# SUBSTANCES INHIBITING THE GROWTH OF *PAENIBACILLUS LARVAE LARVAE* IN HONEYBEE COLONIES

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## 1. Introduction

The honeybee disease known as American foulbrood is a serious economic problem for beekeepers. It is caused by the bacterium *Paenibacillus larvae* subsp. *larvae* (formerly *Bacillus larvae*) (9). Honeybee larvae become infected by swallowing spores of *P. larvae larv*ae that contaminate their food. After spores germinate in the midgut of a larva, the vegetative forms penetrate the tissue of the intestine, start to multiply and finally kill the larva.

In areas of the world where the main role of commercial beekeeping is to provide pollination services, the disease can be controlled with antibiotics. However, where beekeepers harvest honey, pollen, propolis and royal jelly, the possibility of antibiotic residues in food for human consumption is of great concern. The use of antibiotics to control American foulbrood is forbidden in several European countries, and closely regulated in many other regions. In such areas, beekeepers faced with a heavy outbreak of this disease typically burn all infested colonies and suffer heavy economic losses. Thus, alternate control methods would be of great value.

One tactic has been to investigate genetic differences among honeybee colonies, as a basis for selective breeding or some other genetic approach to the problem. In some colonies the adult workers are more likely to cannibalise diseased and dead larvae or remove them from the colony before the pathogen is able to sporulate again, and the cycle of infection is stopped. Adult honeybees never become infected, not even the nurse bees that have extremely close contact with larvae and transfer the bacterial spores to them.

Different lines of honeybees showed different larval mortality rates following inoculation with spores of *P. larvae larvae* (16). Feeding spores to larvae of different ages, Bamrick and Rothenbuhler (3) found that larvae of a resistant line were no longer susceptible after about 36 hours of age, while larvae of a susceptible line did not become resistant until the age of about 48 hours. In general, vegetative stages of the bacterium were found in a higher percentage of young infected larvae than in larvae of intermediate age or older, after approximately the same amount of time for spore germination (5).

Different hypothesis have been proposed to explain the resistance of individual bees. It was assumed that the spores need a low redox-potential for germination, a condition found in the gut of young larvae (2). Also the peritrophic membrane (a protecting tubule between the gut interior and the enterocytes establishing the inner layer of the midgut) plays an important role in inhibiting the multiplication or penetration of the pathogen (4). Pollen as well as spores of *Paenibacillus larvae larvae* are filtered out of the adult crop by the proventricular valve and transported to the midgut (1); the more highly this function is developed in the adults, the lower is the risk for the larvae they feed with crop content to get infected (20).

Our project (7; 14; 19; present study) has investigated the mechanisms of resistance to *P. larvae larvae* and to develop a reliable and inexpensive method of quantifying this resistance in a colony.

Riessberger-Gallé et al. (14) found that one or more non-induced, temperature-stabile substances in the midgut of honeybees have the potential to suppress the germination of spores and the growth of the vegetative stage of *P. larvae larvae*.

Crailsheim and Riessberger-Gallé (7) tested whether these non-induced substances are produced by the bees themselves or originate in food substances. Midgut extract taken from 8d old adults that had been kept in an incubator and fed an artificial diet with no natural bee products did suppress the growth of *P. larvae larvae,* but not as strongly as the extract taken from sisters who lived in a regular colony. It was shown that the midguts of nurse bees (8d old) had a higher growth inhibiting potential for *P. larvae larvae* than midguts of freshly emerged adult bees or foragers.

The differences in the amounts of growth inhibiting substances found in adults (7) and larvae (14) from different colonies might contribute to observed genetic differences between colonies in resistance or susceptibility to American foulbrood.

## 2. Materials and methods

For our experiments we used a wild strain of *P. larvae larvae* form Styria (Austria) (14). Identification of *P. larvae larvae* was carried out by incubation on Columbia sheep blood agar, followed by the catalase and the ”Plagemann” tests (giant whips could be identified in the liquid part of Columbia sheep blood slant agar) (13).

