

## Potential role of gastro-intestinal microbiota in terrestrial snails

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This review provides an overview of the potential impact of the intestinal microbiota of land snails on their survival and functioning in a constantly changing environment. The diverse communities of microorganisms that inhabit both the outside and inside of animals in unique ecosystems help them to survive in constantly changing environmental conditions. A diverse microbial community is critical for digestion and nutrient delivery, but it is also important for animals that are adapting to new ecological niches. Molluscs are stable, multicellular *in vivo* systems that can be used to study the symbiotic relationships between microorganisms and animals, as well as how microbes affect an animal's physiology and ecology. Furthermore, because of the vast number of species and the ecological diversity of molluscs, they allow for a better understanding of the variability of the microbiota associated with both their location in the host organism and the host in the environment. The microbiome is critical to a body's homeostasis and survival in changing environmental conditions. This is true not only for terrestrial molluscs, but also for freshwater and marine species, which use the properties of the microorganisms that inhabit them to counteract the effects of stress.

Key words: molluscs, microbiome, land snails, invertebrates, gut microbes

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Research on biodiversity in a variety of environments often reveals host-microbiota interactions. It turns out that just as the planet's unique ecosystems can support new biodiversity, the organisms in these ecosystems also support specific microbial communities that live both outside and inside animals. These organisms are often essential for digestion and nutrient replenishment, regardless of the specific nature of the host's nutritional strategy (Brune & Ohkuma 2011; Apprill 2017). The collection of genomes of all microorganisms in the environment is defined by the term 'microbiome'. In turn, the term 'microbiota' usually refers to the microorganisms found in a spe-

cific environment, such as e.g. the gastro-intestinal tract; thus, it can refer to all microorganisms found in that environment, including bacteria, viruses and fungi (Marchesi 2014). An animal's microbiota, after years of co-evolution and thanks to its unique host-microorganism relationship, is often described as the 'animal's second genome' or an ecosystem consisting of populations nested in the organism (McFall-Ngai *et al.* 2013).

Molluscs are known as a highly-diverse group, not only in terms of their morphology but also in terms of their habitat and behaviour. They comprise nearly 7% of all living animals. Their chemical composi-

tion and the production of a wide range of secondary metabolites are reflections of the biodiversity that distinguishes these animals (Ahmad *et al.* 2018; Belouhova *et al.* 2022). The majority of molluscs are gastropods, which include snails (Loker 2010). With a transitory change in their feeding patterns, snails have evolved into marine, freshwater and terrestrial species, while transforming from herbivorous to carnivorous feeding, relying on endoparasitism or on chemoautotrophy mediated by symbionts (Dar *et al.* 2017). Slugs and snails make up the majority of Gastropoda species, with snails having coiled shells on their bodies. Along with terrestrial snails, the term 'snail' is frequently used to refer to freshwater and marine species (Ugoh & Ugbenyo 2013). Snails typically flourish in environments with a lot of calcium, limestone, marl, concrete and cement. Despite being hermaphrodites, they need reciprocal copulation in order to create healthy eggs (Skelley *et al.* 2010). The juvenile snails hatch from these eggs, consume their eggshells and spend two weeks underground in cool, moist soil. While the adults are able to digest debris, juveniles eat fragile plant shoots. Under stressful and unfavourable environmental conditions, snails can bury themselves under dirt in an inactive state and go dormant for months or years (Dar *et al.* 2017).

The hosts are supported by the characteristic features of the microorganisms that populate them when adapting to changing environmental factors, which can affect their community structures and diversity, directly or indirectly (Zhao *et al.* 2021). Symbiotic bacteria are involved in the synthesis and degradation of active substances, maintaining homeostasis and building a barrier against pathogenic infections (Belouhova *et al.* 2022), as well as in gene expression regulation, energy metabolism and even tissue development (Hall *et al.* 2017; Li *et al.* 2022). The dynamic balance of the microbial populations inhabiting a host seems to be a kind of evolutionary adaptation that has enabled many animals to capture and use the resources in their environment (McFall-Ngai *et al.* 2013). The gut microbiota is a particularly important subject of observation and research, as it provides insights into subtle changes in the environmental patterns and microbial communities, both host-specific and at the population level (Chalifour *et al.* 2022). It should be noted that it is necessary to understand how the microbiota is subjected to changes over wide temporal and spatial gradients, as these changes may correlate with the host's response to various types of stressors (Neu *et al.* 2021). An analysis of past microbiota trends is also crucial for

