008).

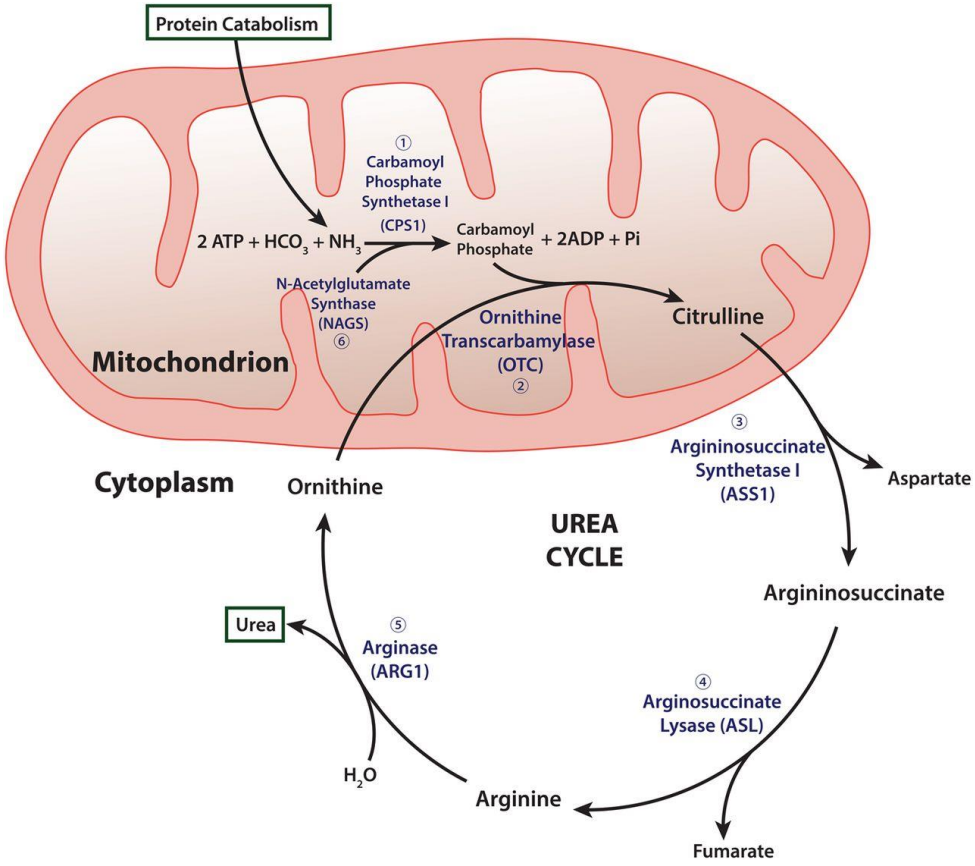

Figure 1. Urea cycle produces urea from the nitrogenous waste of protein catabolism (Blair et al., 2014)

Six enzymes are labeled 1 to 6, with associated gene presented parenthetically.

In mitochondria, ammonia is converted to carbamoyl phosphate by carbamoyl phosphate synthetase 1 (CPS1) and with cofactor-producing enzyme, N-acetyl glutamate synthetase (NAGS); carbamoyl phosphate together with ornithine produce citrulline, the reaction is catalyzed by ornithine transcarbamoylase (OTC); then citrulline is released into cytosol, in which, citrulline and Adenosine triphosphate (ATP, biochemical way to store and use energy, ATP is converted to ADP when one phosphate group is removed) to form citrulline-adenosine monophosphate intermediate (AMP, formed by the removal of one phosphate group from ADP), which reacts with one amino group provided by aspartate to form argininosuccinate, the reaction is catalyzed by argininosuccinate synthetase (ASS); fumarate and arginine produced by the cleavage of argininosuccinate, which is catalyzed by argininosuccinate lyse (ASL); the final step is the hydrolyzation of arginine to produce ornithine and urea, which is catalyzed by arginase 1 (ARG1) (Shambaugh et al., 1977; Mew et al., 2015).

The cycle takes place in the liver primarily, then urea is released into the bloodstream, for some animals, urea is filtered by kidneys and is excreted out of the body in urine (Jonker et al., 1998). However, for elasmobranch, rather than excrete in urine, urea is safely stored in the blood. Marine elasmobranchs contain 2 to 2.5% of urea, while only 0.01-0.03% of blood urea in other vertebrates (Steele et al., 2009; Brown et al., 2013).

B. Urea and Elasmobranch

Urea can assume physical roles other than a waste or toxic product. Urea is the primary osmolyte, together with trimethylamine oxide (TMAO), they are the compounds that exist in the blood and tissues to help maintain the osmotic balance for elasmobranch

(Weber et al., 1983; Vannuccini et al., 1999; Singh et al., 2009). Even urea is less toxic than ammonia, generally, a high concentration of urea is also believed to have a harmful effect on the stability, structure, and function of the protein (Treberg et al., 2006; Gilbert et al., 2008). Urea denatures protein by disrupting water structure, which further weakens hydrophobic interaction and is responsible for the globular structure of the protein, causing proteins to destabilize and thus cease to function properly or at all (Hua et al., 2008). Elasmobranchs accumulate TMAO to counteract and protect against the effect of urea to destabilize protein, not only several functional properties of protein can be activated, but also the structure of protein can be stabilized by TMAO (Treberg et al., 2006; Trischitta et al., 2012). According to a previous study of Yancey et al. (1980) and Treberg et al. (2006), a 2:1 concentration ratio of urea to TMAO is optimal to preserve and protect proper protein function.

For marine animals, one of the biggest challenges they are facing is the osmotic challenge, which requires them to keep the internal balance (homeostasis) against the external osmotic pressures. Equilibrium is reached when internal body fluids and the surrounding fluid have the same osmotic concentration. It is known that cell membranes are permeable to water, and water flows from low to high ion (solute) concentration areas. Depends on the relative ion concentration between cell to the outside environment, water would be absorbed into the body when the body fluids contain a higher solute concentration and leave the body when the outside milieu has higher concentration; it is for sure that no matter where water may flow, it will result in cells bursting or shriveling, which is harmful to the organism either way (Hammerschlag et al., 2006). Most bony fishes are ion regulators, their body fluids are osmotically distinct from the environment

(seawater), which means the ion concentration in fish body is lower than seawater, so the body is constantly losing water, so a small volume of urine is produced; but they work actively to counter the effect of osmotic imbalance by drinking seawater continually and remove the extra salt through chloride pumps (Whittamore et al., 2012).

In contrast to teleosts, elasmobranchs tend to maintain osmotic consistency with their environment, plasma osmolarity is very high, largely due to their body fluid concentration of urea and TMAO is high (Hammerschlag et al., 2006). Elasmobranchs, which include sharks, rays, and skates, have skeletons that made of cartilage (cartilaginous), not calcified bone. Elasmobranchs are predominantly marine, although some are seen with estuarine (10%), euryhaline (2%) and obligate freshwater (1%) lifestyle (Hammerschlag, 2006a).

Marine elasmobranchs accumulate urea to a high level as their osmoregulatory strategy (Treberg et al., 2006), they retain large volumes of urea produced from ammonia via the ornithine-urea cycle, to maintain their body fluids isosmotic (with same osmosis pressure) or moderately hyperosmotic (with greater osmolarity) to surrounding medium (Trischitta et al., 2012; Cramp et al., 2015), that makes water flows slightly into sharks, not surprisingly, shark excretes a great deal of diluted urine. Teleosts begin dying when their blood urea exceeds 200mM, but marine elasmobranchs maintain 300-500 Mm of urea in their body fluid as a major osmolyte (Singh et al., 2009). Elasmobranch kidneys also function in storing urea (Randall & Tsui, 2002). As part of osmoregulatory physiology, elasmobranchs keep urea in their blood and other tissues; urea breaks down to ammonia when they die, that explains the strong smell and odor of the meat, so in

order to avoid this problem, elasmobranchs freshly caught for consumption are normally bled out quickly on the spot (Musick et al., 2002).

Elasmobranchs must minimize the loss of urea across some interfaces, which exist between the body fluids and surrounding medium, in order to maintain the osmotic balance and also reduce or decrease the expense of urea-making process, the main interfaces are gills, kidneys, as well as rectal gland (Trischitta et al., 2012). Both gills and rectal gland of elasmobranch possess unique permeability to allow the water to move but not the urea, it has been detected that a homologue of a renal urea transporter exists in the gills to avoid urea loss by back-transport urea in the basolateral membrane; in kidney, urea is filtered freely by glomerulus, renal tubules can reabsorb as high as 90% - 96% of filtered urea (Trischitta et al., 2012). Sharks excrete urea through gills or from cloaca, once urea concentration is built up too high in the body ("Sharks need to maintain their salt levels", n.d.).

We selected two types of elasmobranchs for this study: Atlantic stingray (*Dasyatis sabina*) and Atlantic sharpnose shark (*Rhizoprionodon terraenovae*). Atlantic stingray is North American fish that is commonly seen in the Gulf of Mexico and it is a small, euryhaline species (Gelsleichter et al., 2006). Atlantic sharpnose shark is a small gray shark, black edges are usually seen on dorsal and caudal fins of their juveniles. Sharpnose sharks prefer high temperature (>30^{o}C) and deep water (> 6m) and they are common in the southern Gulf of Mexico. The two species of elasmobranch represent each of the elasmobranch superorders, Batoidea (Stingrays and skates: Atlantic stingray) and Selachii (Sharks: Atlantic sharpnose shark); the two species also representing two types of habitats of elasmobranch (Atlantic stingray: seafloor, over sediment, Atlantic

sharpnose shark: open water). Both species are abundant in the Gulf of Mexico during summer, they are small-sized and comparatively well-studied elasmobranchs, with a large amount of literature describing their biology and physiology; Furtherly, Atlantic stingray is commonly used as a laboratory model for the examination of elasmobranch physiology.

For this project, we collected and sacrificed 15 Atlantic stingrays with a seine net and 16 Atlantic sharpnose sharks with hook-and-line capture, all the captured animals were checked with health status, parasite load, and they were all visibly healthy; kidney, liver, and blood samples were collected from these 31 animals with aseptic technique.

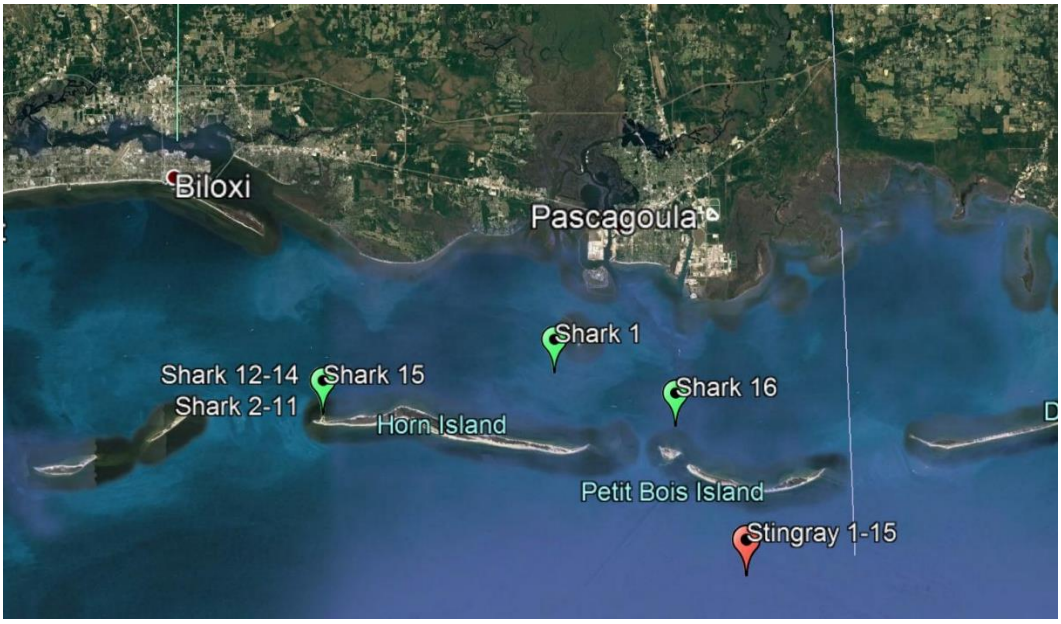

Figure 2. Capture map of 31 elasmobranchs in the Gulf of Mexico

C. Bacterial Richness Analyses

16S ribosomal RNA (16S rRNA) coded by 16S rRNA gene, is an extremely important component of the 30S ribosomal complex in prokaryotes. Due to the relatively slow rates of gene evolution, 16S rRNA genes are mostly used in bacteria identification

and reconstructing phylogenies (Woese et al., 1990). Bacterial 16S rRNA gene sequences generally contain nine “hypervariable regions” (HVR, from V1 to V9) that exhibit appreciable richness of gene sequences and provide species-specific signature sequences that can be used for bacteria identification, because16S rRNA gene is highly conserved between different archaea and bacteria species (Kolbert et al., 1999; Chakravorty et al., 2007; Pereira et al., 2010). 16S rRNA gene (approximately 1,500 base pair) has highly conserved sequences between HVR that enable the universal primer design and primer binding (Chakravorty et al., 2007). 27 forward and 1492 reverse primer are one of the universal primer sets that most frequently used for the aim of phylogenetic study (Janda et al., 2007). Overall, 16S rRNA gene is the robust phylogenetic marker (a fragment of coding or non-coding gene which is used in phylogenetic reconstruction, which is known to have no or predictable variation within all species of a genus), studies show that 16S rRNA gene sequencing (Sanger sequencing), in most cases, provides the identification of genus (90%), to a lesser extent of species (65 to 83%), with from less than 14% of isolates remaining undefined after sequencing (Janda et al.; 2007).

An earlier study of Grimes et al. (1985), examined the bacterial flora of 28 neritic sharks that represented five shark species (lemon, nurse, blacktip, sharpnose and tiger shark), bacterial associated with shark samples were isolated using culture-based method; 59 out of 78 pure cultures were identified as *Vibrio* species, *Vibrio alginolyticus* (26%) was the most frequently isolated species from external surface, followed by *V. harveyi* (15%), *V. furnissii* (9%), *V. damsela* (now as *Photobacterium damselae* subsp. *damselae,* 6%) all isolated from kidney and *Vibrio* spp. (undefined *Vibrio* species, 17%), at the same time, *Proteus* and *Photobacterium* spp. were also collected from inside of the

mouth. *Vibrio* spp. can be readily collected from nearly all tissue samples of free-ranging sharks (Grime et al., 1985). In a later study, Grimes et al. (1993) isolated 197 bacterial strains from 10 carcharhinid sharks when compared with references strains, 14 out of 27 phyla were identified as *Vibrio* species. In this research, we studied elasmobranch tissue-associated bacteria community structure, compared the bacteria richness between Atlantic stingray and Atlantic sharpnose shark, because they represent two superorders of elasmobranch, and compared of bacteria richness in different tissue samples across elasmobranch species as well. Based on previous studies, we hypothesized *Vibrio* would be the predominant species in both Atlantic stingrays and Atlantic sharpnose sharks.

D. Bacterial Urease Activity and ureC Gene

Urease (EC 3.5.1.5) is a nickel-containing metalloenzyme that is able to hydrolyze urea into carbon dioxide and ammonia (Reed 2001). Ureases are found in numerous bacteria, ureA, ureB and ureC genes encode three functional subunits of bacterial ureases, ureD, ureE, ureF and ureG genes generally encode four types of accessory proteins which serve the function to activate and incorporate Ni^{+} (Koper et al., 2004). Reed et al. (2001) observed, that different organisms may have different subunits composition of ureases, but the alignment result of the primary protein structures showed similarity within many amino acid regions. A large variety of organism has been demonstrated to have ureases; urea hydrolysis in shark tissue was first described in the 1950s; ureolytic bacteria isolated from shark tissues and organs were hypothesized to play an essential role in the control of urea storage and flux in where they were collected (Knight et al., 1988).

Urease has been reported as a microbial virulent factor by Rutherford et al. (2014). By hydrolyzing urea, urease derives from ammonia and carbonic acid. Not only ammonia derived by urease is highly toxic to host cells, bicarbonate converted from carbonic acid forms a buffer solution, which keeps the surrounding pH relatively neutral with bicarbonate and ammonia binding and dissociating from hydrogen ions; this is very necessary for bacteria to colonize the stomach where high acidity level (normally pH from 1.5 to 3.5) is required for food digestion; *Helicobacter pylori,* for example, is responsible for stomach infection, and it is widely distributed in tropical and subtropical coastal waters which poses enormous public health and safety risks to human beings (Holman et al., 2014; Rutherford et al., 2014). Urease-positive bacteria produce ammonia and carbonic acid (formed by the hydrolyzation of urea by urease), by binding to precipitated minerals (calcium, magnesium, etc.) can develop infection stones which surround and protect the pathogenic bacteria, such as *Klebsiella* and *Proteus* species associated with urinary tract infections (Rutherford et al., 2014). Pathogenic bacteria are frequently observed with a ureolytic activity which is the main causative factor to result in severe clinical gastric and urinary tract infections, so urease activity is regarded as an important marker of many bacterial infections (Konieczna et al., 2012).

