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STUDIES ON THE MICROBIAL INTERACTIONS INVOLVING BENEFICIAL INSECTS AND ENTOMOPATHOGENS

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ABSTRACT

The relationships between different bacteria and beneficial insects (i.e., honeybee and pest predators) and their interactions were investigated to assess their safety and to evaluate their application potential in beekeeping and agroecosystem management. The secretome of food-borne lactic acid bacteria (LAB) and of a honeybee-borne *Brevibacillus laterosporus* strain, showed significant inhibitory properties against the honeybee pathogen *Paenibacillus larvae*. The antagonistic action of LABs was found to be related to the production of organic acids and antimicrobial peptides. Gene expression analyses on highly active *Lactobacillus plantarum* strains revealed the activation of plantaricin related genes. LC-MS/MS analyses of a protein precipitate from *B. laterosporus* culture supernatant led to the identification of several putative antimicrobials, among which the bacteriocin laterosporulin was found to be the major component. Laboratory bioassays conducted with an entomopathogenic strain of *Brevibacillus laterosporus* did not cause significant lethal effects on larval instars and adults of the green lacewing *Chrysoperla agilis*. The effects of direct bacterial feeding observed on chrysopid egg hatching, development, fecundity and longevity were not significant. Slight sub-lethal effects were instead detected on lacewing larvae fed with *B. laterosporus* treated mealworm beetles (tritrophic interaction).

CHAPTER I

1. INTRODUCTION

1.1 Relationships between bacteria and insects

Bacteria are the first form of life on Earth and are found almost everywhere, insects are the most successful animal class, because of the number of species and abundance of individuals for each species. Part of the success of insects lies in the co-evolution they have established over time with bacteria. They live as endosymbionts (within insects) or ectosymbionts (outside their body), generally in intimate association, providing enzymes, essential amino acids and antibiotics (Douglas, 2009). These associations are defined as symbiotic, in a positive or negative sense for the host. Regardless of the type of qualitative interaction, the associations begin with the entry of the microorganism into the host, facing its immune defences, followed by internal colonization and spread throughout the whole body and, eventually, the outside environment (Richards and Brooks, 1958). Depending on the specific type of host-parasite interaction, different types of associations between organisms can be distinguished. The term symbiosis was first introduced in 1879 by Anton de Bary and Simon Schwendener and defined as "any association between different organisms, provided they are in persistent contact" (Hoffmeister and Martin, 2003). This term incorporates the concepts of:

- commensalism: association between two species in which one benefits from interaction without damaging or benefiting the other;
- mutualism: an association in which both partners benefit from their interaction;
- parasitism: an association in which only one of the two partners benefits at the expense of the other.

Commensalism could be considered as mutualism like the case of many intestinal bacteria, but mutualism itself is a clearer association, in which the microorganism and the insect benefit each other. For this reason, the terms symbiosis and mutualism are often interchangeable. Pathogenetic interactions, from the point of view of the microorganism, are equally characterized by a high degree of specialization and require intimate contact with the host, but are more labile in definition, since the same microorganism may behave differently depending on the host and the surrounding environment, moving from being beneficial to harmful (Sachs et al., 2011). These interactions introduce the concept of balance between bacteria and the host insect, in an unceasing coevolutive race. To simplify the discussion on the various relationships that can be established between a host and a microorganism, the term symbiosis in *sensu stricto* will be divided into two types of extreme relationships, mutualism and pathogenicity, in a semantic categorization, but related to the cases studied.

1.1.1 Mutualistic bacteria

Symbionts can be classified into two groups: primary obligatory endosymbionts and optional secondary symbionts. Primary endosymbionts are obligatory microorganisms that reside in specialised cells and give essential benefits to the life of the host. This type of symbiosis concerns intracellular bacteria, located in specialized cells called bacteriocytes, organized into bodies called bacteriomes. Bacterial transmission occurs vertically, from the mother to the offspring, and since this cellular isolation exists, the evolution of bacteria is more complex, and this association is dominated by a close co-evolution between the host and the bacterium itself. Primary symbionts belong to the phyla of Proteobacteria and Bacteroidetes (Werren and O'Neill, 1997). These bacteria, often implied in the production of numerous nutrients essential for the insect life, are called obligatory symbionts because the insect depends completely on their presence to survive, not having the ability to produce these compounds independently. In particular, the genome of

obligatory symbionts is often small, has low recombination rates and a small number of factors regulating gene expression (Gosalbes et al., 2010). Secondary symbionts are microorganisms that are not strictly necessary for host survival. These symbionts are optional, often extracellular and transmission takes place mainly horizontally. It is more difficult to categorise interactions with secondary symbionts and the role of the microorganism in the host is not always clear. It is generally assumed that primary symbionts have evolved from secondary symbionts and that a secondary symbiont can potentially replace a primary one during the host's evolutionary history. For instance, acetic bacteria, from the Acetobacteraceae family, are a group of important secondary symbionts, studied and associated with various orders of Insects, including Diptera and Hymenoptera (Crotti et al., 2010).

These are extracellular bacteria commonly found in plants, flowers and fruits, as well as being associated with the intestinal tract of insects following a sugary diet. The intestine of these insects is composed of acidic pH regions, aerobic environment and many sugars, which acetic bacteria are able to oxidize into acetic acid. For this reason, such environment represents an optimal niche for these microorganisms. Major insect intestinal symbiotic acetic bacteria belong to the genera *Gluconobacter*, *Gluconoacetobacter* and *Acetobacter*, and have been identified in insects such as *Apis mellifera* L. and *Drosophila* spp (Moran, 2015). The role of these microorganisms was discovered in particular in a study on *Drosophila melanogaster* Meigen, in which they acted as modulators of intestinal homeostasis, in a mechanism based on interaction with the innate immune system of the fruit fly (Ryu et al., 2008), suggesting the importance of symbiotic acetic bacteria in immune stimulation and thus in maintaining the health status of insects (Crotti et al., 2011).

1.1.2 Entomopathogenic bacteria

Entomopathogens are bacteria that have developed different ways to overcome the insect's immune system and cause disease by specifically evolved mechanisms. There are different classes of

entomopathogens, but among all, bacteria are the most significant agents, and include several sporogenic species, even if there are also significant non-spore formers (Ruiu, 2015). The two genera *Clostridium* and *Bacillus* have historically represented the main group and the most important for application purposes. These are Gram-positive bacteria that can reproduce by sporulation. In particular, the genus *Bacillus* include the species with the greatest potential for application in the biological control of insects. *Bacillus thuringiensis* (*Bt*) acts as an insecticide against larvae of Lepidoptera, Diptera, and Coleoptera (Palma et al., 2014), producing protein crystals that, once ingested by sensitive insect larvae, are dissolved in the gut environment and their protoxins are activated. These crystal toxins, also known as Cry proteins, act against insects binding to specific receptors of the intestinal epithelium of larvae, causing an alteration of membrane permeability and consequent disruption and cell lysis, which will lead insect to paralysis and death (Ruiu, 2015). Different strains of *Bt* produce different Cry toxins but may also produce other toxins such as thermolabile α-hexotoxin, thermostable β-hexotoxin or Turingiensin, Insecticide Vegetative Proteins (VIP). Other entomopathogenic bacteria include species in the genus *Streptomyces* and *Saccharopolyspora*, belonging to Actinobacteria, whose metabolites (i.e., avermectins and spinosad, respectively) show significant insecticidal potential. Another entomopathogenic bacterium is *Brevibacillus laterosporus*, an aerobic spore-former featured by a typical canoe-shaped parasporal body at one side of the spore. The insecticidal toxins produced by different strains belonging to this species are active against Lepidopteran (De Oliveira et al., 2004), Coleopteran and Dipteran insect larvae (mosquitoes and flies) (Ruiu, 2013), in addition to other invertebrates like Nematodes and Molluscs (Singer, 1996). This is a very interesting example to define the categorization of bacterial pathogenicity, because *B. laterosporus* strains toxic to some insect species are not active against others, with special regard to non-targets. This bacterium is also mentioned among the natural honeybee residents (Marche et al., 2016). Another entomopathogenic bacterium is *Paenibacillus larvae*, the etiological agent of the American Foulbrood (AFB), a Gram-

positive, optional anaerobic and sporogenic species that, as its name suggests, affects larval bee stages, representing a serious threat to the whole colony (Genersh et al., 2006). Spores degrade larval tissues through the action of some enzymes, such as chitinase and other proteolytic enzymes the bacterium produces (Grady et al., 2016).

1.2. Potential applications with insect-related bacteria and their implications

The management of the entomofauna for the defence of plants and agri-food production can be carried out taking into account the complexity of the insect system, where the community of organisms that interact in symbiosis constitutes a super-individual, defined holobiont (Rosemberg and Zilber-Rosemberg, 2011). This term is used to redefine the living organism in the light of living with its own microbiota and modulates individual subjectivity in a microbial context of interaction on various levels. The diversity of symbiosis between microorganisms and insects and the impact they have on the biology of the host open new scenarios that see insects as a complex system, governed by the interactions between the organisms that make up the holobiont. In the protection against pathogens and harmful insects, symbiotic microorganism properties can be exploited, according to the strategy called Symbiosis Control (SC) (Crotti et al., 2012).

This control method may directly target harmful insects, for example through the production of symbiont molecules with antagonistic activity; or may act against pathogens carried by insect vectors, exploiting the competition between symbionts and these pathogens in the colonization of the host. In addition, this control method could take advantage of the reduction of the insect's ability to transmit the pathogen caused by the symbiont (Alma et al., 2010).

1.2.1 Use of bacteria for insect health. The case of honey bees

In recent decades there has been a progressive decline of pollinators, which heavily involves all bee species (Potts et al., 2010). The phenomenon of colony loss on a planetary scale is due to the

combined effect of a plurality of causes (Roulston and Goodell, 2011). In the last century, there has been a marked decrease in bee activity (Ellis et al., 2010). The intestines of adult bees are dominated by nine groups of bacterial species that include between 95 % and 99.9 % of the bacteria present in almost all individuals at different biological stages. The two omnipresent Gram-negative species are *Snodgrassella alvi* and *Gilliamella apicola*, which are included in the phylum Proteobacteria. Among the Gram-positive bacteria, two groups of species in the phylum Firmicutes are also omnipresent and abundant. These have been referred to as *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5. Although often less abundant, the species *Bifidobacterium asteroides*, which belongs to the phylum Actinobacteria, is also found in most adult worker bees. These five groups of bacterial species form the core microbiota of honey bee intestine (Moran, 2015).

Less numerous, and even less widespread, are some species of the phylum Proteobacteria: *Frischella perrara, Bartonella apis, Parasaccharibacter apium* and a group of species related to *Gluconobacter* called Alpha2.1 (Mohr and Tebbe, 2006). Among honeybee beneficial bacteria, lactic and acetic probiotic species have recently been taken into consideration, since they are believed to play a significant role in immunomodulation, keeping the intestinal microflora healthy. Several species in the genus *Lactobacillus* have been identified in the intestine of *Apis mellifera*, being characterized by high sugar content and acid pH representing an ideal ecological niche for these microorganisms. Among acetic bacteria, different members of the genera *Gluconobacter, Acetobacter, Gluconacetobacter* have also been recognized, and *Saccharibacter* species have been reported as bee symbionts (Mohr and Tebbe, 2007). Probiotics are microorganisms or their components that benefit the host by increasing the beneficial effects of indigenous microflora (Fuller, 1989). Against serious bacterial diseases, recent studies have shown that the use of probiotic bacteria is a promising strategy for their containment. Numerous metabolic products produced by lactic acid bacteria (LABs) have antimicrobial effects, deriving from their metabolites including organic acids, fatty acids, hydrogen peroxide and diacetyl (Ouwehand, 1998). Increasing

attention has been paid to the ability of LABs to act as antagonists and to produce specific antibiotic substances, like bacteriocins, inhibiting the growth of disease agents in the genera *Listeria, Paenibacillus, Clostridium, Staphylococcus, Bacillus*, and *Enterococcus* (Lv et al., 2018; Pei et al., 2018; Wang et al., 2019). Bacteriocins are protein metabolites with antimicrobial activity excreted by different groups of bacteria (Gálvez et al. 2007). Besides insect field of research, bacteriocins produced by LABs are the subject of intensive studies focused on their antibacterial activity against food-borne bacteria. Due to their widespread presence in the environment, the chance to find new LAB strains of different origin, but active against insect targets is gaining interest and is supporting new screening efforts. Among the most interesting species, *Lactobacillus plantarum*, frequently found in food, but also in the body of insects, is characterized by the production of several antibiotic compounds and express a significant antagonistic potential against various microbials (Arena et al., 2016).

