Characterization of the gut and skin microbiomes of wild-caught fishes from Lake Guntersville, Alabama

by

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Abstract

In recent years, a paradigm shift has occurred in the area of organismal health. Where Koch and Hill's fundamental postulates equating to "one microbe—one disease" were once regarded as the rule, research has now shifted to a more holistic view in which whole microbial communities give rise to and participate in complex interactions that can ultimately influence disease processes. The microbial communities that constitute fish microbiomes are now recognized as essential components of host health and defense from invading pathogens. Therefore, a better understanding of the natural bacterial communities of healthy individuals and how they interact with the host and other environmental factors is of critical importance. For this study, I hoped to expand the existing body of research on fish microbiomes to include the skin and gut microbiomes of important freshwater sport fishes. My objectives were to 1) characterize the gut and skin microbiomes of three common freshwater fishes including two important sport fishes, largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus* as well as the distantly-related spotted gar *Lepisosteus oculatus,* 2) compare intra- and interspecies differences in the composition of each microbiome, and 3) identify potential influences of seasonality on core microbial communities. Skin and gut samples were collected in August and November 2014, and May 2015. All samples were sequenced as paired-end reads of the 16S rRNA gene via the Illumina MiSeq platform. More than 5M reads were analyzed representing 4,130 and 2,744 OTUs from gut and skin samples, respectively. Approximately 51.84% of the total OTUs were shared between the skin and gut bacterial

communities. Good's coverage was higher than 98% in all samples. Spotted gar exhibited the most diverse skin microbiome, while largemouth bass was the least diverse species in terms of both the skin and gut microbiome compositions. The highest diversity in the gut microbiome was observed in bluegill; however, the bacterial communities of spotted gar were the most variable across seasons. Seasonal changes in bacterial community structures were also observed. For both the skin and the gut microbiomes, sampling date was found to exert a stronger influence on microbial composition than the species itself; however, season had a lesser impact on the gut microbiome that in the skin indicating the gut microbiomes are more stable. Diversity of the skin was found to be significantly higher in August than in November and May.

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List of Abbreviations

ACE	Abundance-based coverage estimation
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
bp	Base pair
GALT	Gut-associated lymphoid tissue
MDS	Multidimensional scaling
OTU	Operational taxonomic unit
PRIMER	Plymouth Routines In Multivariate Ecological Research
SAS	Statistical Analysis System
SEI	Shannon evenness index
SIMPER	Similarity percentages
ADCNR	Alabama Department of Conservation and Natural Resources
AWFFD	Alabama Wildlife & Freshwater Fisheries Division
SECFPD	Southeastern Cooperative Fish Parasite and Disease Laboratory
QFL	Quantitative Fisheries Laboratory
TRW	Tennessee River Watershed
TVA	Tennessee Valley Authority
USGS	United States Geological Survey

I. INTRODUCTION

1.1. Microbiome

Microbes are ubiquitous in nature and share most of the same environments multicellular organisms call home (Sekirov et al. 2010). Given their close environmental associations, it should not be surprising that many microbes not only inhabit the same spaces but also look to their unsuspecting, multicellular neighbors as prime landscape for colonization. The human body, for example, serves as host to a wide range of microorganisms such as viruses, archaea, bacteria, and other unicellular organisms (Clemente et al. 2012; Hollister et al. 2014; Sekirov et al. 2010). While a great deal is still unknown about the roles of these microorganisms in the body, research in recent years has focused primarily on characterizing the components and interactions of an organism's commensal bacterial communities. These complex commensal communities are commonly referred to as the microbiota or, as we will refer to it henceforth, the microbiome (Hooper et al. 2012; Sekirov et al. 2010).

The term microbiome was first coined by Lederberg and McCray (2001) to describe the ecology of commensal, symbiotic, and pathogenic microbes that share space on and within the body. We have a tendency to associate microorganisms as being disease-causing agents, but as our knowledge has increased over time, we now understand the majority of microbes associated with our external and internal surfaces are actually more symbiotic in nature, often considered to be commensal or mutualistic

organisms that may provide important benefits to our health (Bäckhed et al. 2005; Hooper et al. 2001; Larsen and Arias 2016). In order to understand disease predispositions and pathogenesis, it is first important to better understand the "normal" or healthy states and functions of the microbiome (Turnbaugh et al. 2007). In doing so, it is important not only to characterize the microbiome, but also to learn about the factors that influence the distribution and evolution of these microbial symbionts. Organisms must rely on microbial symbionts to aid in nutrition, resistance of pathogens, and education of the immune system (Dethlefsen et al. 2007).

1.2. Human microbiome

In humans, the microbiome inhabits basically any surface of the body exposed to the external environment (Clemente et al. 2012; Konya et al. 2014; Sekirov et al. 2010). The human gut has long been recognized as playing a significant role in human health and disease (Holzapfel et al. 1998; Tuohy et al. 2003). Even in ancient times, Hippocrates noted the medicinal value of food for the gut and human health (Singer and Bunger 2010). Research indicates the gut microbiome starts out as a relatively blank slate, with establishment beginning during and after birth (Aldenberg and Wu 2014; Koenig et al. 2011, Scholtens et al. 2012). For an infant, the most important source of inoculum is typically provided through the birthing process via the vaginal and fecal microbiomes of the mother. In the first few years of life, bacterial communities are fairly dynamic showing punctuated shifts associated with changes in health and diet. Although bacterial communities may seem chaotic during this time, a nonrandom and gradual colonization of these communities occurs until the microbiome becomes relatively stable at 2-3 years of age. The microbiome continues to increase in both richness and diversity throughout

the life, reaching the highest level of complexity in adulthood. Throughout most of the healthy human life, the "core microbiome" or dominant bacterial phylotypes that help maintain functional stability and homeostasis of the healthy host remain relatively stable (Ottman et al. 2012; Zaura et al. 2009). However, the gut microbiome destabilizes somewhat in older age and again becomes less diverse and more dynamic (Ottman et al. 2012).

At the individual level, the human gut microbiome is composed of between 300-1000 species of bacteria and about two million genes that inhabit the length of the gastrointestinal tract, most of which reside in the colon (Guarner and Malagelada 2003; Holzapfel et al. 1998; Quigley 2010; Sears 2005; Tuohy et al. 2003; Xu and Gordon 2003). Due in part to its large surface area and exposure to external environments, the gastrointestinal tract of humans is estimated to be home to roughly 10^{14} viable bacteria which are about 10 times more than the total eukaryotic cells in the body (Guarner and Malagelada 2003; Holzapfel et al. 1998). The sheer scale of the human microbiome and its importance in healthy function has led to it often being considered as a supraorganism or "forgotten organ" by some (Clemente et al. 2012; O'hara and Shanahan 2006; Quigley 2010; Turnbaugh et al. 2007).

Of the bacterial cells inhabiting the human body, the greatest number of microorganisms can be found at varying concentrations throughout the length of the gastrointestinal tract (Clemente et al. 2012; Konya et al. 2014; Sekirov et al. 2010). The majority of bacteria found within the gut are strict anaerobes and are dominated mostly by two phyla, Bacteroidetes, and Firmicutes. Colonization of the gut microbiome varies by the specific environments and locations along the length of the digestive tract

(Hollister et al. 2014; Sekirov et al. 2010). In general, the number of microbial inhabitants increases as you move downward towards the colon. The structure of the gastrointestinal tract is also important with variations in colonization foci being seen between the lumen and the mucosal surfaces of the gut.

The human gut microbiome is credited for its contribution to important digestive processes, production of metabolites, immune system modulation, and competition with other gastrointestinal microbes (Halzapfel et al. 1998; Ottman et al. 2012; Singh and Bunger 2010). As more information has become available, it has become evident that human health is highly reliant on a delicate balance between the biotic and abiotic compounds that reside in the human gut (Holzapfel et al. 1998). The intestinal epithelium and its normal inhabitants essentially serve as a barrier to protect against invading pathogens and harmful antigens. Multiple factors can lead to disruptions or changes in composition of the gut microbiome such as stressors, age, diet, antibiotic treatments, and varying environmental conditions. When the normal or stable gut microbiome is disrupted, microbial communities enter a state of "dysbiosis" in which distributions and metabolic activities of present microbes may shift within the gut. This disruption often leads to imbalances within the gut that can result in reductions of beneficial bacteria and increases in diseases caused by opportunistic or other pathogens. Imbalances or deviations from the healthy gut microbiome have been linked to diet-related diseases such as allergies, inflammatory bowel disease, and obesity (Ottman et al. 2012).

1.3. Fish microbiomes

Volumes of research and documentation currently exist concerning mammalian microbiomes; however, microbiome research is still relatively new to the realm of fisheries. Although the ways in which mammals and fishes interact with their surrounding environments are quite different, research concerning mammalian microbiomes has helped pave the way and highlight the importance of these microbial communities in aquatic systems. Over the last century, researchers have sought to characterize the microbial communities that inhabit the fish, both inside and out (Peatman et al. 2015; Reed and Spense 1929). Until recently, the characterization of microbial communities in fish was often biased due to the use of culture-dependent methods that vastly underestimated the species richness of the microbiome. As the body of literature continues to build for the study of fish microbiomes, we have come to realize that microbial communities colonize the skin, gills, light-emitting organs, gut, and potentially even the internal organs (previously thought to be relatively sterile) such as the kidney, liver, and spleen of healthy fish (Austin 2006, Tao et al. 2014). Microbiomes have been shown to be of critical importance to host nutrition, energy balance, and development and maintenance of the immune system (Littman and Pamer 2011). Research indicates that colonization of the microbiome may be related to and largely influenced by characteristics of the surrounding environment (Horsley 1977). Despite the dynamic nature of these microbial communities, some studies indicate colonization of microbiomes may show marked species-specificity (Chiarello et al. 2015; Larsen et al. 2013). Furthermore, the skin microbiome of fishes has also demonstrated high interindividual as well as intraindividual variation in diversity and composition (Chiarello

et al. 2015). For purposes of this research, I will focus on the skin and gut microbiomes of wild-caught fishes from a freshwater reservoir in North Alabama.

Gut microbiome

Following on the heels of the growing body of literature surrounding human and mammalian microbiomes, most of the existing research concerning fish microflora has targeted the gut microbiome primarily due to its anticipated importance in digestion, disease control, and the overall health of fishes (Huber et al. 2004). Although many fish microbiome studies have focused on the gut, there is still much that is unknown about the composition and structural function of these communities (Clements et al. 2014; Nayak 2010; Ray et al. 2012). Until recently, most of the work investigating microbial communities of fishes had relied largely on bacterial culture methods and phenotypic characterization by morphological and biochemical techniques (Horsley 1977; Nayak 2010; Onarheim and Raa 1990; Sakata et al. 1990; Sugita et al. 1995). While these studies provided an extremely important foundation for fish microbiome research, the methods used resulted in a somewhat biased and limited view of these communities due primarily to the difficulty of culturing many species of bacteria, a phenomenon commonly referred to as the "great plate count anomaly" (Amman et al. 1995; Staley and Konopka 1985). Studies indicate less than 1% of bacteria found in aquatic habitats are actually culturable (Amann et al. 1995).

The gastrointestinal tract of a fish is a complex and dynamic microbial ecosystem that provides a favorable landscape for the growth of many different types of bacteria, primarily composed of aerobic or facultative anaerobes as well as obligate anaerobes (Nayak 2010; Skrodenyte-Arbaciauskiene et al. 2006). Studies have shown a wide

diversity of bacteria inhabits the intestinal mucosa and feces of freshwater fishes (Austin 2006; Larsen et al. 2014; Nayak 2010; Trust and Sparrow 1974). To date, bacterial genera such as *Acinetobacter, Aeromonas Klebsiella, Plesiomonas, Pseudomonas, Cetobacterium,* and *Clostridium* have been identified in the gut communities of various freshwater fishes.

Historically, most studies concerning the fish gut microbiome have focused on the promotion of growth characteristics, pathogenic bacteria, and disease control in relation to the aquaculture industry (Uchii et al. 2006). Although some studies have indicated that a symbiotic relationship exists involving the host metabolism and bacterial communities of the gut, much is still unknown about host-microbiome interactions at a functional level (Sugita et al. 1991; Sugita et al. 1997). Gut microbiota are now credited as playing important roles in the development of host immune functions, epithelial renewal, nutrition, digestive processes, and xenobiotic metabolism (Mouchet et al. 2012; Nayak 2010; Llewellyn et al. 2014; Uchii et al. 2006). An integrative system for host defense against disease is thought to be formed by a symbiotic partnership between the host's gut epithelium, immune system and the commensal gut communities (Kitano and Oda 2006; Llewellyn et al. 2014). This line of defense helps make up the gut's immune system, more commonly referred to as the gut-associated lymphoid tissues (GALT), which defends the host against invading pathogens and regulates the immune system of the digestive tract (Nayak 2010; Rhee et al. 2004). Thus, the gut microbiome helps defend the host, not only by educating and boosting the immune system, but also by enhancing it via inhibition of invading pathogens by either competitive exclusion or by the production of toxic secondary metabolites that prevent colonization (Llewellyn et al. 2014; Balcazar

et al. 2006; Wells et al. 1988). Anaerobic bacteria have been largely implicated in these mechanisms of "colonization resistance." Therefore, disruption of these important commensal communities can lead to a "dysbiotic" state (Llewellyn et al. 2014; Nayak 2010. Once the balance of the gut microbiome is disturbed, potentially pathogenic transient microbes may colonize the gut resulting in disease for the host. Although used frequently in the literature concerning disease, the dysbiosis is still somewhat subjective since the "natural" or normal gut microbiota is still relatively undefined and misunderstood for host species.

As with mammalian hosts, colonization of the microbiome begins early in life. At the larval stage of fish, bacteria begin to colonize the gastrointestinal tract (Giatsis et al. 2014; Hansen and Olafsen 1999; Uchii et al. 2006). Factors such as the presence of proper receptors for adhesion, defense mechanisms of the host, and appropriate growth conditions for bacteria help to regulate the establishment of microbial communities in fish (Hansen and Olafsen 1989; Hansen and Olafsen 1999). The chorion of a fish egg as well as the intestinal mucosa of the fish contains special glycoproteinaceous receptors that have shown marked species-specific variation. Hansen and Olafsen (1989) found that a clearly discernible growth in bacteria could be seen on eggs 2 h post-fertilization under hatchery-reared conditions. This study also found some of the egg-associated bacteria came from the ovary of the mother prior to spawning. Hence, the establishment of the core gut microbiome of fishes is likely due to bacteria ingestion at the yolk stage (Hansen and Olafsen 1999). The "adult" gut microbiome is thought to develop weeks to months after the first feeding. In addition to the developmental stage, other internal and external factors have been linked to the establishment and colonization of the gut

microbiome such as the anatomical structure of the gut, water temperatures, chemicals and pollutants, antimicrobial treatments, feeding behaviors and conditions, diel fluctuations, and seasonality (Nayak 2010).

The gut microbiome has also been shown to be important for host nutrition and other physiological processes of the host (Nayak 2010). Microbiota colonization of the gastrointestinal tract has been shown to produce various metabolites, amino acids, vitamins, and digestive enzymes that are essential components of growth and digestion in fish (Skrodenyte-Arbaciauskiene et al. 2006; Sugita et al. 1997). For example, under stable conditions, high concentrations of *Aeromonas* can aid in digestion via the secretion of several proteases (Nayak 2010; Pemberton et al. 1997). Anaerobic bacteria also contribute to digestive processes and nutrient absorption by the production of end products such as volatile fatty acids like those that have been reported in the gut of the largemouth bass (Clements 1997; Nayak 2010; Smith et al 1996).

Skin microbiome

Somewhat different from that of terrestrial organisms, the skin microbiome of a fish is constantly in close, intimate contact with its surrounding aquatic environment (Esteban 2012). Unsurprisingly, the mucosal surface regularly encounters a wide array of viruses, archaea, bacteria, and other eukaryotic microorganisms as it traverses its fluid environment. The aquatic environment provides a perfect medium for the proliferation of commensal microorganisms as well as opportunistic and primary pathogens that can colonize the exposed surfaces (i.e. skin, gills, and also entrance to the gut) of other aquatic organisms (Peatman et al. 2015). Under normal circumstances, the fish utilizes a complex innate defense system to maintain a healthy state and ward off potential

pathogens (Ellis 2001). The skin microbiome in conjunction with the skin and mucosal epithelia is thought to serve as the first line of defense providing both a physical and chemical barrier against invading pathogens (Ellis et al 2001; Estaban 2012; Peatman et al. 2015). The mucosal surface along with its commensal bacterial communities helps provide protection by inhibiting the attachment, invasion, and growth of foreign bacteria on or inside host tissues. These protective features are supplied by a variety of mechanisms. Mucus is constantly being produced by goblet cells in the epidermis and sloughing from the skin's epithelial surface. In part, this aids in defense by entrapping and preventing attachment of potentially pathogenic bacteria as well as aiding in the elimination of wastes.

The symbiotic relationship between microbial communities and the host organism requires a somewhat finely tuned response to prevent potentially harsh immune reactions such as inflammatory responses that could ultimately inflict more harm than good for the host where potentially beneficial bacteria are present (Littman and Pamer 2012). The establishment and composition of the microbiome denotes a complex coevolution between the host and its microbial partners which has resulted in a relatively stable and mutually beneficial relationship between the two (Peatman et al. 2015). Research has shown that any disruptions to the skin microbiomes, often referred to as "dysbiosis", can result in an increased host susceptibility to bacterial infections and disease (Cipriano and Dove 2011; Littman and Pamer 2011; Lokesh and Kiron 2015; Mohammed and Arias 2015).

In addition to its role in host defense, the skin microbiome also serves a more functional role that may help fishes more easily navigate their aquatic habitats. Some

bacterial strains that inhabit the skin of fishes have been shown to produce extracellular polymers that reduce frictional drag for fishes as they swim through the water (Sar and Rosenberg 1987; Sar and Rosenberg 1989; Bernardsky and Rosenberg 1992). Bacteria from the Class Gammaproteobacteria such as *Acinetobacter* and *Pseudomonas* have been implicated in their abilities to reduce drag due to the generation of certain compounds or other strain characteristics that facilitate bacterial adhesion by creating a smoother surface to move through aquatic environments. Large fish within a species have been shown to have higher mucus drag reducing activity than smaller fish (Bernadsky et al. 1993).