Bacteria were cultivated for two days in brain-heart-infusion and frozen at –70°C in 1ml aliquots until used. Then 40ml of brain-heart-infusion was inoculated with 1ml of defrosted suspension, heated at 77°C for 10min to eliminate rods that might have survived freezing and to activate spores to germinate, and incubated at 37°C for about 48 hours. The culture was at the beginning of the exponential phase of growth when it was used for inoculation standardised by dilution at an optical extinction of 0,20-0,21 measured at 546nm (11), pathlength 1cm. Optical extinction was measured as a quantification of the cloudiness caused by the growth of the bacteria *P. larvae larvae* (method: 6).

During preliminary trials we tried to test the antibacterial activity of different bee-derived substances. We transferred soaked test carriers on Columbia sheep blood plates inoculated with *P. larvae larvae* or on a Columbia agar two-layer medium (11) containing *P. larvae larvae,* and tried to measure the inhibition zones. As *P. larvae larvae* grew very irregularly (Fig. 1) it was not possible to perform exact inhibition zone measurements. For this reason we used a liquid medium for our further investigations.

To characterise the chronological growth of *P. larvae larvae* (Fig. 2), we inoculated test tubes filled with 1ml of brain-heart-infusion with 30µl of the bacteria suspension with an optical extinction of 0,20-0,21. The optical extinction of the growing bacteria was measured (546nm) every two or three hours until 27 hours, and also after 38, 48 and 72 hours; the higher the extinction, the higher was the growth rate of bacteria.

Before we started the experiments, samples of honey were collected from all honeybee colonies used in the study, and analysis confirmed the absence of spores of *P. larvae larvae* (8; 15 12). This ensured that there was no infection pressure on the colonies tested.

### 2.1 Samples of larvae

For our investigations samples of larvae from two colonies of *Apis mellifera carnica* POLLMANN were taken out of their cells. Age of the larvae was determined by weight (18; 17) and morphology (10).

Samples of 2-day-old larvae and 5-day-old larvae were taken from two different colonies. Each sample consisted of 130mg of tissue. The tissue was put into an Eppendorf vial, 50µl distilled water was added and the sample was homogenized ultrasonically. Finally 400µl ethanol was added and the sample was stored at 4°C overnight. The next day the suspension was centrifuged, and the supernatant was lyophilised and stored at 4°C until used.

### 2.2 Experimental set-up

To test for antibacterial activity, the lyophilised powder of each sample of larvae was dissolved in 200µl sterile distilled water, and different aliquots of the solutions were added to test tubes filled with 1ml of brain-heart-infusion. We tested five different concentrations (according to different aliquots of the stock solution) of larval extract, corresponding to 2.03 – 32.5 mg larval tissue. Sterile $H_2O$ was used as control.

In each test, two test tubes were supplied with the same concentration of extract from the same pool of larvae. One of the tubes was inoculated with 30µl of bacteria suspension, and the other one served as the blank during later photometric measurement (564nm). As the blank remained clear (no cloudiness) after incubation, there were no spores or vegetative forms of bacteria or fungi present in the added extracts. Tubes were incubated aerobically at 37°C for 24 hours and shaken before measurement.

## 3. Results

It takes a rather long time for *P. larvae larvae* to germinate and to grow, and colony development varies considerably. Grown on Columbia sheep blood agar for three days, *P. larvae larvae* developed round, flat, light grey and lustreless colonies with pronounced difference in size (Fig. 1). More than 40% of the colonies were smaller than 4mm² in surface area, while 2.9% of the colonies were larger than 18mm².

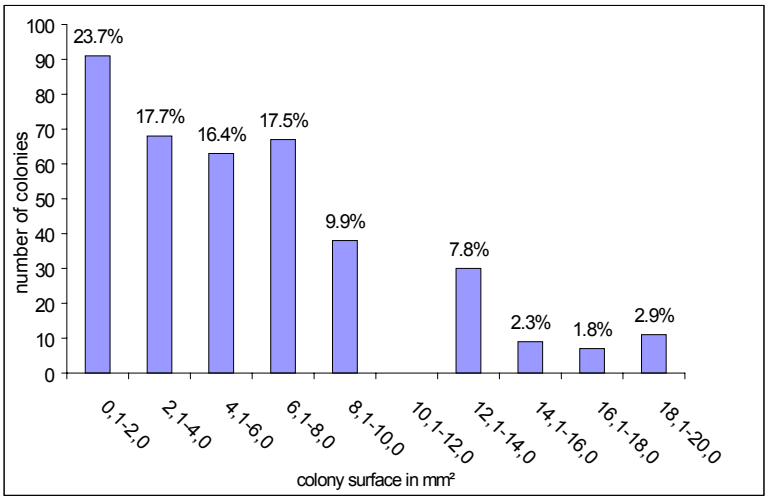


**Fig 1: Distribution of surface areas of *P. larvae larvae* colonies grown on Columbia sheep blood agar and incubated aerobically at 37°C for 72 hours (n=384 colonies).**

