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Authors: Chalifour, Bridget, and Li, Jingchun

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A REVIEW OF THE MOLLUSCAN MICROBIOME: ECOLOGY, METHODOLOGY AND FUTURE

Bridget Chalifour[1*] & Jingchun Li[1,2]

ABSTRACT

Many mollusks host symbiotic microbiota that are tightly involved in molluscan biological functions and ecological interactions. Here, we review the described symbioses between molluscan hosts and their bacterial partners. We focus on associations where the molluscan host is hypothesized to gain an evolutionary advantage because of the role of its symbiont. In addition, we focus only on those relationships that have been established experimentally or at least show strong evidence for symbioses. Along with providing a review of the known molluscan host/microbe mutualistic symbioses, we also outline common methodologies in the study of these relationships. Last, we point out areas of further exploration for molluscan microbiome studies.

Key words: bacteria, mollusk, symbiosis, 16S rRNA, high-throughput sequencing.

INTRODUCTION

Microbiome research has garnered considerable attention in recent years. This research expansion is largely due to the development of affordable high throughput sequencing technology (Tringe & Hugenholtz, 2008; Caporaso et al., 2012; Gómez-Chiarri et al., 2015; Jo et al., 2020), which augments classic *in vitro* cultivation methods and generates more in-depth information on microbial community composition and diversity (Cho & Blaser, 2012; McFall-Ngai, 2014). These technological developments have facilitated an explosion of research pertaining to the human microbiome (Costello et al., 2009; Cho & Blaser, 2012; Huttenhower et al., 2012), allowing researchers to classify risk for diseases, expand cancer research, and advance knowledge of how microbial associations shape human health (Cho & Blaser, 2012; Kostic et al., 2015; Goodrich et al., 2016). Additionally, microbiome research has revealed the largely unknown but vital role that microorganisms play in the ecology and evolution across the animal kingdom (Woese, 2002), even though current research still largely focuses on economically or medically important species (Yildirim et al., 2010; Petri et al., 2013; Stumpf et al., 2013).

Although animal microbiome research tends to focus on vertebrate species, a number of invertebrate species have been investigated. Some invertebrates lack a complex microbiome, while others contain a rich, diverse microbial community (Bahrndorff et al., 2016; Hammer et al., 2017, 2019). For example, tunicates harbor a great number of bacterial symbionts that aid in metabolic interactions and nutrient retention (Donia et al., 2011). Coral microbiomes (in addition to their algal photosymbionts) also play vital roles in the animal's growth, nutrient-cycling, stress-coping, immune responses, and other functions (Nissimov et al., 2009; Ainsworth et al., 2015; Thompson et al., 2015; Zhang et al., 2015; Van Oppen & Blackall, 2019). While some invertebrates have spawned massive research efforts, limited systematic studies have left many invertebrates' microbiome makeup and function as a mystery (Wilson, 1987; Harris, 1992; Lydeard et al., 2004). It is also important to note that the complexity of the microbiome does not necessarily correlate with ecological importance; there are numerous examples of one strain of bacteria having significant influence over its host (see the "squid-*Vibrio*" model mentioned below).

Mollusks, the second largest phylum of invertebrates, are an important component across many ecosystems. They are indicators of habitat quality and pollution hazards and are commercially important as food sources

[1]Department of Ecology and Evolutionary Biology, University of Colorado Boulder, 334 UCB, Boulder, Colorado, 80309, U.S.A.
[2]Museum of Natural History, University of Colorado Boulder, 265 UCB, Boulder, Colorado, 80309, U.S.A.
*Corresponding author: bridget.chalifour@colorado.edu

(Lydeard et al., 2004). Unfortunately, molluscan imperilment is also growing rapidly, representing 42% of documented animal extinctions (Lydeard et al., 2004). Therefore, there are ever-growing needs to understand the full extent of mollusk-microbiome interactions, not only for basic research, but also for developing proper conservation regimes, field relocation, and raising endangered species in a captive environment that enables natural microbiome assembly (West et al., 2019).

While molluscan microbiomes have not been explored fully across diverse lineages, existing studies already show that many mollusks rely on mutualistic microbial associations to survive and fulfill their ecological roles (McFall-Ngai, 2014; Dar et al., 2017). The molluscan microbiome plays important roles in many aspects of the host biology, including nutrition and digestion, metabolism, immune function, reproduction and development, behavior, predator-prey interactions, and surviving challenging abiotic conditions (Dubilier et al., 2008; King et al., 2012). These microbial associations may help to increase host fitness, an essential tool to cope with anthropogenically induced environmental shifts (Wegner et al., 2013). In this review, we summarized current knowledge on mutualistic bacterial associations with mollusks and their ecological roles (Fig. 1).

Many of the studies cited in this article use amplicon sequencing, a widely used technique for microbiome composition assessments. Many authors ask the "who's there" question by microbiome profiling, and then manipulate the molluscan host in different experimental treatments to observe how the original composition changes. While shifts in molluscan microbiome composition may not yet be fully understood, they do aid in authors creating ecological hypotheses regarding microbiome functions. Indeed, many studies can only infer microbial