1.4. Guntersville Reservoir

All host species for the present study were collected from the Lake Guntersville Reservoir located in northern Alabama. Lake Guntersville is part of the fifth largest river system in the United States, the Tennessee River Watershed (TRW) (Bohac and Bowen 2012; Hutson et al. 2004). The TRW extends to seven states, Alabama, Georgia, Kentucky, North Carolina, South Carolina, Tennessee, and Virginia and drains 40,910 square miles (Figure 1). The Tennessee Valley Authority (TVA) manages a series of dams and reservoirs that assist in the regulation of the Tennessee River system in effort to provide power production, improved water quality and supply, reduced flooding, navigation, economic growth, and recreational use throughout the year. Water use from this system is attributed to four primary areas: public supply, irrigation systems, industrial use, and thermoelectric power. For the year 1995, the United States Geological Survey (USGS) reported that TRW accounted for the highest average per day water withdrawals in gallons per square mile in the United States. Interestingly, it also

accounted for the lowest percentage of annual consumptive use in the United States meaning that most of the water coming out of the system was considered to have high reuse potential. The majority of these withdrawals are largely attributed to use for thermoelectric power production.

Formed in 1939 by the completion of the Guntersville Dam, Lake Guntersville is located at the southernmost point of the Tennessee River and is considered the largest impoundment in Alabama (Buchanan et al. 1982). Guntersville reservoir is 75 miles long, covers an area of approximately 27, 500 ha, and boasts a rich diversity of aquatic fauna. Competitive sport fishing is one of the fastest growing uses for inland and marine resources fisheries (Schramm et al. 1991). Since the genesis of organized sport fishing, Lake Guntersville has gained a reputation for being one of the best black bass fishing lakes (consistently ranking in the top 5 for several years by Bassmaster magazine) in the United States attracting a large number of anglers, both competitive and recreational, from foreign and domestic locations (Hall et al. 2012; Snellings 2015). As such, Lake Guntersville and particularly the black bass fishery serve as a huge source of economic gain for local communities and the state of Alabama. For these reasons, in addition to the conservation and ecological importance of many black basses (*Micropterus*), the Alabama Department of Conservation and Natural Resources (ADCNR) Alabama Wildlife & Freshwater Fisheries Division (AWFFD) has invested a great deal of time and money into research and stocking programs to help keep fish populations fit, abundant, and balanced for the overall health of the ecosystem as well as the contentment of angling populations (ADCNR 2016; Gowan 2015).

1.5. Species profiles

All fishes collected from Lake Guntersville are members of the class Actinopterygii, the ray-finned, fishes and are native to freshwater habitats of North America. For the present study, the skin and gut microbiomes of three fish species were examined. Of these, two well-known centrarchid species, largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus,* were chosen primarily due to their high commercial value in terms of ecotourism (i.e. competitive and recreational fisheries) as well for use as food fishes. Bluegill and especially the largemouth bass are considered among the top targeted fish species by anglers in the United States (Leornard 2005). As such, these two species are often the focus of research efforts and stocking programs that aim to help manage healthy and well-balanced ecosystems as well as keeping avid anglers happy. Despite their popularity, economic importance, and the enormous amount of research that has been carried out regarding these two species over the years, few studies have focused on the microbial communities and bacterial diversity of the gut and skin for these two species (Hashizume et al. 2005; Larsen et al. 2013; Uchii et al. 2006). The majority of studies involving bacteria associated with these species have focused on singular bacterial species, often those commonly associated with disease. The spotted gar, *Lepisosteus oculatus,* was also selected to allow for comparison of microbiomes to a more basal lineage of Actinopterygii commonly found in freshwater ecosystems such as that found in Lake Guntersville. To date, this is the first time the skin and gut microbiomes of the spotted gar has been characterized. Profiles for the selected species examined in this project are listed below.

Perciformes

Centrarchidae

Micropterus salmoides (Lacepède, 1802)

The sunfishes (Centrarchidae) range in freshwater habitats across North America (Page and Burr 2011). The fossil record dates Centrarchidae to roughly 35 million years ago (Near and Koppelman 2009). The family is composed of a total of 8 genera including *Ambloplites, Archoplites, Pomoxis, Centrarchus, Enneacanthus, Lepomis, Acantharchus,* and *Micropterus,* many of which are well known for their popularity and value as recreational and competitive sport fishes (Boschung and Mayden 2004; Mayden et al. 1992; Near and Koppelman 2009). Due largely to their immense popularity and value for inland sport fisheries, along with a growing interest for the use of some species in the aquaculture industry as food fishes, many have now been introduced outside of their native ranges across North America and around the world (Heidinger 1976; Heidinger 2000; Page and Burr 2011). Black basses (*Micropterus*) are among the most popular.

Micropterus spp. ran in North American ponds, lakes, streams, and large rivers systems and are considered to be highly important members of these aquatic ecosystems, both in terms of ecological and economical value (DeVries et al. 2014; Gowan 2015). In 2011, black basses were identified as the most popular freshwater fisheries in the country in terms of popularity with both recreational and competitive anglers (USDI 2011). Of these, the largemouth bass, *Micropterus salmoides* is the most highly targeted of all.

The boundaries of the original range of *M. salmoides* have become somewhat blurred over time due primarily to early undocumented introductions to external locations (Boschung and Mayden 2004; Page and Burr 2011). The native distribution of this

species is thought to be from southern Quebec to Minnesota, southward towards the Gulf reaching the southern tip of Florida and then scattered through the Atlantic and Gulf drainages from North Carolina to New Mexico. As a predatory species, largemouth bass depend primarily on sight for food capture and therefore prefer relatively shallow (<3 m), low-flow, clear water habitats over more turbid environments. *Micropterus salmoides* is an ambush predator and typically favors habitats with ample aquatic vegetation, woody or other structures, and brushy environments (Thompson et al. 2005). This species has been shown to have relatively small home ranges with some deviations associated with the size of the water body, size of the fish, water temperatures, overwintering, spring spawning periods, storm events, competition, and human disturbances (Fish and Savitz 1983; Lewis and Flickinger 1967; Love 2009; Thompson et al. 2005). In a study using radio telemetry, Thompson et al. (2005) found that fish moved constantly during diel sampling periods and showed no significant differences in habitat preferences or movement patterns between day and night sampling periods. Fish and Savitz (1983) observed regular movements of *M. salmoides* from weed bed to coverless areas and back again. These constant patrolling type movements are believed to be associated with foraging behaviors. Largemouth bass have also been shown to relocate their home ranges during the winter months, but they return in the spring prior to spawning periods (Thompson et al. 2005; Woodward and Noble 1997).

Depending on the latitude (temperature), the largemouth bass typically spawns from late winter to late spring or when water temperatures fall between 15-30°C (Heidinger 2000; Shultz 2004). In Alabama, the spawning period for largemouth bass typically occurs from around mid-April to mid- June (ACDNR 2016; Boschung and

Mayden 2004; Heidinger 2000; Tidwell et al. 2000). Similar to other centrarchids, the largemouth bass is a nest builder. Once temperatures remain consistently above 15°C, males will build nests on essentially any substrate that is firm and can be swept clean of debris such as tree stumps, roots, or leafy mats; however, sand and gravel beds are preferred. Each male will build a single nest by fanning out an area approximately 6 inches deep and 20 inches across in 1-4 ft of water roughly 7-8 ft from the shoreline. Largemouth bass nesting sites are usually at least 10-20 ft apart and typically contain anywhere from a few hundred to thousands of eggs laid by multiple females. Males will guard the nest and aerate eggs throughout the incubation period which usually lasts 5-10 days depending on temperatures. Once hatched, males will guard fry for an additional 14-28 days until they grow 12-25 mm in length and disperse. These periods can be very stressful for the males resulting in death for those that are not in good condition.

Proper timing of spawning periods can have a major impact on future growth rates since they vary widely and are heavily affected by environmental conditions and food availability (Boschung and Mayden 2004; Tidwell et al. 2000). The diet of largemouth bass varies with age. After absorption of the yolk sac, fry will start out feeding on a constant diet of various types of zooplankton. As fry increase in size, they will gradually begin preying on progressively larger aquatic organisms as they increase in size. Once they reach around 38mm, fingerlings will begin to add insects and other small fishes to their diets. By the time they reach 100 mm, their diets consist almost completely of fishes as well as some crustaceans (Boschung and Mayden 2004; Savino and Stein 1989). The large mouth structure of this species allows them to swallow prey 30-50% or more of their own body length, often utilizing a specialized suction feeding

mechanism to help capture prey (Sanford and Wainwright 2002). Studies have shown that largemouth bass are limited by the body depth of prey being capture rather than length (Boschung and Mayden 2004; Sanford and Wainwright 2002).

Currently, the largemouth bass has two recognized subspecies, the northern largemouth bass, *Micropterus salmoides salmoides*, and the Florida largemouth bass (FLMB), *Micropterus salmoides floridanus* (Page and Burr 2011). The FLMB is prevalent in peninsular Florida and northward towards the St. John's River on the east coast and towards (but not including) the Suwannee River drainage on the Gulf Coast. The centrarchids are notorious for hybridizing so a natural integrade zone exists in the Gulf drainages from the Suwannee River and westward towards Choctawhatchee River as well as the Atlantic drainages (Gowan 2015; Li et al. 2014). Although FLMB are much less aggressive feeders making then harder to catch, anglers often overlook these less desirable traits due to their ability to attain much larger sizes. As such, FLMB has been the subject of extensive stocking and genetic enhancement programs primarily for the purpose of providing larger sized catch for recreational and competitive anglers (ADCNR 2016; Gowan 2015; Li et al. 2014). During the years 1992-1994, Lake Guntersville was selected by biologist to test whether FLMB genes could be successfully introduced into the population if high enough densities of pure-strain FLMB were stocked into the reservoir in hopes of overwhelming and outcompeting naturally spawned northern largemouth bass. These introductions proved to be successful.

Lepomis macrochirus Rafinesque, 1819

The bluegill, *Lepomis macrochirus* (also commonly referred to as bream or panfish), is yet another well-known member of the centrarchid family. Like the largemouth bass, *L. macrochirus* is another commercially valuable sportfish that has been introduced far outside of its native range making the original distribution difficult to pinpoint (Boschung and Mayden 2004). The native range of this popular sunfish species includes the central and eastern portions of North America from the Great Lakes southward to Mexico and eastward to the Atlantic drainages from North Carolina. Anthropogenic introductions have extended this range to encompass the entire United States as well as Europe, South Africa, and other countries. In general, the centrarchid family is considered to be a stenohaline freshwater fish group (Peterson et al. 1993). Although typically associated with freshwater habitats, *L. macrochirus* is fairly tolerant of higher salinities, and abundant populations can be found in more brackish environments such as saline wetlands (Boschung and Mayden 2004; Peterson et al. 1993). Peterson et al. (1993) demonstrated that both freshwater and brackish populations of juvenile bluegill under experimental conditions were able to move through 0 - 10 ‰ salinities without showing any significant preference or noticeable behavioral response to changes in salt concentrations short-term. This suggests that bluegills are more physiologically and behaviorally tolerant of elevated salinities compared to other centrarchid species, especially *Micropterus* spp. Bluegill prefer to live in richly vegetated areas that provide a good source of protective covering to forage and hide from predator species such as the largemouth bass (Savino and Stein 1989).

The home range of the bluegill is considered relatively small in comparison to the largemouth bass (Fish and Savitz 1983). However, studies have shown that home range sizes and site fidelity may be somewhat variable depending on factors such as the type of environment (i.e. lentic vs. lotic systems), fish size, food density, and social hierarchies among the species (Fish and Savitz 1983; Minns 1995; Paukert et al. 2004). Historically, mark-recaptures studies in lotic systems indicated that the majority of bluegills are relatively sedentary, while others had more expanded ranges with some being recaptured as much as 17.6 km from the original capture site (Gatz and Adams 1994; Paukert et al. 2004). In a lentic system, Paukert et al. (2004) found home range territory ranged from 0.13 – 172 ha. This study indicated that core home ranges varied by month with the largest range occurring in April when compared to May, June, July, and September. Site fidelity was also found to be lowest among bluegills in April. In this case, fish lengths were not found to be related to home range sizes; however, home ranges did increase with rises in bluegill movement. Factors such as foraging behaviors, spawning periods, predator avoidance, and environmental conditions are often associated with daily and seasonal movements of fishes. Bluegills are warm water fish and prefer water temperatures that fall within an optimum range of 27-29 °C (Coutant 1977). Activity and movement of fish are typically considered to increase as water temperatures and, subsequently, metabolism increase (Hergenrader and Hasler 1967). However, Paukert et al. (2004) observed the highest movement of bluegills in a Great Plains lake in mid-summer when temperatures averaged 22.8 °C possibly indicating variances in optimal temperatures of bluegills from different populations or other influencing factors. Similar

to *M. salmoides*, movement did not appear to vary significantly between day and night time periods.

Similar to the largemouth bass, bluegills are also nest builders (Boschung and Mayden 2004). However, instead of having relatively isolated nests, most bluegills demonstrate high reproductive synchronization and build their nests in large colonies with very little space between individual nests (Gross and MacMillan 1981; Neff et al. 2004). Parental males typically build nests in open aggregations where water depth, water temperature, and substrates such as gravel or sandy bottoms are relatively similar (Gross and MacMillan 1981). However, on rare occasions, some parental males will construct solitary nests away from the typical colonial spawning sites (Gross and MacMillan 1981; Neff et al. 2004). Females arrive at spawning sites and may deposit eggs into several nests. The close proximity of these nests provides added advantages. As nesting territories of parental males often overlap, these males are better able to protect spawn by demonstrating group mobbing of any invading predators. In addition, group protection effort allows more time for parental males to care for and fan fertilized eggs until they hatch resulting in reduced fungal infections (Côté and Gross 1993; Neff et al. 2004). A disadvantage to colonial nesting is that it allows easier access to sexually mature, cuckold or sneaker males that fertilize eggs at the nesting sites of other males without building nests of their own or providing any parental care to broods. Parental males will remain with the nests for approximately 7 days until free-swimming fry leave the nests. Once fry leave the nests, males move to deeper waters to feed until the next round of spawning begins, usually 3-10 days later during the spawning season. In Alabama, bluegills are

known to have protracted spawning periods that last from around April to September (ADCNR 2016).

Bluegills are sight feeders and are typically known to feed during the day; however, nocturnal feeding behaviors have also been documented (Keast and Welsh 1968; Paukert and Willis 2002; Sarker 1977). Bluegills have small mouths in which they use to capture individual prey items, swallowing them whole in most cases (Mittelbach 1981). Studies indicate that bluegills typically feed on benthic and epiphytic macroinvertebrates as well as zooplankton when predation risk is low (Paukert and Willis 2002). Habitat use and foraging behaviors of *L. macrochirus* are largely dependent on the availability of prey and the risk of predation (Savino and Stein 1982). During its life, the bluegill undergoes ontogenetic habitat shifts between littoral vegetation and pelagic zones (Werner and Hall 1988). In addition, Mittlebach (1981) demonstrated that this species will select prey items and foraging habitats that are the most energetically favorable for the fish. Seasonality can also play a role in habitat and prey selection as larger fish will move from rich, vegetated foraging areas in the spring to pelagic waters in the summer to feed on abundant zooplankton populations. Smaller fish (<100 mm standard length), on the other hand, remain within or near aquatic vegetation to reduce predation risks, only moving to more open waters to feed on larger prey items during the night.

Lepisosteiformes

Lepisosteidae

Lepisosteus oculatus Winchell, 1864

The spotted gar, *Lepisosteus oculatus*, is one of a group of seven basal North American fishes constituting the family Lepisosteidae (Page and Burr 2011). Extant gars are known to be part of two phylogenetically distinct lineages that date back as far as the Cretaceaous period at least 75 million years ago (Boschung and Mayden 2004; Wiley 1976). As such, the family is currently broken down into two recognized genera, *Atractosteus* and *Lepisosteus.* Gars have ganoid scales, a lung-like gas bladder capable of respiration, a spiral valve, and an abridged heterocercal tail (Page and Burr 2011). They have a relatively restricted geographical distribution from North to Central America with some *Atractosteus* spp. being found as far south as Costa Rica and Cuba. Although biogeographic ranges of extant species seem to be focused more in North America, fossils representing both genera have been found in India, southern South America, and parts of Africa including Madagascar (Boschung and Mayden 2004; Gottfried and Krause 1998; Mendoza Alfaro et al. 2008). Historically, *Lepisosteus* spp. have been considered to be nuisance species by many anglers and fisheries managers that regarded them as being highly predaceous and harmful to commercially valuable game fishes (Scarnecchia 1992). However, their high relative abundance and predatory potential in aquatic ecosystems suggest gars may be important in maintaining the trophic webs of these systems (Ostrand et al. 2004; Snedden et al. 1999).

The geographic distribution of *L. oculatus* includes the middle Great Lakes (i.e. Lake Erie, Lake Michigan, and parts of Lake Huron drainage basins), the Mississippi River drainages from Illinois to the Gulf coast, Gulf drainages from the lower Apalachicola River in Florida to the Neuces River in Texas, and as seen in the present study, the Tennessee River drainage (Boschung and Mayden 2004; Love 2004; Pope and

Wilde 2003; Shultz 2004). Spotted gars are commonly found in medium to large river systems characterized by reduced water flow, ample amounts of aquatic vegetation, and relatively clean waters (Boschung and Mayden 2004). However, they are also known to inhabit other freshwater habitats such as swamps, sloughs, lowland creek backwaters, and other aquatic habitats that host abundances of aquatic vegetation or debris (Page and Burr 2011). The specialized physostomous swim bladder allows them to breathe atmospheric air, providing an added advantage over other piscivores, such as the largemouth bass, under more anoxic (< 2.0 mg/L of oxygen) conditions (Burleson et al. 1998; Doerzbacher and Bryan 1983; Snedden et al. 1999). The spotted gar prefers to reside in shallow, brush-covered, open waters between 3-5 m in depth and normally has a home range of roughly 265.1 ha on average. They typically remain relatively stationary throughout the day often basking near fallen trees or other debris and become more active during the night. Studies involving telemetry techniques indicate the home range of *L. oculatus* increases significantly in response to diel and seasonal patterns (Snedden et al. 1999). Spotted gars were observed to utilize a significantly greater home range at night than during daylight hours in association with increased nocturnal foraging behaviors. In addition, seasonality has been shown to have a significant effect on movements with the home range in the spring increasing nearly 30-fold when compared to the summer or winter-fall months. This increase has been associated with rising water temperatures, spawning behaviors, and seasonal flooding patterns.

