COMPARISON OF GUT MICROBIOMES IN LABORATORY CULTURED SEA URCHINS REVEALING SELECTIVE ATTRIBUTES OF MICROBIAL COMPOSITION BASED UPON THEIR FEED AND SURROUNDINGS

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BIOLOGY

ABSTRACT

Bacteria residing in the gastrointestinal tract play important roles in digestive physiology and host health. The advent of NextGen sequencing and bioinformatics has made it possible to establish taxonomic profiles with highest coverage, and map these microbes in the gut ecosystem. Although extensively studied in the context of human health, understanding the microbial profiles associated with other organisms will elucidate the roles of the microbial inhabitants to their respective hosts and environment. The microbes of the sea urchin gut have been linked to digestion, processing, and extraction of nutrients from ingesta while within the gut, and have also been implicated in driving molecular transitions of undigested feed components post egestion. Additionally, the sea urchin may be aquacultured in the laboratory for use as model organisms, and understanding the membership and structure of the microbial profiles associated with the digestive tract is imperative for the comprehensive understanding of the health of the organism. To establish the microbial profiles of the sea urchin gut, community DNA was extracted from the gut and pharynx tissues, the gut digesta and egested fecal pellets, as well as the tank water and feed. NextGen amplicon sequencing of the V4 segment of the bacterial 16S rRNA gene, followed by bioinformatics tools were implemented. The results indicate Proteobacteria to be the dominant taxa of the gut microbiome, with members of Campylobacterales dominating in the gut tissue. Oligotyping analysis

followed by BLAST determined the Campylobacterales sequence oligotype to be related to *Arcobacter* species (identity > 91%), from the likely source of the tank water and feed. In the gut digesta and egested fecal pellets, *Vibrio* was found to be dominant. This study is expected to offer the baseline microbial profile of the sea urchin, *L. variegatus*, as it may pertain to the digestive physiology of the organism, the ecological impact of the microbe-laden egested fecal pellets onto the various marine trophic levels, and the informed culturability of the healthy sea urchin as a model organism.

Keywords: Egested fecal pellet; Illumina MiSeq; 16S rRNA; QIIME; Gulf of Mexico

DEDICATION

I dedicate this thesis to my brothers, Steve and Tony, and my parents, Antoine and Katherine. I was fortunate to have been born into a family of unconditional encouragement, and I owe my success to this love and support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table *Page*

LIST OF FIGURES

Figure *Page*

CHAPTER II: AN ABUNDANCE OF EPSILONPROTEOBACTERIA REVEALED IN THE GUT MICROBIOME OF THE LABORATORY CULTURED SEA URCHIN, *LYTECHINUS VARIEGATUS*

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
bp	Base pair
dNTP	Deoxyribonucleotide Triphosphate
kb	Kilobase pair
MDS	Multi-Dimensional Scaling
MEGA	Molecular Evolutionary Genetics Analysis software
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
OTU	Operational Taxonomic Units
PCR	Polymerase chain reaction
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
ppt	Parts per thousand
QIIME	Quantitative Insights into Microbial Ecology
RDP	Ribosomal Database Program
SRA	Sequence Read Archive
UR	Sea urchin
UV	Ultraviolet
V4	Hypervariable region 4 of 16S rRNA gene in bacteria

CHAPTER I: INTRODUCTION

The Microbiome

Microbes, representing the "unseen majority" of organisms (Whitman et al., 1998), inhabit numerous ecospheres of this planet (Griffin, 2007; Kallmeyer et al., 2012; Charlop-Powers et al., 2014), performing a diversity of ecologically significant processes, such as decomposition, fermentation, and pathogenesis. At the community level, microbes function concurrently and interdependently with adjacent microbes sharing their ecological niche. This interplay provides crucial support to the environment through biogeochemical cycling, which transforms the physical, chemical, and biological characteristics of their surroundings (Stahl et al., 2013; Zarraonaindia et al., 2013; Lebeis, 2015). Historically, examinations of microbial community structure have been restricted to culture dependent methods of characterization, limiting our understanding of an in-depth community structure and interaction between microbes and their environment, as less than 2% of the earth's bacteria can be cultured by traditional methods (Hugenholtz et al., 1998; Makkar and McSweeney, 2005; Guinane and Cotter, 2013; Fisher and Mehta, 2014). However, the current use of NextGen sequencing technology, alongside data mining and bioinformatics, has progressed the customary focus from the "one" cultivable microbe species, to the examination of the whole metacommunity, lending plausibility to an inclusive and comprehensive determination of the microbiota populating a particular ecological niche (Cho and Blaser, 2012).

One such niche exists on and within higher organisms, as part of various systems levels. The integrated microbial communities, known collectively as the microbiota, are recognized to provide crucial function and support to their host (McFall-Ngai et al., 2013). With the recent progress of NextGen sequencing technology targeting the collective microbial genomes, or microbiome, the structure and roles of these microbial assemblages have had the potential to be better understood (Kostic et al., 2013; Shafiei et al., 2015). In fact, these advancements have spurred multiple initiatives, such as the Human Microbiome Project (HMP) (Turnbaugh et al., 2007; Peterson et al., 2009) and recently the Earth Microbiome Project (EMP) (Gilbert et al., 2014), dedicated to categorizing the microbiota of various plant, vertebrate, and invertebrate organisms (Kostic et al., 2013). With over a trillion microorganisms inhabiting the gastrointestinal tracts of some host organisms (Gill et al., 2006), and the majority of which being bacteria (outnumbered only by bacteriophages) (Hoffmann et al., 2013), there has been an increased interest and awareness of the gut microbiome and its connection to the digestive physiology and health of the host.

It has been observed that the gut microbiome is a product of a shared evolution that has been occurring for over 500 million years (Cho and Blaser, 2012). This coevolution between host and microbiome has established core microbial residents, alluding to a selective attribute of the host, or a selection attributed by the host environment, providing a suitable habitat in support of particular microorganisms (Bäckhed et al., 2005; Turnbaugh et al., 2009; Shade and Handelsman, 2012). Nevertheless, gut microbiomes are stochastic, and may contain a number of transient microbes not generally observed in the host species, adding to a unique variability

between the gut microbiomes of different hosts (Grice and Segre, 2011). Such microbial disparity has been attributed to diet, disease, age, geography, host genetic variation, and community (Yatsunenko et al., 2012; Lukens et al., 2014; Blekhman et al., 2015). Despite this variation, it has also been proposed that there exists a conservation of microbiome gene content shared across a host species (Turnbaugh et al., 2009; Cho and Blaser, 2012), eluding to a core functional profile.

The Sea Urchin, *Lytechinus variegatus*

The variegated sea urchin, *Lytechinus variegatus*, can be found along the eastern coast of the United States, off the shores of the Gulf of Mexico into the northern coasts of Brazil (Hendler et al., 1995; Watts et al., 2013). In addition to marine vertebrates, invertebrates, and vegetation, this habitat comprises a robust microbial community, both contributing to the microbiota of higher organisms, as well as affecting the hydrosphere through metabolic cycling of macromolecules and nutrients (MacAvoy et al., 2002). Although this environment confers a diverse assortment of microbiota onto *L. variegatus*, it has been suggested that a particular resident microbiota exists in sea urchins, specific to the organism's phylogeny (Guerinot and Patriquin, 1981a). In its natural setting, the sea urchin *L. variegatus* is considered herbivorous, and will be found grazing seagrass beds, particularly turtlegrass *Thalassia testudinum* – which consists largely of storage and structural carbohydrates such as starch and cellulose (Moore et al., 1963; Zieman, 1982; Pradheeba et al., 2011). Additionally, proteins and lipids are scarce in its environment, though assimilation of such biomolecules is necessary for the health of the sea urchin, observed to be incorporated into the gonadal tissue (Castell et al., 2004; Hammer et al.,

2006; Gibbs et al., 2009; Arafa et al., 2012). Though digestive enzymes have been examined in *L. variegatus*, revealing glucosidases and galactosidases to be responsible for some carbohydrate metabolisms (Klinger, 1984; Klinger et al., 1986), it is unclear as to the extent of which the bacteria are contributing to the digestion of structural plant components and other polysaccharides (Klinger and Lawrence, 1984; Lawrence et al., 2013). Also, there is surmounting evidence to implicate bacteria in the *de novo* assimilation of proteins and lipids, or the molecular scavenge of precursors for amino acid and fatty acid assembly, since proteases and lipases have been scarcely observed in the sea urchin gut (Lawrence et al., 2006).

Historically, most sea urchin gut microbiome examinations centered on the possibility of digestive support provided for the organism via the microbes colonizing onto the gut digesta (Lasker and Giese, 1954; Farmafarmaian and Phillips, 1962; Unkles, 1977), or in disease states of the organism (Becker et al., 2007; Becker et al., 2008; Becker et al., 2009a). In the sea urchin *Strongylocentrotus purpuratus*, Lasker and Geise (1954) identified gut bacteria capable of digesting the naturally occurring agar from algae, a component found in the natural diets of the sea urchin but not readily digested by the enzymes of the sea urchin gut. Sawabe et al. (1995) would determine alginase activity by the bacterial isolates from the guts of the sea urchins *Strongylocentrotus intermedius* and *Strongylocentrotus nudus*, and specifically identify *Vibrio* species (over 96% of alginolytic isolates) to be responsible for such activity in *S. intermedius*, *in vitro* (Sawabe et al., 1995). Fong and Mann (1980) would assign Nitrogen fixing activity to *Vibrio* isolated from the sea urchin, *Strongylocentrotus droebachiensis*, suggesting a necessity for such bacterial activity in the assimilation of gonadal proteins (Fong and Mann, 1980).