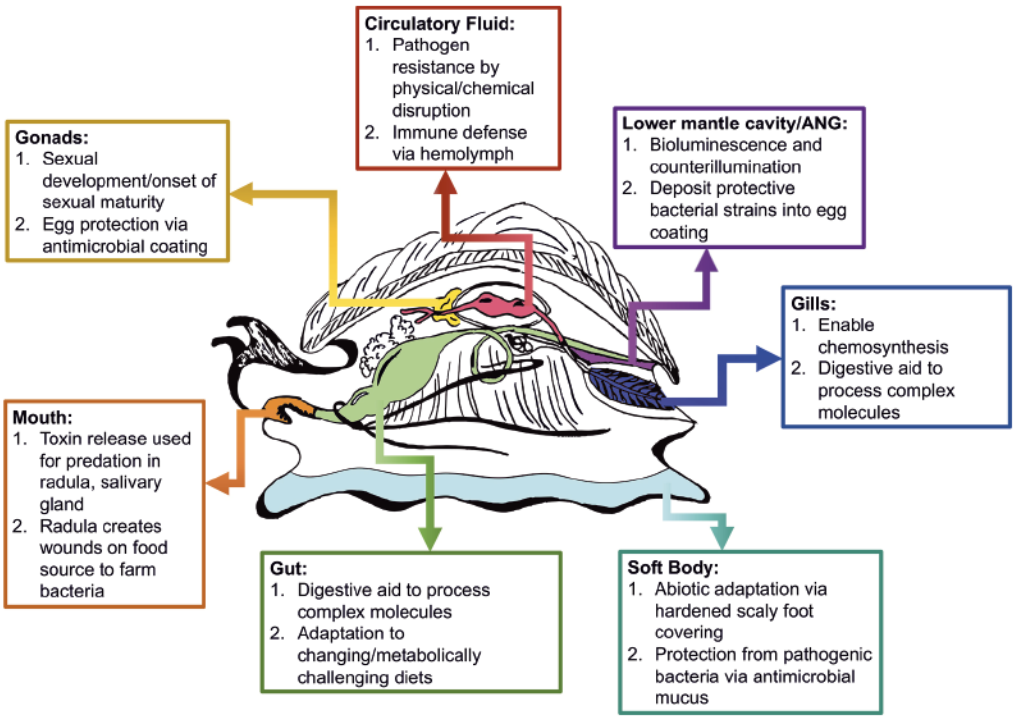

FIG.1. Generalized microbiome presence shown on a representative, nonspecific molluscan host, with arrows indicating the approximate microbiome position on the body. Boxes include hypothesized functions of the bacterial community in that body region. The use of the generalized molluscan body plan here does not mean to represent the microbiome of any single mollusk host, but rather some possible body regions where a microbiome could be found across the phylum. ANG stands for accessory nidamental gland.

functionality in the host based on compositional data, rather than experimentally prove it. We do strive to include studies that provide additional functional assessment when possible. We also provide a brief amplicon sequencing methodology review, along with other methodologies that can shed more light on potential bacterial functionality. Finally, we discuss areas for future molluscan microbiome studies.

MICROBIOMES AND MOLLUSK BIOLOGICAL FUNCTIONS

Nutrition and Metabolism

Microbiomes are crucial for the nutritional health of many mollusks, providing digestion and nutritional supplementation for a wide variety of feeding strategies (Aronson et al., 2017; Russell et al., 2018). Symbiotic bacteria can help the hosts process complex molecules, facilitate highly specialized diets, and chemosynthetically generate energy for their hosts (Prieur et al., 1990; Haygood et al., 1999; Goffredi et al., 2004; Dubilier et al., 2008; Duperron et al., 2013a).

Microbial symbionts facilitate herbivore or detritivore hosts to digest complex molecules. These are especially essential for mollusks that consume predominantly lignocellulosic matter. Tough lignocellulosic molecules can be broken down by microbial enzymes, and sometimes can enhance plant biomass digestion efficiency up to 80% (Larue et al., 2005; Cardoso et al., 2012a, 2012b; Nicolai et al., 2016; Dar et al., 2017). Several species of planorbid snails are known to contain a highly diverse, yet stable community of digestive tract bacteria (Van Horn et al., 2012). Similar associations are found in gastropod families such as Achatinidae, Ampullariidae, Helicidae and Pomatiopsidae (Dar et al., 2017; Hao et al., 2020). These bacteria are found across many regions of the digestive system, including the esophagus, stomach, foregut, hindgut, and cecum (Harris, 1992). Dominant microbial lineages include strains of Proteobacteria (e.g., *Buttiauxella, Citrobacter, Aeromonas* and *Enterobacter*) and Firmicutes (e.g., *Enterococcus, Lactococcus* and *Clostridium*), which are responsible for cellulolytic, proteolytic, and chitinolytic degradation (Charrier et al., 2006; Koleva et al., 2015). Lactic acid bacteria (LAB) are also prevalent in the gut community, as they are responsible for food fermentation. Strains of LAB found in gastropods include those from genera *Lactobacillus, Enterococcus, Lactococcus* and *Leuconostoc* (Dar et al., 2017). In addition to terrestrial gastropods, the eastern oyster (*Crassostrea virginica*) are known to harbor gut microbiomes containing bacterial phyla Firmicutes, Proteobacteria, Tenericutes and Verrucomicrobia (King et al., 2012).

Microbiomes may also enable some mollusks to digest uncommon diets, allowing the hosts to adapt to metabolically challenging habitats. For example, the bone-eating snail *Rubyspira osteovora* is one of very few organisms thought to rely exclusively on enriched bones as a novel form of energy, using its specific microbial community to help digest the nutrient-poor food source (Aronson et al., 2017). *Rubyspira osteovora* relies on a specific and highly selective gut microbiome (*Mycoplasma*, *Psychromonas* and *Psychrilyobacter*) to facilitate its opportunistic colonization of whale falls (sunken, dead whales that provide large amounts of organic material to a very carbon-limited deep-sea environment) (Aronson et al., 2017). Another example comes from the bivalve family Teredinidae, commonly known as "shipworms" (Brito et al., 2018). These bivalves harbor cellulolytic Gammaproteobacterial symbionts (such as those of genus *Teredinibacter)* in their gills that provide critical cellulose-degrading enzymes, including cellulase and dinitrogenase, to digest woody debris (Distel et al., 2002; O'Connor et al., 2014; Flórez et al., 2015; Brito et al., 2018; Shipway et al., 2019).

In addition to digestive roles, gill-associated bacterial communities are essential for some mollusks that rely on the symbionts' chemoautotrophic abilities (Duperron et al., 2013a). These symbionts utilize diverse carbon sources and derive their energy from the oxidation of reduced compounds and include predominantly sulfur-oxidizing and methane-oxidizing bacteria (Ritt et al., 2012). By forming associations with bacterial endosymbionts, chemotrophic mollusks can survive in habitats such as deep-sea vents and cold seeps, which are inhospitable to many other animals due to anoxic conditions and high hydrogen sulfide concentrations (Distel, 1998; Ritt et al., 2012). The chemosynthetic strategy can also be found in mollusks distributed in shallow-water coastal and intertidal systems (König et al., 2016).

