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Effect of alimentary microbial inoculation on freeze tolerance in *Helix pomatia* L. snails

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Original article

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Exogenous bacterial species, which mainly populate the intestines of *Helix pomatia* snails, also affect various biological processes that are crucial to the survival of the host. However, the microbiota undergoes changes during overwintering, as feeding is suspended, leading to serious health consequences for the host. This study aims to investigate whether the food supplementation of snails with a microbiological inoculation containing bacterial strains isolated from the intestines of overwintering snails could improve their cold tolerance in summer. The experiment was conducted on two groups: a control group without supplementation; and an experimental group given the microbiological inoculation. The supercooling point (SCP) of their body fluids was measured to assess the effects of supplementation on the cold tolerance of wild snails in the summer. The results showed that the SCP for the snails in the experimental group was statistically significantly lower than for the snails in the control group, with a mean SCP value of -1.73°C compared to -0.97°C ($p < 0.05$). These findings confirm that the microbiota has a significant impact on cold tolerance in snails, and the microbiological inoculation had a positive impact on lowering the supercooling temperature of *H. pomatia* in the summer. This sheds new light on the mechanism of this species' adaptation to cold, which still remains a mystery.

Key words: microbiota, cold stress, overwintering, supercooling point, terrestrial snails.

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Animal behaviours are shaped by various environmental factors, including the ambient temperature. Prior to winter, invertebrates undergo extensive behavioural, physiological, biochemical and morphological adaptations to prepare for overwintering. Many species spend the winter submerged at temperatures between 0 and 4°C, while others overwinter underground, where they must tolerate freezing conditions.

In land snails, winter torpor is typically induced by decreasing temperature or by a shorter photoperiod. During the autumn, terrestrial snails empty their digestive tracts, reduce their body water content and synthesise cryoprotective substances. They then burrow into the soil and form an epiphragm to seal their shells. However, in temperate climates, the temperatures may still drop low enough to pose a risk of freezing. Although Riddle (1981) suggested that molluscs do not utilise cryoprotectants, our previous studies confirmed their presence in Roman snails (*Helix pomatia* L., 1758) (Nowakowska *et al.* 2006), albeit at much lower concentrations than in

insects (Ansart & Vernon 2003; Loomis 1991). Additionally, *Helix aspersa* exhibits a supercooling ability under short photoperiod conditions (Ansart & Vernon 2003; Ansart *et al.* 2002a; Ansart *et al.* 2001). Consequently, current research is increasingly focusing on enzyme activity, metabolic pathways and the effects of overwintering on the microbiome composition across animal species (Raza *et al.* 2020; Moeller *et al.* 2020; Xiao *et al.* 2019; Sonoyama *et al.* 2009).

The gut microbiota plays a critical role in the majority of biological processes affecting both population dynamics, and the distribution and survival of the host species (Knapp *et al.* 2009a, 2009b). The most important processes that affect the host's fitness include its nutrient absorption, digestive capabilities, immune responses and adaptation to abiotic challenges (Nicolai *et al.* 2015). The majority of the microbiota in a Helicidae intestine is of an exogenous origin. *H. pomatia* eat soil (Dar *et al.* 2017) as well as faeces in order to acquire useful microbes, and scraping the soil is especially important for newly hatched snails (Charrier 1990; Watkins & Simkiss 1990). During the winter inactivity period (overwintering), the bacterial assemblages of the snails undergo changes because feeding is suspended, and thus the exogenous species are not provided (Wang *et al.* 2020; Carey & Assadi-Porter 2017; Dill-McFarland *et al.* 2014; Stevenson *et al.* 2014; Carey *et al.* 2012; Jeuniaux 1955; Pereira & Breckenridge 1981). Moreover, other factors such as the host's genetics, maternal effects, the host's diet, pathogens, interactions with the immune system, diseases, interactions between members of the microbial consortium and seasonality can influence the gut microbiota, exposing the host to serious health consequences (Carey & Assadi-Porter 2017; Carey & Duddleston 2014; Dill-McFarland *et al.* 2014; Carey *et al.* 2013). Therefore, boosting the host's survival under stressful conditions and maintaining its good health is strictly connected with eubiosis.