predicting future changes and protecting rare species that depend heavily on their symbiotic microbes for survival (West *et al.* 2019). More up-to-date studies, e.g. research on *Pomacea canaliculata* (Lamarck 1819) (Chen *et al.* 2021) or *Achatina fulica* (Bowdich 1822) (Pinheiro *et al.* 2015), which are based on identification by 16S rDNA gene sequencing, have confirmed that the diverse intestinal microbiota populates not only different parts of the intestinal tract, but also different developmental stages in their intermediate hosts. Moreover, a more in depth approach by a metagenomic analysis provides a full identification, including uncultivated and novel microbiota, as well as allowing researchers to extract more data about the functionalities and microbial characteristics (Li *et al.* 2023).

Some symbionts are known to alter the physiological properties of holobionts, thus influencing the thermal tolerance of their hosts (Hofmann & Todgham 2010; Brumin *et al.* 2011; Corbin *et al.* 2017; Henry & Colinet 2018). To date, no studies have established the physiological roles of bacteria in modulating the host's thermal tolerance during overwintering in invertebrates. However, such an effect is plausible, because the activation of immune mechanisms can also activate protective mechanisms against abiotic stressors (Jones *et al.* 2015). An interesting example, from the opposite perspective of cold tolerance, comes from tick vectors of the human pathogen *Anaplasma phagocytophilum* (Dumler *et al.* 2001) (Neelakanta *et al.* 2010). This pathogen induces the expression of an antifreeze protein and increases the cold tolerance of the host invertebrate (Mushegian & Tougeron 2019).

In this review, we provide an insight into several aspects of the composition and function of the gut microbiota of terrestrial snails. We focus on research examining the localisation impacts of host microbiota diversity and thermally induced alterations. Molluscs, as a very diverse group of animals, are not only ecologically, but also economically important organisms, with a high biotechnological potential related to their wide range of microbiota. Nowadays, their utility is neglected, but they still dwell in a variety of niches, inhabiting almost every kind of terrain. Among the other topics related to snail microbiota, further research seems to be crucial for understanding the role of symbiotic bacteria in the production and decomposition of molluscan active substances, as well as in maintaining homeostasis and in the development of barriers against infections.

### Effects of the temperature on the composition of the gut microbiota in snails

Environmental temperature is a prominent abiotic variable that influences the composition of gut microbial communities in many animal taxa, including invertebrates and vertebrates (Ferguson *et al.* 2018; Fontaine *et al.* 2018; Li *et al.* 2018; Moghadam *et al.* 2018; Moeller *et al.* 2020; Sepulveda & Moeller 2020). Temperate zone land snails are exposed to extreme thermal conditions throughout the year: temperatures below the freezing point of their body fluids in winter; and high temperatures, often associated with drought, in summer. The most common response of these animals to harmful or stressful environmental conditions is inactivity, also known, depending on the season, as winter torpor or estivation (Nicolai *et al.* 2015). The overwintering snails in this climate zone are subjected to low temperatures for a much longer period than in the case of a tropical climate, which results in significant seasonal changes in food availability and immunity, as well as numerous physiological changes, such as emptying of the intestines, reduction of the body water content and producing an epiphragm before falling into torpor (Nicolai 2010). Overwintering at ambient temperatures below the freezing point of water carries the risk of freezing; however, such overwintering is possible thanks to two opposing but somewhat complementary strategies. The first one consists of the production of cryoprotective substances that prevent the formation of ice in the bodily fluids, which leads to a state of supercooling of the body and thus, an active avoidance of freezing. The second is the use of ice nucleating agents (INAs) to control freezing by seeding tiny ice crystals in precisely defined locations, which is the basis of freezing tolerance (Block 1991; Lee *et al.* 1993; Storey 1997; Zachariassen & Kristiansen 2000; Ansart *et al.* 2010). Riddle (1983) suggested that molluscs do not use cryoprotectants, but this thesis was not confirmed by later studies conducted by Nowakowska *et al.* (2006), which showed the presence of cryoprotectants in *Helix pomatia* (Linnaeus 1758) snails. Interestingly, it was also noted that the concentrations of cryoprotective substances in snails (Nowakowska *et al.* 2006) are much lower than those, for example, in insects (Loomis 1991; Ansart & Vernon 2003). In addition, it has been shown that *Cornu aspersum* (O.F. Müller 1774) snails have the ability to supercool their bodies after exposure to a short photoperiod (Ansart *et al.* 2001; Ansart *et al.* 2002; Ansart & Vernon 2003). Ice nucleating substances are inorganic compounds, such as mineral crystals or organic substances (proteins,