A study of Knight et al. (1988) detected bacterial activity in hydrolyzing urea in liver homogenates in Carcharhinid sharks (lemon and tiger sharks). Each of the shark tissue (kidney, liver, muscle, and blood) homogenates were divided into three subsamples, to each of which was differently added to saline, O/129 (*Vibrio* sp. growth inhibitor) and ampicillin (a type of antibiotics used to kill or inhibit the growth of certain bacteria), then incubated subsamples of tissue homogenates with radiolabeled (^{14}C) urea;

the liver homogenates displayed a significant differences among three treatments, few or on bacterial hydrolysis in O/129 and ampicillin treatments which indicated bacterial activity in urea hydrolyzation; no blood homogenates showed significant difference among three unique treatment, indicating no urea-hydrolyzing occurring in blood homogenates; due to incomplete homogenization, kidney and muscle were not analyzed furtherly (Knight et al., 1988). Blood culture is viewed as a very important clinical test by microbiologist and physicians in the diagnosis of severe infections (such as septicemia and bacteremia) (Weinstein et al., 2003). Generally, shark blood is sterile, while other tissues, such as kidney, liver, and muscle containing tons of bacteria (Grimes et al., 1985; Mylniczenko et al., 2007). In this study, we collected kidney, liver and blood sample and used the traditional method to culture which enabled a direct observation of the existence of microbes.

According to Gresham et al. (2007), among ureA, ureB and ure C gene, ureC gene is the largest one encoding functional urease subunits, and most importantly, there are many highly conserved regions on ureC gene that are suitable for the attachment of PCR (Polymerase chain reaction, used for target gene amplification) primers (binding to target gene to start the chain reaction), thus making ureC gene an ideal target for the purpose of urease analysis. In this study, the ureC gene was selected as a surrogate to detect ureolytic bacteria and to investigate the richness of ureC genes in the ureolytic bacterial community in two types of elasmobranch. As a functional gene encoding urease α subunit of bacteria with various urease activity, we hypothesize that marine microbial ureC gene sequence serves as a functional marker for ureolytic bacteria species.

This study aims to test three key hypotheses: (1) *Vibrio* is the dominant species in kidney, liver and blood samples of marine elasmobranchs; (2) there is no difference in microbial composition and culturable bacteria isolates in tissue samples of two types of elasmobranch; and (3) urease encoding gene-ureC serves as a functional marker to classify ureolytic species with their urease-hydrolyzing performance. In summary, I investigated the microbial community structure of each type of tissue samples of Atlantic stingrays and Atlantic sharpnose sharks, if *Vibrio* predominates, I would expect to see *Vibrio* species take up largest part in microbial composition in kidney and liver samples. If the tissue samples of Atlantic sharpnose shark and Atlantic stingray show no difference in the bacterial composition and number, that indicates in tissue-associated bacteria isolation of shark and stingray are not influenced by where they live and what they prey on, in that case, I would expect to see the same bacteria species appear in the same type of organ in shark and stingray. Additionally, if the ureC gene is a functional maker for urease-positive bacteria, I would expect bacteria with similar urease activity grouped together.

To our knowledge, this is the first study to detect phylogenetic richness of bacteria isolated from both stingrays and sharks, to demonstrate the urea-hydrolyzing ability of elasmobranch tissue-associated bacteria, to explore the utility of ureC gene sequence information as a functional marker for ureolytic bacterial species isolated from elasmobranchs.

CHAPTER II – MATERIALS AND METHODS

A. Sample Collection

Fifteen Atlantic Stingrays were captured in fall in 2014 with seine net near the West Point of Horn Island (Appendix A), in the Gulf of Mexico; 16 Atlantic Sharpnose Sharks were collected with hook and line (with hooks, baits, and chum), at five different locations near Horn Island (Appendix B), sharks from No.1 to 15 were captured in fall in 2014; No.16 was captured in early summer in 2015. Hook-and-line capture is a commonly used metric for verifying elasmobranch health status, animals were captured each one at a time and all captured animals were examined for activity level, parasite load and any evidence of poor health. we excluded animals with unclear health status. All tissue samples were collected aseptically and processed immediately following each capture.

Blood samples: Prior to venipuncture, the area for blood sampling was sanitized with a swipe of isopropyl alcohol pad followed by a minute wait time to eliminate culturable bacteria on the skin of elasmobranch. 1 mL of blood samples were extracted from the caudal vessel of Atlantic sharks with 21-gauge needles, and from wing vessel of the Atlantic stingray with 22-gauge needles. Puncture needle on the syringe hub was replaced after each blood-draw, and then the blood sample was injected into culture tubes containing 5 mL of Zobell Marine Broth 2216 (a medium that mimics seawater, helps with the growth of marine organisms), tubes were stored on ice in cooler.

Kidney and liver samples: After blood sampling, all the animals were euthanized via submersion in tricaine methanesulfonate (MS-222) before tissue collection. Incision sites were sterilized with betadine and sampling instruments were flame-sterilized with

70% EtOH. All tissue samples (e.g., kidney and liver) were washed adequately in freshly-made Phosphate Buffer Saline (PBS) solution prior to tissue sampling. 5-mm section of kidney and liver tissues were cut and preserved in a culture tube containing 5 mL of Marine Broth, all tubes were stored on ice in a cooler not longer than 5 hours prior to lab processing.

B. Bacterial Cultivation, Isolation, and Preservation

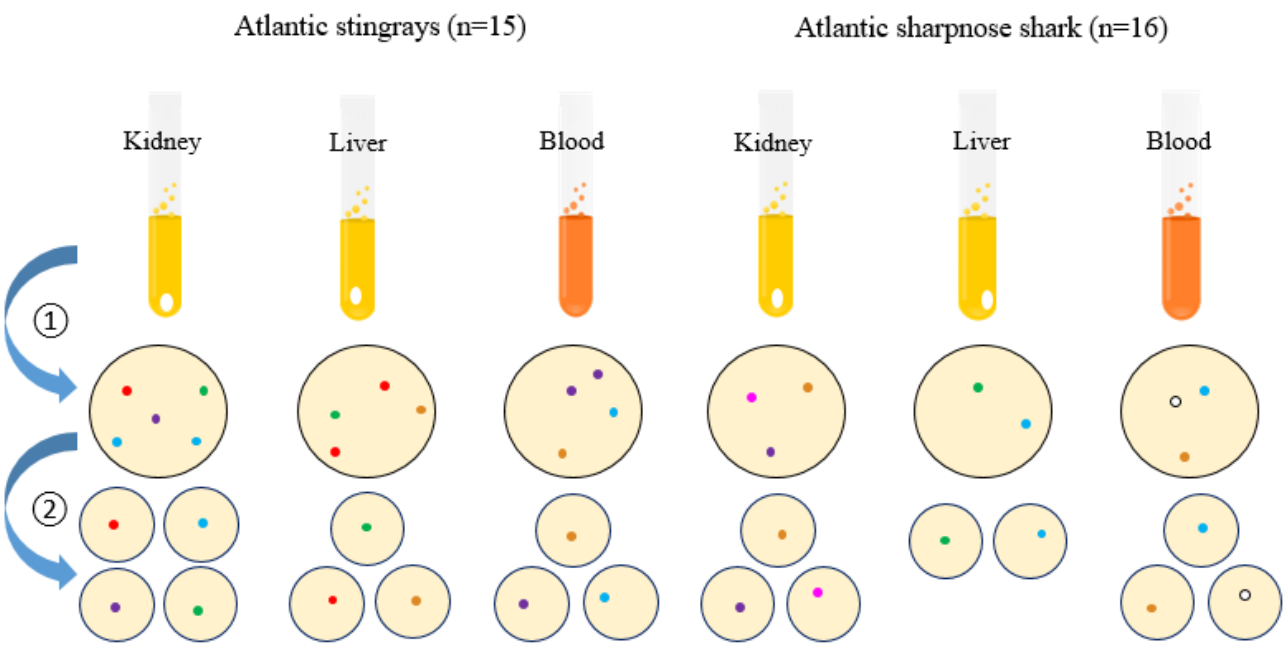

Figure 3. Isolation and purification of elasmobranch tissue-associated micro-organism schematic illustration

① represents pour plate (spread plate) method to grow micro-organisms (mixed cultured); ② indicates to pick up each of the visibly unique colonies, inoculate bacterial culture onto new petri dish and streak the plate (to sufficiently thin out the inoculum) to produce isolated colonies of an organism, as well as to obtain pure strain from a single species of bacteria (a re-streaking may need for complete purification)

Culture tubes containing tissue samples were incubated in a shaker incubator at 35ºC overnight (to encourage the multiplication of bacterial cells) for bacteria enrichment. 200-µL evenly mixed enrichment broth was spread onto Marine Agar 2216

and plates were incubated at 35ºC for 24 to 72 hours. Not like in seawater, fewer and countable bacteria reside in elasmobranch tissues, which made visibly unique bacterial colonies easily to spot, then the unique colony was picked out to re-streak onto new Marine Agar plates for colony isolation and purification. Isolated cultures were stored both in Marine Agar slants at room temperature and in 1.5-mL cryotube containing glycerol and skim mile as cryoprotectants preserved at - 80ºC freezer, for the purpose of long-term storage.

C. Urease Test

Stuart's urea broth (20.0 g urea, 9.5 g Na_2HPO_4, 9.1 g KH_2PO_4, 0.1 g yeast extract, 0.01 g phenol red and 1000 ml demineralized water) was used to test the ability of organisms to produce the urea degrading enzyme, urease. The indicator phenol red remains original color (orange) at neutral pH and changes to pink or magenta once pH is above 8.4. Urease-positive organisms catalyze the hydrolysis of urea to ammonia and carbon dioxide, which creates an alkaline environment, leading the indicator to turn pink (Brink et al., 2010). Controls were *Escherichia coli* ATCC 11775 acquired from the American Type Culture Collection (negative control) and a *Proteus* sp. (positive control) isolated from the Grimes lab.

A loopful pure culture were taken out aseptically from marine agar with sterile disposable inoculating loop, a sterile urea broth test tube was taken, the cap was removed, and the neck of the tube was quickly flamed (passing the neck through the flame forward and back several times) to avoid possible contamination (prevent the entry of non-related organisms). The loopful organism was then inoculated in the urea broth, the neck of the tube was once again flamed and put back in the tube rack, the tube rack was then

incubated at 37°C for 24 to 48 hours. After incubation, urease-positive and -negative organisms were differentiated by color, negative control (*E. coli*) should remain orange (or slightly yellowish), positive control (*Proteus* sp.) should change the urea broth to deep pink.

D. Urease Activity Test

The urease activity of each urease-positive isolate was tested using both a Urease Activity Assay Kit (catalog # K38-100) (Biovision Incorporated, Milpitas, CA, USA) and BCA Protein Assay Kit (Biovision Incorporated, Milpitas, CA, USA). A loopful of isolated colonies from a pure culture was homogenized in ice-cold PBS buffer containing Protease Inhibitor Cocktail (Biovision Incorporated, Milpitas, CA, USA). The lysate was centrifuged at 10,000×g for 10 min at 4^{o}C with a refrigerated centrifuge (Eppendorf Biotools, CA, USA) and the supernatant was collected. A 10-µL sample was added to a flat-bottom 96-well plate, 90 µL of the reaction mix (88 µL Urease Assay Buffer, 2 µL 1× Urea) was added into the same well and mixed well. Urease was diluted by adding 10 µL urease into 90 µL Urease Assay Buffer, then 10 µL of diluted urease and 90 µL reaction mix to the desired well as a positive control (Biovision, 2015). Add 10 µL Urease Assay Buffer and 90 µL reaction mix into desired well, for reagent background control. To prepare the standard curve, ammonium chloride was diluted from 100 mM to 1mM with de-ionized water and mix thoroughly (Biovision, 2015). Then pipette 0, 4, 8, 12, 16, and 20 µL of the standard into a series of desired wells to produce 0, 4, 8, 12, 16, 20 nmol standards per well, and adjust the volume of each well with de-ionized water to 100 µL. The 96-well plate was incubated for 30 min at 37^{o}C. And reagents 1 and 2 were dispensed into each well (except standards) and incubated at 37^{o}C for 30 min

(https://www.biovision.com/documentation/datasheets/K378.pdf, Biovision, 2015). The optical density (OD) of ammonia produced through the hydrolysis of urea was then measured at 670 nm in a multi-well spectrophotometer (BioTek Instruments, Winooski, VT, USA).

The total protein is the total protein content presents in the sample, it can be detected and quantified by using BCA Protein Assay Kit (catalog # K813-2500) (Biovision Incorporated, Milpitas, CA, USA). With the measurement of the absorbance of a series of known concentrations of Bovine Serum Albumin (BSA) standards, together with the standard curve, we are able to calculate the concentration of total protein.

Prepare different concentration of samples and dilute with de-ionized water within the assay range (25-2000 μg/mL), add 25 μL of the sample into 96-well plate; BSA standards were diluted with de-ionized water to generate the final BSA concentrations as 2000, 1500, 1000, 500, 250, 125 and 0 μg/mL; 25 μL of each BSA standard was added to desired wells. Then 200 μL of the BCA working reagent was added to all the samples and standards. The plate was incubated at 37°C for 30 min and then read using a spectrophotometer at 562 nm (Biovision, 2014). During the reaction process, chelate complex (Cu^{+1}- BCA chelate) is generated from the chelation of bicinchoninic acid (BCA) with a cuprous cation (Cu^{+1}), which has a strong absorbance at 562 nm. Cu^{+1} is produced by the reduction of protein with a cupric cation (Cu^{+2}) under the alkaline environment (https://www.biovision.com/documentation/datasheets/K813.pdf, Biovision, 2014). One unit of urease activity is 1 umol of ammonia released per min per mg of microbial cytoplasmic protein, according to Mirbod-Donovan et al. (2006).

E. Microbial DNA Extraction

DNA from all the visibly unique pure colonies were extracted with a simple method called heat treatment. Heat treatment of bacteria cell is an easy and swift way of DNA extraction furtherly used to perform PCR, and DNA sequencing (Dashti et al., 2009). Exposure to high temperature is widely known to damage cell membranes and cell walls, a two-minute heating is able to denature the cell wall (Lou et al., 1993). Similarly, exposure to low temperature is also found to cause damage to cell membranes and cell walls, as crystallization of water inside cells is induced by freezing treatment which furtherly destructs the cytoplasmic structure (Lou et al., 1993; Dashti et al., 2009), a few repeats of freezing and thawing is tested by Tell et al. (2003) as a simple method to obtain bacterial DNA. To examine the effect of heat treatment method to extract bacterial DNA, Dashiti et al. (2009) put two colonies of bacteria into a test tube that contained 1 mL of distilled water and boiled the tube in a water bath for 10 min, then centrifuged the tube at 1,000 rpm for five minutes and collected supernatant for PCR; they found out the heat treatment of bacteria yielded enough DNA molecules to perform the following molecular research. Heat treatment method is cheap, simple and quick, it also minimizes time and the need for reagents, most importantly, it shows excellent results of DNA extraction (Dashti et al., 2009). In this study, for each of the Marine Agar plates (each contained purified bacterial colony which morphology was unique within the tissue sample is isolated from), we picked up one purified colony and inoculated it into a 1.5-mL centrifuge tube containing 200 μL de-ionized water and mixed well. We adjusted the method by combining the heat treatment method of Dashiti et al. (2009) and freeze treatment method of Tell et al. (2003), we placed the tube in a heat block at 100^{o}C for 5

min, then cooled down in - 20°C freezer for 5 min. The heat-cool treatment was repeated and the tubes were centrifuged (Eppendorf, NY, USA) at 4°C for 5 min, 2800×g and the supernatant were collected for Polymerase Chain Reaction (PCR).

F. PCR Amplification of 16S rRNA gene

16S rRNA genes were amplified with the universal primer set 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTT ACGACTT-3') (Brosius et al., 1978). Each reaction contained: 1× PCR buffer, 2 mM $MgCl_2$, 1.6 units Taq Polymerase, 0.2 mM dNTP mixture, 1 µM each forward and reverse primer, 20 to 30 ng of genomic DNA template. PCR amplification began with 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 55°C, extension for 90 s at 72°C; followed with a final extension for 10 min at 72°C; The reactions were performed in Thermal Cycler (Bio-Rad Laboratory, CA, USA). An approximate 1500-bp single band PCR product was visualized in the 2% agarose gel. PCR products were purified with DNA Purification Kit (DNALand Scientific, Baton Rouge, LA, USA) and then shipped to Eurofins Genomics Company (Louisville, KY, USA) for gene sequencing.

G. PCR Amplification of ureC gene

ureC genes extracted from urease-positive individuals were amplified with four primer sets. L2F/ L2R, ureC-F/ ureC-R, SF-3/ SR were designed by Gresham et al. (2007); UC-F/ UC-R were found in the study of Collier et al. (1999) (Table 1). Each reaction contained: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP mixture, 1.5 µM each forward and reverse primer, 1.25 units Taq Polymerase, 125 to 130 ng of genomic DNA template. PCR amplification started with 5 min initial denaturation at 94°C,

followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 90 s at 55°C, an extension for 2 min at 72°C; with a final extension for 15 min at 72°C. Due to the various primer sets, 250-350 bp single band PCR product shown on the 2% agarose gel. PCR products were purified with DNA Purification Kit (DNALand Scientific, Baton Rouge, LA, USA). Purified amplicons were sent to Eurofins Genomics Company (Louisville, KY, USA) for sequencing.

