University of South Bohemia in České Budějovice, Faculty of Science

Biocontrol of slugs: Effects of slug parasitic nematodes and bacterial metabolites on harmful slugs

Bachelor Thesis

Victoria Weijler

Supervisor: Ing. Jiří Nermuť, Ph.D.

České Budějovice, 2023

Weijler V., 2023: Biocontrol of slugs: Effects of slug parasitic nematodes and bacterial metabolites on harmful slugs. Bc. Thesis, in English. - 32p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The effects of combining secondary metabolites produced by *Xenorhabdus* bacteria with slug parasitic nematodes belonging to the genus *Phasmarhabditis* on the pestiferous slug species *Arion vulgaris* were investigated. Secondary metabolites from ten different *Xenorhabdus* strains were tested and their slug repellent effects as well as their impact on the slugs' feeding behavior were observed.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 13.08.2023

Victoria Weijler

Abstract

Slugs, notably *Deroceras reticulatum* and *Arion vulgaris*, are considered harmful pests in agriculture and horticulture. With the increasing restrictions in chemical pest control, the need for alternative control forms is growing. *Phasmarhabditis hermaphrodita*, a slug parasitic nematode is already used as a biological control agent. For the control of insects, entomopathogenic nematodes of the genus *Steinernema*, which live in symbiosis with *Xenorhabdus* bacteria are utilized. The secondary metabolites of *Xenorhabdus* bacteria show repellent and lethal effects towards scavengers of the host's cadavers, leading to the hypothesis, that these compounds could impact slugs as well. This study aimed to investigate the combined effects of *Xenorhabdus sp*. secondary metabolites and three *Phasmarhabditis* species (*P. apuliae, P. bohemica, P. hermaphrodita*) on the slugs' positional and feeding preferences. Plastic boxes were divided into equal halves and treated on one side with isolated secondary metabolites of *Xenorhabdus* bacteria and nematode cultures with *Enterobacter* sp. strain DERc. A round cut out of a lettuce leaf was placed in both sides. Over five days, slug positions and feeding behavior were observed. Statistical analysis showed significant effects of the combination of nematodes and metabolites on the slugs' side preferences and the amount of lettuce consumed. No significant statistical difference was found in the repellent effect among the various nematode and metabolite combinations. However, some significant differences in the feeding activity among the combinations were observed. In conclusion, the combination of secondary metabolites (*Xenorhabdus sp*.) and slug parasitic nematodes (*Phasmarhabditis sp*.) exhibit great effects against the slug species *A. vulgaris*, highlighting the potential of secondary metabolites as control agents in agriculture and horticulture.

Content

1. Introduction

Many species of slugs are considered serious pests in agriculture and horticulture around the world. Slugs belong to the phylum Molusca and to the class of Gastropoda, which stems from the Greek word “gaster” (Genitive: gastros) meaning “stomach” and “pous” (Genitive: podos) meaning “foot”. Slugs are hermaphrodites with a high reproduction rate, making population control very difficult. In Europe, slugs of the genera *Arion* and *Deroceras* are considered to be the most harmful pests in agriculture. Their feeding on plants and contamination of the harvest with their eggs, secreted mucus and their bodies leads to a large loss of yield of agricultural products, such as e.g., field crops, vegetables and ornamental plants. For a long time, chemicals have been used for slug population control. Metaldehyde and methiocarb, which are known as molluscicides, are a way of such chemical slug control (Kumar, 2020). However, those chemicals are toxic to the environment as well as harmful to some vertebrates and invertebrates. As a result, methiocarb was banned in the European Union as well as in the UK (Pieterse, et al. 2017). Metaldehyde has been banned in the UK since March 2022. Therefore, biological control, which refers to the use of natural enemies of slugs to control their population, and bio-rational control, meaning the use of products coming from natural sources, are gaining importance. Biocontrol and bio-rational control are beneficial since they are non-hazardous and do not affect organisms that should not be targeted (Barua et al., 2021). A form of biological control is the use of various families of entomopathogenic and slug parasitic nematodes (Pieterse, et al., 2017). The term entomopathogenic nematodes (EPNs) refers to insect parasitic round worms. Nematodes of the families *Steinernematidae* and *Heterorhabditidae* are EPNs, and they are used against insect pests in agriculture (Banu et al., 2017). Thus far, eight families of nematodes are known to have mollusks as a definitive host, which are *Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae*, *Diplogastridae, Mermithidae* and *Rhabditidae* (Pieterse, et al., 2017). The nematode species *Phasmarhabditis hermaphrodita* and *Phasmarhabditis californica* (family: *Rhabditidae*, genus: *Phasmarhabditis*) are the only commercially used species of slug parasitic nematodes in slug control. They are used in agriculture due to their easy production in solid or liquid media and are available on the market under the trade name Nemaslug® and Nemaslug 2.0® respectively. Other Nematodes of the genus *Phasmarhabditis* also have the potential to become biological control agents for slugs but are not yet commercially available (Nermuť

and Půža, 2017; Mc Donnell et al., 2023). Growth and pathogenicity of the species *P. hermaphrodita* can be influenced by monoxenic bacterial cultures in the liquid medium (Wilson, 2007). The parasitic nematodes can infect the slug through the slug's body wall, during mating and through oral infection. The stage of the nematodes that infect the slugs are the so called dauer juveniles or infective juveniles and an infection shows through characteristic symptoms such as swelling of the mantle (Nermuť and Půža, 2017). When used in agriculture the dauer juveniles of *P. hermaphrodita* are applied with water to moist soil via a watering can or hydraulic spraying equipment. Usually, 3×10^9 infective juveniles are applied per 1 ha (Rae, et al., 2007).

Interestingly, it has been shown that entomopathogenic nematodes of the genus *Steinernema* enhance their lethality by living in symbiosis with Bacteria of the genus *Xenorhabdus*. A species of *Steinernema* is always associated with a certain species of *Xenorhabdus* bacteria (Dreyer, et al., 2018). When infective larvae of the family *Steinernema* locate a suitable host, they make their way into the hemocoel of the host and release their symbiotic bacteria. Both the bacteria and the nematodes release compounds leading to the death of the host, which creates good living conditions for the nematodes. After the food supply is used up and the nematodes have reached their infective juvenile state again, they take up some of their bacterial symbionts again. After this, the search for a new host begins (Blanco-Pérez, et al. 2019). The secondary metabolites of *Xenorhabdus spp*. show activity against bacteria and fungi as well as insects, nematodes, and protozoa (Dreyer, et al., 2018).
This prior knowledge about the bioactivity of the secondary metabolites of *Xenorhabdus spp.* leads to the hypothesis that their cell metabolites show activity against slugs as well and would be a helpful tool in the control of slug populations. In this study the cell metabolites of ten strains of *Xenorhabdus* in combination with different nematode strains (*P. hermaphrodita, P. bohemica, P. apuliae*) are tested on their effect on the feeding behavior of the target slug *Arion vulgaris*.

2. Literature review

2.1. Slugs

The term “slug” is commonly used to describe terrestrial mollusks that possess a diminutive shell in comparison to their body size or lack a shell completely. Slugs developed from snails, which are terrestrial gastropod mollusks that possess a shell (Barua et al., 2021; Wilson, 2007).

2.1.1. Slugs as pests

In Europe slugs of the genera *Arion, Deroceras, Limax, Milax* and *Tandonia* are considered pests in agriculture. Among them, the slug species *Deroceras reticulatum*, commonly known as the “grey garden slug” and *Arion vulgaris*, often referred to as the “spanish slug” are particularly harmful in both agriculture and horticulture (Kumar, 2020). Both *D. reticulatum* and *A. vulgaris* are hermaphroditic slug species, meaning they possess male and female reproductive organs. Self-fertilization, however, is rare in these two species and reproduction primarily occurs through outcrossing, also referred to as amphimixis, which implies that genetic material is exchanged between the mating individuals (Wilson, 2007). While the species *A. vulgaris* undergoes an annual life cycle, *D. reticulatum* has the ability to complete multiple lifecycles within one year under favorable conditions, such as a suitable temperature and humidity. Therefore, they are often referred to as opportunistic breeders. The peak seasons for reproduction in *D. reticulatum,* however, were found to be spring and autumn. Except for slight variations in coloration, the juveniles of the two slug species closely resemble the adult slugs. (Wilson, 2007; Shirley et al., 2020).