The spotted gar does not actively pursue its prey but rather utilizes more of an ambush or lie-in-wait predatory foraging strategy (Kammerer et al. 2005; Ostrand et al. 2004; Snedden et al. 1999). The diet of this species varies as the size of the gar increases,

with smaller fish feeding on small aquatic animals such as mosquito larvae and small crustaceans, juveniles feeding on small fishes, and adults feeding primarily on larger fish and crustaceans (Boschung and Mayden 2004). Although their slow movements and more camouflaged appearance may give them advantage over unsuspecting prey in more densely vegetated environments, their success as predators seems to be more related to behaviors of the species of prey, particularly those that exhibit less evasive behaviors and residing in shallower habitats. (Ostrand et al. 2004).

Lepisosteus oculatus is typically not a gregarious species except during the spawning season when they aggregate for reproduction purposes in shallow flood plains or areas with adequate aquatic vegetation that provide protective nurseries for progeny (Boschung and Mayden 2004; Love 2004; Mendoza Alfaro et al. 2008). The spawning season is primarily associated with spring, but various studies have shown spawning periods ranging from February to June depending on location and temperature (Love 2004; Tyler and Granger 1984). Spawning behaviors typically involved aggregations of one female closely accompanied by 2-8 smaller males at spawning sites. Tyler and Granger (1984) noted interruptions in spawning behaviors associated with temperature declines and increased turbidity associated with spring rains.

II. OBJECTIVES

The bacterial communities that constitute fish microbiomes are now recognized as essential components of host health and defense from invading pathogens. Therefore, a better understanding of the natural bacterial communities of healthy individuals and how they interact with the host and other environmental factors is of critical importance. For this study, I wanted to expand the existing body of research on fish microbiomes to include the skin and gut microbiomes of important freshwater sport fishes. My working hypothesis was that the species of each fish would exert the highest influence on microbiome composition particularly in more stable gut communities. My specific objectives were as follows:

1) Characterize the gut and skin microbiomes of three common freshwater fishes including two commercially valuable sport fishes, largemouth bass *Micropterus salmoides* and bluegill *Lepomis macrochirus* as well as the more primitive spotted gar *Lepisosteus oculatus*.
2) Compare intra- and interspecies differences in the composition of each microbiome.
3) Identify potential influences of seasonality on core microbial communities.

III. MATERIALS AND METHODS

3.1. Sample collection.

All fish species were collected from the Lake Guntersville reservoir on the Tennessee River in northern Alabama. Sample collections took place in August and November 2014 as well as May 2015 in effort to survey fish microbiomes at seasonal time points for Summer, Fall, and Spring, respectively (Figure 2). Fishes were captured via electrofishing techniques (7.5 GPP Smith Root electrofishing boat) and identified on site by students of the Auburn University Quantitative Fisheries Laboratory (QFL). In order to help ensure viability of microbiome samples, all fishes were maintained in live-wells until they could be returned to the shoreline for harvesting and preservation of target tissues. At least five individuals per species were collected during each sampling event in effort to account for possible absent or inadequate microbiome samples.

Upon arrival to the docking site, fishes were humanely euthanized, segregated by species, and placed on ice for immediate processing of tissues. Prior to harvesting of selected tissues, total lengths (mm) were recorded for each individual (Table 1). After each skin sample had been collected and stored in the appropriate preservation media, the ventral and lateral sides of the fish, particularly surrounding the urogenital pore, were sprayed with 70% isopropanol and wiped clean repeatedly (x3) in effort to eliminate the contamination of gut microbiome samples with remnant communities from the external

skin. Once excess isopropanol had been sufficiently dried from the skin, fish were first squeezed in an anterior to posterior direction to allow gut contents to be expelled from the urogenital pore onto a sterilized spatula. If adequate samples could not be obtained by external pressure alone, sterile scissors were used to expose the body cavity, and the gut contents were aseptically squeezed directly from the intestine onto the sterile spatula. Gut or fecal contents were carefully transferred into a labelled 2 ml microcentrifuge tube containing 800 µl of RNA*later*®. Since we were interested in communities not only at the species level but also at the individual level, all materials (i.e. gloves, surgical pads, surgical tools, etc.) were sterilized or replaced between each individual sampled.

After all fishes had been processed, microbiome samples were placed on ice and transported to the Southeastern Cooperative Fish Parasite and Disease Laboratory (SECFPD). Samples were held at 4°C for a minimum of 6 hours to allow for thorough penetration of the preservation media into tissues. Samples were then transferred to -80°C storage until DNA extractions could be performed.

3.2. DNA extraction.

All DNA extractions were performed at the SECFPD. Before beginning DNA extractions, selected samples were transferred from -80°C storage to -20°C for temporary storage and partial thawing of tissues frozen in RNA*later*®.

Skin samples were removed from the -20°C freezer, placed immediately on ice, and allowed to thaw for DNA extractions. Once the tissue samples had thawed in RNA*later*®, skin samples were removed from the storage medium, cut into smaller pieces, and transferred into sterile, pre-weighed 1.7 ml microcentrifuge tubes. Total

weights were taken for tissues in tubes, and adjustments were made as needed to meet target weights of 25 mg of tissue per sample as specified by the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA; tissue kit). In effort to reduce potential problems in downstream microbial analyses associated with excess salts found in RNA*later*®, samples were first gently washed in 750 µl of ice cold, sterile phosphate buffered solution (PBS) three times. After the final wash, DNA extractions were performed following the purification of total DNA from animal tissues protocol, including pre-treatment steps to aid in lysis of Gram-positive bacteria. The manufacturer's instructions were used with only slight deviations from the standard protocols. During the elution step, only 50 µl of buffer AE was used rather than the 200 µl suggested by the manufacturer in effort to reduce potential over dilution of the total DNA product (Qiagen 2001). This step was repeated a second time in a separate 1.7 ml microcentrifuge tube for a total elution of 100 µl of total DNA (Refer to Appendix A for more detailed information of modified DNA extraction protocols for the Qiagen DNeasy® Blood and Tissue Kit).

Fecal samples were allowed to thaw slowly and were carefully washed using ice cold, sterile PBS (three times) to remove excess salts found in RNA*later*® from the samples. Total weights were taken for tissues in tubes and adjustments were made as needed to meet target weights of 180-200 mg (or as the highest volume possible) of stool per individual sample as specified by the QIAmp® DNA Stool Mini Kit (Qiagen, Valencia, CA; stool kit). In effort to reduce potential problems in downstream microbial analyses associated with excess salts found in RNA*later*®. During the elution step, a total of 50 µl of buffer AE was added to the QIAmp® spin column (Refer to Appendix B for more detailed information of modified DNA extraction protocols for the Qiagen

QIAmp® DNA Stool Mini Kit). Total DNA concentrations were then quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Nanodrop Technologies, Wilmington, DE, USA).

3.3. PCR Amplification and Sequencing.

Based on DNA quality, yield, and availability of replicates, a total of 70 samples were submitted to MR DNA® (Shallowater, TX, USA) for PCR amplification and next-generation sequencing. For each tissue type, a total of 4 replicates were chosen for each fish species, for each of the 3 sampling events for a total of 12 samples per species per tissue. Unfortunately, adequate fecal samples were not successfully acquired for the spotted gar in November so only 11 total samples were able to be submitted for sequencing. Universal bacterial primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with a barcode on the forward primer were used to target the 16S rRNA gene V4 variable region. The HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used to run all samples under the following PCR conditions: an initial denaturation step for 3 minutes at 94 °C followed by 28 cycles of 94 °C for 30 s (denaturing), 53 °C for 40 s (annealing), and 72 °C for 1 min (extension) before performing a final elongation step for 5 min at 72 °C. Following amplification, PCR products for all samples were run through a 2% agarose gel to verify successful amplification and relative band intensity of the target DNA. Multiple samples were pooled together and purified using calibrated Ampure XP beads to prepare the Illumina DNA library prior to sequencing.

All samples were sequenced as paired-end reads on the Illumina MiSeq platform following the manufacturer's instructions. Resulting sequences were processed using a

proprietary pipeline (MR DNA, Shallowater, TX, USA). Sequencing data were joined, and all barcodes, primers, and sequences <150 bp were removed. Additionally, sequences with ambiguous base calls and spans of identical monomer units longer than 6 bp were removed. Denoising of sequences was also performed, and operational taxonomic units (OTUs) were generated. Cut-offs for OTU assignment were defined at a 97% similarity (<3% sequence variation) in concurrence with the prokaryotic species concept (Rossello-Mora and Amann 2001). Taxonomic classifications were obtained using BLASTN against the GreenGenes database (DeSantis et al. 2006).

3.4. Data analysis.

Since species richness and evenness can only be compared between samples of equal size, all sequences were randomly selected in order to standardize to the least number of sequences found for the skin (N=11,574) and gut (N=52,205) microbiomes. After standardization of each sample type to the sample yielding the least number of total sequences, Mothur v.1.33.3 (Schloss et al., 2009) was used to generate rarefaction curves and to calculate diversity statistics including Good's coverage, Shannon Evenness Index (SEI), abundance-based coverage estimation (ACE), Chao1, observed OTUs, and shared OTUs. SAS 9.2 (Statistical Analysis System, SAS Institute, Cary, NC) was used to run both one-way ANOVAs with Tukey multiple comparison tests ($\alpha = 0.05$) as well as two-way ANOVAs in order to determine differences in the observed species richness (in observed OTUs), the total predicted species richness (ACE and Chao1), and species evenness (SEI) between samples. One-way ANOVAs were run first to determine potential differences between species and sampling dates, followed by two-ANOVAs to determine if a significant interaction variable existed. OTU tables including all samples

were loaded into PRIMER v6 for clustering using the similarity matrix and analysis of similarities (ANOSIM). Genera tables were also loaded into PRIMER for similarity percentages (SIMPER) analysis in order to determine specific taxonomical differences between communities. The cut-off for low contributions was set to the default at 90%.

IV. RESULTS

4.1. Analysis of the skin and gut microbiomes.

Prior to standardization of each sample to the lowest number of sequences, sequencing of the 16S rRNA gene using the Illumina MiSeq platform resulted in a total of 5,419,640 sequences for combined the skin and gut microbiome samples. Overall, 4527 OTUs were identified for the skin and the gut. Of these, 2744 OTUs were identified for skin, and 4130 OTUs were identified for the gut. Approximately 51.84% of the total OTUs were shared between the skin and gut bacterial communities (Figure 3). A total of 397 OTUs were found to be unique to the skin communities, while 1783 OTUs were found exclusively in the gut for overall sampling of the three species over time.

In order to accurately compare species richness and evenness, sequences were standardized to the lowest number of obtained sequences for both the skin (n= 11,574) and the gut (n= 52,205) samples for 35 individuals (i.e. Bluegill (n=12); Spotted Gar (n=11); Largemouth bass (n=12)) for totals of 405, 090 sequences with 2441 OTUs and 1,827,175 sequences with 3066 OTUs, respectively. Regardless of sequence and OTU losses associated with standardization of samples, sequence coverage was ≥ 98% for all samples.

Skin microbiome

After standardization to the lowest number of obtained sequences (n= 11, 574), a total of 405,090 out of 1,311,450 sequences and 2441 OTUs remained for all skin samples. Although sequences were reduced to approximately 31% of the original values, sequence coverage remained ≥ 98% for all samples (Table 2). High sequence coverage for skin OTUs is reflected by rarefaction curves generated by Mothur for each species (Figure 4). Total expected richness as calculated by ACE and Chao1 showed no significant differences among species. However, the total observed OTUs and the SEI for bacterial communities of the skin were found to be significantly higher in *L. macrochirus* than in *M. salmoides*. For the sampling date, the number of observed OTUs, SEI, and the number of predicted OTUs as calculated by Chao1 were significantly higher for August than for November or May. Of the three sampling periods, May represented the least observed species richness. Statistical analyses using two-way ANOVAs indicated a significant interaction variable existed between the species and the sampling month for all diversity indices in association with the skin microbiome.

Gut microbiome

For the gut microbiome, standardization to the lowest number of obtained sequences (n= 52,205) resulted in a decline from 4,108,190 sequences to 1,827,175 with 4130 OTUs remaining for all gut samples. Despite a drop of over 44% of the total sequences, sequence coverage remained ≥ 99% for all samples (Table 3). High sequence coverage for skin OTUs is reflected by rarefaction curves generated by Mothur for each species (Figure 5). All diversity indices (i.e. observed OTUs, ACE, Chao1, and SEI) showed significant differences among the three species. Observed OTUs, SEI, and the predicted OTUs as calculated by Chao1 were significantly higher in *L. macrochirus* than

in *L. oculatus* and *M. salmoides.* Of the three species, *M. salmoides* again represented the lowest observed species richness. For the sampling date, the total expected richness as calculated by ACE and Chao1 were significantly higher for August than for November or May. However, the observed richness and SEI showed no significant differences. Statistical analyses using two-way ANOVAs indicated significant differences between the species and sampling period; however, no significant interaction was identified between these two variables and the gut microbiome.

4.2. Composition of the skin microbiome

Overall, analysis of the sequence data revealed that the skin microbiomes for the three species consisted of 27 bacterial phyla with an additional 0.01% of unidentified phyla (Figure 6). At 37.33%, Proteobacteria was the predominant phylum found in the skin microbiome for all three species. Within the phylum Proteobacteria, the most abundant class of bacteria found was Gammaproteobacteria (16.69%) followed in succession by Betaproteobacteria (11.39%), Alphaproteobacteria (8.48%), Deltaproteobacteria (0.72%), and Epsilonproteobacteria (0.05%). Twenty-one phyla (these counts not including representatives found in all from the "Spring Alpine Meadow" candidate division and other unidentified bacterial phyla, were present in varying abundances for all three species over time. *L. macrochirus* and *L. oculatus* each had one unique phylum when compared to the other species. The phylum Ignavibacteriae was found in some of the skin samples from bluegill, while Dictyoglomi was present in a single spotted gar sample. Both of these phyla were collected from skin samples in November and are typically associated with thermophilic bacteria.

Overall, 626 genera were found to populate the skin microbiomes for the three species. For the overall bacterial communities of the three species, the top 10 genera found were *Cetobacterium* (19.33%), *Clostridium* (10.39%), *Deinococcus* (9.36%), *Plesiomonas* (2.89%), *Pseudomonas* (2.89%), *Cloacibacterium* (2.51%), *Bacteroides* (2.32%), *Aeromonas* (2.06%), *Vibrio* (1.72%), and *Acinetobacter* (1.54%). The relative percent abundances in which these genera constituted the skin microbiomes varied between the species and across sampling points within each species (Table 4). Variations in abundances could also be seen between individuals both across sampling dates and within, but the specifics of this data are not reported herein.

Multidimensional scaling (MDS) plots based on skin OTU abundances were generated in effort to better visualize clustering patterns for each factor, fish species and sampling date. MDS plots indicated the bacterial composition of the skin was influenced more by sampling date than by fish species (Figure 7). These results were supported my ANOSIM for both fish species and sampling date (Table 5). MDS was also used to visualize grouping of individuals by sampling date within each fish species (Figure 8). Within each species, all groupings were well separated by date as confirmed by ANOSIM with global R values of 1.000, 0.817, and 0.867 for bluegill, largemouth bass, and spotted gar, respectively. A two-way crossed ANOSIM (Table 6) was also run to pick up on possible interactions between the fish species and the sampling date. Overall analysis and pairwise tests indicate some type of interaction does exist between these two factors resulting in a higher level of separation between groups. This can be better visualized by an MDS for all groupings of fish species over time (Figure 9).

One-way crossed SIMPER analyses by bacterial genera found the largest differences in the composition of skin communities between fish species (Table 7) for the *L. macrochirus* and *M. salmoides* and sampling dates (Table 8) for November and May. However, a two-way cross SIMPER analysis accounting for interactions between the two factors indicated the highest dissimilarity was found between *L. oculatus* and *M. salmoides. Cetobacterium* contributed to the highest percentages of dissimilarity for all pairwise groupings of fish species and sampling dates.

4.3. Composition of the gut microbiome

Similar to the skin microbiome, the overall gut microbiome was again found to consist of 27 bacterial phyla with a minute percentage (0.0003%) of unidentified phyla (Figure 10). Not counting the incidence of unidentified bacterial taxa, 19 phyla were found to be present in the gut communities of all three species. Over 97% of the overall bacterial communities of the gut were composed primarily of four phyla: Fusobacteria (35.13%), Firmicutes (32.52%), Proteobacteria (15.87%), and Bacteroidetes (14.62%). Each species of fish had at least one phylum present that was unique to its gut microbiome. *L. macrochirus* had the highest incidence of unique phyla present including the phyla Chlorobi (1 individual/month sampled), Elusimicrobia (1 individual in May), and Synergistetes (2 individuals in May). Other unique phyla found were Deferribacteres and Candidatus saccharibacteria (part of the "Spring Alpine Meadow" candidate division) identified in the gut communities of spotted gar and largemouth bass, respectively. Ignavibacteriae and Thermotogae were also found in minute abundances in a few gut samples from both the bluegill and the spotted gar during November and May sampling dates.

