COLD TOLERANCE AND BACTERIAL COMPOSITION IN PREDATORY MITES *Amblydromalus limonicus* AND *Iphiseius degenerans* UNDER LOW AND SUBZERO TEMPERATURES

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DECLARATION

This thesis is my original work and has not been presented elsewhere for a degree or any other award.

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DEDICATION

I dedicate this work to my family.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS/ACRONYMS

ERA – Environmental Risk Assessment

DNA – Deoxyribonucleic acid

LTime – lethal time

LTemp – lethal temperature

LTime$_{50}$ – Lethal time at 50%

LTemp$_{50}$ – Lethal temperature at 50%

Exotic - Non-native

IPM – Integrated pest management

UK – United Kingdom

SMHI – Swedish meteorological and hydrological institute

GLM – Generalized linear models

GLMM – Generalized linear mixed models

ANOVA – Analysis of variance

PCOA - Principal Coordinate Analysis

ASVs – Amplicon sequence variants

ABSTRACT

Risk assessment is a critical prerequisite for the introduction of non-native biological control agents, as it ensures that the benefits of pest management do not come at the expense of irreversible ecological damage and unintended non-target impacts. In the context of biological control, comprehensive risk evaluations integrate laboratory cold tolerance testing, host-specificity testing and ecological modeling to predict an agent's establishment potential and its subsequent effects on native biodiversity and ecosystem balance. *Amblydromalus limonicus* and *Iphiseius degenerans* are targeted as biological control agents of various pests and phytophagous mites such as thrips and whiteflies. Knowledge about cold tolerance of non-native biological control agents is critical to avoid permanently establishing them in new temperate areas outside of their native range. The cold tolerance of the predatory mites, *A. limonicus* and *I. degenerans*, was investigated in the laboratory to assess their establishment potential in northern Europe, particularly Sweden. The lethal time of *I. degenerans* (the number of days until 100% mortality was reached) declined steeply from 5°C to 0°C and was almost zero at -5°C. The lethal time of *A. limonicus* did not differ between 5°C and 0°C, but was reduced at -5°C. For both species, $LTime_{50}$ (the number of days until 50% of the mites died) was longer for fed than for unfed mites. The lethal temperature of *A. limonicus* (the temperature at which 100% mortality was reached) was -17.75°C, whereas most *I. degenerans* died at -8.5°C. $LTemp_{50}$ (the temperature at which 50% of the mites died) was lower for *A. limonicus* (-9.8°C) than for *I. degenerans* (-0.1°C). Complementing the cold tolerance assessments, bacterial communities within both mites were profiled across selected low and sub-zero temperatures. Both mites, were dominated by Firmicutes (most abundant phylum), Actinobacteria Bacteroidetes and Proteobacteria and the order Bacillales was consistently dominant consistently prevalent. Notably, *A. limonicus* displayed higher microbial diversity at -5°C, as indicated by Shannon index 3.01 which might confer additional adaptive advantages under cold stress. Collectively, the data support a robust risk assessment framework for these non-native biological control agents. The enhanced cold tolerance of *A. limonicus* signals a significant risk regarding its potential establishment in temperate regions such as Sweden, where it might persist and affect non target species and ecosystem dynamics. However, the observed lower cold tolerance of *I. degenerans* suggests that its survival through Swedish winter is unlikely, thereby reducing its establishment risk.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Biological control involves control of pests by use of beneficial organisms (Eilenberg et al., 2001; Allen, 2009; Basso Valeria, 2022). For many years, the framework has been used to manage pests by using natural enemies including exotic species (Basso Valeria, 2022). It largely relies on parasites, predators and pathogens to regulate pest populations (Eilenberg et al., 2001). In protected cultivation such as in greenhouses, the use of arthropod predators for crop pest management is advantageous compared to use of pesticides in that, there is no chemical residues on produce, no pollution to the environment and no buildup of resistance (Carrillo et al., 2015)..

Although biological control provides a sustainable alternative to synthetic pesticides, there are concerns about the environmental safety particularly when using arthropods as natural enemies (Meurisse et al., 2021). The environmental risks associated with proposed and potential natural enemies aimed for biological control agents in new areas where they are not native have been overlooked which has led to negative effects on resident species of that environment (Van Lenteren et al., 2006). Environmental risk assessments including studies on cold tolerance, dispersal potential and host range are proposed for determining the biology and ecological effects of potential biological control agents before their release (Van Lenteren et al., 2006; Meurisse et al., 2021).

For most arthropod species proposed for biological control agents, their cold tolerance are measured as part of environmental risk assessments before release to new areas to avoid negative effects on local flora and fauna (Maes et al., 2015). Cold tolerance describes whether an organism has ability to tolerate cold (freeze tolerant) or dies quickly after exposure to low temperatures (freeze intolerant) (Bale, 1987; Storey & Storey, 2005). Cold tolerance studies are a key first step in evaluating whether non-native biological control agents can survive and establish in new environments (Hart et al., 2002; Van Lenteren et al., 2003; Tullett et al., 2004; Berkvens et al., 2010).

Family Phytoseiidae hosts important mites used in biological control programs (Carrillo et al., 2015; Kreiter & Douin, 2021). Predatory mites are now commercialized and used in many parts of the world for crop pest management (Knapp et al., 2018; Tixier, 2018; Souza & Vázquez, 2019). Similarly, the predatory mites *A. limonicus* and *I. degenerans* are produced and commercially available and play a crucial role in pest control in different countries (Bolckmans et al., 2005 Vantornhout et al., 2005; Messelink et al., 2006; Hoogerbrugge et al., 2011). Although the predatory mites *A. limonicus* and *I. degenerans* play a crucial role in pest control their cold tolerance is unknown.

Consequently, arthropods host numerous microorganisms which play important roles in their hosts. Many organisms including predatory mites form relationships with microorganisms. The microbiota contribute to various functions including improving host's physiology, adaptation to the environment, reproduction and overall host survival (Engel & Moran, 2013; Zhang et al., 2019; Zhang et al., 2020). Majority of arthropods maintain symbiosis with bacteria that provide them with nutrition and this may raise the question as to whether the dependence on symbionts in a way promotes temperature tolerances and geographic ranges. According to (Fortuna et al., 2023), temperature fluctuations significantly alter host microbiota in invertebrates. While the composition and abundance of microorganisms such as bacteria in various predatory mites are known, no data exists on bacterial composition of *A. limonicus* and *I. degenerans*. Additionally no research has investigated how temperature changes may influence microbial abundance and affect the tolerance of these mites to thermal stress.

To assess the establishment potential of *A. limonicus* and *I. degenerans* in Sweden, their cold tolerance were assessed through assays of lethal time and lethal temperature in the laboratory. Additionally, the microbiome of *A. limonicus* and *I. degenerans* particularly their bacterial communities were characterized and compared at different low and subzero temperatures through 16S rRNA sequencing to understand the effect of temperature on the mites microbiome.

1.2 Statement of the problem

Biological control using predatory mites is an essential pest management strategy. For an exotic biological control agent, knowledge of its biology and cold tolerance is a crucial component in evaluating the environmental risk assessment at its introduction in new regions (Maes et al., 2015). Environmental risk assessment are used to inform the establishment potential in a new area and to ensure safety to biodiversity (Barratt & Ehlers, 2017). Historically, the introduction of biological control agents before proper cold tolerance assessment has led to unintended ecological consequences on native biodiversity as cold-adapted species have a higher likelihood of establishing permanent populations outside their native environments (Allen, 2009; Hughes, 2010; Maes et al., 2015). Predatory mites *A. limonicus* and *Iphiseius degenerans* have been proposed for biological control use in Sweden but their cold tolerance features are unknown. As there are risks of *A. limonicus* and *I. degenerans* spreading in the environment, releasing these mites without proper assessment poses a risk of permanent establishment and therefore the risk of possible establishment needs to be investigated.

Consequently, temperature is a major determinant of establishment potential in temperate areas and is reported to alter host microbiota in invertebrates. Predatory mites are associated with microbial symbionts which can influence host survival, stress tolerance and adaptations and adaptations to extreme environments (Engel & Moran, 2013). Different bacterial symbionts have been characterized in several predatory mites and associated with different roles in mites (Gotoh et al., 2007; Schütte et al., 2008; Sumner-Kalkun et al., 2023) However, the bacterial symbionts of predatory mites *A. limonicus* and *I. degenerans* are unknown and their response to fluctuating low and subzero temperatures. This may form a baseline for testing hypothesis of whether bacterial communities are contributing to the overall cold tolerance in *A. limonicus* and *I. degenerans.*

1.3 Justification

The widespread use of predatory mites as biological control agents has improved pest management efficacy in controlled environments, yet significant uncertainties remain regarding their survival and establishment outside the controlled areas. Predatory mites *A. limonicus* and *I. degenerans* have been used and proven to be excellent biological control agents in agricultural settings in various countries worldwide. They are generalist predators important in the control of phytophagous motes, thrips, whiteflies, cassava green mite. Predatory mites have been used in augmentative biological control in Sweden since the 1970s (Kjellstrom, 2019), particularly in the greenhouses. Winter in Sweden acts as an establishment barrier and ability to overwinter plays a major role in the establishment potential of a species. *A. limonicus* and *I. degenerans* are potential biological control agents in Sweden, but their cold tolerance is largely unknown. Lack of cold tolerance data limits our ability to predict whether these species could overwinter, establish and spread beyond unintended areas.

Existing studies on related predatory mites such as *Amblyseius swirskii* and *Phytoseiulus longipes* have demonstrated that cold tolerance assessed through lethal time and lethal temperature assays quantify their level of cold tolerance. Conversely, microbiome associated with predatory mites can significantly influence host fitness, stress tolerance and reproductive capacity. Despite the known role of symbionts in modulating responses to environmental challenges, there is little data of the microbiome associated with *A. limonicus* and *I. degenerans*. This knowledge is critical because temperature -induced alterations in the symbiont profile could affect the mites' cold tolerance and efficacy in pest control applications. This research addresses the gap in knowledge by evaluating the cold tolerance of *A. limonicus* and *I. degenerans* through lethal time and lethal temperature assays and characterizing their bacterial communities and response to temperatures changes using 16S rRNA sequencing

1.4 Research Questions

1. To what extent do *A. limonicus* and *I. degenerans* survive exposure to low and subzero temperatures?
2. What is the composition of the bacterial communities associated with *A. limonicus* and *I. degenerans* and how do they compare when exposed to different low and subzero temperatures?

1.5 Objectives

1.5.1 Main Objective

To quantify the cold tolerance of predatory mites *Amblydromalus limonicus* and *Iphiseius degenerans* and investigate the composition and diversity of their associated bacterial communities in response to low and subzero temperatures.

1.5.2 Specific Objective

1. To quantify the cold tolerance of *Amblydromalus limonicus* and *Iphiseius degenerans* using lethal time and lethal temperature metrics.
2. To analyze the bacterial community composition and diversity of *Amblydromalus limonicus* and *Iphiseius degenerans* in response to low and subzero temperature stress using 16S rRNA sequencing.

CHAPTER 2

LITERATURE REVIEW

2.1 Biological control

To address the growing global population, efforts have been made to enhance food production and mitigate the negative environmental impacts of conventional farming practices and the need for sustainable food production (Sönmez et al, 2017). Biological control is one promising method within this framework known to be an environment friendly approach of managing agricultural pests (Sönmez et al, 2017; Eilenberg et al., 2001). It involves using living organisms to reduce pest populations thereby minimizing their damage and reducing the reliance on synthetic pesticides that harm the environment (Eilenberg et al., 2001; Allen, 2009; Basso Valeria, 2022; Tyagi et al., 2024).

2.1.1 History of Biological control

The introduction of predatory ants to control larval Lepidoptera in citrus orchards is known as the first use of arthropods for pest management (Huang and Yang 1987). Earlier on, synthetic pesticides were recognized and used to control crop pests but overtime, they were associated with negative environmental side-effects (Hajek et al., 2016). This encouraged the shift from use of chemical pesticide to use of biological control with the aim of protecting the environment (Hajek et al., 2016; Sönmez et al, 2017). Biological control agents, naturally present in agricultural ecosystems, regulate pest numbers through predation but they can be introduced to enhance pest control when they are insufficient as done in augmentation (Bale et al., 2008).