1.2.2 Use of bacteria for pest management

Protections of crops, farm animals and humans against insect pests have historically required an intense use of insecticidal substances, mostly chemicals, normally formulated in commercial products with co-formulants and coadjuvant to improve their efficacy (Green, 2000). However, do to the risks associated with the use of chemical compounds such as environmental pollution, contamination of food and water, intoxication of operators, the development of insect resistance, health effects on non-target organisms, including insect predators and pollinators, the use of low-impact insecticidal products is strongly encouraged. Such eco-sustainable approach has led to the implementation of integrated pest management (IPM) programs including the combined use of modern chemical products and biocontrol strategies (Ruiu, 2018). The latter include the use of biological control agents like macro-organisms (i.e., predators and parasitoids) and microorganisms, like Bacteria, Fungi, Virus, Nematodes (Tanada and Kaya, 2012). Today, different

entomopathogenic bacteria and their metabolites are the active substance of a variety of phytosanitary products and biocides used in agriculture against crop pests, and in the animal farming sector to contain the population of different parasites, including flies and mosquitoes. The effectiveness of entomopathogenic bacteria is often associated with a proper application of commercial formulations in the field, that should ensure adequate coverage of target crops. This has led to the development of a specialized industry of bacterial processing and formulation with the aim of maximizing shelf-life, improve dispersion and adhesion, reduce spray drift and especially increase effectiveness (Lacey et al., 2001).

Among bacteria, *Bacillus thuringiensis* strains represent the most studied and used active substances in field applications. However, due to their very specific mode of action and the limited range of targets associated with a strain, their employment is restricted to niche market segments (Glare et al., 2012). For these reasons, continuous efforts of industry and academia are focused on the discovery and development of new species and strains with a wider range of action and an adequate efficacy. On the other hand, the introduction of new microbial products in the market requires their safety for the environment and non-target organisms. Accordingly, pre-market registration procedures involve risk-assessment trials on beneficial insects like bees, ladybirds and chrysopids (Ruiu, 2018). The side-effects possibly deriving from the application of a bioinsecticide are not limited to the acute toxicity they may have against some non-target, but significant effects might be related to the ingestion of sub-lethal doses causing deleterious effects on immature development or reduced longevity and reproductive performance (Cloyd, 2012).

For these reasons, specific studies investigating the possible action of entomopathogenic bacteria and their metabolites on these non-target species are assuming increasing importance.

1.3 Thesis objectives and achievements

The main thesis objective was to study the relationships between different bacteria and beneficial insects with special regard to honeybees and pest predators, and their possible utility.

More in details, the following three objectives were pursued:

- screen and characterize the potential of food-borne lactic acid bacteria (LAB) against the honeybee pathogen *Paenibacillus larvae*
- study and characterize the antagonistic activity of a honeybee-borne strain of *B. laterosporus* against the honeybee pathogen *Paenibacillus larvae*
- assess the safety of an entomopathogenic strain of *B. laterosporus* with potential for pest management for the non-target insect predator *Chrysoperla agilis*.

These studies led to the production of the following co-authored manuscripts already published, submitted, or ready for submission to peer-reviewed international scientific journals:

1) Anna Marta Lazzeri, Nicoletta P. Mangia, Maria Elena Mura, Ignazio Floris, Alberto Satta, Luca Ruiu, 2020. Potential of novel food-borne lactic acid bacteria (LAB) isolates against the honeybee pathogen *Paenibacillus larvae*. [Ready for submission]
2) Maria Giovanna Marche, Alberto Satta, Ignazio Floris, Anna Marta Lazzeri, Luca Ruiu, 2019. Inhibition of *Paenibacillus larvae* by an extracellular protein fraction from a honeybee-borne *Brevibacillus laterosporus* strain. [Published on Microbiological Research (2019), Vol. 227, 126303]
3) Luca Ruiu, Anna Marta Lazzeri, Maria Tiziana Nuvoli, Ignazio Floris, Alberto Satta, 2019. Safety evaluation of the entomopathogenic bacterium *Brevibacillus laterosporus* for the green lacewing *Chrysoperla agilis* (Neuroptera: *Chrysopidae*). [Published on Journal of Invertebrate Pathology (2020), Vol. 169, 107281].

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CHAPTER II

Potential of novel food-borne lactic acid bacteria (LAB) isolates against the honeybee pathogen *Paenibacillus larvae*

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(Manuscript ready for submission to a scientific journal)

2.1 Abstract

An *in vitro* study was conducted to screen for the inhibitory potential of lactic acid bacteria (LAB), isolated from fermented food matrices, against the honeybee American Foulbrood agent *Paenibacillus larvae*. Various strains of *Lactobacillus plantarum* showed significant antagonism associated with their cell-free culture supernatant (CFS). The characterization of this inhibition was based on assays and analyses involving different fraction and extracts. A significant effect was associated with media acidification resulting from LAB growth. However, this antimicrobial activity was found to be heat-stable and specific experiments suggested the implication of small antimicrobial peptides and antibiotics. Accordingly, a possible role of plantaricins released by *L. plantarum* in the culture supernatant was confirmed by the expression of different plantaricin-related genes. The results obtained in this study support further investigations on LAB strains as biological control agents for application in the apiary to preserve and improve honeybee health.

Key words: Insect; Antibiotic; Antimicrobial; Plantaricin; *Lactobacillus plantarum*.

2.2 Introduction

The American Foulbrood (AFB) etiological agent, *Paenibacillus larvae* (Firmicutes), is a deleterious bacterial species threatening the hive and determining significant honeybee colony losses worldwide (Genersch, 2010). The spores of this microorganism, normally brought and spread in the colony by workers, once ingested by young larvae with food, germinate in the gut environment where the degradative action of replicating vegetative cells, associated with the release of enzymes and virulence factors, leads to the spread of the infection through the gut barrier to the whole insect body (Garcia-Gonzalez et al., 2014). The death of septicemic larvae will cause further spread of the disease in the colony (Ebeling et al., 2016). Despite the susceptibility of this microorganism to a variety of antibiotics, due to the restrictions associated with their employment in the hive context, and the lack of other active substances compatible with the colony ecosystem, the management of this disease is still a challenge (Evans, 2003; Murray et al., 2007).

Studies on the social and individual honeybee mechanisms of defense against pathogens are providing new insights on their possible exploitation to protect colony health (Marche et al., 2019a). In addition to the innate immune response and specific hygienic behaviors of honeybees (Chan et al., 2009), the interest in the apparent immune-related role of their core bacterial community, mostly residing in the gut, is significantly increasing (Engel and Moran, 2013; Marche et al., 2019b). Among these microbials, a remarkable role is played by lactic acid bacteria (LAB), whose activity in the bee gut appears to be significantly related to maintaining good bee health conditions, reducing pathogen prevalence (Royan, 2019). The antimicrobial properties of this group of bacteria are in relation with their ability to produce organic acids, reactive oxygen species, and antimicrobial peptides (Cintas et al., 2001). Due to this antagonistic potential against a wide range of target microbes, LABs are exploited for application in different sectors, including food preservation (Cleveland et al., 2001) and medicine (Gorbach, 1990). The susceptibility of *P. larvae* to LAB

species isolated from the honeybee body was highlighted, supporting their employment for colony health preservation (Forsgren et al., 2010). On the other hand, growth inhibition of *P. larvae* may result also from the action of LABs of different origin, including isolates form fermented feeds and foods (Yoshiyama et al., 2013). Accordingly, alongside the numerous studies aimed at discovering new LAB species and strains with potential of industrial exploitation in different sectors, new actives against *P. larvae* are expected to be identified within bacterial colonies associated with niche food ecosystems (Parvez et al., 2006).

This study investigated the inhibitory potential of different LABs isolated from fermented food matrices (i.e., sausage and cheese niche products) against *P. larvae*, in order to identify new isolates with application potential. Specific experiments were conducted with the most promising isolates with the aim of gaining preliminary information on their mechanism of action. Observations on a protein fraction obtained from the culture supernatant and the expression of plantaricin-related genes were also conducted.

2.3 Materials and methods

2.3.1 Bacterial strains and preparations

Lactic acid bacteria (LAB) strains used in this study are maintained in the collection of the Microbiology section of the Department of Agriculture (University of Sassari, Italy) and were isolated form a variety of fermented food matrices, including sheep sausage, pork sausage, Casizolu cheese, and Fiore Sardo cheese (Mangia et al., 2013; Mangia et al., 2016; Mangia et al., 2019). These included different strains of *Lactobacillus plantarum* (n = 52), *L. brevis* (n = 9), *L. paracasei* (n = 4), *L. curvatus* (n = 1), and *L. fermentum* (n = 1). LABs were routinely grown in MRS (Man Rogosa Sharpe) broth for 24 h at 30 °C to collect the cell free culture supernatant (CFS) by

centrifugation (14,000 *x g* for 10 min, at 4 °C) to be used in bioassays, after being filter-sterilized using a 0.2 µm pore size filter *(Minisart®).*

Reference strain DMS 7030 (=ATCC 9045) of *Paenibacillus larvae* **(**genotype *ERIC* I), gently provided by Istituto Zooprofilattico Sperimentale delle Venezie (Italy), was routinely cultured at 35 °C on J medium, including Tryptone (0.5 %), K_2HPO_4 (0.3 %), yeast extract (1.5 %), agar for plates (2 %), 10 % glucose (Sigma Aldrich) (20 ml/l), with pH adjusted to 7.3 (Hornitzky and Nicholls, 1993).

2.3.2 Screening bioassays

Screening bioassays aiming at evaluating the antagonistic potential of different LAB strains, were based on agar well diffusion tests (Valgas et al., 2007). An aliquot (300 µl) of an overnight *P. larvae* culture (> 10^6 cells/ml) at exponential phase, was preliminarily inoculated on Brain Heart Infusion (BHI) soft agar (0,75 %) into Petri dishes. After drying, holes (9 mm diameter) were made aseptically on agar using the reverse end of a tip, and an aliquot (100 µl) of each LAB culture supernatant or MRS broth (negative control) was introduced into the well. After incubation at 35 °C for 48-72 h, the eventual inhibition of *P. larvae* growth was checked measuring the diameter of the inhibition halo crossing the well (Balouiri et al., 2016). Tetracycline (10 µg/ml) was used as a positive control, and each LAB strain was tested in triplicates.

2.3.3 Liquid culture bioassays

A selection of LAB strains that showed different potential against *P. larvae* was involved in liquid culture bioassays. Liquid cultures in J broth (9 ml), after being added with 1 ml of cell free supernatant (CFS) of each LAB isolate or J medium (negative control), was inoculated with an aliquot (100 µl) of an overnight *P. larvae* culture at the exponential phase (> 10^6 cells/ml) and incubated at 35 °C, shaking at 150 rpm. Turbidity (absorbance) at 600 nm (OD) was measured to

monitor bacterial growth during the following 48 h. Three different experiments, each involving three replicates, were conducted.

The first experiment had the purpose to confirm the inhibitory properties of the secretome of *L. plantarum* isolates PT23-1, PD57, S7-10, S7-12, S12-9, and S20-3, that showed high potential in agar well diffusion assays. In this experiment, the activity of CFS was compared with untreated control and with CFS adjusted to pH 6.5, in order to evaluate the possible effect of media acidification due to LAB growth.

A second experiment was conducted to evaluate the heat-stability of the secretome inhibitory properties from selected LAB strains. For this purpose, CFS of PT23-1, S7-10, S7-12, was either treated at 80 °C for 10 min, 95 °C for 30 min, or 121 °C for 15 min, before being used in bioassays in comparison with untreated cell free supernatant.

The third experiment had the purpose of evaluating *P. larvae* growth at different initial pH levels. Accordingly, liquid J broth was titrated before *P. larvae* inoculation, and growth response effect was determined assaying progressive pH levels within the range 4.0-7.3.

2.3.4 CFS protein precipitation and assays

In order to evaluate the possible involvement of medium/highly sized proteins in CFS of differently active LAB strains, filter-sterilized culture supernatant of *L. plantarum* PT23-1 (highly active), *L. plantarum* T03 (moderately active), *L. plantarum* T2-3, *L. paracasei* FS103 and FS109 (low active) was mixed with ammonium sulfate up to 85 % saturation (w/v) and incubated overnight at 4 °C with gentle stirring, before being centrifuged at 15,000 *x g* for 20 min. The harvested protein precipitate was resuspended and dialyzed against phosphate-buffered saline (PBS) to remove ammonium sulfate residues, using a 12 kDa cut-off dialyses tubing (Sigma), which did not retain small peptides. Protein concentration was determined by Bradford dye-binding method using Bio-

Rad Protein Assay (Bradford, 1976). Protein solution with a concentration of around 1 mg/ml was used in liquid bioassays against *P. larvae* according to the previously described methods.