Guerinot and Patriquin (1981a) also determined dinitrogen fixing bacteria, classified in the genus *Vibrio*, to be present in two sea urchin species, *Strongylocentrotus droebachiensis* and *Tripneustes ventricosus*, which was expounded upon in later studies (Guerinot and Patriquin, 1981a; Guerinot and Patriquin, 1981b; Guerinot et al., 1982). Molecular based culture-independent approaches by Becker et al. (2007), and the subsequent studies to follow (Becker et al., 2007; Becker et al., 2008; Becker et al., 2009a), would identify the microbiota associated with sea urchin disease, and eventually address a contributory role of microbiota to assist in wood feeding in the echinoid, *Asterechinus elegans* (Becker et al., 2009b). Regarding their role in digestive physiology, symbiotic relationships have been proposed to the bacteria of the digestive tract. However, these accounts have relied largely on metabolic characteristics of isolates *in vitro*, which limits this understanding to a relative few cultivable taxa of bacteria.

NextGen Sequencing and Bioinformatics

As molecular based approaches revolutionized the methods by which prokaryotes are delineated to phylogeny, the 16S rRNA gene has become the standard target for the identification of bacteria (Woese et al., 1990; Sharpton, 2014). Though the gene product is conserved due to the necessity of its function in translation, the gene itself contains nine hypervariable regions (V1-V9) within the 1.5 kb nucleotide sequence (Chakravorty et al., 2007). When sequenced, these hypervariable regions reveal adequate nucleotide variation between phylogeny to designate separate microorganisms. Interspersed amongst these variable regions are conserved sequences, which are exploited for microbial ecology (Petrosino et al., 2009; Soergel et al., 2012), as universal oligonucleotide primers

may be constructed for the amplification of diverse bacterial populations (Mizrahi-Man et al., 2013). Of the nine hypervariable regions, the V4 region is 254 bp in length, and offers sufficient variation for classification up to the genus level (Griffen et al., 2012).

The Illumina MiSeq™ platform (Caporaso et al., 2012; Kozich et al., 2013; Kumar et al., 2014) is a NextGen technology capable of community amplicon sequencing in parallel, utilizing a reversible terminator sequencing by synthesis chemistry, which can achieve approximately 9 Gb of data from a paired end 250 bp run (Caporaso et al., 2012; Liu et al., 2012). To prepare for sequencing, purified microbial DNA is isolated, and amplicon libraries are prepared using unique barcoded primers (Kozich et al., 2013; Kumar et al., 2014), which contain a sequence primer binding site as well as a region to compliment forward and reverse oligonucleotide primers prepared on a solid surface flow cell (Quail et al., 2012). Fragment strands are amplified through a bridge PCR concurrently, to create amplified DNA colonies (Adessi et al., 2000; Fedurco et al., 2006; Shendure and Ji, 2008), which is followed by a removal the reverse strands. The remaining forward strands receive a 3' protectant, and remain as a template along the flow cell, which are sequenced by the addition of four reversibly terminating fluorescently tagged nucleotides, allowed to compete for complementation, which occurs in parallel to the many DNA colonies along the flow cell (Mardis, 2008). Each subsequently base-pairing nucleotide releases a signal signifying complementation, which is recorded by a charge coupled device, and the read product is then removed in preparation for sequencing of the reverse strand (Liu et al., 2012). To do this, the existing template is de-protected, bridged to the reverse oligonucleotide primer (Mardis, 2008), and a reverse sequence is generated. The original template strands are washed from the

reaction, leaving the reverse strand as a template for sequencing by synthesis chemistry (Liu et al., 2012; Quail et al., 2012), again through fluorescently labeled nucleotide complementation and base calling through a charged couple device. These corresponding forward and reverse reads can then be aligned, to ensure the fidelity of the sequenced product (Edgar, 2010; Kozich et al., 2013; Nelson et al., 2014). When applied to segments of the bacterial 16S rRNA gene, the generated NextGen sequences can then be analyzed using bioinformatics software, such as the Quantitative Insights into Microbial Ecology (QIIME v1.7.0 and v1.8.0) (Caporaso et al., 2010), assigning Operational Taxonomic Units (OTUs) through programs such as UCLUST (Edgar, 2010) at specified similarity thresholds, and achieving taxa identities through 16S rRNA gene databases such as Greengenes (McDonald et al., 2011). In particular, the V4 hypervariable region of the 16S rRNA gene of the bacterial genome, which offers sufficient variation to distinguish phylogenetic identity, was the segment of microbial DNA targeted for NextGen sequencing for the execution of this thesis study.

Indeed, a comprehensive examination of the gut microbial communities of the sea urchin using NextGen sequencing by the Illumina MiSeq platform will resolve the inhabiting taxa at the highest coverage in various components of the sea urchin digestive system. However, modern bioinformatics software, such as the recently developed oligotyping technique (Eren et al., 2013; Eren et al., 2014) has made it possible to determine the source, selection, and distribution of microbial taxa at the species level (Schmidt et al., 2014). In this technique, Shannon entropy (Shannon, 1948) is used to define single nucleotide variations between closely related sequences, relying on those nucleotide positions that offer the highest entropy (Eren et al., 2013). Once these variable

nucleotides are determined along a sequence, identical sequences are grouped as an "oligotype" (Eren et al., 2013; Eren et al., 2014). Though designed to differentiate closely related taxa, this technology may be used to determine the source and selection of ecological dispersal of particular lineages of bacteria within the gut environment of the sea urchin *L. variegatus* (Schmidt et al., 2014). By using this technique on the sequence data generated from the sea urchin microbiome along with its feed and surrounding environment, the distribution of bacteria between the different compartments of the digestive tract within the sea urchin, as well as the incorporation of bacteria from the environment, can be determined.

Laboratory Cultured Sea Urchin

When used for research, the sea urchin may be aquacultured in the laboratory (Lawrence et al., 2001). Traditionally, the sea urchin is used to study embryology and early development (Moore et al., 1963; Kominami and Takata, 2008), and a recent genome sequencing effort has qualified the sea urchin as a model organism with many evolutionarily conserved genes shared with higher chordates (Sodergren et al., 2006). With the gut microbiome showing increased influence on host health, unfavorable conditions or poor diet may modulate the microbiome, and consequently lessen the quality of the organism for research. For that, there exists the potential for the informed culturability of the sea urchin (Nayak, 2010). This means understanding the impact that the culture conditions (tank water and feed) may have on shaping, or contributing to, the gut microbiome. Additionally, as the sea urchin gonad (uni) continues to be a delicacy and export worldwide (Muraoka, 1990; Andrew et al., 2002), and over-fishing has caused

a decline in the availability of the organism (Reynolds and Wilen, 2000), there is usefulness to culturing the sea urchin for economic value (Keesing and Hall, 1998). Understanding how the gut microbiome is impacted under aquaculture conditions can lead to the optimization and improved delicacy of uni (McBride, 1997). For these reasons, determining the highly abundant taxa in the gut microbiomes of the laboratory raised sea urchins will not only elucidate the selective attributes of the sea urchin gut from that environment, but help understand the overall impact of the culture environment on shaping the gut microbiome. This also helps determine a baseline to the selected microbes that reside and proliferate in the various components of the sea urchin gut environment (gut tissue, pharynx tissue, gut digesta, and egested fecal pellets), when fed a formulated feed in laboratory culture (Hammer, 2006; Hammer et al., 2006).

Microbes residing on and within marine organisms (fish, crustaceans, shellfish, and echinoderms) will impact the biogeochemical structure of the community through nutrient cycling, an event that occurs through the microbiota of egested fecal pellets of the sea urchin, post digestion (Johannes and Satomi, 1966; Koike et al., 1987; Wotton and Malmqvist, 2001; Sauchyn et al., 2011). As food is ingested by the sea urchin, a mucosal membrane develops around the gut digesta, which remains intact after egestion – an event that is accompanied by microbe enrichment and proliferation within the mucous film (Sauchyn et al., 2011; Holland, 2013). Because of this microbial colonization, egested fecal pellets from sea urchins undergo microbe driven molecular transitions, such as increasing the lipid availability from within the pellet, mineralizing organic nitrogen, incorporating nitrogen from the surrounding marine water into the fecal pellet, as well as the increasing in organic carbon availability from the remnants of the sea urchin diet

(Koike et al., 1987; Sauchyn et al., 2011). As a transformed food source with readily available energy, it has been suggested that suspended fecal pellets provide a source of food for neighboring marine organisms. Additionally, egested fecal pellets allowed to settle will cycle the remnants of the egested sea urchin diet back into the environment, and the role of the microbes associated with the egested fecal pellets may be better understood once these microbial inhabitants are identified and characterized in the laboratory culture environment.

Thesis Research

The primary objective of this thesis research was to use culture-independent NextGen sequencing approaches and bioinformatics tools to identify and characterize, at the highest coverage, the microbial communities associated with the gut microbiome of the laboratory cultured sea urchin *L. variegatus* when fed a standard reference diet, and define the contributory role of the culture environment to shaping the gut microbiota using oligotyping (Eren et al., 2013; Eren et al., 2014). In Chapter II, *L. variegatus* sea urchins were collected from Port Saint Joseph, Florida (29.80° N 85.36° W), held in aquaculture for 6 months, and fed a formulated diet daily (Hammer, 2006; Hammer et al., 2006), after which the resultant gut microbial ecology was evaluated. The gut environment was designated as the pharynx tissue, gut tissue, gut digesta, and egested fecal pellets, to achieve a definitive look into the bacterial profiles that are establishing themselves in each gut component. The microbial ecology of the feed and the tank water aquaculture system were also considered, to achieve a comprehensive view of the contribution of the environment to the gut microbial ecology. By identifying those

bacterial taxa supported in the gut, an insight into the selective attribute of the sea urchin *L. variegatu*s gut can be achieved. The overall results of Chapter II indicated members of the phylum Proteobacteria to be the dominant taxa in the gut and pharynx tissues, as well as the gut digesta and egested fecal pellets. The gut tissue revealed order Campylobacterales to be selected from the culture environment. As the order Campylobacterales dominated the gut tissue, and resolution to the genus level could not be established, oligotyping (Eren et al., 2013; Eren et al., 2014) enabled us to resolve the highly abundant Campylobacterales bacterial sequences of the gut tissue to an olygotype, which was found to occur in the tank water and feed alike. An alignment of this highly represented gut tissue sequence of Campylobacterales to the NCBI non-redundant (nr) database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed members of an uncultured *Arcobacter* sp. clone, as well as other *Arcobacter* related microorganisms (identities > 91%). The gut tissue maintained a unique ecology as compared to the other samples of the study, which was shown using multidimensional scaling analysis (MDS). The gut digesta and egested fecal pellets both appeared to be closely related in microbial ecology through MDS, and expressed a high relative abundance of the genus *Vibrio*. This study elaborates the distinct overall distribution of the bacterial community in *L. variegatus* gut ecosystem, while considering the likely source for the selective bacterial enrichment in the laboratory raised sea urchin.