These two pestiferous slug species cause extensive damage to plants, especially young seedlings, and lead to significant losses in crops every year. Their feeding behavior poses a significant threat to various crops including vegetables, ornamental plants, rapeseed, and legumes such as red clover, yellow lupins and field beans (Kozlowski et al., 2018). Additionally, slugs can be directly harmful to animals and humans as they can be carriers of diseases and parasites such as the rat lungworm (*Angiostrongylus cantonensis*). Rat lungworms are an invasive species originally endemic in parts of Asia and were found for the first time in continental Europe in Valencia, Spain, in 2021. In humans, the rat lungworm can cause eosinophilic meningitis. Slugs act as intermediate hosts for *A. cantonensi,* and the consumption of slugs or contact with their slime, in which the stage three larvae are excreted, can cause a parasitic infection. While primarily rodents are definitive hosts for the parasite, humans can also be infected through, for instance, the consumption of infected slug slime on

vegetables (Galán-Puchades et al., 2023). Suitable and effective slug control is therefore of great importance for both the agricultural sector and home gardeners alike.

2.1.2. Chemical molluscicides

One way of slug control is the use of chemical molluscicides, which are synthetic pesticides specifically designed for the use against mollusks. In 2014, methiocarb, a commonly used chemical in many slug pellets, was banned in the EU and in the UK, due to its effects on the environment and other soil organisms (Pieterse, et al. 2017). Metaldehyde was therefore the most frequently used chemical in slug control and was typically distributed in gardens and on fields in the form of pellets (Grubišić et al., 2018). These pellets paralyze the slugs, as they induce higher mucus production and therefore the dehydration of the slug, making it unable to move and they work as both dermal irritants as well as a poison for the slug's gastrointestinal tract (Campbell et al., 2021). Despite its high effectiveness, the use of metaldehyde is losing significance and was prohibited in the UK starting in 2022 (Barua et al., 2021). In organic farming the use of metaldehyde is highly regulated or even prohibited, due to its significant environmental problems, such as the pollution of drinking water. In the UK the concentration of metaldehyde frequently exceeded the thresholds set by the EU for pesticides in drinking water throughout the seasons in which the chemical molluscicides are applied, as metaldehyde spreads quite easily through the soil. Additionally due to its chemical properties, the removal of metaldehyde from drinking water poses a great challenge as well (Castle et al., 2017).

Iron phosphate is a chemical that is widely used in agriculture, horticulture as well as private gardens and currently it is the only available chemical pesticide against slugs in the EU. However, it poses its own problems, such as affecting important soil organisms like earthworms and therefore disturbing the natural ecosystem. Edwards and colleagues (2009) state in their paper that iron phosphate, even though it has no significant effect on earthworms by itself, does in combination with the chelating agents EDDS (Ethylenediamine disuccinic acid) and EDTA (Ethylenediaminetetraacetic acid), which are often found in iron phosphate pellets, reduce the number of earthworms in the soil significantly when applied in concentrations between 100 mg kg^{-1} and 1000 mg kg^{-1}. Due to the negative effects of chemical molluscicides and their restrictions in use, alternative forms of pest control become increasingly important.

2.1.3. Cultural and Mechanical control of slugs

Other ways of slug management lie in cultural and mechanical control. Cultural control involves modifying the slug's habitat to make it less hospitable. Examples of cultural control methods are right tillage timing and a suitable choice of green manure crops. When tillage is performed during cold months, slug eggs can freeze and therefore die. Additionally, proper soil preparation can help dry out the ground which is unfavorable for slugs. When using green manure, it is important to choose plants that have no effects or negative effects on slug populations. For example, perennial ryegrass (*Lolium perenne*) in regions with temperate climate, can promote slug growth in the following year and would therefore be an unsuitable choice as far as slug control is considered (van Rozen et al., 2009; Rosenfeld and Rayns, 2011). In a study conducted by Brooks et al. (2003) it was suggested that certain plants like wild spinach (*Chenopodium album*), shepherd's purse (*Capsella bursa-pastoris*), or wild white clover (*Trifolium repens*) can be used as trap crops in fields with winter wheat, as they are more attractive to slugs compared to winter wheat itself.

Mechanical control of slugs on the other hand refers to measures like handpicking slugs, which is sometimes applicable in small home gardens, or barriers like copper or wooden ash barriers (Watz and Nyqvist, 2021). Research conducted by Laznik and colleagues (2020), demonstrated that ashes from beech, oak, fir and spruce trees showed to be effective as physical barriers. These ashes contain for example silicone dioxide, which forms crystals that scratch the slugs' bodies leading to their dehydration (Laznik et al., 2020). Copper tape is thought to be an effective barrier against slugs, as it is believed that the reaction of slug slime and copper produces an electric current repelling the slug from crawling over the barrier. However, studies suggest that it only delays the passage of slugs like *D. reticulatum* or only slightly reduces numbers of slugs and snails crossing the barrier (Watz and Nyqvist, 2021). Furthermore, the research conducted by Watz and Nyqvist (2021) showed that in controlled settings, copper foil only slightly delayed the passage of arionid slugs and in semi-field validation, did not hinder them at all when compared to control conditions without a barrier.

Even though cultural and mechanical control can be effective in slug management, they are mostly suitable for small settings such as home gardens and are not as successful on their own as they are when combined with other control methods such as chemical molluscicides (Speiser et al., 2001).

2.1.4. Biological control of slugs

As previously stated, due to unsatisfactory performance of other control types and environmental problems caused by chemical molluscicides, the use of alternative forms of pest control, one of which is biological control, is steadily increasing. Biological control or biocontrol refers to the use of living organisms, for instance nematodes, against harmful pests such as slugs or insects (Stenberg et al., 2021). Besides nematodes, which will be focused on later in this review, there are two other natural enemies of slugs worth mentioning: Carabid beetles and Sciomyzid flies.

Carabid beetles are commonly found in large populations in the soil all over the world and they are known to feed on insects, their eggs, as well as slugs and slug eggs. Therefore, they are considered to be natural pest control agents with a high potential (Kromp, 1999). *Pterostichus melanarius* and *Poecilus cuperus* are two carabid species that are found in high numbers in arable sites in Europe. Therefore, they were subject of investigation of Oberholzer and Frank in their study published in 2003. In laboratory conditions they tested the predatory behavior of these carabid beetle species towards slugs of the species *D. reticulatum* and *A. vulgaris* as well as their eggs and concluded that both species showed potential for natural slug control. For a long time, the feeding behavior of carabid beetles could only be recorded under laboratory conditions (Kromp, 1999), but tools like multiplex PCR systems, such as the assay for the identification of pestiferous slugs developed by Guenay-Greunke and colleagues (2021), make it possible to identify the prey of carabid beetles in agricultural ecosystems and therefore assess their importance for biological control. However, even though carabid beetles are considered to be natural enemies of slugs they are not a type of biological control in the sense of the purposeful introduction of a slug enemy into the ecosystem (Howlett, 2012).

Sciomyzid flies belong to the family of *Sciomyzidae,* and are commonly known as marsh flies. The family of *Sciomyzidae* comprises of about 550 species. Out of these 550 species, while many are considered mollusk feeding, only nine species are known to be capable of killing slugs. Out of these, *Tetanocera elata*, *Tetanocera valida* and *Euthycera chaerophylli* solely feed on slugs. *T. elata*, as it is a slug-killing marsh fly species and common in central and western Europe, could be suitable biocontrol agents for European countries in settings like greenhouses (Barua et al., 2021; Hynes et al., 2014). Studies have been conducted on the optimal temperature conditions for larvae of *T. elata* (Hynes et al., 2014), egg production in laboratory settings, feeding behavior strategies and prey preferences of the different larvae instars (Ahmed et al., 2019). Furthermore, Ahmed and colleagues (2019) investigated how

starvation periods affect the development of *T. elata* larvae and the possibility of using *T. elata* and *P. hermaphrodita* in combination with each other. However, further research has to be conducted before *T. elata* can be considered a biological control agent.

2.2. Nematodes

Nematodes are roundworms of the phylum Nematoda that can be found in almost every ecosystem on the planet (Stock and Goodrich-Blair, 2012). Even though there are numerous unfavorable nematode species, such as plant parasitic nematodes, when it comes to biological control, there is a great abundance of nematodes such as mermithids, fungivorous nematodes, entomopathogenic nematodes (EPNs) and slug parasitic nematodes that hold a great potential for being effective biocontrol agents (Askary and Abd-Elgawad, 2017). Seven families of nematodes are the main focus of research for biocontrol agents: *Steinerenmatidae, Heterorhabditidae, Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae* and *Rhabditidae* (Stock and Goodrich-Blair, 2012).