In total, 804 genera were identified to inhabit the gut communities for the three species. The top 10 genera found in the gut microbiome for all samples were as follows: *Cetobacterium* (35.02%), *Clostridium* (26.97%), *Bacteroides* (13.66%), *Plesiomonas* (7.90%), *Aeromonas* (3.58%), *Romboutsia* (1.73%), *Phyloobacterium* (1.63%), *Mycoplasma* (1.42%), *Turibacter* (1.03%), and *Ferrovum* (1.00%). Although *Cetobacterium* was found to make up the highest percentages of the gut microbiome overall, the overall gut communities of the bluegill were dominated by primarily by *Clostridium* (43.75%). *Clostridium* constituted over 51% of the gut microbiome during August and November; however, a shift was seen in May where *Cetobacterium and Clostridium* constituted 28.42% and 28.09% of bacterial communities of the gut, respectively. The relative percent abundances in which these genera constituted the gut microbiomes varied between the species and across sampling points within each species (Table 9). Variations in abundances could also be seen between individuals both across sampling dates and within, but the specifics of this data are not reported herein.

Multidimensional scaling (MDS) plots based on gut OTU abundances were generated in effort to better visualize clustering patterns for the designated factors, fish species and sampling date. MDS plots indicated the bacterial composition of the gut was again influenced more by sampling date than by fish species (Figure 11). These results were supported my ANOSIM for both fish species and sampling date (Table 10). MDS was also used to visualize grouping of individuals by sampling date within each fish species (Figure 12). Within each species, the bluegill and largemouth bass showed moderate to high separation by sampling date as supported by ANOSIM with global R values of 0.690 (separated but overlapping groups) and 0.813 (well separated),

respectively. However, the spotted gar had a lot more variability between replicates and showed no significant differences between sampling dates (R=0.187: barely separated). A two-way crossed ANOSIM (Table 11) was also run to pick up on possible interactions between the fish species and the sampling date. Overall analysis and pairwise tests indicate some type of interaction does exist between these two factors resulting in a higher level of separation between groups. This is demonstrated by an MDS for all groupings of fish species over time (Figure 13).

Similar to analyses for the skin microbiome, one-way crossed SIMPER analyses by bacterial genera found the largest differences in the composition of gut communities between fish species (Table 12) for *L. macrochirus* and *M. salmoides* as well as sampling dates (Table 13) for November and May. The bacterial genera *Cetobacterium, Clostridium, Bacteroides,* and *Plesiomonas* accounted for the highest percentages of dissimilarity for all pairwise groupings between fish species.

V. DISCUSSION

In recent years, a paradigm shift has occurred in the area of organismal health and pathogenesis (Vayssier-Taussat 2014). Where Koch and Hill's fundamental postulates equating to "one microbe—one disease" were once regarded as the rule, research has now shifted to a more holistic view in which whole microbial communities give rise to and participate in complex interactions that can ultimately impact and fuel disease processes. Due largely to the ever growing body of research concerning mammalian species, the bacterial communities that comprise the microbiomes of various internal and external surfaces of the body are now recognized as integral components to the overall health of the host. Historically somewhat hampered by culture-dependent methods that often produced incomplete or biased results, research regarding microbiomes is now conceivably one of the fastest developing fields in biology (Christian et al. 2015). With the advent of newer, more advanced sequencing technologies and data processing platforms, researchers are now able to more fully unearth the cryptic diversity and function of these microbial communities much more quickly and at a fraction of the cost (Llewellyn et al. 2014; Zimmerman et al. 2014). Although research lags far behind mammalian microbiome studies, the bacterial communities that constitute fish microbiomes are now considered to be essential components in host health, nutrition, growth and development, and defense against invading pathogens (Austin 2006; Nayak 2010). This study aimed to expand on existing information concerning fish microbiomes by characterizing the skin and gut microbiomes of three well-known freshwater fishes

from the Lake Guntersville reservoir in northern Alabama. I also hoped to identify potential influences of the environment, in this case primarily targeting seasonal impacts, on the composition of these communities.

For the skin microbiomes, Proteobacteria represented the most abundant phylum found for largemouth bass, bluegill, and spotted gar. More specifically, Gammaproteobacteria constituted the highest overall percentage of Proteobacteria found in the three species. These results are consistent with previous findings from the skin and mucus of various fish taxa from both freshwater and saltwater environments (Arias et al. 2013; Cipriano 2011; Larsen et al. 2013; Larsen et al. 2015; Mohammed and Arias 2015). While this overall finding was consistent with past research at the phylum level, the genus that accounted for the highest percentage of overall bacterial sequences of the skin was not. *Cetobacterium* (19.33%) was found to be the most abundant genus overall for skin communities of the three species (Table 4). Within each species, the highest abundance of this genus was seen in *M. salmoides* (32.25%), with *Cetobacterium* representing the highest percentages of bacterial sequences for August (36.24%) and November (43.52%). For May, on the other hand, *Cetobacterium* percentages in largemouth bass dropped to 1.76% of total sequences. Finding this bacterial genus in such appreciable numbers as part of the skin microbiome is surprising since the vast majority of reports for this genus have typically been associated with the gastrointestinal tracts of mammals and other fishes (Finegold et al. 2003; Foster et al. 1995; Larsen et al. 2014; Li et al. 2014; Sugita et al. 1991; Tsuchiya et al. 2008).

Cetobacterium is typically described as a genus of non-motile, short, pleomorphic, gram-negative, non-sporeforming rods that are microaerotolerant (Finegold

et al. 2003). While considered to be mostly anaerobic, some bacterial species in this genus have been shown to be able to grow at as much as 6% oxygen. In the gut environment, some strains of *Cetobacterium* are capable of producing high amounts of vitamin B12 and also inhibiting growth of some other bacterial taxa (Sugita et al. 1991; Sugita et al. 1996). So why are they so abundant in the overall skin in this study? Could samples have been contaminated during sample prep and DNA extractions? This possibility is very unlikely. Fin and fecal samples from individuals were performed completely independently of each other on separate days using two different Qiagen DNA extraction kits. Could the fin and fecal samples have been cross contaminated somehow during on-site sample collections? Possible but not very probable. In all cases, fin samples were taken prior to collection of fecal samples for each fish. Surgical tools were soaked in 70% isopropanol and repeatedly cleaned between each sample collection in effort to eliminate or reduce unintentional carryover of bacterial cells between samples. If contamination is indeed a factor, one of the most likely sources of contamination could be through transmission in live wells. Fishes were collected via electrofishing techniques and kept alive in onboard live wells until they could be returned to the shore for sample processing. This step was taken in effort to help ensure freshness and viability of skin and gut samples until tissues could be extracted. Although most reports of this genus have been associated with the gastrointestinal tracts of various organisms, *Cetobacterium* has also been reported in small percentages (2.19% of sequence abundance) in earthen pond water samples from an aquaculture facility in China (Li et al. 2014). Perhaps maintaining multiple fishes within a live well has the potential (even with fresh water being run into them every few minutes) to create an environment

in which conditions are favorable for increased concentrations and consequently inoculation of mucoid surfaces by *Cetobacterium.* Of course, the latter scenario is entirely speculative since water samples were not taken in this trial but may warrant further investigations into live well conditions as well as the effects of increased densities and proximity on the structure of the skin microbiomes of fishes.

The possibility also exists that these findings are correct, and the heightened abundances of *Cetobacterium* in the skin microbiome are reflective of certain, unaccounted for environmental characteristics or behavioral aspects of the host. While *Cetobacterium* accounted for the highest abundances overall for the three species, percentages varied across species, across sampling dates, and across individuals. In August, *Deinococcus* accounted for the highest number of sequences in both the bluegill and the spotted gar, while *Clostridium* and *Holospora* made up the highest percentages for bluegill and spotted gar, respectively, in November.

When looking at the overall diversity and richness for each species over time (Figure 4), it is also noteworthy to mention that a noticeable trend was observed in the skin microbiomes of bluegill and the spotted gar. Over the course of the project, an overall decrease in the diversity was seen from August through May of the sequential sampling year. This trend was confirmed by statistical analyses which found that diversity as significantly higher in August than in November and then May.

Interestingly, the skin communities of largemouth bass also seemed to shift somewhat during the month of May. Where *Cetobacterium* and *Clostridium* were found in the highest percentages of skin communities for largemouth bass in August and November, *Cloacibacterium* (9.32%), *Aeromonas* (8.86%), and *Hylemonella* (5.15%)

made up the highest percentages in May. In Alabama, largemouth bass typically spawn from around mid-April to mid- June when water temperatures increase to roughly 17 – 20 °C (ACDNR 2016; Boschung and Mayden 2004; Heidinger 2000; Tidwell et al. 2000). During this time, males often build nests in relatively still, shallow waters with weedy or wooded areas often near the shoreline. After spawning occurs, males will remain with the nest to care for eggs and ward off potential predators until broods are able to leave the nest. During this period, males could potentially become immuno-compromised as they do not leave the nest to feed during this time but continue to expend energy to protect their eggs. Therefore, one might speculate that this overall shift in community structure could potentially be related to changes in habitat use and spawning behaviors. However, sexes of individual largemouth bass were not obtained for this particular study.

Micropterus salmoides was shown to have the least bacterial diversity for both the skin and gut microbiomes when compared to *L. macrochirus* and *L. oculatus*, with significant differences being found in the diversity of *L. macrochirus and M. salmoides* in both cases. Larsen et al. (2014) found similar results in an experimental recreational fishing pond from which fecal contents of channel catfish (*Ictalurus punctatus*), bluegill, and largemouth bass were sequenced via pyrosequencing techniques and compared. In both cases, bluegills were observed to have significantly higher observed OTUS, expected richness, and evenness when compared to largemouth bass. While the specific stomach and fecal contents of the fish species used in this study were not analyzed, bluegills (generalists) were regularly observed to have greater incidences of plant materials in fecal samples, while the contents of the largemouth bass (piscivorous) were typically more consistent with digested vertebrate species. In mammalian studies, both

the host phylogeny and the diet have been implicated as potential factors influencing the bacterial diversity of gut microbiomes, with herbivores typically demonstrating higher bacterial diversity than carnivores (Ley et al. 2008). Also worth mentioning, similar to Larsen et al. (2014), largemouth bass were shown to have high abundances of *Cetobacterium* in the gut, averaging close to 52% composition over the course of the study. The gut microbiomes of bluegill from this reservoir, on the other hand, demonstrated a higher proportion of *Clostridium.*

For purposes of this study, Lake Guntersville was considered to be a single hydrological unit. In this respect, all collection sites were compared equally over the course of the study based on the idea that fishes in their dynamic, natural habitats are not confined to static locations. From this perspective, seasonality was shown to significantly influence both the skin and gut microbiome structures of fishes in Lake Guntersville. Sampling date resulted in a higher degree of separation between samples than the species level in skin communities (R=0.583) and to a lesser extent in the gut communities (R=0.391) indicating these communities may be more stable than in the skin (Figures 7 & 11). In addition, analyses using two-way ANOVAs indicated that a significant interaction variable existed between the species and the sampling date for the skin microbiome. This may indicate changes in behavior, habitat use, or feeding patterns associated with seasonal shifts for each species. Although sampling locations were not factored into these analyses, the possibility for complex interactions between the gut microbiome, species, season, and geographic locations cannot be ignored.

Home ranges for these species have been shown to vary with the size of the water body, size of the fish, water temperatures, overwintering, spring spawning periods, storm

events, competition, diel movements, foraging shifts, and human disturbances (Fish and Savitz 1983; Lewis and Flickinger 1967; Love 2009; Snedden 1999; Thompson et al. 2005). Lake Guntersville has consistently been ranked as one of the best bass fishing lakes in the United States, generating millions of dollars in revenue each year associated primarily with recreational and competitive fisheries (Hall et al. 2012; Snellings 2015). As such, the high anthropogenic use of Lake Guntersville along with the high number of catch and release tournaments that take place there annually creates unique problems with defining specific site locations and home ranges for largemouth bass in some cases. Telemetry studies have suggested that many surviving largemouth bass displaced by catch and release fishing events often return to their home ranges (Phillip and Ridgeway 2002; Richardson-Helt 2000; Ridgeway 2002; Stang et al. 1996). However, Wilde (2016) compiled both published and unpublished estimates concerning dispersal distances of *Micropterus* spp. that were captured and released during fishing tournaments that indicated otherwise. Data from this research suggested only around 14% of *M. salmoides* that were released following tournaments or other catch and release events returned to their original capture sights. Furthermore, roughly 51% of the largemouth bass released did not move any farther than 1600 m from the site of release. Although sampling occurred at different sampling sites for each of the three seasonal time points, the above reasons are used as justification for the elimination of location as a factor for purposes of this research. However, future research would benefit from seasonal samplings of the microbiome at multiple sampling locations in effort to account for potential influences of both factors.

In summary, this is the first time both skin and gut microbiome samples from *Micropterus salmoides, Lepomis macrochirus,* and *Lepisosteus oculatus* have been characterized at multiple seasonal time points using high-throughput Illumina MiSeq techniques. This research also represents the first ever characterization of *Lepisosteus oculatus* microbiomes. All samples were wild-caught from the Lake Guntersville reservoir in northern Alabama, and all tissue samples were processed individually to allow for observation of both intra- and interspecies differences in bacterial composition over time. *Micropterus salmoides* had the least bacterial diversity of the three species. For both the skin and the gut microbiomes, sampling date was found to exert a stronger influence on microbial composition than the species itself; however, season had a lesser impact on the gut microbiome that in the skin indicating the gut microbiomes are more stable. Analyses using two-way ANOVAs indicate a significant interaction variable exists between the species and the sampling date for the skin but not for the gut reflecting the more stable nature of gut communities. The greatest dissimilarity for the skin and the gut was found between the largemouth bass and the bluegill, while the greatest differences in sampling dates were seen between November and May.

Future Directions:

As is common within the realm of scientific research, when seeking to answer certain questions or gain a better understanding of a particular problem, we typically end up with just as many (if not more) questions than we started with. This particular project is no exception to that rule. The overall hope for this project was to gain a better understanding of the bacterial communities that comprise the gut and skin microbiomes of wild-caught fishes and learn how certain environmental factors may influence their

colonization and structure. Below, I have outlined a few questions and relevant issues for further study and future consideration.

- Can the microbiome serve as a potential sentinel for environmental conditions?
- Do mucosal surfaces of fish exert chemotaxis effects that influence microbial composition?
- Potential introductions of foreign bacteria or pathogens associated with stocking programs, catch and release fisheries, or invasive introductions.
- Potential variations in the microbiomes of hybrid fishes (i.e. Northern Largemouth Bass vs. Florida Largemouth Bass).

Table 1. Total length (mm) of individual sampled along with average lengths (mm) for each species by season and overall average lengths (mm) for each species. Fish IDs consist of two letters and a number. The first letter represents the species, B: Bluegill, G: Spotted Gar, and L: Largemouth Bass. The second letter represents the sample month, A: August, N: November, and M: May. The number in each Fish ID represents the replicate number (1-4) for that particular sampling event.

Fish ID	Length (mm)	Month Averages (mm)	Species Averages (mm)
BA1	138		
BA2	145		
BA3	136		
BA4	128	**Average:** 136.75 ± 6.99	
BN1	195		
BN2	184		
BN3	192		
BN4	182	**Average:** 188.25 ± 6.24	
BM1	225		
BM2	205		
BM3	229		
BM4	192	**Average:** 212.75 ± 17.37	**Total Average:** 179.25 ± 34.65
GA1	706		
GA2	579		
GA3	456		
GA4	600	**Average:** 585.25 ± 102.54	
GN1	504		
GN2	620		
GN3	395	**Average:** 506.33 ± 112.52	
GM1	580		
GM2	650		
GM3	515		
GM4	536	**Average:** 570.25 ± 59.67	**Total Average:** 558.27 ± 88.95
LA1	444		
LA2	369		
LA3	345		
LA4	362	**Average:** 380.00 ± 43.84	
LN1	377		
LN2	360		
LN3	346		
LN4	255	**Average:** 334.50 ± 54.49	
LM1	385		
LM2	432		
LM3	374		
LM4	351	**Average:** 385.50 ± 34.08	**Total Average:** 366.67 ± 47.13

Table 2. Diversity indices for the skin microbiome as calculated by Mothur (v. 1.33.3). Operational taxonomic units (OTUs) are defined at 97% similarity. Significance among total values for each fish species and sampling periods were determined by one-way ANOVAs followed by Tukey's post hoc tests.

Diversity of the Skin Microbiome

Sample ID	Fish Species	Month sampled	# Observed OTUs	# of Predicted OTUS		Shannon Evenness	Good's Coverage
				ACE	Chao1		
BAF1	*L. macrochirus*	August	510	1009	778	0.780	0.984
BAF2	*L. macrochirus*	August	665	1118	992	0.803	0.981
BAF3	*L. macrochirus*	August	556	1196	877	0.802	0.983
BAF4	*L. macrochirus*	August	520	1081	855	0.741	0.982
BNF1	*L. macrochirus*	November	439	1388	942	0.793	0.983
BNF2	*L. macrochirus*	November	503	1079	838	0.762	0.983
BNF3	*L. macrochirus*	November	585	1008	885	0.749	0.983
BNF4	*L. macrochirus*	November	522	1179	886	0.756	0.981
BMF1	*L. macrochirus*	May	359	906	626	0.616	0.986
BMF2	*L. macrochirus*	May	410	871	649	0.602	0.986
BMF3	*L. macrochirus*	May	462	894	722	0.691	0.985
BMF4	*L. macrochirus*	May	429	854	695	0.685	0.985
GAF1	*L. oculatus*	August	707	926	1015	0.765	0.982
GAF2	*L. oculatus*	August	552	1040	854	0.794	0.983
GAF3	*L. oculatus*	August	422	861	649	0.734	0.986
GAF4	*L. oculatus*	August	656	1010	982	0.775	0.982
GNF1	*L. oculatus*	November	392	1150	770	0.673	0.985
GNF2	*L. oculatus*	November	449	1120	799	0.733	0.983
GNF3	*L. oculatus*	November	401	1152	723	0.779	0.986
GMF1	*L. oculatus*	May	350	745	557	0.742	0.988
GMF2	*L. oculatus*	May	317	783	642	0.572	0.987
GMF3	*L. oculatus*	May	379	875	651	0.719	0.986
GMF4	*L. oculatus*	May	387	800	653	0.633	0.986
LAF1	*M. salmoides*	August	422	1218	861	0.759	0.984
LAF2	*M. salmoides*	August	366	1304	753	0.693	0.984
LAF3	*M. salmoides*	August	397	710	640	0.589	0.987
LAF4	*M. salmoides*	August	401	855	704	0.674	0.986
LNF1	*M. salmoides*	November	515	867	792	0.691	0.985
LNF2	*M. salmoides*	November	375	796	643	0.574	0.986
LNF3	*M. salmoides*	November	337	630	615	0.564	0.988
LNF4	*M. salmoides*	November	370	691	567	0.622	0.988
LMF1	*M. salmoides*	May	335	1014	621	0.703	0.986
LMF2	*M. salmoides*	May	384	939	702	0.738	0.986
LMF3	*M. salmoides*	May	337	997	738	0.594	0.985
LMF4	*M. salmoides*	May	452	1147	828	0.724	0.983
TOTALS							
	Lepomis macrochirus	August	563	1101	876	0.782	0.983
		November	512	1164	888	0.765	0.983
		May	415	881	673	0.649	0.986
		Overall	497	1049	812	0.732	0.984
	Lepisosteus oculatus	August	584	959	875	0.767	0.983
		November	414	1141	764	0.728	0.985
		May	358	801	626	0.667	0.987
		Overall	456	951	754	0.720	0.985
	Micropterus salmoides	August	397	1022	740	0.679	0.985
		November	399	746	654	0.613	0.987
		May	377	1024	722	0.690	0.985
		Overall	391	931	705	0.660*	0.986

Table 3. Diversity indices for the gut microbiome as calculated by Mothur (v. 1.33.3) software. Operational taxonomic units (OTUs) are defined at 97% similarity. Significance among values for each fish species and sampling periods were determined by one-way ANOVAs followed by Tukey's post hoc tests.