Transferred into a liquid medium, *P. larvae larvae* developed as usual for bacteria. The exponential phase of growth, characterised by continuous division of cells, lasted for about 24 hours in our experimental set-up, then the culture reached the stationary phase. Further measurements were carried out: after 27, 38, 48, 65 and 72 hours. As nutritive substances were used up and waste products increased, the stationary phase gave way to the mortification phase after 48 hours of cultivation. Results are summarised in Fig. 2.

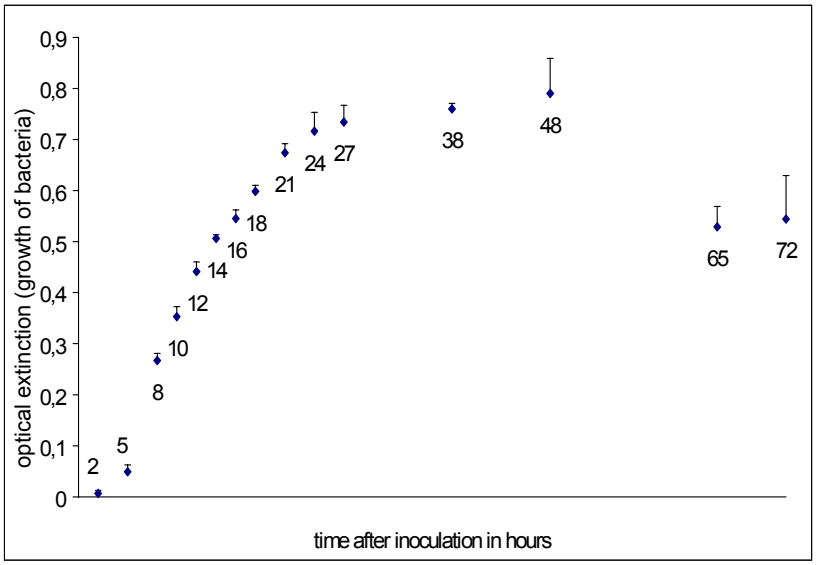


**Fig 2: Chronological growth of *P. larvae larvae* measured at $E_{546}$ of bacterial suspension after different lengths of time of aerobic incubation at 37°C.**

The larval extracts differed in the inhibition of growth of *Paenibacillus larvae larvae*. For diluted extracts of young (2d) larvae, the most extreme difference, between colonies A and B, was significant ($P<0.05$; Mann – Whitney U-test; n=6) (Fig 3).