There is a growing body of evidence that the microbiota is involved in a snail's cold hardiness during overwintering, due to limiting ice formation to the snail's extracellular compartments (Ansart *et al.* 2010; Flari & Charrier 1992), and subsequently enhancing their fitness and survival. Moreover, Nicolai *et al.* (2015) have described the bacterial community structure of the gut in the activity and inactivity states in three geographically distinct populations of *H. pomatia*, and have shown that some strains of bacteria remain in the gut even during overwintering and they are known to have an ice-nucleating activity. Interestingly, snails that showed dysbiosis or had a less diverse bacterial community did not enter dormancy, despite being exposed to winter conditions (Nicolai *et al.* 2015). Bacteria, as a source of ice nucleation, were also found in the intertidal bivalve mollusc *Geukensia demissa* (Loomis & Zinser 2001). These microorganisms help to protect these cold-tolerant invertebrates against freeze damage by ensuring that ice formation is limited to the host's extracellular spaces. Fluctuating changes in the microbiota composition of *H. pomatia* not only during the seasons, but also throughout overwintering from the beginning (in October) to the end of winter torpor (in April), corelate with seasonal changes in the environmental temperature from acute heat stress to cold below the freezing point of water.

To the best of our knowledge, the influence of *H. pomatia's* microbiota on its freeze tolerance and surviving cold stress during the seasons of the snails' activity has never been characterised. Therefore, the main aim of our study was to check whether the alimentary supplementation of the snails' feed with a microbial preparation containing bacteria isolated during the winter from the snails' gut could improve the host's fitness in summer. We evaluated the effects of the supplementation on the wild snails' cold tolerance in summer, estimated by measurements of shifts in the supercooling point (SCP) of their body fluids. Our study should contribute to understanding the mechanisms of cold adaptation, which still holds many secrets. Moreover, we would like to point out the important role of the microbiota in a land snail's biology.

Materials and Methods

Animals and experimental groups

Experiments focused on measurements of the supercooling point of body fluids were performed on adult specimens of *Helix pomatia* L. snails. Specimens (n = 24) characterised by well-defined labium on the shell and roughly the same body mass (25.0 ± 0.5 g) were gathered from their natural habitat in the vicinity of Toruń (central Poland, 53°02′N, 18°35′E) in summer (licence WOP.6401.3.2.2023.DB.1 from the Regional Directorate for Environmental Protection in Bydgoszcz, Poland). The animals used in this part of the project were divided into two equal experimental groups: C – control (n = 12); and E – experimental (n = 12). They were housed in spacious plastic containers filled with a fine layer of soil and plant debris from their habitat, in a room with a standard laboratory temperature of 22°C and humidity of 45%

under natural light-dark conditions, for a month each. During that time, the animals were fed with leaves and plant debris from the collection site (like in their natural environment), and additionally every two days were supplemented with sterilised cellulose scraps (control group) or sterile cellulose scraps with a microbial preparation (experimental group), and sprinkled daily with water. After a month, both groups were subjected to measurements of the supercooling point of their tissues at the Institute of Nature Conservation, Polish Academy of Sciences in Krakow, Poland.

The microbiological preparation was prepared from the microbiota collected from adult *H. pomatia* snails, known by a well-defined labium on the shell and roughly the same body mass (25.0 ± 0.5 g), from their natural environment during the previous winter. Due to the fact that the snails were dead after collecting the material for the microbiota identification, the rest of the research was carried out on new individuals. The procedure requires the killing of these animals, so it is necessary to continue with new individuals.

Isolation of gut bacteria

The bacterial strains used to prepare the microbial preparation were isolated from the digestive tracts of overwintering snails in their natural habitat during the winter in the previous year. For this purpose, the digestive tracts of the animals were dissected, according to the method described by Ziętek *et al.* (2019). Briefly, to obtain the gastrointestinal tract, an incision was made from the pneumostome, about 2 mm from the visible edge of the distal part of the intestine, to the end of the pallial lung. Next, a cut was made from the back edge of the mantle (about 2 mm from its end) at a 90-degree angle to the first incision, and to the opposite end of the pallial lung. To gain easier access to the foot's dorsal part, the mantle's muscular prominence was removed in the following step. by cutting the dorsal wall towards the head in the sagittal plane and opening the frontal part of the foot up to the head. After exposing the internal tissues, the gastrointestinal tract was collected by severing and securing both the distal and proximal parts of the large intestine from the surrounding tissues (around 20 mm of the free intestine). The secured part was gently pulled out of the tract, then cut out and placed in a sterile Petri dish.