2.2.1. Slug parasitic nematodes

From the seven families under research, *Rhabditidae* are considered to be the most important in the control of pestiferous slugs and the nematode species *P. hermaphrodita* is especially effective in the biological control of slugs (Stock and Hunt, 2005). In their study published in 1993, Wilson and colleagues proposed for the first time a parasitic relationship between *P. hermaphrodita* and *D. reticulatum*. Based on their findings they suggested that *P. hermaphrodita* has the potential to be a biocontrol agent against pestiferous slugs. *P. hermaphrodita* and *P. californica*, available on the market under the tradename Nemaslug® and Nemaslug 2.0® respectively, are the only commercially available and widely used nematode species in biological control (Nermuť and Půža, 2017; Mc Donnell et al., 2023). However, other nematode species such as *P. apuliae*, *P. bohemica* and *P. bonaquaense*, were shown to exhibit great potential as biological control agents as well (Nermuť et al., 2020). The dauer larvae, which are the third stage larvae of the slug parasitic nematode, infect the slugs through a canal that is connected to the shell sac located above the mantle cavity. An infection of the slug with *P. hermaphrodita* is in most cases indicated by swelling of the mantle, or more precisely the rear part of it, which is caused by fluid accumulation in the cavity. After 7 to 21 days the infection usually leads to the death of the slug and the nematodes spread from the shell cavity to all parts of the body, replicate further and feed on the slug's remains until the food sources are used up and new dauer larvae are formed (Wilson et al., 1993). Besides this parasitic life cycle, *P. hermaphrodita* are able to complete their lifecycle on dead slugs, slug

feces as well as for example pig kidney or leaf compost in a non-parasitic way and are therefore considered facultative parasites (Tan and Grewal, 2001; Nermuť et al., 2014). Furthermore, *P. hermaphrodita* are nematodes that feed on bacteria, although they can feed and grow on many different bacterial species and do not have one particular bacterial symbiont like EPNs do (Nermuť et al., 2014). *P. hermaphrodita* are commercially produced in fermenters with a monoxenic liquid culture containing the bacterium *Moraxella osloensis*, as the growth of the nematodes on this bacterium proved to yield the highest number of dauer larvae. With this method an amount of up to 100 000 dauer larvae mL^{-1} can be produced and the typical rate of application in agriculture is 3 billion larvae per hectare. While there have been numerous reports of *P. hermaphrodita* showing high success rates as biological control agents, there are some shortcomings. For example, the costs of slug parasitic nematodes as control agents are much higher compared to chemical control methods and they can be stored for a maximum of six months only. Furthermore, when nematodes are not handled properly or environmental conditions are not favorable for the nematodes, the success rate could decline. In some cases, soil predators like the collembola species *Isotoma viridis* or mesostigmatid mites were found to feed on the slug parasitic nematodes applied to fields and, similarly to EPNs, it is believed that the population of nematodes decreases rapidly shortly after application (Rae et al., 2007).

2.2.2. Entomopathogenic nematodes

The term entomopathogenic nematodes refers to nematodes that are insect parasitic, causing disease to their hosts. These types of nematodes live in symbiosis with bacteria, that play a crucial role in the nematodes' pathogenicity (Grewal et al., 2005). From the seven previously introduced families under research *Steinernematidae* and *Heterorhabditidae* are considered to be most important in the control of insects and they are examples of EPNs (Stock and Goodrich-Blair, 2012). Nematodes of the genus *Steinernema* live in a symbiotic relationship with bacteria of the genus *Xenorhabdus*, while nematodes of the genus *Heterorhabditis* live in symbiosis with bacteria of the genus *Photorhabdus*. Like in most nematode species, the life cycle of EPNs consists of an egg stage, four larval stages, whereby the larvae of the third larval stage are also known as infective or dauer juveniles, and an adult stage. All stages, except for the dauer larvae, that can survive freely in the soil to find new insect hosts, are only found inside of insects or their cadavers (Chitra et al., 2017). EPNs are effective biological control agents that can be used to manage a wide range of soilborne insects. Different species of entomopathogenic nematodes have their own specific preferences for insect hosts. Examples of insects that can be managed by EPNs are the corn root worm, craneflies, and different types

of beetles (Chitra et al., 2017). When a suitable host is found, the nematodes infect it through openings such as the mouth, anus or the insect's respiratory system and make their way into the haemocoel. Once the larvae have infected the insect the symbiotic bacteria spread throughout its body. To kill the host and to prevent other microorganisms and pathogens from attacking the same host, the bacteria release certain toxins. Furthermore, they secrete enzymes that break down the insect cadaver through hydrolysis making it an ideal medium for the nematodes to grow and reproduce (Javed et al., 2017). EPNs can be produced for commercial use through both *in vitro* and *in vivo* methods. In *in vivo* production the nematodes are cultivated on insect hosts, such as *Galleria mellonella*, which are the larvae of the great wax moth. The choice of a suitable host for the nematode species being produced is of great importance as it can scientifically impact the production yield. Different methods including the white trap method, the LOTEK technique or the cadaver application method are used in *in vivo* production. While these methods are generally low-cost methods, the white trap method is labor intensive and is therefore not suitable for large-scale applications. *In vitro* production is done both on solid and liquid medium, whereby the cultivation of nematodes in the liquid culture method is achieved in large scale fermenters. The solid culture method, like the *in vivo* white trap method, results in higher labor costs. The liquid method on the other hand is very cost-effective and is currently the most widely used method in the production of EPNs (Askary and Ahmad, 2017).

2.3. Secondary Metabolites of *Xenorhabdus*

The gram-negative bacteria of the genus *Xenorhabdus* both show mutualistic interactions as well as pathogenic interactions with their hosts as they live in a beneficial relationship with EPNs and show pathogenicity against various host insects (Goodrich-Blair and Clark, 2007). The mutualistic relationship with the host nematodes lies therein, that the nematodes serve as safe transporters of the bacteria into the haemocoel of the host insects. This is important because bacteria of the genus *Xenorhabdus* cannot survive independently in the soil and they cannot infect insects through their digestive system when ingested. In return the bacteria provide a nutrient-rich environment inside the insect cadaver, which serves as a food source and suitable growing grounds for the nematodes (Akhurst and Boemare, 1990). As previously stated, the symbiotic bacteria of EPNs excrete certain secondary metabolites to not only kill the hosts but also to repel other organisms in the soil that would normally prey on the insect cadavers as well. This is of great importance as the development of the new infective larvae takes time and scavengers would consume the infected insect cadavers much faster (Javed et

al, 2017; Foltan and Puza, 2009). Besides their repellent antimicrobial activity, the secondary metabolites of *Xenorhabdus* bacteria show antibacterial, antifungal, antiprotozoal and insecticidal activity as well, thus making them a possible control agent for disease vectors such as mosquitos as well as insect crop pests, harmful oomycetes, and fungi (Cimen et al., 2022). For instance, a study of Fang and colleagues (2014) showed that the secondary metabolites of *X. nematophila* showed strong negative effects toward spore germination and mycelial growth of *Botrytis cinerea* and *Phytophtora capsisi*, a fungus and an oomycete respectively, that can cause great agricultural loses. Furthermore, it was shown that the compounds produced by *Xenorhabdus* bacteria show activity against the yellow fever mosquito (*Aedes aegypti*) as well as the Asian tiger mosquito (*Aedes albopictus*) (da Silva et al., 2020).

Due to the various effects of secondary metabolites on scavengers of insect cadavers, the hypothesis arose that, as slugs are scavengers themselves, secondary metabolites of bacteria of the genus *Xenorhabdus* could also have an impact on pestiferous slugs.

3. Materials and Methods

3.1. Slugs

For this study the slug species *A. vulgaris* was used. The slugs as well as their eggs were collected on wooden boards laid out on the grass in front of the Biology center CAS in České Budějovice. After collection the slugs were stored in plastic boxes measuring 17 cm in length and 12.5 in width, which were closed with lids with air holes. Moistened garden soil and a food source such as carrot slices or lettuce leaves were placed in the boxes. Adequate lightning (12 hours day) to guarantee good survival conditions for the slugs was provided.

3.2. Isolation of bacterial strains

In order to obtain the cell metabolites of the symbiotic bacteria of specific nematodes, first the bacterial strains needed to be isolated.

