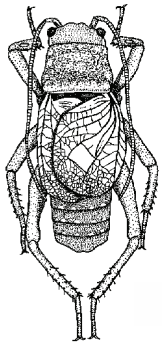

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EFFECTS OF DIET AND FEED COMPOSITION ON ANTIBACTERIAL ACTIVITY OF HEMOLYMPH OF SAPROXYLIC BEETLES: A CASE STUDY OF *ZOPHOBAS ATRATUS* (COLEOPTERA: TENEBRIONIDAE)

V. S. Veremenko[1)], M. T. Khandy[1,2)], E. A. Shevchenko[1)], A. V. Grinchenko[1,3)], V. V. Kumeiko[1,3)], A. V. Kuprin[2*)]

1) *Far Eastern Federal University, Russky Island, Vladivostok, 690922, Russia.*

2) *Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, 690022, Russia. *Corresponding author, E-mail: kyprins@mail.ru*

3) *A.V. Zhirmunsky National Scientific Center of Marine Biology, Far East Branch of the Russian Academy of Sciences, Vladivostok, 690041, Russia.*

Summary. Effects of the diet on the antibacterial activity of hemolymph of *Zophobas atratus* (Fabricius, 1775) larvae cultivated in laboratory conditions are described. It is shown that plasma specimens of *Z. atratus* larvae grown on an artificial fungi-based diet have a prominent antibacterial activity as compared to plasma specimens of larvae grown on a diet of standard feed, so it can be suggested that the diet composition intensifies the synthesis of efficient concentrations of bactericide molecules to the hemolymph. It is found that the hemolymph fraction below 30 kDa inhibits the growth of gram-negative bacteria *E. coli* ATCC 25922, but has no activity towards gram-positive bacteria *B. subtilis* ATCC 6633=DSM 347.

Key words: saproxylic beetles, mass rearing, antibacterial activity, hemolymph, Coleptera, *Zophobas atratus*.

В. С. Веременко, М. Т. Ханды, Е. А. Шевченко, А. В. Гринченко, В. В. Кумейко, А. В. Куприн. Влияние режима питания и состава корма на антибактериальную активность гемолимфы жуков сапро-ксилофагов на примере *Zophobas atratus* (Coleoptera: Tenebrionidae) // Дальневосточный энтомолог. 2022. N 458. С. 1-12.

Резюме. Приводятся данные о влиянии режима питания на антибактериальную активность гемолимфы личинок *Zophobas atratus* (Fabricius, 1775), культивируемых в лабораторных условиях. Показано, что образцы плазмы личинок *Z. atratus*, выращенных на искусственной «грибковой» диете, обладают выраженной антибактериальной активностью по сравнению с образцами плазмы личинок, выращенных на стандартном корме, поэтому, можно предположить, что состав диеты, вызывает усиление процесса синтеза эффективных концентраций бактерицидных молекул в гемолимфу. Установлено, что фракция гемолимфы ниже 30 кДа ингибирует рост грамотрицательных бактерий *E. coli* ATCC 25922, но не проявляет активность в отношении грамположительной бактерии *B. subtilis* ATCC 6633=DSM 347.

INTRODUCTION

There are multiple data concerning the effects of various parameters of the environment and the feed composition on the development of many groups of insects in laboratory conditions (Nakamura, 2002; Saska *et al*., 2014; Lopatina, 2018; Lopatina & Gusev, 2019). There is no doubt that nutrition is the primary factor affecting the growth and development as well as weight gain of insects (Tschinkel, 1981; Ichikawa & Kurauchi, 2009; Zaelor & Kitthawee, 2018; Yi *et al*., 2019). An important internal factor affecting the viability of insects is immunity (Sheldon & Verhulst, 1996; Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2005; Siva-Jothy *et al*., 2005; Wilson, 2005). It is believed that the immune system of xylobiont coleoptera is well developed since they live in decaying timber and detritus rich in all types of decomposers including pathogen microorganisms (Jönsson *et al*., 2004; Bhawane *et al*., 2015; Filipia, 2018).