The middle part of the intestine was chosen for the identification of the snails' gut microbiota and was trimmed to about 10 mm with an incision exposing the inside of the intestine. The prepared piece of the gut was then homogenised (IKA T18 basic Ultra-Turrax homogeniser, IKA, Staufen, Germany) in a saline solution (0.85% NaCl) and 10-fold dilutions to 1:1000 were prepared. After that, each diluted intestine sample was spread onto the bacterial growth media (1 ml).

In order to retrieve the most diverse bacterial representatives of the gut microbiota, three kinds of media were applied: (I) PCA (Plate Count Agar, Biomaxima, Warsaw, Poland); (II) CMC medium (carboxymethyl cellulose) containing (g/l): CMC – 10, yeast extract 1, $MgSO_4 \times 7H_2O$ – 0.2, NaCl – 0.2, $NH_4H_2PO_4$ 1 and agar 3; and (III) CHIT medium (chitin medium) containing (g/l): colloidal chitin 20, yeast extract 1, $MgSO_4 \times 7H_2O$ – 0.2, NaCl – 0.2, $NH_4H_2PO_4$ 1 and agar 3. After incubation at 10°C for 14 days, the bacterial colonies from different media that differed in their morphology and showed rapid growth were selected for further identification and antagonistic activity to prepare the microbial preparation.

Identification of bacterial strains

Microorganisms isolated from the snails' intestines were identified on the basis of the 16S rDNA gene sequence. The identification of the bacterial strains was performed according to Kalwasińska *et al.* (2019). The Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit (EURx, Gdańsk, Poland) were used for extracting the total genomic DNA from the selected bacterial strains. All of the procedure was performed using the manufacturer's instruction manual. The 16S rDNA gene was amplified by PCR in a reaction mixture (1 U Taq DNA polymerase (EURx), 0.2 mM dNTP mixture, Polbuffer B with 1.5 mM $MgCl_2$, 0.25µM of universal primers 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') (Lane 1991) and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Polz & Cavanaugh 1998) with 1µl of genomic DNA in a total volume of 20µl. The thermocycling conditions (TProfesional Basic Gradient, Biometra) were as follows: 95ºC for 3 mins (initial degradation), 30 amplification cycles (denaturation at 95ºC for 30s, annealing at 52ºC for 20 s and an extension at 72ºC for 1 min 40s) and a final extension at 72ºC for 5 mins. 1% (w/v) agarose gel stained with Midori Green DNA Stain was used to check the PCR amplicons.

In order to sequence the PCR products, the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) with universal primers 27 F and 1492 R was used. This procedure was performed using the

manufacturer's instruction manual. Capillary electrophoresis was performed by the Oligo Sequencing Laboratory (IBB PAS, Warsaw, Poland). Nucleotide sequences of the bacterial strains obtained in this study were deposited in GenBank under the accession numbers MN911420-MN911430. The taxonomic affiliation of the bacterial strains was determined using the EzBioCloud online tool (Yoon *et al.* 2017).

During the cultivation stage on three different media, the strains exhibiting the fastest growth and the most diversity among the colonies were selected. In total, 35 strains were isolated and preliminarily identified, among which particular attention was paid to the strains that were identified as *Microbacterium algeriense*, *Buttiauxella agrestis*, *Luteimonas* sp., *Chryseobacterium balustinum*, *Sphingobacterium faecium*, *Prolinoborus fasciculus, Buttiauxella chrysanthemi* and *Klebsiella huaxiensis*.

Bacterial antagonism

Four morphologically different strains were selected for the further examination, showing rapid growth on the nutrient broth and having both chitinolytic and cellulolytic properties. Antagonism between the selected strains (*Microbacterium algeriense*, *Buttiauxella agrestis*, *Luteimonas* sp. and *Chryseobacterium balustinum*) was checked prior to the preparation of the microbiological formulation. The antagonism was checked by the well method on a solid Nutrient Growth medium (Biomaxima, Warsaw, Poland) inoculated with the test strain. Wells with a diameter of 10 mm were cut out of this medium and 24-hour liquid cultures of the antagonistic strains were introduced, before the wells were solidified by the addition of 1% (v/v) agar. After 3-5 days of incubation at 26°C, the growth inhibition diameters were measured. An antagonism checkup was performed only for 4 strains, because only these were components of the consortium supplemented to the snails.