Bivalves are the most widespread users of the chemosymbiosis strategy, both phylogenetically and geographically (Distel, 1998; Taylor & Glover, 2006, 2010). In particular, the bivalve family Lucinidae is one of the most diverse groups of chemoautotrophic mollusks (Taylor &

Glover, 2006). Lucinids exist in a variety of environments including the sub-oxic zone of marine sediments, mangrove muds, and seagrass beds (Taylor & Glover, 2006). They predominantly use thiotrophic, or sulfur-oxidizing, bacterial symbionts which might include bacterial species from Epsilonproteobacteria, but have also been shown to use methanotrophic symbionts, hypothesized to be closely related to Gammaproteobacterial genera like *Methylobacter* and *Methylomicrobium*. Not limited to lucinids, other molluscan chemosynthesizers include the mussel *Bathymodiolus thermophilus*, the giant white clam *Calyptogena magnifica,* the scaly-footed snail *Chrysomallon squamiferum*, and the peltospirid gastropod *Gigantopelta chessoia* (Chen et al., 2015), all found in deep-sea vent environments, well-known chemosynthetic habitats (Fiala-Médioni et al., 1993; Belkin et al., 2007; Newton et al., 2007; Dubilier et al., 2008). In shallow waters, the clam *Solemya velum* utilizes uncharacterized chemosynthetic Gammaproteobacterial bacteria to fix carbon dioxide, which provides critical nutritional support to the host (Russell et al., 2018). Similarly, *Codakia orbicularis,* a tropical shallow-water bivalve commonly found in seagrass sediments, hosts sulfur-oxidizing symbionts in its gill filaments (Gros et al., 2012; König et al., 2016). These symbionts, while yet uncultured, appear to be related to other diazotrophic Gammaproteobacteria that are known to be sulfur oxidizers, including *Sedimenticola thiotaurini*, *Thiorhodococcus drewsii* and *Allochromatium vinosum* (König et al., 2016).

Some grazing gastropods may use bacteria as a food source itself. The Hawaiian tree snail *Achatinella mustelina* feeds on microbial communities growing on leaf surfaces and is a generalist feeder, though the majority of bacteria come from orders Oceanospirillales and Enterobacteriales (O'Rorke et al., 2014). Questions arise regarding how to determine if bacteria in host guts are indicative of food source or resident symbionts. One may starve the host before dissection/extraction of gut content in order to remove all transient bacteria (Cardoso et al., 2012b), or compare bacterial strains found in the gut, feces, and environment before and after host introduction (O'Rorke et al., 2014, 2017). Grazing snails may also "farm" microbial communities, acting as ecosystem engineers to promote or constrain microbial activities which may accelerate the decomposition of other food sources, like leaf litter (Meyer et al., 2011). A prominent example is fungal farming by the salt marsh periwinkle, *Littoraria irrorata*. *Littoraria irrorata* uses and morphologically manipulates its radula to create wounds on cordgrass that in turn become infected with microscopic fungi that the snail returns to consume (Silliman & Newell, 2003; Hensel & Silliman, 2013; Chalifour et al., 2019). Fungal farming is a known feeding strategy, but whether snails are also farming bacterial strains is unknown as of yet (O'Rorke et al., 2016; Gilbertson et al., 2019). Understanding which bacterial strains are favored as food is useful for creating captive breeding programs for endangered molluscan species and appropriate field reintroduction (O'Rorke et al., 2014, 2016).

Overall, microbial symbionts may be vital components in many mollusks' metabolic and growth processes. These examples of various bivalves and gastropods are by no means exhaustive. With more in-depth investigations, we may find that the diverse microbiomes in other molluscan classes (e.g., Polyplacophorans – Duperron et al., 2013b) also play integrative roles in the hosts' energy metabolism.

Immunology

In many species across animal phyla, including mollusks, the immune system serves to regulate and maintain microbial communities and prevent pathogen colonization (McFall-Ngai, 2007; West et al., 2019). Recent research has shown that the molluscan microbiomes may potentially impact the hosts' immunobiology and pathogen resistance abilities by physically or chemically disrupting pathogens or hosting beneficial symbionts in the host's body (Romalde & Barja, 2010; Lokmer & Wegner, 2015; Allan et al., 2018).

Molluscan microbiomes may strengthen host pathogen resistance in various ways. For example, beneficial symbionts can take up space and resources on both internal and external host surfaces for which pathogenic bacteria must compete (Loker et al., 2004). This specifi conglomeration of symbiotic bacteria – unique to the host's body rather than the surrounding environment – prevents pathogen colonization of the host (Engel et al., 2002). An example of mutualistic symbiont presence on epithelial surfaces driving away pathogenic colonizers occurs in the squid *Euprymna scolopes*, famous for its mutualistic association with the luminous bacterium *Vibrio fischeri* (Loker et al., 2004; Mandel & Dunn, 2016). In addition to *V. fischeri*, it has been suggested that *E. scolopes* houses uncharacterized symbiotic bacteria in its gill tissues. These

mutualistic bacteria facilitate the hosts' defensive measures against opportunistic pathogens which invade the circulatory system by sequestering pathogenic bacteria in cysts after they are redirected to the gills (Small & McFall-Ngai, 1999). However, this contradicts a recent study that found no bacterial antigen presence in octopus gills but hypothesized that digestive glands may instead clear out pathogens (Bakopoulos et al., 2017). In *Crassostrea virginica*, microbial symbionts are also hypothesized to protect the hosts from pathogenic bacteria by disrupting biofilm growth and inhibiting their settlement (Braun et al., 2019). Selective and well-managed bacterial populations may be essential to warding off pathogens in more molluscan host species than currently known. While microbiomes may help to strengthen resistance to pathogens through physical disruptive measures, chemical protection may also be aided by symbiotic bacteria.

Molluscan microbial communities may produce anti-pathogenic chemicals that benefit the host species. For example, *Streptomyces* strains isolated from the snail genus *Conus* produce several benzyl thiazole and thiazoline compounds that exhibit antimicrobial, anti-inflammatory and antihypotensive properties (Peraud et al., 2009; Lin et al., 2010). A bacterial strain (*Lactobacillus mesentereoides*) found in the land snail *Cornu aspersum* displays antibacterial activity, inhibiting the growth of a pathogen *Propionibacterium acnes* (Koleva et al., 2014). These microbial symbionts can cluster externally in order to provide the most direct antagonistic defense against unwanted colonizers before they can breach the host's surfaces (Flórez et al., 2015).

Some mollusks can host symbiotic bacteria within the circulatory system (Rubiolo et al., 2019). The hemolymph, or circulatory fluid, allows bacterial entrance either through invasion or filter feeding. Symbiotic bacterial strains are hypothesized to protect the molluscan hosts from pathogens, with many belonging to the same genera as the beneficial symbionts (Rubiolo et al., 2019). In many healthy marine bivalves, like mussels and oysters, the hemolymph contains bacteria of the genera, *Vibrio, Pseudomonas*, *Alteromonas*, *Pseudoalteromonas*, *Stappia* and *Aeromonas* (Olafsen et al., 1993; Ivanova et al., 1996; Pujalte et al., 2005; Antunes et al., 2010), which exhibit antimicrobial activities towards many pathogenic bacterial strains, including *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Roseovarius crassostreae* and *Salmonella typhi* (Boettcher et al., 2000; Pujalte et al., 2005; Braun et al., 2019). Further studies looking at bacterial function within the host are needed, as bacteria may show defensive properties *in vitro* that do not necessarily translate to *in vivo*.