Diversity of the Gut Microbiome

Sample ID	Fish Species	Month sampled	# Observed OTUs	# of Predicted OTUS		Shannon Evenness	Good's Coverage
				ACE	Chao1		
BAS1	*L. macrochirus*	August	834	1511	1270	0.574	0.994
BAS2	*L. macrochirus*	August	553	923	816	0.510	0.996
BAS3	*L. macrochirus*	August	713	1169	1000	0.580	0.995
BAS4	*L. macrochirus*	August	720	1318	1119	0.554	0.995
BNS1	*L. macrochirus*	November	732	1291	1086	0.569	0.995
BNS2	*L. macrochirus*	November	784	1297	1135	0.587	0.995
BNS3	*L. macrochirus*	November	720	1734	1336	0.470	0.994
BNS4	*L. macrochirus*	November	586	1208	1005	0.481	0.995
BMS1	*L. macrochirus*	May	688	1298	1077	0.508	0.995
BMS2	*L. macrochirus*	May	605	1107	922	0.420	0.996
BMS3	*L. macrochirus*	May	787	1439	1237	0.567	0.994
BMS4	*L. macrochirus*	May	748	1610	1214	0.489	0.994
GAS1	*L. oculatus*	August	404	679	639	0.413	0.997
GAS2	*L. oculatus*	August	453	840	644	0.406	0.997
GAS3	*L. oculatus*	August	394	808	706	0.363	0.997
GAS4	*L. oculatus*	August	487	1046	815	0.436	0.996
GNS1	*L. oculatus*	November	693	1373	1091	0.552	0.995
GNS2	*L. oculatus*	November	558	1121	895	0.444	0.996
GNS3	*L. oculatus*	November	658	1107	922	0.467	0.995
GMS1	*L. oculatus*	May	624	1369	1200	0.503	0.995
GMS2	*L. oculatus*	May	610	1301	1129	0.371	0.995
GMS3	*L. oculatus*	May	532	1297	956	0.388	0.995
GMS4	*L. oculatus*	May	562	1375	1038	0.307	0.995
LAS1	*M. salmoides*	August	549	1098	884	0.455	0.996
LAS2	*M. salmoides*	August	530	886	752	0.462	0.996
LAS3	*M. salmoides*	August	405	694	581	0.418	0.997
LAS4	*M. salmoides*	August	437	787	648	0.388	0.997
LNS1	*M. salmoides*	November	590	1320	1083	0.450	0.995
LNS2	*M. salmoides*	November	547	1131	871	0.513	0.996
LNS3	*M. salmoides*	November	589	1126	965	0.508	0.995
LNS4	*M. salmoides*	November	423	868	678	0.431	0.997
LMS1	*M. salmoides*	May	524	1189	899	0.417	0.996
LMS2	*M. salmoides*	May	541	990	900	0.475	0.996
LMS3	*M. salmoides*	May	561	1324	915	0.404	0.995
LMS4	*M. salmoides*	May	576	1123	912	0.478	0.996
			TOTALS				
	Lepomis macrochirus	August	705	1230	1051	0.555	0.995
		November	706	1175	1005	0.553	0.995
		May	707	1269	1085	0.573	0.995
		Overall	706	1410	1169	0.545	0.995
	Lepisosteus oculatus	August	435	1383	1141	0.527	0.995
		November	636	1384	1138	0.512	0.995
		May	582	1337	1085	0.470	0.995
		Overall	599	1263	1060	0.494	0.995
	Micropterus salmoides	August	480	1364	1113	0.496	0.995
		November	537	1209	1003	0.472	0.995
		May	551	1142	934	0.469	0.995
		Overall	523	984	801	0.418	0.996

Table 4: Top 10 genera found in the skin microbiome for each fish species, both overall and per sampling date. Each genus is given as a percent of the overall abundance for that grouping.

Bluegill Skin		Bluegill Skin-August		Bluegill Skin-November		Bluegill Skin-May	
# Genus	%	# Genus	%	# Genus	%	# Genus	%
1 *Clostridium*	17.64%	1 *Deinococcus*	17.03%	1 *Clostridium*	38.22%	1 *Cetobacterium*	17.11%
2 *Cetobacterium*	7.71%	2 *Clostridium*	7.82%	2 *Turicibacter*	4.16%	2 *Bacteroides*	10.75%
3 *Deinococcus*	7.07%	3 *Pseudomonas*	7.81%	3 *Pseudomonas*	3.87%	3 *Clostridium*	10.60%
4 *Pseudomonas*	4.86%	4 *Cetobacterium*	5.39%	4 *Cyanobacterium*	2.78%	4 *Lysobacter*	6.81%
5 *Bacteroides*	4.06%	5 *Cloacibacterium*	2.75%	5 *Acinetobacter*	2.55%	5 *Cloacibacterium*	6.53%
6 *Cloacibacterium*	3.71%	6 *Janthinobacterium*	2.71%	6 *Cloacibacterium*	2.09%	6 *Thauera*	5.55%
7 *Acinetobacter*	2.73%	7 *Exiguobacterium*	2.45%	7 *Shewanella*	1.78%	7 *Acinetobacter*	3.96%
8 *Lysobacter*	2.48%	8 *Acinetobacter*	1.93%	8 *Microcystis*	1.55%	8 *Aeromonas*	3.94%
9 *Aeromonas*	2.01%	9 *Sphingomonas*	1.92%	9 *Bacillus*	1.43%	9 *Acidovorax*	2.34%
10 *Thauera*	1.97%	10 *Beijerinckia*	1.72%	10 *Janthinobacterium*	1.42%	10 *Hylemonella*	2.23%

Gar Skin		Gar Skin-August		Gar Skin-November		Gar Skin-May	
# Genus	%	# Genus	%	# Genus	%	# Genus	%
1 *Cetobacterium*	19.76%	1 *Deinococcus*	25.84%	1 *Holospora*	7.63%	1 *Cetobacterium*	36.86%
2 *Deinococcus*	15.93%	2 *Cetobacterium*	14.83%	2 *Oenococcus*	7.36%	2 *Plesiomonas*	5.03%
3 *Clostridium*	4.24%	3 *Clostridium*	5.52%	3 *Vibrio*	6.04%	3 *Aeromonas*	4.06%
4 *Plesiomonas*	2.87%	4 *Hymenobacter*	3.14%	4 *Deinococcus*	5.77%	4 *Cloacibacterium*	3.74%
5 *Hymenobacter*	2.46%	5 *Knoellia*	3.01%	5 *Achromobacter*	5.46%	5 *Clostridium*	2.96%
6 *Vibrio*	2.36%	6 *Sphingomonas*	2.53%	6 *Orientia*	4.63%	6 *Polynucleobacter*	2.90%
7 *Sphingomonas*	2.02%	7 *Vibrio*	2.53%	7 *Hymenobacter*	4.19%	7 *Pseudomonas*	2.87%
8 *Pseudomonas*	1.94%	8 *Plesiomonas*	2.34%	8 *Erythrobacter*	3.46%	8 *Hylemonella*	2.51%
9 *Knoellia*	1.79%	9 *Bacteroides*	2.15%	9 *Cetobacterium*	2.69%	9 *Acinetobacter*	2.14%
10 *Cloacibacterium*	1.59%	10 *Arthrobacter*	1.69%	10 *Candidatus odyssella*	2.41%	10 *Exiguobacterium*	2.11%

LMB Skin		LMB Skin-August		LMB Skin-November		LMB Skin-May	
# Genus	%	# Genus	%	# Genus	%	# Genus	%
1 *Cetobacterium*	32.25%	1 *Cetobacterium*	36.24%	1 *Cetobacterium*	43.52%	1 *Cloacibacterium*	9.32%
2 *Clostridium*	11.23%	2 *Clostridium*	7.77%	2 *Clostridium*	16.52%	2 *Aeromonas*	8.86%
3 *Plesiomonas*	8.20%	3 *Vibrio*	6.73%	3 *Plesiomonas*	13.00%	3 *Hylemonella*	5.15%
4 *Mycoplasma*	4.42%	4 *Plesiomonas*	5.75%	4 *Mycoplasma*	7.71%	4 *Acidovorax*	4.63%
5 *Aeromonas*	3.06%	5 *Deinococcus*	5.29%	5 *Ferrovum*	2.01%	5 *Vogesella*	4.50%
6 *Cloacibacterium*	2.52%	6 *Bacteroides*	4.53%	6 *Aeromonas*	2.00%	6 *Clostridium*	4.28%
7 *Vibrio*	2.40%	7 *Pseudomonas*	2.50%	7 *Candidatus soleaferrea*	1.55%	7 *Dechloromonas*	3.91%
8 *Deinococcus*	2.10%	8 *Mycoplasma*	1.74%	8 *Pseudomonas*	0.96%	8 *Pseudomonas*	3.77%
9 *Pseudomonas*	2.04%	9 *Tetrasphaera*	1.25%	9 *Cyanobacterium*	0.70%	9 *Arthrobacter*	2.88%
10 *Bacteroides*	1.62%	10 *Kocuria*	0.95%	10 *Cloacibacterium*	0.69%	10 *Acinetobacter*	2.87%

Table 5. One-way ANOSIM results for global tests and pairwise comparisons of skin samples separated by factor.

One-way ANOSIM of the Skin Microbiome		
Pairwise tests	**R value**	***p value***
By species		
Global	0.179	0.002
Spotted gar vs. Largemouth bass	0.081	0.110
Spotted gar vs. Bluegill	0.326	0.001
Bluegill vs. largemouth bass	0.142	0.036
By month		
Global	0.583	0.001
August vs. November	0.545	0.001
August vs. May	0.609	0.001
November vs. May	0.606	0.001

Table 6. Two-way ANOSIM results for global tests and pairwise comparisons of skin samples separated by factor.

Two-way ANOSIM of the Skin Microbiome		
Pairwise tests	**R value**	***p value***
By species		
Global	0.664	0.001
Spotted gar vs. Largemouth bass	0.559	0.001
Spotted gar vs. Bluegill	0.729	0.001
Bluegill vs. largemouth bass	0.764	0.001
By month		
Global	0.877	0.001
August vs. November	0.883	0.001
August vs. May	0.837	0.001
November vs. May	0.975	0.001

Table 7. SIMPER analysis comparing gut communities by species. Only genera accounting for at least 2% of dissimilarity between each combination of fish species is given including average abundances for each fish species sampled as well as the percent contribution to dissimilarity for each genus.

One-way SIMPER Analysis of the Skin Microbiome: Genus level by Species				
		Average Abundance		
Fish Species	Bacteria Genus	Species 1	Species 2	Contribution to dissimilarity (%)
1. *Lepomis macrochirus*	*Cetobacterium*	7.3	23.89	15.57
2. *Micropterus salmoides*	*Clostridium*	16.49	9.27	8.66
	Deinococcus	6.01	2.93	4.62
	Plesiomonas	0.78	6.09	3.83
	Cloacibacterium	3.47	3.69	3.04
	Bacteroides	4.13	1.51	2.82
	Acinetobacter	4.04	1.35	2.66
	Vibrio	0.36	3.63	2.48
	Mycoplasma	0.69	3.54	2.47
	Pseudomonas	4.9	2.75	*2.45*
Ave. diss.= 70.99%	*Aeromonas*	2.07	3.75	2.39
1. *Lepisosteus oculatus*	*Cetobacterium*	19.21	23.89	17.45
2. *Micropterus salmoides*	*Deinococcus*	11.42	2.93	7.55
	Clostridium	3.71	9.27	4.67
	Plesiomonas	2.98	6.09	3.97
	Vibrio	3.02	3.63	3.06
	Cloacibacterium	2.33	3.69	2.93
	Aeromonas	1.75	3.75	2.74
Ave. diss.= 70.43%	*Mycoplasma*	0.04	3.54	2.50
1. *Lepisosteus oculatus*	*Cetobacterium*	19.21	7.3	11.84
2. *Lepomis macrochirus*	*Clostridium*	3.71	16.49	9.45
	Deinococcus	11.42	6.01	8.18
	Bacteroides	1.31	4.13	2.85
	Acinetobacter	1.13	4.04	2.52
	Pseudomonas	2.21	4.9	2.29
	Cloacibacterium	2.33	3.47	2.21
Ave. diss.= 69.96%	*Vibrio*	3.02	0.36	2.03

Table 8. SIMPER analysis comparing gut communities by sampling date. Only genera accounting for at least 2% of dissimilarity between each combination of sampling month is given including average abundances for each month sampled as well as the percent contribution to dissimilarity for each genus.

One-way SIMPER Analysis of the Skin Microbiome: Genus level by Sampling Date				
		Average Abundance		
Fish Species	**Bacteria Genus**	**Month 1**	**Month 2**	**Contribution to dissimilarity (%)**
1. November	*Cetobacterium*	16.8	17.56	14.61
2. May	*Clostridium*	17.98	5.7	10.25
	Cloacibacterium	1.68	6.47	3.64
	Plesiomonas	4.94	2.2	3.4
	Aeromonas	1.26	5.6	3.23
	Acinetobacter	1.25	4.19	2.57
	Bacteroides	0.32	3.88	2.52
	Lysobacter	0.19	3.42	2.22
Ave. diss.= 73.36%	*Mycoplasma*	3.07	0.89	2.21
1. August	*Cetobacterium*	15.84	16.8	14.07
2. November	*Deinococcus*	16.17	2.47	10.02
	Clostridium	6.97	17.98	9.77
	Plesiomonas	2.87	4.94	3.63
	Vibrio	4.19	2.06	3.08
Ave. diss.= 71.21%	*Mycoplasma*	0.56	3.07	2.22
1. August	*Cetobacterium*	15.84	17.56	13.31
2. May	*Deinococcus*	16.17	0.97	10.73
	Cloacibacterium	1.28	6.47	3.75
	Aeromonas	0.67	5.6	3.55
	Clostridium	6.97	5.7	3.32
	Bacteroides	2.67	3.88	2.87
	Vibrio	4.19	0.68	2.73
	Acinetobacter	1.08	4.19	2.63
	Pseudomonas	4.51	2.85	2.27
	Lysobacter	0.36	3.42	2.23
	Plesiomonas	2.87	2.2	2.08
Ave. diss.= 71.46%	*Acidovorax*	0.27	3.14	2.01

Table 9: Top 10 genera found in the gut microbiome for each fish species, both overall and per sampling date. Each genus is given as a percent of the overall abundance for that grouping.