Microbiological preparation

Before the strains were applied to the snails in the form of the microbial preparation, the abundance of each isolate was checked using the spread plate method. The abundance of each isolate in the 24-h liquid culture was 10^9 CFU/ml.

The microbiological preparation contained the following strains: *Microbacterium algeriense*, *Buttiauxella agrestis*, *Luteimonas* sp. and *Chryseobacterium balustinum.* The Nutrient Broth (Biomaxima, Warsaw, Poland) (1 l) was inoculated with each bacterial strain (OD = 1). The incubation was carried out for 4 days at 20°C. Then, the culture was centrifuged for 10 minutes at 10 000 × g (Rottina, Hettich, Germany). The biomass was suspended in 30% glycerol and was stored at 4°C.

The isolates' biomass was then mixed in equal proportions and applied to the snails on sterile cellulose scraps as a food supplement.

Exposure to low temperature and measurement of the supercooling point (SCP)

Prior to the measurements, both groups (n = 12 each) of animals were exposed to a temperature of 5°C for 12 hours (to restrict animal movement during the thermocouple placement and measurements). This allowed measurements of the time of the crystallisation temperature to be significantly shortened, and also limited the mobility of the animals during the experiment. In *H. pomatia* snails, the umbilicus is not visible, so the method involving drilling a hole in the shell was chosen.

After the adaptation, small holes were drilled in the upper part of the shells of the individuals, through which k-type thermocouples were inserted so that their tips touched the hepatopancreatic tissue inside the shell, but without damaging any internal structures of the animal. Each animal was handled with care, with a focus on not harming any internal structures during the process.

Then, the animals were placed on plastic trays covered with a layer of paper inside the freezer at 22°C with a cooling rate of 0.5-1.0°C/per min, until the temperature reached -10°C (see the next paragraph).

The thermocouples were connected to a recorder (Delta OHM HD 32.8 Thermocouple Datalogger) so that it could measure the temperatures of eight individuals at a time. The sampling rate was set very densely at approximately 1 measurement per second for over 90 minutes to capture each individual variation, which provided a huge amount of raw data from each animal (approx. 3 thousand measurements for each individual). The animals were placed inside a freezer and the temperature was brought down over the course of the experiment. According to recommendations obtained from the literature (Ansart & Vernon 2004; Salt 1961), the rate of cooling was between 0.5 and 1.0°C per minute, and the visualised graph in the result sections starts from 5°C until the SCP was recorded, to focus solely on this specific reaction. The SCP was reached at the moment when the exothermic reaction (due to the formation of ice) appeared, and any delay in its appearance was noted. A prolonged observation of the exothermic reaction led to the death of the animal (the mortality rate was 100%).

In each group, we observed a nadir as a result of the exothermic reaction reaching the SCP. Approximate-

ly 80% of individuals showed a correct exothermic reaction, which allowed us to assume that 20% are unable to survive freezing. Since all the individuals had a very similar size and weight, reactions within the group occurred at the same time, which allowed us to assume that the water content in their bodies was at a similar level, excluding potential major differences in the rate of freezing.

Statistical methodology

The results were averaged at an interval of 30 seconds and within the group, due to the very large amount of raw data. Statistical analyses were performed in PAST ver. 4.03 software (Hammer 2020). The data followed a normal distribution (Shapiro-Wilk test, $p > 0.05$). The Student's t-test for independent samples showed a significant difference between the groups ($p = 0.016$). This result was confirmed by a nonparametric test (Mann-Whitney, $p = 0.021$).

Results

Identification of H. pomatias' intestinal microbiota and microbiological preparation

During the early stage of the research project, several strains found in the H. pomatias' intestines and cultivated *in vitro* were identified as *Microbacterium algeriense*, *Buttiauxella agrestis*, *Luteimonas* sp., *Chryseobacterium balustinum*, *Sphingobacterium faecium*, *Prolinoborus fasciculus, Buttiauxella chrysanthemi* and *Klebsiella huaxiensis*.