Molluscan immunology and disease protection research are particularly important for aquaculture. These businesses are threatened by emerging and repeated disease outbreaks attributed to a wide variety of pathogens (Prieur et al., 1990; Bachère et al., 1995; Romalde & Barja, 2010). These pathogens include, but are not limited to, bacteria in genera *Achromobacter, Pseudomonas, Flavobacterium* and *Vibrio* (Bachère et al., 1995). While antibiotics are traditionally used to treat these diseases, issues with increased antibiotic resistant strains and side effects on the environment have been raised (Dubert et al., 2016, 2017). Therefore, probiotics are emerging as a sustainable alternative to prevent disease in mollusk aquaculture (Romalde & Baria, 2010; Karim et al., 2013; Li et al., 2017). Future studies may promote the utilization in aquaculture of beneficial microbiomes isolated from known disease-resistant mollusks to prevent disease outbreak (King et al., 2019).

Although no cases have been directly established in mollusks, many other vertebrate and invertebrate hosts utilize bacteria to prevent macroparasite invasion (Biron et al., 2015). The interactions between molluscan bacterial symbionts and macroparasites should be further investigated to elucidate if molluscan symbionts can protect them from larger parasites.

Reproduction & Development

The reproductive and developmental success of mollusks may be influe ced by their microbiomes. In some taxa, bacterial symbionts produce secondary metabolites for host chemical defense and may explain why the eggs and larvae of many marine mollusks are unpalatable to predators (Lindquist, 2002; Barbieri et al., 2001). For example, tetrodotoxin of blue-ringed octopuses, likely produced by microbial symbionts, is used to coat octopus egg masses and potentially protect them from predation (Hwang et al., 1989; Williams et al., 2011).

Microbial symbionts may also be responsible for the antimicrobial and anti-fouling properties found in egg masses of many mollusks (Benkendorff et al., 2001). For instance, diverse bacteria strains with antimicrobial activities can be isolated from different sea slugs' egg masses, indicating a potentially microbial-driven egg protection mechanism (Böhringer et al., 2017). A more thoroughly studied class is the cephalopods

(Flórez et al., 2015). They lay large clutches of eggs that can go unsupervised for weeks or months before hatching. In some cephalopods, these egg masses do not fall victim to algal, fungal or bacterial infections and seem to resist fouling despite being constantly surrounded by the high density of microbes present in seawater (Biggs & Epel, 1991; Kerwin et al., 2019). Evidence indicates that female cephalopods deposit microbiomes from the accessory nidamental gland (ANG) into the jelly coating of the eggs to protect them from infection and fouling (Barbieri et al., 2001; Kerwin & Nyholm, 2017, 2018; Kerwin et al., 2019), as these two entities (the ANG and egg coating) show highly similar bacterial compositions (Lutz et al., 2019; Li et al., 2019). The ANG contains a dense bacterial community, found to be common across many cephalopod species and environmental backgrounds (Pichon et al., 2005; Kerwin & Nyholm, 2018). This community includes members of bacterial classes Alphaproteobacteria (e.g., *Roseobacter* and *Phaeobacter*), Gammaproteobacteria (e.g., *Vibrio*, *Pseudoalteromonas*, and *Pseudomonas*), and phylum Bacteroidetes (e.g., *Flavobacterium*). Some of them are shown to have antifungal properties or can produce neurotoxins (Barbieri et al., 2001; Collins et al., 2012; Kerwin & Nyholm, 2017). Indeed, eggs from the Hawaiian bobtail squid (*Euprymna scolopes*) treated with an antibiotic were completely enveloped by fouling in 15 days, while untreated eggs fully resisted fouling (Kerwin et al., 2019). Given the rich microbiome in the ANG, further research is needed to investigate additional functions of ANG-associated microbial communities (Collins et al., 2012; Kerwin et al., 2019).

Along with egg protection, sexual maturity in several cephalopods is associated with ANG microbiome activities, where certain unidentified, but likely gram-negative bacteria, are hypothesized to produce carotenoids to change ANGs from white to red, signaling the onset of sexual maturity (Van den Branden et al., 1980; Lum-Kong & Hastings, 1992; Flórez et al., 2015). However, further research is necessary to explore if the host induces the bacteria to produce carotenoids or if the bacteria triggers female maturity (Flórez et al., 2015).

It is currently unclear whether microbiomes play crucial roles in mollusk development. However, we do know that bacterial community compositions can change through different host life stages. For instance, cellulolytic bacteria are not detected in juvenile snails of *Cornu aspersum*, while strains from the genus *Aeromonas* were only found in juveniles (Koleva et al., 2015). This may be because amylolytic bacteria are vertically transmitted in these snails, whereas transient proteolytic and cellulolytic bacteria are gained through environmental augmentation when the adult snail is active (Dar et al., 2017). Similarly, in the freshwater snail *Radix auricularia*, juvenile snails' gut microbiomes are more enriched with *Faecalibacterium* and *Subdoligranulum* compared to adults, likely meeting the juveniles' digestive needs (Hu et al., 2018). Overall, though the microbiome is often persistent throughout mollusk life stages, its makeup shifts likely due to the changing needs of the host.

MICROBIOMES AND MOLLUSK ECOLOGICAL INTERACTIONS

Predator/Prey Interactions

Use of Bacterial By-Products for Protection

Microbial associations may play important roles in molluscan anti-predator activities. In particular, microbial symbionts can produce bioactive compounds to enhance hosts' chemical defense (Flórez et al., 2015). For example, Tetrodotoxin (TTX) is a defense chemical that blocks sodium channels in nerve and muscle tissues of many predatory species (Yasumoto et al., 1986; Narita et al., 1987; Noguchi et al., 1987). It is now widely supported, as TTX is found in a wide variety of phylogenetically distinct animal phyla, that TTX in animals is produced by symbiotic bacteria from genera including *Bacillus, Vibrio, Shewanella, Aeromonas, Alteromonas, Plesiomonas* and *Pseudomonas* (Hwang et al., 1989; Cheng et al., 1995; Wang et al., 2008; Chau et al., 2011; Magarlamov et al., 2017). Further research into unculturable bacteria may provide more insight into the mechanisms surrounding the biosynthesis of TTX (Chau et al., 2011). It is important to note that host protection is not always demonstrated in the following studies, and further investigation is needed to demonstrate how secondary metabolite productions translates to host protection *in vivo.*

TTX-producing bacteria can live in a variety of mollusks, including bivalves, gastropods, and cephalopods (Silva et al., 2012; Cassiday, 2008; Magarlamov et al., 2017). A prominent example is the blue-ringed octopus (*Octopus*

maculosus), where TTX is found primarily in the posterior salivary gland (PSG) and other soft parts of the animal (Hwang et al., 1989; Williams et al., 2011). TTX may provide defense not only to the adult octopus, but also to their eggs (see Reproduction & Development), protecting them from predation (Hwang et al., 1989). TTX-producing bacteria are also found in the grey side-gilled slug (*Pleurobranchaea maculate*) and other gastropods, such as *Nassarius conoidalis* and *Nassarius semiplicatus* (Cheng et al., 1995; Wang et al., 2008).