There are different biological control strategies which are used for pest control (Bale et al., 2008; Allen, 2009; Hughes, 2010; Handoko & Latifah, 2020) This include classical, augmentative and conservation biological controls. These approaches are used worldwide in agriculture, forestry and greenhouse horticulture to protect crops and the environment (Perdikis et al., 2008; Handoko & Latifah, 2020).

2.1.2 Types of biological control

2.1.2.1 Classical biological control

Pest management by classical biological control aims to regulate invasive pests by finding effective natural enemies, introducing them to the pest's area and establishing a self-sustaining population for ongoing control (Eilenberg et al., 2001; Bale et al., 2008; Carrillo et al., 2015). These biological control agents include parasites, mites, nematodes and predatory insects (Hajek et al., 2016). Implementing classical biological control requires a search for natural enemies, followed by quarantine in the receiving country. Rearing methods are developed and colonies are cleansed of parasites and pathogens. Evaluations of potential predators include developmental time, feeding, and mating behaviors. Release occurs only after permits are secured (Cock et al., 2012).

Classical biological control mainly targets economically significant agricultural and forestry pests but increasingly addresses invasive species affecting natural and managed systems (Bale et al., 2008; Cock et al., 2012). The parasitoid *Epidinocarsis lopezi* introduced to control the mealy bug *Phenacoccus manihoti* in different parts of Africa has been recognized as a successful example of classical biological control (Bale et al., 2008).

2.1.2.2 Augmentative biological control

Augmentation as a framework of biological control involves periodic release of biological control agents by man (Bale et al., 2008). Most of the natural enemies used in this strategy are mass produced and commercialized. The practice has targeted arthropod crop pests in both open field and protected cultivation (Allen, 2009; Cock et al., 2012). Two primary strategies exist in augmentation namely inundative and inoculative releases (Allen, 2009; Bale et al., 2008; Hughes, 2010). The former is aimed at immediate suppression of pests while the latter releases small numbers of natural enemies at intervals, allowing them to reproduce for longer-term control (Bale et al., 2008).

Current augmentative programs favor specialized parasitoids or indigenous species which minimizes potential adverse effects on biodiversity and the environment. If an exotic augmentative biological control agent is considered as a potential for biological control, its initial release is subject to review in line with the regulations and international standards of that country (Cock et al., 2012). As part of augmentation *Trichogramma* spp. have been used against exotic Lepidoptera pests of food crops such as the European and Asian corn borers (Bale et al., 2008; Cock et al., 2012).

2.1.2.3 Conservation biological control

This approach uses native natural enemies to regulate indigenous or exotic pests (Bale et al., 2008; Allen, 2009). Success of conservation biological control hinges on conserving all natural enemies, both native and introduced. To achieve this, specific cultural practices are implemented to promote natural enemy populations (Randall & Tu, 2000; Allen, 2009).

2.2 Ecological risks and risk assessment of introducing exotic species for biological control.

2.2.1 Ecological risks

Biological control of invasive pests using invertebrate biological control agents is a popular pest management strategy. However, it has faced significant criticism due to reported ecological risks such as attack on non-target species and disruption of local biodiversity (Simberloff & Stiling, 1996; Wright et al., 2005). Before the1980s, exotic biological control agents were used in pest management to control pests reducing the need for synthetic chemical pesticides. However, environmental safety concerns about these introductions emerged later (Howarth, 1983).

After several studies focusing on impacts of introduced biological control agents, proponents of biological control began developing new methods and regulations to ensure only safe species are released for biological control (Delfosse, 2005; Van Lenteren et al., 2006). Initially, broad host ranges for released biological control agents was seen as beneficial but this approach negatively impacted native biodiversity (Van Lenteren et al., 2006).

Natural enemies introduced in new regions for biological control have the potential to directly affect the population size of non-target species through competition and predation. The multicolored Asian beetle, *Harmonia axyridis*, has been associated with significant non-target impacts. This voracious, generalist predator has become a nuisance in Europe and North America, damaging fruit, contaminating grapes (leading to undesirable wine flavors) and displacing native species (Majerus et al., 2006; Hughes et al., 2011).

The above example highlights the fact that agents released for biological control are not restricted to the area of introduction which makes it difficult to quantify and predict their potential impact once released (Hughes, 2010).

2.2.2 Risk assessment

Following the growing awareness of ecological risks associated with biological control particularly when using exotic natural enemies, effective risks assessment has become crucial to ensure that biological control agents used in new environments do not affect non-target species or native ecosystems (Van Lenteren et al., 2006). This is particularly relevant in greenhouse environments which although enclosed are not entirely isolated from the external environment.

Greenhouse agriculture, developed in the 1950s in the UK, USA and New Zealand, facilitates the intensive cultivation of vegetables among other different crops (Zhang, 2003). However, the stable conditions within the greenhouse system creates optimal environments for rapid pest development in the absence of natural enemies. To manage such situations, predatory mites and other beneficial arthropods are frequently introduced (Zhang, 2003).

In contrast to classical biological control in the open fields, where long-term establishment is desired, augmentative biological control which is largely used in greenhouses presents a distinct challenge. The primary concern is not whether the introduced agents will survive but rather the implications of their escape and potential establishment in the open field (Hart et al., 2002; Hughes, 2010). The above concerns are significant given that previous assumptions regarding climatic mismatches such as the belief that species from warmer climates would not endure cooler ones which have been proven as unreliable. For example the unexpected outdoor establishment of *Neoseiulus californicus* in the UK raised concerns as it had been assumed that this species lacked sufficient cold tolerance to survive outside the greenhouse (Hart et al., 2002).

Such occurrences have prompted regulatory authorities in several countries to enhance controls and mandate formal risk assessments before granting release permits for exotic biological control agents (Allen, 2009; Hughes, 2010). Risk assessments aim to quantify the likelihood and consequences of non-target effects by evaluating critical factors such as accurate identification of the biological control agent using DNA-based tools, quantifying the agents hosts range especially its potential to attack non-target or native species and investigating dispersal ability and survival potential in outside the enclosed environments (Van Lenteren et al., 2003, 2006).

While winter conditions in Sweden differ from those in the UK, the above example emphasizes the need to transition from the belief that most introductions are harmless to an understanding that comprehensive research is required to ensure proper use of exotic biological control (Simberloff & Florida, 1996; Allen, 2009).

2.3 Cold tolerance in arthropods

Temperature significantly influences the geographic distribution and seasonal activity of arthropods impacting the spread of arthropods used for biological control (Fortuna et al., 2023a). Moreover, low temperature biology is crucial for insect fitness and a primary factor that determines insect distribution (Bale, 1996; Sinclair et al., 2015). As ectotherms most arthropods have body temperatures that closely match their surroundings, thus fluctuations in ambient temperature can have lethal effects on their physiology. Therefore, assessing cold tolerance of arthropods is an excellent way to understand the widespread influence of temperature on the establishment potential of arthropod biological control agents (Sinclair et al., 2015; Block & Somme, 2016).

Low temperature effects can be both harmful and lethal to these organisms. Freezing results to death which requires adaptive strategies to survive low and subzero conditions during cold seasons. There are different ways organisms can protect themselves against extreme cold during the winter season, these include insulation, migrating to warmer climates and the developing cold hardiness (Storey & Storey, 2005). For arthropods, there are mechanisms associated with the ability to endure low temperatures, which determine species' cold tolerance (Bale, 1987; Sinclair et al., 2015).

2.3.1 Strategies of cold tolerance in arthropods

Strategies of cold hardiness of arthropods can be summarized as follows: arthropods can either be freezing-tolerant or intolerant (Leather et al., 1995; Storey & Storey, 2005).

2.3.1.1 Freeze tolerance

While most terrestrial arthropods can't survive freezing temperatures, some have developed the capacity to endure ice formation within their body tissues and haemolymph (Allen, 2009). This remarkable adaptation allows them to tolerate conditions that would be lethal to most arthropods. The freeze tolerance group can manage ice formation in their extracellular fluids, thereby protecting their intracellular water from freezing and preserving their cell function (Somme, 1999; Storey & Storey, 2005). As a way to survive lethal cold temperatures during the winter, species in this category accumulate ice nucleating agents (proteins or peptides) to inhibit lethal intracellular freezing and minimize freeze damage (Bale, 1987; Storey & Storey, 2005; Allen, 2009; Ghazy et al., 2016). The cranefly *Tipula trivittat* is an excellent example of a well-studied freeze-intolerant species (Bale et al. 2002).

Many of these species inhabit North America, Scandinavia, Russia and China (Leather et al., 1995; Bale, 1996). While freeze-tolerant species have advantages over their freeze-intolerant counterparts, it is crucial to recognize that this strategy is not without risks; mortality rates increase with decreasing temperatures and prolonged exposure to frozen conditions.

2.3.1.2 Freeze intolerance

Freeze avoidant species inhabit cool climates (Storey & Storey, 2005; Allen, 2009). The transition to a winter temperatures for freeze avoidant species occurs in two stages. Initially, these species undergo various behavioral and physiological such as selecting insulated that shield them from severe low temperatures, ceasing feeding and evacuating gut contents that serve as ice nucleators. The second stage involves synthesis of anti-freezing proteins and polyols (Leather et al., 1995; Hart et al., 2002; Storey & Storey, 2005; Andreadis et al., 2008; Allen, 2009; Ghazy et al., 2016).

Bale (1996) proposed a reclassification system for insects based on their level of cold-hardiness These reclassification added three other mechanisms based on cold tolerance of various organism namely chill susceptible, chill tolerant and opportunistic survival. The advantages of this categories lie in its consideration of species based on their observed of cold-hardiness limits, its differentiation between freezing and chilling as stressors and potential causes of mortality and its applicability worldwide (Bale, 1996; Andreadis et al., 2008). Phytoseiid mites are considered freeze intolerant (Ghazy et al. 2016).

2.4 Determination of cold tolerance in arthropods

Low temperatures significantly affect animal life, influencing their distribution and survival (Fortuna et al., 2023a). Nevertheless, arthropods have colonized large cold zones including polar regions. In temperate zones, insects often face temperatures below their optimal growth conditions. As a result, the ability of insects to endure low temperature has become a key focus of ecophysiological research. Cold tolerance is largely supported by various physiological and biochemical adaptations that protect organisms from cold injury (Storey & Storey, 2005).

To determine the cold tolerance of proposed species for biological control, several methodologies have been developed and tested (Hart et al., 2002; Tullett et al., 2004; Allen, 2009;; Sinclair et al., 2015). Understanding how a biological control tolerates cold, is an important factor which contributes to knowledge about the risks associated with its release in new environments (Maes et al., 2015). To determine the cold tolerance of *Amblyseius californicus* a predatory mite of greenhouse spider mite, Hart et al., (2002) developed a series of assays which have been replicated in subsequent studies (Tullett et al., 2004; Hughes et al., 2009;). These experimental approaches have led to classification of arthropods into based on their ability to tolerate cold (Block, 1990)

2.4.1 Lethal time

Lethal time measures the survival of a population by determining the period (in terms of days, weeks or months) it survives following long term exposure to set temperature (e.g. 5°C, 0°C, -5°C) (Allen, 2009; Bale & Walters, 2001). During the study of lethal time, LTime_{50} is assessed to determine the number of days, weeks or months it takes to kill 50% of the sample population (Hatherly et al., 2004). Several studies on the thermal biology of predatory mites in the UK, have found a strong correlative relationship between the period of time needed to kill half of the sample at 5°C and the duration of winter survival in the field (Hatherly et al., 2005). However, for countries experiencing lower average temperatures such as Sweden 0 and -5°C are most appropriate (White et al., 2018).

Using lethal time to quantify the cold tolerance is reliable because experimental samples are exposed to low temperatures for prolonged periods which represents naturally occurring stress (Allen, 2009; Hughes, 2010).

2.4.2 Lethal temperature

Lethal temperature measures the mortality rate of a sample following a brief exposure (e.g. one minute) to low and subzero temperatures (Allen, 2009). The lethal temperatures are expressed as proportional percentages to express the temperature at which a certain percentage of exposed individuals die. For example, the LTemp_{50} is the median temperature that kills half of the sample population exposed to different temperatures after a short period.