This was done by treating the larvae of *Galleria mellonella* with dauer larvae of different strains of nematodes (Table 1). Through this, the *G. mellonella* larvae were infected by the symbiotic bacteria of the nematodes. Ten different strains of bacteria were used. To isolate the bacterial strains, five larvae of *G. mellonella* were placed into ten prepared Petri dishes. The larvae were then treated with the infective juveniles of the Nematode strains and in turn infected by the bacteria. After one to two days signs of bacterial infection, such as decreased reaction time or darker coloring, could be observed. At this point, it was possible to proceed with the isolation of the bacteria. Under sterile conditions in the flow box, the larva with the slowest reaction time was chosen and washed in 70% ethanol. With scissors a foot of the larva was cut, and a drop of the excreted hemolymph placed on a previously prepared NBTA agar plate (37 g Standard nutrient agar I, 25 mg bromothymol blue, 1 L distilled water, after sterilization cooled to 50 °C 4 mL 1 % 2,3,5-triphenil-tetracoliumchoride solution). The drop of hemolymph was then spread on the agar using the streak plate method. The Petri dishes were closed with parafilm and left for the bacteria to grow and to form colonies. After 48 hours, a single colony of each bacterial strain was transferred into an Erlenmeyer flask containing YS-medium and the flask was closed with a cellulose plug. The medium with the bacteria was then put on a shaker for the bacteria to grow (96 hours, 180 rpm). The YS-medium was prepared as follows: To 400 mL of water 2 g of Yeast extract (Merck), 2 g of Sodium chloride, 0.2 g of $NH_4H_2PO_4$, 0.2 g of K_2HPO_4, 0.08 g of $MgSO_4 \cdot 7H_2O$ were added and mixed. The medium was then divided into 50ml Erlenmeyer flasks (20 mL of media per one flask). The flasks were closed with a cellulose plug and the medium sterilized using autoclave.

Figure 1: Liquid bacterial cultures of strains SGI 197 - *S. beitlechemi* and CHIN - *S. ceratophorum* (Photo: J. Nermuť).

Table 1 : Bacterial Strains. Symbiotic Bacteria of the genus Xenorhabdus of nematodes of the genus Steinernema.

Strain designation	**bacterium**	**host**	**origin**
HOS2	*X. bovienii*	*S. affine*	Czech Republic
V. AF	*X. bovienii*	*S. affine*	Czech Republic
JAKUT	*X. bovienii*	*S. feltiae*	Russia
NFUST	*X. bovienii*	*S. feltiae*	Russia
CHIN	*X. budapestensis*	*S. ceratophorum*	China
1298	*X. budapestensis*	*S. bicornutum*	Czech Republic
DIA	*X. doucetiae*	*S. diaprepesi*	USA
SGI 197	*X. khoisanae*	*S. beitlechemi*	South Africa
JEGOR	*X. kozodoii*	*S. arenarium*	Ukraine
SLOV	*X. kozodoii*	*S. arenarium*	Slovakia

3.3. Isolation of bacterial metabolites

For the isolation of the actual cell metabolites used, the Erlenmeyer flasks containing the YS-medium with the different strains of bacteria were autoclaved. The resulting metabolites in the medium were transferred into 50 mL tubes and stored in the fridge (5 °C) for further use (see Table 2).

Table 2: Known secondary metabolites of the bacteria (after Dreyer, et al., 2018 and Dreyer, et al. 2019).

Bacterium	**Secondary metabolic compounds**
X. bovienii	amicoumacin, xeomin, xenorxid, xenorhabdin, xenematide
X. budapestensis	fabclavine, bicornitun, unnamed peptide
X. doucetiae	xenoamicin, xenocoumacin, xenorhabdin, phenylethylamine, tryptamide
X. khoisanae	xenocoumacin
X. kozodoii	xenocoumacin

3.4. Cultivation of monoxenic nematode cultures

In this experiment, three different kinds of nematodes were used (Table 3). For the cultivation of the monoxenic nematode cultures, three 9 cm Petri dishes were prepared with a small piece of pig kidney placed on a round wettened filter paper. To each dish, larvae of one species of nematodes were added and the petri dishes were put in the fridge for the nematodes to grow. After sufficient growth the eggs of the nematodes were isolated. To accomplish this, the male and female nematodes were washed off the pig kidney with deionized water and collected in a test tube. The male nematodes were separated from the female nematodes, once the larger female nematodes settled on the bottom of the tube. The supernatant, containing the smaller male nematodes, was discarded. The female nematodes were subsequently transferred into a 1.5 mL Eppendorf tube and washed with deionized water. The supernatant was discarded again. With a plastic rod the females were then homogenized gently to obtain the eggs. Through a filter (Uhelon 130T with loops 42 µm), which let the eggs pass, the solution was transferred into a new Eppendorf tube. The solution was then centrifuged (4000 rpm), the water discarded, and a sterilization solution (10 mL H_2O, 1.5 mL 12% NaClO, 0.5 mL 4M NaOH) added. The following steps were all conducted under sterile conditions in the flow box. The sterile solution was discarded, and the eggs washed with sterile YS-medium. Afterwards, YS-medium was added to the eggs again and the medium with the eggs was divided into three wells of a 24-well multi well plate. This procedure was done for all three nematode strains. After two days the new nematodes were checked for contamination (turbidity of YS medium) and then transferred into 250 ml Erlenmeyer flasks containing liquid

growth medium and bacteria of the strain DER c (*Enterobacter sp*.). The flasks were stored on a shaker (18°C, 165 rpm) for further use. The liquid growth medium was prepared as follows: For 1 L of medium 9 g of pig kidney, 17.4 g of yeast extract, 8.6 g egg yolk powder, 52.6 g sunflower oil were weighed in and the mixture was autoclaved for sterilization. Table 3 shows the three nematode species that were used in the experiment.

To find the appropriate amount of the medium with the nematodes to be placed on the treated side, the approximate number of nematodes in one mL of medium had to be estimated by counting. Under sterile conditions, one mL of the medium was put into a test tube which was then filled up to 10 mL with deionized water. Three drops of 10 µL were put on a microscope slide and the number of nematodes in each drop counted. Using this procedure, the approximate number of nematodes in 1 mL of medium and therefore the amount of medium to be put on the treated side of the boxes could be calculated.

Table 3: Species and strains of monoxenic nematodes used in the experiment.

Nematode	**Strain**	**Origin**
Phasmarhabditis hermaphrodita	B1	Czech Republic
Phasmarhabditis apuliae	BAR	Italy
Phasmarhabditis bohemica	CH1	Czech Republic

3.5. Experimental setup

The purpose of the experiment was to test the impact of a specific metabolite of symbiotic bacteria on the feeding behavior of the target slugs, when combined with different strains of nematodes.

3.5.1. Preparation of the experiment

For the experiment plastic boxes were used (17cm x 12.5cm), each box equipped with a plastic wall that divides the space into two equal halves. A plastic circle was positioned at the center of each plastic wall and served as the starting point for the slugs at the beginning of the experiment. The two sides of the boxes were marked with a plus and a minus, the plus indicating the treated side and the minus the untreated side. Different combinations of metabolites and nematodes or nematodes only were tested, each in seven boxes.

Approximately 100 g of 1.5 mm sand was added to each half of a box. In the prepared boxes (nematodes or metabolites and nematodes) deionized water was added to the sides labeled with a minus (untreated). To the plus side (treated) of the Test-Boxes, 10 mL of the chosen bacterial metabolites (Table 1) with 3187 nematodes (=300 000 nematodes per m^2) or nematodes only in 10 mL of water were pipetted. Ultimately, same size circles (diameter of 2 cm) were cut out of a leave of an iceberg salad (*Lactuca sativa*) and one circle put in each side of the boxes. A slug was then placed onto the plastic center of each box and the boxes were closed using lids that had small holes in them.

For the next five days the position and the feeding behavior of the slugs was observed and documented. At the end of each experiment the amount of lettuce consumed by each slug was determined and documented as well and the obtained data was used for further analysis.

Figure 2: Example of a plastic box (17 cm x 12.5 cm) used in the experiments (Photo: J. Nermuť).

3.6. Statistical Analysis

The statistical analysis was done using Statistica 10 (StatSoft, Inc.), a data analysis software. A factorial ANOVA was employed to analyze the statistical differences between the combinations of metabolites and nematodes. As the ANOVA model assumes a normal distribution of the data, the data was transformed to a logarithmic scale before analysis. The specific differences within each combination were tested by performing a Tukey HSD post-hoc Test. The graphs were drawn using the data in its untransformed form, not in its logarithmically transformed form.

4. Results

Throughout the experiment, the position of the slugs during the five days, as well as their feeding behavior and the amount of salad consumed by the slugs, were recorded. The results of the statistical analysis of the obtained data throughout the experiments are summarized in this section.

4.1. Position of the slugs

The presence of nematodes (Figure 3) or the combination of nematodes and bacterial metabolites (Figure 4) had a significant influence on the positions of the slugs during the experiment ($F_{1, 564} = 1594.385$, $p < 0.001$). However, no significant difference was observed between the strains of nematodes ($F_{2, 564} = 2.071$, $p > 0.05$), or the metabolites produced by the different bacterial strains ($F_{10, 564} = 0.782$, $p > 0.05$). Figure 3 shows the differences in the repellent effect on the slugs among the three different *Phasmarhabditis* strains. Figure 4 illustrates the differences in the repellent effect resulting from the metabolites of the *Xenorhabdus* strains.