2.3.5 Plantaricin gene detection and expression

While no inhibitory effects against *P. larvae* were associated with the protein precipitate obtained from *L. plantarum* isolates, because dialysis conditions did not retain small peptides, specific analyses were conducted to evaluate the possible role of bacteriocin genes typically harbored by this bacterial species and encoding for low size peptides. For this purpose, the expression of plantaricin genes during LAB growth in MRS broth was determined by RT-PCR on a LAB strain selection. Consistently, cells of *L. plantarum* PT23-1 (highly active), *L. plantarum* T03 (moderately active), *L. plantarum* T2-3, *L. paracasei* FS103 and FS109 (low active) were harvested after 24 h growth in MRS, resuspended in TRIzol®Reagent (Life Technologies) and subjected to 12 cycles (30 s) of sonication (100 W, 40 kHz) and cooling in ice. RNA was extracted according with Chomczynski and Sacchi (2016) and an aliquot (1 μg) was reverse-transcribed to cDNA using the GoScript™ Reverse Transcription System Protocol as recommended by manufacturer (Promega). PCR analyses with cDNA targeting *plantaricin* (*pln*) genes (Tai et al., 2015) and using primer pairs indicated on Table 1, were conducted using EasyTaq DNA Polymerase protocol according with manufacturer's recommendations (Transbionovo). PCR conditions were 95 °C for 5', followed by 30 cycles of 95 °C for 30'', 53 °C for 30'', 72 °C for 1', followed by 72 °C for 10'. 16S rRNA was used as a control and cDNA amplification was verified by electrophoresis on agarose gel (1 %) using SimplySafe (EURx) for visualization.

2.4 Statistical analysis

Data on *P. larvae* growth inhibition involving treatments with different CFS preparations were subjected to analysis of variance followed by least square means comparison (LSD test).

Over time analysis of *P. larvae* growth in liquid medium treated with CFS at different initial pH levels was analyzed using repeated measures ANOVA (PROC MIXED), and means were separated using LSMEANS comparison (adjust = Tukey).

Linear regression analyses were used for analyzing the relationship between initial pH level and *P. larvae* growth (OD) in liquid culture.

All analyses were performed using R (R Development Core Team, 2016).

2.5 Results

2.5.1 Screening bioassays

The outcome of screening experiments on agar plates evaluating the inhibitory activity of different LAB strains against *P. larvae* is summarized in Table 2. As expected, a significant variability between strains was observed. While no inhibition was detected in several cases, the best performing LAB strains produced an inhibition halo of more than 15 mm.

2.5.2 Liquid culture bioassays

The inhibition of *P. larvae* growth in J broth treated with culture supernatant of a selection of *L. plantarum* active strains is shown in Fig. 1. Percentage inhibition in respect to growth in the untreated control ranged on average between 55 and 85 %, and between 10 and 35 %, in cultures treated with CFS and CFS adjusted to pH 6.5 from different LAB strains, respectively ($F_{12,38}$ = 53.99, $P < 0.0001$). Growth of *P. larvae* in liquid media was significantly affected by LAB strain ($F_{6,38}$ = 23.37, $P < 0.0001$), pH adjustment ($F_{1,38}$ = 338.84, $P < 0.0001$), and the interaction strain-pH adjustment ($F_{5,38}$ = 33.78, $P < 0.0001$).

P. larvae growth in liquid medium J adjusted to progressive pH levels within the range 4.0-7.3 is shown in Fig. 2. The level of pH at the start of the culture caused a significant effect on bacterial

growth and a positive relationship between these factors was found (adjusted R^2 = 0.6266, F = 49.65, P < 0.0001).

pH values of liquid medium J treated with CFS of *L. plantarum* strains before and after *P. larvae* growth is shown in Table 3 in correspondence of different inhibition levels, in comparison with untreated control. The average pH value changed significantly as a result of treatment ($F_{7,48}$ = 448.02, P < 0.0001) and time (before and after *P. larvae* growth) ($F_{1,48}$ = 5420.63, P = 0.0002). A significant interaction treatment x time was also observed ($F_{7,48}$ = 21.85, P < 0.0001). No differences between *L. plantarum* strains in terms of pH values reached before or after growth, were instead observed. As a result of linear regression analysis, *P. larvae* growth inhibition was found to be not significantly correlated with the pH of cultural broth treated with supernatant from different *L. plantarum* strain cultures (adjusted R^2 = 0.12, F = 1.682, P = 0.2644).

Heat-treatment at 80, 95 and 121 °C of culture supernatants from *L. plantarum* isolates PT23-2, S7-10, S7-12 did not cause a significant reduction in their inhibitory properties against *P. larvae*. Growth of *P. larvae* in heat-treated samples was significantly reduced (> 80 %) compared with growth in untreated control ($F_{9,29}$ = 211.27, P < 0.0001).

2.5.3 Protein precipitate

No significant inhibition of *P. larvae* growth in J broth treated with a medium/high molecular weight protein fraction precipitated with ammonium sulphate from culture supernatant of a selection of progressively active LAB isolates (PT 23-1, T03, T2-3, FS103, FS109) was observed ($F_{5,17}$ = 1.18, P = 0.3753).

2.5.4 Plantaricin gene detection and expression

The activation of *plantaricin* genes in a selection of *L. plantarum* strains showing significant inhibition against *P. larvae* is shown in Table 4. Based on RT-PCR analysis, expression of plnN

during growth in MRS broth was confirmed for strains S7-10, S 20-3, and PT 23-1, while plnE and plnF were expressed by strain S7-10. No expression was observed for strains PD57 and S12-9.

2.6 Discussion

American foulbrood management is a major challenge for modern beekeeping worldwide, due to the unavailability of active substances effective against *P. larvae*, especially against its highly resistant spores, and compatible with the colony ecosystem and the outside environment (Genersch, 2010). While the use of antibiotics against bee pathogens in apiaries is banned in different parts of the world, including Europe, their employment in countries where it is allowed is leading to the development of resistance by this pathogen, which raises further concerns (Tian et al., 2012).

An innovative and very promising approach to the biocontainment of this pathogen leverages the beneficial bacteria, in particular the main components of the microbial community of the honeybee, among which a major role is played by lactic acid bacteria (Mudroňová et al., 2011). Several studies have highlighted the potential of honeybee-specific *Lactobacillus* and other LAB species to enhance honeybee innate immunity and to exert a direct action of inhibition against *P. larvae in vitro* and under field-conditions (Daisley et al., 2019; Lamei et al., 2019). On the other hand, there is a great diversity of lactic acid bacteria associated with beneficial probiotic effects in several ecological niches, including animal and human intestines (Heilig et al., 2002). Many of these bacteria also play a key role in the fermentation and preservation of food (Cleveland et al., 2001). Such properties are related with highly conserved genetic traits shared among LAB species of different origin and with the release of antimicrobials such as organic acids, reactive oxygen species, and small peptides determining inhibitory properties against a wide range of bacteria (Cintas et al., 2001; Devi et al., 2015). Accordingly, the filter-sterilized culture supernatant of LAB strains isolated from fermented food (i.e., sausages and cheese) showed different degrees of *P. larvae* growth inhibition in our experiments in agar plates and liquid cultures, corroborating similar

observations conducted with LABs isolated from the honeybee body (Lamei et al., 2019). These inhibitory effects, mostly associated with *L. plantarum* strains, were attenuated when the pH of the supernatant of the LABs culture (normally around 4.1) was adjusted to 6.5 before being used for treatments, which ensured suitable conditions for *P. larvae* growth, as confirmed by our experiments assaying the pH range of growth for this honeybee pathogen. It is well known that the acidification of the culture medium, resulting from the production of organic acids, is one of the factors determining inhibition by LABs. However, in our case, such acidification was not sufficient to explain the observed effects, as demonstrated by the variability of inhibitory power shown by different strains of lactic acid bacteria, which however resulted in the same level of acidification of the culture medium. In addition, pH adjustment of LAB culture supernatants used for *P. larvae* medium treatments caused a reduction, but not an elimination of the inhibitory effect, showing that the likely effect of organic acids was only a co-factor and that other explanations should be sought in other components released by the LABs during their growth.

This statement is in line with the results of experiments conducted with a newly isolated *Lactobacillus bombicola* strain producing lactic acid and inhibiting the parasite *Crithidia bombi*, in which the pH variation was not sufficient to explain the observed effects, suggesting the additional contribution of other metabolites released by the bacterium (Palmer-Young et al., 2019). On the other hand, the activation/inactivation of antimicrobial compounds like bacteriocins might be *per se* related to pH levels (Garcia-Garcera et al., 1993).

Heating cell free supernatants (CFS) of highly active LAB strains at different temperatures up to 121 °C did not cause a significant reduction in their inhibitory properties at the concentrations we assayed, which is in line with the expected heat stability of small antimicrobial peptides (Fernandes et al., 2017). A possible role of small peptides is also reinforced by the lack of effects on *P. larvae* growth observed after treatments with CFS precipitate containing proteins of a size exceeding 12 kDa. The probable involvement in the inhibitory action of antimicrobial peptides produced by the

most active strains is also supported by a significant expression of some *plantaricin* (*pln*)-related genes during the growth of the most active *L. plantarum* strains. These antimicrobial peptides having a size around 5 kDa, are commonly produced by *L. plantarum* and show activity against a wide range of bacterial species including *Staphylococcus aureus*, *Escherichia coli*, *Saccharomyces cerevisiae* (Pei et al., 2018), *Bacillus cereus* (Lv et al., 2018), and *Listeria monocytogenes* (Wang et al., 2019).

Lactobacillus plantarum is a ubiquitous species and a very versatile lactic acid bacterium applied in different industrial sectors, especially exploiting the heterogeneity in functional properties related to genetic variability among different strains (Guidone et al., 2014). Probiotic functions and antagonism against honeybee pathogens have been associated with *L. plantarum* strains isolated from this insect body (Javorský et al., 2017). However, the effectiveness of *L. plantarum* strains we isolated from fermented food matrices support a parallel effort for the discovery of novel probiotic strains, suitable for use in beekeeping, in environments other than the hive. Although the introduction into the hive of an exogenous LAB strain should primarily ensure compatibility with honeybees and the hive ecosystem, in the case of probiotic food-borne Lactobacilli, the potential risks of contamination of the hive products (i.e., honey) would be minimised (Zhou et al., 2000). The results of our study encourage a continuous search for probiotic bacterial strains with potential application in the hive to protect honeybee health. Likewise, further studies are needed to clarify the *in vivo* mechanism of action of promising *P. larvae* antagonists.

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2.9 Tables and figures

Table 2.9.1 Primer pairs used for RT-PCR of *plantaricin* genes of *L. plantarum* strains.

Plantaricin gene	Primer sequence*	
	Sense 5'-3'	Antisense 5'-3'
plnE	GGTTCAGTTGCAGGCATTCGTGGTAT	GACAGCGCTAATGACCCAATCGGC
plnF	ATGCTACAGTTTGAGAAGTTACAATATTCCAG	GCATGGAAAACGCCACCTGAAATAGCA
plnA	GTAATAAGGAAATGCAAAAAATAGTAGGTGGAAAG	GCCCCCATCTGCAAAGAATACGC
plnK	GAAGAATTAACTGCTGACGCTGAAAAG	CGGCTGTAAGCATTGCTAACCAATC
plnJ	TAACGACGGATTGCTCTGCCAGC	GATTTGGATGTAGTAGATGCATTTGCACC
plnN	ATTGCCGGGTTAGGTATCG	CCTAAACCATGCCATGCAC
16S rDNA	ATCATGATTTACATTTGAGTG	CGACGACCATGAACCACCTGT

*Primers were designed on sequences of *L. plantarum* bacteriocin loci (Tai et al., 2015)

Table 2.9.2 *Paenibacillus larvae* inhibition haloes* in BHI soft agar plates by different food-borne lactic acid bacteria strains

LAB strain	Inhibition halo (mm)	LAB strain	Inhibition halo(mm)	LAB strain	Inhibition halo(mm)
Lactobacillus plantarum		*Lactobacillus plantarum*		*Lactobacillus brevis*	
T01	+	PT18-5	-	PT7-5	+
T02	+	PT18-6	-	PT7-10	+++
T03	++	PT18-8	-	PT9-5	+
T04	++	PT18-9	-	PT9-7	+
PMT03	+	PT23	-	PT9-10	++
T1-6	++	PT23-1	+++	T1-5	++
T1-7	++	S05-1	++	T2-6	++
T1-8	+	S05-4	++	T5-1	++
T2-1	++	S05-8	++	6BC2	-
T2-3	+	S7-1	++		
T3-9	++	S7-7	++	*L. paracasei*	
PT5-5	-	S7-10	+++	FS103	+
PT5-6	-	S7-12	+++	FS109	+
PT5-9	+	S12-2	++	7ACU2	-
PT5-10	+	S12-7	++	24AC3	-
PT7-3	++	S12-9	+++		
PT9-1	-	S16-7	++	*L. curvatus*	
PT9-2	-	S20-1	+	S05-5	+
PT9-3	++	S20-3	+++		
PT9-8	+++	S20-4	++	*L. fermentum*	
PT9-11	+	S20-5	++	5BC2	-
PT13-3	+	S20-7	++		
PT13-5	+	S20-9	++		
PT18-1	+	S20-10	-		
PT18-2	+	PD57	+++		
PT18-3	-	5BC2	-		

*halo < 10 mm (+), between 10-15 mm (++), > 15 mm (+++); no halo (-)

Table 2.9.3 Mean (± S.E.) of pH values detected in the *P. larvae* culture broth after treatment with *L. plantarum* strain culture supernatants (CFS) and 48 h growth.