To determine lethal temperature, individual samples must be acclimated to a favorable temperature from which the temperatures will be lowered to a range of target temperatures (e.g., 10 to -20°C), each for a set constant time. After exposure, sample are rewarmed to the acclimation temperature and allowed to recover before assessing the survival (Bale & Walters, 2001; Allen, 2009; Sinclair, Alvarado, et al., 2015). Lethal temperatures are often estimated graphically by plotting the survival proportions by temperature and comparing the sample mortality by temperatures tested (Sinclair, Alvarado, et al., 2015).

2.5 Predatory mites of family Phytoseiidae and their role in biological control

The Phytoseiidae family consists of fast growing predatory mites which are found on land and plants. Phytoseiid mites play a crucial role in crop protection against pests such as thrips, mealy bug and whiteflies (McMurtry , Sourassou, 2015; Knapp et al., 2018; Tixier, 2018; Souza & Vázquez, 2019). The family consists of predators with various feeding habits, some are specialist predators while some are generalist predators. These are further grouped as Type I mites which are specialized predators of *Tetranychid* spp. such as *Phytoseiulus* spp. Type II lifestyle are selective predators of tetranychid mites represented by *Neoseiulus* species who have an acceptable wider range of prey compared to the Type I including Tarsonemidae and pollen. Type III lifestyle are generalist predators feeding on a range of prey including pollen, plant exudates and honeydew during times of food or prey scarcity. Majority of these species belong to genera *Amblyseius*, *Neoseiulus* and *Typhlodromus*. Type IV are lifestyle consist of specialized pollen feeders mainly genera *Euseius* spp (McMurtry , Sourassou, 2015).

Predatory mites are recognized worldwide, largely present in USA, China, India, Brazil and Pakistan but absent in Antarctica (Demite et al., 2014). The life cycle of Phytoseiids is split into five life stages (Handoko & Latifah, 2020; Zhang, 2003). In warmer zones, development and reproduction can occur year-round but in temperate zones development can only occur all year round in greenhouses (Zhang, 2003). Majority of predatory mites are presently found in plants (McMurtry, Sourassou, 2015) and have been categorized based on their feeding preferences, where groups are either specialist or generalist predators (McMurtry et al., 2013; Souza & Vázquez, 2019). Alternative food in absence of prey include honeydew and pollen

(McMurtry et al., 2013). Predatory mites are now mass reared and have been commercialized for biological control (Knapp et al., 2018).

2.5.1 *Iphiseius degenerans*

Iphiseius degenerans is dark brown mite belonging to the order Mesostigmata. The predatory mite is described as a generalist predator (McMurtry & Croft, 1997). It plays a crucial role in the control of different crop pests such as whiteflies, flower thrips in protected hortcultural crops such as cucumber, sweet pepper, strawberry and ornamentals (Vantornhout et al., 2005; Perdikis et al., 2008; Pijnakker et al., 2017; Döker et al., 2018). This predatory mite has been recorded widely across Europe, Africa and Asia (Zannou et al., 2005; Vantornhout, 2006; Döker et al., 2018; Kreiter et al., 2018;).

The mite is native in cassava crops in southern part of Africa known to predate on *Mononychellus tanajoa* (Vantornhout et al., 2005). *Iphiseius degenerans* has been suggested to be an effective predator of Western flower thrips in sweet pepper (van Houten et al., 1995). The predatory mite has been tested against *Amblyseius swirskii* for the control of whitefly and thrips. *I. degenerans,* developed faster and significantly reduced thrips compared to *Amblyseius swirskii* (Pijnakker et al., 2017). In another study, the predatory mite has been proposed as a biological control of *Eutetranychus orientalis* (Kreiter et al., 2018).

Having been tested in different occasions as an excellent pest control of crop pests in greenhouses as shown above, the predatory mite *I. degenerans* may have further applications in greenhouses within northern Europe especially Sweden. However, despite its commercial value, the cold tolerance of *I. degenerans* and its potential for establishment in temperate regions like Sweden have not been studied. . Cold temperatures during the winter act as an establishment barrier, therefore ability to overwinter plays a major role in the establishment potential of a species.

2.5.2 *Amblydromalus limonicus*

Amblydromalus limonicus, initially identified as *Amblyseius limonicus* by Garman & McGregor was first identified from citrus trees in California (Knapp et al., 2013).

This predatory mite prefers high humidity and moderate temperature and is native to New Zealand, Hawaii, Australia and North and South America (Knapp et al., 2013; Xu & Zhang, 2018). It is a generalist predator and can feed and reproduce on different sources including whiteflies, spider mites, thrips and pollen of various plant species (McMurtry & Croft, 1997). *A. limonicus* is commercialized for control of spider mites, thrips and whiteflies (Knapp et al., 2013). The predatory mites *A. limonicus* has been used for biological control of red mites (McMurtry & Scriven, 1965) and according to Hoogerbrugge (2011) the predatory mite outperforms *Amblyseius swirskii* in the control of thrips and whiteflies in strawberries. When *A. limonicus* effectiveness to control pest was compared with that of native predators *Kampimodromus aberrans* and *Euseius finlandicus* in Austria, the mites was the most aggressive predator among the three. *A. limonicus* had a high killing efficiency due to its larger body size relative to the natives. According to Dittmann, *A. limonicus* constitutes a high risk predator capable of influencing the efficacy of native predators through aggressive behaviour and effective consumption of prey (Dittmann et al., 2016). Despite its commercial value, the cold tolerance of *A. limonicus* and its potential for establishment in temperate regions like Sweden have not been studied. Since establishment in temperate climates depends on the survival ability at low temperatures, understanding their cold tolerance is essential for predicting their potential use in Northern Sweden particularly, Sweden.

2.6 Mite symbionts and associated bacteria

Arthropods are largely represented globally in terms of numbers and most of their success and diversity is attributed to their relationships with microorganisms (Engel & Moran, 2013). For most arthropods, microbial communities provide nutrients and protection to their hosts against pathogens and determine the reproduction of their host among other functions (Engel & Moran, 2013; Gurung et al., 2019; Holt et al., 2024). In addition, microbial communities in arthropods may harbor the full potential of arthropods if they are pathogenic (Engel & Moran, 2013; Gurung et al., 2019).

High-throughput sequencing now enables microbiome profiling (Pekas et al., 2017). For several prey and predatory mites, their microbial communities such as bacteria have been characterized (Pekas et al., 2017). Endosymbiotic and pathogenic bacteria have been identified in phytoseiid mites (Weeks & Stouthamer, 2004; Enigl & Schausberger, 2007; Goodacre, 2011; Famah Sourassou et al., 2014; Pekas et al., 2017;) with *Metaseiulus occidentalis* serving as a well-studied example. This mite hosts a variety of bacteria, including gut symbionts and endosymbionts (*Wolbachia* and *Cardinium* (Hoy & Jeyaprakash, 2005). Bacterium *Acaricomes phytoseiuli* has been identified in commercial populations of *Phytoseiulus persimilis* populations (Enigl & Schausberger, 2007; Bjørnson, 2008;).

Recent studies have characterized bacterial communities of predatory mites *Phytoseiulus persimilis, Neoseiulus cucumeris* and *Typhlodromips swirskii*, typically dominated by phylum Proteobacteria (Sumner-Kalkun et al., 2023). However, no microbial exists for predatory mites *A. limonicus* and *I. degenerans* despite their important role as biological control agents. While bacterial communities influence mite reproductive traits (Kikuchi, 2009; Pekas et al., 2016; Sumner-Kalkun et al., 2023), their role in arthropod thermal adaptation remain poorly explored. Temperature fluctuations significantly alter host microbiota in invertebrates (Fortuna et al., 2023), for instance, *Buchnera* in aphids regulates thermal tolerance (Zhu et al., 2021) but similar roles in predatory mites are unexplored. In spider mites (*Tetranychus truncatus*) co-infection with *Wolbachia* and *Spiroplasma* was associated with stable survival under thermal stress compared to uninfected strains (Zhu et al., 2021).

Psychrophiles are microorganisms which tolerate cold environments and include bacteria and archaea. Their activity at -20°C in Arctic seas ice recycles nutrients in cold environments. Early molecular work linked psychrophiles to a few genera Brevibacterium*, Microbacterium, Micrococcus, Flavobacterium, Bacillus and Clostridium, Pseudomonas and vibrio.* Current orders lists at least 96 bacterial genera belonging Micrococcales, Bacteroidales, Cytophagales, Flavobacteriales, Sphingobacteriales, Bacillales and Clostridiales, Oscillatoriales,, Pseudomonadales, Rhizobiales, Rhodobacterales, Sphingomonadales, Vibrionales, Xanthomonadales. Although psychrophiles occur throughout the bacterial domain, most isolates belong to Bacteroidetes and Proteobacteria (Moyer et al., 2017).

Based on the above literature, whether similar mechanisms labeled above occur in predatory or whether mites harbor cryoprotective symbionts remains unknown. This study provides the first characterization of bacterial communities in predatory mites *A. limonicus* and *I. degenerans* at low and subzero temperatures (10°C, 0°C, -5°C and -15°C) using 16S rRNA sequencing. The justification for characterizing the bacterial communities via 16S rRNA sequencing is twofold. First comprehensive microbial profiling enables the detection and quantification of bacterial taxa, establishing baseline core microbiota for each mite species and to determine if any shifts occur when the mites are exposed to low and subzero temperatures. Such shifts may inform patterns in activation or suppression of cold adaptive microbial functions.

2.7 Use of molecular techniques in microbial ecology

Molecular techniques became essential in microbial ecology in the early 90s (Pickup, 1991). Before that time, sequencing DNA sequences was costly for processing large number of samples, prompting the development of alternative molecular approaches. DNA-based analyses have been employed to analyze whole communities, individual isolates, and clones of genes. Techniques with low resolution and broad based scope such as DNA reassociation can estimate the overall diversity present in a community. In contrast, modern next-generation high-throughput sequencing technologuies sequence all genetic material found in an environment (Thomas et al., 2012). This enables discovery of interactions between microorganisms and the environment, and assignment of ecosystem functions to various communities (López-García & Moreira, 2008).

CHAPTER 3

MATERIALS AND METHODS

This chapter uses lethal time and lethal temperature assays to assess the cold tolerance of *A. limonicus* and *I. degenerans* under laboratory conditions at low and sub-zero temperatures. Additionally, it details the methods used to characterize their bacterial communities and comparing them at different low and subzero temperatures.

3.1 Source of mites

Samples of *A. limonicus* were ordered from Biobasiq (Koppert) and delivered in plastic bottles filled with wheat bran and over 1000 *A. limonicus* individuals in each container. *I. degenerans* were obtained from Lindesro AB and transported in plastic bottles of 500 pieces per unit, with vermiculite as carrier. Biobasiq (Koppert) and Lindesro AB are biological control agent providers in Sweden. The Predatory mites were stored at acclimation temperature 10°C upon arrival.

For easy transfer, to glass vials, the mixture was transferred to a collecting glassware placed on a sticky band to prevent the escaping mites from being dispersed. An order of mites was made every week and individual mites were tested after 24 hours. Batch was included to the models in the analysis to control for batch-specific effects. The mites were delivered in one container (batch) per week, which might have introduced some variability in the conditions of the mites tested. To ensure consistency within the experiments, only healthy, actively motile adult mites were selected. After exposure, the mites were considered to be alive if they moved away when touched. Photosynthetic photon flux density (PPFD) during the experiments was 100 µmol/m²/s.

3.2 Experimental procedures for objective one: Cold tolerance assays

3.2.1 Measurement of lethal time

Lethal time was measured at 5°C, 0°C and -5°C (Bale & Walters, 2001; Hart et al., 2002). For each temperature regime, 10 mites of each species were placed in individual transparent glass vials and fed (Nutrimite, Biobasiq), while another 10 mites had no access to food. A piece of moist filter was used in the glass as a source of water (Hart et al., 2002) and fresh moist papers were added after recording survival. This procedure was replicated for three batches of *A. limonicus* and two of *I. degenerans*. A batch consisted of one container of mites ordered each week, each batch had 10 samples for each treatment (fed/unfed) and temperature, resulting in 180 individuals of *A. limonicus* and 120 of *I. degenerans* tested.

Mortality was recorded at 24-hour intervals by examining the mites under a microscope (Nikon SMZ 1500). Glass vials were removed from the climate chambers and warmed to room temperature by hand rolling for one minute to allow for mite recovery before survival was assessed. Motile mites were considered alive and non-motile mites were considered dead. The glass vials containing mites considered dead were maintained in the climate chambers for 24 hours and examined again to confirm mortality.