All three nematode strains had the same significant repellent effect on the slugs (Figure 3). The combinations of *P. bohemica* CH1 with metabolites of the bacterial strains NFUST (*S. feltiae*), SLOV (*S. arenarium*), and JEGOR (*S. arenarium*), as well as the combination of *P. apuliae* BAR with SGI-197 (*S. beitlechemi*), showed the best repellent effects as can be seen in Figure 2. For *P. hermaphrodita* B1 the best results were achieved when combined with V.AF (*S. affine*). However, it is important to note that the differences in the effects of the bacterial metabolites are, as stated before, not statistically significant.

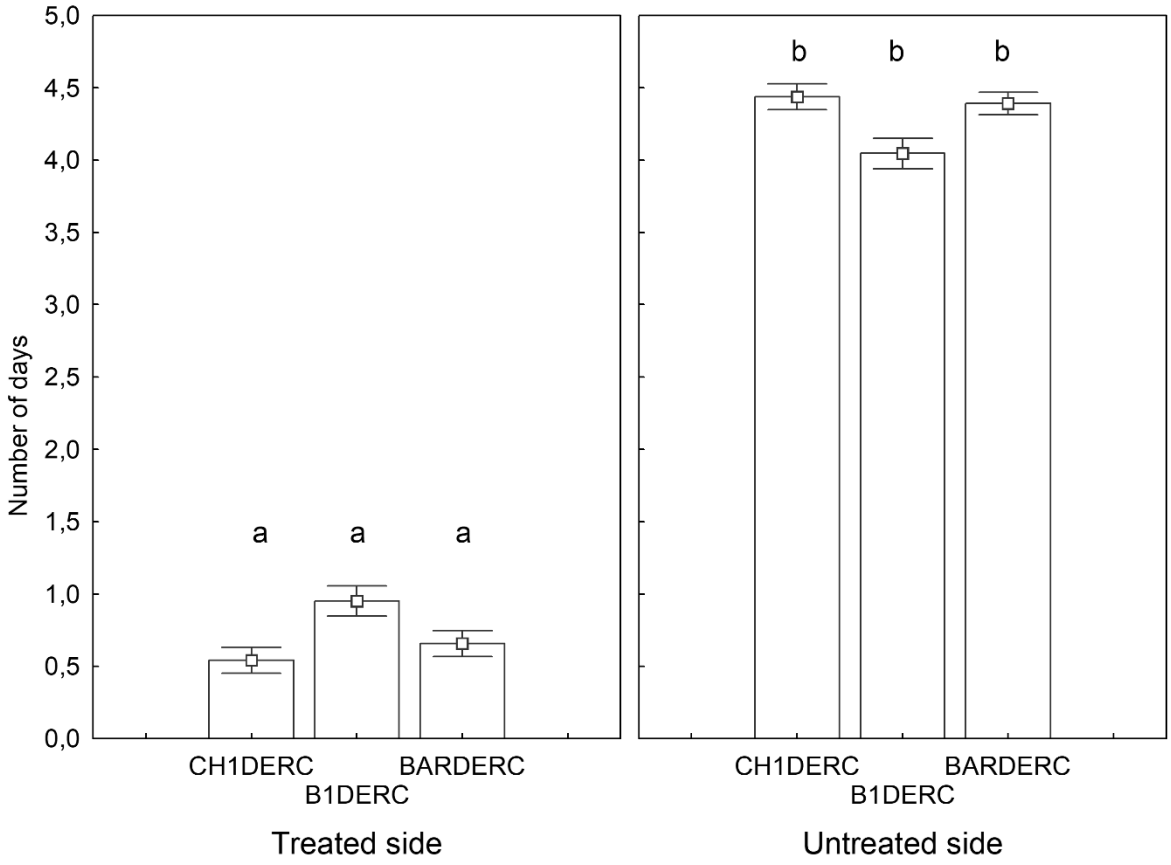

Figure 3: Comparison of the slugs' side preference between the treated and untreated side in days among the three nematode species *P. bohemica* (CH1), *P. hermaphrodita* (B1) or *P. apuliae* (BAR). The slug parasitic nematodes were grown in monoxenic cultures containing *Enterobacter sp*. of the strain DERc. The letters indicate a significant difference between the treated and untreated side.

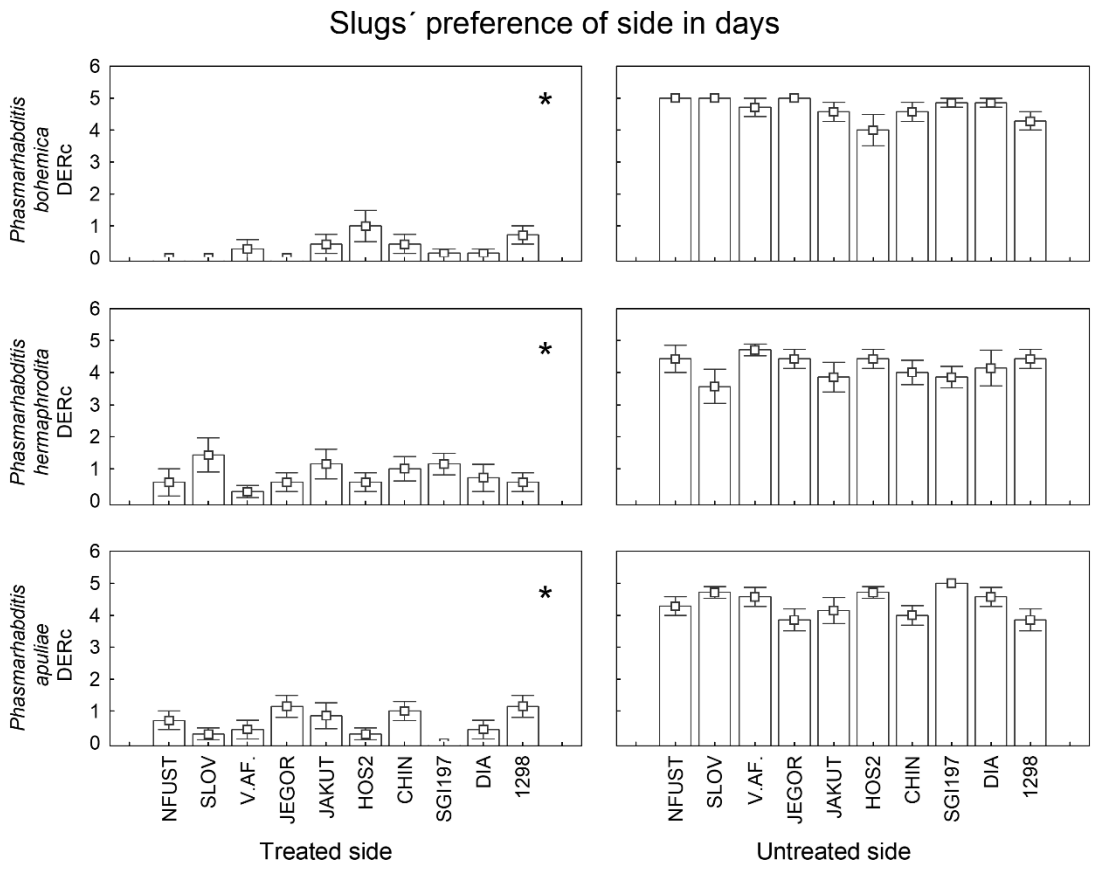

Figure 4: Comparison of the slugs' side preference between treated and untreated sides in days, when treated with secondary bacterial metabolites in combination with *P. bohemica* (CH1), *P. hermaphrodita* (B1) or *P. apuliae* (BAR). The slug parasitic nematodes were grown in monoxenic cultures containing *Enterobacter sp*. of the strain DERc. The Asterix indicates a significant difference between the treated and the untreated side.

4.2. Feeding behavior

The feeding behavior and the amount of lettuce consumed by the slugs was significantly influenced by the presence of nematodes (Figure 5) or the combination of metabolites and nematodes (Figure 6) ($F_{1, 564} = 318.605$, $p < 0.001$). Additionally, significant differences were observed between the effectiveness of the different *Phasmarhabditis* strains ($F_{2, 564} = 6.801$, $p < 0.05$) as well as the metabolites of the individual bacterial strains ($F_{10, 564} = 21.067$, $p < 0.001$). Figure 5 depicts a comparison of the nematode strains while Figure 6 compares the differences between the metabolites produced by bacteria of the genus *Xenorhabdus*.