Later, four morphologically different strains were selected for a further examination and microbiological preparation: *Microbacterium algeriense*, *Buttiauxella agrestis*, *Luteimonas* sp. and *Chryseobacterium balustinum* (Table 1).

Effect of the microbial inoculation on the SCP

During our experimental procedures, both the control and the experimental groups showed similar behavioural responses to cold. After the overnight acclimation at 5°C, the animals were lethargic and motionless.

The results presented on the graph (Fig.1) show that alimentary microbial supplementation significantly affected the cold response of *H. pomatia*. As is shown in Fig. 1A, both the control and experimental groups exhibited a gradual decline in body temperature during cooling; however, this decrease was more rapid in the supplemented snails. In the experimental group, the exothermic event associated with the onset of freezing occurred earlier and was more pronounced, indicating a sharper transition at the supercooling point (SCP). By contrast, the control individuals displayed a more gradual temperature decline and a less distinct exothermic peak.

The quantitative analysis confirmed a significant difference in the SCP between the groups (Fig. 1B). The mean SCP was -0.97°C in the control group and -1.73°C in the experimental group, with the latter being significantly lower ($p < 0.05$; $n = 8$ per group). The relatively small standard errors indicate consistent responses within each group.

These results demonstrate that a microbial supplementation enhances the supercooling capacity in *H. pomatia*, lowering the temperature at which spontaneous freezing occurs and altering the dynamics of the freezing process.

The entire SCP measurement lasted for about 1.5 hours in each group, at a rate of lowering the ambient temperature between 0.5 and 1°C/minute. The starting temperature of the chamber was 22°C, and the final temperature was -10°C. The temperatures recorded inside the snail shells at the beginning of the experiment (after an overnight exposure to 5°C) were all between 7 to 8°C. Due to the large amount of data, we decided to show the averaged measurements for both groups, ranging from 5°C to -3°C of their body temperature for approximately 23 minutes of the measurements to visualise the supercooling point. The control group showed a mellow and slow decrease in temperature, while the experimental group showed a significantly quicker decrease in temperature. The average difference in the SCP achievement was approximately 3-5 minutes between the two groups.

Table 1

Results of 16S rRNA gene sequence identification of *Helix pomatia'*s gut bacteria

Symbols	Top-hit taxon	Top-hit strain	Similarity (%)	Completeness (%)
11	*Luteimonas* sp.	9C	99.86	94.4
17	*Microbacterium algeriense*	G1	99.77	91.8
19	*Buttiauxella agrestis*	ATCC 33320	99.50	94.9
27	*Chryseobacterium balustinum*	DSM 16775	100.00	92.1