3.2.2 Measurement of lethal temperature

Predatory mites were exposed to a range of predetermined temperatures (5, 0, -5, -10, -15 and -20°C) (Sinclair et al., 2015). However, the temperatures were adjusted to 3.88°C, 0.75°C, -3.88°C, -8.5°C, -13.13°C and -17.75°C, i.e. the actual temperature experienced in the vials to account for buffering provided by glass vials against rapid change in temperature. This adjustment was based on a study where temperatures inside vials were recorded (Kjellstrom, 2019). The mites were individually transferred to transparent glass vials and placed in climate chambers at an acclimation temperature of 10°C. The temperature was lowered by 0.3°C/min to the target temperatures and held there for one minute (Kjellstrom, 2019). This cooling rate was used to ensure a balance between ecological relevance (cooling rates in nature) and the efficient use of time (Sinclair et al., 2015). The temperature was then raised again at the same rate to 10°C which was maintained for 24 hours to allow samples to recover before survival was assessed.

Mite mortality was assessed directly afterwards at room temperature. The glass vials were kept at the acclimation temperature of 10°C for an additional 24 hours and examined again to confirm mortality. Batch was not considered in the lethal temperature experiment because not all temperatures could be tested in each batch due to some batches arriving with a delay. Groups of 20 mites were tested in five replications for each temperature regime, resulting in 700 tested mites of each species.

3.3 Comparison of laboratory findings with Swedish winter conditions

To support interpretation of the laboratory results, monthly temperature data for 2010-2023 from the Swedish Meteorological and Hydrological Institute (SMHI) for seven localities ranging from North to South Sweden were obtained. The average, minimum and maximum temperatures for each month were calculated (Table 4.2). Laboratory lethal time and lethal temperature results were compared with regional temperature data to evaluate the potential winter survival of *A. limonicus* and *I. degenerans* ability across different parts of Sweden.

3.4 Experimental procedures for objective two: Characterization of bacterial communities associated with *A. limonicus* and *I. degenerans.*

The bacterial community analysis aimed to characterize bacterial communities associated with predatory mites *A. limonicus* and *I. degenerans* exposed to different temperatures. Mites used in this analysis were retrieved from the lethal temperature experiments. Only mites that had survived were sampled. Bacterial communities were characterized from 10°C, 0.75°C, -3.88°C and -13.13°C.

3.4.1 Preparation of mite samples and total DNA extraction

For DNA extraction mites from four temperatures namely 10°C, 0.75°C, -3.88°C and -13.13°C were selected as samples. Each mite was rinsed with 75% ethanol for 1 min (Liu et al., 2023). Samples of both *A. limonicus* and *I. degenerans* from each of the different temperature treatments were selected. For *A. limonicus*, ten mites each from 10° C (L1), 0.75°C (L2), -3.88°C (L3) and -13.13°C (L4) were separately pooled into 2 ml micro-centrifuge tubes. The procedure above was repeated for *I. degenerans* 10° C (D1), 0.75°C (D2), -3.88°C (D3) and -13.13°C (D4). The pooled samples in their separate micro-centrifuge were crushed using a clean sterilized plastic pestle and the DNA for each sample was extracted using a DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer’s standard protocols (Liu et al., 2023; Pekas et al., 2017). Pooling samples increases DNA yield from pooled samples as compared to DNA extracted from one mite. DNA samples quality were checked by electrophoresis on a 1% agarose gel and stored at -20°C awaiting sequencing. (Karanja et al., 2020).

3.5 Data analysis

Objective 1: Cold tolerance assays

Generalized linear models (GLMs) modeled the effects of temperature (5°C/0°C/-5°C), treatment (fed/unfed), and the interaction between them, on lethal time (number of days) of both species. The lethal time of *A. limonicus* was modelled with a Poisson error distribution (Brenard, 2020). A negative binomial distribution was used for *I. degenerans* due to overdispersion in the data (Brenard, 2020). Batch was included to control for batch-specific effects. When a significant effect was detected, post-hoc pairwise comparisons were performed using the package “emmeans” (Lenth, 2023)

To assess lethal temperature, binomial GLM were used to relate the temperature regimes (10°C/3.88°C/0.75°C/-3.88°C/-8.5°C/-13.13°C/-17.75°C) to mite mortality. Post-hoc pairwise comparisons of mite mortality between temperature regimes were performed. The two lowest temperatures (-13.13°C/-17.75°C) were excluded from the *I. degenerans* analysis because none of the mites survived.

To estimate LTime$_{50}$, mortality curves were constructed using binomial GLMs with a probit link function. In the models, mite mortality (response variable) related and time (log-transformed number of days; explanatory variable) were related for each combination of temperature and treatment (Karanja et al., 2020). The LTime$_{50}$ values were then estimated with the dose.p function in R package MASS (Brian et al., 2023). The number of days in the GLM relating time (number of days) to mortality were log transformed because the time to death relationship is rarely linear on the raw scale and log transformation normalizes the data.

For LTemp$_{50}$, binomial GLMMs with an observation-level random effect were used to relate mite mortality and temperature (converted to a continuous variable).

$LTemp_{50}$ was then estimated using an adaptation of the dose.p function for GLMM. All analyses were conducted using R software version 4.22 (R Development Core & Team, 2021)

Objective 2: Bacterial composition in predatory mites *A. limonicus* and *I. degenerans*

Sample sequencing was performed using the Pacbio Sequel Ile system. For initial quality filtering, SMRTlink (v11.0) was used to prepare high accurate reads (>QV40). Raw amplicon sequence data generated from Pacbio Sequel Ile system were processed using DADA2 pipeline (Callahan et al., 2016). Quality filtering was applied to remove reads containing ambiguous bases. Error models were learned from filtered data and amplicon sequence variants (ASVs) were inferred using DADA2 algorithm. Dereplication was performed at the sample level and chimeric sequences were identified and removed with consensus method. The resulting ASV table was constructed and used for downstream analyses. Taxonomic classification of ASVs was carried out against the SILVA v138.1 reference database with the dada2 naive Bayesian classifier. Final ASV table was used for downstream diversity and community composition analysis in phyloseq and vegan. The raw 16S rRNA sequences obtained from this study have been deposited at National Center for Biotechnology Information (NCBI) under accession number PRJNA1242186.

All analyses were conducted using R software version 4.22 (R Development Core & Team, 2021). Diversity of the bacterial communities in the samples was determined by computing Simpson and Shannon indices (Hill et al., 2003; Hughes et al., 2016). To determine whether diversity measures were significantly different between samples from the different two-way ANOVAs were used after validation of the normal distribution of the residuals (Diyes, 2024). Variation in bacterial taxonomic composition among samples was visualized using principal coordinates analyses (PCoA). PCoA was performed using the R package vegan (Karanja et al., 2020).

CHAPTER FOUR

RESULTS

4.1 Evaluation of cold tolerance of *A. limonicus* and *I. degenerans* through lethal time and lethal temperature experiments.

Table 4.1. Effects of temperature (5°C/0°C/-5°C), treatment (fed/unfed) and their interaction, on the number of days until mites died (lethal time), and the effect of temperature (10°C/3.88°C/0.75°C/-3.88°C/-8.5°C/-13.13°C/-17.75°C) on mite mortality rate

Response variable	Predictor	*I. degenerans*			*A. limonicus*		
		χ^2	Df	*P* value	χ^2	Df	*P* value
Number of days (Lethal time)	Treatment (fed/unfed)	8.61	1	**0.003**	9.84	1	**0.002**
	Temperature	86.98	2	**<0.001**	15.86	2	**<0.001**
	Batch	0.98	1	0.322	10.78	2	**0.005**
	Treatment (fed/unfed) × Temperature	9.02	2	**0.011**	1.53	2	0.467
Mortality rate (%) (Lethal temperature)	Temperature	200.63	4	**<0.001**	357.76	6	**<0.001**

4.1.1 Lethal time

The lethal time of *I. degenerans* declined steeply from 5°C to 0°C (Figure 4.1a). It was influenced by an interaction between temperature and treatment (Table 4.1), where unfed mites had a shorter lethal time than those that were fed , except at -5°C where the lethal time of both groups were almost zero and thus did not differ (Figure 4.1a).

The lethal time of *A. limonicus* did not change between 5°C and 0°C, but declined at -5°C (Table 1, Figure 4.1b). Unfed mites had a shorter lethal time than mites that were fed (Table 4.1, Figure 4.1c). There was no interaction between temperature and treatment (Table 4.1).

For both species, the time it took for 50% of the population to die was longer for fed than for unfed mites (Figure 4.2). As the variable "Day" was log-transformed, the corresponding LTime_{50} values in days are indicated in the plots. LTime_{50} of *I. degenerans* at 0°C and -5°C are not presented because the individuals only survived 1-2 days at those temperatures.

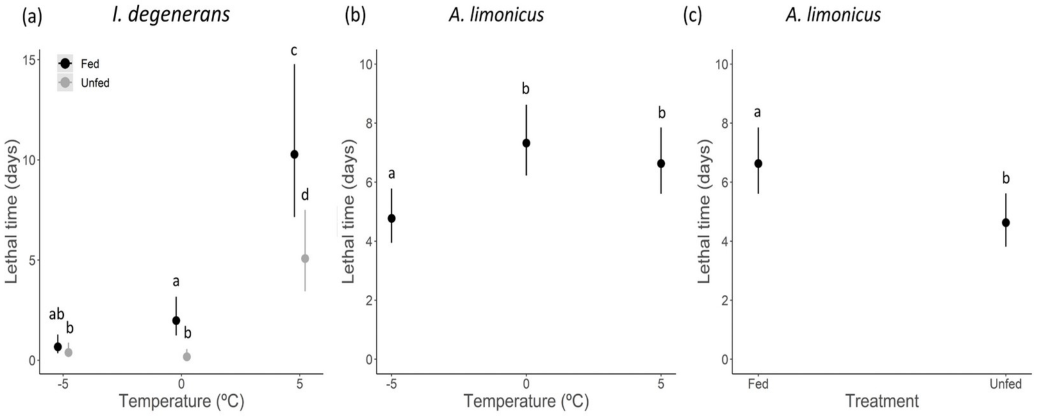

Figure 4.1. Effect of a) the interaction between temperature and treatment on lethal time of *I. degenerans* and of b) temperature and c) treatment on lethal time of *A. limonicus*. A shared letter indicates no significant differences ($P < 0.05$). The dots represent the model estimates and the lines are 95% confidence intervals.