When comparing the results, it becomes evident that the nematode strains *P. hermaphrodita* B1 and *P. apuliae* BAR show a significantly stronger antifeedant effect compared to *P. bohemica* CH1, but no significant difference is noticeable between *P. hermaphrodita* and *P. apuliae* (Figure 5). The statistical analysis of the metabolites' effects on the feeding behavior of the slugs (Figure 6) suggests that the combinations of *P. hermaphrodita* with metabolites of bacterial strains of the nematode strains V.AF (*S. affine*) and SGI-197 (*S. beitlechemi*) as well as combinations of *P. apuliae* with metabolites from the bacterial strains DIA (*S. diaprepesi*), V.AF and HOS2 (*S. affine*), NFUST (*S. feltiae*) and CHIN (*S. ceratophorum*) results in the greatest reduction in slug feeding activity. For *P. bohemica,* the best antifeedant effect was achieved in combination with metabolites of NFUST (*S. feltiae*).

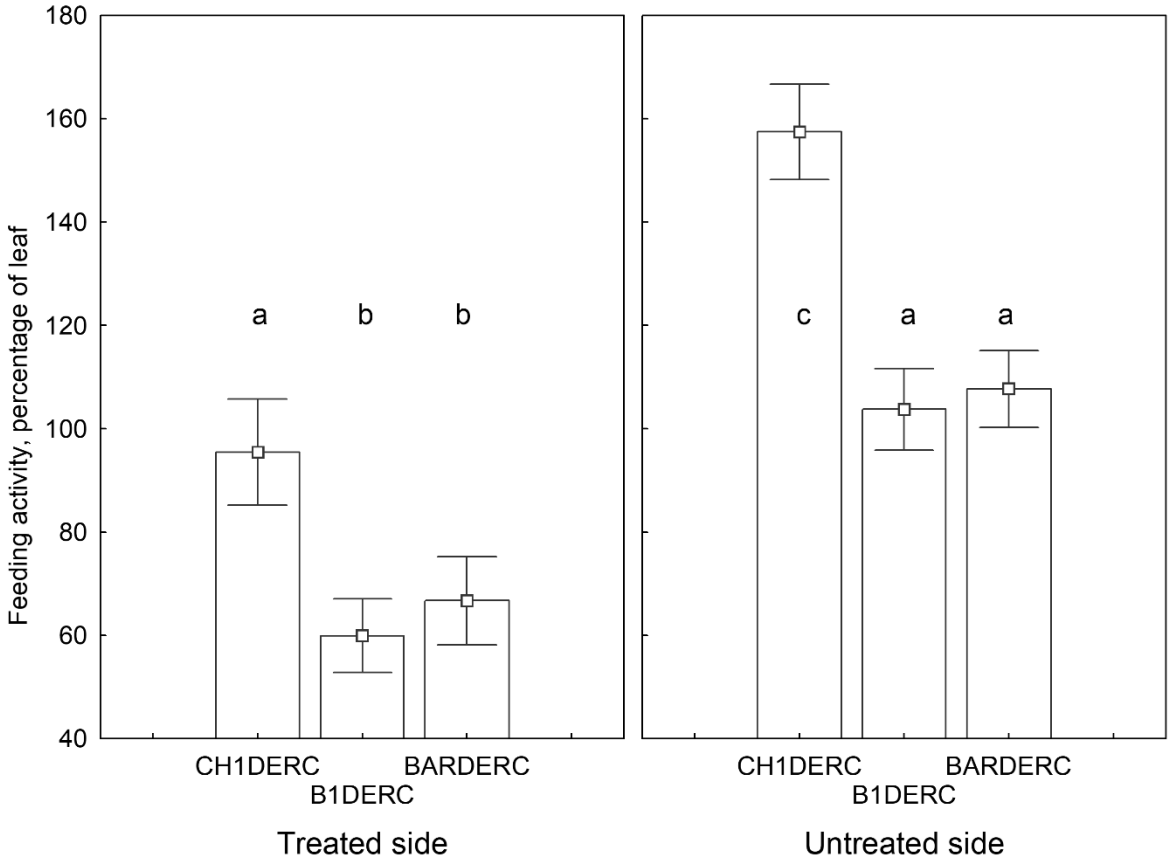

Figure 5: Comparison of the effects on the slugs' feeding behavior among the three different nematode species *P. bohemica* (CH1), *P. hermaphrodita* (B1) or *P. apuliae* (BAR). The feeding activity was measured in the percentage amount of lettuce consumed. The slug parasitic nematodes were grown in monoxenic cultures containing *Enterobacter sp*. of the strain DERc. The letters indicate a significant difference between the treated and untreated side as well as between the different nematode species.

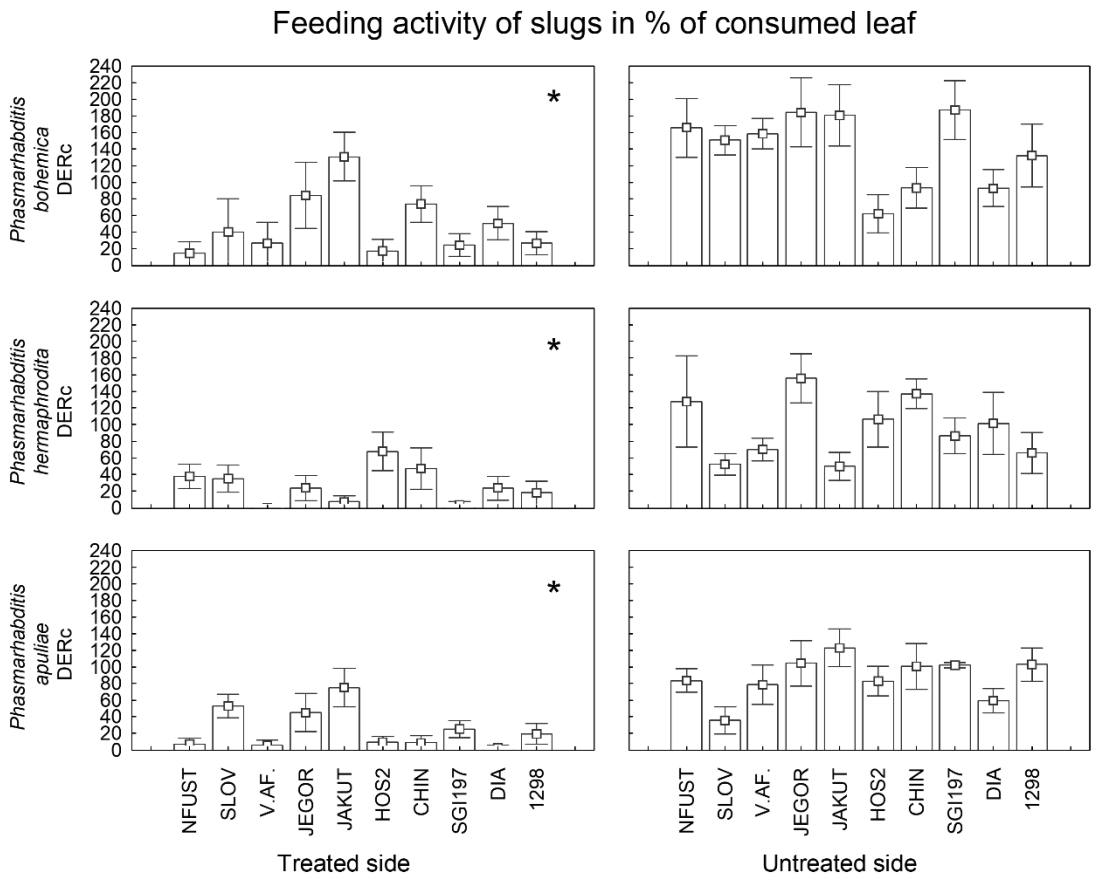

Figure 6: Comparison of the differences in the slugs' feeding behavior between treated and untreated sides, when treated with secondary bacterial metabolites in combination with *P. bohemica* (CH1), *P. hermaphrodita* (B1) or *P. apuliae* (BAR). The feeding activity was measured in the percentage amount of lettuce consumed. The slug parasitic nematodes were grown in monoxenic cultures containing *Enterobacter sp*. of the strain DERc. The Asterix indicates a significant difference between the treated and the untreated side.

5. Discussion

The slug parasitic nematode *Phasmarhabditis hermaphrodita* is a commonly used biocontrol agent in agriculture and a recent study has shown that the two species *P. apuliae* and *P. bohemica* show a great potential as control agents as well (Nermuť et al., 2022). As the secondary metabolites of *Xenorhabdus* bacteria (symbionts of EPNs) show repellent and antifeedant effects against various microorganisms, pathogens, and insects, which scavenge on EPN infected cadavers (Cimen at al., 2022), the hypothesis arose that these metabolites show the same impact against slugs. In the context of the growing demand for alternative control agents in agriculture, this study investigated the impacts of slug parasitic nematodes – *P. hermaphrodita* (B1)*, P. apuliae* (BAR) or *P. bohemica* (CH1) – as well as the combination of secondary metabolites from ten bacterial strains and these three nematode species on the slug species *A. vulgaris*.