In addition to chemical defense, some cephalopods may form symbioses with bioluminescent bacteria as an anti-predation adaptation. These associations are well studied in the famous "squid-*Vibrio*" model, consisting of a symbiotic relationship between the Hawaiian bobtail squid (*E. scolopes*) and a luminous bacterial species *Vibrio fischeri* (McFall-Ngai, 2014). The squid has specialized light organs containing the light-producing bacteria. Light production in the lower mantle cavity is used for counter-shading down-welling moonlight, also known as counterillumination, a tactic used to avoid being detected by predators (Jones & Nishiguchi, 2004). *Vibrio fischeri* maintains luminescence through a day-night rhythm consistent with the foraging habits and prime active hours of the squid (Heath-Heckman et al., 2013; McFall-Ngai, 2014). Further, it is an association that appears to begin almost immediately from birth. No bacteria are found on hatched embryos, but they are discovered shortly after, showing they are acquired directly from the environment (McFall-Ngai, 2014). This relationship is maintained by *V. fischeri's* ability to effectively colonize the host and the squid's ability to eliminate non-functional bacterial cells (McFall-Ngai et al., 2012).

Though arguably the most well-known molluscan bacterial symbiosis, the bobtail squid is not the only cephalopod that houses bioluminescent bacteria (Guerrero-Ferreira & Nishiguchi, 2009). Since the discovery of this relationship, many studies have shown similar perceived symbiotic associations between bioluminescent bacteria and other squid genera, including *Loligo*, *Sepia* and *Euprymna* (Flórez et al., 2015; Guerrero-Ferreira & Nishiguchi, 2007). Molecular research has also revealed undescribed lineages of the luminescent bacteria (Guerrero-Ferreira & Nishiguchi, 2007), demonstrating that the diversity and function of symbiotic bioluminescent bacteria still need further exploration.

Use of Bacterial By-Products for Predation

Though fewer studies consider the microbiome as a tool to facilitate mollusks as predators, one possible example resides in the cone snails (family Conidae). The venomous cone snails prey upon marine worms, fish and other invertebrates, using their neurotoxic venom to immobilize their prey (Lin et al., 2010; Torres et al., 2017). Studies show that the microbiomes of some cone snails are relatively unique. They contain diverse actinomycetes and other bacteria that produce secondary metabolites, which exhibit neurological activity (Peraud et al., 2009; Lin et al., 2010). In addition, abundant and universal presence of *Stenotrophomonas*-like bacteria are found in the venom ducts of diverse cone snail species (Torres et al., 2017). Currently, it is unclear whether these seemingly unique microbiomes actually contribute to the venom cocktail in cone snails. Therefore, further work is needed to assess the functional importance of the microbiome and their metabolites, as well as *in vivo* studies to demonstrate that these bacterial secondary metabolites function similarly in the host.

Adaptation to Abiotic Challenges

Mollusks may utilize symbiotic relationships for protection against harsh environments. One interesting case of structural protection due to microbial facilitation has been described in the scaly-footed snail, *Crysomallon squamiferum*, a gastropod occurring at hydrothermal vents (Goffredi et al., 2004; Flórez et al., 2015). The snail's foot is covered in hardened scale-shaped sclerites of multiple layers, which likely aid in their adaptation to a physically and chemically challenging (e.g., thermal fluctuation) deep-sea habitat (Goffredi et al., 2004). The outer covering of the sclerites is composed of iron sulfides and is colonized by a rich microflora, including Delta- and Gammaproteobacteria (e.g., genus *Desulfobulbus)* known to recycle sulfur and mineralize iron sulfides. It is therefore hypothesized that the microbiome on the surface of the snail sclerites is responsible for producing the iron sulfides veneer, although more direct evidence is still needed to further support this hypothesis (Goffredi et al., 2004)

Although direct studies on microbial-aided abiotic adaptation are largely lacking in mollusks, many studies have shown that mollusk associated microbiomes shift significantly with the changing abiotic environment. For example, some terres-

trial snails cope with fluctuating climates by altering their physiological state and entering periods of aestivation or hibernation, a behavioral adaptation that ensures survival under adverse conditions (Cardoso et al., 2012a, 2012b; Nicolai et al., 2016; Koleva et al., 2015). When in an altered physiological state, the host microbiome undergoes dramatic compositional changes (Nicolai et al., 2016). These changes are seasonally dynamic and restructure the gut community. Proposed reasons for microbiome change include: lacking exogenous or allochthonous sources of microbiota; host expulsion of gut contents; and lacking water content within the body (Pawar et al., 2012; Nicolai et al., 2016; Koleva et al., 2015; Dar et al., 2017). In addition, expunging some bacterial strains can create space for other strains that are less dominant during host active periods, such as ice-nucleating bacteria (Nicolai et al., 2016). However, few studies have investigated how these shifting microbiomes functionally interact with host physiology.

MOLLUSK MICROBIOME TRANSMISSION AND ASSEMBLY

Transmission

The microbiome can be made up of either or both horizontally and vertically transmitted microbiota. Vertical transmission refers to symbionts passed maternally or via an egg coating (McFall-Ngai et al., 2014). Core bacteria found in mussels, for instance, are theorized to be vertically transmitted, including pathogenic bacteria which may account for higher rates of mortality in juvenile mussels (Rubiolo et al., 2019). See the above section on Reproduction & Development for more examples of vertical transmission. Further study of vertical microbial transmission may require examining egg-laying behavior across more molluscan species, for example, slugs may transmit microbiota from parent to offspring using an extracellular substance (Sommer, 2018).