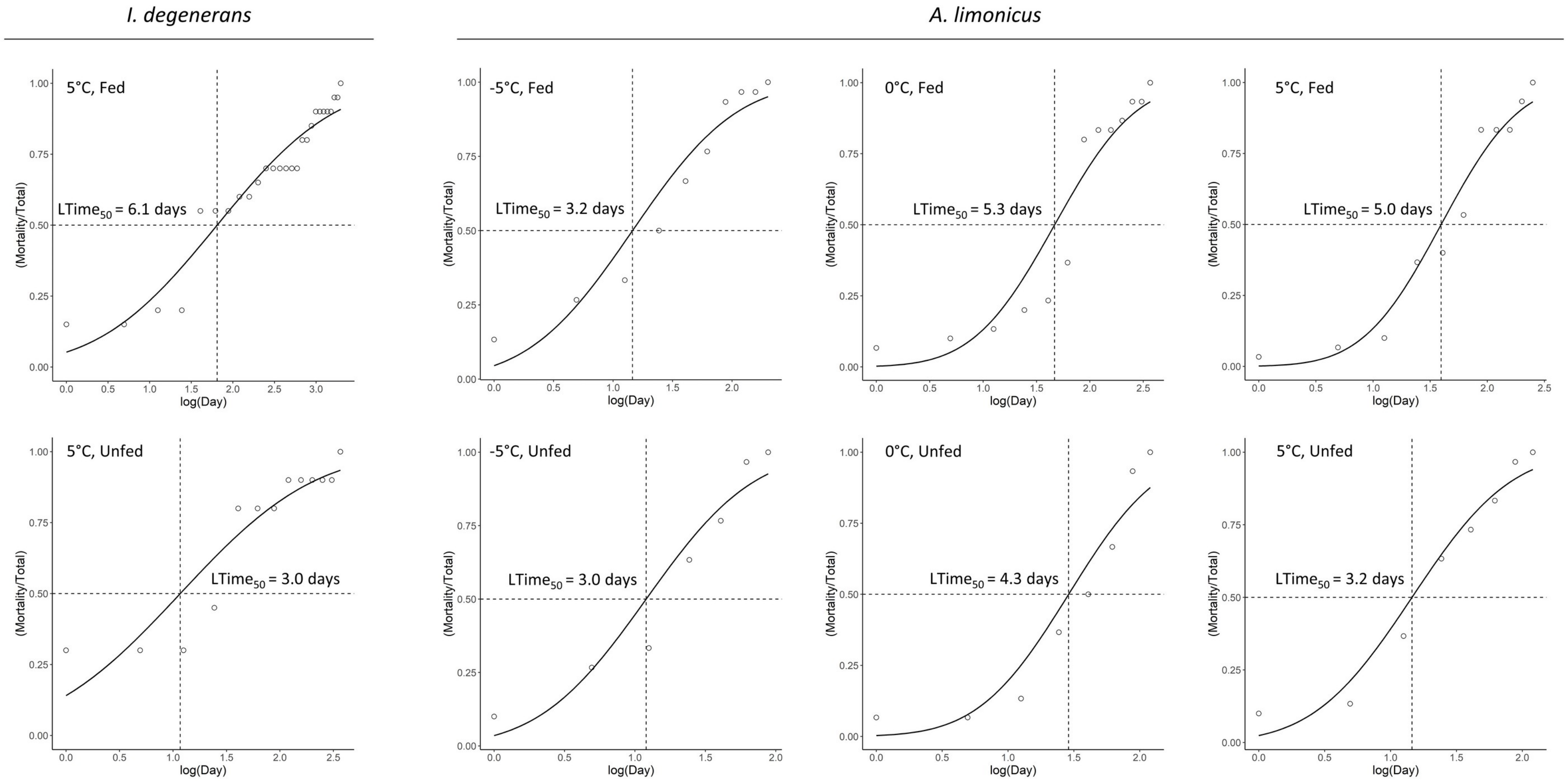

Figure 4.2. The number of days at which 50% of *I. degenerans* and *A. limonicus* individuals died (LTime$_{50}$) at -5°C, 0°C and 5°C.

4.1.2 Lethal temperature

The mortality rate of *I. degenerans* increased from 10°C to -8.5°C. It was less than 25% at 10°C, between 25-50% at 3.88°C, 0.75°C and -3.88°C, and increased to almost 100% at -8.5°C (Table 4.1, Figure 4.3). The mortality rate of *A. limonicus* increased from 10°C to -17.75°C, remaining low between 10°C and -8.5°C (<25%), then increasing sharply to approximately 60% at -13.13°C and to almost 100% at -17.75°C (Table 4.1, Figure 4.3). The temperature at which 50% of the sample population died was lower for *A. limonicus* (-9.8°C) than *I. degenerans* (-0.1°C) (Figure 4.4)

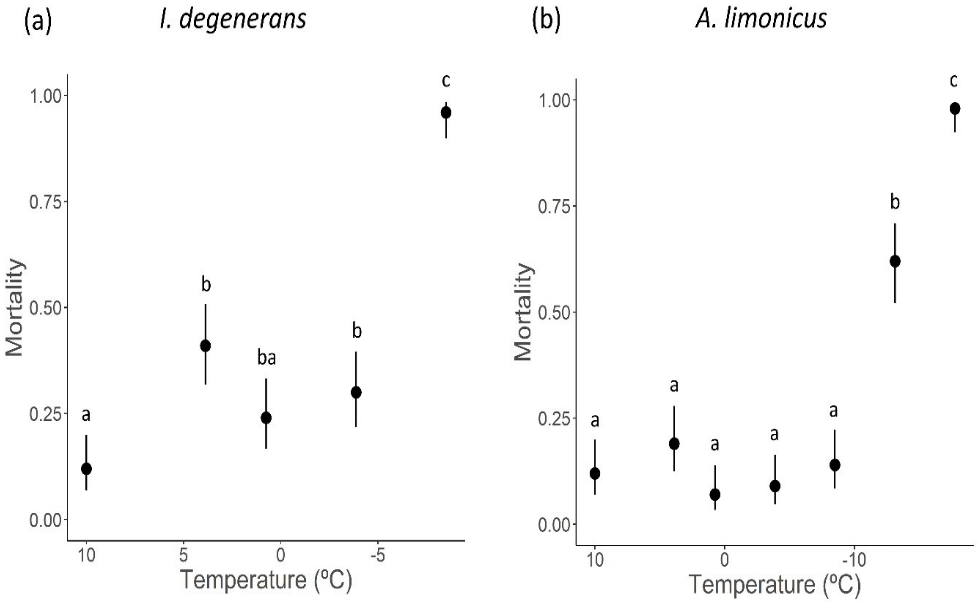

Figure 4.3. Effect of temperature on the mortality rate of a) *I. degenerans* and b) *A. limonicus*. A shared letter indicates no significant differences (P < 0.05). The dots represent the model estimates and the lines are 95% confidence intervals.

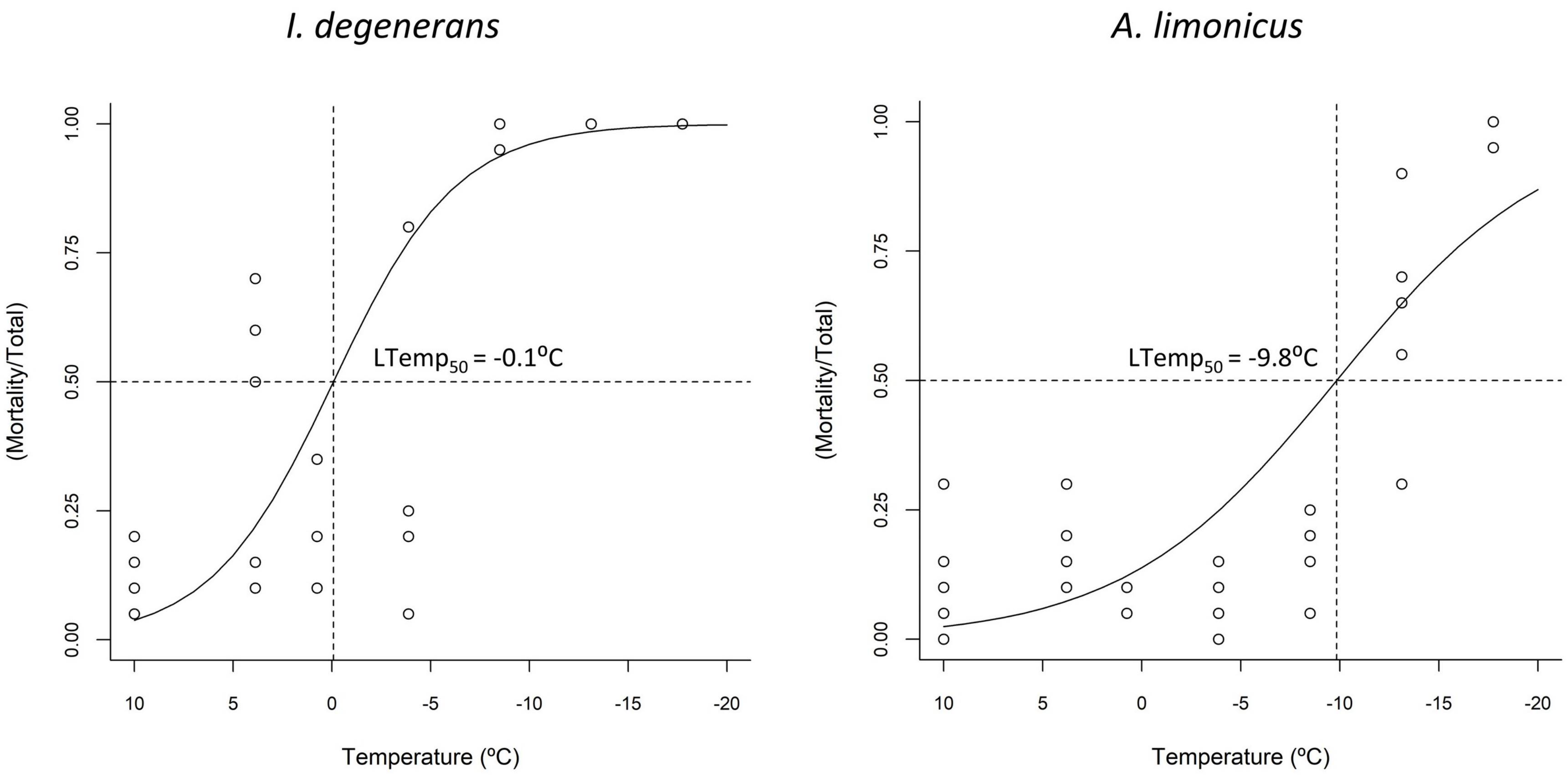

Figure 4.4. The temperature at which 50% of *I. degenerans* and *A. limonicus* mites died (LTemp$_{50}$).

4.1.3 Winter temperature patterns across Sweden

Table 4.2. Winter temperatures from November to March (spanning 2010-2023) for seven Swedish localities used to evaluate potential field survival of *A. limonicus* and *I. degenerans* through comparison with laboratory findings.

	November			December			January			February			March		
	Average	Max	Min	Average	Max	Min	Average	Max	Min	Average	Max	Min	Average	Max	Min
Site (N to S)															
Skellefteå	-1.1	2.9	-7.0	-4.9	-0.4	-13.2	-7.8	-1.2	-12.3	-6.9	-0.7	-14.1	-2.6	0.6	-8.3
Hudiksvall	1.5	4.0	-3.0	-2.2	1.8	-10.0	-4.1	2.1	-9.6	-3.2	0.9	-9.4	0.5	3.8	-4.2
Stockholm	4.3	6.9	0.3	0.7	4.1	-6.6	-0.6	4.0	-4.2	-0.2	2.9	-4.2	2.2	4.5	-2.2
Jönköping	3.4	5.9	-0.3	-0.1	3.3	-8.0	-1.8	3.2	-7.7	-1.5	1.9	-5.6	1.1	3.8	-4.4
Visby	5.3	7.5	2.8	2.0	4.7	-3.6	0.0	4.2	-4.9	0.0	3.1	-4.1	1.8	3.9	-3.0
Kalmar	5.1	7.2	2.5	1.4	4.8	-6.5	0.1	4.6	-5.0	0.2	3.6	-3.1	2.7	5.2	-2.3
Malmö	6.3	8.0	3.3	3.0	6.2	-4.4	1.4	5.3	-3.6	1.5	5.0	-1.6	3.8	5.8	-1.1

Data from SMHI from November to March demonstrates a clear north to south gradient in temperature. Northern Sweden (Skellefteå) is consistently colder across all months with average temperatures below freezing. January and February are the coldest months during winter in Sweden (average temperatures below -6°C). In Central Sweden (Stockholm) the average temperatures in the same months range from 4.3°C to -0.6°C, with minimum temperatures ranging from 0.3°C to -6.6°C and maximum temperatures ranging from 6.9°C to 2.9°C. In Southern Sweden

(Malmö), the average temperature varies between 6.3°C and 1.4°C, with minimum temperatures ranging from 3.3°C to -4.4°C and maximum temperatures ranging from 8.0°C to 5.0°C (https://www.smhi.se/en/weather) (Table 4.2)

4.2 Overview of the diversity of bacterial communities associated with predatory mites *A. limonicus* and *I. degenerans.*

Analyses of 16S rRNA amplicon sequences yielded 242,172 high quality sequences after demultiplexing, quality filtering, denoising and removal of potential chimeras. These were clustered into 561 ASVs. The bacterial taxa at each temperature were visualized using rarefaction curves. A steep slope that flattened to the right was observed in the rarefaction curves indicating that majority of the rarefaction curves approached saturation, which indicated that the sampling depth accurately characterized the bacterial diversity of the majority of these samples (Figure 4.5).