Bluegill gut		Bluegill Gut-August		Bluegill Gut-November		Bluegill Skin-May	
# Genus	%	# Genus	%	# Genus	%	# Genus	%
1 *Clostridium*	43.75%	1 *Clostridium*	53.76%	1 *Clostridium*	51.44%	1 *Cetobacterium*	28.42%
2 *Cetobacterium*	19.63%	2 *Cetobacterium*	22.22%	2 *Aeromonas*	16.30%	2 *Clostridium*	28.09%
3 *Bacteroides*	8.86%	3 *Turicibacter*	6.34%	3 *Cetobacterium*	7.56%	3 *Bacteroides*	17.13%
4 *Aeromonas*	6.15%	4 *Romboutsia*	2.86%	4 *Bacteroides*	6.00%	4 *Romboutsia*	6.90%
5 *Romboutsia*	3.95%	5 *Bacteroides*	2.30%	5 *Mycoplasma*	4.32%	5 *Plesiomonas*	5.06%
6 *Plesiomonas*	2.58%	6 *Aeromonas*	1.65%	6 *Ferrovum*	3.54%	6 *Peptoclostridium*	4.21%
7 *Turicibacter*	2.49%	7 *Ureibacillus*	1.59%	7 *Romboutsia*	1.74%	7 *Dysgonomonas*	2.18%
8 *Peptoclostridium*	1.60%	8 *Candidatus rhabdochlamydia*	1.46%	8 *Bacillus*	1.58%	8 *Aeromonas*	0.77%
9 *Ferrovum*	1.45%	9 *Limibacter*	1.30%	9 *Paludibacter*	1.41%	9 *Ferrovum*	0.70%
10 *Mycoplasma*	1.44%	10 *Bacillus*	1.13%	10 *Plesiomonas*	1.32%	10 *Turicibacter*	0.67%

Gar Gut		Gar Gut-August		Gar Gut-November		Gar Gut-May	
# Genus	%	# Genus	%	# Genus	%	# Genus	%
1 *Cetobacterium*	33.60%	1 *Cetobacterium*	48.79%	1 *Cetobacterium*	31.62%	1 *Clostridium*	29.20%
2 *Clostridium*	21.89%	2 *Bacteroides*	22.89%	2 *Clostridium*	31.61%	2 *Cetobacterium*	15.69%
3 *Bacteroides*	15.80%	3 *Plesiomonas*	13.13%	3 *Plesiomonas*	16.38%	3 *Plesiomonas*	15.30%
4 *Plesiomonas*	14.58%	4 *Clostridium*	11.28%	4 *Ruminiclostridium*	6.24%	4 *Bacteroides*	13.02%
5 *Phyllobacterium*	4.75%	5 *Aeromonas*	0.92%	5 *Bacteroides*	5.89%	5 *Phyllobacterium*	12.58%
6 *Aeromonas*	2.27%	6 *Phyllobacterium*	0.48%	6 *Ferrovum*	2.20%	6 *Aeromonas*	4.32%
7 *Ruminiclostridium*	1.44%	7 *Eubacterium*	0.46%	7 *Aeromonas*	1.70%	7 *Pantoea*	3.11%
8 *Pantoea*	1.18%	8 *Cellulosilyticum*	0.31%	8 *Phyllobacterium*	0.83%	8 *Romboutsia*	1.55%
9 *Romboutsia*	0.73%	9 *Ruminiclostridium*	0.22%	9 *Romboutsia*	0.58%	9 *Peptoclostridium*	1.09%
10 *Ferrovum*	0.60%	10 *Romboutsia*	0.16%	10 *Bacillus*	0.38%	10 *Cellulosilyticum*	0.54%

LMB Gut		LMB Gut-August		LMB Gut-November		LMB Gut-May	
# Genus	%	# Genus	%	# Genus	%	# Genus	%
1 *Cetobacterium*	51.80%	1 *Cetobacterium*	52.02%	1 *Cetobacterium*	59.41%	1 *Cetobacterium*	43.84%
2 *Bacteroides*	16.71%	2 *Bacteroides*	33.02%	2 *Clostridium*	17.21%	2 *Clostridium*	20.37%
3 *Clostridium*	14.30%	3 *Plesiomonas*	6.44%	3 *Mycoplasma*	6.69%	3 *Bacteroides*	14.70%
4 *Plesiomonas*	7.58%	4 *Clostridium*	5.27%	4 *Aeromonas*	5.44%	4 *Plesiomonas*	13.49%
5 *Mycoplasma*	2.60%	5 *Dysgonomonas*	0.40%	5 *Plesiomonas*	2.91%	5 *Phyllobacterium*	1.07%
6 *Aeromonas*	2.09%	6 *Asaccharospora*	0.38%	6 *Bacteroides*	2.64%	6 *Mycoplasma*	0.85%
7 *Ferrovum*	0.87%	7 *Peptoclostridium*	0.24%	7 *Ferrovum*	2.29%	7 *Turicibacter*	0.84%
8 *Phyllobacterium*	0.56%	8 *Mycoplasma*	0.18%	8 *Fusobacterium*	0.65%	8 *Romboutsia*	0.66%
9 *Romboutsia*	0.35%	9 *Vogesella*	0.15%	9 *Phyllobacterium*	0.59%	9 *Aeromonas*	0.63%
10 *Turicibacter*	0.34%	10 *Aeromonas*	0.15%	10 *Candidatus soleaferrea*	0.30%	10 *Dysgonomonas*	0.42%

Table 10. One-way ANOSIM results for global tests and pairwise comparisons of gut samples separated by factor.

One-way ANOSIM of the Gut Microbiome		
Pairwise tests	**R value**	***p value***
By species		
Global	0.209	0.001
Spotted gar vs. Largemouth bass	0.063	0.123
Spotted gar vs. Bluegill	0.216	0.010
Bluegill vs. largemouth bass	0.350	0.002
By month		
Global	0.391	0.001
August vs. November	0.692	0.001
August vs. May	0.110	0.039
November vs. May	0.441	0.001

Table 11. Two-way ANOSIM results for global tests and pairwise comparisons of gut samples separated by factor.

Two-way ANOSIM of the Gut Microbiome		
Pairwise tests	**R value**	***p value***
By species		
Global	0.448	0.001
Spotted gar vs. Largemouth bass	0.106	0.160
Spotted gar vs. Bluegill	0.507	0.002
Bluegill vs. largemouth bass	0.694	0.001
By month		
Global	0.585	0.001
August vs. November	0.847	0.001
August vs. May	0.406	0.001
November vs. May	0.622	0.002

Table 12. SIMPER analysis comparing gut communities by species. Only genera accounting for at least 2% of dissimilarity between each combination of fish species is given including average abundances for each fish species sampled as well as the percent contribution to dissimilarity for each genus.

One-way SIMPER Analysis of the Gut Microbiome: Genus level by Species

		Average Abundance		
Fish Species	**Bacteria Genus**	**Species 1**	**Species 2**	**Contribution to dissimilarity (%)**
1. *Lepomis macrochirus*	*Cetobacterium*	18.81	52.47	28.72
2. *Micropterus salmoides*	*Clostridium*	44.99	12.47	28.51
	Bacteroides	8.33	17.35	13.13
	Plesiomonas	2.44	8.38	5.75
	Aeromonas	5.99	1.87	5.2
	Romboutsia	4.08	0.29	3.22
	Mycoplasma	1.64	2.55	3.02
Ave. diss.= 60.61%	*Turicibacter*	2.62	0.29	2.07
1. Lepisosteus oculatus	*Clostridium*	24.89	44.99	24.91
2. *Lepomis macrochirus*	*Cetobacterium*	32.18	18.81	21.15
	Bacteroides	12.86	8.33	11.6
	Plesiomonas	14.22	2.44	11.43
	Aeromonas	1.87	5.99	5.29
	Phyllobacterium	5.36	0.07	4.68
	Romboutsia	0.98	4.08	3.56
Ave. diss.= 57.17%	*Turicibacter*	0.1	2.62	2.21
1. *Lepisosteus oculatus*	*Cetobacterium*	32.18	52.47	26.33
2. *Micropterus salmoides*	*Clostridium*	24.89	12.47	20.75
	Bacteroides	12.86	17.35	16.71
	Plesiomonas	14.22	8.38	13.7
	Phyllobacterium	5.36	0.83	6.17
	Aeromonas	1.87	1.87	2.76
Ave. diss.= 47.40%	*Mycoplasma*	0.01	2.55	2.67

Table 13. SIMPER analysis comparing gut communities by sampling date. Only genera accounting for at least 2% of dissimilarity between each combination of sampling month is given including average abundances for each month sampled as well as the percent contribution to dissimilarity for each genus.

One-way SIMPER Analysis of the Gut Microbiome: Genus level by Sampling Date

		Average Abundance		
Fish Species	**Bacteria Genus**	**Month 1**	**Month 2**	**Contribution to dissimilarity (%)**
1. November	*Cetobacterium*	34.04	27.70	23.49
2. May	*Clostridium*	33.72	26.39	21.95
	Bacteroides	4.67	13.80	11.57
	Plesiomonas	5.09	12.78	11.31
	Aeromonas	7.75	1.56	6.61
	Phyllobacterium	0.59	5.12	4.88
	Mycoplasma	4.13	0.33	3.71
	Romboutsia	0.93	3.37	3.09
Ave. diss.= 55.40%	*Ferrovum*	2.72	0.46	2.18
1. August	*Clostridium*	22.98	33.72	26.23
2. November	*Cetobacterium*	41.88	34.04	24.46
	Bacteroides	19.39	4.67	16.22
	Plesiomonas	6.41	5.09	7.25
	Aeromonas	0.91	7.75	6.93
	Mycoplasma	0.09	4.13	3.87
	Ferrovum	0.07	2.72	2.50
Ave. diss.= 53.01%	*Turicibacter*	2.14	0.40	2.07
1. August	*Clostridium*	22.98	26.39	24.15
2. May	*Cetobacterium*	41.88	27.7	23.82
	Bacteroides	19.39	13.8	16.57
	Plesiomonas	6.41	12.78	11.00
	Phyllobacterium	0.16	5.12	5.19
	Romboutsia	1.05	3.37	3.62
Ave. diss.= 49.89%	*Turicibacter*	2.14	0.50	2.23

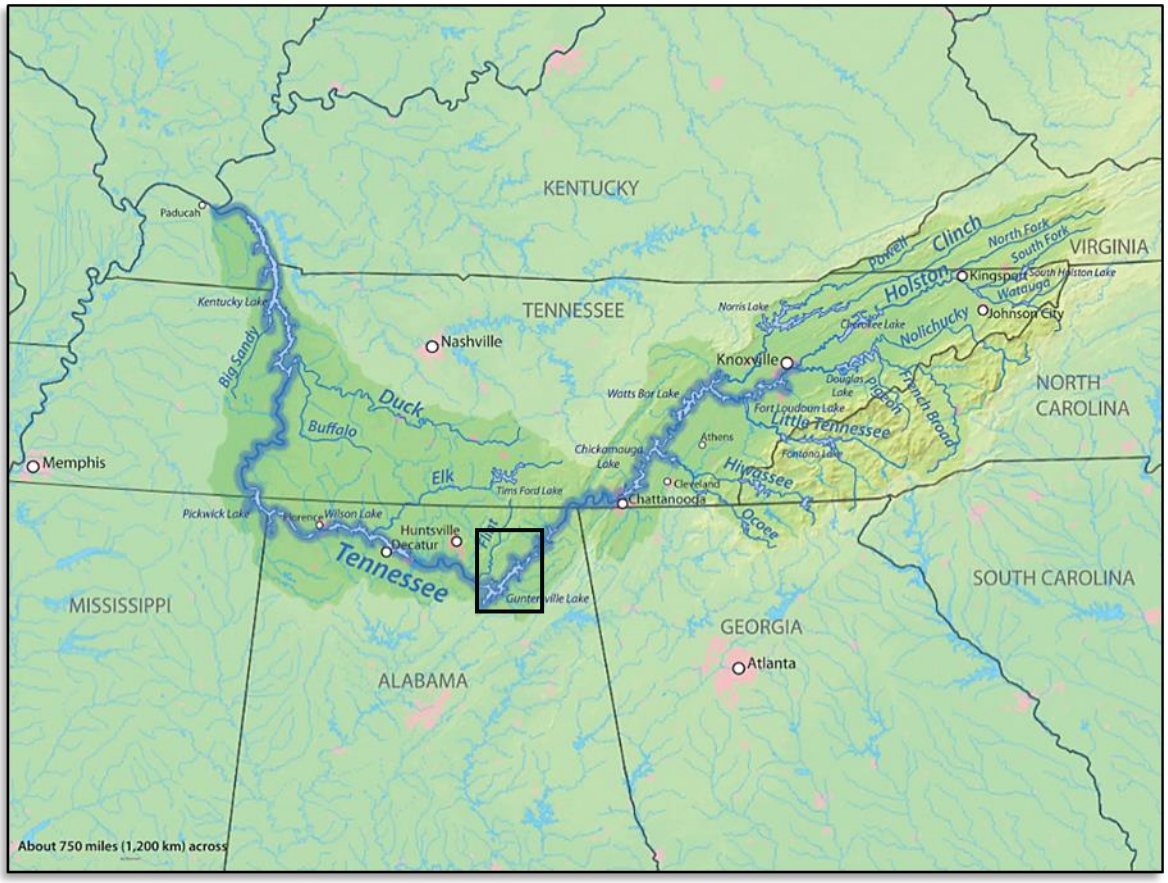
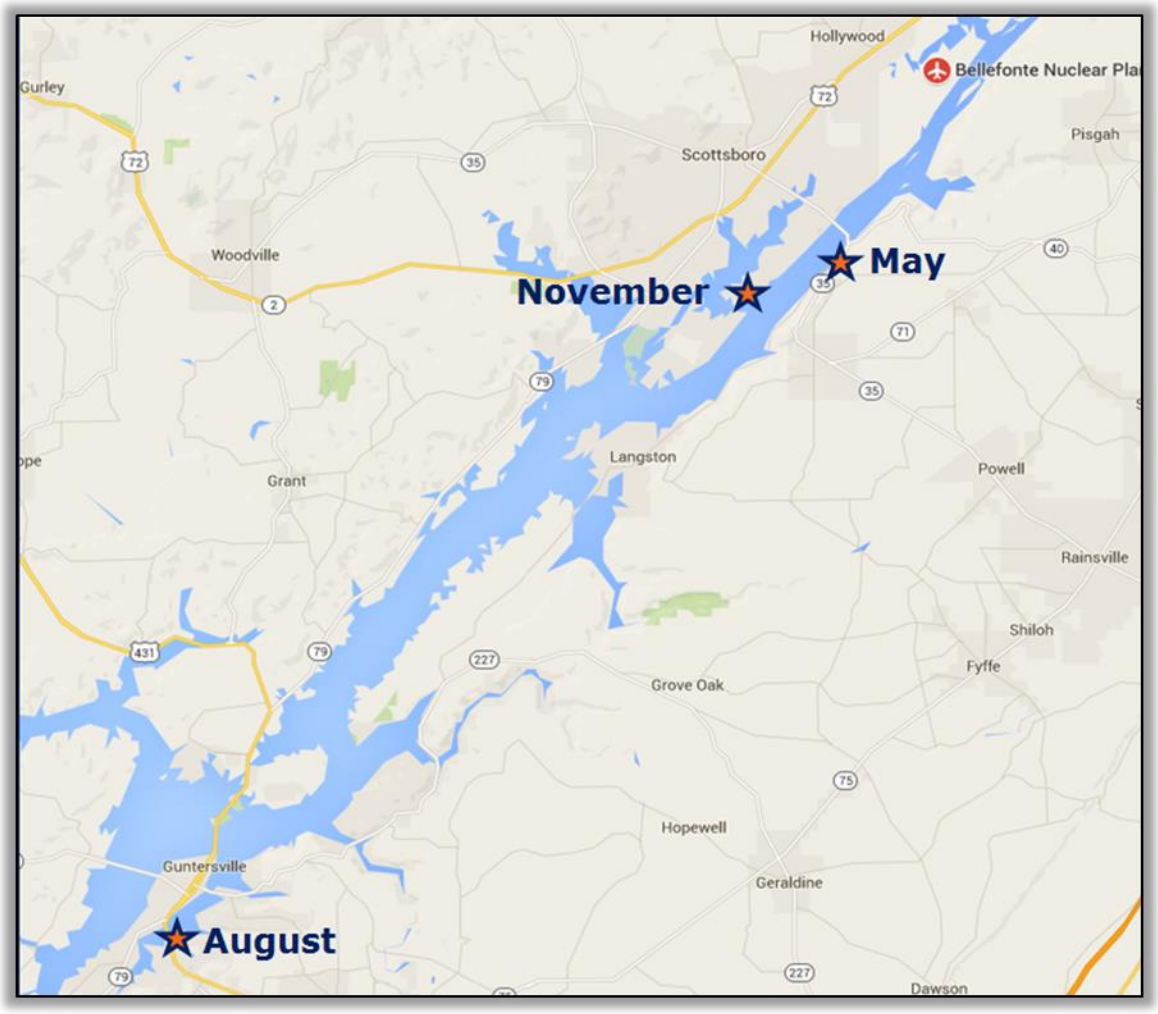


Figure 1. Map of the Tennessee River Watershed. Lake Guntersville is highlighted by the box

(Photo credit: https://commons.wikimedia.org/wiki/File:Tennesseermfinal.jpg).

Figure 2. Map of Lake Guntersville sample sites from August 2014 to May 2015. Sample sites and time points are indicated by stars. Image created by google maps (©2016 Google).



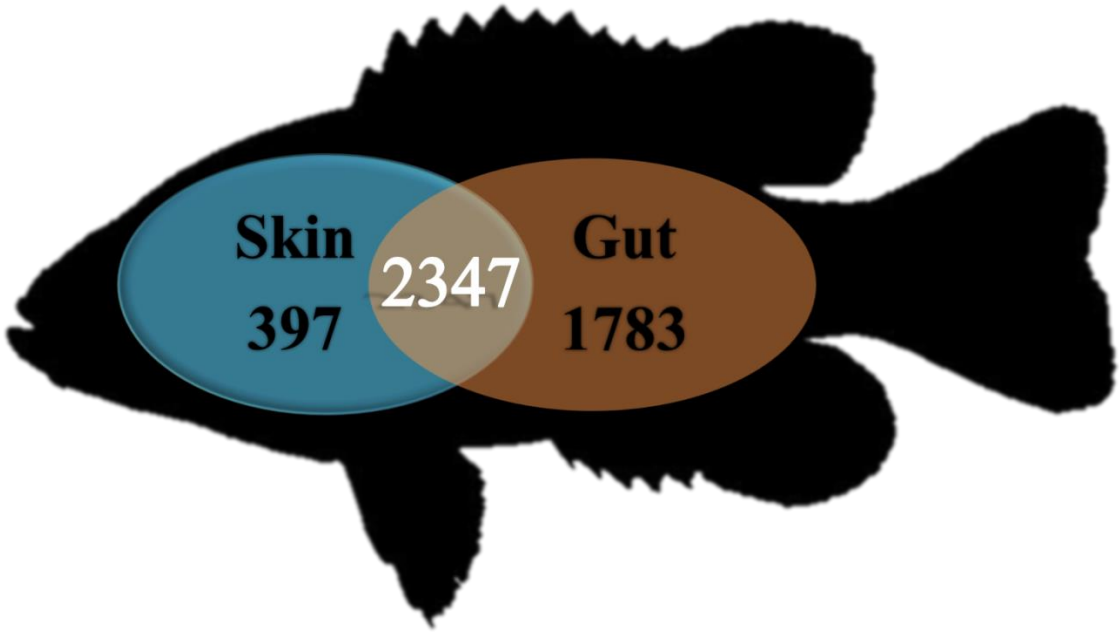


Figure 3. Venn diagram demonstrating the operational taxonomic units (OTUs) shared between the overall skin and gut samples for all three species. Blue, skin (Total OTUs = 2744) and orange, gut (Total OTUs = 4130).