lipoproteins and microorganisms or their metabolites), that cause the heterogeneous nucleation of water molecules and the formation of ice. However, this term is usually reserved for substances that induce nucleation at temperatures above  $-10^{\circ}\text{C}$ , i.e. ‘biological nuclei’ (Lee *et al.* 1995a; Lundheim 2002). Naturally occurring INAs have been found in the haemolymphs (Neven *et al.* 1986; Loomis 1991) and in the guts (Lee *et al.* 1991; Lee *et al.* 1995b) of both freeze-tolerant and freeze-avoiding organisms. The ice nuclei in the haemolymph are usually proteins or lipoproteins, while the intestinal germs are food particles or bacteria with the ability to nucleate ice. Ice-nucleating bacteria, which have been known since the 1970s, make it possible to lower the ice nucleation point to a temperature of about 2 degrees below  $0^{\circ}\text{C}$  (Hirano & Upper 1995). Many such species have been identified in the last few decades, especially in the atmosphere, soil and in decaying vegetation (Lindow *et al.* 1982; Ansart *et al.* 2010), suggesting that they are widely present in the environment. Interestingly, there have been reports that INAs are generally removed or inactivated before winter in species that avoid freezing, while they are maintained or synthesised and stored in species that can tolerate freezing (Zachariassen 1985; Toxopeus & Sinclair 2018).

Although *H. pomatia* snails burrow into the ground to a depth of about 0.2 m before the onset of winter, they cannot avoid the risk of freezing, because in temperate climates the ground freezes to a much deeper level. Therefore, *H. pomatia* is considered to be a semi-hardy species that tolerates temperatures below the freezing point of their bodily fluids for short periods, due to the multiple mechanisms responsible for controlling the formation of ice crystals in their bodies (Ansart *et al.* 2010). Although a short-term crystallisation of the body’s fluids is not lethal, it can result in numerous types of cell and tissue damage, which in turn leads to irreversible changes in the organism’s physiology, development and reproductive capacity (Lee Jr & Costanzo 1998; Ansart *et al.* 2002; Ansart *et al.* 2010; Nicolai & Ansart 2017). The wintering of snails exhibiting frost resistance is a complex phenomenon that is based not only on the storage of cryoprotective substances, such as amino acids, glycerol and triglycerides (Nowakowska *et al.* 2006), as well as the synthesis of heat shock (Mizrahi *et al.* 2015) and cold shock proteins (Idczak-Figiel *et al.* 2023), but also on maintaining a diverse psychrophilic bacterial microflora that induces and/or controls the nucleation of ice crystals (Ansart *et al.* 2010) and allows for a lowering of the freezing point of bodily fluids. Nicolai