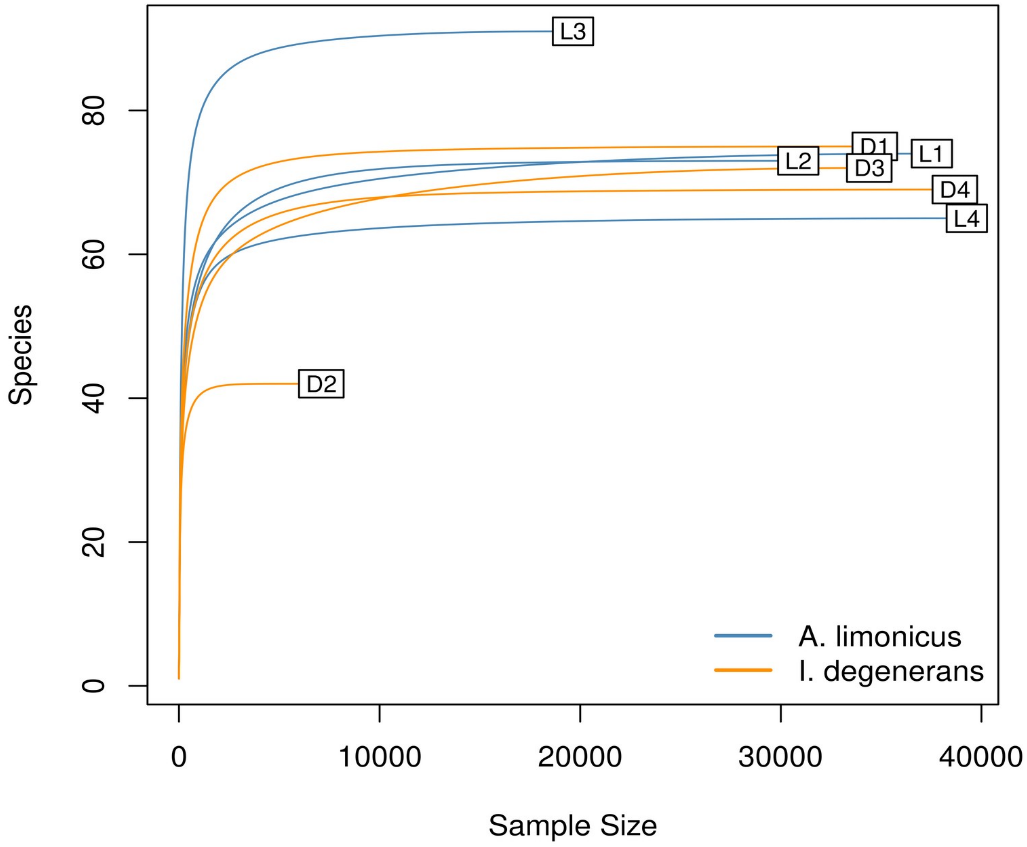

Figure 4.5. Rarefaction curves indicating level of sequence coverage within 16S rRNA for both *A. limonicus* and *I. degenerans* at different temperatures.

Overall most of the sequences from the bacterial communities associated with *A. limonicus* and *I. degenerans* belonged to phylum Firmicutes (Figure 4.6).

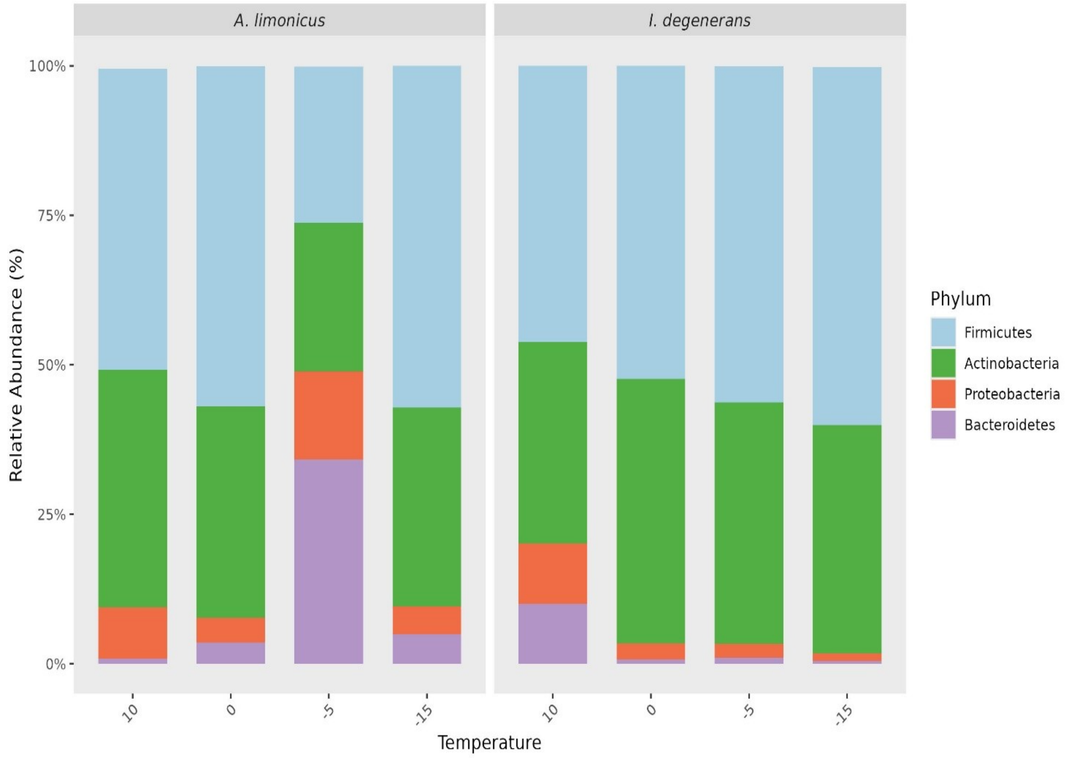

Figure 4.6. Relative abundance of the most dominant phyla observed at low and subzero temperatures for *A. limonicus* and *I. degenerans.*

Members of Bacteroidetes were relatively more abundant at -5°C within *A. limonicus.* The two mites shared a number of orders including Bacillales, Micrococcales, Lactobacillales, Enterobacteriales, Sphingobacteriales, Flavobacteriales, Corynebacteriales, Betaproteobacteriales, Rhizobiales and Pseudomonadales (Table 4.3). Order Bacillales was the most dominant for both mites. Micrococcales, Lactobacillales and Corynebacteriales were more abundant in *I. degenerans* than in *A. limonicus* (Figure 4.7, Table 4.3). *Staphylococcus, Brevibacterium, Weissella, Yersinia* and *Micromonospora,* were the top most dominant genera (Figure 4.8, Table 4.4*).*

Table 4.3. Most dominant orders for each Species (*A. limonicus* and *I. degenerans) and their percentages*

Source	**Dominant orders by source**	**Percentage (%)**
A. limonicus	Bacillales	39.76
	Micrococcales	24.41
	Lactobacillales	11.55
	Corynebacteriales	6.10
I. degenerans	Bacillales	38.90
	Micrococcales	28.04
	Lactobacillales	15.57
	Corynebacteriales	6.63

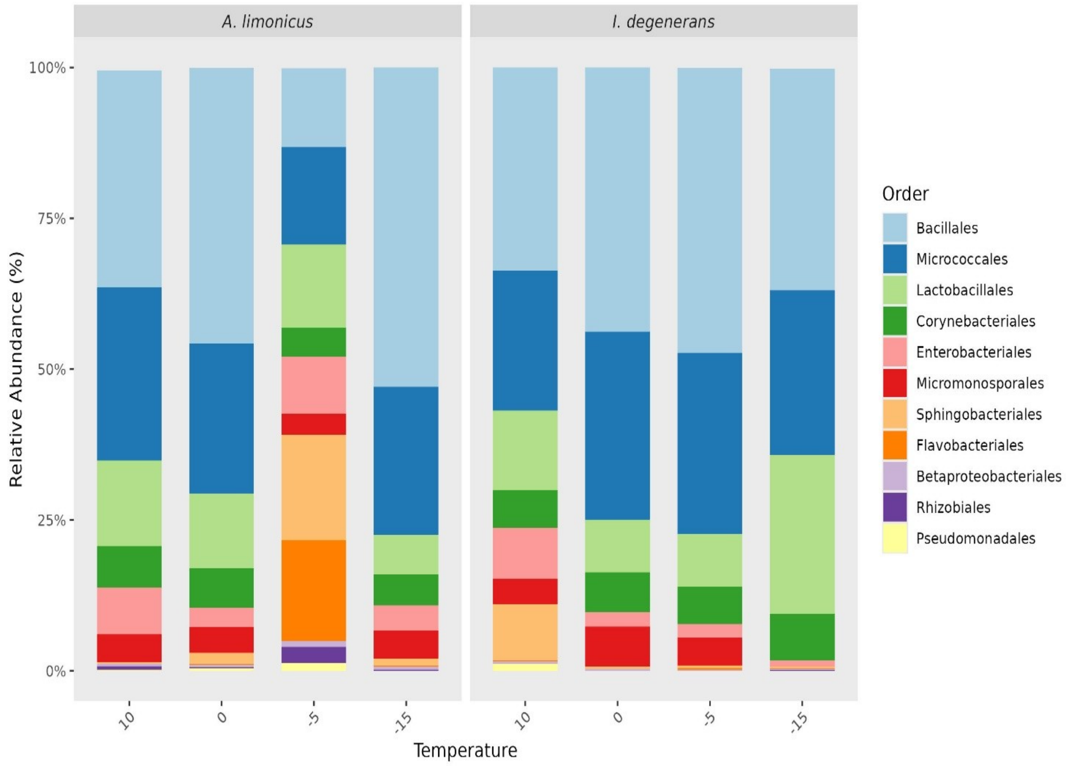

Figure 4.7. Relative abundance of the most dominant orders observed at low and subzero temperatures for *A. limonicus* and *I. degenerans*.

Table 4.4. Most dominant genera for each Species (*A. limonicus* and *I. degenerans) and their percentages*

Source	**Dominant genus by source**	**Percentage (%)**
A. limonicus	*Brevibacterium*	16.31
	Elizabethkingia	5.05
	Staphylococcus	59.49
	Weissella	6.74
I. degenerans	*Brevibacterium*	18.15
	Staphylococcus	53.16
	Weissella	17.22

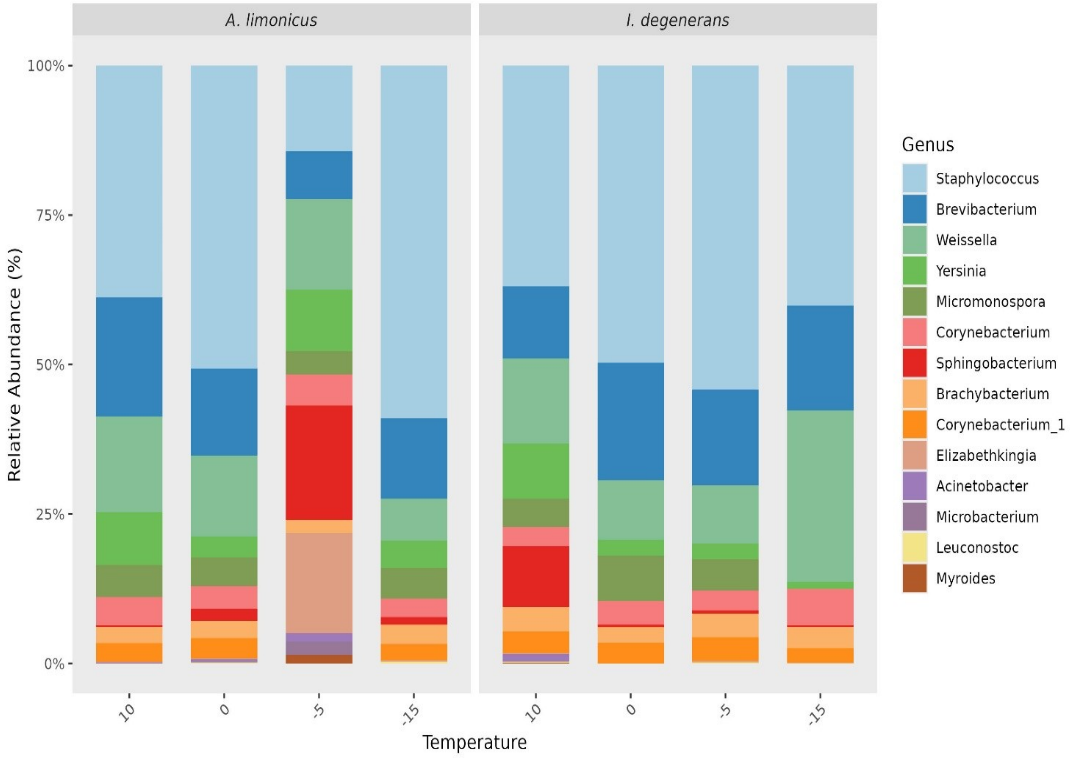

Figure 4.8. Relative abundance of the most dominant genera observed at low and subzero temperatures.