Table 1

ureC gene-specific PCR primers retrieved from previous studies.

Primer	Primer Sequence (5' to 3')	Amplicon size (bp)	Source
L2F	ATHGGYAARGCNGGNAAYCC	394	Gresham 2007
L2R	GTBSHNCCCCARTCYTCRTG		Gresham 2007
SF-3	GGYGGBGGMCAYGCHCCNGA	277	Gresham 2007
SR	TCWCCDACDCGBCCCATBGC		Gresham 2007
ureC-F	TGGGCCTTAAAATHCAYGARGAYTGGG	323	Reed 2001
ureC-R	GGTGGTGGCACACCATNANCATRTC		Reed 2001
UCF	AAGSTSCACGAGGACTGGGG	316	Collier 1999
UCR	AGGTGGTGGCASACCATSAGCAT		Collier 1999

H. 16S rRNA Gene Phylogenetic Analysis

Elasmobranch tissue-sample isolates were collected and used to determine the closest relatives of isolated bacterial gene sequences by using BLAST (Basic Local Alignment Search Tool) analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), 16S rRNA gene sequences were obtained from NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) for sequence alignment. Mega 5.0 was adopted for sequence alignment and the generation of the phylogenetic tree to study the bacterial richness. Nucleotide sequences of bacterial DNA were aligned by Clustal W, neighbor-

joining statistical method (1,000 replications) was adopted with maximum composite likelihood model for the analyzation of the distance. One hundred and forty-one 16S rRNA gene sequences were retrieved from Genbank database; all bacteria sequences of phylum Bacteroidetes were assigned as monophyletic out-group. In the phylogenetic study, an out-group consists of a group of organisms, when studying the evolutionary relationship among monophyletic groups of organisms, out-group can be seen as a reference group to compare with the in-group (Farris et al., 1982). An out-group can either be in-group's sister group or a bit more distantly related group (Morrison et al., 2013). To better understand the traits evolution along a phylogeny, the selection of out-group is necessary.

I. ureC Gene Phylogenetic Analysis

Twenty-seven ureC gene sequences (~228 base pair) obtained from elasmobranch tissue-isolate ureolytic bacteria, together with 64 bacterial ureC gene sequences (ureolytic bacteria species selected from the 141-species used for the construction of 16S rRNA phylogeny) retrieved from GenBank database and UniProt (http://www.uniprot.org/) were collected and aligned with respect to amino acid codons (substitution as amino acid) by using mega 5.0. Neighbor-joining method (1,000 replications) with the p-distance model was adopted to study the richness of ureC gene. All bacteria gene sequences from phylum Bacteroidetes were assigned as monophyletic out-group.

J. Statistical Analysis

Analysis of variance (ANOVA): factors that impact microbial richness were analyzed by split-plot experiment with R studio. The analysis yielded a p-value < 0.1 (significance level $\alpha=0.1$) was considered as the statistically significant difference.

Cluster analysis: IBM SPSS Statistics 22 was used to classify animal individuals into groups according to their tissue-associate bacteria isolation results, k-means cluster analysis was adopted to generate clusters.

CHAPTER III - RESULTS

A. Culture Isolation and Bacteria Species Identification

Colony morphology is cultural characteristics of an organism presented on an agar plate, features of colonies can be utilized to pinpoint the bacterium identity; generally, different bacteria species present different colonies (Austin Community College, 2005; Washington State University, 2005; Reynolds, 2011). With consideration of the edge, size, chromogenesis (color), consistency, opacity, elevation, surface of the colony, we isolated distinct colonies from each tissue sample (Atlantic stingrays: 3×15; Atlantic sharpnose sharks: 3×16), 73 colonies were cultured and isolated with Marine Agar plates and classified into 58 bacteria species. DS10 K-3 and DS10 K-4 are both *Vibrio sinaloensis,* which were isolated from the kidney sample of same the individual of Atlantic stingray (no. 10), so we ruled out DS10 K-4 and kept DS10 K-3. The same thing happened with DS1 B-4 and DS1 B-6, they are both *Bacillus alkalogaya* collected from the blood sample of the first-captured stingray, we only kept DS1 B-4 to perform the following analyzation. Valid (effective) number of isolated colonies are 71. From those, we classified 58 distinct bacteria species.

Forty-seven isolates (42 distinct bacteria species) obtained from tissue samples of 15 Atlantic stingrays, 23 distinct bacteria species were found in the kidney. From the heat map (Figure 4), bacteria richness is pretty high in stingray kidney, and *Vibrio* spp. predominate (nine *Vibrio* species) among the micro-organism species in the kidney. Other species included *V. harveyi, V. azureus, V. campbellii, V. communis, V. owensii, V. panuliri, V. parahaemolyticus,* and *V. sinaloensis,* with *V. harveyi* isolated twice from kidneys of two stingrays; Bacillus spp. were also isolated frequently (five *Bacillus*

species), including *B. flexus, B. subtilis, B. tequilensis, B. velezensis* and an undefined *Bacillus* species which might be *B. licheniformis;* three *Shewanella* species, *S. corallii, S. fidelis, S. japonica* were found to reside in the kidney; *Pseudomonas stutzeri, Micrococcus terreus, Photobacterium damsela* and *Psychrobacter* sp. were collected from stingray kidney as well. Six bacteria species were acquired in stingray liver, *Bacillus hwajinpoensis, Bacillus megaterium, Kistimonas scapharcae, Micrococcus yunnanensis, Oceanobacillus caeni* and *Rothia amarae.* In stingray blood sample, 15 bacteria species were collected, *Micrococcus* was the dominant species (five *Micrococcus* species in stingray blood), *M. yunnanensis, M. luteus* and *M. aloeverae,* with *M. yunnanensis* isolated twice from blood samples of two different stingrays; followed by three Bacillus species, *B. alkalogaya, B. infantis* and *B. safensis;* two *Thalassospira* species were obtained, *T. tepidiphila* (isolated from two stingrays) and *T. profundimaris;* the rest of isolated species were *Pseudoalteromonas piscicida, Pseudoalteromonas* sp., *Acinetobacter radioresistens, Oceanobacillus caeni, Rothia amarae/ mucilaginosa* and *Stenotrophomonas* sp. (See Table 2)

Twenty-four isolates (24 distinct bacteria species) were collected from tissue samples of 16 Atlantic sharpnose sharks (See Table 3); eight bacteria species were isolated from shark kidneys, two species from genus *Exiguobacterium*, they were *E. aestuarii* and *E. profundum; Bacillus fordii, Oceanobacillus caeni, Psychrobacter* sp., *Shinella granuli, Sporosarcina contaminans* and *Vibrio* sp. also present in shark kidney samples. Nine distinct bacteria species observed in shark livers, the predominating species was *Pseudomonas, P. hibiscicola, P. parafulva and Pseudomonas* sp., other species were *Brachybacterium paraconglomeratum, Micrococcus yunnanensis,*

Photobacterium damsela, *Psychrobacter celer*, *Roseomonas cervicalis* and *Serratia marcescens.* In blood samples of shark, seven species were obtained, *Bacillus* species *as B. koreensis*, *B. tequilensis*, and *Micrococcus* species as *M. luteus*, *M. yunnanensis* were relatively frequent isolated; the rest were *Staphylococcus saprophyticus/ xylosus*, *Stenotrophomonas maltophilia*, and *Vibrio nigripulchritudo.*

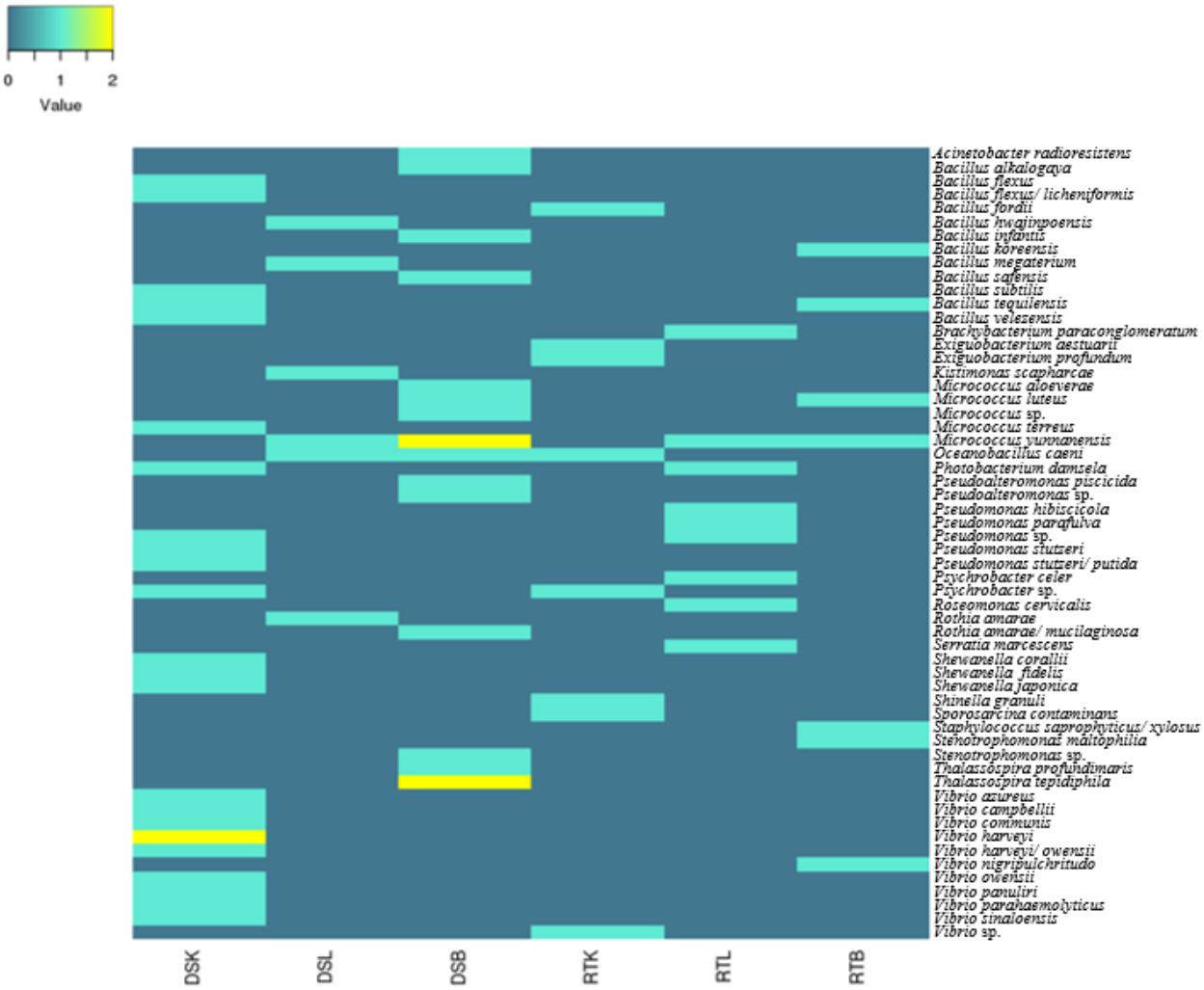

Figure 4. The species-level richness of bacterial sequence and microbial community structure of each tissue sample of two types of elasmobranch.

Seventy-one bacteria isolates classified into 58 bacteria species. Value ”0” indicates no bacteria (right column) isolated from tissue samples of all individuals of Atlantic stingray or Atlantic sharpnose shark, “1” indicates one isolate of certain bacteria species was isolated, “2” indicates two isolates acquired. “DSK”, “DSL” and “DSB” represent kidney, liver and blood samples acquired from 15

Atlantic stingrays, respectively; “RTK”, “RTL” and “RTB” represent kidney, liver and blood samples collected from 16 Atlantic sharpnose sharks, respectively.

Table 2

Culturable bacteria isolation from tissue samples of each individual of 16 Atlantic sharpnose sharks.

RT	Kidney	Liver	Blood
1	/	*Serratia marcescens*	*Micrococcus luteus*
2	/	*Pseudomonas hibiscicola*	*Vibrio nigripulchritudo*
3	/	/	/
4	*Vibrio sp.* *Sporosarcina contaminans* *Bacillus fordii*	*Photobacterium damsela subsp. damsela*	/
5	/	/	/
6	/	/	/
7	/	/	/
8	/	/	*Stenotrophomonas maltophilia*
9	*Exiguobacterium aestuarii*	/	/
10	/	*Micrococcus yunnanensis* *Roseomonas cervicalis*	/
11	/	/	/
12	/	/	*Micrococcus yunnanensis*
13	*Oceanobacillus caeni* *Exiguobacterium profundum*	/	*Staphylococcus saprophyticus/ xylosus*
14	/	/	*Bacillus koreensis*
15	/	/	/
16	*Shinella granuli* *Psychrobacter sp.*	*Pseudomonas sp.* *Pseudomonas parafulva* *Brachybacterium paraconglomeratum* *Psychrobacter celer*	*Bacillus tequilensis*

[RT] Atlantic sharpnose shark

No culturable bacterial strain was recovered from tissue samples of Atlantic sharpnose shark No. 3, 5, 6, 7, 11 and 15. Eight strains were recovered from kidney

samples of four individuals, two strains of *Exiguobacterium* sp. were collected from shark No. 9 and 13. Nine strains were recovered from liver samples of five individuals, three strains of *Pseudomonas* sp. were isolated from shark No.2 and 16. Seven strains were recovered from blood samples of seven individuals, two strains of *Micrococcus* sp. were collected from shark No. 1 and 12; two strains of *Bacillus* sp. were isolated from shark No. 14 and 16.

Table 3

Culturable bacteria isolation from tissue samples of each individual of 15 Atlantic stingrays.

DS	Kidney	Liver	Blood
1	*Vibrio azureus*	*Kistimonas scapharcae*	*Bacillus alkalogaya*
			Pseudoalteromonas piscicida
			Pseudoalteromonas sp.
			Stenotrophomonas sp.
			Oceanobacillus caeni
2	*Vibrio harveyi*	*Micrococcus yunnanensis*	*Rothia amarae/ mucilaginosa*
	Vibro harveyi/ owensii		
	Bacillus velezensis		
	Pseudomonas sp.		
	Pseudomonas stutzeri		
	Pseudomonas stutzeri/ putida		
	Bacillus tequilensis		
	Shewanella corallii		
	Shewanella fidelis		
	Shewanella japonica		
3	/	/	/
4	*Vibrio communis*	*Bacillus megaterium*	/
	Vibrio campbellii		
5	*Bacillus flexus/ licheniformis*	/	/
6	*Photobacterium damsela*	/	/
7	/	/	/
8	*Vibrio harveyi*	*Rothia amarae*	/
		Oceanobacillus caeni	

Table 3 (continued).

9	*Vibrio sp./ panuliri*	/	*Thalassospira profundimaris*
			Acinetobacter radioresistens
10	*Vibrio sinaloensis*	/	*Bacillus infantis*
	Bacillus flexus		
11	*Vibrio parahaemolyticus*	/	/
12	/	/	*Micrococcus luteus*
			Micrococcus sp.
			Micrococcus yunnanensis
13	*Psychrobacter sp.*	/	*Thalassospira tepidiphila*
	Bacillus subtilis		*Micrococcus aloeverae*
14	*Micrococcus terreus*	/	*Thalassospira tepidiphila*
15	*Vibrio owensii*	*Bacillus hwajinpoensis*	*Micrococcus yunnanensis*
			Bacillus safensis

[DS] Atlantic stingray

No culturable bacterial strain was recovered from tissue samples of Atlantic stingray No. 3 and 7. Twenty-five strains were recovered from kidney samples of 12 individuals, 10 strains of *Vibrio* sp. were collected from stingray No. 1, 2, 4, 8, 9, 10, 11 and 15; five strains of *Bacillus* sp. were collected from stingray No. 2, 5, 10 and 13; three strains of *Pseudomonas* sp. and three strains of *Shewanella* sp. were isolated from stingray No. 2. Six strains were recovered from liver samples of five individuals, two strains of *Bacillus* sp. were isolated from stingray No. 4 and 15. Seventeen strains were recovered from blood samples of eight stingray individuals, five strains of *Micrococcus* sp. were collected from stingray No. 12, 13 and 15; three strains of *Bacillus* were collected from stingray No. 1, 10 and 15; three strains of *Thalassospira* sp. were isolated from stingray No. 9, 13 and 14.

For sharks, *Exiguobacterium* sp. appeared more than other species in kidney samples; *Pseudomonas* sp. were more to be seen in liver samples; *Micrococcus* sp. and

Bacillus sp. were most seen species in blood samples. For stingrays, *Vibrio* sp., *Bacillus* sp., *Pseudomonas* sp. *Shewanella* sp. were mostly isolated from kidney samples; *Bacillus* sp. was more likely to be observed in livers; *Micrococcus* sp., *Bacillus* sp. and *Thalassospira* sp. were most seen species in blood samples.