The results of the experiments show that the slug parasitic nematodes on their own and all combinations of the secondary metabolites with the three nematode species exhibit a significant repellent effect against slugs of the species *A. vulgaris*. There were no significant differences among the nematodes and among the metabolites in regard to the days that the slugs spent on the treated side, which indicates that the repellent properties were similar in all nematodes and combinations of metabolites and nematodes. Interestingly, although all combinations showed a significant impact on the slugs' feeding behavior as well, there were some significant differences among the nematode species and the different metabolites indicating differences in their antifeedant effects.

All three nematode strains tested showed a significant repellent and antifeedant effect. For the species *P. hermaphrodita* these results correspond to other studies conducted on the repellent effect of this slug parasitic nematode (Wilson et al., 1999; Wynne et al., 2016). However, for the two species *P. apuliae* and *P. bohemica* these results are the first of their kind and as such quite important as not all *Phasmarhabditis* species show such a repellent effect and are therefore not suitable for biological slug control. *P. neopapillosa* for instance attracts certain slug species rather than repelling them and would consequently not be a suitable biocontrol agent for said slug species (Rae, 2023). A study of Wynne and colleagues (2016) suggested that the repellent effect on slugs of *P. hermaphrodita* does not rely on chemical cues but more likely relies on the mechanical penetration of the slug by the dauer larvae. The similar repellent effects of the three nematodes species used in this experiment could indicate that the mode of repellence is similar as well and could rely on mechanical stimulus in not only *P.*

hermaphrodita but also *P. apuliae* and *P. bohemica*. Furthermore, it could be interesting to investigate if the type of bacteria used in the monoxenic cultures, in this case *Enterobacter sp*., has an effect on the repellence of slugs by the slug parasitic nematodes.

Interestingly, the feeding behavior of the slugs was influenced in different ways by *P. bohemica* in contrast to the two other *Phasmarhabditis* species. Therefore, it would be interesting to investigate if factors other than the mechanical stimulation of the slugs by the dauer larvae play a role in the mode of action of nematodes against slugs especially concerning the antifeedant effects of the nematodes.

When comparing the results of the nematodes only and the combinations of nematodes and metabolites it becomes evident that the addition of secondary metabolites of *Xenorhabdus* bacteria greatly increases the repellence of slugs as well as their feeding behavior. These findings show for the first time that the secondary metabolites of *Xenorhabdus* bacteria could be a highly promising slug control agent in agriculture, providing an environmentally friendly alternative to chemical pesticides. The combination of *P. bohemica* and metabolites of the bacterial species *Xenorhabdus bovienii* (NFUST) and *X. kozodoii* (JEGOR, SLOV), showed the greatest effect in the repellence of *A. vulgaris*. Xenorhabdins that are found in the secondary metabolites of *X. bovienii* are not only antibiotics, but are known to show insecticidal activities too (McInerney et al., 1991). Xenematide, which also occurs in the secondary metabolites of *X. bovienii*, was shown to exhibit insecticidal properties as well (Lang et al., 2008). A closer examination of the secondary metabolites of the most effective bacterial species could shed some light on the mechanisms of repellence. It would be interesting to identify active compounds of the bacterial species and to investigate if and which compounds of the secondary metabolites show molluscicidal activities.

Even though these results are greatly promising, it is important to note that the experiments were conducted under controlled laboratory conditions. Environmental factors, which could greatly influence the results, were not considered in this study. Future studies aiming to replicate these experiments in outdoor settings such as raised beds, small gardens as well as fields could shed light on how environmental factors could influence the effectiveness of metabolites in the use as slug control agents. Factors like seasonal changes and weather conditions could be considered for finding the appropriate application time as well as the effects of the metabolites against slugs for different crops. In addition, research could be conducted in a similar way for other pestiferous slug species and in different geographic

regions. Another important area to investigate is the optimization of metabolite production for larger amounts of medium as well as the development of an effective method of applying the metabolites on the field. Further developments in the use of metabolites could therefore potentially ease slug control for farmers and provide a more environmentally friendly control agent for slugs as opposed to chemical pesticides.

6. Conclusion

In conclusion this study shows that in addition to *P. hermaphrodita*, both *P. apuliae* and *P. bohemica* exhibit significant slug repellent effects as well. The combination of secondary metabolites from the ten *Xenorhabdus* strains with the three slug parasitic nematodes show a great repellent effect and effects on the feeding behavior of the slug species *A. vulgaris*. The addition of metabolites to the nematodes greatly enhanced the effectiveness against slugs when compared to nematodes only. This study shows the great potential of secondary metabolites to be used as biocontrol agents against slugs in agriculture, providing farmers with a more environmentally friendly option for slug control.

7. References

Akhurst, R. J., & Boemare, N. E. (1990). Biology and taxonomy of Xenorhabdus. Entomopathogenic nematodes in biological control., 75-90.

Askary, T. H., & Abd-Elgawad, M. M. M. (2017). Beneficial nematodes in agroecosystems: a global perspective. Biocontrol Agents: Entomopathogenic and Slug Parasitic Nematodes. CABI, Wallingford, UK, Boston, USA, 3-25.

Askary, T. H., & Ahmad, M. J. (2017). Entomopathogenic nematodes: mass production, formulation and application. Biocontrol Agents: Entomopathogenic and Slug Parasitic Nematodes. CABI, Wallingford, UK, Boston, USA, 261-286.

Banu, J. G., Gannayane, I., & Meena, K. S. (2017). Entomopathogenic nematodes: General biology and behaviour. Biocontrol Agents: Entomopathogenic and Slug Parasitic Nematodes. CABI, Wallingford, UK, Boston, USA, 63-87.

Barua, A., Williams, C. D., & Ross, J. L. (2021). A literature review of biological and bio-rational control strategies for slugs: Current research and future prospects. Insects, 12(6), 541. Insects 2021, 12, 541.

Blanco-Pérez, R., Bueno-Pallero, F. Á., Vicente-Díez, I., Marco-Mancebón, V. S., Pérez-Moreno, I., & Campos-Herrera, R. (2019). Scavenging behavior and interspecific competition decrease offspring fitness of the entomopathogenic nematode *Steinernema feltiae*. Journal of invertebrate pathology, 164, 5-15.

Brooks, A. S., Crook, M. J., Wilcox, A., & Cook, R. T. (2003). A laboratory evaluation of the palatability of legumes to the field slug, *Deroceras reticulatum* Müller. Pest Management Science: formerly Pesticide Science, 59(3), 245-251.

Campbell, A., Audsley, N., & Port, G. (2021). The fate of *Deroceras reticulatum* following metaldehyde poisoning. *Insects*, *12*(4), 344.

Castle, G. D., Mills, G. A., Gravell, A., Jones, L., Townsend, I., Cameron, D. G., & Fones, G. R. (2017). Review of the molluscicide metaldehyde in the environment. Environmental Science: Water Research & Technology, *3*(3), 415-428.

Chitra, P., Sujatha, K., & Jeyasankar, A. (2017). Entomopathogenic nematode as a biocontrol agent: recent trends—a review. International Journal of Advanced Research in Biological Sciences, 4(1), 9-20.

Cimen, H., Touray, M., Gulsen, S. H., & Hazir, S. (2022). Natural products from *Photorhabdus* and *Xenorhabdus:* mechanisms and impacts. Applied Microbiology and Biotechnology, 106(12), 4387-4399.

da Silva, W. J., Pilz-Júnior, H. L., Heermann, R., & da Silva, O. S. (2020). The great potential of entomopathogenic bacteria *Xenorhabdus* and *Photorhabdus* for mosquito control: a review. Parasites & vectors, 13, 1-14.

Dreyer, J., Malan, A. P., & Dicks, L. M. (2018). Bacteria of the Genus *Xenorhabdus*, a Novel Source of Bioactive Compounds. Frontiers in microbiology, 9, 3177.

Dreyer, J., Rautenbach, M., Booysen, E., Van Staden, A. D., Deane, S. M., & Dicks, L. M. T. (2019). *Xenorhabdus khoisanae* SB10 produces Lys-rich PAX lipopeptides and a Xenocoumacin in its antimicrobial complex. BMC microbiology, 19(1), 1-11.

Edwards, C. A., Arancon, N. Q., Vasko-Bennett, M., Little, B., & Askar, A. (2009). The relative toxicity of metaldehyde and iron phosphate-based molluscicides to earthworms. Crop Protection, 28(4), 289-294.