At 5°C, *A. limonicus* demonstrated the highest Shannon diversity (3.01) and Simpson index (0.92), indicating a richer and more even microbial community (Table 4.5). In contrast, *I. degenerans* at the same temperature had slightly lower diversity (Shannon: 2.50, Simpson: 0.87) (Table 4.5).

Table 4.5. Richness and diversity estimation of the bacteria communities collected at low and subzero temperatures in two predatory mites *A. limonicus* and *I. degenerans*

Sample_id	**Temperature (°C)**	**Source**	**High quality sequences**	**Simpson**	**Shannon**	**Observed ASVs**	**Goods coverage**
L1	10	*A. limonicus*	37528	0.90	2.66	74	1
L2	0	*A. limonicus*	30869	0.89	2.61	73	1
L3	-5	*A. limonicus*	19640	0.92	3.01	91	1
L4	-15	*A. limonicus*	39271	0.86	2.44	65	1
D1	10	*I. degenerans*	34684	0.91	2.79	75	1
D2	0	*I. degenerans*	7096	0.87	2.34	42	1
D3	-5	*I. degenerans*	34410	0.87	2.5	72	1
D4	-15	*I. degenerans*	38674	0.90	2.71	69	1

Alpha diversity was compared across temperature. Shannon and Simpson did not differ significantly across temperature treatments ($p>0.05$) (Table 4.6). PERMANOVA was used to assess whether temperature explained variation in community composition. While the model explained 36% variation, the result was not statistically significant ($p=0.85$) (Table 4.7). A dispersion test showed highly unequal within group variability ($p<001$). While PERMANOVA model explained 36% of the variation, the lack of statistical significance is likely attributable to the highly unequal dispersion among temperature group as revealed by the significant dispersion. So here the discussion of the findings from the PERMANOVA are explanatory.

Table 4.6. Anova summary showing the effect of temperature on the alpha diversity

Metric	**Factor**	**Df**	**Sum_Sq**	**F_value**	**P_value**
Shannon	Temperature	3	0.10	0.52	0.70
Simpson	Temperature	3	0.02	0.28	0.63

Table 4.7. Summary of PERMANOVA analysis showing the effect of temperature treatments on microbial community composition, including degrees of freedom (Df), proportion of variance (R^2), F-statistic (F) and p-value.

Factor	**Df**	**R2**	**F. value**	**P. value**
Treatment	3	0.36	0.74	0.85

To visualize variation in bacterial community structure on different samples, PCoA plot was used based on Bray-Curtis dissimilarity distances (Figure 4.9). Additionally, since the model showed non-significance, any visual clustering observed in the PCoA is likely driven by dispersion differences rather that true community differences. Nevertheless assemblages from different samples show some level of differentiation. For instance sample -15°C for *I. degenerans* and 10°C are dispersed in the ordination while taxa from temperatures 0 and 10°C in *A. limonicus* and those of temperatures -5 and 0°C grouped together on the ordination (Mbaka, S. (2025). Metagenomic profiling reveals temperature-stable bacterial symbionts in cold-tolerant predatory mites (phytoseiidae).

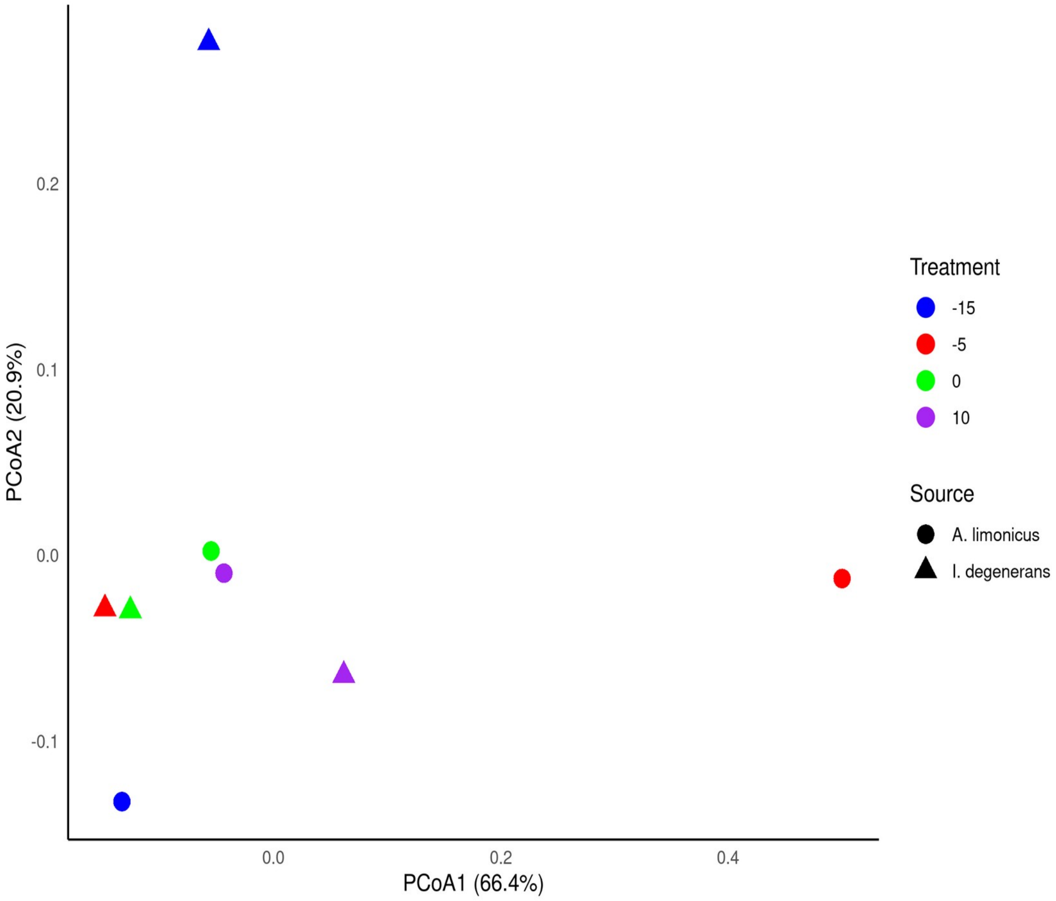

Figure 4.9. Principal coordinate analysis of microbial composition across mite species and temperature treatments.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

In this study, laboratory-based experiments of lethal time and lethal temperature were used to demonstrate the cold tolerance of predatory mites, *A. limonicus* and *I. degenerans*. High throughput sequencing of 16S rRNA amplicon were also used to demonstrate whether temperature changes had an effect on the microbial abundance in predatory mites.

It was observed that the predatory mite *A. limonicus* was more cold tolerant than *I. degenerans*. The lethal time of *A. limonicus* declined between 0°C and −5°C, whereas the lethal time of *I. degenerans* was reduced already between 5°C and 0°C indicating high mortality even at low temperatures above freezing point. Furthermore, in the lethal temperature experiments, temperatures as low as −17.75°C were required to kill the majority of *A. limonicus* individuals, while most *I. degenerans* individuals died already at −8.5°C. Mites from both species survived longer when they had access to food, and some feeding was, therefore apparent.

From November to March, temperatures vary significantly across Sweden, with northern localities such as Skellefteå experiencing the coldest temperatures (averages ranging from −1.1°C to −7.8°C) and central localities such as Stockholm ranging from 4.3°C to −0.6°C. The results from this study results suggest that *I. degenerans* will quickly die after exposure to temperatures north of Stockholm, as individuals declined rapidly at 0°C, and the majority died at −8.5°C after a short exposure to low and subzero temperatures. The $LTemp_{50}$ of *A. limonicus* was −9.8°C, where the majority of the mites died at −17.75°C and their lethal time did not differ between 5°C and 0°C, although it was reduced at −5°C. Despite exhibiting higher cold tolerance, *A. limonicus* appears unlikely to persist for long around Skellefteå but could potentially persist around Stockholm. In southern Sweden (Malmö), where average temperatures vary between 6.3°C and 1.4°C. It is likely that *I. degenerans* will not withstand prolonged exposure compared to *A. limonicus* as individuals of *I. degenerans* rapidly died between 5°C and 0°C. On the other hand, this study indicates that *A. limonicus* might persist for a longer time in southern Sweden (Table 1). Survival during the entire winter is uncertain though, given the scarcity of food sources and unlikely reproduction. As such, for a complete picture of the cold tolerance of *A. limonicus*, as well as that of *I. degenerans*, it is necessary to test survival in field conditions (Hatherly et al., 2004).

This study aligns with the previously described cold tolerance of *A. limonicus* (Dittmann et al., 2016) and suggests that the species is in the mid-range of cold tolerance among commercial phytoseiids. For example, LTime_{50} of *Neoseiulus californicus* and *Phytoseiulus longipes* at 5°C, used to assess survival in the UK, ranges from approximately 40 to 60 days and 12.3 to 34.9 days, respectively (Hart et al., 2002; Allen, 2009). In this study LTime_{50} values for *A. limonicus* at 5°C were considerably lower (3.2 and 5 days in the respective treatments). Furthermore, LTemp_{50} of *A. limonicus* (−9.8°C) is higher than that of *N. californicus* (approximately −14 to −18°C) and *P. longipes* (approximately −8.0 to −13.1°C). On the other hand, LTime_{50} of *A. limonicus* is longer than that of *Amblyseius swirskii* (1.1 to 3.9 days; Allen, 2009) and *Phytoseiulus macropilis* Banks (1.8 to 2.6 days; (Coombs & Bale, 2014) at 5°C. The LTemp_{50} values of *A. swirskii* (−1.0 to −4.7°C) and *P. macropilis* (−3.9 to −5.7°C) for all life stages and treatment groups are also higher compared to *A. limonicus*. This study noted that *I. degenerans* exhibited LTime_{50} values at 5°C (3.0 and 6.1 days) similar to those of *A. limonicus*, its much higher LTemp_{50} (−0.1°C) suggests it is in the lower range of cold tolerance.

Although this study recommend further experimentation to confirm these findings, it is notable that it is unlikely that *I. degenerans* will establish in northern Europe. *A. limonicus*, on the other hand, exhibits high cold tolerance and has been reported to be an aggressive predator of native predatory mites in Austria (Walzer et al., 2017). This raises concerns about the risks of its establishment as it may compete with native natural enemies and prevent more specialized predators from effectively controlling pest populations (Palevsky et al., 2013). Further research on the risks and side effects of the establishment of *A. limonicus* is thus needed.

A non-native species in a new environment possess a risks to native species (Simberloff & Stiling, 1996; de Clercq et al., 2011). In the past, researchers have reported examples of generalists predators which have caused damage to non-target organisms (Majerus et al., 2006). In some cases some non-native natural enemies readily consumes other natural enemies (Koch, 2003). Intraguild predation between different natural enemies may prevent the more specialized predator from controlling pests (Palevsky et al., 2013).

High throughput sequencing of 16S rRNA amplicon were used to to determine the bacterial composition of predatory mites *A. limonicus* and *I. degenerans* exposed to low or subzero temperatures. This study provides the first characterization of the bacterial communities of both mites.

Major phyla observed in predatory mites *A. limonicus* and *I. degenerans* were Firmicutes (dominant for both species), Actinobacteria, Bacteroidetes and Proteobacteria. Order Bacillales was the most dominant for both mites. Micrococcales, Lactobacillales and Corynebacteriales were more abundant in *I. degenerans* than in *A. limonicus*. *Staphylococcus, Brevibacterium, Weissella, Yersinia* and *Micromonospora,* were the top most dominant genera. Members of Bacteroidetes were relatively more abundant at -5°C within *A. limonicus.* Genus *Elizabethkingia* is only present in *A. limonicus* (Mbaka, S. (2025). Metagenomic profiling reveals temperature-stable bacterial symbionts in cold-tolerant predatory mites (phytoseiidae). Moreover, drawing parallels from studies in other arthropods for example the modulation of thermal tolerance by *Buchnera* in aphids supports the hypothesis, bacterial communities particularly studied psychrophiles may influence mites’ ability to withstand low temperatures but also their overall field performance as biological control agents. Although Shannon and Simpson diversity did not show statistically significant differences across temperature treatments ($p>0.05$, Table 4.6), closer inspection of the data suggests a meaningful biological pattern. At 5°C, *A. limonicus* demonstrated highest Shannon (3.01) and Simpson (0.92) diversity, indicating a richer and more even microbial community compared to *I. degenerans* at the same temperatures (Table 4.5). This suggests a potential interspecific difference in microbial stability under mild cold stress.