Sample	% Growth inhibition[a]	pH value[b]	
		Post-treatment[c]	Post-growth[d]
Control (J broth)	-	7.4 ± 0.00 a[f]	6.2 ± 0.07 b
L. plantarum PT23-1	84.9 ± 7.99 b[e]	6.3 ± 0.03 b	5.5 ± 0.07 c
L. plantarum S7-10	78.2 ± 3.77 b	6.4 ± 0.05 b	5.5 ± 0.05 c
L. plantarum S7-12	72.2 ± 4.23 b	6.3 ± 0.04 b	5.5 ± 0.07 c
L. plantarum S12-9	54.1 ±3.15 a	6.3 ± 0.03 b	5.5 ± 0.04 c
L. plantarum S20-3	71.6 ± 2.88 b	6.3 ± 0.01 b	5.5 ± 0.02 c
L. plantarum PD57	56.2 ± 0.80 a	6.3 ± 0.03 b	5.6 ± 0.02 c

[a] *P. larvae* growth inhibition was calculated in respect to growth in the control (J broth).
[b] Average pH of culture supernatant of different *L. plantarum* strains was 4.1 ± 0.14. J medium pH was 7.4
[c] Culture supernatant (1 ml) of different *L. plantarum* strains were added to J broth (9 ml) before *P. larvae* inoculum.
[d] Post-growth data were recorded 48 h after *P. larvae* inoculum
[e] Means in growth inhibition column followed by different letters are significantly different (ANOVA, LSD test, $P < 0.05$).
[f] pH value means followed by different letters are significantly different (Mixed Proc. ANOVA, Tukey test, $P < 0.05$).

Table 2.9.4 *Plantaricin* gene expression in different *L. plantarum* strains during growth in liquid broth (MRS)

L. plantarum strain	Plantaricin genes*					
	plnA	*plnE*	*plnF*	*plnJ*	*plnK*	*plnN*
PT 23-1	-	-	-	-	-	+
S7-10	-	+	+	-	-	+
fS12-9	-	-	-	-	-	-
S20-3	-	-	-	-	-	+
PD57	-	-	-	-	-	-

* Gene expression was verified by mRNA extraction and RT-PCR analysis.
+ = expressed; - = non expressed.

Fig. 2.9.1 Growth inhibition of *Paenibacillus larvae* in liquid media treated with different *Lactobacillus plantarum* strain culture supernatants (CFS) or CFS adjusted to pH 6.5. Inhibition percentages are calculated in respect to growth in the untreated control. Different letters above bars indicate significantly different means, while an asterisk (*) indicates a significant difference with untreated control (2 ways-ANOVA, followed by LSD test).

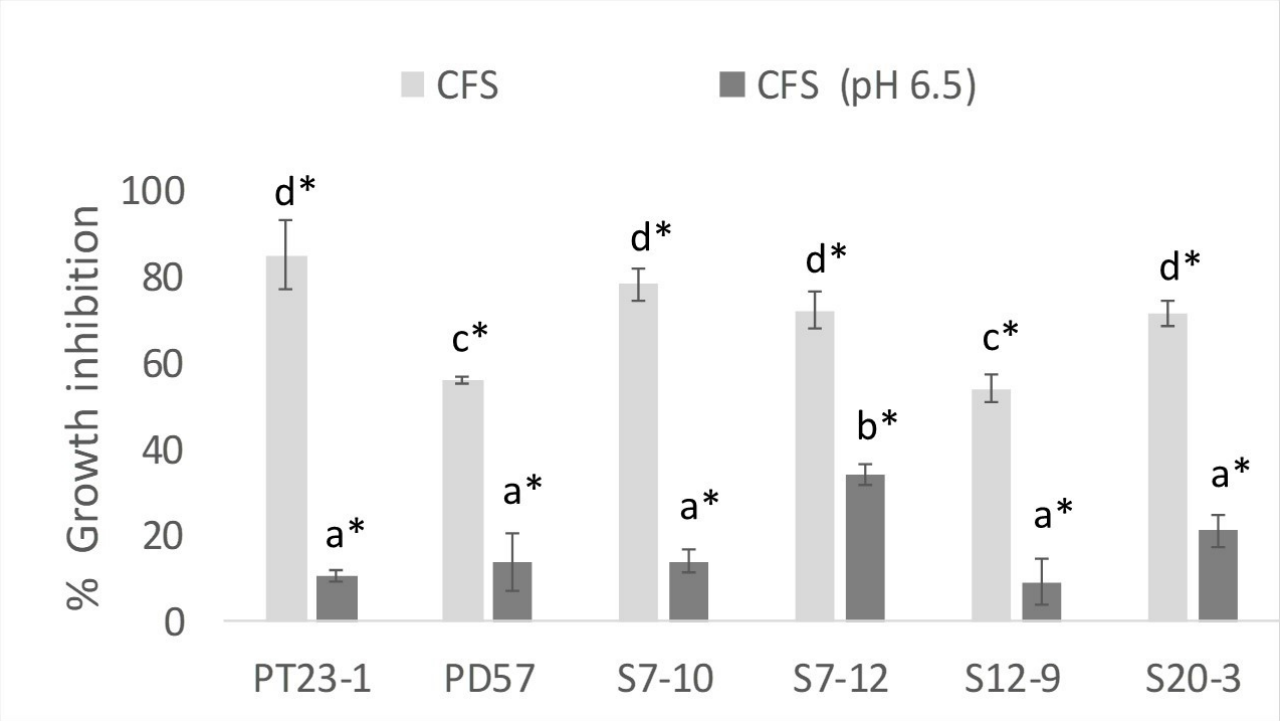
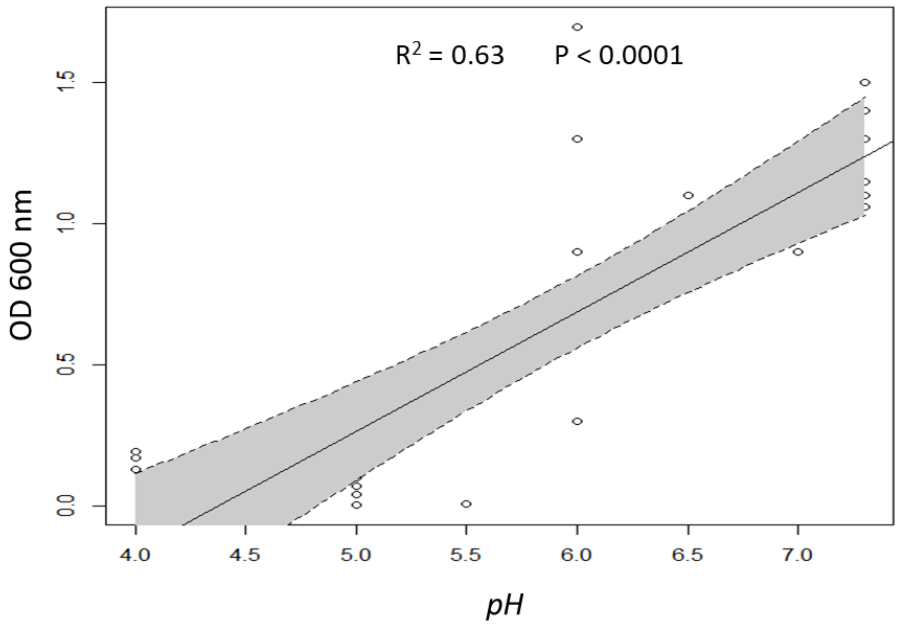


Fig. 2.9.2 Linear regression plot with 95 % confidence intervals (shaded areas) representing the pH range of *Paenibacillus larvae* growth measured as optical density (OD) at 600 nm.



CHAPTER III

Inhibition of *Paenibacillus larvae* by an extracellular protein fraction from a honeybee-borne *Brevibacillus laterosporus* strain

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3.1 Abstract

The inhibitory action that a *Brevibacillus laterosporus* strain isolated from the honeybee body causes against the American Foulbrood (AFB) etiological agent *Paenibacillus larvae* was studied by *in vitro* experiments. A protein fraction isolated from *B. laterosporus* culture supernatant was involved in the observed inhibition of *P. larvae* vegetative growth and spore germination. As a result of LC-MS/MS proteomic analyses, the bacteriocin laterosporulin was found to be the major component of this fraction, followed by other antimicrobial proteins and substances including lectins, chaperonins, various enzymes and a number of putative uncharacterized proteins. The results obtained in this study highlight the potential of *B. laterosporus* as a biological control agent for preserving and improving honeybee health.

Key words: *Apis mellifera*; American Foulbrood; laterosporulin; antimicrobial; bacteria.

3.2 Introduction

A variety of bacterial species have developed unique and host-specific relationships with the honeybee *Apis mellifera* (Moran, 2015). These include the Gammaproteobacteria *Gilliamella apicola* and *Frischella perrara* (Orbaceae), the Betaproteobacterium *Snodgrassella alvi* (Neisseriaceae), *Lactobacillus* (Firmicutes) and *Bifidobacterium* (Actinobacteria) species, residing in the bee gut and representing its core bacterial community (Kwong and Moran, 2016). Such bacterial species are believed to play a significant role in maintaining a good health status counteracting against stress factors like insect pathogens and parasites (Engel and Moran, 2013). Other bacterial species found in the bee hive ecosystem have instead specifically evolved as pathogens. This is the case of *Paenibacillus larvae* (Firmicutes), the causal agent of the American Foulbrood (AFB), a destructive disease of worldwide importance, affecting the colony (Genersch, 2010). The management of this bacterium is a main issue for the beekeeper, especially because of the limitations associated with the use of antibiotics in the beehive ecosystem and the resistance development in diverse *P. larvae* strains (Evans, 2003; Murray et al., 2007). Hence, research studies are being carried out to find alternative and effective natural antimicrobial substances (Isidorov et al., 2018). This pathogen, typically enter the body of a neonate larva by the ingestion of food contaminated by its spores. Once in the midgut, spores germinate producing new vegetative cells releasing several toxins, enzymes and virulence factors supporting the infection process. After the degradation of the peritrophic matrix, probably by the action of proteases and chitinases (Garcia-Gonzalez, 2014), bacterial cells interact with the midgut epithelial cells causing damages and invading the haemocoel where they proliferate causing a widespread septicaemia. At this infection phase, dead larvae appear flaccid, with glue-like consistency (ropy stage), which will contribute to further spread of new bacterial spores within the colony and outside the hive (Ebeling et al., 2016).

The honeybee defense mechanisms against this pathogen are primarily based on social immunity (hygienic behavior) and on the innate humoral and cellular response (Chan et al., 2009), but may also rely on additional immune-related functions performed by components of its core bacterial community (Zheng et al., 2018). Among the bacterial species commonly inhabiting the honeybee body, there is the spore former *Brevibacillus laterosporus* (Marche et al., 2016), whose antimicrobial potential is well documented (Miljkovic et al., 2019). Despite this bacterium was found as a secondary invader in bee colonies affected by the European foulbrood caused by *Melissococcus pluton* (Firmicutes) (White, 1912), it has more recently been reported to have beneficial effects on bees, showing a specific inhibitory action against *P. larvae* (Alippi and Reynaldi, 2006; Hamdi and Daffonchio, 2011). However, the mechanism leading to such inhibition has not yet been clarified (Khaled et al., 2018).

The purpose of this study was to investigate the *in vitro* inhibition that a *B. laterosporus* strain isolated from the honeybee body causes against *P. larvae*. The proteomic characterization of an extracellular bacterial fraction involved in such effects is for the first time presented.