B. 16S rRNA Gene Phylogenetic Analysis

The neighbor-joining tree presented with 1000 replicates. Seventy-one tissue sample isolates fell into three phyla and 21 genera, shows a relatively large richness of microflora in elasmobranch liver, kidney, and blood. All 71 bacteria species fell in three phyla, they are Proteobacteria, Firmicutes, and Actinobacteria.

Proteobacteria is the predominating phylum in both Atlantic stingray and Atlantic sharpnose shark tissue samples, as the data shown (Figure 6), 55.3% of bacteria species isolated from stingrays and 50% of sharks were Proteobacteria. Likely, Proteobacteria is also the most dominating bacterial phylum found in marine sponge *Xestospongia testudinaria* (Su et al., 2013). In accordance, Firmicutes was a less dominating phylum in both stingrays (25.5%) and sharks (33.3%), followed by Actinobacteria, 19.2% in stingrays and 16.7% in sharks.

16S rRNA gene sequence phylogeny shows most of bacteria species were grouped with bacteria from the same genus or to another genus (from the same phylum), DS2 K-8 (Firmicutes) was the only one that had been misgrouped with Gammaproteobacteria, a class of Proteobacteria with a bootstrap value of 34%.

Figure 5. Cladogram phylogenetically showing the distribution of bacterial lineages associated with three types of tissue samples of two types of elasmobranchs.

Bacterial isolates collected from kidney (K), liver (L) and blood (B) of Stingrays (DS) and Sharks (RT). The neighbor-joining method used to generate the phylogenetic tree, numbers at nodes show bootstrap values with 1000 replicates, red triangles indicate values no less than 70% (≥ 0.7), larger triangles indicate higher bootstrap values. Scale bar represents 0.1 substitutions per nucleotide position.

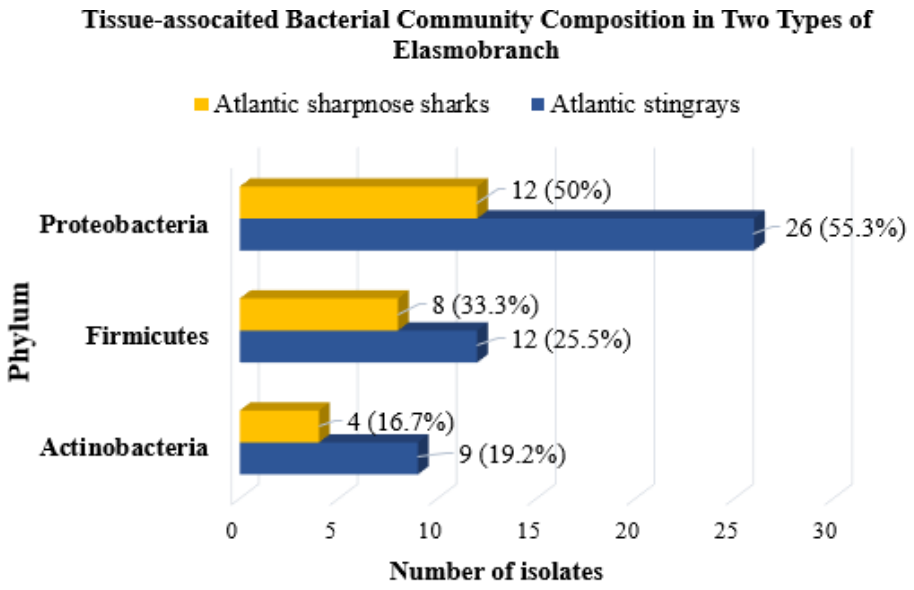

Figure 6. Chart of tissue-associated bacterial community composition of Atlantic stingrays and Atlantic sharpnose sharks

Forty-seven isolates (blue bar) from 15×3 tissue samples of Atlantic stingray, 24 isolates (yellow bar) collected from 16×3 tissue samples of Atlantic sharpnose shark.

C. Urease Assay

71 valid isolates were used to screen for urease-positive individuals. 29 isolates were determined to be ureolytic bacteria by virtue of turning the orange urea broth to dark pink. During the test, negative control *E. coli* ATCC 11775 did not change the color of urea broth (stay yellowish orange); the positive control *Proteus* sp. changed the color to deep pink in a short period of time. Color changes recorded after 48 hours of incubation at 35ºC. Within all the tissue samples (kidney, liver and blood) of Atlantic stingray, 54.2% (13 out of 24) of the kidney isolates, 16.7% (1 out of 6) of the liver isolates and 29.4% (5 out of 17) of the blood isolates were proved to be urease-positive (Table 2, Table 3). As for Atlantic sharpnose shark, 25% (2 out of 8) of kidney isolates, 44.4% (4 out of 9) of the liver isolates and 57.1% (4 out of 7) of blood isolates were ureolytic strains (Table 2, Table 3).

Table 4

Identification of all isolated ureolytic bacterial strains. 16S rRNA gene sequences from bacterial isolates revealed that those are related to the known species closely.

Isolate	Bacteria species	UA mU/mg protein	Isolate	Bacteria species	UA mU/mg protein
DS2 K-1	**Vibrio harveyi*	14.99	DS12 B-1	**Micrococcus luteus*	24.36
DS2 K-5	**Pseudomonas stutzeri*	15.03	DS13 B-2	*Thalassospira tepidiphila*	10.44
DS2 K-6	**Pseudomonas putida/ stutzeri*	14.45	DS14 B-1	*Thalassospira tepidiphila*	10.67
DS2 K-7	*Bacillus tequilensis*	15.09	DS8 L-1	**Rothia amarae*	13.91
DS2 K-8	*Bacillus velezensis*	6.92	RT13 K-2	**Exiguobacterium profundum*	15.14
DS2 K-9	**Vibrio owensii/ harveyi*	13.02	RT16 K-1	*Shinella granuli*	9.32
DS4 K-1	**Vibrio campbellii*	11.96	RT2 L	**Pseudomonas hibiscicola*	20.1
DS5 K	**Bacillus licheniformis/ flexus*	10.41	RT4 L	**Photobacterium damselae* subsp. *damselae*	7.89
DS6 K	**Photobacterium damselae*	9.97	RT10 L-2	**Roseomonas cervicalis*	7.91
DS8 K	**Vibrio harveyi*	11.06	RT16 L-4	*Psychrobacter celer*	6.00
DS10 K-1	*Bacillus flexus*	17.34	RT1 B-1	**Micrococcus luteus*	46.84
DS11 K	**Vibrio parahaemolyticus*	16.73	RT8 B-1	**Stenotrophomonas maltophilia*	16.48
DS13 K-2	*Bacillus subtilis*	13.58	RT13 B	**Staphylococcus saprophyticus/ *xylosus*	66.46
DS2 B	**Rothia mucilaginosa/ amarae*	10.52	RT16 B-2	**Bacillus cereus/ subtilis*	11.64
DS9 B-2	*Thalassospira profundimaris*	3.77			
PC	*Proteus* sp.	5.54	NC	*E. coli* ATCC 11775	3.06

"*" (asterisk) indicates the bacteria species is an opportunistic pathogen, "UA" is short for urease activity; "PC", positive control; "NC", negative control; "K" for kidney; "B" for blood; "L" for liver

D. Urease Activity Assay

Twenty-nine ureolytic bacterial strains were tested for their urease activity. Nineteen urease-positive bacteria species isolated from 15 Atlantic stingrays were classified into seven genera, with genus *Vibrio*, *Bacillus*, *Pseudomonas* and *Photobacterium* were isolated from kidney, genus *Rothia* was isolated from liver, genus *Thalassospira* and *Micrococcus* were isolated from blood.

Bacteria with the highest and lowest urease activity among all 19 isolates were both observed in stingray blood samples. *Micrococcus luteus* has the highest urease activity as 24.36 mU/mg protein (Figure 8), while *Thalassospira profundimaris* has the lowest urease activity as 3.77 mU/mg protein, unlike *T. tepidiphila* (10.44 and 10.67 mU/mg protein, separately) which was also isolated from blood samples of two distinct stingrays, has much higher ability hydrolyzing urea.

In stingray kidney samples, *Vibrio* species showed excellent capacity of urea utilization, urease activity of five isolates from three (or four, with one stays unidentified) species all above 11 mU/mg protein, *V. parahaemolyticus* ranks the top with activity of 16.73 mU/mg protein; V. harveyi shows 3 units difference from two different stingray individuals. Urease activity of *Bacillus* species varies a lot within the genus, *B. flexus* has the second highest ability (17.34 mU/mg protein) utilizing urea, with *B. velezensis* the second lowest. *Pseudomonas* species possess the relatively high ability, Photobacterium damselae relatively low. *Rothia amarae* is the only one urease-positive bacterial isolate obtained from stingray liver, compared with *R. amarae* (also possible to be *R. mucilaginosa*, 10.52 mU/mg protein) isolated from blood, this one (kidney isolate) is of higher urease activity as 13.91 mU/mg protein.

10 ureolytic strains isolated from 16 Atlantic sharpnose sharks were divided into 10 genera; genus *Exiguobacteriu, Shinella* were isolated from kidney, genus *Pseudomonas, Photobacterium, Psychrobacter,* and *Roseomonas* were isolated from liver, genus *Micrococcus*, *Stenotrophomonas*, *Staphylococcus,* and *Bacillus* were isolated from blood.

Isolates with the highest and second highest urease activity were observed in shark blood samples; *Staphylococcus saprophyticus* (could also be *Staphylococcus xylosus*) ranks the first with 66.46 mU/mg protein and followed by *Micrococcus luteus* with 46.84 mU/mg protein. *Bacillus subtilis* (could also be *Bacillus cereus*) and *Stenotrophomonas maltophilia* possess relatively high urease activity.

Bacteria with the lowest activity of urea-hydrolyzing was recorded in shark liver; *Psychrobacter celer*, as 6.00 mU/mg protein, *Photobacterium damselae* (7.89 mU/mg protein) and *Roseomonas cervicalis* (7.91 mU/mg protein) were the second and third lowest species in urease activity. *Pseudomonas hibiscicola* (20.1 mU/mg protein) has high urea utilization ability.

Two isolates from shark kidney samples were *Exiguobacterium profundum* and *Shinella granuli*, with the urease activity of 15.14 and 9.32 mU/mg protein, respectively.

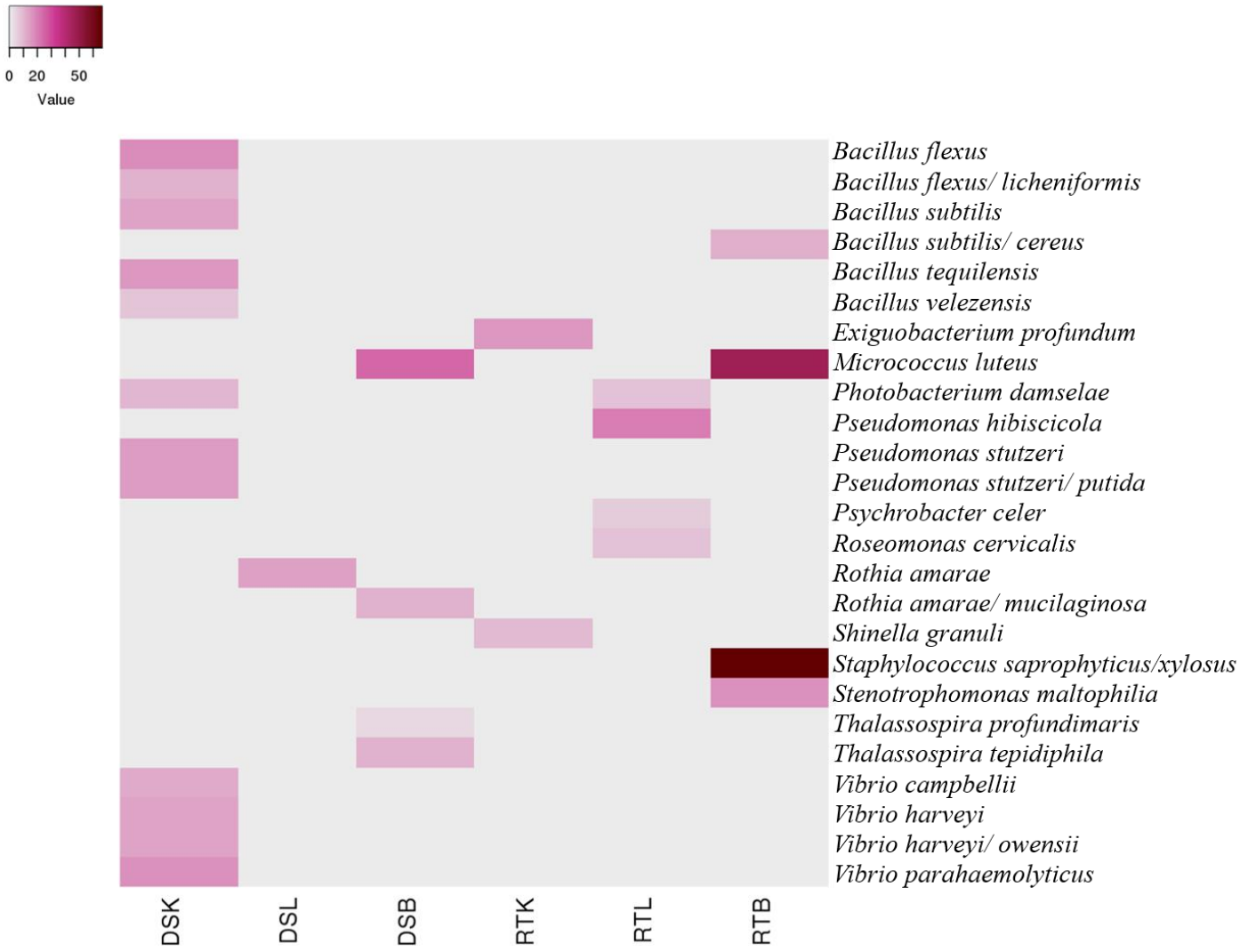

Figure 7. Heatmap that shows species isolation and the urease activity of certain bacteria species.

Species on the right column are all ureolytic species that isolated from tissue samples of 31 elasmobranchs, the bottom row is where they were collected; “RTB”, shark blood sample; “DSK”, stingray kidney sample; “DSB”, stingray blood sample; “RTL”, shark liver sample; “DSL”, stingray liver samples; “RTK”, shark kidney sample. Color range on top indicates the value of urease activity of certain bacteria species in certain elasmobranch tissue sample, value “0” indicates no certain species (to the row) isolated from certain elasmobranch tissue sample (to the column)

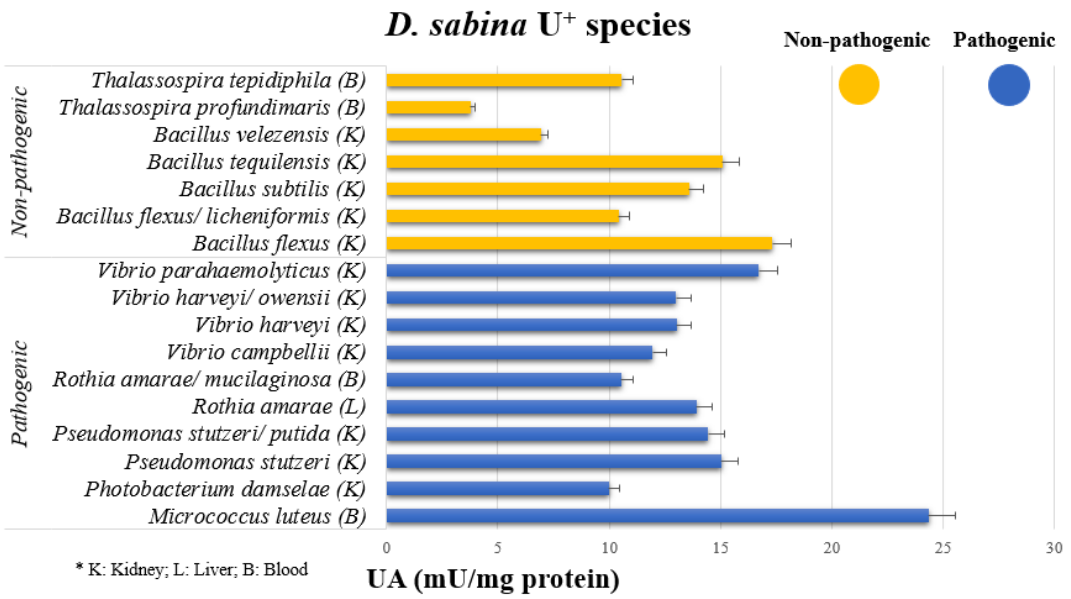

Figure 8. Urease activity of ureolytic bacterial isolates from Atlantic stingray tissue samples.

"K", "L" and "B" represent kidney, liver, and blood, respectively. Here, the "Non-pathogenic" group indicates no harmful influence on the host has yet been detected; "Pathogenic" group indicates the harmful impact on the host has been reported from previous studies.