Horizontal transmission refers to symbionts that are acquired *de novo* from the environment (Mc-Fall-Ngai et al., 2014). The gut microbiome, for example, can reflect the flora and fauna that mollusks consume from their environment. For terrestrial mollusks, these include the plant and fungal species present in their habitat, as well as soils eaten to augment their microbial composition (Dishaw et al., 2014; Nicolai et al., 2016). The exogenous sources of microbiota that make up the gut microbiome can change in respect to micro-habitat conditions like soil composition, flora diversity, and climatic factors (Nicolai et al., 2016). In other systems, such as deep-sea hydrothermal vents, bivalves gain chemosynthetic bacteria nearly exclusively each generation from the environment (Funkhouser & Bordenstein, 2013).

Intraspecies Variation in Microbiome Assembly

Within mollusks, some bacteria are transitive, and some are stable, core members of the microbiome. A core microbiome refers to a population of bacterial species that are consistently present in large numbers (Rosenberg & Zilber-Rosenberg, 2016). The variation of molluscan microbiome makeup can be attributed to several factors that also fluctuate; these include environmental, life history, and genetic effects. For instance, the microbiome composition of marine bivalves is influenced by host genetics, environmental conditions, and host infections and stress (Gómez-Chiarri et al., 2015).

Some molluscan gut microbiomes contain core members, but the overall composition is readily altered by environmental changes, such as alterations in diet, physiological state triggered by seasonal changes (i.e., hibernation, aestivation), and habitat (Cardoso et al., 2012b; Nicolai et al., 2016; Sommer, 2018). For example, in the freshwater snail *Oncomelania hupensis*, Actinobacteria dominates the gut microbiome of snails in mountainous regions, and Firmicutes dominates those from marshlands (Hao et al., 2020). The giant African land snail, *Achatina fulica,* harbors a diverse gut community that is able to adapt to changing diets introduced *in vitro*, suggesting that mollusks can selectively choose their core microbiota to survive and adapt to the demands of a changing diet (Cardoso et al., 2012b). This indicates that the presence of core and non-core microbiota are a result of recent, environmental horizontal acquisition and can be changeable by external manipulation (Rosenberg & Zilber-Rosenberg, 2016).

Variation in the host's life history, particularly reproductive strategy, can influence the makeup of microbial communities in mollusks. For some gastropods, the transition from sexual to asexual reproduction triggers a change in microbiome composition (Takacs-Vesbach et al., 2016). This shift is apparent in the New Zealand mud snail, *Potamopyrgus antipodarum,* in

which individuals in these populations can either use obligately sexual or obligately asexual means of reproduction. These two means of reproduction are associated with two different dominant bacteria found in both the head and body tissues. Asexually reproducing individuals are dominated by a strain from the genus *Rhodobacter*, while sexual individuals harbor a strain form the order Rickettsiales. This association suggests the snail's reproductive strategy has certain levels of influence on the assembly of its microbiota (Takacs-Vesbach et al., 2016).

Genetic effects, such as allelic variation and the presence or absence of a gene, can alter the microbial composition within a host (Allan et al., 2018). In other species like mice, host genotype has been found to have a strong effect on individual gut microbiome composition (Benson et al., 2010). In planorbid snails, changes in allelic variation are thought to infl - ence pathogenic immunity and host defense by affecting the snail microbiome (Allan et al. 2018). Allelic variation, particularly in the Guadeloupe resistance complex (GRC), is thought to influence the composition of the snails' microbiomes, which in turn is hypothesized to influence resistance to schistosome pathogens (Allan et al., 2018).

To better understand the mechanisms of molluscan microbiome assembly, we can borrow concepts from community ecology (Costello et al., 2012). Assemblages of microbiomes in other species, for instance, are shaped by factors like priority effects (Sprockett et al., 2018), resources (Larue, 2005; Ahmed et al., 2019), immigration (Manichanh et al., 2010), and disturbance (Dethlefsen & Relman, 2011). It is likely that these same principles can be applied to molluscan host/microbe interactions. Prosser et al. (2007), Konopka (2009), Costello et al. (2012) and Pepper & Rosenfeld (2012) all provide more detailed analysis and expertise into the community ecology of microbiome research.

COMMON METHODS IN MOLLUSK MICROBIOME RESEARCH

While understanding host microbiome composition (the "who's there" question) is the focus of most research cited in this paper, other methodologies can better infer potential bacterial symbiont functionality within the host (the "what are they doing" question) and where these bacteria are localized ("where are they doing it") (Apprill, 2017). However, often the very first step to analyze host-associated microbiomes are studies comparing microbial community composition between treatment groups based on manipulated factors.

Experimental manipulation in the animal host's natural environment is an ideal way to study host-microbiome interactions (Apprill, 2017). However, natural systems offer a limited selection of variables that may be manipulated or are natural occurring, such as various life history events (Apprill, 2017). Common garden experiments allow another way to compare between molluscan host conditions and their associated microbiomes. These experiments can control variables that are difficult to change in nature, such as ambient temperature, humidity, and diet, etc. The experiments also allow for antibiotic experiments that are concerning to dose in the wild. One limitation of common garden experiments is their narrow study systems. They can be useful for some organisms (e.g., smaller gastropods, bivalves, etc.), but are not realistic ways to study many host species that are hard to culture (e.g., hydrothermal vent species or others in extreme environments).

Recent developments in environmental DNA (eDNA) sampling strategy allow us to collect information from environmental samples to assess biotic interactions instead of directly from the host. In these studies, DNA is extracted from environmental samples and species present are identified using trace DNA. This is becoming a popular way to identify the presence of exotic and invasive molluscan species, for example, by extracting eDNA from ballast water (Ardura et al., 2015; Clusa et al., 2017; Cowart et al., 2018; Klymus et al., 2017). One can also use eDNA to gain information on bacterial strains present in soil or water samples, informing how mollusks acquire bacteria from their environment.

After obtaining microbial samples from the hosts/abiotic environments, genetic analyses are commonly used to assess which specific bacteria are present in the microbiome. Recent advances in next-generation sequencing technologies have dramatically improved both the speed and accuracy of genomic DNA sequencing, while continuing to reduce overall effort and cost. Outlined below is a brief summary of amplicon sequencing and analyses, one of the more common diversity-based survey methodologies. Detailed information on next-generation sequencing technology and methods can also be found in published review papers and project protocols (e.g., Zhou et al.,

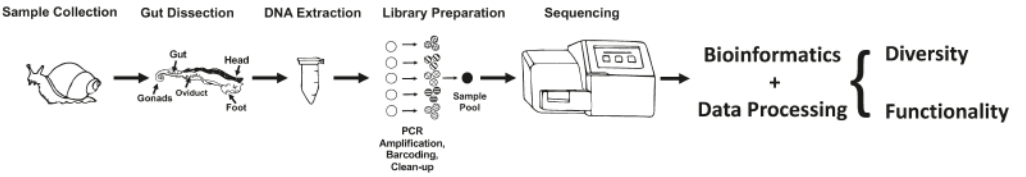

FIG. 2. Generalized protocol from sample collection through data processing for mollusk microbiome analysis. First, mollusk specimen samples are collected, the example used here is a snail. The mollusk is dissected to take tissue from the desired section, in this case, the gut. DNA is extracted from the dissected gut tissue. Extracted DNA samples undergo PCR with primers optimized for microbial amplicons. Samples from multiple individuals are then barcoded (represented by the patterns shown here), cleaned, and pooled. PCR products are sequenced using high-throughput technology. Resulting community sequencing data are analyzed using bioinformatics pipelines to run diversity analyses. Functional analyses may be run using other methodologies, for example, metagenomic shotgun sequencing pipelines or *in vivo* studies.