*et al.* (2005) identified a weak ice nucleator bacterial strain *Kluyvera* sp. (Farmer *et al.* 1981) in *H. pomatia* snails; however, it still induces the nucleation of ice crystals at temperatures well above the crystallisation temperature ( $T_c$ ) of active or inactive snails (Ansart *et al.* 2010). Moreover, Ansart *et al.* (2010) have confirmed that the digestive tract is the first place that ice nucleation takes place in *C. aspersum* snails, similarly to that in the insects *Pyrrhocoris apterus* (Linnaeus 1758) (Hodkova & Hodek 1997) and *Trichiocampus populi* (Hartig 1837) (Shimada 1989). Organic ice nucleating agents have also been found previously in the mucous ribbon of *C. aspersum* that had their intestines emptied as a result of starvation (Ansart *et al.* 2008). The microbiota of snails has a wide impact on the physiological state of the host, providing protection against pathogens and regulating the immune functions, as well as enabling the digestion of various substances in the host's diet (Dar *et al.* 2017; Chalifour & Li 2021). This fits in with the thesis about the important role of microbiota in the host's adaptation, tolerance of environmental changes and proper functioning of its immune system (Amato 2013; Bahrndorff *et al.* 2016). The environmental temperature is an important abiotic variable that affects the composition of the gut microbial community of many animal taxa (Ferguson *et al.* 2018; Fontaine *et al.* 2018; Li *et al.* 2018; Moghadam *et al.* 2018; Moeller *et al.* 2020; Sepulveda & Moeller 2020). Furthermore, microorganisms inhabiting ectothermic organisms are subjected to the same temperature changes as their hosts. Therefore, changes related to the overwintering of the host may be less critical for microorganisms than those caused by freezing itself (Mushegian & Tougeron 2019). In addition, recent studies in endothermic animals have shown that temperature-induced changes in the microbiota may have a cascading effect on the host's phenotypes related to thermal tolerance and potential consequences for the host in response to climate change (Moeller *et al.* 2020; Sepulveda & Moeller 2020). Extreme temperature changes can also disrupt and destabilise alpha diversity within the gut microbiota of individual organisms and initiate dominant changes in the creation of beta diversity in the community (alpha diversity is a measure of the microbiome diversity applicable to a 'single sample'; while beta diversity is a measure of the similarity or dissimilarity between two communities). During periods of host inactivity, such as winter torpor, bacterial communities undergo changes as feeding is suspended and thus exogenous species are not supplied (Pereira & Breckenridge 1981). In addition, various factors such as the host's genetics, maternal effects,

diet, diseases, pathogens, interactions with the immune system and between members of the microbial community, and seasonality can affect the animal's microbiota, exposing the host organism to serious health consequences (Carey *et al.* 2013; Carey & Duddleston 2014). Therefore, the increased likelihood of a host's survival under stress conditions and its good health is closely related to eubiosis, i.e. the microbial balance in the body.

Evidence for the presence of different types of bacteria in snails suggests that the symbiotic relationship between the host and the microbes evolves over time. Many eukaryotic symbionts were isolated from snails in the families *Achatinidae*, *Ampullariidae*, *Helicidae*, and *Planorbidae* (Koch *et al.* 2014). Furthermore, isolated gut bacteria have been identified in vetigastropods of the genus *Haliotis* as well as in several other pulmonates, e.g. *Biomphalaria*, *Bulinus*, *Helisoma*, *Helix*, *Cornu* (Charrier *et al.* 1998; Charrier *et al.* 2006) and *Achatina* (Pawar *et al.* 2012; Dar *et al.* 2015; Pinheiro *et al.* 2015).

### Metagenomic analyses

Utilising cutting-edge in-silico and meta-genomic techniques, an enormous diversity of microbes has been found in the guts of snails, including uncultivated and novel microbial populations (Li *et al.* 2023). Therefore, more research is required to better understand the bacterial diversity of snails and their characteristics. Metagenomic techniques have been used in a few studies to examine the bacterial populations present in snails (Dar *et al.* 2017). Metagenomics and other advanced techniques have also provided evidence that gut bacteria perform various beneficial functions for the host. For example, these resident bacteria aid the host in processes such as the digestion of complex molecules into simpler forms, the generation of energy, synthesising cofactors and amino acids for primary metabolism, and preventing the growth of pathogens. In addition, some bacteria isolated from the snails induce the fermentation of sugars such as glucose, lactose, mannitol, rhamnose, arabinose, maltose and others, demonstrating the snails' positive interaction with their gut flora (Mahejabin & Tarannum 2015). Cardoso *et al.* (2012a, 2012b) examined the giant snail *Achatina fulica* and its biotechnological potential by a metagenomic analysis to potentially expand the enzyme sources for the biofuel industry. Members of the bacteria phylum present in the snail samples mostly belonged to the *Proteobacteria*, followed by *Bacteroides*, *Firmicutes* and many others. The results of



the functional analysis showed a wide range of microbial genes possibly responsible for processes related to the degradation of recalcitrant lignocellulose, the synthesis of essential amino acids and vitamins, or the detoxification of xenobiotics. Furthermore, many novel glycoside hydrolase genes, as well as cellulase and hemicellulase coding sequences were discovered, representing the unique diversity and complexity of this symbiotic relationship (Cardoso *et al.* 2012a, 2012b).