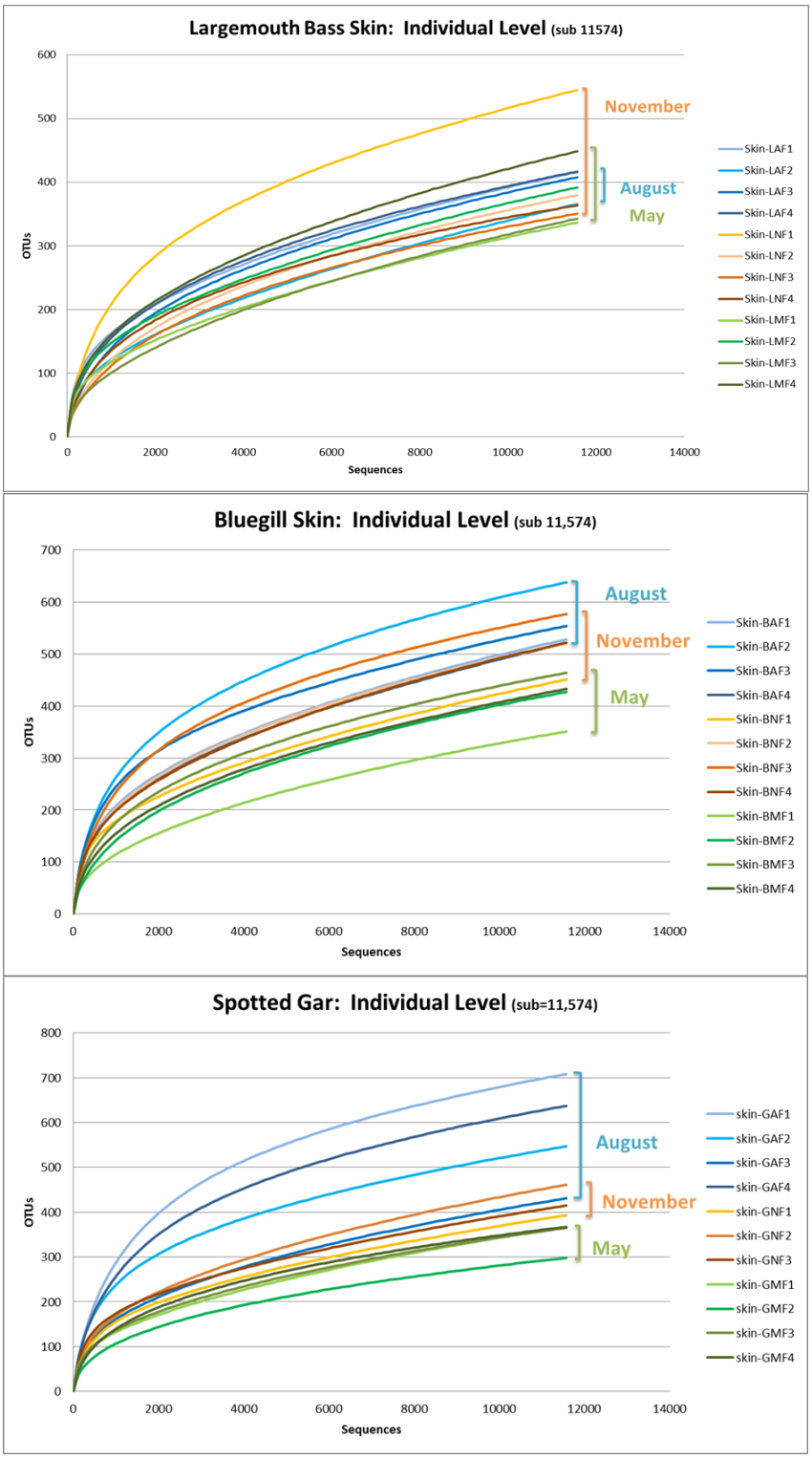


Figure 4. Rarefaction curves reflect species richness for the skin communities of each species. All sequences were standardized to the least number of obtained sequences (n=11,574) for direct comparison. Curves are bracketed to show individual variations in richness by seasonal time points.

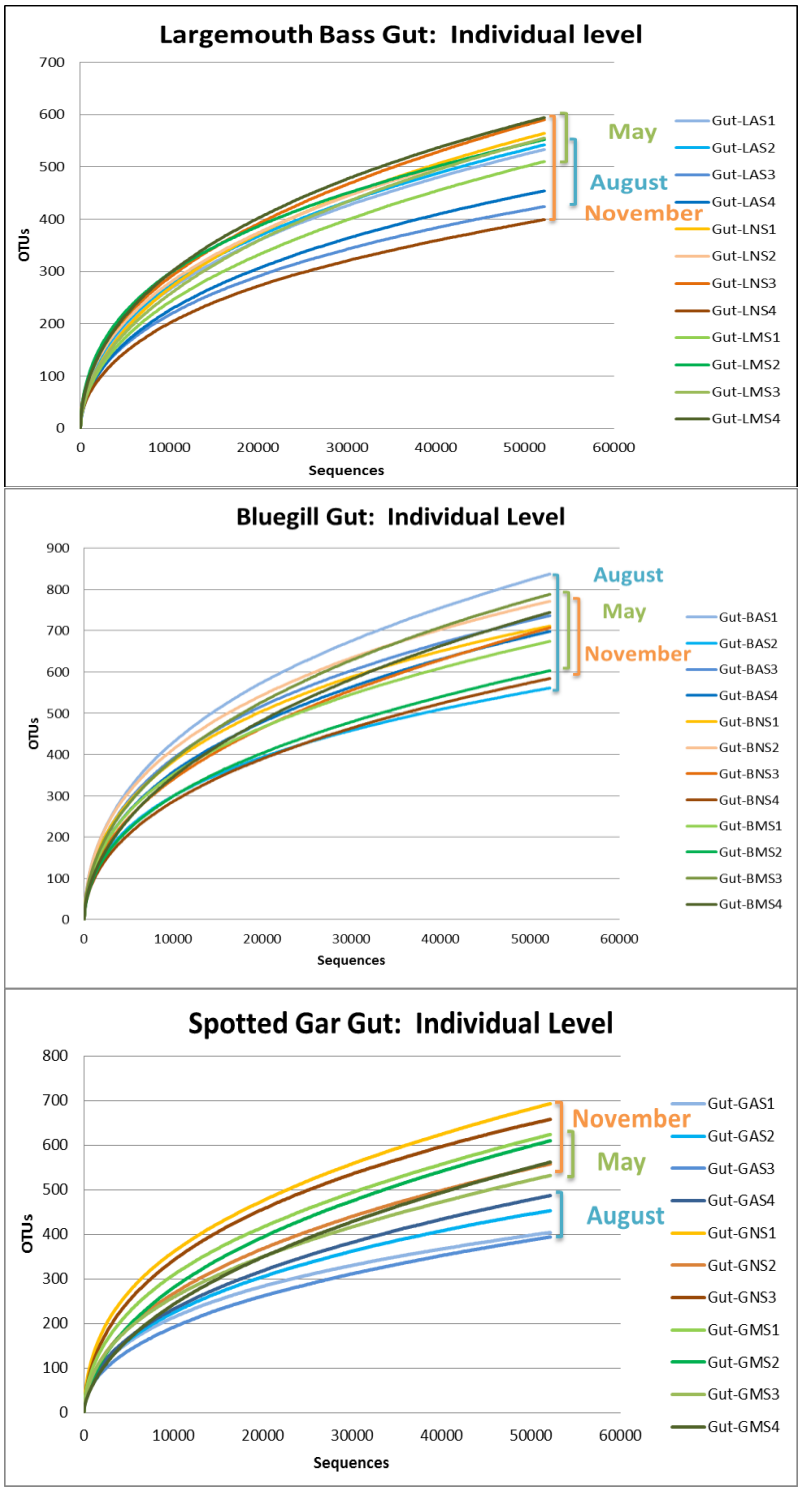


Figure 5. Rarefaction curves reflect species richness for the gut communities of each species. All sequences were standardized to the least number of obtained sequences (n=52,205) for direct comparison. Curves are bracketed to show individual variation at seasonal time points.

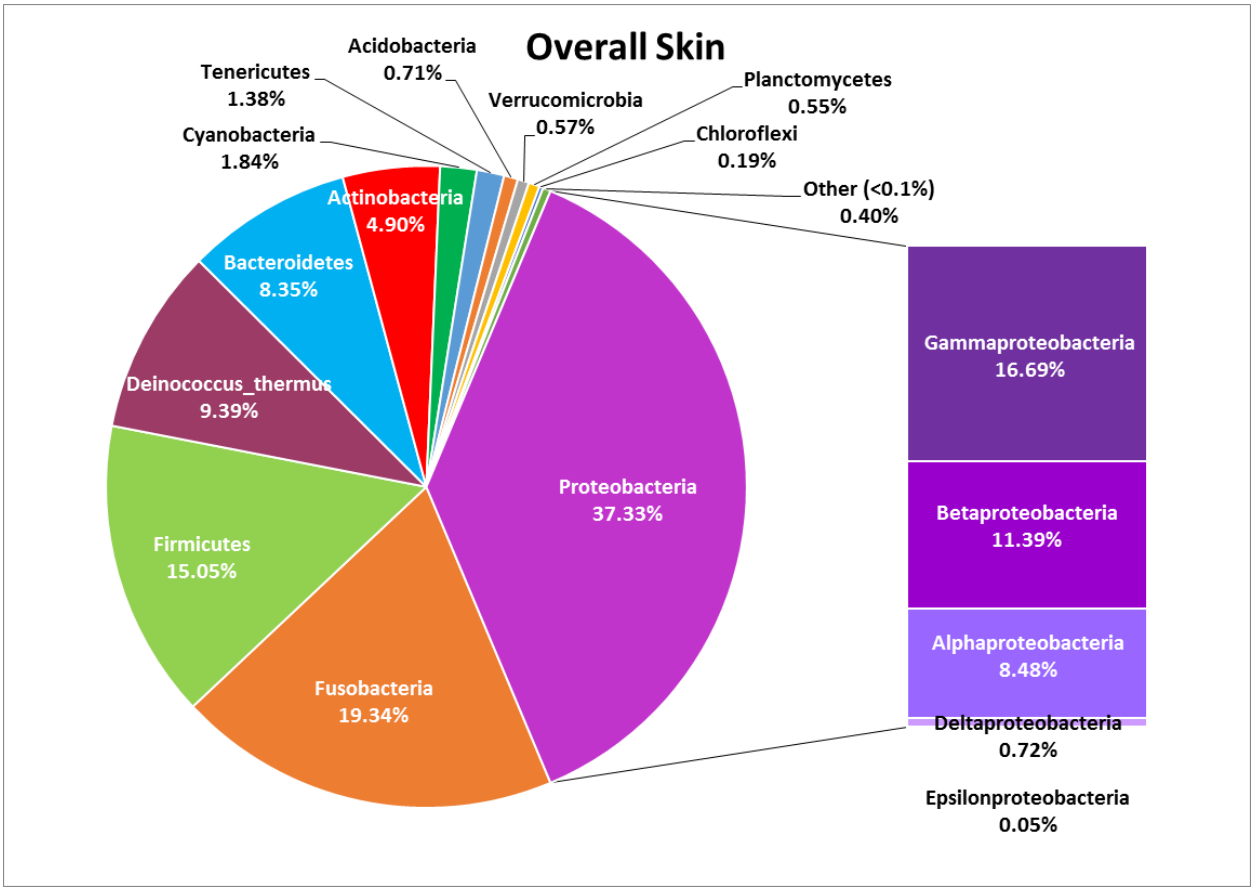
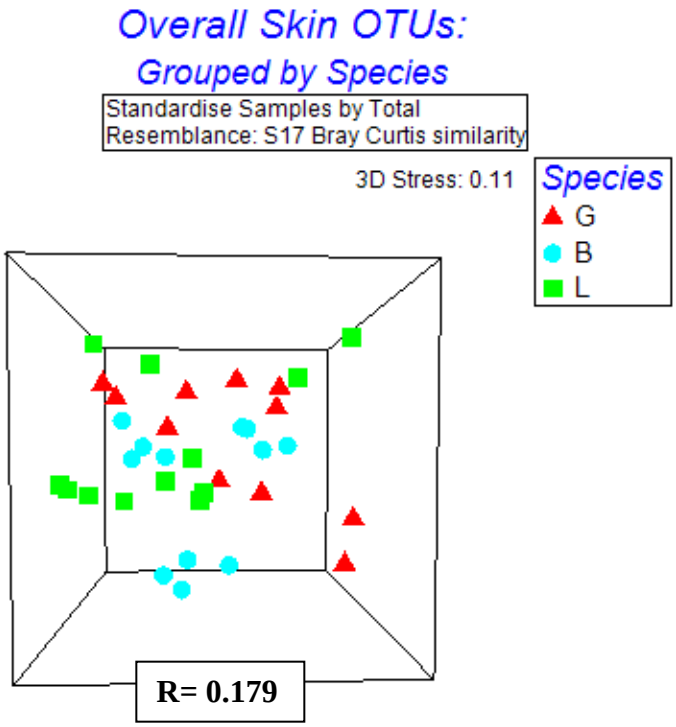


Figure 6. Pie graph showing overall phylum level composition of the skin microbiome for all species sampled across all time points. Percentages reflect relative abundances of phyla for overall sequences present in skin communities for the three species. All Phyla present in abundances of $\leq 0.1\%$ are included as other. A Class breakdown for the Phylum Proteobacteria is also given.

A


Overall Skin OTUs:
Grouped by Species
Standardise Samples by Total
Resemblance: S17 Bray Curtis similarity
3D Stress: 0.11
Species
G
B
L
R= 0.179

B

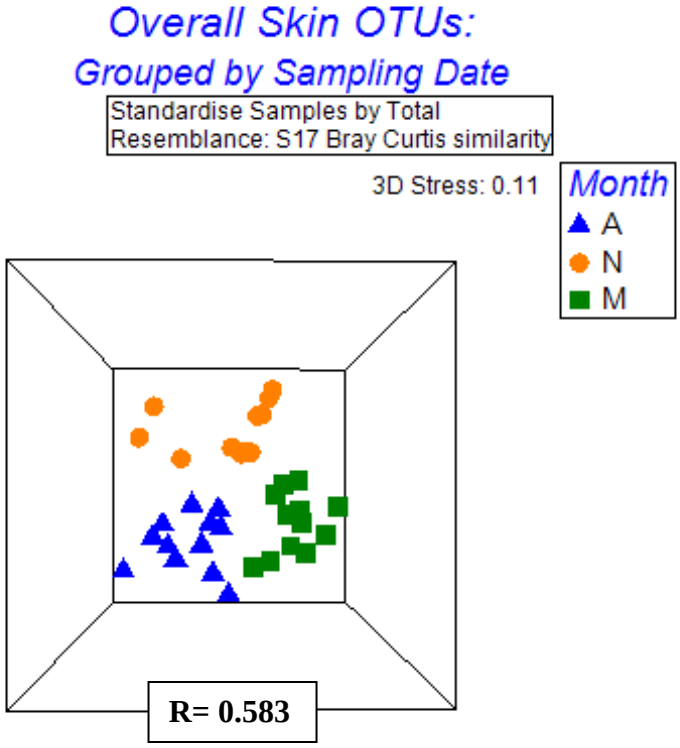

Overall Skin OTUs:
Grouped by Sampling Date
Standardise Samples by Total
Resemblance: S17 Bray Curtis similarity
3D Stress: 0.11
Month
A
N
M
R= 0.583

Figure 7. Multidimensional scaling (MDS) plots separated by factor for skin samples. ANOSIM results are indicated by R values for each factor. A, Fish species: Largemouth bass (L), Bluegill (B), and Spotted Gar (G); B, sampling date: August (A), November (N), and May (M).

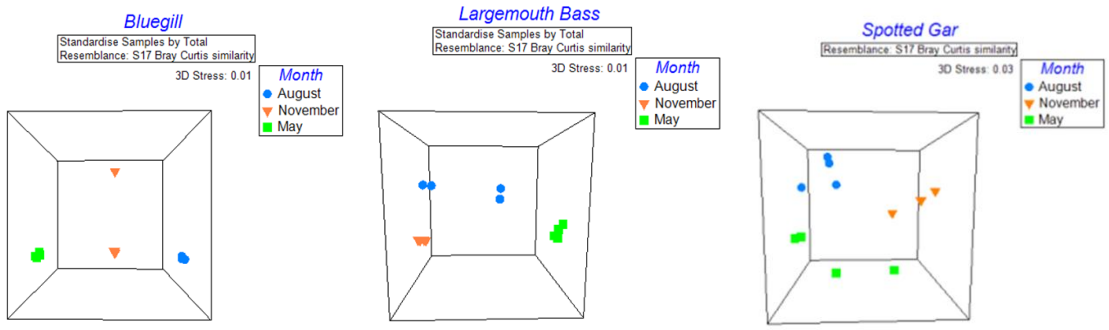


Figure 8. Multidimensional scaling (MDS) plots for each fish species demonstrating grouping of individual skin samples by sampling date. August, Blue; November, Orange; May, Green.

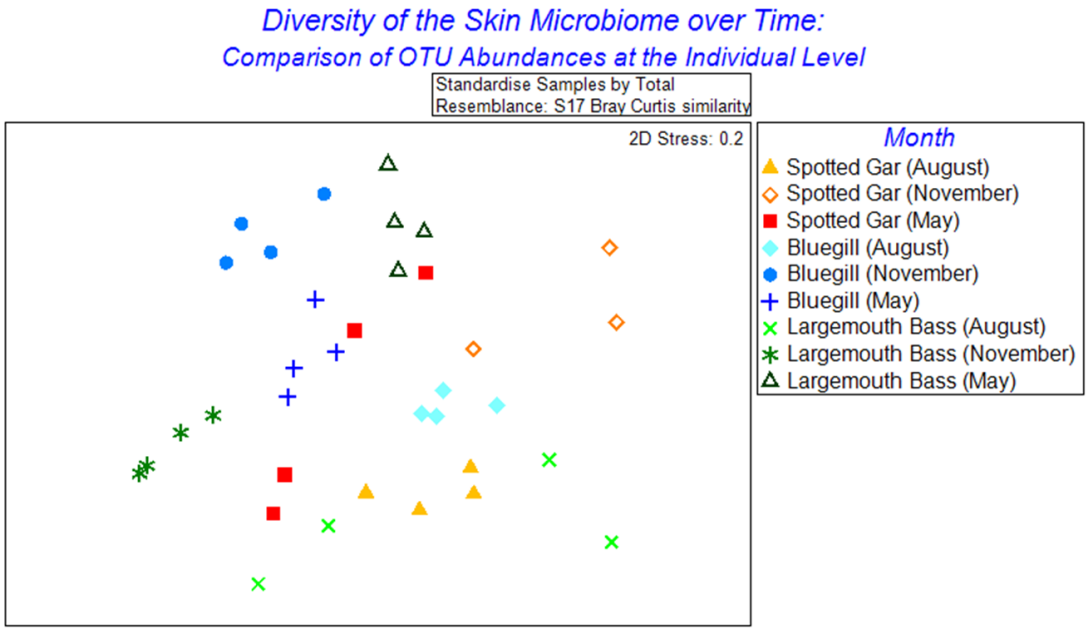


Figure 9. Multidimensional scaling (MDS) plot for all species at all sampling points.