3.3. Materials and methods

3.3.1 Bacterial strains and growth conditions

Paenibacillus larvae reference strain DMS 7030 (= ATCC 9045) corresponding to genotype *ERIC* I, gently provided by Istituto Zooprofilattico Sperimentale delle Venezie (Italy), was used in this study. Solid and liquid cultures were routinely conducted at 35 °C on agar plates or liquid broth containing J medium: Tryptone (Fluka) (5 g/l), K_2HPO_4 (Sigma Aldrich) (3 g/l), yeast extract (Sigma Aldrich) (15 g/l), agar (Sigma Aldrich) (20 g/l) (only for plates), 10 % glucose (Sigma Aldrich) (20 ml/l), pH adjusted to 7.3 (Hornitzky and Nicholls, 1993). This medium was preliminarily tested for its suitability for our *in vitro* bioassay model (De Graaf et al., 1993).

Vegetative cells or sporulated cultures were obtained by inoculating liquid J medium with fresh *P. larvae* cells and the presence of different stages of growth was checked under phase-contrast microscopy (Forsgren et al., 2008).

Brevibacillus laterosporus strain F5 maintained in glycerol at -80 °C at the University of Sassari, was employed in this study. This honeybee-borne strain was isolated and identified in a previous work (Marche et al., 2016). *Brevibacillus laterosporus* was routinely cultivated on LB (Luria Bertani) agar, while bacterial cell culture for bioassays and analyses was conducted on J broth shaking at 180 rpm at 30 °C for 72 h. Culture supernatant of a whole sporulated culture was collected by centrifugation at 15,000 *x g* at 4 °C for 15 min (Marche et al., 2017). Supernatant used in bioassays or processed for protein precipitation was preliminarily sterilized through 0.2 μm pore size filter (Minisart®).

3.3.2 Protein precipitation and analyses

For protein precipitation, filter-sterilized culture supernatant of *B. laterosporus* was mixed with ammonium sulfate up to 85 % saturation (w/v) before being incubated overnight at 4 °C with gentle stirring. The solution was centrifuged at 15,000 *x g* for 20 min and the protein pellet obtained was resuspended in phosphate-buffered saline (PBS) and dialyzed against the same buffer to remove ammonium sulfate residues. Protein concentration of different samples was routinely measured by Bio-Rad Protein Assay according to Bradford dye-binding method (Bradford, 1976).

Aliquots of the same protein solutions used in bioassay were supplied to the proteomic facility of Porto Conte Ricerche Srl (Tramariglio, Alghero, Italy) for LC-MS/MS analyses. The protein fraction was subjected to on-filter reduction, alkylation, and trypsin digestion according to the filter-aided sample preparation (FASP) protocol, with slight modifications (Addis et al., 2009). Peptide mixtures were analyzed through a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) interfaced with an UltiMate 3000 RSLCnano LC system (Thermo Scientific) (Tanca et al., 2013).

Mass spectrometry output data were analyzed through Proteome Discoverer (version 1.4; Thermo Scientific), using Sequest-HT (Eng et al., 1994), the database UniprotKB and Mascot (Matrix Science, Boston, MA) as search engine for protein identification. Protein relative abundance was expressed by means of the normalized spectral abundance factor (NSAF) (McIlwain et al., 2012). Peptide sequence data were further processed against the NCBI database (http://www.ncbi.nlm.nih.gov).

3.3.3 Antimicrobial bioassays

The inhibitory effects of *B. laterosporus* on *P. larvae* were preliminarily studied by agar well diffusion tests and subsequently by liquid culture bioassays. Culture supernatant of *B. laterosporus* to be employed in bioassays was fresh prepared and filter-sterilized as described above.

Preliminary experiments were conducted on agar plates according to the agar well diffusion method (Valgas et al., 2007). Briefly, the whole agar plate surface was inoculated by spreading 500 µl of a *P. larvae* liquid cell culture with a concentration of at least 10^6 cells/ml. After leaving plates to dry inside a laminar flow cabinet, a hole (8 mm diameter) was made aseptically using a tip, and *B. laterosporus* supernatant (100 µl) or sterile J broth (control) was introduced into the well. After incubation at 35 °C for 48 h, the diameter of the *P. larvae* growth inhibition halo crossing the well was measured (Balouiri et al., 2016).

Secondly, three different liquid culture bioassays were conducted to study the inhibitory action of *B. laterosporus* on *P. larvae* vegetative growth. For this purpose, *P. larvae* liquid cultures (5ml) at the exponential phase, with a concentration of 10^6 cells/ml, were inoculated into flasks containing 20 ml J broth incubated at 35 °C shaking at 120 rpm. Bacterial growth was monitored by measuring optical density (OD) at 600 nm every hour. In the first experiment, when *P. larvae* exponential vegetative growth reached 0.5-0.6 OD in the flask, an aliquot (5 ml) of *B. laterosporus* supernatant (treated) or sterile J broth (control) was added to the culture, and bacterial growth was monitored

during the next hours. In the second experiment conducted under the same conditions, the following three bacterial preparations were used to treat *P. larvae* culture at the exponential phase: heat-treated (100 °C for 10 minutes) and untreated *B. laterosporus* culture supernatant (at the same proportion as in the previous experiment) and the protein fraction (at a concentration of 1 µg/µl) obtained by precipitation from the supernatant. Bacterial growth was assessed for 6 hours after treatment. In the third experiment conducted under the same experimental conditions, the concentration-response effect of the protein fraction was determined assaying progressive protein concentrations within the range 0.1-1 µg/µl. Growth inhibition percentage in treated flasks was calculated against growth in the control.

A fourth experiment in liquid culture was conducted to assay the possible inhibitory properties of *B. laterosporus* preparations on *P. larvae* spore germination. For this purpose, aliquots (500 µl) of sporulated cultures of *P. larvae* with a concentration of 10^6 spores/ml were inoculated into tubes containing fresh J broth (2 ml) and 500 µl of *B. laterosporus* culture supernatant or protein fraction at a concentration of 1 µg/µl, according to the above described protocol conditions. During the next 12 hours, spore germination was checked under phase microscopy and vegetative cell growth was monitored by OD measurements.

The design of all the above experiments involved 3-5 replicates and each experiment was repeated at least three times.

3.4 Statistical analysis

Statistical analyses were performed with SAS software (version 9.1) with significance level set at $\alpha = 0.05$ (SAS, 2004).

Data on *P larvae* growth inhibition and spore germination in liquid cultures, involving different *B. laterosporus* preparations (i.e., supernatant and protein fraction) were subjected to analysis of

variance (one factor design: bacterial preparation) followed by multiple comparison of means (adjust = Tukey).

Overtime *P. larvae* growth in liquid medium was analyzed using repeated measures ANOVA (Proc Mixed), and means were separated using lsmeans comparison (adjust = Tukey).

Linear regression analyses were used for analyzing the relationship between protein concentration and bacterial growth (OD) in liquid culture.

3.5. Results

3.5.1 Antimicrobial bioassays

Preliminary experiments on agar plates inoculated with *P. larvae* showed a clear inhibition zone around wells filled up with *B. laterosporus* supernatant (Fig. S1). An average inhibition halo of 13.30 ± 3.78 mm was obtained employing supernatants from different preparations of *B. laterosporus* cultures, showing a clear difference in comparison with controls in which no inhibition halo was detected.

The growth of *P. larvae* in J broth treated with *B. laterosporus* culture supernatant in the first experiment in liquid culture is shown in Fig. 1. Growth was significantly affected by treatment ($F_{1,8}$ = 32.96, P = 0.0004) and time ($F_{11,88}$ = 209.99, P < 0.0001). A significant interaction treatment x time was also observed ($F_{11,88}$ = 72.57, P < 0.0001). Treatment caused a significant slowing or interruption of bacterial growth, while *P. larvae* cellular replication continued exponentially in the control.

Inhibition of *P. larvae* growth in the second experiment in liquid cultures treated with *B. laterosporus* culture supernatant or protein fraction at a concentration of 1 µg/µl is shown in Table 1. A high inhibition, over 80 % in respect to growth in the control, was achieved by both culture supernatant and the protein fraction obtained from the same supernatant. On the other hand, heat treatments caused a significant reduction of their inhibitory properties ($F_{3,8}$ = 63.58, P < 0.0001).

The inhibition effect caused by the protein fraction as determined in the third experiment was concentration dependent (Fig. 2). As a result of linear regression analysis, the percentage of *P. larvae* growth inhibition was shown to be positively correlated with the concentration of the *B. laterosporus* protein fraction in the culture broth (adjusted $R^2 = 0.8801$, $F = 610.1$, $P < 0.0001$). Germination of *P. larvae* spores in liquid cultures treated with *B. laterosporus* culture supernatant or protein fraction at a concentration of 1 µg/µl (fourth experiment) was significantly inhibited or slowed down in comparison with post-germination growth in the control. No significant *P. larvae* growth was observed in the 12 h following spore inoculation in J medium treated with *B. laterosporus* culture supernatant or protein fraction ($F_{2,8} = 39.48$, $P = 0.0004$) (Fig. S2).

3.5.2 Proteomic analysis

The proteome of the *B. laterosporus* culture supernatant showing inhibitory properties against *P. larvae* appeared to be a complex protein mixture. As a result of mass spectrometry, including the analysis of 4502 internal peptides, a variety of proteins were identified in this fraction. Major proteins were found within the 5-121 kDa range (Table 2). The antimicrobial peptide laterosporulin appeared to be the relatively most abundant, based on the normalized spectral abundance factor (NSAF) determination. Proteins involved in the bacterial cell structure, including surface layer and wall proteins were also well represented. Several enzymes and stress related proteins were detected. In addition, a lectin domain protein and a 60 kDa chaperonin were significantly represented. Among others, a number of putative uncharacterized proteins were found.

3.6 Discussion

Among the variety of bacteria found in the beehive ecosystem, the family Paenibacillaceae include the pathogenic species *P. larvae* (Genersch, 2010) and the honey bee body resident *B. laterosporus* (Marche et al., 2016). While they are likely to have developed strategies to compete through

evolution in the same environment, the inhibitory properties of *B. laterosporus* against the American Foulbrood etiological agent have only recently been outlined (Alippi and Reynaldi, 2006). Such antagonistic potential is in line with the reported production of several antimicrobial peptides and antibiotics by different *B. laterosporus* strains (Ruiu, 2013). In this regard, the availability of genes related to the antimicrobial activity in the genome of *B. laterosporus* was found to be highly conserved in this species (Djukic et al., 2011; Camiolo et al., 2017). Most of the deriving bioactive compounds have been isolated from the *B. laterosporus* culture supernatant (Zhao et al., 2012; Chawawisit and Lertcanawanichakul, 2014; Yang et al., 2016; Yang and Yousef, 2018). Accordingly, the inhibitory properties of the honeybee-borne strain F5 of *B. laterosporus* against *P. larvae* were associated with the culture supernatant, and this finding is in line with previous observations conducted employing other hive-isolated bacterial strains (Alippi and Reynaldi, 2006; Bartel et al., 2018). Inhibition of both *P. larvae* vegetative growth and spore germination was demonstrated in our experiments. These effects were analogously observed when ammonium sulfate precipitated proteins from the culture supernatant of *B. laterosporus* were employed in bioassays, thus demonstrating a main role of the proteins released during bacterial growth. Mass spectrometry analyses revealed a protein complex in which the 5.7 kDa bacteriocin, laterosporulin, was the major component, followed by higher molecular weight proteins representing the different layers that make up the cell wall, and other functional (i.e., enzymes) and stress-related peptides.

While the presence of cell wall and other cell proteins in the culture supernatant is substantially due to the normal events characterizing the stationary phase of the bacterial growth such as lysis of old cells and sporangia, the detection of laterosporulin and other bioactive compounds like a lectin domain protein and a 60 kDa chaperonin among major proteins, might be related to the observed antimicrobial activity. In addition, several putative uncharacterized proteins detected in this

fraction, may also represent important factors, whose specific role would however have to be specifically evaluated.