CHAPTER 2: ANABUNDANCE OF EPSILONPROTEOBACTERIA REVEALED IN THE GUT MICROBIOME OF THE LABORATORY CULTURED SEA URCHIN, *LYTECHINUS VARIEGATUS*

by

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ABSTRACT

In this study, we have examined the bacterial community composition of the laboratory cultured sea urchin *Lytechinus variegatus* gut microbiome and its culture environment using NextGen amplicon sequencing of the V4 segment of the 16S rRNA gene, and downstream bioinformatics tools. Overall, the gut and tank water was dominated by Proteobacteria, whereas the feed consisted of a co-occurrence of Proteobacteria and Firmicutes at a high abundance. The gut tissue represented Epsilonproteobacteria as dominant, with order Campylobacterales at the highest relative abundance (>95%). However, the pharynx tissue was dominated by class Alphaproteobacteria. The gut digesta and egested fecal pellets had a high abundance of class Gammaproteobacteria, from which *Vibrio* was found to be the primary genus, and Epsilonproteobacteria, with genus Arcobacter occurring at a moderate level. At the class level, the tank water was dominated by Gammaproteobacteria, and the feed by Alphaproteobacteria. Multi-Dimensional Scaling analysis showed that the microbial community of the gut tissue clustered together, as did the pharynx tissue to the feed. The gut digesta and egested fecal pellets showed a similarity relationship to the tank water. Further analysis of Campylobacterales at a lower taxonomic level using the oligotyping method revealed 37 unique types across the 10 samples, where Oligotype 1 was primarily represented in the gut tissue. BLAST analysis identified Oligotype 1 to be *Arcobacter* sp., *Sulfuricurvum* sp., and *Arcobacter bivalviorum* at an identity level >90%. This study showed that although distinct microbial communities are evident across multiple components of the sea urchin gut ecosystem, there is a noticeable correlation between the

overall microbial communities of the gut with the sea urchin *L. variegatus* culture environment.

Introduction

Recent advancements in the discovery of gut microbial communities in the animal kingdom has offered a glimpse into the supportive role of various microbial taxa in growth, development, metabolism, and digestive physiology of the host, as well as protection from predators, and adaptive fitness to the environment they inhabit (Shin et al., 2011; Gomez et al., 2012; Nguyen and Clarke, 2012; Guinane and Cotter, 2013; Kostic et al., 2013; Heintz and Mair, 2014). Conventional microbiological culture-based methods, and more recently the advent of the culture-independent NextGen sequencing approach, has enhanced our capability to understand the gut microbial composition of many animals with the highest coverage, and in particular, a number of invertebrates such as Crustacea, Mollusca, and some Echinodermata (Harris, 1993; King et al., 2012; Gerdts et al., 2013; Kostic et al., 2013; Chauhan et al., 2014). Besides determining the microbial community profile of these invertebrates, the predictive roles of various microbial taxa in both the digestive health of the host, as well as the ecological importance of those bacteria to the host's community has been proposed. Among many ecologically and commercially important invertebrates, the sea urchin has received attention for its importance in the seafood industry (Muraoka, 1990; Andrew et al., 2002), as a model organism for developmental biology (McClay, 2011), and its role in nutrient cycling effecting the community structure and dynamics in the ecosystem they inhabit (Sauchyn and Scheibling, 2009a,b; Sauchyn et al., 2011). Yet, relatively little attention has been

given to the sea urchin gut microbial ecology, and the potential role of those microbes in host health and other facets of its natural community (Becker et al., 2007, 2008, 2009; Lawrence et al., 2013).

Lasker and Giese (1954) first proposed a role of microbiota in nutrient digestion and absorption in sea urchins (Lasker and Giese, 1954), and in fact, most of the previous microbial analysis work on the sea urchin has focused on a generalized role of microbes in digestive support (Lawrence et al., 2013), or in disease progression (Becker et al., 2007, 2008, 2009). Later examinations would suggest involvement of the sea urchin gut egesta bacteria in nutrient transfer among trophic levels in their communities (Sauchyn and Scheibling, 2009a,b). Nevertheless, as the microbial ecosystems of the sea urchin gut continue to foretell a relationship between the microbial community and nutrient intake, determining the bacterial composition within the gut of the sea urchin fed a formulated diet in an aquaculture environment would provide valuable insights into sea urchin digestive physiology and health.

The variegated sea urchin, *Lytechinus variegatus* is often found in nearshore seagrass communities in the Gulf of Mexico, and consumes a wide variety of plant and animal material (Watts et al., 2013). In the laboratory culture environment, *L. variegatus* can process formulated diets containing macronutrients from a variety of sources (Hammer et al., 2012). Since gut microbiota has previously been implicated in the digestive process of sea urchins (Lasker and Giese, 1954; Fong and Mann, 1980; Sawabe et al., 1995), understanding the microbial composition of the sea urchin digestive system may elucidate the role of the gut microbiome in conferring host health through formulated diet. In this study, we describe the microbiome composition in the lumen of

the digestive tract and gut digesta, along with egested fecal pellets, feeds, and the culture environment with high taxonomic coverage using a culture-independent method of NextGen sequencing technology and bioinformatics tools. The results from this study will help establish the microbial population that is conferred onto the sea urchin through the aquaculture conditions, as well as the trends of distribution and selective enrichment of the microbial community associated with the sea urchin, *L. variegatus*.

Materials and Methods

Collection and Culture of L. variegatus

Adult sea urchins were collected on April 2013, from Port Saint Joseph, Florida (29.80◦ N 85.36◦ W), and transported in seawater to a recirculating salt water system within the laboratory at the University of Alabama at Birmingham. Water conditions were maintained at 22 ± 2◦ C, with a pH of 8.2 ± 0.2 and a salinity of 32 ± 1 ppt. using synthetic sea salt (Instant Ocean; Spectrum Brands, Inc., Blacksburg, VA) added to treated municipal water. Prior to use, municipal water was filtered by 5 micron sediment, charcoal, and reverse osmosis membranes, followed by an ion exchange resin, with the final addition of Instant Ocean sea salts to achieve the desired salinity of 32 ppt. Water was replaced in the recirculating seawater culture system at a rate of ca. 5% water exchange per day. Water quality was maintained using a dolomite mechanical gravel filter, followed by biological filtration using Bioballs biological media (Foster and Smith, Inc., Rhinelander, WI), and UV sterilization of water exiting the recirculating filter. The sea urchins were fed a formulated feed (Hammer et al., 2006) *ad libitum*, consisting of a

relative percentage of 6% lipid, 28% protein, and 36% carbohydrate, once every 24–48 h for a 6 month period prior to analysis.

Sample and DNA Preparation

Two laboratory-cultivated sea urchins were used for the study (UR1 d = 50 mm, wet wt = 60.3 g, and UR2 d = 49 mm, wet wt = 63.2 g during the time described in the previous section). Sample collection from each sea urchin began 22 ± 1 h after feeding. Prior to dissection, the sea urchins were relocated to a temporary container containing sterile (autoclaved at 121º C for 20 min at 103.42 kPa) sea water, from which the egested fecal pellets from each sea urchin were collected. After fecal pellet collection, the sea urchins were then removed from the water and dissected immediately. Briefly, an incision was made with sterilized scissors into the test surrounding the peristomial membrane, and a dissection was performed circumnavigating the mouth. The peristomial membrane, along with the nested mouth (the Aristotle's lantern) (Sodergren et al., 2006), was lifted from the sea urchin, while still maintaining the integrity of the digestive tract (Watts et al., 2013).

The pharynx enclosed by the lantern was separated from the digestive tract, collected intact, and rinsed with autoclaved sea water. The remaining segment of the digestive tract (gut tissue), which included the esophagus, stomach, and intestine (Holland, 2013), was then removed from the sea urchin. The gut was rinsed with autoclaved sea water, and voided of gut food pellets by gentle shaking. The gut tissue was collected separately from the gut food pellets and both were rinsed with autoclaved sea water. The microbiota obtained from the seawater within the closed recirculating

system where the sea urchins were maintained was collected via vacuum filtration through Millipore 0.22µm filtration paper (EMD Millipore Corporation, Danvers, MA), and feeds were collected from the stock sea urchin food source (Hammer et al., 2006). All samples were divided into 3 separate sub-samples, flash frozen in liquid nitrogen, and preserved at -80 ºC until used for DNA purification and preparation for sequencing of the 16S rRNA gene. Food samples and whole filter paper containing water system microbes were also divided into three subsamples, frozen in liquid nitrogen, and preserved at -80 ºC until used.