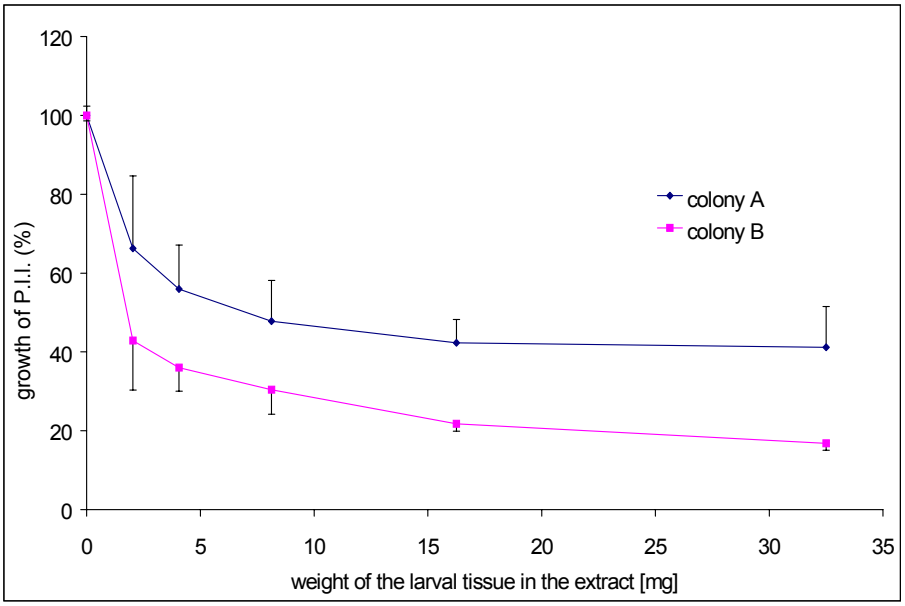


**Fig 3: Inhibiting effect (measured at $E_{546}$ of bacterial suspension after 24 hours of aerobic incubation at 37°C) of extract of 2-day-old larvae from two different colonies on the growth of the vegetative stage of *P. larvae larvae* (for each weight of larval tissue, growth inhibition was significantly different between the two colonies).**

Significant differences between some of the tested colonies were also observed in extracts from 5-day-old larvae ($P<0.05$; Mann – Whitney U-test; n=6) (Fig 4).

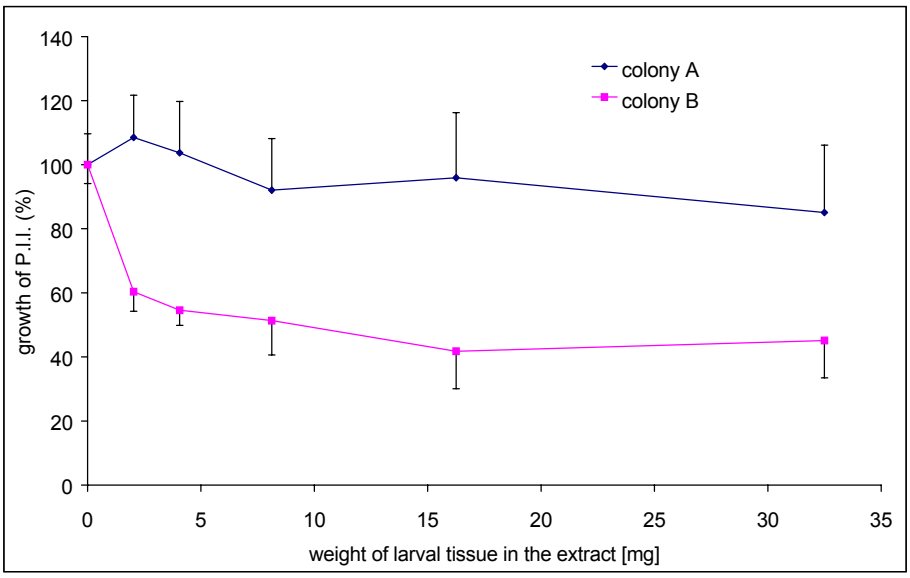


**Fig 4: Inhibiting effect (measured at $E_{546}$ of bacterial suspension after 24 hours of aerobic incubation at 37°C) of extract of 5-day-old larvae from two different colonies on the growth of the vegetative stage of *P. larvae larvae* (for each weight of larval tissue, growth inhibition was significantly different between the two colonies).**

## 4. Discussion

Our results demonstrate a method of testing a colony's resistance to *P. larvae larvae* without infection of brood cells in the laboratory or in the colony. The level of resistance of each larval stage can be tested separately, and results are available within a few days. The only requirement is a laboratory that can maintain sterile conditions and is equipped with a flow bench and an incubator.

As in the adults, in the larvae there is a non-induced mechanism (honey samples of all colonies were free of *P. larvae larvae* spores) that inhibits the development of the bacterium. This was demonstrated for larval stages from the age of 30 h to 5 days (7; and present study). The growth inhibition is much weaker in the older larvae, and is not significant in capped larvae, but the protection is less important as a larva nears pupation, when new infection through feeding is impossible.

In the future we will test differences in resistance in larvae of colonies of various origins and life history (for example, larvae from apiaries with colonies with clinical symptoms, as well as *P.l.l.*-free apiaries). We also plan to breed relevant lines to test heritability of this kind of resistance. We also hope to determine chemical nature of the substance in the gut that inhibits bacterial growth.

## 5. Acknowledgements

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1 Amadeus Grant (France)
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