2010; Rajesh & Jaya, 2017; Thompson et al., 2017). Importantly, since amplicon sequencing usually focus on one or only a few genes, they cannot directly inform the functional capabilities of the microbiome (Langille et al., 2013).

The general workflow from microbial DNA extraction through data processing and analysis via amplicon sequencing is given in Figure 2. The tissue of interest from a host organism is dissected from the body. Total genomic DNA is extracted from the tissue using the appropriate DNA extraction kit. Choosing an appropriate kit may depend on the tissue that DNA is extracted from. For example, molluscan tissue benefits from a rigorous lysing step. The general process of DNA extraction involves isolating DNA from the cell. This is done through disrupting cell membranes to release their DNA contents, separating the DNA from cellular debris, and precipitating and cleaning the DNA. Follow-up steps include confirming the quality and quantity of the extracted genomic DNA. DNA purity can be determined using optical density readings taken by a spectrophotometer, and the DNA concentration quantified by a flu - rescent dye-based assay (for example, pico assay or Qubit).

Genomic DNA can then be used for downstream applications such as metagenomic library preparation and sequencing. Primers that target bacterial and archaeal marker genes from the extracted samples are used to assess bacterial and archaeal community composition of the host species. One common primer pair used for targeting bacterial gene markers is the combination of 515F and 806R (Forward: GTGYCAGCMGCCGCGGTAA; Reverse: GGACTACNVGGGTWTCTAAT), which targets the V4 hypervariable region of the 16S rRNA gene (Caporaso et al., 2012; Thompson et al., 2017). The PCR amplifica ion protocols for this primer set can be found via the Earth Microbiome Project (Thompson et al., 2017). Gel electrophoresis is used to confirm the success of the PCR step. The expected fragment length for 515F/806R is around 390 base pairs. The PCR amplification and barcoding should use a series of controls to ensure no foreign or contaminant DNA has been introduced into the samples during extraction and library preparation. This includes negative extraction controls (blanks) and negative PCR controls. Minimizing the effects of both contaminant DNA and cross-contamination, especially in low microbial biomass samples, is key to correctly interpreting microbial data (de Goffau et al., 2018; Eisenhofer et al., 2019). More detailed methods and principles of next-generation sequencing library prep and sequencing can be found in review papers such as Li (2015) and Rajesh & Jaya (2017).

Software like QIIME (Quantitative Insights into Microbial Ecology), mothur, USEARCH, and DADA2 provide a standardized pipeline for 16S rRNA gene sequence data processing and analysis from raw sequences to interpretation, and to deposition into databases (Caporaso et al., 2010; Allali et al., 2017; Galloway-Peña & Hanson, 2020). As more sequences are added to databases, especially molluscan microbial-associated community sequences, efficiencies and accuracies within these pipelines will improve across diverse molluscan groups. This is why submission to public databases (these including QIIME, MG-RAST, NCBI, among others) of both sequence files and available metadata is vital to the progression of the field (Goodrich et al., 2014). Other downstream

analyses often quantify and compare the abundance, alpha-, and beta- diversity of samples. Popular multivariate analyses include PERMANOVAs, ANOSIMs, hierarchical clustering, random forests, Kruskal-Wallis tests, and principal coordinate analyses (Li, 2015; Callahan et al., 2016). Goodrich et al. (2014) provide a foundational guideline for how to visualize microbiome data. However, deciding which tests are appropriate is also dependent on the metadata associated with the project and researchers should investigate the methods of relevant literature to find appropriate tests

Following microbiome composition assessments, microbiome functions should be investigated (Apprill, 2017). Many "omics" methods can be used to infer microbiome functionality. These typically include metagenomics, transcriptomics, proteomics, and metabolomics (Fondi & Liò, 2015; Beale et al., 2016). Metagenomics in particular can be useful in assessing potential functionality of microbial communities. For example, the shotgun sequencing approach can be used to capture snapshots of genomes and their predicted functions in the microbiome, although this method is limited by available reference genomes, coverage, and host DNA overshadowing that coverage (Galloway-Peña & Hanson, 2020). Certain bioinformatics pipelines, such as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), can predict the microbiome functionality based on marker gene data and reference genomes (Langille et al., 2013). In order to compliment the genomics data, a multi-omics approach can be used to discover and validate functional assignments by integrating other types of information (Santos et al., 2011; Fondi & Liò, 2015), such as protein production, gene expression, and metabolic profile (Baran et al., 2009; Beale et al., 2016). The multi-omics approach is an expensive option and integrating these large and diverse datasets may be mathematically and computationally challenging. However, experimental and computational pipelines have been developed to allow multi-omics modelling (Fondi & Liò, 2015). More pipelines and downstream tools for microbiome analysis are summarized in Galloway-Peña & Hanson (2020).

Bacteria cultivation methods can also help characterize functionality of certain bacterial strains *in vitro*. Although it is long believed that culturing can only provide information on the limited number of culturable bacteria (Salmonová & Bunešová, 2016; Nazir, 2016), recent research indicates that more bacteria are culturable than previously thought (Martiny, 2019). While an inexpensive option, culturing is both a time-consuming and labor-intensive process. As with other methods, information gained through culturing may not translate into when the bacteria are present in the host (Salmonová & Bunešová, 2016). Some studies co-culture bacteria along with host tissues to better simulate an *in-vivo* environment, although this will not inform where bacteria perform their functions in/on the host (Galloway-Peña & Hanson, 2020).