Metacommunity DNA Purification and Generation of 16S rRNA Amplicon Library

Microbial community DNA was isolated using the Fecal DNA isolation kit from Zymo Research (Irvine, CA; catalog # D6010) following the manufacturer's instructions. Once the sample DNA was prepared, PCR was used with unique bar coded primers to amplify the hyper variable region 4 (V4) of the 16S rRNA gene, to create an amplicon library from metacommunity DNA samples (Kozich et al., 2013; Kumar et al., 2014). The oligonucleotide primers used for the PCR amplification of the V4 region of the 16S rRNA gene were as follows: Forward primer V4: 5'-AATGATACGGCGACCACCGAG ATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3'; and Reverse primer V4: 5'-CAAGAGAAGACGGCATACGAGATNNNNNNAGTCAGTCAGCCG GACTACHVGGGTWTCTAAT-3' (Eurofins Genomics, Inc., Huntsville, AL) (Kumar et al., 2014). The individual PCR reactions were set up as follows: 10 µL of 5X Reaction Buffer; 1.5 µL (200 µM) of each of the dNTPs; 2 µL (1.5 µM) of each of the oligonucleotide primers; 1.5 µL (5 U) of the "LongAmp" enzyme kit (New England

Biolabs, Ipswich, MA; cat # E5200S); 30 µL (2–5 ng/µl) of the template DNA; and 3 µL of sterile H_2O to a total reaction volume of 50 µL. The PCR cycling parameters were as follows: initial denature 94 ºC for 1 min; 32 cycles of amplification in which each cycle consisted of 94 ºC for 30 s, 50 ºC for 1 min, 65 ºC for 1 min; followed by final extension of 65 ºC for 3 min; then a final hold at 4 ºC. Following PCR amplification of the targeted gene, the entire PCR reaction was electrophoresed on a 1.0% (w/v) Tris-borate-EDTA/agarose gel. The PCR product (approximately 380 bp predicted product size) was visualized by UV illumination. The amplified DNA band was excised with a sterile scalpel, and purified from the agarose matrix using QIAquick Gel Extraction Kit according to manufacturer's instructions (Qiagen, Inc., Venlo, Limburg; cat # 28704).

Nextgen Sequencing and Bioinformatics Tools

The PCR products were sequenced using the NextGen sequencing Illumina MiSeq™ platform (Caporaso et al., 2012; Kozich et al., 2013; Kumar et al., 2014). We used a 250 bp paired-end kit from Illumina for the microbiome analysis. The samples were first quantified using Pico Green dye (Life Technologies, Grand Island, NY), adjusted to a concentration of 4 nM, then used for sequencing on the Illumina MiSeq (Kumar et al., 2014). The raw sequence data was then de-multiplexed and converted to FASTQ format (http://maq.sourceforge.net/fastq. shtml). The FASTQ files were subjected to quality assessment using FASTQC (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/), prior to merging and trimming of the raw sequence data, which was followed by quality filtering using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Since the overlap between the

paired reads from each 16S fragment was approximately 245 bases, the overlapping paired end regions were merged to generate a single high quality read, using the "fastq_mergepairs" module of USEARCH (Edgar, 2010). Read pairs with an overlap of less than 50 bases or with mismatches (>20) in the overlapping region were discarded. The sequences were again checked for quality using FASTQC, which was followed by chimeric filtering using the "identify_chimeric_seqs.py" module of USEARCH (Edgar, 2010). The remainder of the steps were performed with the Quantitative Insights into Microbial Ecology microbiome analysis package (QIIME, v1.7.0) (http://qiime.org/) (Lozupone et al., 2007; Caporaso et al., 2010b; Navas-Molina et al., 2013; Kumar et al., 2014). Sequences were grouped into Operational Taxonomic Units (OTUs) using the clustering program UCLUST at a similarity threshold of 97% (Edgar, 2010). The Ribosomal Database Program (RDP) classifier was used to make taxonomic assignments (to the species level wherever possible) for all OTUs at a confidence threshold of 80% (0.8) (Wang et al., 2007). The RDP classifier (http://rdp.cme.msu.edu/) was trained using the Greengenes (v13.8) 16S rRNA database (http://greengenes. lbl.gov/cgi-bin/nph-index.cgi) (McDonald et al., 2011). The resulting OTU table included all OTUs, their taxonomic identification and abundance information. Additionally, OTUs whose average abundance was less than 0.0005% were filtered out. Remaining OTUs were then grouped together to summarize taxon abundance at different hierarchical levels of taxonomic classification (e.g. phylum, class, order, family, and genus). These taxonomy tables were also used to generate stacked column bar charts of taxon abundance using Microsoft Excel software (Microsoft, Seattle, WA). Multiple sequence alignment of OTUs was performed with PyNAST (Caporaso et al., 2010a). Subsampling was performed using the

"single_rarefaction.py" module of QIIME (v1.7.0), to account for variation in read depth across samples, (Gotelli and Colwell, 2011), at an even sampling depth of 77,194 reads per sample. The subsampled OTU table was used for downstream Beta and Alpha diversity analyses. A heatmap with the top 25 most highly abundant (>1% in any sample) taxa at the order level was generated using the "heatmap.2" function in R package (available at http://CRAN. R-project.org/package=gplots). The raw sequence files from this study are deposited in the NCBI SRA (http://www.ncbi.nlm.nih. gov/sra), under the accession number SRP062365.

Oligotyping of the V4 Hypervariable Region of the Campylobacterales 16S rRNA Gene

Oligotyping utilizes informative nucleotide variations between similarly clustered reads to designate an oligotype identity (Eren et al., 2013, 2014; Schmidt et al., 2014). After assignment of taxonomy for the total 1,137,478 quality reads, 296,777 sequences from the 10 samples were aligned using MUSCLE, which was implemented in MEGA software (Tamura et al., 2013). The aligned sequences were then used for oligotyping (Eren et al., 2013). After the initial Shannon entropy analysis, 29 variable sites were identified for oligotyping. The parameters required that each oligotype must (1) appear in at least one sample and (2) have a minimum abundance of 100 sequences for each unique oligotype. After elimination of oligotypes not meeting these parameters, 275,566 reads (92.85%) were retained. Each oligotype representative sequence was aligned to the NCBI non-redundant (nr) database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical Analyses of Bacterial Diversity

The alpha diversity (diversity within the samples) of the sea urchin microbiome and the culture environment was determined using QIIME (v1.7.0). The alpha-diversity was estimated using observed OTUs, Shannon diversity index (Shannon, 1948; Hill et al., 2003; Marcon et al., 2014), and Simpson diversity index (Simpson, 1949; Hill et al., 2003). In order to estimate the beta diversity (differences between the samples), the OTUs of the bacterial communities were analyzed using Primer-6 analytical software (Primer-E Ltd., Plymouth Marine Laboratory, Plymouth U.K., v6.1.2) (www.primer-e.com). Discrete OTU counts per sample were standardized, and then transformed to the square root values (Clarke and Gorley, 2001). Multidimensional scale plots (Kruskal and Wish, 1978; Clarke, 1993; Clarke and Gorley,2001), were generated according to Bray–Curtis similarity values (Bray and Curtis, 1957; Clarke and Gorley, 2001).

Results

Total Illumina Sequence Reads, Quality Trimming, and OTU Designation

A total of 1,481,476 raw sequence reads of the V4 segment of the 16S rRNA gene from 10 samples of the two sea urchin (UR1 and UR2) gastrointestinal tracts, feeds, and tank water, were generated on an Illumina Miseq sequencing platform (Table 1). The sea urchin microbiome samples consisted of the gut tissues, pharynx tissues, gut digesta, and egested fecal pellets. After high stringent quality-based trimming, 1,137,478 quality sequence reads were used for further bioinformatics analyses. Within these reads, 81,169 sequences clustered into 609 OTUs from the gut tissue; 221,150 sequences clustered into 2,455 OTUs from the pharynx tissue; 219,512 sequences clustered into 926 OTUs from

the egested fecal pellets; 204,048 sequences clustered into 1,562 OTUs from the gut digesta; 164,930 sequences clustered into 1,654 distinct OTUs from the sea urchin feed; and lastly 146,669 reads clustered into 1,511 OTUs from the tank water (Table 1). All OTUs were clustered at a 97% sequence similarity from the trimmed sequences of the respective samples using UCLUST (Edgar, 2010; Koo et al., 2014).

Microbial Diversity across Different Samples

The relative abundances of taxa identified to the most resolvable taxa (phylum, class, order, family, and genus) across all 10 samples are elaborated in Figure 1. In the gut tissue samples of the sea urchins, microorganisms belonging to phylum Proteobacteria represented the highest relative abundance. Further analysis revealed class Epsilonproteobacteria to be dominant, and from within this class, order Campylobacterales was the most abundant taxon. Resolution to the genus level could not be achieved in the gut tissue samples. The pharynx tissue of the sea urchins was also dominated by Proteobacteria, and at the class level, Alpha-, Beta-, Epsilon-, and Gammaproteobacteria were presented. *Arcobacter, Mycoplana*, and *Vibrio* appeared as the highly represented genera from phylum Proteobacteria. Phylum Firmicutes was represented by a high relative abundance of the genera *Bacillus* and *Allobaculum*.

The gut digesta consisted mainly of bacteria belonging to phylum Proteobacteria, with class Gammaproteobacteria being distinguishably elevated. The dominant genera were *Agarivorans*, *Arcobacter*, *Shewanella*, and *Vibrio*, all of which belonging to phylum Proteobacteria. The bacterial composition in the egested fecal pellets consisted of many of the same taxa observed in the gut digesta. In the egested fecal pellets, Proteobacteria

accounted for the highest abundance, and at the class level, Gammaproteobacteria was dominant. At the genus level, *Agarivorans*, *Arcobacter*, *Shewanella*, and *Vibrio* were detected as dominant taxa.