Laterosporulin was initially discovered in the soil-borne *B. laterosporus* strain GI-9 (Sharma et al., 2012) and was shown to be produced and released in the culture supernatant during the stationary phase of growth (Singh et al., 2012). In the same study, this bacteriocin exhibited significant inhibitory activity against a wide range of Gram-positive and Gram-negative bacteria including *Bacillus subtilis* and *Staphylococcus aureus*. However, its potential against insect pathogens is not known. Laterosporulin produced by the honey-bee borne *B. laterosporus* strain F5, showed 100 % homology with lateroporulin produced by strain GI-9 and strain LMG 15441 (Djukic et al., 2011), confirming that this bacteriocin is highly conserved in this broad-spectrum antimicrobial species. Crystallography studies showed that cysteines are disulfide-bonded, so that laterosporulin keeps its peptide structure in solution, revealing a human defensin-like structural module (Singh et al., 2015; Baindara et al., 2016). These studies suggested that bacterial growth inhibition is determined by membrane permeability increase, which if confirmed would be in line with the inhibitory activity against *P. larvae* vegetative cells we observed in liquid cultures. On the other hand, different mechanisms or components of the protein mixture may regulate the spore germination inhibition we observed. Among other putative inhibition factors, we may speculate that a jackalin-like lectin found in this mixture, could also be involved in the observed antagonism against *P. larvae*. Accordingly, this class of carbohydrate binding proteins is counted among potential virulence factors and antimicrobial compounds (Sharon, 1987; Ziółkowska et al., 2006; Breitenbach Barroso Coelho, 2018). Another protein suspected of being involved in the action against *P. larvae* is the *B. laterosporus* 60 kDa chaperonin found in the bio-active culture supernatant protein precipitate. Consistently, in addition to their primary protein folding function, bacterial chaperones have recently been associated with either mutualistic and pathogenic interactions between bacteria and their host (Kupper et al., 2014). Besides a possible direct action of all these proteins against *P.*

larvae, we cannot exclude that *B. laterosporus* proteome arsenal could at the same time interact with honeybees stimulating their innate immune system, but such a hypothesis should be confirmed by specific experiments.

The exploitation of *B. laterosporus* antagonistic potential in biological control programs against *P. larvae* has previously been suggested (Hamdi and Daffonchio, 2011). More recently, the production of antimicrobial products by naturally occurring *B. laterosporus* strains was successfully induced in the hive through the application of selected bacteriophages (Brady et al. 2018). As a result, a significant action against *P. larvae* in AFB infected hives was obtained. Advances in the knowledge of *B. laterosporus* factors specifically inhibiting *P. larvae* development in the hive, is expected to contribute to further improvement of such biological control methods, ensuring a more targeted action against this honeybee pathogen.

Another aspect arising from this study and deserving further investigation is the actual *B. laterosporus* ecological significance in the beehive ecosystem and within the honeybee body (Berg et al., 2018). Recent microbiome studies revealed that a natural selection have led to the establishment of a core honeybee bacterial community, mostly residing in the gut, represented by few and beneficial species (Kwong and Moran, 2016). Beyond evolutionary origin, such bacterial community is thought to take part in the complex of defense mechanisms against pathogens and stress factors (Engel et al*.,* 2016). Similarly, *B. laterosporus* might be naturally involved in defense mechanisms of the hive, exploiting its own competitive potential (Marche et al., 2019). The promising results obtained in experiments with *B. laterosporus* against *P. larvae*, promote further studies to clarify the role of each putative antimicrobial component and to evaluate its actual potential contribution to honeybee health preservation and improvement within the complex hive ecosystem.

3.7 Acknowledgements

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3.9 Tables and figures

Table 3.9.1 - Inhibition of *Paenibacillus larvae* growth by heat-treated and untreated *Brevibacillus laterosporus* culture supernatant and protein fraction.

Preparation[a]	Inhibition %[b] (means ± S.E.)
Culture supernatant	81.1 ± 12.0 a[c]
Protein fraction	86.9 ± 7.6 a
Heat-treated supernatant	25.7 ± 11.5 b
Heat-treated protein fraction	0.57 ± 1.0 c

[a] Culture supernatant was collected from a 48 h *B. laterosporus* liquid culture in J broth. Protein fraction was obtained by protein precipitation from supernatant. Heat treatments were performed at 100 °C for 10 minutes.

[b] *Paenibacillus larvae* growth inhibition percentages were calculated against growth in the control.

[c] Means followed by different letters, are significantly different (ANOVA, followed by Tukey's test).

Table 3.9.2 - Mass spectrometry identification of major proteins of the *Brevibacillus laterosporus* culture supernatant

UniprotKB Acc. No.	Protein description[a]	Coverage (%)	PSMs	Score	PI	MW (kDa)	NSAF
A0A075R4 × 6	Putative laterosporulin	50	43	70.39	6.71	5.7	272.87
A0A075RBQ6	Hexagonal wall protein	67.22	485	586.32	5.45	121.2	142.28
H0UAR9	Surface layer protein	56.68	368	445.09	5.40	121.2	108.19
A0A075RA20	Uncharacterized protein	63.11	142	362.87	5.86	49.9	100.00
A0A075R7D3	Thioredoxin OS	61.54	30	36.67	4.88	11.5	91.36
H0U9 × 0	Putative uncharacterized protein	66.67	18	22.11	8.37	8.3	81.83
A0A075RAM3	Alkyl hydroperoxide reductase subunit C	55.25	44	49.98	4.97	19.8	77.00
A0A075R7C5	Jacalin-like lectin domain protein OS	48.55	33	50.86	6.54	15.3	75.71
A0A0F7C0H0	General stress protein OS	44.59	35	36.8	4.92	16.8	74.92
H0UCY7	Peptidylprolyl isomerase OS	50.42	81	109.77	7.39	38.7	72.71
A0A075R7T2	Outer cell wall protein	44.15	254	301.34	4.86	115.8	72.37
A0A075R7N0	Cold shock-like protein CspD	60.61	14	15.96	4.94	7.4	67.13
A0A075QVU7	50S ribosomal protein L7/L12	32.77	25	27.51	4.70	12.4	66.50
A0A075RCX3	Uncharacterized protein	60	24	33.34	8.94	12.8	66.14
H0UH15	Putative uncharacterized protein	47.33	31	41.22	5.58	16.8	65.43
A0A075R7P5	Major cold shock protein CspB	61.54	13	13.09	4.72	7.2	63.33
A0A075R3Y6	Ribosomal protein S1	51.65	77	113.59	5.19	43.5	62.07
A0A0F7C0U1	Polyamine aminopropyltransferase	46.21	52	69.94	5.52	31.2	59.45
A0A075RGL0	Fe/S biogenesis protein NfuA	43.84	13	23.00	4.61	8.0	56.39
A0A075R8T7	4-hydroxyphenylpyruvate dioxygenase [A0A075R8T7_BRELA]	39.67	64	85.78	5.08	41.1	55.89
A0A075QYC0	30S ribosomal protein S13	44.26	21	29.6	11.31	14.0	54.58
A0A075Q2Z9	60 kDa chaperonin	43.93	92	125.68	4.98	57.6	53.55
A0A075R0L2	Alanine dehydrogenase	87.17	63	79.71	5.82	39.2	53.41

[a] Proteins with a concentration > 0.5 ng/ml based on NSAF determination are indicated.

Fig. 3.9.1 – Comparison of progressive *P. larvae* growth (mean OD ± SE) in J broth treated vs non-treated with *B. laterosporus* culture supernatant. Treatment was performed after 6 h growth. Different letters indicate significant differences among means (ANOVA PROC MIXED, followed by Tukey test).

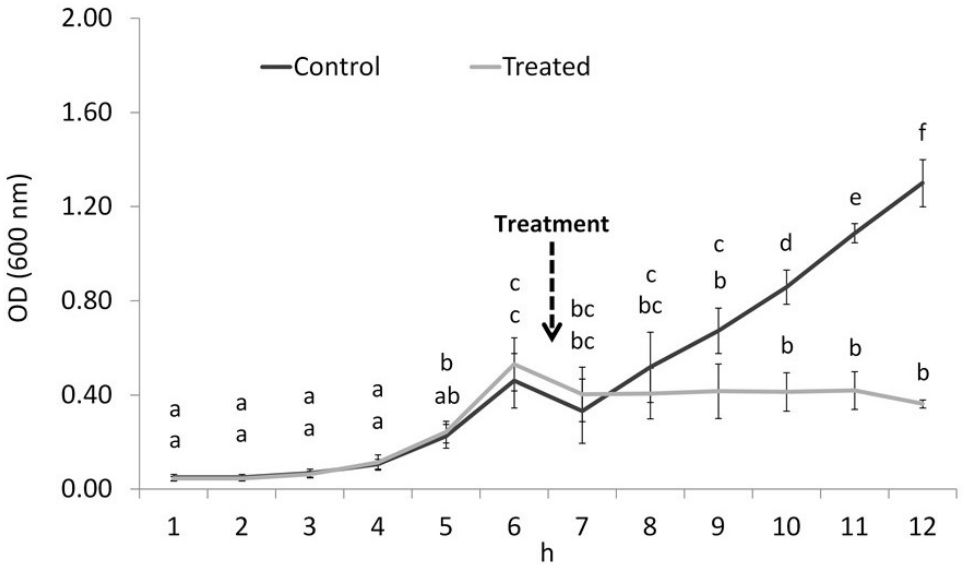


Fig. 3.9.2 - Linear regression plot with 95 % confidence intervals (shaded areas) showing the predicted relationship between concentration of *Brevibacillus laterosporus* proteins isolated from culture supernatant and *Paenibacillus larvae* growth inhibition.

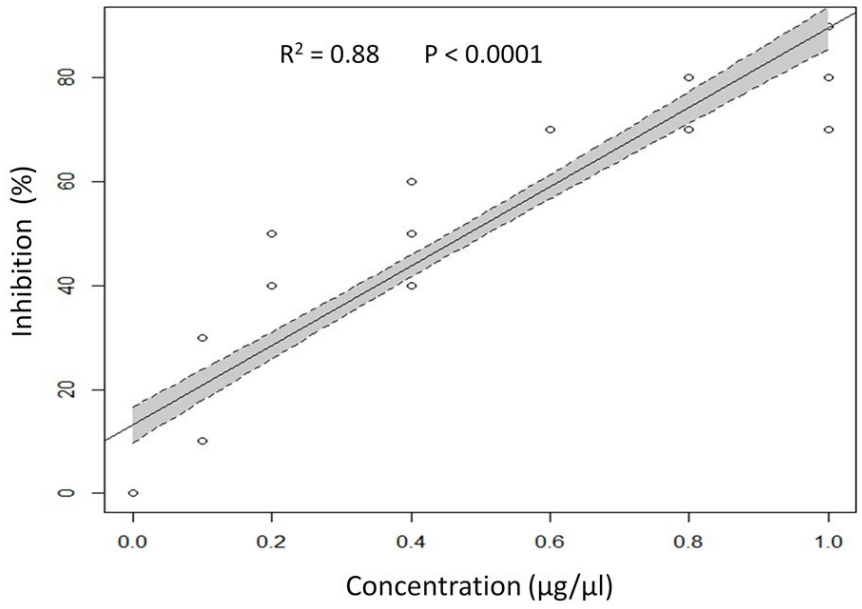


Fig. 3.9 S1 – Representative inhibition zone (h) for *P. larvae* in an agar diffusion test where treatment with *B. laterosporus* culture supernatant (B) is compared with control (A).

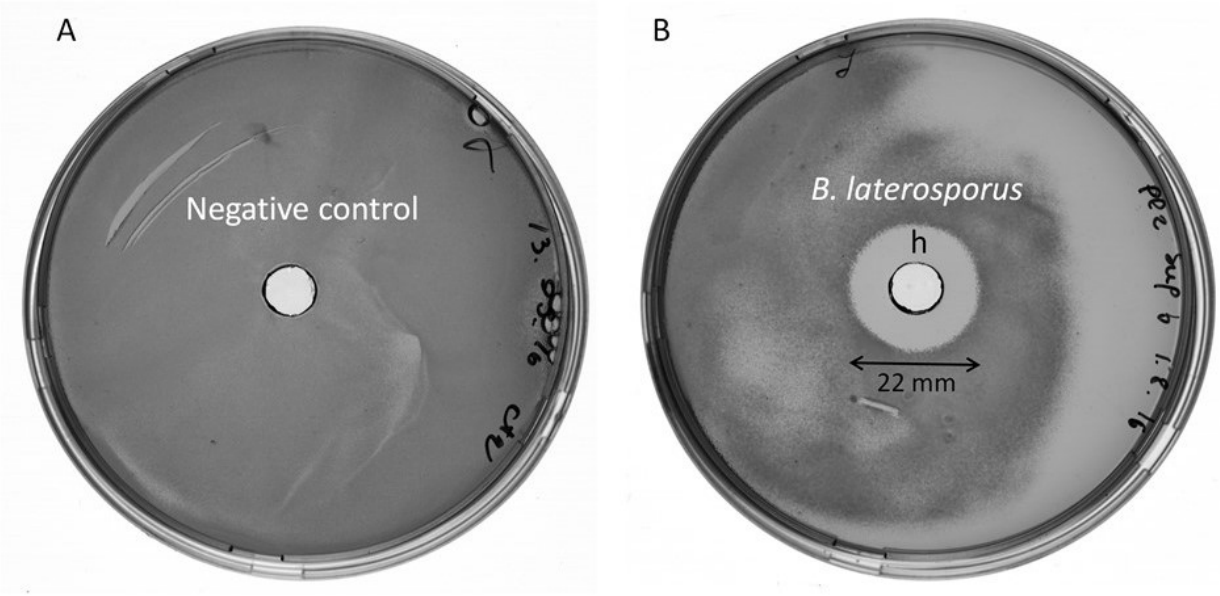
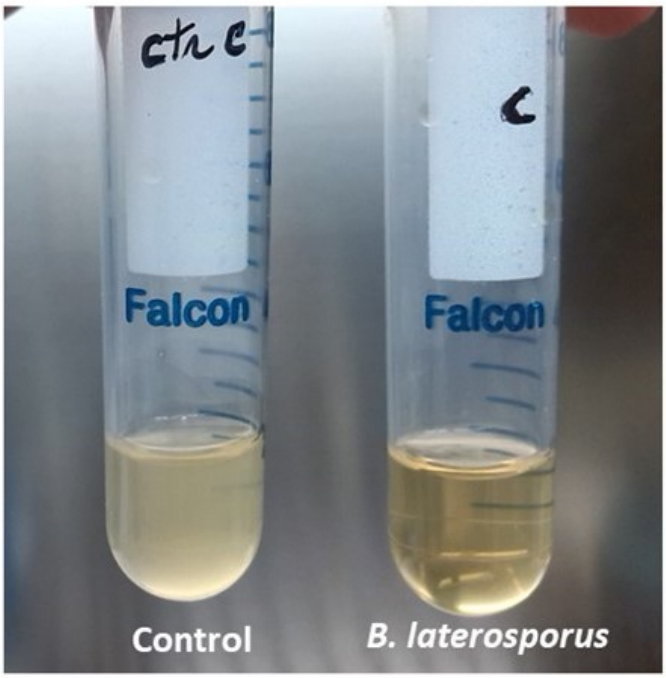