### Environmental and dietary-related changes in microbial diversity

Herbivorous animals that primarily consume lignocellulosic-rich plant materials are perceived as rich sources of bacteria of industrial importance, because their intestines function as natural bioreactors for the decomposition of plant biomass (Devendran *et al.* 2016). The digestive systems of animals change according to their nutritional needs and their physiological adaptations. The digestive tract is usually long and the bacteria inhabiting it provide the host with a special set of enzymes required for the decomposition of materials (Breznak & Brune 1994; Dar *et al.* 2017). As a result, the bacterial flora in the digestive tracts of these animals may be critical to the digestive processes. It is suspected that all functionally separate areas of the digestive tract may constitute characteristic microenvironments that support the various bacterial communities inhabiting a given organism (Dar *et al.* 2017). Studies on the dynamics of the faecal microflora of *C. aspersum* have shown that microorganisms belonging to the Gammaproteobacteria group are abundantly present in various populations of wild snails (Smith *et al.* 2019). The genera *Pseudomonas* and *Buttiauxella*, which are closely related to the soil, showed the highest level of abundance. In addition, the diversity of Gammaproteobacteria in the experimental group that received penicillin decreased during the treatment period, but began to recover over time. After three weeks, faeces samples of the snails still contained Gammaproteobacteria. These results confirmed that *C. aspersum* maintains an endogenous gut microbiome (Smith *et al.* 2019).

The host's physiology and diet are the primary factors that influence an organism's community structure. Many animals' gut microbiomes, including those of snails, have recently been studied (Pawar *et al.* 2012; Loomis 1991). Animals are known to select their gut microbes based on their functions, with the microbial cells outnumbering their hosts

by orders of magnitude (Turnbaugh *et al.* 2007; Zilber-Rosenberg & Rosenberg 2008). Snails, like many other invertebrates, mostly eat soil to obtain beneficial microbes that aid in the digestion process. In turn, the microbiota has important implications for the host's immune system (McFall-Ngai 2007), preventing exogenous pathogenic microbe invasions. In other words, changes in the snail's microbial flora could have a negative impact, causing them to stop feeding and eventually die (Dar *et al.* 2017). Research about the dietary and environmental influences on a snail's microbiota is also important in the preparation of new habitats and the protection of endangered species, e.g. the leaf-eating snail *Achatinella mustelina* (Mighels 1845), which grazes for its microbes on the leaf surfaces of trees. In the results obtained to identify the microbiota in both their faeces and guts, over 60% were shared with leaf samples from the same location. Furthermore, the researchers concluded that the snails are not selective eaters but tend to consume every plant within their range (O'Rorke *et al.* 2015). However, like many other species, they can alter the community structure of the plants that they feed on (Aizenberg-Gershtein *et al.* 2013; Sieg *et al.* 2013) and influence the biosphere succession and nutrient cycling, and so they can alter the antimicrobial layers or barriers on plants (Lindow & Brandl 2003; Yadav *et al.* 2005).

It is well known that one of the main components in the diet of herbivores is cellulose. Scientists have long been interested in the bacteria that release cellulose hydrolysing enzymes, enabling the full use of plant foods in the host's diet. Seillière (1906) was the first to isolate bacterial cellulases from the gastrointestinal tract of *H. pomatia*; while Florkin and Lozet (1949) followed suit, studying cellulases in a similar way (Myers & Northcote 1959; Dar *et al.* 2017). Jeuniaux (1955) then postulated that chitinases of a microbial origin isolated from *H. pomatia* are crucial in the digestion of plant materials in all phytophagous snails. Despite the emptying of the intestines before the onset of winter, and the lack of a supply of exogenous strains during the overwintering and aestivation of the snails, certain strains of bacteria are still active in their intestines. Although the numbers of the bacteria always decline over time (Charrier *et al.* 2006; Watkins & Simkiss 1990), they can be considered as indigenous to the snail's intestine. In addition, the age of the individual also affects the diversity of the bacterial population. Amylolytic bacteria inhabiting the digestive tract of *C. aspersum* inhabit immature individuals, while proteolytic and cellulolytic bacteria have only been observed in mature individuals (Koleva *et al.* 2015). Strains with a higher

cellulolytic and proteolytic activity were observed in snails in their active state, which indicates that these bacteria are introduced into the body along with food, thereby strengthening and accelerating the digestive process. However, during aestivation, overwintering and the immature period, proteolytic bacteria are absent. In addition, it has also been observed that the number of cellulolytic bacteria begin to decrease during the snails' winter torpor (Dar *et al.* 2017).