The microbiota of the sea urchin feed consisted of phylum Proteobacteria, as well as Firmicutes at the highest abundance. Classes Alpha- and Betaproteobacteria were dominant in the feed, and at the genus level, *Agrobacterium*, *Acinetobacter*, *Limnohabitans*, and *Mycoplana* were observed. From phylum Firmicutes, order Lactobacillales dominated in the feed, and at the genus level, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* were observed. The microbial composition of the tank water was found to be more diverse as compared to the other samples. Of the represented phyla, Proteobacteria was found to be dominant, followed by Chloroflexi, and to a lesser extent Bacteroidetes. Classes Gamma- and Alphaproteobacteria were dominant, and at the order level, Alteromonadales and Vibrionales were represented at relatively high abundances. In addition, significant abundances of genera *Arcobacter*, *Agarivorans*, *Shewanella*, *Pseudoalteromonas*, and *Vibrio* were identified within phylum Proteobacteria.

Differentiation of Distinct Taxa using Oligotyping

Oligotyping analysis of those sequences corresponding to order Campylobacterales in the 10 samples of this study revealed 37 different oligotypes (Figure 3; UR1, sea urchin 1, UR2, sea urchin 2). Of these oligotypes, 21 were found in the UR1 and 11 in the UR2 gut tissues; 21 in the UR1 and 30 in the UR2 pharynx tissues; 17 in the UR1 and 26 in the UR2 gut digesta; 18 in the UR1 and 17 in UR2 egested fecal

pellets. The tank water and feed contained 18 and 6 oligotypes, respectively. Of all the identified oligotypes, Oligotype 1 was found to be overrepresented in the gut tissues of the sea urchins, with a relative abundance of 92.7% for UR1 and 91% for UR2. This oligotype was detected in the tank water at 0.3%, and the sea urchin feed at 22.8% (Figure 3). Across all samples, Oligotype 2 (which ranged from 8.5% to 88.36%) and Oligotype 3 (2.3% to 60%) were highly abundant, except for the gut tissues (Figure 3). A MEGABLAST search of the representative sequence of Oligotype 1 displayed a close match to an uncultured *Arcobacter* sp. clone (Identity: 91%, E- value: 1.82E–87), *Arcobacter bivalviorum* (Identity: 91%, E-value: 2.00E–89), *Sulfuricurvum* sp. (Identity: 90%, E-value: 4.00E–86), and an uncultured bacterium clone (Identity: 90%, E-value: 2.00E-89). A MEGABLAST search was performed on the other 36 identified oligotypes, revealing most to be closely related to uncultured *Arcobacter* sp., or uncultured bacterium clones.

Statistical Analysis

Rarefaction curves representing the number of unique OTUs from the normalized 16S rRNA sequences obtained from two sea urchins and their environments (total of 10 samples) reached or approached a plateau, indicating that a sufficient sequencing depth was used to assess community diversity (Figure 2). Shannon (Shannon, 1948; Hill et al., 2003; Marcon et al., 2014) and Simpson diversity indices (Simpson, 1949; Hill et al., 2003) displayed relatively low diversity within the gut tissue samples, whereas moderate diversity within egested fecal pellet and gut digesta samples; and high diversity within pharynx tissue, sea urchin feeds, and tank water samples (Table 1). The

multidimensional-scaling (MDS) plot (Kruskal and Wish, 1978; Clarke, 1993; Clarke and Gorley, 2001) revealed three distinct clusters of similarity among corresponding samples from the two sea urchins (Figure 4). In the MDS plot, the first dimension of gut tissues were differentiated from all other samples, and the second dimension separated the pharynges and feeds from the rest of the samples, i.e., the egested fecal pellet, gut digesta, and tank water (Figure 4). Subsampling of OTUs showed no significant differences in the cluster patterns of microbial communities in the respective samples.

Inter-sample microbial community compositions showed a similarity between samples (Figure 5). The gut tissue revealed a significant abundance of members from order Campylobacterales. The presence of Campylobacterales was also observed to be highly abundant in the gut digesta and egested fecal pellets, along with a significant presence of order Vibrionales. In the pharynx tissue, orders Burkholderiales and Caulobacterales were found to be abundant, whereas the tank water had high representation of order Alteromonadales, and the feed had a significant presence of Lactobacillales. The feed also presented orders Burkholderiales and Caulobacterales (Figure 5).

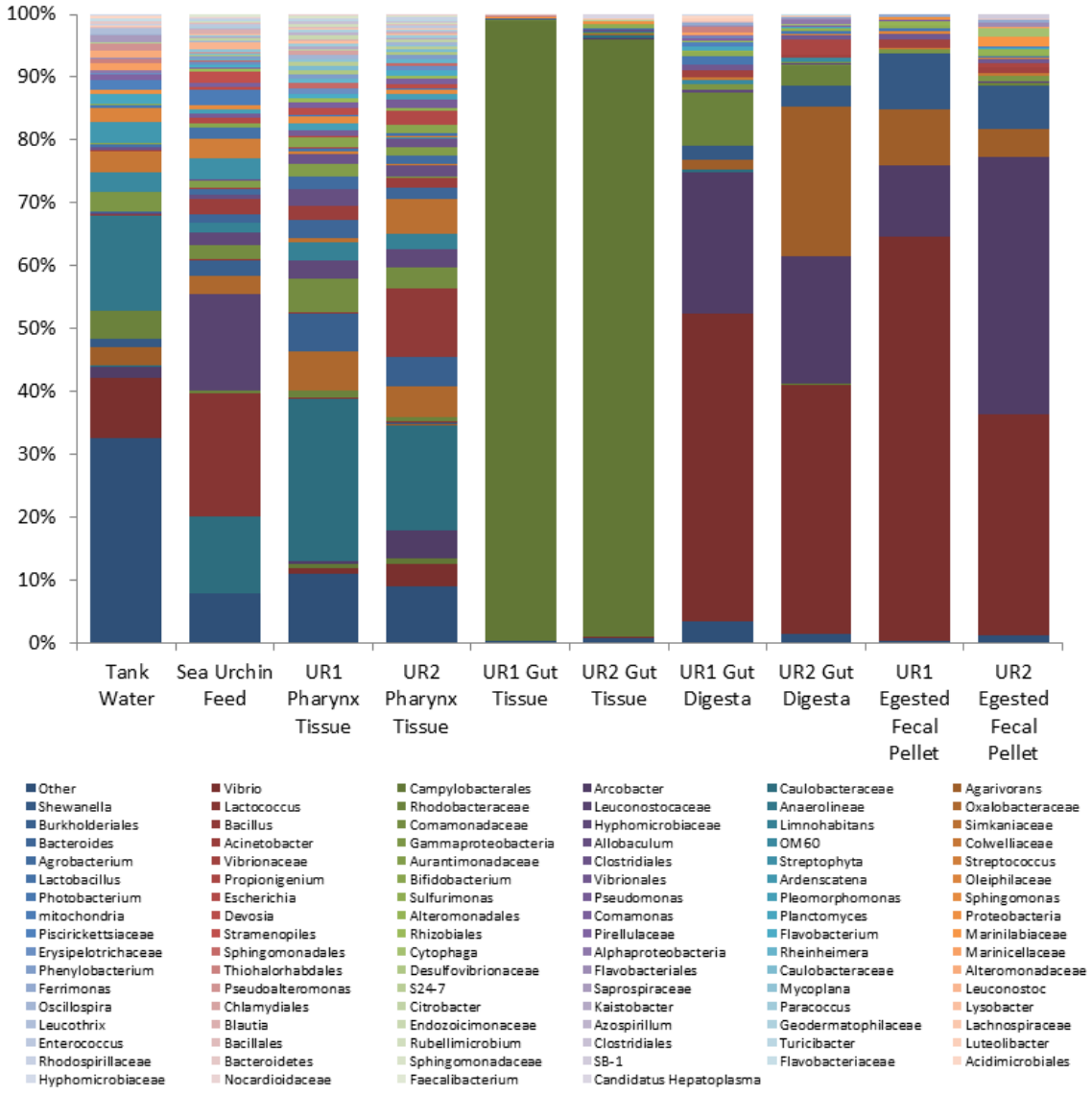

Figure 1. Stacked column bar graph depicting the relative abundances and distribution of the most highly abundant resolved taxa across the 10 samples of this study. The gut microbiome consisted mainly of Phylum Proteobacteria, whereas the sea urchin feed was dominated by both Firmicutes and Proteobacteria. At the highest resolution, order Campylobacterales was determined to be the most abundant taxa in the gut tissue. In the gut digesta and egested fecal pellets, *Vibrio*, *Arcobacter*, and *Agarivorans* were observed. Relative abundances were performed through QIIME (v1.7.0), and graphs were generated using Microsoft Excel software (Microsoft, Seattle, WA). UR1, sea urchin 1; UR2, sea urchin 2.