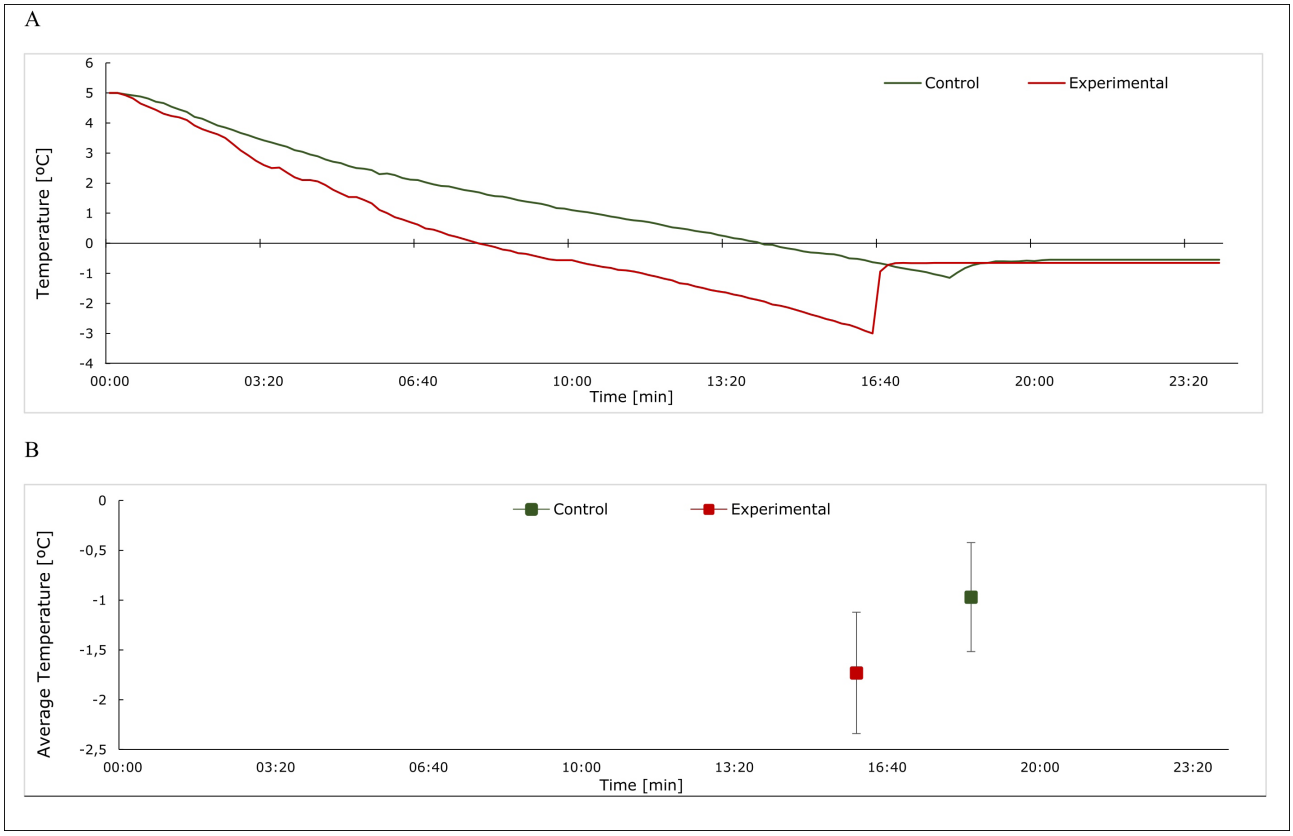

Fig. 1. A – Representative examples of time courses of the control (green line) and experimental (red line) *H. pomatia* snails' body temperature during testing for the supercooling point (the nadirs). B – Mean values (± SE) of the SCP and the times of reaching it in the control (green point) and experimental (red point) snails. The SCP mean value for the control group was -0.97°C, and for the experimental group was -1.73°C ($p < 0.05$).

Discussion

During the winter, *H. pomatia* snails burrow into the ground to a depth of about 0.2 m; however, this does not mean that they can avoid the risk of freezing, because in a temperate climate the ground freezes to a much deeper level. Therefore, *H. pomatia*, which is considered a partially freeze-tolerant species, tolerates temperatures below their body fluid freezing point for short periods, partially by controlling the nucleation of ice crystals inside their bodies. While the short-term crystallisation of body fluids is not lethal, it can still lead to a lot of cell and tissue damage, which ultimately lead to irreversible changes in an animal's physiology, development and reproduction capability (Nicolai & Ansart 2017; Ansart *et al.* 2002a, 2002b; Lee Jr. & Costanzo 1998). This leads to a complex cold shock response, which is based not only on the storage of cryoprotective substances such as amino acids, glycerol and triglycerides (Nowakowska *et al.* 2006), and on the production of heat and cold shock proteins (Idczak-Figiel *et al.* unpublished) as well as ice-binding proteins (Duman 2015), but also on the maintenance of psychrophilic bacterial microbiota, which induce the nucleation of ice crystals (Ansart *et al.* 2010). Our ongoing research showed that during the activity, the *H. pomatia* microbiota is predominantly colonised by *Proteobacteria, Firmicutes* and *Bacteroidetes* with a large variety of different families, but during the period of torpor, the intestines contain only some *Bacteroidetes* and *Proteobacteria* with a reduced diversity of strains (Idczak-Figiel *et al.* unpublished). This may indicate the existence of the core microbiome *of H. pomatia*, which was also discussed by Apostolou *et al.* (2025) in their recent literature review.