The PERMANOVA metric is highly sensitive to violations of the homogeneity of dispersion assumption due to a low sample size (Anderson, 2017). A subsequent dispersion test confirmed that within-group variability was highly unequal (p<0.001) meaning the groups were not similarly variable. This violation likely masked he detection of true differences between temperature groups in inflating the p-value. The high percentage of explained variance 36% is ecologically meaningful and suggests that temperature may indeed be an important driver of microbial community structure in these mites. The visual clustering observed in the PCoA (Figure 4.9) is likely influenced by these dispersion differences but still reflects meaningful variation in the data that is associated with the temperatures. Previous studies have noted the prevalence of these phyla in three predatory mites, with Proteobacteria being the predominant phylum in many cases (Liu et al., 2023; Sumner-Kalkun et al., 2023). In this study phylum Firmicutes was the most abundant (Mbaka, S. (2025). Metagenomic profiling reveals temperature-stable bacterial symbionts in cold-tolerant predatory mites (phytoseiidae).

5.2 Conclusions

1. The study demonstrates clear differences in cold tolerance between *A. limonicus* and *I. degenerans*. *A. limonicus* exhibits substantially higher resilience with lethal temperature experiments showing that 1-7.75°C is required to kill the majority of individuals, whereas *I. degenerans* experiences severe mortality at -8.5°C.
2. The lethal time experiments reveal that access to food prolongs survival in both species under cold conditions, highlighting that nutritional status is a critical determinant of winter survival. Despite this benefit, the overall survival of both species is strongly governed by temperature (Table 4.1).
3. When contextualized within the climatic conditions of Sweden, the results suggest that *I. degenerans* is unlikely to persist in regions with colder temperatures such as Skellefteå and Stockholm. However, *A. limonicus* could potentially persist in central and southern Sweden where average temperatures are not as cold as up north. Food scarcity during winter may limit overwintering success of *A. limonicus.*
4. An additional component of the study involved characterizing the bacterial communities through 16S rRNA sequencing. Both *A. limonicus* and *I. degenerans* are dominated by the phylum Firmicutes. Order Bacillales,

Flavobacteriales, Sphingobacteriales, Pseudomonadales and Rhizobiales profiled in this study have been documented psychrophilic bacteria, which may be associated in cold adaptation.

5. Notably, the genus *Elizabethkingia, Myroides,* orders Flavobacteriales and Rhizobiales were exclusively detected in *A. limonicus,* suggesting species-specific microbial associations that might have implications for cold tolerance or other physiological traits.

5.3 Recommendations

To ensure sustainable and responsible augmentative biological control practices, a comprehensive approach that integrates rigorous risk assessments is advocated before releasing non-native species to avoid environmental disasters witnessed in the past. Assessment of cold tolerance should be a major part of such risk assessments in temperate regions. Further field-based experiments are needed to assess the winter survival of both species under fluctuating environmental conditions and natural food availability, which will provide a more complete picture of their cold tolerance. The relatively low cold tolerance of *I. degenerans* positions it as a safer candidate for augmentative biological control in Sweden, while the high cold tolerance and aggressive predatory behavior of *A. limonicus* raise concerns regarding its establishment, potential competition with native predatory mites, and non-target effects. Finally, further research should investigate the role of the bacterial communities in conferring temperature resilience, as they will elucidate whether beneficial microbial associations contribute to enhanced cold tolerance.

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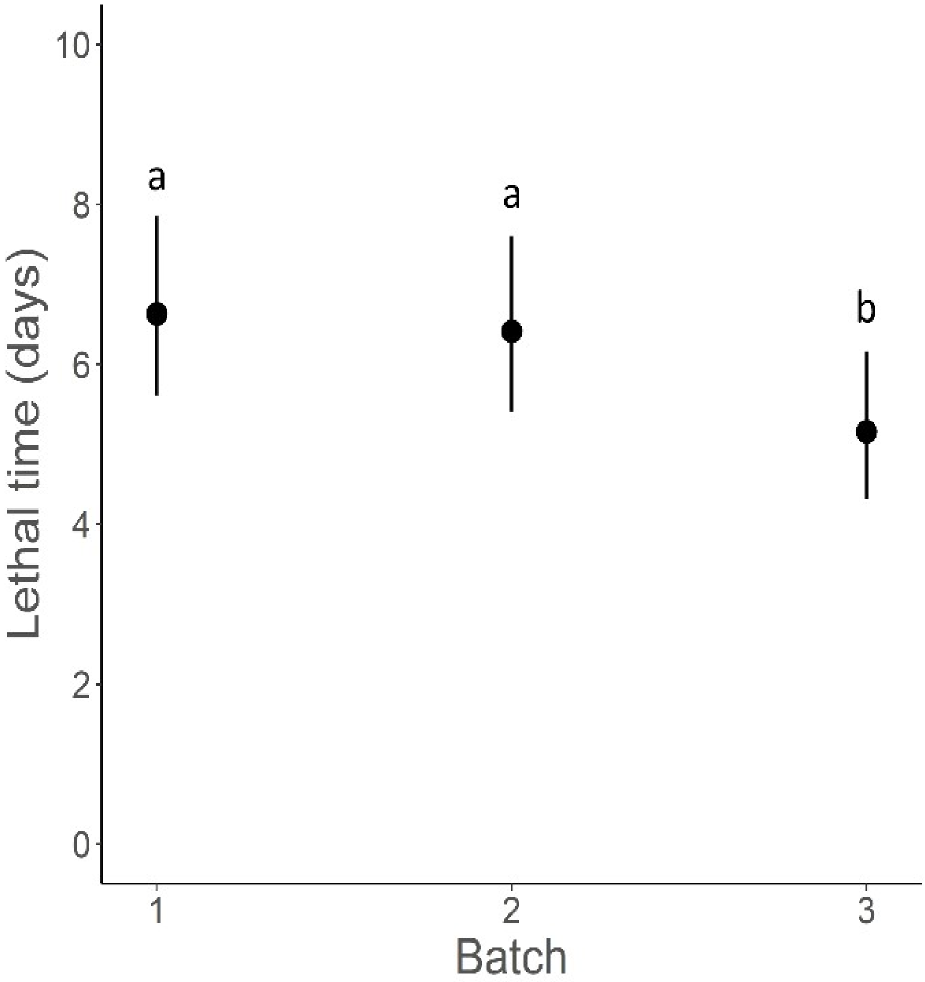
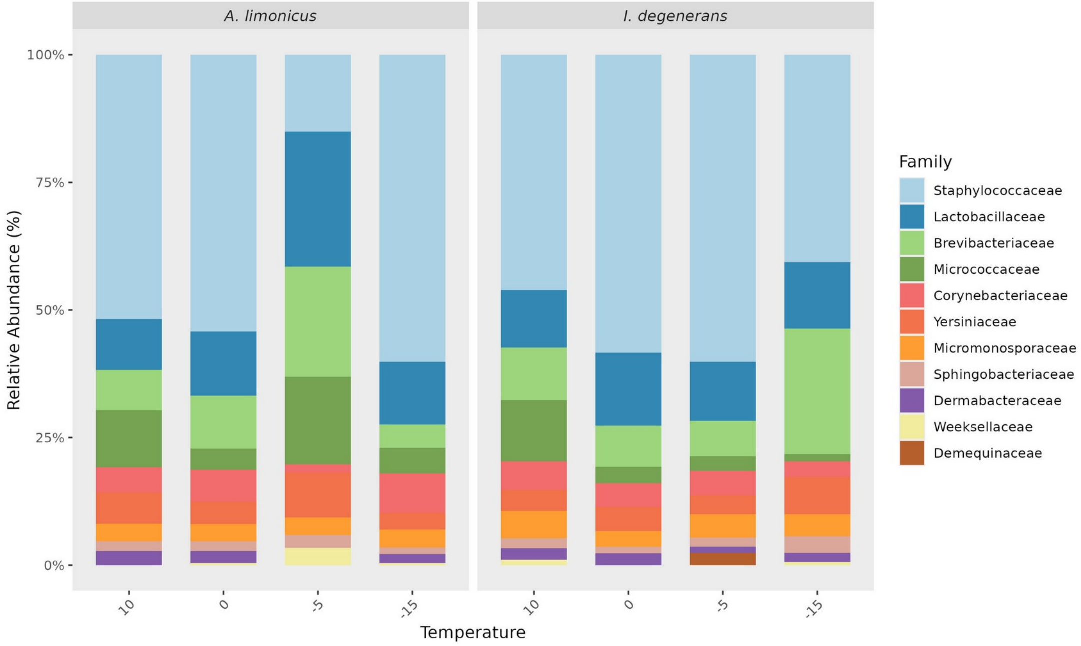
APPENDICES

Appendix 1. DNA extraction reagents

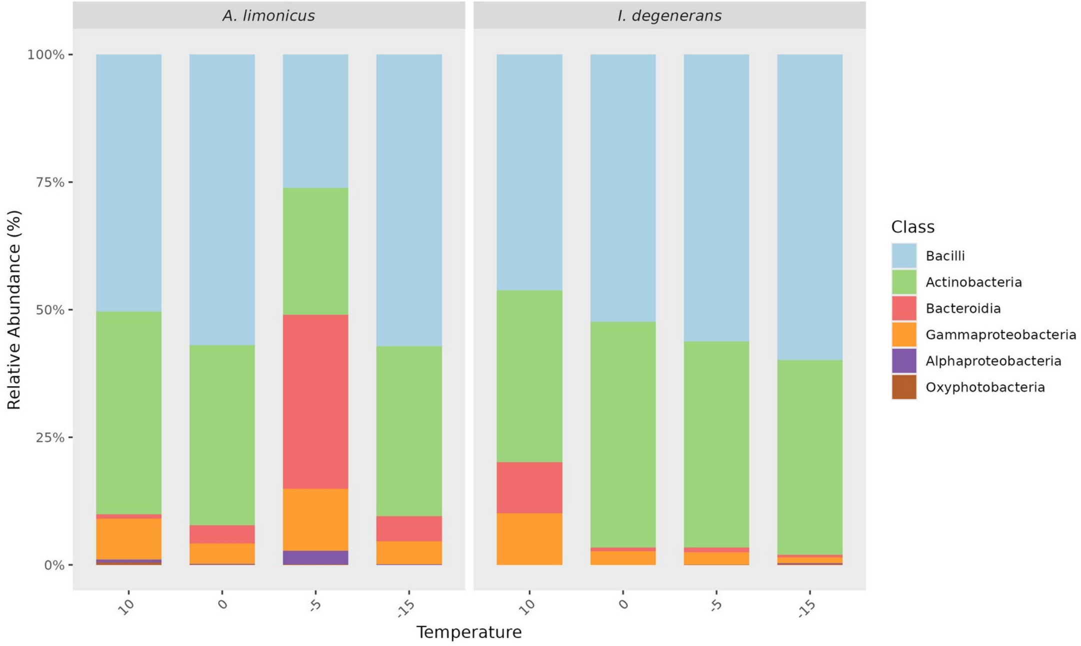
- Buffer ATL 180 µl
- Proteinase K µl
- Buffer AL 200 µl
- Ethanol 200 µl
- Buffer AW1 500 µl
- Buffer AW2 500 µl
- Buffer AE 100 µl



Appendix 2. Effect of batch on the mortality rate of *A. limonicus*. A shared letter indicates no significant differences ($P < 0.05$). The dots represent the model estimates and the lines are 95% confidence intervals.



Appendix 3. Relative abundance of bacterial families in predatory mites *A. limonicus and I. degenerans* across temperatures



Appendix 4. Relative abundance of bacterial classes in predatory mites *A. limonicus* and *I. degenerans* across temperatures.