The invading apple snail *Pomacea canaliculata* has specialised tissues mapped as deposits of uric acid, which upon their release act as a protectant from the damaging effects of tissue reoxygenation following estivation (Giraud-Billoud *et al.* 2013). Koch *et al.* (2014) for the first time identified several uricase-positive bacteria in the guts of this snail species, and speculated that they could be involved in the conversion of uric acid into simpler molecules in the recycling of uric acid nitrogen.

In the western Rocky Mountains of the United States and Canada, there is an ecologically important terrestrial gastropod called the Rocky Mountainsnail *Oreohelix strigosa* (A. Gould 1846). Chalifour and Li (2021) were the first to examine samples of the gut microbiomes of adult, foetal and starving *O. strigosa*, and their results showed that the internal conditions of the host affect the composition of the bacterial community and that the bacteria may be passed vertically from a parent to its child before birth. The rich and diverse microbial community found in *O. strigosa* potentially assists in the organism's cellulolytic digestion and aids in acting as an environmental bioindicator (Chalifour & Li 2021).

Shifts in both the microbial community and the host's physiology (e.g. physical fitness, cold tolerance), could help us to understand the functional links between them, as well as the potential consequences of climate change or the conditions of animal husbandry. If the microbiome are directly affected by the external temperature, then the warmer or more variable winters predicted for many temperate regions due to climate change could modify the microbiome. By contrast, if the microbiome structure is directly regulated by the host, the host could either continue to regulate the microbiome, and thus maintain a normal performance under novel conditions, or the regulation of the microbiome under the novel conditions could be insufficient and the animal's performance would be compromised by the conditions.

Research concerning the microbiomes in animal organisms deals with two important problems. First, the processes underlying hibernation's characteristic plasticity enable the hibernator's internal environ-

ment to adjust seamlessly to the changing external environment over the circannual cycle. Moreover, these processes, if translated into human biology, may enhance an individual's resilience to an array of external factors, just as they function in hibernators. The analysis of hibernation's underlying processes, therefore, provides opportunities to both obtain a glimpse into these 'mechanisms and adaptations of exquisite beauty' (Krogh 1929), and to prepare therapies and techniques that may improve human health and fitness. Second, understanding these host-symbiont relationships in wildlife is likely to be useful in order to predict and mitigate the effects of global changes on wildlife. Temperature is a prominent abiotic environmental variable that drives the adaptive trajectories of animal lineages and structures the composition of animal communities. Cumulatively, the available data indicates that a disruption of the microbiome may be a mechanism by which changing temperatures will impact animal fitness in wild-living populations.

The proper functioning of snails is closely related to the diversity of their microbiota, which interacts with many of the mechanisms responsible for maintaining homeostasis and survival under changing environmental conditions. This applies not only to land snails, but also to species inhabiting freshwater and marine territories which, when exposed to various types of stressors, use various properties of the microorganisms inhabiting them to counteract the effects of the stress. Molluscs are the subject of research in various fields, including food production, cosmetology and biomedicine. They are also gaining recognition as potential animal models for a wide range of biological research. The relationship between the host and the gut microbiota may contribute to the understanding of longevity, metabolism, development and the physiology of invertebrates. Finding the optimal approach to sampling the snail microbiota will be crucial for the success of future research. So far, standard sampling strategies can mask or miss important microbes that we are not yet able to culture *ex vivo*. Therefore, more work is needed to determine how the snail microbiota differs between different organs and tissues within the same species.

### Authors' Contributions

Research concept and design: A.N.; Collection and/or assembly of data: P.A.I.-F.; Data analysis and interpretation: P.A.I.-F.; Writing the article: P.A.I.-F.; Critical revision of the article: A.N.; Final approval of article: A.N.

## Conflict of Interest

The authors declare no conflict of interest.

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