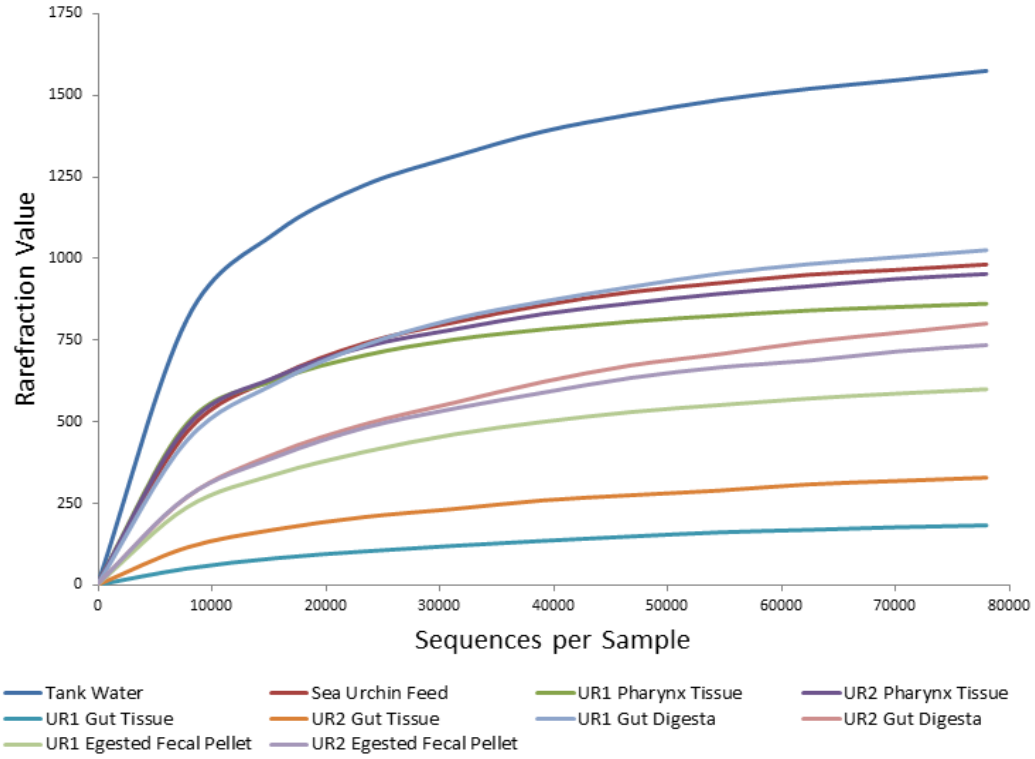

Figure 2. Rarefaction curves based upon the 16S rRNA genes generated from the 10 samples used in this study. The rarefaction curve was generated using QIIME (v1.7.0), and plotted using Microsoft Excel software (Microsoft, Seattle, WA). UR1= sea urchin 1; UR2= sea urchin 2.

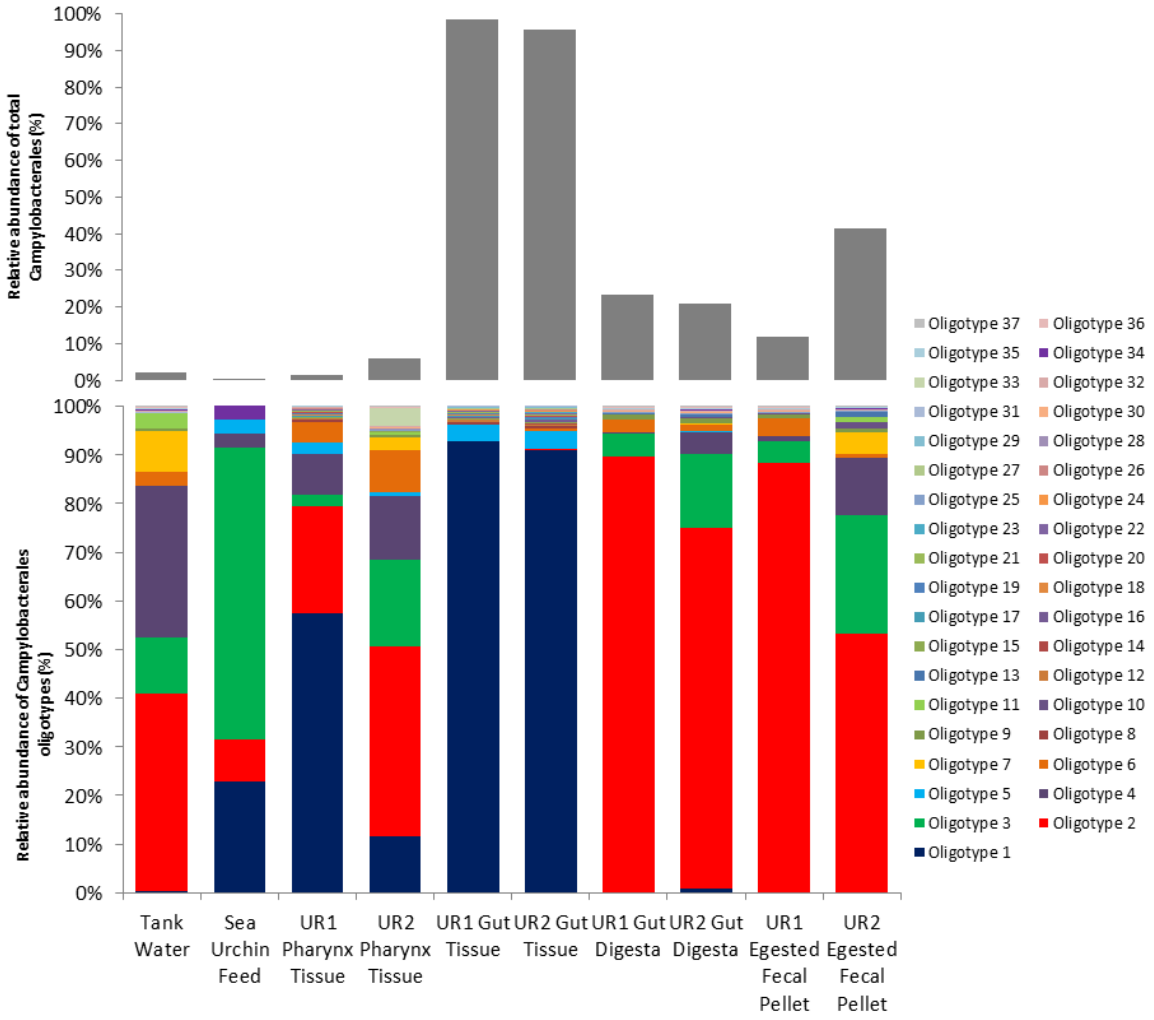

Figure 3. Oligotype distributions for the 10 samples used in this study. The relative abundance of each oligotype within the total Campylobacterales diversity for each sample is presented in stacked column bar graphs (bottom), and the proportion of the relative abundance of total Campylobacterales within all bacterial diversity for each sample is shown with light gray bars (top). Oligotyping analyses were performed using the open-source pipeline for oligotyping, available at http:// oligotyping.org. The stacked column bar graphs were generated using Microsoft Excel software (Microsoft, Seattle, WA). UR1, sea urchin 1; UR2, sea urchin 2.

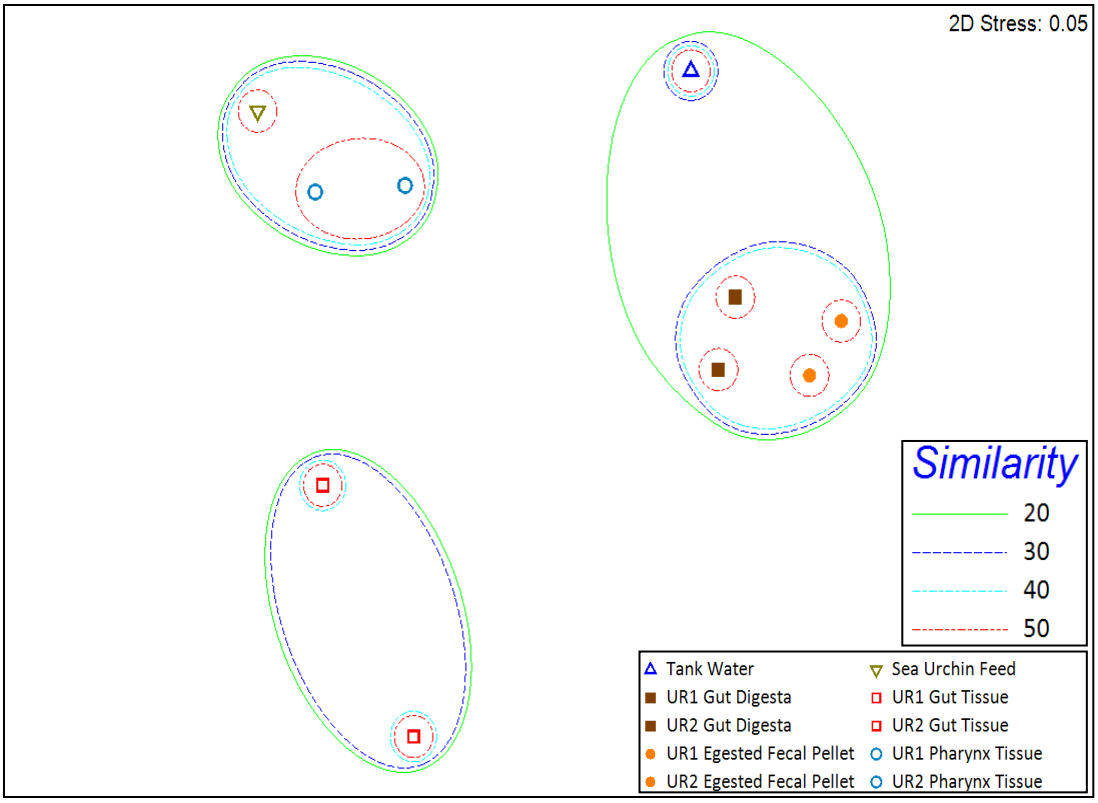

Figure 4. 2D multidimensional scaling (MDS) graph generated through PRIMER-6 (www.primer-e.com). Overlay of similarity clusters were produced according to Bray–Curtis Similarity values, set at 10% intervals from 20% to 50%. The pharynx tissue and sea urchin feed sample microbial ecologies clustered with a similarity greater than 40%. The tank water, gut digesta, and egested fecal pellet samples also clustered together at a similarity greater than 20%. The gut tissue samples from the two sea urchins showed a divergent cluster pattern, illustrating a reduced degree of similarity to the other samples of the study. UR1, sea urchin 1; UR2, sea urchin 2. Similarity= Bray–Curtis Similarity (scaled to 100).