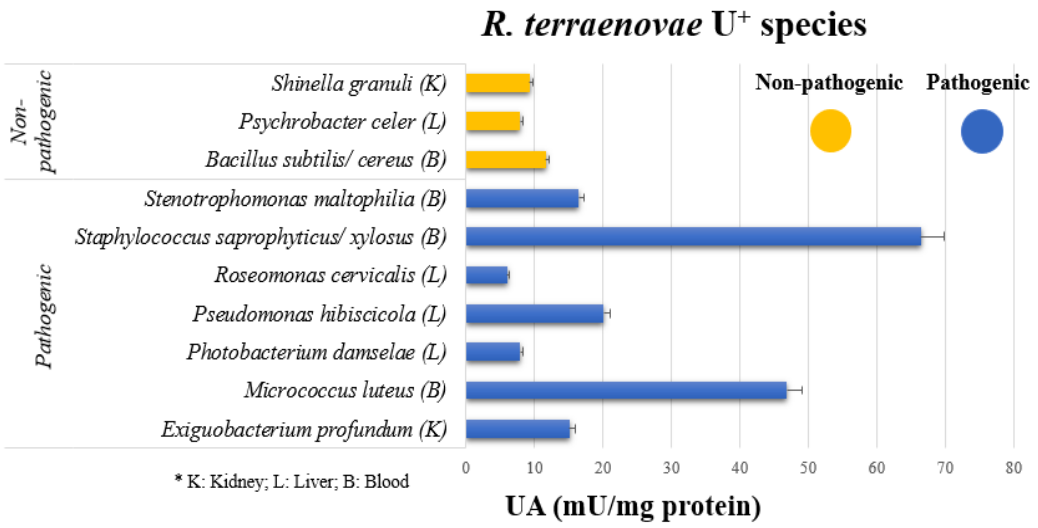

Figure 9. Urease activity of ureolytic bacterial isolates from Atlantic sharpnose shark tissue samples.

"K", "L" and "B" represent kidney, liver, and blood, respectively. Here, the "Non-pathogenic" group indicates no harmful influence on the host has yet been detected; "Pathogenic" group indicates the harmful impact on the host has been reported from previous studies.

In the study, pathogenic group was defined as the opportunistic pathogen (an infectious microorganism that are normally commensal and does not do harm to the host; but cause disease when the resistance of host becomes low), which has been previously reported and well-studied to be able to take advantages of certain opportunities to cause disease. Non-pathogenic was defined as bacteria species has not been well-proven to cause disease so far.

Micrococcus luteus isolated from the blood samples of two types of elasmobranchs showed high but different urease activity; *Staphylococcus saprophyticus* collected from shark blood sample presented the highest urea-utilizing ability among all the urease-positive isolates. *Photobacterium damselae* from pathogenic group isolated from different samples showed low urease activity. Over half of the ureolytic isolates from both stingray and shark were opportunistic pathogens.

E. ureC Gene Detection and Phylogenetic Analysis

Among 29 ureolytic bacterial strains, 27 were amplified using four different types of primer pairs (Table 1) with different amplicon sizes (Figure 10), two remained undetermined. 15 strains (nine genera) were amplified with L2F/L2R, which suggested the ureC-specific primer set is also a good fit to the amplification of marine bacteria and show a broad detection range of urease-positive bacterial species. No ureC gene band showed for *E. coli* ATCC 11775 (negative control).

Aside from 27 ureC genes of our bacterial isolates collected from elasmobranch tissues, 64 more bacterial ureC gene sequences were retrieved from GenBank database

and UniProt. ureC gene as a functional gene encoding urease was translated and aligned with respect to amino acid codons. The 64 species were the same group of species that were constructed and analyzed in 16S rRNA phylogeny (Figure 5). *Blattabacterium* spp. and *Flavobacterium* spp. of phylum Bacteroidetes served as outgroup taxa, the neighbor-joining method was adopted for the generation of the phylogenetic tree.

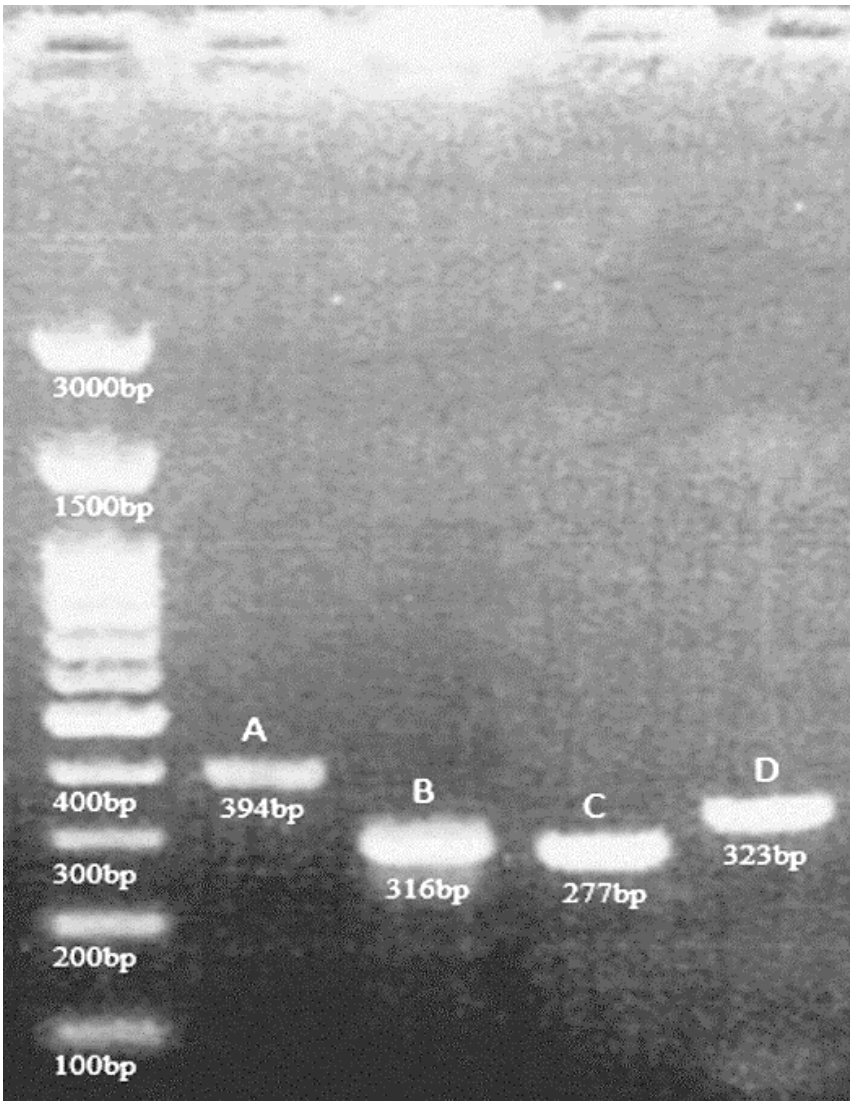

Figure 10. Agarose gel electrophoresis show specific ureC gene amplification with four types of primer pairs (noted as A, B, C, D)

From Left to right: 100 base pair DNA Marker (Bioland Scientific LLC); the next four lanes were ureC gene bands amplified by A (L2F/ L2R); B (UCF/ UCR); C (SF-3/ SR); D (ureC-F/ ureC-R), respectively.

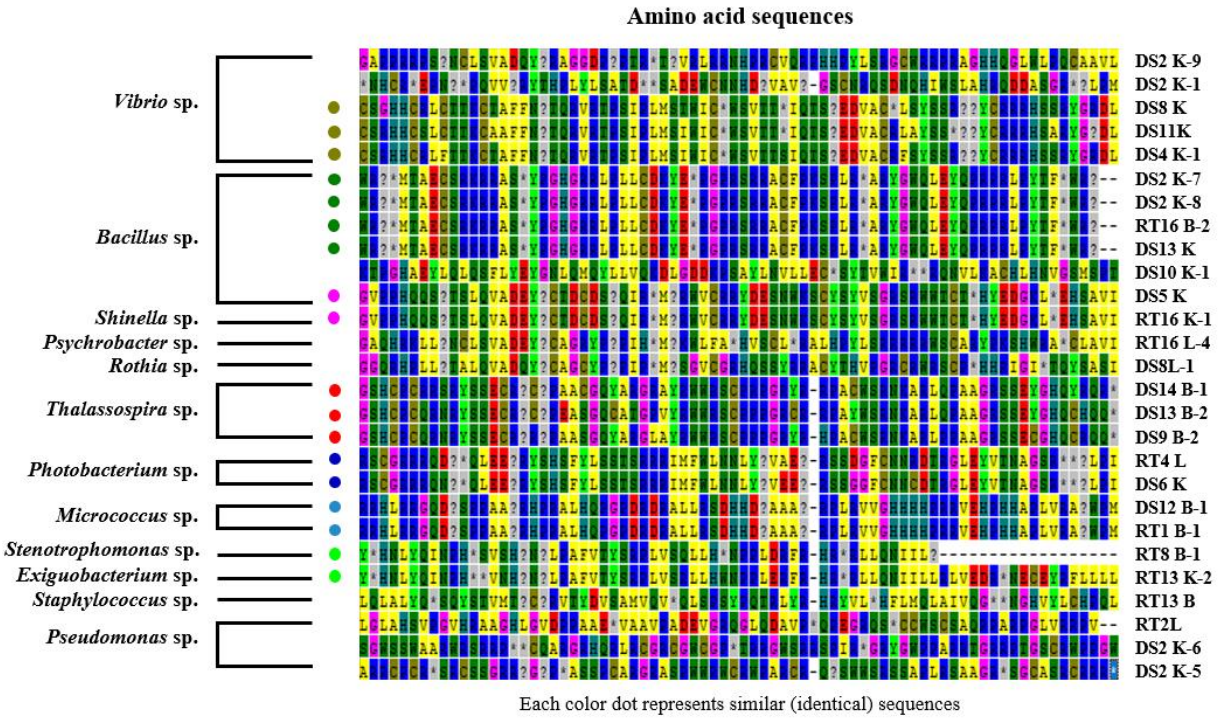

Figure 11. Functional (urease-coding) ureC gene nucleotides (~ 228 base pair) deduced amino acid sequences from 27 ureolytic bacteria isolates from tissue samples of two types of elasmobranchs (presented only bacteria genus)

Sample ID was presented to the right of each sequence.

Among 27 urease-positive bacteria isolates, 14 (51.9%) bacteria isolates showed species-specific ureC gene sequences. 60% of *Vibrio* spp., 66% of *Bacillus* spp., 100% of *Thalassospira* spp., *Photobacterium* spp. and *Micrococcus* spp. were observed with highly similar or identical amino acid sequences within their own genus. Interestingly, bacteria from a different genus, such as DS5 K (*Bacillus flexus/ licheniformis*) and RT16 K-1 (*Shinella granuli*), had shown the identical ureC amino acid sequence; also as RT8 B-1 (*Stenotrophomonas maltophilia*) and RT13 K-2 (*Exiguobacterium profundum*) showed similar sequences.

ureC gene-based phylogenetic tree (Figure 12) indicated that, among 27 bacteria isolates, ureC gene of 22 isolates (81.5%) were grouped with bacteria species according

to their phyla, such as some species from *Bacillus*, *Pseudomonas*, *Streptomyces*, *Arthrobacter*, *Providencia*, *Vibrio*, *Blattabacterium*, and *Flavobacterium*, they were regrouped with species exactly from their own phyla with supportive bootstrap values. Isolates of *Bacillus* sp., DS13 K-2 (*Bacillus* sp.), DS2 K-7 (*Bacillus* sp.), RT16 B-2 (*Bacillus* sp.) from Firmicutes were grouped with species of Firmicutes with high bootstrap values at 94%, so as DS10 K-1 (*Bacillus* sp.) with 100% bootstrap value; isolates DS2 K-5 and DS2 K-6 (*Pseudomonas* sp.); RT16 L-4 (*Psychrobacter* sp.); DS4 K-1 and DS2 K-9 (*Vibrio* sp.), DS13 B-2 (*Thalassospira* sp.); RT4 L (*Photobacterium* sp.) and RT8 B-1 (*Stenotrophomonas* sp.) of phylum Proteobacteria were grouped with species of the same phylum with the support of high bootstrap values; isolates DS12 B-1 and RT1 B-1 (*Micrococcus* sp.) from phylum Actinobacteria fell in the groups with species of Actinobacteria. However, ureC gene phylogeny had 13 out of 27 (48.1%) were re-grouped with species from the same genus, and the rest of the bacterial species seemed not to be divided into relevant groups (across genus), such as DS5 K (*Bacillus* sp.), DS8 L-1 (*Rothia* sp.), RT13 K-2 (*Bacillus* sp.) probably because the unavailable of certain ureC gene sequences from the same genera on the tree that can closely relate to our isolates.

K. Statistical Analysis

We ran three split-plot models, to analyze which factor significantly impact bacteria richness of 31 elasmobranch individuals. We have three factors, they are: elasmobranch superorders (Batoidea: Atlantic stingray; Selachii: Atlantic sharpnose shark), tissue types (kidney, liver, and blood), the interaction of elasmobranch superorders and tissue types, our random effect is elasmobranch individual.

In model 1, we included all three factors and found the interaction between elasmobranch superorder and tissue type is not significant (P= 0.1395), therefore we reduced model 1 to model 2.

In model 2, we involved superorder and tissue type, but not their interaction, and we found the factor tissue type is also not significant (P= 0.2477).

We reduced model 2 to model 3, which only have one factor, elasmobranch superorder. Model 3 cannot be reduced any more. We found that bacteria richness is significantly different (P= 0.0814, P< α) based on elasmobranch superorder difference.

Akaike information criterion (AIC) for the three models are 327.4994 (model 1), 329.1354 (model 2) and 326.8743 (model 3). AIC is the quality estimator of each model, used for model selection, the lower the AIC value, the better the model. Among our three models, model 3 is the best.

Table 5

Split-plot experiment results with three models

		Sum Sq	Mean Sq	NumDF	DenDF	F.value	Pr (>F)
Model 1	Superorder	5.15	5.15	1.00	29.00	3.26	0.0814
	Tissue type	5.02	2.51	2.00	58.00	1.59	0.2129
	Interaction (S &T)	6.44	3.22	2.00	58.00	2.04	0.1395
Model 2	Superorder	5.32	5.32	1.00	29.00	3.26	0.0814
	Tissue type	4.67	2.33	2.00	60.00	1.43	0.2477
Model 3	Superorder	5.40	5.40	1.00	29.00	3.26	0.0814

Sum Sq: Sum of squares

Mean Sq: Mean square

NumDF: Numerator degrees of freedom

DenDF: Denominator degrees of freedom

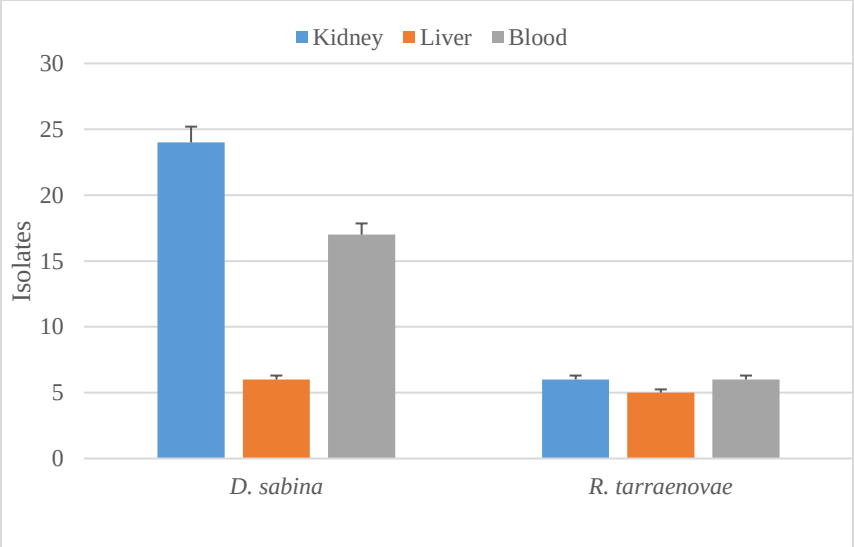

Figure 12. The number of bacteria isolates in each type of elasmobranch tissue samples.

Error bar indicates 5% of the value of the data point.

By using SPSS Statistics hierarchical cluster analysis (Ward's cluster method and squared Euclidean distance measurement), we generated two dendrograms according to tissue-associated bacterial isolations to regroup elasmobranch individuals (31 animals) within their species in groups to make each group has more similar individuals.

Table 6

Details of bacteria isolates from each elasmobranch individual.