The knowledge concerning the potential role of the microbiota of *C. aspersum* cannot be applied directly to other terrestrial snail species, mainly because of the differences in their microbiota composition and the fact that their territorial range does not overlap, so they need different adaptation mechanisms. However, there has been a piece of relevant information (Ansart *et al.* 2008) stating that overwintering *C. aspersum* snails in their natural habitats seek out rock crevices or dig in the soil, in this way avoiding extreme temperatures. In their immediate environment, the temperatures rarely drop below

-5ºC. The supercooling ability of *C. aspersum* varies seasonally, ranging from -2°C in the spring and autumn (active periods) to -5°C in the winter (inactive periods); in winter, this species has a limited capacity to produce ice in its tissues without affecting its growth or reproduction abilities (Ansart *et al.* 2002b, 2001). It must be emphasised that *C. aspersum* experiments (Ansart *et al.* 2010) did not reveal the presence of ice-nucleating bacteria in dormant and estivating snails when mixed bacterial solutions were obtained from their mucous ribbon cultures (Ansart *et al.* 2010). During torpor, the aerobic microbiota of *C. aspersum* revealed the sporadic presence of *Pseudomonas* species (Kiebre-Toe *et al.* 2003). Charrier *et al.* (2006) identified *Clostridium* sp. as the sole strictly anaerobe in *C. aspersum*. Such an irregular presence of ice-nucleating factors in the digestive tracts of active individuals may partially account for the high degree of variation observed between samples. Given the propensity of ice-nucleating microbes to lose activity in a culture, some authors have noted the difficulty of establishing nucleating activity in culture samples (Costanzo *et al.* 2000; Worland & Block 1999). Thus, changes in temperature, the culture age, culture media and diet can affect the ice-nucleating activity of the bacteria (Ansart *et al.* 2010).

Within the winter samples, we identified and chose cultivable strains of *Microbacterium algeriense*, *Buttiauxella agrestis*, *Luteimonas* sp. and *Chryseobacterium balustinum* in order to check their antagonism (which was not the case), and further to prepare the microbial preparation. The microbial strains that we identified partially correspond to the diversity review described by Apostolou *et al.* (2025), where a repeating core microbiome belonged mainly to the Pseudomonadales (core genera: *Acinetobacter, Citrobacter, Enterobacter, Escherichia, Kluyvera, Pantoea* and *Pseudomonas*); however, the main aim of this work was focused on the differences between the microbiome of snails of freshwater, oceanic and terrestrial environments, where the significant factor turned out to be the phylogeny of cellulolytic bacteria. Despite the growing interest in gastropod-associated microbiota, terrestrial snail microbiomes remain insufficiently characterised, particularly in the context of their physiological adaptations to low temperatures.

The supercooling ability is associated with a snail's size and their body water content (Ansart *et al.* 2008; Ansart & Vernon 2003; Ansart *et al.* 2001; Riddle 1981). Larger species with a higher body water content tend to have a lower supercooling ability and to rely more on the ice-nucleating properties of their microbiota. Proteins with cryoprotective properties may also contribute to their survival (Ansart *et al.* 2014). The overwintering of snails can be analysed on many levels, starting from low molecular mass cryoprotectants accumulation, as well as a reduction of the body water content (Ansart & Vernon 2003), and ending on an elevation of the production of HSPs and CSPs (Idczak-Figiel *et al.* unpublished), resulting in an intricate mechanism that allows them to survive temperatures below the freezing point of their body fluids. Coping with environmental cold stress is essential for the survival of land snails, since drastic changes such as freezing and osmotic shocks are lethal. During cold stress, the cell membrane fluidity, enzymatic activity and various physiological responses change. Seasonal variations in haemolymph metabolites are likely to reflect the seasonal changes in a snail's diet. The ingestion of vegetal food could also indirectly affect dormancy by the absorption of ice-nucleating active bacteria that limit cold hardiness. In 1974, Douzou *et al.* suggested the possibility of the hemocyanin subunits dissociation as a mechanism responsible for the enhancement of the hemolymph viscosity in molluscs. A similar mechanism proved to increase the energy outputs and to alter the respiratory circulation in insects during the lowering the temperature process, enabling them to maintain a stable circulation (Kenny *et al.* 2018).