Fang, X., Zhang, M., Tang, Q., Wang, Y., & Zhang, X. (2014). Inhibitory effect of *Xenorhabdus nematophila* TB on plant pathogens *Phytophthora capsici* and *Botrytis cinerea in vitro* and *in planta*. Scientific Reports, 4(1), 4300.

Foltan, P., & Puza, V. (2009). To complete their life cycle, pathogenic nematode–bacteria complexes deter scavengers from feeding on their host cadaver. Behavioural Processes, 80(1), 76-79.

Galán-Puchades, M. T., Gómez-Samblás, M., Osuna, A., Sáez-Durán, S., Bueno-Marí, R., & Fuentes, M. V. (2023). Update on the first finding of the rat lungworm, *Angiostrongylus cantonensis*, in *Rattus* spp. in continental Europe, Valencia, Spain, 2022. Pathogens, 12(4), 567.

Goodrich-Blair, H., & Clarke, D. J. (2007). Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Molecular microbiology, 64(2), 260-268.

Grewal, P. S., Ehlers, R. U., & Shapiro-Ilan, D. I. (Eds.). (2005). Nematodes as biocontrol agents. Cabi publishing.

Grubišić, D., Gotlin Čuljak, T., Mešić, A., Juran, I., Loparić, A., Starčević, D., Bremz, M., & Benković Lačić, T. (2018). Slug control in leafy vegetable using nematode *Phasmarhabditis hermaphrodita* (Schneider). Applied Ecology & Environmental Research, 16(2).

Guenay-Greunke, Y., Bohan, D. A., Traugott, M., & Wallinger, C. (2022). A multiplex PCR assay for detecting slug species common in European arable land in the diet of carabid beetles. Entomologia Generalis, 42(1).

Howlett, S. A. (2012). Terrestrial slug problems: classical biological control and beyond. CABI Reviews, (2012), 1-10.

Hynes, T. M., Mc Donnell, R. J., Kirsch, A., Dillon, R. J., O'Hora, R., & Gormally, M. J. (2014). Effect of temperature on the larval stage of *Tetanocera elata* (Diptera: Sciomyzidae)–Potential biological control agent of pestiferous slugs. Biological control, 74, 45-51.

Javed, N., Kamran, M., & Abbas, H. (2017). Toxic secretions of *Xenorhabdus* and their efficacy against crop insect pests. Biocontrol Agents: Entomopathogenic and Slug Parasitic Nematodes. CABI, Wallingford, UK, Boston, USA, 223–230.

Kozlowski, J., JasKulsKa, M., & Kozlowska, M. (2018). Damage to the legume (Fabaceae) and rapeseed (Brassicaceae) plants caused by *Arion vulgaris* Moquin-Tandon, 1855, *A. rufus* (Linnaeus, 1758) and *Deroceras reticulatum* (OF Müller, 1774). Folia Malacologica, 26(4).

Kromp, B. (1999). Carabid beetles in sustainable agriculture: a review on pest control efficacy, cultivation impacts and enhancement. Agriculture, Ecosystems & Environment, 74(1-3), 187-228.

Kumar, P. (2020). A review—on molluscs as an agricultural pest and their control. International Journal of Food Science and Agriculture, 4(4), 383-389.

Lang, G., Kalvelage, T., Peters, A., Wiese, J., & Imhoff, J. F. (2008). Linear and cyclic peptides from the entomopathogenic bacterium *Xenorhabdus nematophilus*. Journal of natural products, 71(6), 1074-1077.

Laznik, Ž., Majić, I., Horvat, A., & Trdan, S. (2020). Contact Efficacy of Different Wood Ashes against Spanish Slug, Arion vulgaris (Gastropoda: Arionidae). Applied Sciences, 10(23), 8564.

Mc Donnell, R. J., Howe, D. K., & Denver, D. R. (2023). First Report of the Gastropod-Killing Nematode, *Phasmarhabditis californica,* in Washington State, USA. Journal of Nematology, 55(1).

McInerney, B. V., Gregson, R. P., Lacey, M. J., Akhurst, R. J., Lyons, G. R., Rhodes, S. H., Smith D. R. J., Engelhardt L. M., & White, A. H. (1991). Biologically active metabolites from *Xenorhabdus* spp., Part 1. Dithiolopyrrolone derivatives with antibiotic activity. Journal of Natural Products, 54(3), 774-784.

Nermuť, J., Holley, M., & Půža, V. (2020). *Phasmarhabditis hermaphrodita* is not the only slug killing nematode. Microbial Nematode Control Invertebrate Pests 150, 152–156.

Nermuť, J., & Půža, V. (2017). Slug parasitic nematodes: biology, parasitism, production and application. Biocontrol Agents: Entomopathogenic and Slug Parasitic Nematodes. CABI, Wallingford, UK, Boston, USA, 533-547.

Nermuť, J., Půža, V., & Mráček, Z. (2014). The effect of different growing substrates on the development and quality of *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae). Biocontrol Science and Technology, 24(9), 1026-1038.

Oberholzer, F., & Frank, T. (2003). Predation by the carabid beetles *Pterostichus melanarius* and *Poecilus cupreus* on slugs and slug eggs. Biocontrol Science and Technology, 13(1), 99-110.

Pieterse, A., Malan, A., & Ross, J. (2017). Nematodes that associate with terrestrial molluscs as definitive hosts, including *Phasmarhabditis hermaphrodita* (Rhabditida: Rhabditidae) and its development as a biological molluscicide. Journal of Helminthology, 91(5), 517-527.

Rae, R. (2023). Avoidance and attraction behaviour of slugs exposed to parasitic nematodes. Journal of Invertebrate Pathology, 197, 107896.

Rae, R., Verdun, C., Grewal, P. S., Robertson, J. F. and Wilson, M. J. (2007), Biological control of terrestrial molluscs using *Phasmarhabditis hermaphrodita*—progress and prospects. Pest. Manag. Sci., 63: 1153-1164.

Rosenfeld, A., & Rayns, F. (2011). Sort out Your Soil: A practical guide to green manures.

Shirley, M., Howlett, S., & Port, G. (2020). Not All Slugs Are the Same: Variation in Growth and Development of the Slug *Deroceras reticulatum*. Insects, 11(11), 742.

Speiser, B., Glen, D., Piggott, S., Ester, A., Davies, K., Castillejo, J., & Coupland, J. (2001). Slug damage and control of slugs in horticultural crops. Research Institute of Organic Agriculture (FiBL).

Stenberg, J. A., Sundh, I., Becher, P. G., Björkman, C., Dubey, M., Egan, P. A., Friberg, H., Gil, J. F., Jensen, D. F., Jonsson, M., Karlsson, M., Khalil, S., Ninkovic, V., Rehermann, G., Vetukuri, R. R., & Viketoft, M. (2021). When is it biological control? A framework of definitions, mechanisms, and classifications. *Journal of Pest Science*, *94*(3), 665-676.

Stock, S. P., & Goodrich-Blair, H. E. I. D. I. (2012). Nematode parasites, pathogens and associates of insects and invertebrates of economic importance. Manual of techniques in invertebrate pathology, 2.

Stock, S. P., & Hunt, D. J. (2005). Morphology and systematics of nematodes used in biocontrol. Nematodes as biocontrol agents, Wallingford UK: CABI Publishing, 3-43.

Tan, L., & Grewal, P. S. (2001). Infection behavior of the rhabditid nematode *Phasmarhabditis hermaphrodita* to the grey garden slug *Deroceras reticulatum*. Journal of Parasitology, 87(6), 1349-1354.

van Rozen, K., van Balen, D. J. M., & Holwerda, J. (2009). Preventie en bestrijding van slakken [prevention and control of slugs] (No. 24). Wageningen UR etc.

Watz, J., & Nyqvist, D. (2021). Artificial barriers against arionid slug movement. Crop Protection, 142, 105525.

Wilson, M. J. (2007). Terrestrial mollusc pests. In Field manual of techniques in invertebrate pathology, Springer, Dordrecht, 751-765.

Wilson, M. J., Glen, D. M., & George, S. K. (1993). The rhabditid nematode *Phasmarhabditis hermaphrodita* as a potential biological control agent for slugs. Biocontrol Science and Technology, 3(4), 503-511.

Wilson, M. J., Hughes, L. A., Jefferies, D., & Glen, D. M. (1999). Slugs (*Deroceras reticulatum* and *Arion ater* agg.) avoid soil treated with the rhabditid nematode *Phasmarhabditis hermaphrodita*. Biological Control, 16(2), 170-176.

Wynne, R., Morris, A., & Rae, R. (2016). Behavioural avoidance by slugs and snails of the parasitic nematode *Phasmarhabditis hermaphrodita*. Biocontrol Science and Technology, 26(8), 1129-1138.