Elasmobranch types	Kidney isolate	Liver isolate	Blood isolate
RT1	0	1	1
RT2	0	1	1
RT3	0	0	0
RT4	3	1	0
RT5	0	0	0
RT6	0	0	0
RT7	0	0	0
RT8	0	0	1

RT9	1	0	0
RT10	0	2	0
RT11	0	0	0
RT12	0	0	1
RT13	2	0	1
RT14	0	0	1
RT15	0	0	0
RT16	2	4	1
DS1	1	1	5
DS2	10	1	1
DS3	0	0	0
DS4	2	1	0
DS5	1	0	0
DS6	1	0	0
DS7	0	0	0
DS8	1	2	0
DS9	1	0	2
DS10	2	0	1
DS11	1	0	0
DS12	0	0	3
DS13	2	0	2
DS14	1	0	1
DS15	1	1	2

"RT" represents Atlantic sharpnose shark, "DS" represents Atlantic stingrays

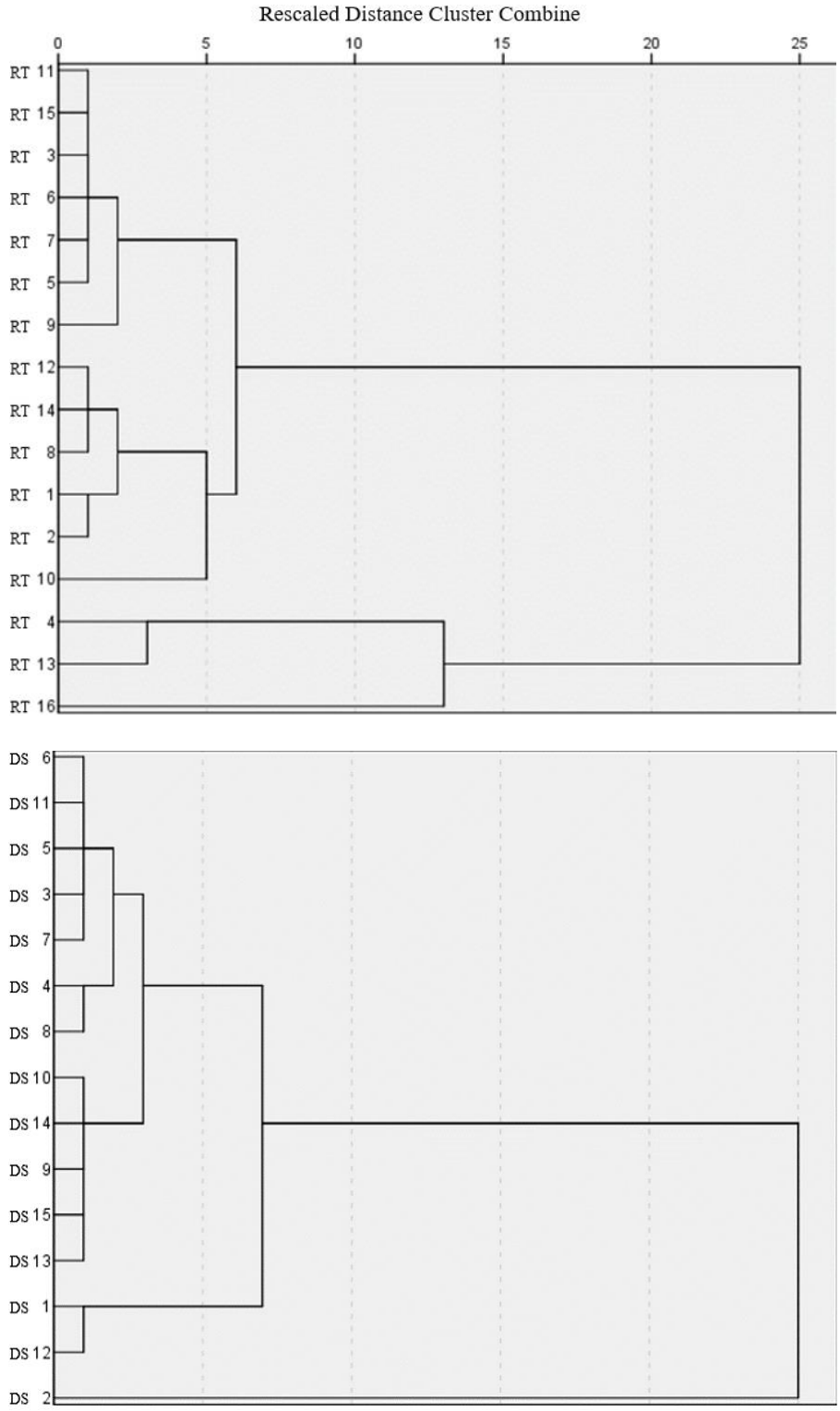

Figure 13. Dendrogram using Ward Linkage to classify shark (top figure) and stingray (bottom figure) individuals according to the tissue-associated bacterial isolations.

Individual IDs were shown on the left; "RT", "DS" represent sharpnose shark and stingray, respectively.

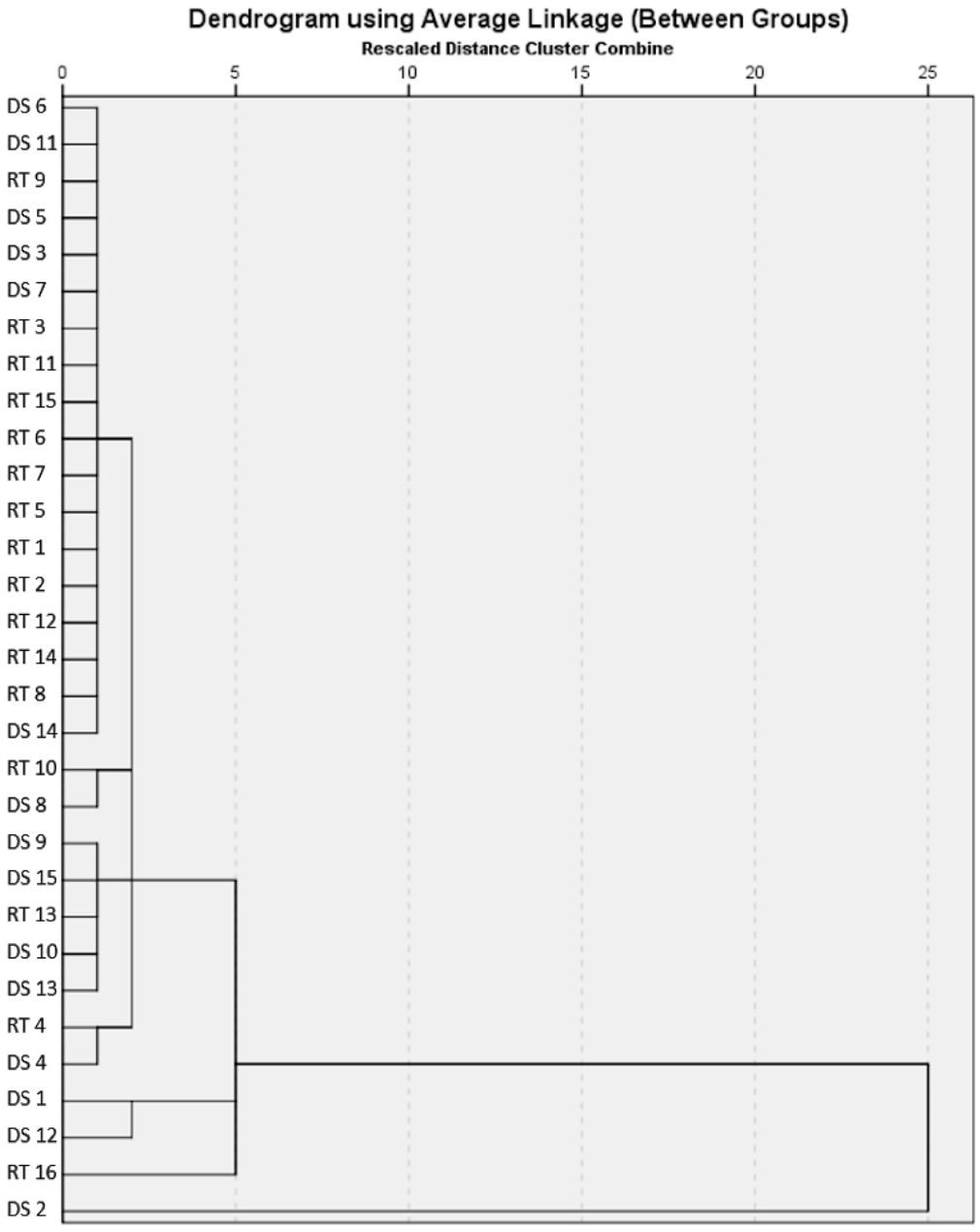

Figure 14. Dendrogram using Ward Linkage to classify 30 elasmobranch individuals (both sharks and stingrays) according to the tissue-associated bacterial isolations.

Individual IDs were shown on the left; “RT”, “DS” represent sharpnose shark and stingray, respectively.

Table 7

Cluster analysis of elasmobranch individuals at the distance of five.

Group	Shark No.
A	16
B	4, 13
C	10
D	1, 2, 8, 12, 14
E	3, 5, 6, 7, 9, 11, 15
Group	Stingray No.
A	2
B	1, 12
C	3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15
Group	Elasmobranch No.
A	DS2
B	RT16
C	DS1 DS12
D	RT: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 DS: 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15

Cluster analysis: Similar individual were divided into groups according to bacteria isolation results (isolated from kidney, liver and blood samples). We see the individual difference in the amounts of bacteria isolates, but 16 sharks were mainly five groups (at the distance of 5). Group A indicated most bacteria isolates from individual and shark No. 16 is the only individual in this group, as we mentioned before, No. 16 was captured the year after the other 15 sharks were collected, and they were from different seasons as well, which indicated the water parameters (such as temperature, salinity) are different, and that may have impact on bacteria growth and the number of bacteria isolated from elasmobranch tissues samples. Sharks in group B and C had more bacteria isolates than group D and E. Fifteen Atlantic stingrays were divided into three groups (at the distance of 5), from group A to C, the number of bacteria isolates decreased gradually. At the

distance of 5, we clustered 16 sharks and 15 stingrays to four groups according to the number of tissue isolates of each individual. With relatively high bacteria richness, DS2 and RT16 are from group A, group B, respectively. Group 3 contains DS1 and DS12, with the rest of 27 elasmobranch individuals belong to group D which indicates lowest bacteria richness.

Figure 15. Unrooted phylogenetic tree based on ureolytic bacterial species.

Bootstrap values (1,000 replicates) no less than 50% are presented. Inside the parenthesis shows he species accession number. Abbreviations indicate the phylum of certain species next to parenthesis are as follows: Fm for Firmicutes; Ac for Actinobacteria; Cy for Cyanobacteria; Bac for Bacteroidetes; AP, BP, GP are further divided under phylum Proteobacteria; AP for Alpha Proteobacteria; BP for Beta Proteobacteria; GP for Gamma Proteobacteria. The scale bar under indicates 5% substitutions per amino acids position.

CHAPTER IV – DISCUSSION

A. Bacterial Richness and Microbial Community Structure

Compared with Atlantic sharpnose shark, Atlantic stingray tissue samples show higher richness in bacteria species (Figure 4 & 5). Two distinct types of habitats and feeding habits preferred by stingray and shark are likely to contribute to the difference. 10,000 to 200,000 viable bacteria were estimated to be in a liter of surface seawater (Lewin et al., 1974). In open sea water, a milliliter of seawater contains 10^6 bacteria cells; in marine surface sediments, the average abundance of bacteria cells is 10^8 to 10^9 per gram (Amaral- Zettler et al., 2010). Bacteria in open sea water tend to adsorb suspending organic or inorganic particles which would finally settled, be deposited on the bottom, and then accumulate in sediment; sediments provide solid surfaces and complex nutrients matrix for the growth and proliferation of microbes, marine sediments are widely known to be high in microbial richness (Carlucci et al., 1959; Wang et al., 2012). Carlucci et al. (1959) pointed out, compared with overlying water, there were a great number of bacteria settled in marine sediments. Similarly, a study nowadays also shows the richness of taxon and biomass of micro-organisms in sediments outcompetes those of corresponding water bodies (Wang et al., 2012), which makes stingray inhabiting in shallow coastal waters over silty and sandy bottoms exposed to bacteria enriched shallow water; also, not like shark feeds on fish and shrimps, stingray preys on benthic invertebrates which have close association with the marine sediment, in that case, bacteria can be ingested into gastrointestinal tract (GI tract), make the way to bloodstream through intestine and then cause the colonization of internal organs later on (Ribet et al., 2015). It is considered that the ultraviolet light from the sun might be an unfavorable effect on bacteria reside in

shallow, but no evidence shows the number of bacteria from surface water sample varies with the amount of sunlight during summer when it is most intensive (Carlucci et al., 1959). Zobell et al. (1935) reported that no evidence was found that bacteria occurrence influenced by sunlight, even bacteria in shallow layers of seawater were observed to die quickly when exposed to intense midsummer sunlight, bacteria 20 cm under the surface or deeper would not be affected lethally. In this study, we only chose two types of elasmobranchs to represent two different kinds of living habitats as in marine sediments and overlying waterbody to explore the bacteria abundance within their habitats; future work needs to involve more species of stingray and shark that inhabit spatially differently to better prove the relation between different habitats and bacteria richness.

Proteobacteria (Gammaproteobacteria), Firmicutes and Actinobacteria were three phyla we observed, and they present in the tissue samples of both Atlantic stingray and Atlantic sharpnose shark. Proteobacteria is the phylum that has been constantly acquired in marine-related samples, da Silva et al. (2013) cultured the sediment samples retrieved from South Atlantic Ocean with the depth ranges from 1905 to 5560m, likely, they isolated and classified the strains into phylum Gammaproteobacteria, Firmicutes, and Actinobacteria. Proteobacteria and Bacteroidetes are two bacterial phyla that predominate in seawater, their abundances were observed varying seasonally; the high levels of light and primary production (chemical energy produced by plants in ecosystem) and the decent concentration of nutrients facilitate the growth of Proteobacteria, due to the combination factors, Proteobacteria peaks in summer and fall; in contrary, Bacteroidetes reaches its maximum in winter, and minimum in summer (Suh et al., 2015). Based on the former studies, this dynamic microbial community shift, not only regionally, but globally

(Giovannoni et al., 2012; Suh et al., 2015). The test animals we captured for this study is in August and September, that makes good sense that Proteobacteria is the dominant phylum in both stingray and shark, also, that explains the reason why no bacteria species from phylum Bacteroidetes was isolated in this research. Firmicutes are very abundant in marine sediment (Hamdan et al., 2013); among our isolates from phylum Firmicutes, more than half were collected from tissue samples of stingrays which inhabit over sediment. Actinobacteria are ubiquitous in the ocean and tend to present during spring and fall, they have been isolated from lots of marine creatures (Valliappan et al., 2013; Suh et al., 2015). Interestingly, Actinobacteria have been regarded as a potential source for marine drugs (bioactive compounds) and have the potential to produce natural pharmacy products (Manivasagan et al., 2013; Valliappan et al., 2013).

The study showed preliminary observation of culturable bacteria from elasmobranch tissue samples. There is actually no obvious consistency of bacteria species observed in the same type of tissue sample among different individuals (vertically comparison) or in the same individual across different tissue types (horizontally comparison). *Exiguobacterium* spp., *Shinella granuli*, and *Sporosarcina contaminan* were only isolated from kidney samples of shark No. 9, 16 and 4, these three bacteria genera are not well-studied. *Serratia marcescens, Roseomonas cervicalis*, and *Brachybacterium paraconglomeratum* were only collected from the liver samples of shark No. 1 and 10. *Serratia marcescens* is considered to be a human pathogen which responsible for wound and urinary tract infection (UTI), and present abundantly in the environment; similarly, *Roseomonas cervicalis* is also pathogenic for humans to cause eye, urogenital infections (Rihs et al., 1993). *Staphylococcus saprophyticu*s (could also be *S. xylosus*) was only

collected from the liver sample of shark No. 13; Gram-positive *Staphylococcus* from phylum Firmicutes, shape in grape-like clusters under a microscope. Over 40 species included in this genus (Harris et al., 2002). Many of them are not harmful and usually be found on the skin, mucous membranes of humans and as well as other organisms (Madigan et al., 2005). Based on the observation of female patients, it is believed that acute UTI is mostly caused by *S. saprophyticu*s (Wallmark et al., 1978). Shark No.16 was capture in late spring of 2016, the rest of sharks were captured in early autumn of 2014, more culturable bacteria species were recovered from shark No.16 in kidney and liver samples, which may indicate the marine bacterial community shift in a different season or in a different year. Seasonal succession in microbial community composition is robust and is largely driven by temperature and nutrient concentration (Gilbert et al., 2012). Gilbert (2012) found that the seasonal variations of bacteria community are significant, but there are strong repeating patterns in each year.