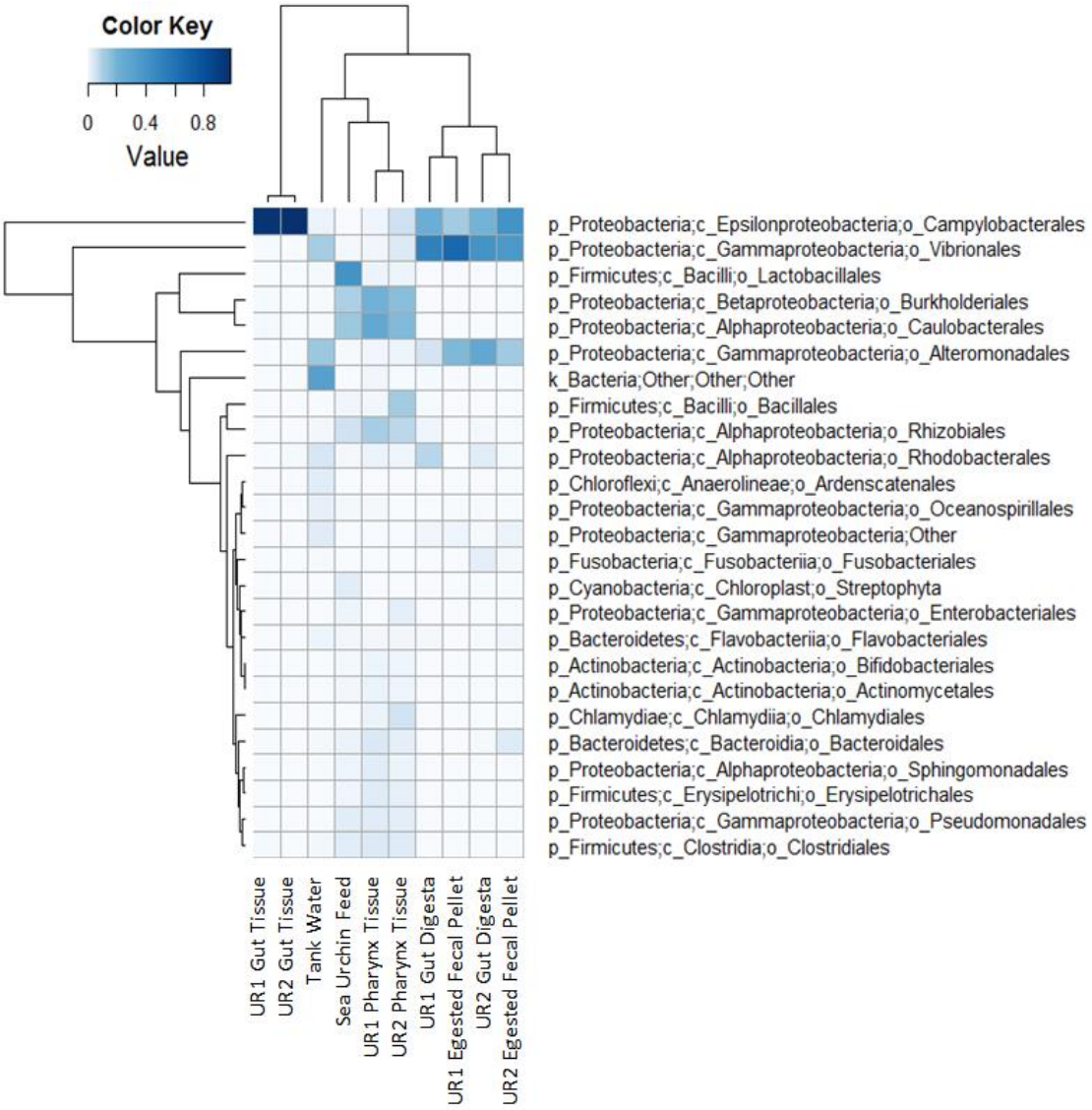

Figure 5. Heatmap of microbial compositions at the order level. The rows represent the bacterial taxa and the columns represent the 10 samples used in this study. Both dendrograms were created using hierarchical clustering (complete linkage) of the compositional data. The heatmap was generated using the "heatmap.2" function in R package (available at http://CRAN.R- project.org/package=gplots). UR1, sea urchin 1; UR2, sea urchin 2.

Sample	Raw Sequences	Trimmed Sequences	OTUs Identified	Shannon	Simpson
Tank water	181,387	146,669	1,511	6.51	0.95
Sea Urchin Feed	205,651	164,930	1,654	5.68	0.93
UR1 Pharynx Tissue	138,911	97,670	1,190	6.21	0.95
UR2 Pharynx Tissue	180,891	123,480	1,265	6.16	0.96
UR1 Gut Tissue	90,693	77,194	188	0.17	0.02
UR2 Gut Tissue	127,431	103,975	421	0.56	0.09
UR1 Gut Digesta	120,424	100,073	861	3.87	0.76
UR2 Gut Digesta	176,771	103,975	701	3.39	0.78
UR1 Egested Fecal Pellet	128,082	110,922	384	2.79	0.65
UR2 Egested Fecal Pellet	131,235	108,590	542	3.71	0.81

Table 1. Sample statistics following NextGen sequencing and the diversity values, as determined by QIIME (v1.7.0), are listed. Included are the number of raw sequences, trimmed sequences, and unique OTUs. Shannon, and Simpson diversity indices are also presented. UR1= sea urchin 1; UR2= sea urchin 2.

Discussion

Our study revealed that, although the sea urchin *L. variegatus* has a primitive gut as compared to the highly compartmentalized digestive systems in higher order deuterostomes (Sauchyn et al., 2011; Holland, 2013), distinct microbial compositions and abundances were noticed in the gut tissue, pharynx and the gut digesta, which shared a striking similarity with the food and culture environments. Additionally, it appears that the microbiota of the sea urchin consisted of a high abundance of Proteobacteria, which is comparable to observations of previously examined marine invertebrate gut microbiota (Van Horn et al., 2011). For example, in the sea slug, members of Alpha-, Beta-, and Gammaproteobacteria have been observed as overrepresented (Devine et al., 2012), and in the gut of the sea cucumber *Apostichopus japonicus*, an echinoderm, it was shown that members of Delta- and Gammaproteobacteria are dominant (Gao et al., 2014).

The luminal surface of the gut contained a low overall bacterial diversity, but a high relative abundance of order Campylobacterales of class Epsilonproteobacteria (Figure 1). It has been reported that representatives from this class have been found to inhabit many ecological niches, both terrestrial and marine, performing a diversity of metabolic functions (Eppinger et al., 2004; Gupta, 2006). In the marine environment, members of Epsilonproteobacteria have been associated as gill symbionts of hydrothermal vent dwellers such as the bivalve *Bathymodiolus azoricus* (On, 2001) and gastropod *Cyathermia naticoides* (Zbinden et al., 2014); as residents of other bivalves such as *mussels Brachidontes* sp. of marine lakes (Cleary et al., 2015) and the Chilean oyster *Tiostrea chilensis* (Romero et al., 2002); as epibionts of crustaceans such as *Kiwa puravida* (Goffredi et al., 2014); and lastly, as gut microbial inhabitants of the

aquacultured Norway lobster *Nephrops norvegicus* (Meziti et al., 2012) and hydrothermal vent dwelling shrimp, *Rimicaris exoculata* (Durand et al., 2010). Therefore the commonality of the occurrence of Epsilonproteobacteria in marine invertebrates and the sea urchins in our study may indicate a mutual benefit between the bacterial taxa and the host, perhaps at the physiological and nutritional level.

Further analysis of the lower level of taxonomic groups within Campylobacterales showed 37 oligotypes across all ten samples, with Oligotype 1 displaying a dominant presence in the gut tissue (Figure 3). This suggests that Oligotype 1 is the preferred bacterial group in the sea urchin gut. Additionally, a MEGABLAST search of the representative sequence of the highly abundant gut tissue Oligotype 1 revealed an uncultured species of *Arcobacter* sp., as well as *Sulfuricurvum* sp., and *Arcobacter bivalviorum* (Identities >90%). In a previous study, Epsilonproteobacteria clones identified as *Arcobacter* sp. were found to be associated with marine organisms, including shrimp species (*Rimicaris exoculata*) and the Chilean oyster (*Tiostrea chilensis*) (Romero et al., 2002; Durand et al., 2010). Taxonomic groups similar to Oligotype 1 were also found in the sea urchin feed and water samples, although to a much lesser extent, suggesting that the culture environment may have contributed to the high abundance of Oligotype 1 in the gut tissue microbial ecosystem following proliferation (Figure 3).

As food enters the digestive tract of sea urchins, it is enveloped in a mucosal film that remains intact even after egestion, as a microbial-enriched fecal pellet (Sauchyn et al., 2011; Holland, 2013). The microbiota of the gut digesta and egested fecal pellets both contained a high abundance of Gammaproteobacteria, specifically *Vibrio* of family

Vibrionaceae (Figure 1). In as early as 1954, Lasker and Geise reported colonization of bacteria in the gut digesta through microscopic observation (Lasker and Giese, 1954). Similarly in our study, a preliminary examination of the egested fecal pellets using transmission electron microscopy showed comma, round, and rod shaped structures, which appeared to be bacteria resembling *Vibrio*, *Arcobacter* and *Agarivorans*, genera later determined by NextGen sequencing using the Illumina MiSeq sequencing platform (data not shown). Besides morphological studies, much attention has been allotted to the bacteria colonizing the ingested feed of the sea urchin, with many investigations implicating those bacteria as both crucial to the digestive physiology of the sea urchin, as well as an enriched source of nutrients to organisms at various trophic levels in the hydrosphere (Johannes and Satomi, 1966; Koike et al., 1987; Sauchyn et al., 2011). Previous studies on the gut related microbiota of sea urchins have described the potential symbiotic support of certain strains of *Vibrio* to the sea urchin *Strongylocentrotus droebachiensis*, specifically nitrogenase activity, which is necessary for nitrogen fixing in the assimilation of proteins in sea urchin gonad (Fong and Mann, 1980; Guerinot et al., 1982).