Fig. 3.9 S2 – Inhibition of *P. larvae* spore germination. A: Representative overnight liquid media inoculated with *P. larvae* spores after treatment with *B. laterosporus* proteins (right) or untreated (left). B: Inhibition of *Paenibacillus larvae* growth in the next 12 h following treatment with *B. laterosporus* culture supernatant and protein fraction.

A



B

Preparation	Growth inhibition %[a] (means ± S.E.)
Culture supernatant	84.2 ± 5.3 a[b]
Protein fraction	72.3 ± 9.9 a

[a]*Paenibacillus larvae* growth inhibition percentages were calculated against growth in the control.
[b]Means were not significantly different (ANOVA and Tukey's test).

CHAPTER IV

Safety evaluation of the entomopathogenic bacterium *Brevibacillus laterosporus* for the green lacewing *Chrysoperla agilis* (Neuroptera: Chrysopidae)

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4.1 Abstract

The safety of the entomopathogenic bacterium *Brevibacillus laterosporus* for the natural insect predator *Chrysoperla agilis* was evaluated in this study. For this purpose, laboratory bioassays were conducted exposing different larval instars and adults of the chrysopid to bacterial spore preparations, in order to evaluate the possible effects on survival, longevity, immature development, and adult reproductive performance. The sub-lethal effects were investigated by feeding the bacterium directly to adults and larvae of *C. agilis* or to mealworm beetles (*Tenebrio molitor*) used as hosts for chrysopids (tritrophic interaction). Direct feeding of *B. laterosporus* spores to different lacewing larvae instars and to adults did not cause mean mortality levels significantly different from untreated control, and slight though not significant effects of treatments were generally observed on insect longevity, development, fecundity and egg hatching. In the case of lacewing larvae feeding on treated mealworm beetles, adult emergence percentage was reduced approximately 12 %, in comparison with untreated control. Based on these results, the use of *B. laterosporus* for pest management in the agroecosystem appears to be compatible with chrysopids.

Key words: bioinsecticide; side-effects; sub-lethal effects; pest management; bacteria.

4.2 Introduction

The need to identify effective and eco-sustainable active substances for the management of crop pests is leading to the discovery of new species and strains of entomopathogenic Bacteria, Fungi, Virus, Microsporidia, and Nematodes, with novel bioinsecticidal properties and target range (Ruiu, 2018). On the other hand, for a more complete evaluation of a new entomopathogen and its potential, effects in the agricultural ecosystem should be considered in the broadest sense. This involves the evaluation of the possible side-effects against non-target organisms, including natural predators and parasites (Lacey et al., 2001).

Brevibacillus laterosporus is a bacterial species represented by several strains exhibiting varying levels of pathogenicity against a variety of insect targets in different orders, including Coleoptera, Lepidoptera, and Diptera (Ruiu, 2013). The insecticidal action is mostly related to the production of diverse toxins, most of which act in the insect gut after ingestion (Marche et al., 2018; Glare et al., 2019).

While future applications of this microorganism and its products in agriculture appear promising, knowledge about the possible effects on non-target organisms is still limited. Previous studies have demonstrated a slight susceptibility of the muscoid fly parasitoid wasp *Muscidifurax raptor* Girault and Sanders (Hymenoptera: Pteromalidae) exposed to higher doses than those active against target pests (Ruiu et al., 2007), but no information on the possible effects on other beneficial insects is available.

Chrysopid species are primary predators against several plant pests including aphids, lepidopterans, mites, thrips, and whiteflies (Principi and Canard, 1984; Senior and McEwen, 2001). In addition to predation by natural populations, different species belonging to the *Chrysoperla carnea* group have been employed successfully in pest management programs that involve augmentation releases in the

field (Pappas et al., 2011). Appropriate integrated pest management strategies should ensure protection and enhancement of their populations in the agricultural ecosystem (Cordeiro et al., 2010) and the safety of plant protection products, including entomopathogens, for chrysopids is therefore necessary. While several studies considering the side-effects of several chemically active substances against these beneficial insects have been conducted (Michaud and Grant, 2003), knowledge about the possible non-target toxicity or pathogenicity associated with entomopathogenic microbials is still limited. On the other hand, some studies have been dedicated to evaluating the safety of some insect pathogens for chrysopids, including fungi, for example, *Beauveria bassiana* (Donegan and Lighthart, 1989) and *Metharizium anisopliae* (Ríos-Moreno et al., 2018), and bacteria, including *Bacillus thuringiensis* (Rodrigo-Simón et al., 2006; Lövei et al., 2009). No information on susceptibility of chrysopids to *B. laterosporus* is available.

Chrysoperla agilis Henry et al. (Neuroptera: Chrysopidae) is a natural biocontrol agent belonging to the *Chrysoperla carnea* group. *C. agilis* populations are distributed in Western Europe, overlapping with other lacewing species within the same phylogenetic group, including *C. carnea sensu stricto* (Stephens), *Chrysoperla lucasina* (Lacroix), *Chrysoperla mediterranea* (Hölzel) and *Chrysoperla pallida* Henry et al. (Noh and Henry, 2010). Because of its wide distribution in agricultural ecosystems and a broad range of hosts, the predatory role of *C. agilis* in the bio-containment of pests in agriculture is considered significant (Pappas et al., 2013).

The purpose of this study was to evaluate the susceptibility of *C. agilis* to a *B. laterosporus* strain that shows significant pathogenicity against different insect pests. In particular, our work investigated the lethal and sublethal effects (development of immatures, adult emergence and reproductive performance) in lacewings exposed to a diet containing bacterial spores. To evaluate possible indirect effects on *C. agilis* larvae feeding on insect prey previously exposed to *B. laterosporus*, the tritrophic interaction of host-predator-bacterium was also considered.

4.3 Materials and methods

4.3.1 Bacterial preparations

Entomopathogenic *B. laterosporus* strain UNISS 18 (= NCIMB 41419) was selected for this work because of its well-documented insect pathogenic properties (Marche et al., 2018). Spore suspensions used in bioassays were routinely produced by cultivation in LB broth, shaken at 180 rpm at 30 °C for 48-72 h to achieve culture sporulation.

For this purpose, a pre-culture (25 ml) was inoculated with a heat-activated spore suspension (1 ml), and then used to inoculate a second culture in sporulation medium T3, as described elsewhere (Marche et al., 2017). Spores were harvested by centrifugation at 15,000 *x g* at 4 °C for 15 minutes and resuspended in water to achieve the concentration needed in bioassays.

4.3.2 Insect bioassays

All insect bioassays were conducted in a climatic room at 25 °C with a photoperiod of L16:D8.

Lacewings used in bioassays were provided by the insect rearing facility of the University of Sassari where a colony of *C. agilis* established in 2016 from field-collected individuals is maintained according to methods described by Pasqualini (1975) with some adaptations (Loru et al., 2013). *Tenebrio molitor* L. (Coleoptera*:* Tenebrionidae) larvae (mealworm beetles) and *Musca domestica* L. (Diptera: Muscidae) adults employed in bioassays were furnished by the same insect facility.

4.3.2.1 Toxicity bioassays

Ingestion assays were conducted exposing lacewing adults and larvae to bacterial suspension drops because it represents the way they may contact a bioinsecticide sprayed on plants against pests.

C. agilis larvae of each instar (1st, 2nd, and 3rd), immediately after moult, were placed individually into plastic jars (2 cm diameter x 3 cm high) where a 4-µl drop of a 20 % fructose solution

incorporating *B. laterosporus* spores (10^9 spores/ml) was administered daily for 5 d. From the sixth day on, larvae of *T. molitor* (1 larva per day) were provided to lacewing larvae as food. Mortality was assessed daily for 10 d.

Newly emerged male and female *C. agilis* adults were maintained individually in plastic jars (2 cm diameter and 3 cm high) and exposed daily to a 4-µl drop of a 20 % fructose solution incorporating *B. laterosporus* spores (10^9 spores/ml). Honeybee pollen was provided *ad libitum* to each adult beginning on the sixth day and insect viability was verified daily for 10 d. These experiments included 50-60 individual *C. agilis* adults and larvae.

Experiments also were conducted on *M. domestica* adults in order to verify the pathogenicity of the spore suspensions used in experiments with lacewings. Newly emerged *M. domestica* adults were grouped into four groups of 10 individuals per cage (10 x 10 x10 cm) and exposed to drops (10 µl/fly/day) of a 30 % saccharose solution containing *B. laterosporus* spores (10^9 spores/ml) from the same spore culture used in the lacewing experiments. Insect mortality was checked daily for 7 d, comparing treated flies and untreated controls (Mura and Ruiu, 2017).

4.3.2.2 Sub-lethal bioassays

Lacewing larvae and adults were challenged for 5 d with *B. laterosporus* spores as previously described in the toxicity bioassays. Surviving individuals were maintained in the laboratory to investigate the possible sub-lethal effects.

To test immature stages, second and third instar larvae surviving treatments (n = 20-30), were maintained individually in jars, fed on *T. molitor* larvae (1 larva/individual/day), through pupation and adult emergence. Treated and control insects were checked daily, and the dates of larval moult, pupation, adult emergence, and death during the bioassay, were recorded.

Individual adult insects surviving *B. laterosporus* challenge (n = 20) were transferred into larger plastic cages (10 cm diameter x 10 cm high) with two sides covered with gauze to allow ventilation.

Treated and control females were paired with treated and control males (1 pair per cage), respectively, to allow mating and oviposition. The inner surface of the cage was covered with paper on which females could lay their eggs, which were removed and counted daily. During this period, lacewing adults in the cage were provided *ad libitum* with honeybee pollen and a piece of cotton soaked in water. Death of each male or female individual in a cage was recorded during the bioassay to assess insect longevity. A group of eggs (n=10) from each control and treated cages was analysed to evaluate hatching rate. For this purpose, eggs were kept individually in a jar for a week up to hatching. This analysis was repeated 5 times during the bioassay with different cohorts of eggs.

4.3.2.3 Tritrophic bioassays

In this experiment, mealworm beetles were used to evaluate the possible indirect effects on *C. agilis* larvae feeding on an insect prey previously exposed to *B. laterosporus*. Mealworm beetles were maintained for minimum 1 week on sterile dry wheat bran without a source of water to encourage their subsequent feeding on a 2-cm diameter ball of bran moistened with a *B. laterosporus* suspension (10^9 spores/ml) or water (untreated control), replaced daily. Treated mealworm beetles were maintained under these conditions for at least 3 d before being administered to lacewing larvae.

Second instar *C. agilis* larvae (n = 30) were maintained individually in plastic jars and fed treated or untreated (control) mealworm beetles, replaced daily (1 larva/individual/day) for 7 days. From the eighth day on, larvae were fed on untreated mealworm beetles and were inspected daily to assess mortality, date of moult, pupation, and adult emergence. Pupal weight was also recorded.