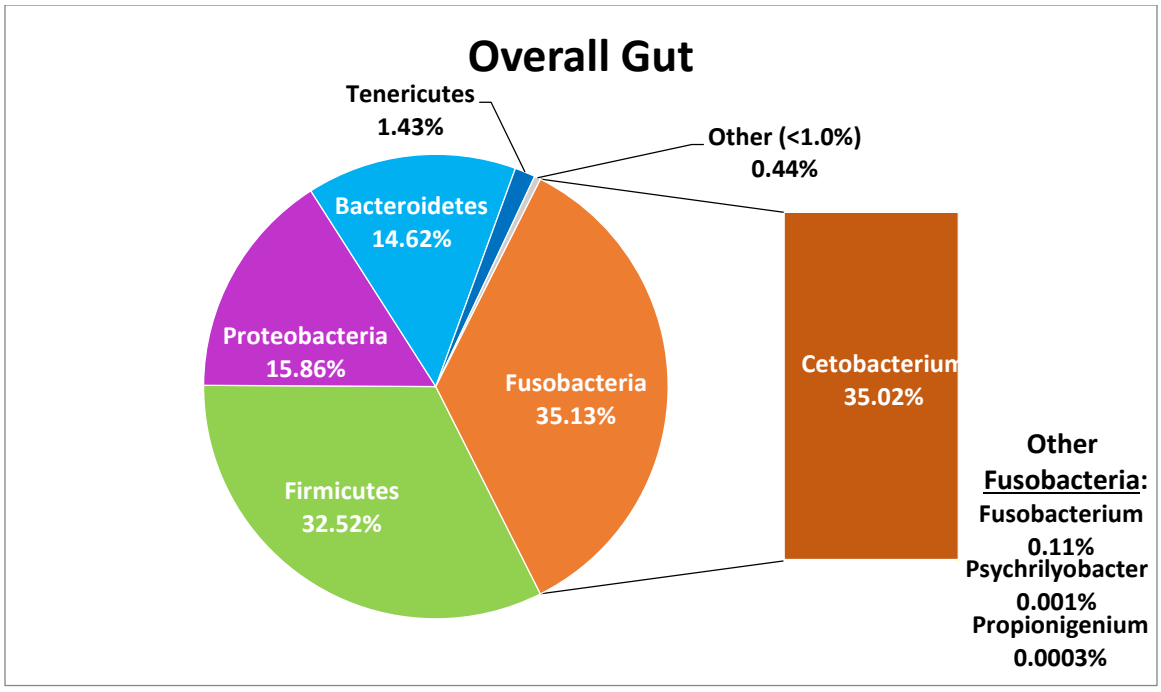
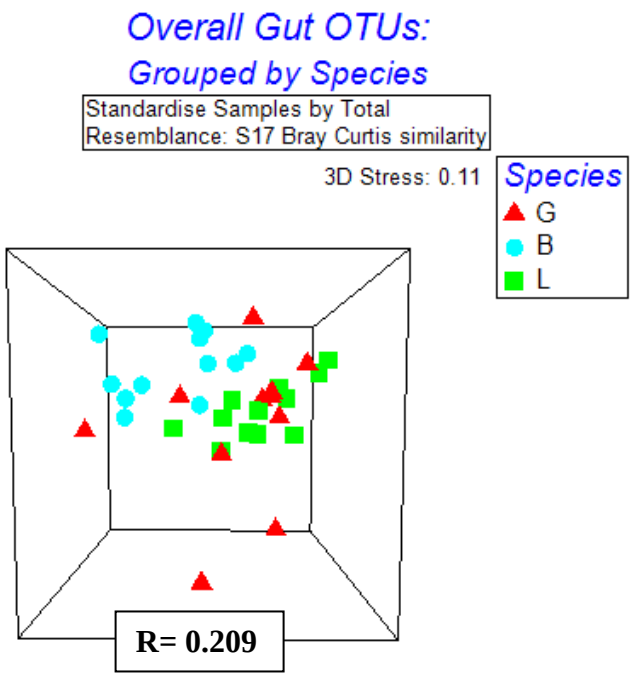


Figure 10. Pie graph showing overall phylum level composition of the gut microbiome for all species sampled across all time points. Percentages reflect relative abundances of phyla for overall sequences present in gut communities. All Phyla present in abundances of ≤ 1.0% are included as other. A Class breakdown for the Phylum Fusobacteria is also given.

A


Overall Gut OTUs:
Grouped by Species
Standardise Samples by Total
Resemblance: S17 Bray Curtis similarity
3D Stress: 0.11
Species
G
B
L
R= 0.209

B

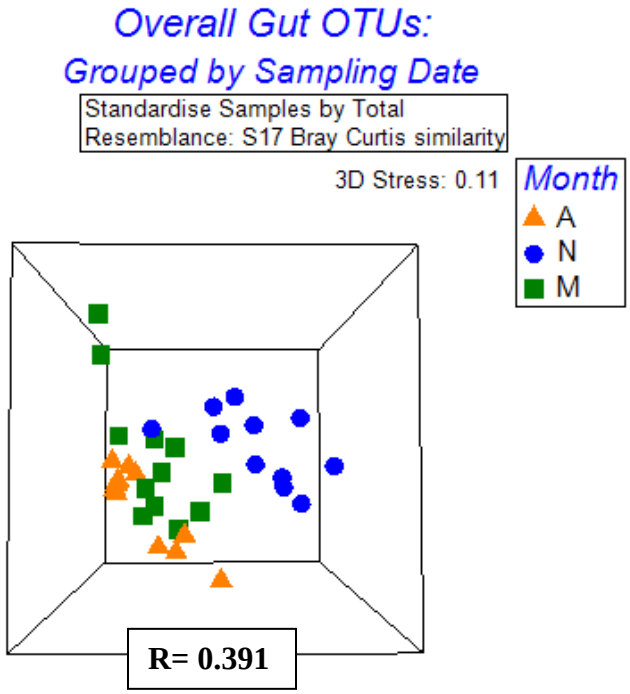

Overall Gut OTUs:
Grouped by Sampling Date
Standardise Samples by Total
Resemblance: S17 Bray Curtis similarity
3D Stress: 0.11
Month
A
N
M
R= 0.391

Figure 11. Multidimensional scaling (MDS) plots separated by factor for skin samples. ANOSIM results are indicated by R values for each factor. A, Fish species: Largemouth bass (L), Bluegill (B), and Spotted Gar (G); B, sampling date: August (A), November (N), and May (M).

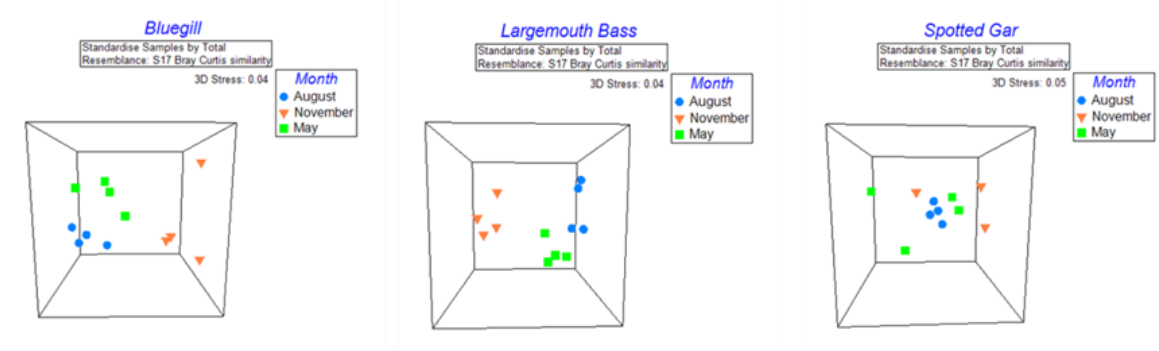


Figure 12. Multidimensional scaling (MDS) plots for each fish species demonstrating grouping of individual gut samples by sampling date. August, Blue; November, Orange; May, Green.

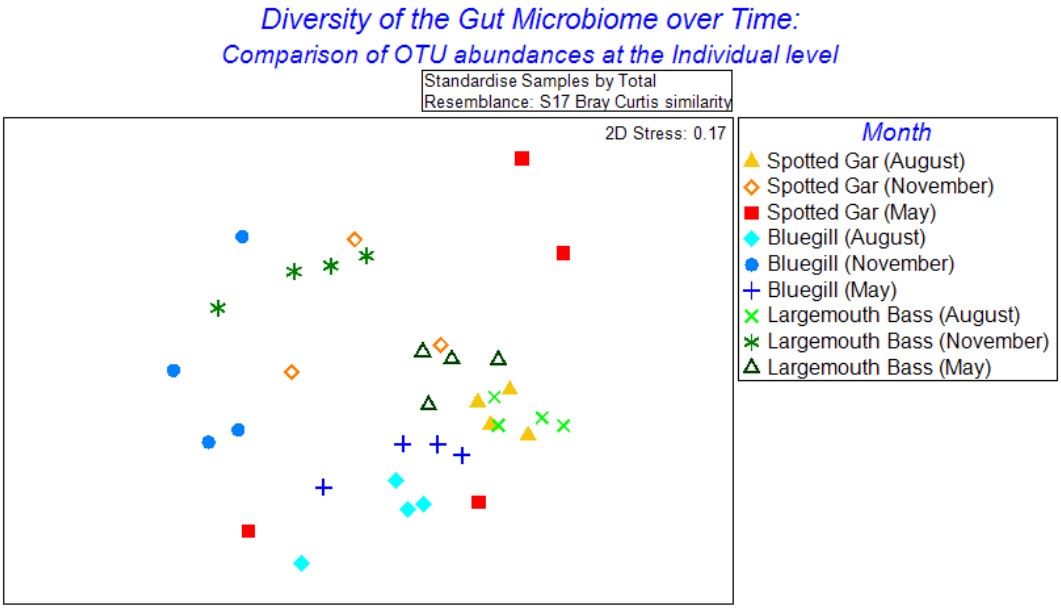


Figure 13. Multidimensional scaling (MDS) plot for all species at all sampling points.

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Appendix A.

Qiagen DNeasy® Blood & Tissue Kit

Gram (+) Fin clip DNA extraction checklist

Sample Source: _____ Sample Date: _____

Sample Type & Storage: _____ Species: _____

Extraction Date: _____

Prep Steps:

_____ 1) Check waterbath to make sure it has an adequate volume of water and temperature is around 56°C

_____ 2) Prepare hood with clean tools, sterile petri dishes, oil burner, and flame ethanol.

_____ 3) Calculate volume of lysis buffer needed, and make sure there is enough pre-prepared for all samples being extracted

_____ 5) Heat microcentrifuge incubator to 37°C

Gram (+) Protocols:

_____ 1) Aseptically cut fin clips and weigh samples to 25mg ± 1mg in 1.5ml microcentrifuge tubes.

-Sterilize forceps and scissors between each sample using flame ethanol sterilization.

_____ 2) Wash weighed samples in microcentrifuge tubes w/750µl sterile PBS to remove excess salts (**3 washes total**)

_____ - Centrifuge full speed (13,200 rpm) for 3min

_____ - Repeat these steps **two times** pouring off/removing supernatant after each centrifugation

_____ - On 3rd wash, centrifuge @7500rpm for 10min

_____ - Remove as much supernatant as possible without disrupting pelleted sample.

_____ 3) Resuspend pellet in 180µl enzymatic lysis buffer w/lysozyme (20mg/ml)

_____ - ******Add lysozyme to measured volume of pre-prepared buffer immediately before use

_____ 4) Incubate for 30min at 37°C.

_____ 5) Add 25µl proteinase K and 200µl Buffer AL (w/out ethanol)

_____ - Mix by vortexing for 1min

_____ 6) Incubate at 56°C in waterbath overnight for complete lysis (~14-16hrs)

_____ - Start time ( )

_____ - End time ( )

_____ - Mix by vortexing for 1min

_____ 7) Add 200µl ethanol (95-100%) to lysed sample

_____ - Mix by vortexing for 1min

_____ 8) Pipet entire mixture into DNeasy mini spin column

_____ - Centrifuge at 8000rpm for 1min

_____ - Discard collection tube and replace

_____ 9) Add 500µl Buffer AW1 to spin column

_____ - Centrifuge at 8000rpm for 1min

_____ - Discard collection tube and replace

_____ 10) Add 500µl Buffer AW2 to spin column

_____ - Centrifuge at 13,200rpm for 3min

_____ - Discard collection tube and replace

_____ - Repeat (optional) to ensure membrane is dry

_____ 11) Place DNeasy mini spin column into new 1.7ml microcentrifuge tube

_____ - Add 50µl Buffer AE directly to membrane

_____ - **Incubate at Room Temperature for 1min**

_____ - Centrifuge at 8000rpm for 1min

_____ - Repeat for 2 total elutions.

Appendix B.

Qiagen QIAamp® DNA Stool Mini Kit

DNA extraction checklist

Sample Source: _____ Sample Date: _____

Sample Type & Storage: _____ Species: _____

Extraction Date: _____

Prep Steps and supplies:

_____ 1) Prepare hood with sterile pipettes and clean spatulas.

_____ 2) Waterbath at ~60°C

_____ 3) Heat microcentrifuge incubator to 70°C

_____ 4) Bucket of ice for tubes

_____ 5) Pre-sterilized 2 ml and 1.7 ml microcentrifuge tubes

Gram (+) Protocols:

_____ 1) Wash/dilute samples in microcentrifuge tubes w/ ice cold, sterile PBS to remove excess salts (**3 washes total**)

_____ - May invert to mix

_____ - Centrifuge full speed (13,200 rpm) for 3 min in Eppendorf Centrifuge

_____ - Repeat these steps removing roughly half of supernatant after each centrifugation

_____ - On 3rd wash, centrifuge @13,200 rpm for 10 min

_____ - Remove as much supernatant as possible without disrupting pelleted sample.

_____ 2) Weigh samples out to roughly 180-200 mg (or as much as available if less)

_____ - Samples containing large chunks or tissue may require homogenization with handheld homogenizer

_____ 3) Add 1.4ml Buffer ASL to each stool sample.

_____ - Vortex continuously for 1 min or until the stool sample is thoroughly homogenized

_____ - ******Make sure to keep samples as cold as possible until this step to avoid degradation

_____ 4) Heat the suspension for 5 min at 70°C.

_____-**May be increased to up to 95°C for difficult to lyse cells such as Gram positives **(*optional*)**

_____ - Vortex for 15s

_____ - Centrifuge sample at full speed (13,200 rpm) for 1min

_____ 5) Pipet 1.2ml of the supernatant into a new 2 ml microcentrifuge tube and discard the pellet

_____ - Mix by vortexing for 1 min

_____ 6) Add 1 InhibitEX tablet to each sample

_____ - Vortex immediately and continuously for 1 min (or until tablet completely suspended)

_____ - Incubate suspension for 1 min at RT (**allows inhibitors to absorb to InhibitEX matrix)

_____ 7) Centrifuge sample at full speed (13,200 rpm) for 3 min to pellet inhibitors

_____ 8) Pipet all of the supernatant into a new 1.7 ml microcentrifuge tube and discard pellet

_____ - Centrifuge the sample full speed (13,200 rpm) for 3 min

_____ - **Small amounts of remaining pelleted material will not affect procedure

_____ 9) Pipet 15µl Proteinase K into a new 1.7ml microcentrifuge tube.

_____ 10) Pipet 200µl supernatant from step 8 into the 1.7ml microcentrifuge tube containing Proteinase K.

_____ - Freeze remaining volume of supernatant from step 8 in -20°C.

_____ 11) Add 200µl Buffer AL

_____ - Vortex for 15s (must mix thoroughly)

_____ - ****Do not add Proteinase K directly to Buffer AL**

_____ 12) Incubate at 70°C for 10 min

_____ - (optional) May choose to centrifuge briefly to remove drops from cap

_____ 13) Add 200µl of ice cold ethanol (96-100%) to the lysate

_____ - Mix by vortexing (1min)

_____ - (optional) May choose to centrifuge briefly to remove drops from cap

_____ 14) Place QIAamp spin column (be sure to label lids) in 2ml collection tube

_____ - Carefully apply complete lysate from step 13 to the spin column

_____ - **<u>**Be careful not to moisten rim</u>→close cap**

_____ - Centrifuge at full speed (13,200 rpm) for 1 min

_____ - Discard collection tube and place spin column in a new one

_____ - ****Make sure lysate has completely passed through the spin column**

_____ 15) Add 500µl Buffer AW1 to spin column

_____ - Centrifuge at 13,200 rpm for 1 min

_____ - Discard collection tube and replace

_____ 16) Add 500µl Buffer AW2 to spin column

_____ - Centrifuge at 13,200 rpm for 3min

_____ - Discard collection tube and replace

_____ - Repeat (optional) to ensure membrane is dry

_____ 17) Place QIAamp spin column into new 1.7ml microcentrifuge tube

_____ - Add 50µl of warm Buffer AE directly to membrane

_____ - **<u>Incubate at Room Temperature for 2 min</u>**

_____ - Centrifuge at 13,200 rpm for 1min