Trends of microbial ecology in the sea urchin have been suggested by Guerinot and Patriquin (1981), who proposed a possibility of an endemic microbiota that will not dissociate from the gut wall of the sea urchin as food transits through the digestive tract (Guerinot and Patriquin, 1981; Lawrence et al., 2013). Evidence of this can be observed in the current study, as the gut digesta and egested fecal pellets were heavily dominated by *Vibrio* species, which were not observed to be significant in the gut tissue (Figure 1). Moreover, a unique oligotype (Oligotype 1) was observed in the gut tissue, which did not

appear to be as significant in the gut digesta and egested fecal pellets. This indicates that there is a preference by the host to select specific microbial taxa, perhaps necessary for their nutrition and health (Thorsen, 1998). Moreover, the pharynx tissue shared many of the bacterial taxa of the sea urchin feed (Figure 1), suggesting a likely influence and transmittance of microbes from the food source, which is supported through oligotype analysis (Figure 3), a trend also observed by Meziti et al. (2007) in *P. lividus* (Meziti et al., 2007). The outcome of this study has established for the first time the microbial community composition in the sea urchin *L. variegatus* gut ecosystem, as well as its culture environments, using NextGen sequencing and bioinformatics to achieve taxonomic coverage at the highest level. Future evaluation of the functional metagenomics of the gut microbiome of *L. variegatus* is warranted to establish the role of the microbial community associated with the digestive physiology, nutritional and other health benefits of this animal.

Conflict of Interest Statement

The authors declare no conflict of interest associated with this this study and this study was conducted solely for the purpose of scientific investigation.

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CHAPTER IV: CONCLUSIONS

We determined the microbial composition of sea urchin *L. variegatus* when aquacultured in the laboratory and fed a formulated feed, which has established a baseline for future investigations of the sea urchin microbiome. To do this, oligonucleotide primers designed for the V4 hypervariable region of bacterial 16S rDNA were implemented in NextGen sequencing, followed by bioinformatics analysis. It was determined that phylum Proteobacteria constituted the majority of the gut microbiome (gut tissue, pharynx tissue, gut digesta and egested fecal pellets), which was also observed in the tank water and sea urchin feed. Resolution in the gut tissue revealed order Campylobacterales of class Epsilonproteobacteria to be the most abundant taxa, from which representatives inhabit many ecological niches, both terrestrial and marine, as chemolitho- or chemoorganotrophic bacteria (Eppinger et al., 2004; Gupta, 2006). Order Campylobacterales were found to a lesser extent in the feed and the tank water, which were the likely sources of this taxon and presumably selected to be integrated in the lumen of the gut. Oligotype analysis (Eren et al., 2013; Eren et al., 2014; Schmidt et al., 2014) of the highly represented sequences corresponding to Campylobacterales in the gut tissue revealed a specific oligotype to be near dominant, alluding to the preference of the specific bacterial strain. BLAST analysis of this dominant oligotype produced identities related to uncultured *Arcobacter* sp. clones (identity: 91%), as well as *Arcobacter bivalviorum* (identity: 91%) and at a lesser match, *Sulfuricurvum* sp. (identity: 90%).

Arcobacter spp. have been observed to be associated with other marine organisms, including shrimp (*Rimicaris exoculata*) and oysters (*Tiostrea chilensis*) (Romero et al., 2002; Durand et al., 2010), though the isolation and examination of this bacterium would be necessary to verify the identity, and subsequently the functional role of this bacterium in the digestive tract of the sea urchin, *L. variegatus*.

Though distinct, the pharynx tissue shared a similar microbial ecology to the sea urchin feeds, suggesting a transmittance of microbes from the food source. Similar findings have been observed previously in the sea urchin *Paracentrotus lividus* by Meziti et al. (2007), who determined that bacterial profiles of the gut will not be greatly influenced by the microcosm of the environment, while the pharynx of the sea urchin will receive a microbial contribution from the environment (Meziti et al., 2007). The gut digesta and egested fecal pellets were dominated by Proteobacteria, specifically genus *Vibrio*, of family Vibrionaceae, and genus *Arcobacter*, of family Campylobacteraceae. Previous studies on the sea urchin *Strongylocentrotus droebachiensis* have described the potential symbiotic support of certain strains of *Vibrio*, namely nitrogenase activity, which is necessary for nitrogen fixing in the assimilation of proteins in sea urchin gonad (Fong and Mann, 1980; Guerinot et al., 1982).

In future studies, an investigation of the microbiome associated with naturally occurring sea urchins *L. variegatus* for comparison against the corresponding laboratory raised organisms would be beneficial. An evaluation of shared OTUs between the complementary organisms would shed light on the possibility of a selected core microbiome in the sea urchin *L. variegatus*. Lastly, an examination at the lowest possible

taxonomic level of co-occurring OTUs would offer a more determinant glimpse into the identities of potential endemic microbiota.

As food is scarce in the environment, and the nutrient profile of seagrass is heavily dominated by insoluble polysaccharides, there have been many studies implicating the gut associated bacteria of the sea urchin in the processing and extracting of necessary biomolecules for the digestion of structural carbohydrates, as well as the assimilation of proteins and lipids into gonadal tissue (Lasker and Giese, 1954; Tysskt et al., 1961; García-Tello and Baya, 1973; Unkles, 1977; Fong and Mann, 1980; Guerinot and Patriquin, 1981b; Becker et al., 2009b). Although particular glucosidases and galactosidases have been documented to be innate digestive enzymes in the sea urchin *L. variegatus* (Klinger and Lawrence, 1984; Klinger et al., 1986), proteases and lipases are yet to be determined with certainty (Lawrence et al., 2006). This has alluded to the necessity for bacteria in digestive physiology, due to limmited capability and efficiency by which the sea urchin can digest structural components of seagrass cell walls, and process proteins and lipids. The sea urchin *L. variegatus* grazes marine seagrass in its natural habitat, and has been identified to be significant in the cycling of nutrients throughout the marine communities off the coast of the United States and other countries (Eklöf et al., 2008; Miyata, 2010). As the seagrass is ingested, a mucosal envelop will begin to form around the feed, and bacteria will colonize. Although the bacteria involved in this pelleted food have been implicated in the aiding of digestion, it is unclear as to what occurs within this pellet post egestion (Sauchyn et al., 2011; Holland, 2013). It has been previously shown that the egesta of sea urchins (Sauchyn et al., 2011), as well as other organisms (Johannes and Satomi, 1966), will continue to carry the microbiota into

the marine communities. These pellets have been suggested as valuable sources of nutrient to many neighboring organisms (Johannes and Satomi, 1966; Koike et al., 1987; Sauchyn et al., 2011). Particular studies have noted the molecular changes in the nutrient profile of the egested fecal pellet, noting increases in lipid availability and the fixing and incorporation of organic nitrogen from the marine environment, which has been attributed to the bacteria of the pellet (Koike et al., 1987; Sauchyn et al., 2011). In this study, *Vibrio* was found to be heightened in the gut digesta. Members of these genera have been observed to perform such metabolic functions as nitrogen fixation, which was demonstrated in species of *Vibrio* isolated from the guts of *Strongylocentrotus droebachiensis* and *Tripneustes ventricosus* (Guerinot and Patriquin, 1981a), However, the metabolic role and function of the heightened *Vibrio* observed in the laboratory raised sea urchin *L. variegatus* is yet to be determined.

Importantly, identifying the selected microbial profile of the sea urchin gut environment may help define the role of microbes in the digestive physiology and health of the sea urchin, *L. variegatus*. As it may pertain to aquaculture for both laboratory research and enhancing uni quality as a seafood export, understanding the selected microbial profiles in the laboratory cultured sea urchin can lead to informed culturability, though future investigations would be necessary to determine those taxa which are beneficial to the sea urchin host. Lastly, identifying the microbiota associated with the egested fecal pellets may clarify to the role of the egested fecal pellet microbiome in nature, addressing both the microbial transmittance to neighboring organisms that feed on the fecal pellets, and the biogeochemical cycling pertaining to nutrient incorporation into the pellet, or the efficient processing of the undigested components of the sea urchin diet

back into the environment. A determinant investigation using metagenomics would provide the functional profiles of the various microbial populations relegated to compartments of the sea urchin gut ecosystem. Nevertheless, current advancements in bioinformatics tools have established techniques, such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt; v1.0.0) (Langille et al., 2013), which may be useful in revealing functional profiles of bacteria in the context of digestive physiology or as egesta in the surrounding environment, on the basis of inferred gene content derived from phylogeny (Langille et al., 2013). Future application of PICRUSt (v1.0.0) in the sea urchin microbiome would show trends of metabolic function expressed by bacterial profiles under various circumstances – either well fed a formulated feed daily in the laboratory, or under naturally occurring conditions in the Gulf of Mexico. This information could be compared to determine the metabolic shifts, if any, occurring between the microbial populations of various life circumstances. The outcome of this study, however, is expected to establish the baseline microbial community composition in the laboratory cultured sea urchin gut ecosystem at the highest coverage, along with its culture environments in the laboratory, which will help future investigations address the role and dynamics of the sea urchin gut microbiome, as it relates to understanding the digestive physiology, improving the health and quality of the organisms, and determining the ecological impact that sea urchin associated bacteria may have in the natural community.

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