4.4 Statistical analysis

Data were analysed using R software (R Development Core Team, 2016).

We used *t*-tests to compare data means of treated and control groups of different experiments.

General Linear Models (GLM) of ANOVA, followed by Least Significant Difference (LSD) tests for post-hoc comparison of means when needed, were used to analyse data on immature (larval and pupal) development time and percentage of adult emergence in experiments involving direct feeding of *B. laterosporus* to either second or third instar lacewing larvae.

4.5. Results

4.5.1 Toxicity bioassays

Direct feeding by different lacewing larvae instars and adults for 5 d on a fructose solution containing *B. laterosporus* spores did not result in mean mortality levels that were significantly different from untreated controls ($F_{7,33}$ = 0.53; P = 0.8091). Larval mortality ranged on average between 7 and 10 % for treated insects and between 3 and 6 % for the controls, while mean adult mortality was 9-13 %, for both treated and control insects (Fig. 1).

The insecticidal properties of the spore suspensions were confirmed in bioassays with *M. domestica*, producing 100 % adult mortality within 5 d in comparison with the control mortality of approximately 5 % (t = 39.19; df = 4; P < 0.001).

4.5.2 Sub-lethal bioassays

The sublethal effects on lacewings that survived exposure to *B. laterosporus* for 5 d during the second or third larval instar are shown in Table 1. Larval development from egg hatch to pupation was not affected by treatment in comparison with control ($F_{3,59}$ = 0.24; P = 0.8699). Pupal development was significantly influenced by exposure of second instar larvae to *B. laterosporus* ($F_{3,59}$ = 5.04; P = 0.0037). An average reduction in pupal development of approximately 1 d was observed (Treatment: $F_{1,59}$ = 6.00; P = 0.0176; interaction Instar x Treatment: $F_{1,59}$ = 9.11; P = 0.0039). Adult emergence of treated insects did not differ from control ($F_{3,12}$ = 0.12; P = 0.9458).

Viability and reproductive performance of lacewing *C. agilis* adults fed *B. laerosporus* spores incorporated in a fructose solution are shown in Table 2. Exposure to the bacterium did not cause significant changes in the longevity of either sex (males: t = 1.088; df = 30; P = 0.1426; females: t = -0.56; df = 24; P = 0.2900). Treated lacewing females that survived exposure to the bacterium exhibited fecundity levels comparable with control (t = 0.939; df = 15; P = 0.1955). Similarly, no differences in egg hatch rate were observed in treated and control groups (t = 0.138; df = 10; P = 0.4466).

4.5.3 Tritrophic bioassays

The sub-lethal effects on immature lacewing development after second instar larvae were exposed to mealworm beetles treated with *B. laterosporus* are reported in Table 3. Treatment did not result in significant changes in development time of second (t = 0.535; df = 23; P = 0.2988) and third (t = 0.713; df = 20; P = 0.2421) instar larvae, nor were there differences in pupal weight (t = -1.141; df = 17; P = 0.1349). A slight though not significant slowing of pupal development time was observed in treated lacewings (t = -1.637; df = 11; P = 0.0649). Treatment of larvae reduced the average percentage of adult emergence by approximately 12 % in comparison with the untreated control (t = 1.964; df = 6; P = 0.0400).

4.6 Discussion

Brevibacillus laterosporus showed only slight effects on *C. agilis* larvae and adults directly exposed to bacterial spores at concentrations that cause high mortality in Diptera (Lethal $Concentration_{100}$) (Ruiu et al., 2007a; Marche et al., 2017). The bioassay methods used in this study involved the administration of spores incorporated in a fructose solution to simulate the way in which chrysopids would contact *B. laterosporus* as an insecticide sprayed in the field. Additionally, the continuous exposure to bacterial drops in the laboratory can be considered an extreme condition, given that

natural feeding behavior of *C. agilis* larvae and adults may significantly reduce possible contacts with a bioinsecticide applied in the field. Chrysopid adults typically feed on plant resources such as pollen, nectar, and honeydew, so could be attracted by sugary baits, but the insecticidal formulations might be less attractive for these non-target insects (Wang et al., 2011). In addition to food preferences, contact between non-target insects and the active ingredient in the field may be affected by specific behaviors related to repellency or, more generally, to avoidance (Cordeiro et al., 2010).

Lacewing larvae feed on insect prey, which may pose an additional barrier to direct contact between the beneficial insect and the entomopathogen. When *C. agilis* larvae were offered mealworm beetles treated with *B. laterosporus* as food, no significant effects on development until pupation were observed. However, slight changes in adult emergence rate were detected, suggesting a possible indirect effect of treated mealworms, due to the bacterium or, possibly due to deteriorated quality of the treated prey as food (Eubanks and Denno, 2000). Previous laboratory studies showed that development and survival of beneficial arthropods feeding on hosts that were pre-fed with a diet containing entomopathogenic bacteria like *Bacillus thuringiensis* could be affected under specific conditions (Blumberg et al., 1997; Ruiu, et al., 2007b; Salama et al., 1991; Sterk et al., 1999).

The general safety of *B. laterosporus* for *C. agilis* we recorded for this study is in line with results of several investigations conducted with *B. thuringiensis* and its insecticidal toxins against different chrysopid species. Given its highly specific mode of action, crystal toxin Cry1Ab from *B. thuringiensis* was found to have no direct effects on *C. carnea* after ingestion (Romeis et al., 2004), which is corroborated with no detected interaction of Cry toxins with midgut receptors of this non-target species (Rodrigo-Simón et al., 2006). On the other hand, significant differences between treated and control insects were observed in different laboratory conditions (Hilbeck et al., 1998). Similarly, several laboratory experiments assessing the safety of genetically modified (GM) plants,

mostly expressing Cry proteins from *Bt*, for beneficial arthropods, have led to variable and sometimes conflicting results, probably in relation to the use of different non-target species and experimental conditions (Lövei et al., 2009). However, such effects observed in the laboratory are expected to be attenuated in natural field conditions (Bourguet et al., 2002).

The entomopathogenic action of bacteria on their targets is often complex and involves the combined effect of several toxins and virulence factors (Glare et al., 2019). Accordingly, the pathogenicity of *B. laterosporus* against invertebrate pests may rely on a wide range of molecules including enzymes (i.e., chitinases, proteases), insecticidal toxins homologous to Cry proteins, polyketides, and nonribosomal peptides (Glare et al., 2019). The insecticidal potential of the *B. laterosporus* strain employed in this study and the range of toxins and virulence factors it can express are well documented (Marche et al., 2018). The lack of toxicity and only slight sublethal effects we observed on *C. agilis* fed extreme spore concentrations support the compatibility of *B. laterosporus* with this non-target species. According to our findings, the use of this bacterium in the pest management context appears promising.

It is not fully understood how *B. laterosporus* specifically evolved as a pathogen for certain insect species, while it is weakly active or inactive in others (Ruiu et al., 2007b). Among beneficial insects, microbiome studies revealed that *B. laterosporus* is a common resident of the honeybee body (Marche et al., 2017) and a beneficial role in favour of bee health was proposed, as a result of its antagonism against honeybee pathogens (Bartel et al., 2018; Marche et al., 2019 a and b).

Based on the present knowledge, *B. laterosporus* can be considered a selective microbial species with potential for integrated pest management programs. Further research in the laboratory and in the field is needed to screen a wider range of non-target species in order to evaluate the safety of this microorganism in different agroecosystem contexts.

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4.9 Tables and figures

Table 4.9.1 – Means (± SE) of larval development time, pupal development time, and percentage of adult emergence of *Chrysoperla agilis* exposed to *Brevibacillus laterosporus* at different developmental stages.

Treated lacewing larval instar	Development time (days)		Adult emergence[c] %
	Larvae[a]	Pupae[b]	
2nd instar			
Treated	18.4 ± 0.47 a[d]	9.8 ± 0.16 a	82.5 ± 4.74 a
Control	18.8 ± 0.37 a	11.1 ± 0.37 b	80.0 ± 2.36 a
3rd instar			
Treated	18.9 ± 0.64 a	10.7 ± 0.33 ab	82.5 ± 3.03 a
Control	18.7 ± 0.58 a	10.2 ± 0.25 ab	85.0 ± 3.73 a

[a] calculated from egg hatching to pupation

[b] calculated from pupation to adult emergence

[c] calculated on the initial number of larvae

[d] Different letters in a column indicate significantly different means (GLM ANOVA, followed by LSD test, $P < 0.05$).

Table 4.9.2 - Means (± SE) of longevity, fecundity, and percentage of egg hatching of *Chrysoperla agilis* adults surviving exposure to *Brevibacillus laterosporus*

Treatment[a]	Longevity (days)[b]		n[c]	Eggs/female	Egg hatching[d] %
	Male	Female			
Treated	26.5 ± 2.02 a[e]	27.9 ± 1.98 a	19	352.9 ± 32.85 a	77.1 ± 5.65 a
Control	24.7 ± 2.46 a	31.1 ± 2.28 a	19	291.9 ± 56.14 a	78.3 ± 6.54 a

[a] Newly emerged adults were exposed for 5 days to a 20 % fructose solution containing *B. laterosporus*.

[b] Days from adult emergence to death.

[c] Number of ovipositing females.

[d] Egg hatching was evaluated at different time intervals during the oviposition period. Mean values are reported.

[e] Means in each column followed by different letters, are significantly different (*t*-test, $p < 0.05$).

Table 4.9.3 - Means (± SE) of larval instar and pupal development time, pupal weight, and percentage of emergence of *Chrysoperla agilis* exposed to mealworm beetles treated with *Brevibacillus laterosporus*

Treatment	Larval development time[a] (days)		Pupal weight (mg)	Pupal development time[b] (days)	Adult emergence[c] %
	2nd instar	3rd instar			
Treated	4.8 ± 0.22 a[d]	4.2 ± 0.20 a	7.6 ± 0.20 a	11.2 ± 0.32 a	72.5 ± 1.58 a
Control	4.9 ± 0.24 a	4.4 ± 0.23 a	7.1 ± 0.36 a	10.6 ± 0.15 a	82.5 ± 1.44 b

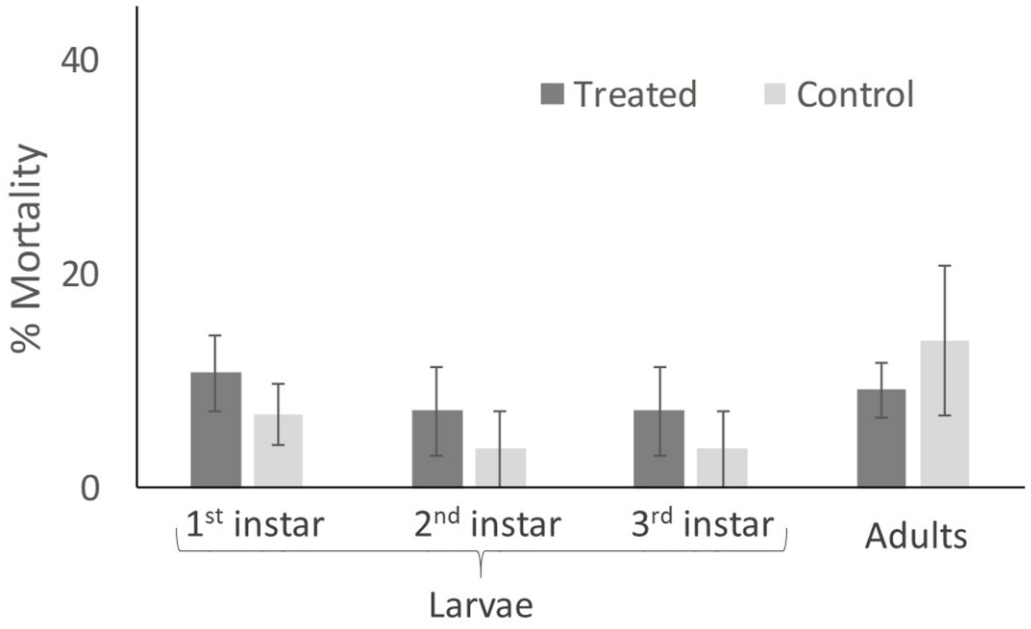
[a] time between moults

[b] calculated from pupation to adult emergence

[c] calculated on the initial number of larvae

[e] Means in each column followed by different letters, are significantly different (*t*-test, $p < 0.05$).

Fig. 4.9.1 – Mean (± SE) percentage mortality of *Chrysoperla agilis* larvae and adults after 5 days exposure to *Brevibacillus laterosporus* spores. No significant differences between means were found (GLM ANOVA, followed by LSD test: P > 0.05).



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