Visualization methods can be used to determine where bacterial consortia arise in a host, and how the bacteria's function is connected and varies with the host environment (Tropini et al., 2017). Simple observations using scanning electron microscopy (SET) or transmission electron microscopy (TEM) can be used to confirm symbiont presence within host tissues. Observations within the embryo or larvae, for example, can indicate that vertical transmission may be happening between parent and offspring (Chen et al., 2017). Other microscopy methods, such as fluorescence *in situ* hybridization (FISH) and catalyzed reporter deposition-FISH (CARD-FISH), may also be used to visualize bacteria (Chen et al., 2017). These approaches can be used in conjunction with experimental design to observe bacterial distribution, activity, and interactions with the host. The development of more advanced microscopy techniques, such as light sheet microscopy (Legant et al., 2016) and electron cryotomography (Oikonomou et al., 2016), provides the possibility to observe living bacteria at the molecular level. These methods may shed light on molecular mechanisms of symbiont-host interactions. Limitations exist with these visualization methods as well, mostly caused by difficultie in developing viable protocols to work with diverse living systems (Tropini et al., 2017) and access to specialized equipment.

A multi-approach pipeline, including comparative studies and investigating the diversity, potential function, and host-microbe relationship *in vivo* is needed to further study the ecology of mollusk microbiomes. No perfect methodology exists, and studies must be designed based on realistic financial budgets and equipment accessibility. Combining a variety of investigative tools may help researchers better understand the composition of molluscan microbiomes and the complexity of the symbiotic relationships between bacteria and host.

FUTURE DIRECTIONS OF MOLLUSCAN MICROBIOME RESEARCH

As shown in this review, diverse molluscan microbiome research is already being conducted. However, there are certainly areas in which research is lacking. Many of the works cited here have examined the identity of bacterial strains exist within different molluscan host. This is relatively easy to accomplish using the amplicon sequencing pipeline. Future research should move on to experimentally assess the microbiomes' functional roles, shedding more light on the complex interactions among the molluscan hosts, their microbiomes, and the environment (Callahan et al., 2016). In particular, knowledge of other invertebrate microbiome functions may inform specific roles of molluscan symbionts (Newton et al., 2013; Petersen & Osvatic, 2018; Van Oppen & Blackall, 2019). These might include how bacteria influence the host mollusk's biochemical processes (for example, signaling pathways), immunological responses within the gut, and behaviors (Torres et al., 2017). Additionally, one common challenge is how to assess microbial functions in the hosts' natural environment (Apprill, 2017). For example, many defense-related microbial compounds are only obvious in the presence of the host's antagonists, which is varied and difficult to replicate in laboratory conditions (Flórez et al., 2015). Therefore, to truly understand the ecological function of many microbial symbionts, experiments and methodologies need to be designed to properly capture microbial gene expressions/metabolites *in situ*.

Future research should also move beyond species level investigations and start to elucidate how microbiomes vary within a population or across populations (King et al., 2012). While many studies cited in this review give insight into a survey of bacteria present in certain mollusk species' microbiomes, few look at microbiome variations across host populations, such as examining microbial compositional changes across geographic gradients, seasonal differences, varying environmental factors, or human impacts. Only by gathering this population-level information can we reveal more integrative interactions between mollusks and their microbiomes.

Mollusk microbiome research will also likely grow in the industrial and applied direction. For example, deciphering the digesting processes of mollusks that can quickly and efficiently break down recalcitrant materials may greatly impact industries that need to convert plant biomass into products like biofuels, feeds, textiles, and paper products (O'Connor et al., 2014). Understanding how microbiomes impact the health of commercially important mollusks may improve the efficiency and safety of aquaculture (Rubiolo et al., 2019). Preventing the spread of invasive species is economically viable, and vital in terrestrial systems for productive agricultural practices worldwide (one famous pest example is the *Achatina fulica*, the giant African snail). Microbiome research is valuable in providing insight to more effective control of invasive molluscan species, especially as they often diminish existing, imperiled populations of native mollusks (Sommer, 2018).

Mollusks and their symbionts hold potential for drug development, as they contain diverse and unique natural products that may be exploited for pharmaceutical purposes (Haygood et al., 1999; Peraud et al., 2009). Bioactive compounds utilized for predator deterrence and defense in the wild may also have potential in pharmaceuticals (Peraud et al., 2009; Lopanik, 2014). Additionally, freshwater snails are intermediate hosts for many vertebrate parasites that affect human health. Therefore, understanding their immunology can help researchers better comprehend the transmission of these parasites (Allan et al., 2018). Future studies may help us understand the effec of the microbiome in protecting the mollusk host from pathogens that can be passed to humans, for example, schistosomiasis (Huot et al., 2020). Research investigating these intermediate hosts may prove that their microbiome can contribute to the compatibility of the parasite with the host, and host sensitivity to molluscicides (Hao et al., 2020).

Diverse microbial associations can influenc the health, behavior, and ecology of mollusks, and in turn affect how they respond to anthropogenically-induced challenges, such as climate change (Apprill, 2017; Rubiolo et al., 2019). In order to predict how mollusks will respond to human-induced challenges, it is vital to understand how their symbiotic associations will affect their fitness. For example, in vertebrate hosts, mislocalization of symbiotic bacteria can be a sign of disease, and this may translate to invertebrate hosts as well (Tropini et al., 2017). Research that can potentially aid molluscan survival is imperative due to Mollusca's conservation status; they are

one of the most imperiled animal groups on Earth (Lydeard et al., 2004).

Microbiome research also needs to expand to more diverse molluscan hosts. Within this review, case studies largely came from three molluscan classes: Bivalvia, Cephalopoda and Gastropoda. Within these groups, a large percentage of studies focus on commercially valuable species. The less well-studied classes, such as Polyplacophora, Scaphopoda, and others, should also be investigated through the lens of their microbiomes. These classes may be ecologically unique, and some, like Polyplacophora, are quite abundant, allowing for wide sampling capabilities. While the phenomenon of a stable microbiome appears to exist in many mollusks, there is a great need to investigate many more and a wider spectrum of mollusks before arriving at general conclusions. Expansion of microbiome research across the entire scope of Mollusca will allow comparative studies among diverse morphologies, lifestyle, diets, and habitats, etc. Further examination of extant mollusks will also be key to determining other unexplored, and potentially anthropogenically benefi ial symbiotic microbes.

CONCLUSIONS

While some invertebrate species do not host more than a transient bacterial community (Hammer et al., 2017, 2019), many molluscan species rely heavily on their mutualistic microbiomes. These microbiomes provide nutritional benefits, disease prevention, defense mechanisms, and more. Increased research into the microbiome across the entire molluscan phylogeny will continue to uncover the true nature of these relationships, and what other symbiotic associations exist in phylum Mollusca.

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