Vibrio species were isolated from kidney samples of eight stingrays (No. 1, 2, 4, 8, 9, 10, 11 and 15). In common with a previous research of Grimes et al. (1985), *Vibrio* spp. are the most frequently encountered species in marine-associated samples, *Vibrio* spp. predominated kidney samples of Atlantic stingrays and most of them are considered to be opportunistic pathogens (organism that is able to cause disease when the resistance of the host decreased). When faced with exogenous or endogenous stressors, fish generally compromise to those pathogens (DeGuzman and shots 1988). The phenomenon has also been noticed in elasmobranchs, stress or concurrent disease can turn opportunistic flora to pathogenic ones (Grimes et al., 1984; Bertone et al., 1996; Pedersen et al., 1997; Mylniczenko et al., 2007). It is also well established that *Vibrio* spp. are

indigenous (autochthonous) flora in neritic sharks (Grimes et al., 1985). Among the *Vibrio* isolates we collected, *V. harveyi* as an opportunistic pathogen which may cause shrimp infection, especially when the animal density and nutrients concentrations reach high, together with closely related *V. parahaemolyticus* and *V. campbellii*, they are notable pathogens in finfish and mollusk intensive rearing (Rungrassamee et al., 2014). *Bacillus* species were collected from kidney samples of four stingrays (No. 2, 5, 10 and 13), *B. subtilis* and *B. licheniformis* we isolated were known to be common inhabitants of marine environment; *Pseudomonas* sp. (No.2), *Shewanella* sp. (No.2), *Photobacterium* sp. (No. 6), *Psychrobacter* sp. (No. 13) and *Micrococcus* sp. (No. 14) were also isolated from kidney samples, but only within one stingray individual. The presence of *Shewanella* spp. were observed in stingray kidney, some members from this genus were reported to be commonly isolated in aquatic environment, as well as marine sediments (Horikoshi et al., 2010); *Shewanella* strains probably serve a role of protecting in marine environment, because they have been found to have weak antifungal and antimicrobial activity (Shnit-Orland et al., 2010). *Photobacterium damsela*, previously known as *Vibrio damsela* or *Listonella damsela* (stingray kidney and shark liver isolates) was reported to cause severe acute renal failure (Asato et al., 2004). *Photobacterium damselae* subsp. *damselae* (shark liver isolate) contains fish-virulent strains, was firstly isolated from diseased fish and clinical samples; the strains can cause septicemia in brown shark (*Carcharhinus plumbeus*), eels (*Anguilla anguilla*) and damselfish (*Chromis punctipinnis*), skin lesions and extensive haemorrhages are the main external symptoms of the infection with *Photobacterium damselae* subsp. *damselae* (Fouz et al., 2000). *Micrococcus* spp. predominated in blood samples of stingrays, and it has been known that

Micrococcus can be an opportunistic pathogen, especially in hosts with broken and compromised immune system (Smith et al., 1999). *M. luteus* is Gram-positive, ureolytic bacteria which belongs to *Micrococcaceae*. *M. luteus* is the normal flora on mammalian skin, and also the common species isolated in the environment. According to Gillespie et al. (1975), among all the other bacterial populations, Micrococci predominate in the marine fish located on the South Australian coasts. *Pseudomonads* sp. and *Micrococcus* sp. were reported by Evelyn et al. (1961) that they frequently encountered in both fresh-water and marine fish and *Pseudomonads* species can be opportunistic pathogens as well. *P. stutzeri* (stingray kidney isolate) is widely distributed in nature, even it caused rare infections, it still an opportunistic pathogen (Sader et al., 2005). *P. putida* (stingray kidney isolate) was proved to be able to produce a very powerful antimicrobial product, which is effectively work against bacteria that possess multi-drug resistance (Marinho et al., 2009). Except *Bacillus* and *Micrococcus*, other bacteria species were not observed in liver and blood samples of stingrays. Positive liver cultures were found in five stingrays, Bacillus species were collected from the liver samples of two stingrays (No. 4 and 15); *Kistimonas scapharcae* and *Rothia amarae* were only recovered from stingray (individual No. 1, 8, respectively), *Kistimonas scapharcae* was firstly collected from dead ark clam acquired on the south coast of Korea (Lee et al., 2012). Most bivalves bury themselves in sediment to protect their lives from predators, stingrays inhabit over sediment and prey on bivalves, that may explain why *Kistimonas scapharcae* was only observed in stingray individual. *Rothia amarae* was a novel species that firstly acquired from sludge samples from a foul water sewer (Fan et al., 2002). Each of *Bacillus*, *Micrococcus* and *Thalassospira* was isolated from three stingray individuals of their blood samples,

Thalassospira species have the potent against harmful algal bloom (algicidal) by producing active substance, and are mostly present in summer (Suh et al., 2015; Lu et al., 2016). *Thalassospira profundimaris* was previously collected from West Pacific Ocean deep-sea sediment (Lai et al., 2012); Thalassospira tepidiphila was firstly isolated from seawater, is a polycyclic aromatic hydrocarbon-degrading bacteria species (Kodama et al., 2008). *Pseudoalteromonas* species are widely distributed in nature and are abundant during spring and summer in marine environment (Suh et al., 2015; Richards et al., 2017). In this study, two *Pseudoalteromonas* strains were isolated only from blood sample of one stingray individual, including *P. piscicida*. It is reported that *P. piscicida* may possess antimicrobial potential by being capable of secreting cell-associated proteolytic enzymes; most surprisingly, *P. piscicida* was observed to be able to kill *Vibrio* species and other bacterial pathogens with two mechanisms: secrete antimicrobial product and direct transfer lytic (digestive) vesicles to bacterial pathogens surface to surface to create holes in cell walls to destroy the cell (Richards et al., 2017). *Stenotrophomonas* was isolated from the blood samples of shark No. 8 and stingray No. 1. *Stenotrophomonas maltophilia* is a nosocomial pathogen in patient who has compromised immune system, the isolation from blood (or other normally sterile sites) may indicate infection (Cho et al., 2015).

Among our isolates in two types of elasmobranchs, some of them are previously reported as opportunistic pathogens, and some are serving a protective role, the function of the rest species remains underexplored. It is possible that the bacteria species which are able to produce antimicrobial substance are autochthonous flora that resides in tissues of elasmobranchs to combat against the pathogenic factors by producing a bioactive

antimicrobial product, to keep the internal balance of the animals. To prove this hypothesis, the level of presence of bacteria of interest in their characteristic localization should be kept on track throughout the whole lifespan of healthy animals, the amount of secretion of antimicrobial substance need to be examined *in vivo*, as well as the microbial activities.

B. Analysis of Positive Blood Culture

The observation of positive kidney and liver cultures is very common, kidney and liver are also not the first time to be known as tissues to inhabited by some of the ureolytic bacteria. The elasmobranch kidney functions to store urea (Randall et al., 2002), kidney, liver, muscle, and other tissues have an autochthonous flora; these tissues and organs contain bacteria ranging from 10^2 to 10^5 per gram, wet weight (Grimes et al., 1988).

Blood of marine elasmobranch has a high content of urea. Without usual urinary tract, sharks concentrate and enrich urea in their blood (Vannuccini et al., 1999), urea is also kept in other tissues as part of the osmoregulatory strategy (Musick et al 2002). Blood cultures are used to diagnose and confirm septicemia and bacteremia in animals clinically ill, a positive blood culture may indicate physical disease in normal animals (Nostrandtet al., 1990; Mylniczenko et al., 2007), elasmobranchs captured for this study were visibly healthy without obvious lesion. According to Grimes et al. (1985), based on examination of lemon and tiger sharks, the blood of sharks is typically sterile. Healthy sharks are usually pre-colonized by urease-positive bacteria which are shown to be active in liver but not present in the blood (Grimes et al., 1985). However, positive blood

cultures were observed from both Atlantic stingray and Atlantic sharpnose shark in this study.

Sharks generally react to acute stress exaggeratedly and dramatically, such as handling and capture stress (Hoffmayer et al., 2001; Manire et al., 2001). Hoffmayer et al. (2001) carried a research on 24 Atlantic sharpnose sharks to study their physiological response to the capture and handling stress, the study examined the parameters of blood samples with 15-minute intervals from 0 to 60 minutes; they found out that the blood glucose, lactate, and plasma osmolality were all increase after capture, from 9.2 to 13.1 $mmol^{-1}$, 1.5 to 28.9 $mmol^{-1}$ and 871 to 929 mOsm kg^{-1}, respectively; while the blood pH declined from 6.86 to 6.78. In that case, swift systemic invasion may happen due to capture stress related compromisation of the immune system (Grimes et al., 1985), because some bacteria can cross mucosal barriers, alter the permeability of endothelial and finally access the bloodstream (Ribet et al., 2015). In blood cultures, contamination is considered to be the reason for false positives (Hall et al., 2006). Given those, one factor that caused the presence of bacteria in the elasmobranch blood sample could be the acute stress of capture and handling, which might have an effect on the test animals, cause the bacterial invasion and the entry of bacteria into the bloodstream and show the false positive result.

It is suggested that over 40% of all positive blood cultures are more likely contaminants; coagulase-negative staphylococci (CNS), *Micrococcus* spp. and *Bacillus* spp. are normally regarded as potential contaminants when isolated from blood cultures (Richter et al., 2002). The contamination of blood samples could be the penetration of the needle through elasmobranch muscle which is known to have normal flora (Grimes et al.,

1985; Knight et al., 1988), the needle penetrating introduced bacteria from muscle into the bloodstream that caused false positive result eventually. To verify the blood sample is actually contaminated by the needle penetration through muscle, the future study needs to be conducted with the needle passing through muscle without penetrating bloodstream, and culture the needle tip, then compare bacteria culture result with blood sample result (needle penetrating through the bloodstream). The two types collection should be carried out in the sample test animal at the same time, and repeated in different individuals; if same species of bacteria present in both muscle and blood collections, bacteria might be introduced to blood samples though needle penetration to cause false positive blood culture; if bacteria species cultured from blood are different from muscle collection, then bacteria cultured from blood are less likely to be introduced from muscle collection.

Another possible reason contributed to positive blood culture could be some of the animals were visibly healthy, but physically not. Even health status of each captured animals was examined based on appearance (activity level & parasite loads) and appetite, and only visibly healthy individuals were kept for the research; but it is still not sufficient to regard them as physically healthy, sick animals may still show the same living patterns as healthy ones under certain condition. Hematologic and serum analysis need to be adopted in the future work to precisely analyze animal health status, cerebrospinal fluid bacterial culture can also serve as a good tool to diagnose the neurological disease of elasmobranch (Terrell, 2004).

It is less likely that bacteria in the bloodstream came from the skin via the needle, which normally considered as a likely source of the positive blood cultures. The previous study evaluated the skin source contamination scenario by taking the skin cultures before

and after disinfection (a firm swipe with an alcohol-soaked gauze) with culturette swabs where the blood collection (venipuncture site) was intended. The study concluded that elasmobranchs are sensitive to commonly used disinfectants and, as well as the vigorous skin swiping; as a result, 100% negative skin culture rate was shown based on the simple disinfection (Mylniczenko et al. 2007). In this study, we used isopropyl alcohol to disinfect the skin area of intended venipuncture site prior to the blood-drawing to reduce the risk of infection from external contamination to a large extent, in that case, the bacteria isolated from blood culture were unlikely introduced from elasmobranch skin.

With the observation of positive blood culture from healthy captive and free-ranging elasmobranchs in the study, Mylniczenko (2007) suggested that it is possible that some certain benign resident microbes colonize in the bloodstream, the evidence needs to be further studied. However, without supporting diagnostics, it is insufficient to conclude bacteremia and septicemia in elasmobranchs with positive blood cultures.

C. Bacterial Urease Activity Analyzation

Among the bacteria isolated from sharks, many were capable of hydrolyzing urea; and some of them utilized the products of urea hydrolysis, CO_2, and NH_3, as carbon and nitrogen sources (Grimes et al., 1984). Konieczna et al. (2012) reported urease-positive is more likely to be observed in pathogenic bacteria, such as pathogenic *Staphylococcus* strains. Among our isolates, Atlantic sharpnose shark blood isolate *Staphylococcus saprophyticus* (could also be *xylosus*) possesses the highest urease activity. *Staphylococcus saprophyticus* produces urease and has also been proved to cause bacteremia which can happen in elasmobranchs (Gatermann et al., 1989; Mylniczenko et

al., 2007). A study of Gatermann et al. (1989) found that the urease of *S. saprophyticus* is the virulence factor of the organism.

Micrococcus luteus (stingray blood and shark blood isolates), has the high urea-utilizing ability, however, the ability varies between stingray and shark blood culture (approximately 22 units of difference). It could be the blood urea concentration in Atlantic sharpnose shark is higher than Atlantic stingray, the phenomenon of bacterial acclimatization (micro-organism adapts to certain change in the environment, and it maintains the performance across other environmental conditions) emerges (El-Bestawy et al., 2013). Same bacteria species reside in tissue samples from different host provided with distinct urea concentrations with a period of time, bacteria may acclimatize to certain condition and maintain the performance and living pattern even given with the same concentration of urea solution, they tend to show differentiation. To test the theory, the blood urea nitrogen (BUN) of the two types of elasmobranchs need to be monitored, colonies of the same purified bacteria species need to be added in to serially diluted urea solutions, cultured for few generations (period is unknown, need further test), and then test their urease activity to see if any difference appear. This study revealed the possible relation between pathogeny and urease activity, further research needed to provide corroborating examination.

D. Phylogeny analysis and comparison between 16S rRNA gene and ureC gene

One misgrouping of bacteria species happened in 16S rRNA gene cladogram, DS2 K-8 (*Bacillus velezensis*) which belongs to phylum Firmicutes was grouped mistakenly with Gammaproteobacteria (a class of phylum Proteobacteria), with bootstrap (1000 replicates) value of 34%. This is the only one species that was misgrouped among

73 (1.36%), it was likely due to the uneven coverage of bacteria species. A study by Fan et al. (2017) showed that *B. velezensis* is closely related to *B. amyloliquefaciens* ssp. *plantarum* and *B. methylotrophicus*, without a full coverage of related species, that caused the misgroup of *B. velezensis.*

Two urease-positive bacteria isolates, DS2 B (*Rothia amarae/ mucilaginosa*) and RT10 L-2 (Roseomonas cervicalis) showed decent urease activity (Table 3) but failed to yield ureC gene with all four types of primer sets. It could be the detection range of the primer sets we used did not fairly cover those two isolates or the urease-encoding gene of the two isolates are not ureC gene (ureC gene does not exist). ureC gene is the largest urease-encoding gene, but not the only gene; ureA, ureB, ureD gene were also proved to be able to harvest urease-positive phenotype when they were introduced to previously urease-negative *Campylobacter jejuni* (Cussac et al., 1992). Similarly, other urease-encoding genes may contribute to urease production, which it seemed to be the reason why *B. tequilensis*, *B. velezensis*, *B. subtilis* had same ureC amino acid sequence (Figure 11), but different urease activity (Table 3); another possible reason could be urease of bacteria species evolved independently, not genus- or phylum-relatedly.

Sixty-four ureolytic bacteria species retrieved from Genbank and Uniprot were selected and presented on 16S rRNA gene cladogram and ureC gene phylogenetic tree for better comparison. From the ureC gene phylogenetic tree (Figure 13), we see some bacteria genera, *Vibrio* sp., *Streptomyces* sp., *Staphylococcus* sp., *Synechococcus* sp. for instance, are divided into separate clades, grouped with genus- or even phylum-unrelated bacteria, that could possibly be unavailable of certain ureC gene sequences from the same genera on the tree that can closely relate to our isolates or suggest the ureases produced

have evolved independently (Gresham et al., 2007; Su et al., 2013). We observed that, isolates DS13 K-2, DS2 K-7, and RT16 B-2 were grouped into one cluster supported by bootstrap value of 94%, the urease activity of these three isolates are 13.58, 15.09 and 11.64 mU/mg protein respectively; meanwhile, we found DS8 K and DS11K were in one clade, with urease activity as 11.06 and 16.73 mU/mg protein; RT1 B-1 (46.84 mU/mg protein) and RT13 B (66.46 mU/mg protein), are top two species in urea-hydrolyzing, they were also classified in one cluster. We found that the ureolytic bacterial ureC gene phylogeny presented above doesn't quite identify with their 16S rRNA gene phylogeny. As Gresham et al. (2007) and Klein et al. (2001) found out in their study, ureC genes are generally not showing a strict congruence to the 16S rRNA-based phylogeny. This phenomenon could possibly be induced by horizontal gene transfer (HGT) of ureC gene among ureolytic bacteria, instead of the transmission of genetic material from one generation to the next; it is more of a transmission of genes between unicellular or multicellular (Keeling et al., 2008), which is also an important and necessary factor for many organisms to evolve (Gyles et al., 2014). The HGT can be examined by the study of ureC gene GC content and insertion-deletion sequences, a study found that bacteria species observed that were divided into separate clades were mainly from divisions Actinobacillus and Firmicutes (Gresham et al., 2007). Similarly, Su et al. (2013) reported that the investigation of 16S rRNA gene only gives a full picture of the community structure of the elasmobranch tissues-related bacterial species, however, it may not serve good function to investigate urease-positive bacterial species; ureC gene is able to better estimate the urea utilization potential of those ureolytic bacteria. With this, we conclude that instead of being a phylogenetic marker, the ureC gene has the potential as a function

indicator to furtherly group species according to their certain function, the phylogeny provides useful information towards urease-positive populations and demonstrates a variety of functional gene. Although the ureC gene sequences are not usually as strong as 16S rRNA gene sequences analysis, but as a potential functional marker, combined with the phylogenetic maker (16S rRNA gene), urease positive bacteria can be analyzed and studied in a more accurate way.