In ectothermic organisms, both vertebrates and invertebrates, microbes are exposed to the same temperature fluctuations as those of their hosts; consequently, due to behavioural responses, the host's overwintering-related changes may be less significant than those encountered by the same microbes outside the animal's body (Mushegian & Tougeron 2019; Dill-McFarland *et al.* 2014). In addition, recent research on endothermic animals has demonstrated that temperature-induced alterations in the microbiome can have cascading effects on the host's phenotypes related to thermal tolerance (Moeller *et al.* 2020; Doremus *et al.* 2018; Dunbar *et al.* 2007). For instance, the transplantation of gut microbiota from mice acclimated to cold into germ-free mice resulted in a modification of the gut morphology that increased cold tolerance in the recipient hosts (Chevalier *et al.* 2015). Moreover, experiments involving the transplantation of bear gut microbiota have demonstrated that the summer-associated bear gut microbiota increases the adiposity of germ-free mice. That indicates that seasonal shifts in the gut microbiota may contribute to the adaptive phenotypic plasticity of their hosts. In active mammalian heterotherms, such as hamsters (Sonoyama *et al.* 2009) and bats (Xiao *et al.* 2019), the thermal conditions

experienced by bacteria during winter are likely to change less dramatically than in overwintering poikilothermic ectotherms.

Nicolai *et al.* (2005) demonstrated that in *H. pomatia* snails, the bacterial strain *Kluyvera* sp. is a weak ice nucleator; however, it induces nucleation at temperatures well above the crystallisation temperature (Tc) of active or dormant snails (Ansart *et al.* 2010). Furthermore, Ansart *et al.* (2010) confirmed that the digestive tract is the first site of nucleation in *Cornu aspersum* snails, similarly to that in insects such as *Pyrrhocoris apterus* (Hodkova & Hodek 1997) and the *Trichiocampus populi* (Shimada 1989). Organic ice-nucleating factors have also been found previously in the mucous ribbons of starved *C. aspersum* (Ansart *et al.* 2008).

Unlike terrestrial molluscs, all of the intertidal gastropods are freeze-tolerant species, utilising the 'active freezing tolerance' mechanisms in their salty environment (Ansart & Vernon 2003). In general, *H. pomatia* snails show more flexibility in their adaptation to rapidly changing environmental conditions.

Our investigation confirmed that the microbial preparation isolated from overwintering snails, used as a diet supplement, had an impact on lowering the supercooling point of *H. pomatia* snails. According to the generally available literature, it should be possible to measure the moment of crystallisation within the supercooling point in snails by inserting a thermocouple through the umbilicus into the shell, as Riddle (1981) described. Another method involves drilling the shell and inserting the thermocouple with a tiny hole inwards so that it contacts the tissues inside (Ansart & Vernon 2004; Ansart *et al.* 2001; Biannic & Daguzan 1993). So far, none of the bacterial strains isolated in these investigations were reported to have ice-nucleating activity, and they were not cultivated in a 10°C temperature either. However, to fully understand this outcome, further research is still needed. We consider these results to be of application significance, because they can be used in the future as a reference for creating a supplement for breeders in snail farms, where an elevated mortality of animals occurs during the winter season. However, despite the success in conducting this research project, we still do not know what mechanisms lie behind our results. It is possible that supplemented bacterial strains work as ice nucleating agents, but they could also overproduce cryoprotective substances. Moreover, it is also possible that their metabolites enter the reaction cascades or cycles, leading to a decrease in the supercooling point temperature. However, at present we are not able to verify this on the basis of the obtained results. Nonetheless, this will allow us to plan further projects aimed at understanding the mechanisms responsible for the partial freezing tolerance of *H. pomatia* snails on many different levels of their unit organisation.

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Author Contributions

Research concept and design: P.A.I.-F., A.N.; Collection and/or assembly of data: P.A.I.-F., K.D., J.U., A.M.L.,A.K.; Data analysis and interpretation: P.A.I.-F. K.D., J.U., A.M.L.,A.K., M.S.B.; Writing the article: P.A.I.-F.; Critical revision of the article: A.M.L.,A.K., M.S.B., A.N.; Final approval of article: P.A.I.-F., A.N.

Conflict of Interest

The authors declare no conflict of interest.

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