E. Statistical Analysis

Cluster analysis: We acquired four groups among 31 elasmobranch individuals at the distance (rescaled distance cluster combine) of five (see Figure 14). According to the data (Table 6), bacteria richness of individual is highest in group A, then group B and C. Group A and group C only have stingray individuals, group B has RT16 (RT16 was captured in the different season compared to the rest of 30 elasmobranch individuals). Group D has shark and stingray individuals. From the cluster, we see elasmobranchs with high bacteria richness are commonly seen in Atlantic stingrays, which may suggest the elasmobranch superorder plays a role in the richness of bacteria, and we adopted split-plot experiment to test this.

Split-plot ANOVA: there was no significant difference in bacteria richness on tissue types (kidney, liver and blood samples), but between two elasmobranch superorders (Batoidea and Selachii), which suggests the difference of bacteria richness exist in the two types of elasmobranchs in this study. The difference could due to their habitats and feeding habits, as we discussed earlier, stingray inhabits over silty sediment; compared with overlying water, sea sediments contain larger amounts of bacteria, the biomass-rich habitat enables bacteria access to stingray in a large extent. Stingray preys

on benthic invertebrates (bivalves, crustacean), bacteria carried by daily food can be ingested into GI tract, then make way to deeper organs through the bloodstream (Ribet et al.,2015).

Conclusion

This study explored the microbiome community structure in each tissue sample of two types of elasmobranchs, Atlantic stingray (*Dasyatis sabina*) and Atlantic sharpnose shark (*Rhizoprionodon terraenovae*). By conducting split-plot ANOVA, we found the bacteria richness is significant different between elasmobranch superorders (P=0.0814), the difference may largely due to their preferred habitats and feeding habits. ureC genes (urease subunit alpha) of 27 ureolytic bacteria isolates were detected, amplified and compared with respect to amino acid codons. We also broadened the detection range of primer set L2F and L2R from groundwater to marine elasmobranch tissue-associated microbiomes. Bacterial 16S rRNA genes, as well as ureC genes phylogenetic richness of ureolytic bacterial strains, were analyzed and compared and we found ureC gene as a potential functional indicator (marker). This study confirmed the fundamental idea of the capacity of urea hydrolysis in some marine microorganisms living under the condition of high urea concentration. The study researched enzymatic (urease) activity and ureC gene-based phylogeny provides a better understanding of ureolytic bacteria for their urea-utilizing potential, enables the further study of highly-effective urease encoding ureC gene on bioengineering and bioremediating of marine urea eutrophication in a larger scale; and meanwhile we provided the insight that bacterial pathogeny may relate to their urea hydrolyzing activity.

APPENDIX A – ATLANTIC STINGRAY CAPTURE DATA

Table A1.

Water parameters of Atlantic stingrays (Dasyatis sabina) capture cites.

DS[a] No.	GPS Coordinates	DO[b] (mg/L)	Salinity (ppt)	Temp[c] (ºC)
1-11	N30.14545	8.01	30	29.1
12-15	W088.46410	8.13	29.6	24.9

[a] DS= *Dasyatis sabina*

[b] DO= Dissolved Oxygen

[c] Temp= Temperature

Table A2.

Detailed characteristics of 15 captured Atlantic stingrays (Dasyatis sabina).

DS[a] No.	Sex	Mass (kg)	Disc Width (cm)
1	Male	1.15	29.5
2	Female	1.1	28.5
3	Male	0.85	26.5
4	Female	0.7	25.5
5	Female	1.675	34.5
6	Male	0.75	26
7	Male	0.775	26
8	Male	0.85	27
9	Female	1.45	33.5
10	Female	0.525	24
11	Male	1.025	29
12	Female	1.15	30.5
13	Female	0.95	28.5
14	Male	0.9	27.5
15	Female	0.8	27

[a] DS= *Dasyatis sabina*

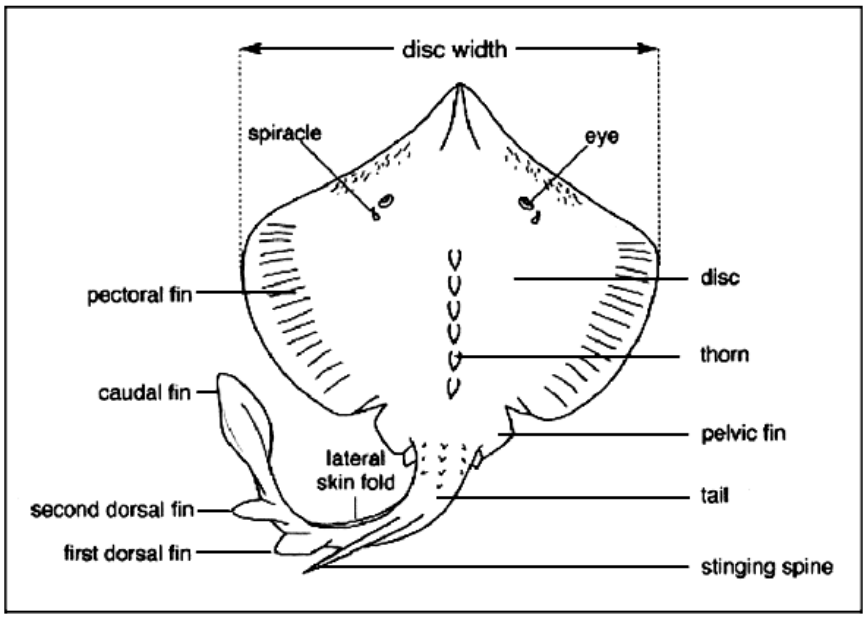

Figure A1. The terminology of the ray.

Adapted from: *Taxonomy and field techniques for identification and available regional guides* (p. 15), by J. D. Stevens, 2005, Rome: FAO FISHERIES TECHNICAL PAPER. Copyright 2005 by FAO FISHERIES TECHNICAL PAPER.

APPENDIX B - ATLANTIC SHARPNOSE SHARK CAPTURE DATA

Table B1.

Water parameters of Atlantic sharpnose shark (Rhizoprionodon terraenovae) capture sites.

RT[a] NO.	GPS Coordinates	DO[b] (mg/L)	Salinity (ppt)	Temp[c] (°C)
1	N30.27376 W088.60532	S:6.40 B:5.64	S:30 B:31.8	S:31.3 B:31.2
2-11	N30.24702 W088.77499	S:5.19 B:4.72	S:31.3 B:29.7	S:30.2 B:29.9
12-14	N30.24708 W088.77494	S:5.19 B:4.72	S:31.3 B:29.7	S:30.2 B:29.9
15	N30.24712 W088.77489	S:5.19 B:4.72	S:31.3 B:29.7	S:30.2 B:29.9
16	N30.24009 W088.51636	S:7.30 B:7.48	S:24.57 B:25.99	S:26.2 B:26.0

[a] RT= *Rhizoprionodon terraenovae*

[b] DO= Dissolved Oxygen

[c] Temp= Temperature

S= Surface

B= Bottom

Table B2.

Detailed characteristic of 16 Atlantic sharpnose shark (Rhizoprionodon terraenovae) capture cites.

RT[a] No.	Sex	Maturity	Mass (kg)	PCL[b] (cm)	FL[c] (cm)	STL[d] (cm)
1	Male	Adult	2.55	65.5	71.2	86.6
2	Male	Adult	2.7	67.0	72.8	88.3
3	Male	Adult	2.2	61.6	67.3	82.3
4	Male	Adult	2.4	63.8	69.8	83.8
5	Male	Adult	2.1	62.9	68.3	83.6
6	Male	Adult	2	61.6	67.0	82.4
7	Male	Adult	2.2	64.4	70.1	85.7
8	Male	Adult	3	70.3	76.1	93.5
9	Male	Adult	2.9	67.9	74.1	NR
10	Male	Adult	2.4	64.0	69.6	84.9
11	Male	Adult	2.7	64.9	70.8	86.1
12	Male	Adult	2	59.8	65.7	80.6
13	Male	Transitional	2.9	68.9	75.0	91.8
14	Male	Adult	2.6	65.3	71.4	86.3
15	Male	Adult	3	69.7	76.1	92.2
16	Male	Adult	2.46	70.0	74.5	88

[a] RT= *Rhizoprionodon terraenovae*

[b] PCL= Pre-caudal Length

[c] FL= Fork Length

[d] STL= Stretch Total Length

NR= No Record

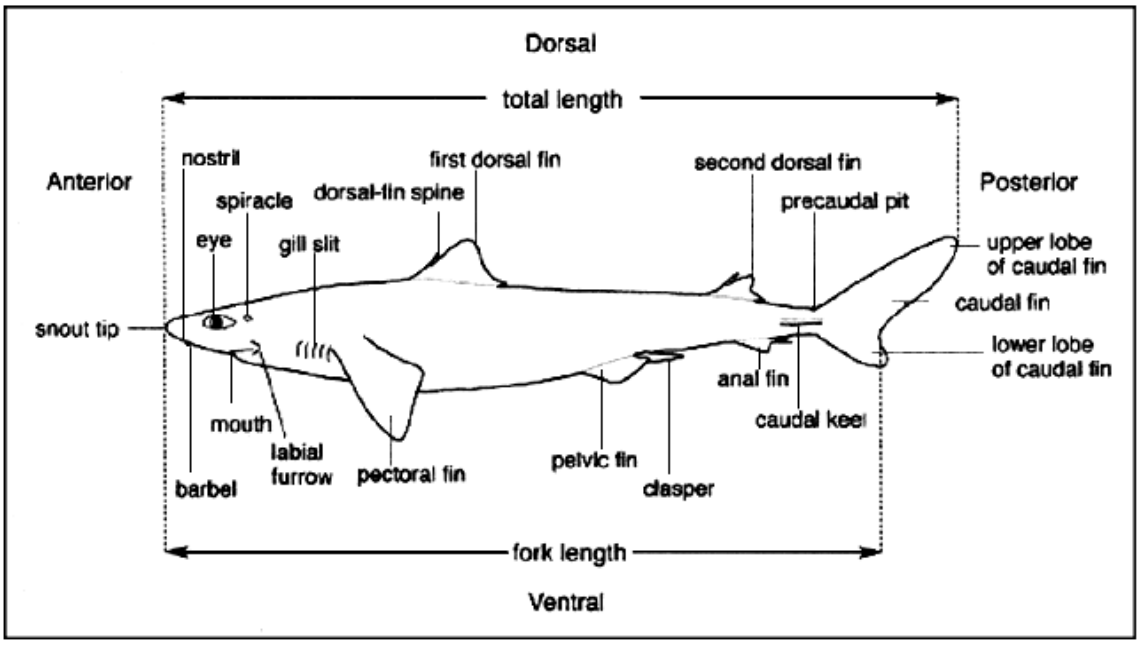

Figure A1. The terminology of shark.

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APPENDIX C – BACTERIA ISOLATION DATA

Table 8

Bacteria cultured from kidney, liver and blood samples of free-ranging Atlantic stingrays and Atlantic sharpnose sharks.

Isolates	Microorganism	Number of isolates	Total isolates
DS9 B-1	*Acinetobacter radioresistens*	1^{DSB}	1
DS1 B-4	*Bacillus alkalogaya*	1^{DSB}	1
DS10 K-1	*Bacillus flexus*	1^{DSK}	1
DS5 K	*Bacillus flexus/ licheniformis*	1^{DSK}	1
RT4 K-3	*Bacillus fordii*	1^{RTK}	1
DS15 L	*Bacillus hwajinpoensis*	1^{DSL}	1
DS10 B-1	*Bacillus infantis*	1^{DSB}	1
RT14 B	*Bacillus koreensis*	1^{RTB}	1
DS4 L	*Bacillus megaterium*	1^{DSL}	1
DS15 B-1	*Bacillus safensis*	1^{DSB}	1
DS13 K-2	*Bacillus subtilis*	1^{DSK}	1
DS2 K-7	*Bacillus tequilensis*	$1^{DSK\ (DS2\ K\text{-}7)}$ $1^{RTB\ (RT16\ B\text{-}2)}$	2
DS2 K-8	*Bacillus velezensis*	1^{DSK}	1
RT16 L-3	*Brachybacterium paraconglomeratum*	1^{RTL}	1
RT9 K	*Exiguobacterium aestuarii*	1^{RTK}	1
RT13 K-2	*Exiguobacterium profundum*	1^{RTK}	1
DS1 L	*Kistimonas scapharcae*	1^{DSL}	1
DS13 B-1	*Micrococcus aloeverae*	1^{DSB}	1
DS12 B-1	*Micrococcus luteus*	1^{DSB} $1^{RTB\ (RT1\ B\text{-}1)}$	2
DS12 B-4	*Micrococcus* sp.	1^{DSB}	1
DS14 K	*Micrococcus terreus*	1^{DSK}	1
RT12 B	*Micrococcus yunnanensis*	$2^{DSB\ (DS12\ B\text{-}3)\ (DS15\ B\text{-}2)}$ 1^{RTB} $1^{DSL\ (DS2\ L)}$ $1^{RTL\ (RT10\ L\text{-}1)}$	5
RT13 K-1	*Oceanobacillus caeni*	$1^{DSB\ (DS1\ B\text{-}5)}$ $1^{DSL\ (DS8\ L\text{-}2)}$ 1^{RTK}	3
DS6 K	*Photobacterium damsela*	1^{DSK} $1^{RTL\ (RT4\ L)\ (subspecies\ damsela)}$	2
DS1 B-2	*Pseudoalteromonas piscicida*	1^{DSB}	1

Table 9 (continued).

Isolates	Microorganism	Number of isolates	Total isolates
DS1 B-3	*Pseudoalteromonas* sp.	1^{DSB}	1
RT2 L	*Pseudomonas hibiscicola*	1^{RTL}	1
RT16 L-2	*Pseudomonas parafulva*	1^{RTL}	1
DS2 K-10	*Pseudomonas* sp.	1^{DSK} $1^{RTL\ (RT16\ L-1)}$	2
DS2 K-5	*Pseudomonas stutzeri*	1^{DSK}	1
DS2 K-6	*Pseudomonas stutzeri/ putida*	1^{DSK}	1
RT16 L-4	*Psychrobacter celer*	1^{RTL}	1
DS13 K-1	*Psychrobacter* sp.	1^{DSK} $1^{RTK\ (RT16\ K-2)}$	2
RT10 L-2	*Roseomonas cervicalis*	1^{RTL}	1
DS8 L-1	*Rothia amarae*	1^{DSL}	1
DS2 B	*Rothia mucilaginosa/ amarae*	1^{DSB}	1
RT1 L	*Serratia marcescens*	1^{RTL}	1
DS2 K-2	*Shewanella corallii*	1^{DSK}	1
DS2 K-3	*Shewanella fidelis*	1^{DSK}	1
DS2 K-4	*Shewanella japonica*	1^{DSK}	1
RT16 K-1	*Shinella granuli*	1^{RTK}	1
RT4 K-2	*Sporosarcina contaminans*	1^{RTK}	1
RT13 B	*Staphylococcus saprophyticus/ xylosus*	1^{RTB}	1
RT8 B-1	*Stenotrophomonas maltophilia*	1^{RTB}	1
DS1 B-1	*Stenotrophomonas* sp.	1^{DSB}	1
DS9 B-2	*Thalassospira profundimaris*	1^{DSB}	1
DS14 B-1	*Thalassospira tepidiphila*	$2^{DSB\ (DS14\ B-1,\ DS13\ B-2)}$	2
DS1 K	*Vibrio azureus*	1^{DSK}	1
DS4 K-1	*Vibrio campbellii*	1^{DSK}	1
DS4 K-2	*Vibrio communis*	1^{DSK}	1
DS2 K-1	*Vibrio harveyi*	$2^{DSK\ (DS2\ K-1,\ DS8\ K)}$	2
DS2 K-9	*Vibrio harveyi/ owensii*	1^{DSK}	1
RT2 B	*Vibrio nigripulchritudo*	1^{RTB}	1

Table 9 (continued).

Isolates	Microorganism	Number of isolates	Total isolates
DS15 K	*Vibrio owensii*	1[DSK]	1
DS9 K	*Vibrio panuliri*	1[DSK]	1
DS11 K	*Vibrio parahaemolyticus*	1[DSK]	1
DS10 K-3	*Vibrio sinaloensis*	1[DSK]	1
RT4 K-3	*Vibrio* sp.	1[RTK]	1
Total isolates		71	71

[DSK] Stingray kidney sample

[DSL] Stingray liver sample

[DSB] Stingray blood sample

[RTK] Shark kidney sample

[RTL] Shark liver sample

[RTB] Shark blood sample

APPENDIX D – IACUC Approval Letter

THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER:	15101510 (Replaces 13121201)
PROJECT TITLE:	Initial Characterization of the Elasmobranch Microbiome and Potential Roles in Host Physiology
PROPOSED PROJECT DATES:	10/2015 - 09/2018
PROJECT TYPE:	Renewal
PRINCIPAL INVESTIGATOR(S):	Andrew Evans
DEPARTMENT:	Coastal Sciences
FUNDING AGENCY/ SPONSOR:	N/A
IACUC COMMITTEE ACTION:	Full Committee Approval
PROTOCOL EXPIRATON DATE:	September 30, 2018

Frank Moore, PhD
IACUC Chair

10/01/2015
Date

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