Recent studies of compounds protecting beetles against pathogen infections have shown that higher orders of holometabolous insects: Lepidoptera, Diptera, Hymenoptera and Coleoptera and some species of heterometabolous insects of the order Hemiptera (Hoffmann *et al*., 1996) accumulate a wide range of antimicrobial peptides (AMP), small bioactive compounds (usually ≤ 20 kDa) (Vilcinskas *et al*., 2012; Chowanski *et al*., 2017; Adamski *et al*., 2019). The primary source of AMPs is the fat body (Hoffmann & Reicchart, 2002). The large size and central position in the body cavity make it a key organ of the immune system, capable of not only synthesizing AMP but also providing efficient concentrations of bactericide molecules to the hemolymph. The hemolymph is the primary indicator of an insect's physiological condition (Blow *et al*., 2019). It is suggested that changes in the diet will have an effect on hemolymph components. In this connection, it is proposed to consider feed a critical factor in the immunity studies of saproxylic insects.

Zophobas atratus (Fabricius, 1775) is a representative of a large family of darkling beetles (Tenebrionidae) including about 20,000 described species. *Z. atratus* are found in tropical regions of the Central and Southern America, while larvae (saproxylic beetles) are trophically associated with large timber remnants of hard wood, and there is indication to feeding by bat excretions (Tschinkel, 1981, 1984; Marcuzzi, 1984). The species has currently been brought to different regions of Europe and Asia, and beetle larvae are used as animal feed in insectaries and zoos, or as a model object in research to solve various problems (Bulet *et al*., 1991; Yuan *et al*., 2012; Fursov & Cherney, 2018; VandenBrooks *et al*., 2020).

This paper is aimed at evaluating the effects of the diet and the feed composition on the antibacterial activity of hemolymph of *Zophobas atratus* larvae cultivated in laboratory conditions.

MATERIALS AND METHODS

Cultivation of Z. *atratus* larvae biomass with various diets. The stock culture was procured in a zoo shop (Vladivostok, Primorsky Krai). Ten pairs of imago were kept in laboratory conditions, in 5L plastic tanks. The bottom of the tanks was lined with 5 cm of Japanese elm sawdust, leaf debris (to maintain moisture) and large branches with bark at the 4th decomposition stage in order to allow beetles to build shelters and lay eggs. Various fruit purees, fresh fruits or vegetables (rarely) were used for additional feeding of imago. The stock culture was sprayed with filtered or distilled water two or three times per week to maintain the moisture level of 60–75%. The laid eggs were transferred to boxes with substrates having two different compositions.

Composition 1. Fungi-based diet (FD) that corresponds to the trophic preferences of darkling beetles in natural conditions of their larvae (saproxylic beetles). To make the artificial diet, a mycelium of *Pleurotus citrinopileatus* Singer was used, deposited in the bioresource collection of the Federal Scientific Center of Biodiversity (Vladivostok). Crushed Japanese elm timber with a relative humidity of 60 to 70%. was used as sawdust. Moistened sawdust was autoclaved at 121°C for 2 hours at the steam pressure of 1 atm. The treated substrate was transferred to sterile containers, and other diet components were introduced as described in detail in our paper (Kuprin *et al*., 2022). The prepared containers with all components of the nutritious diet were cultivated in darkness in a climate incubator (manufactured by Sanyo, MIR-154, Japan) at 25°C and air humidity above 70%, for about 20 days according to the procedure developed by us for *Callipogon relictus* (Yi *et al*., 2017).

The prepared substrate was transferred to sterile tanks (Ferplast Geo Large, China) 30x20x20.3 cm in size, where 1st age larvae were placed in groups of 100 pieces. The substrate surface was sprayed with distilled water 2–3 times per week.

Composition 2. The standard diet (SD) containing wheat flakes, bran, and additionally, fruit skin, carrots and other vegetable remnants were offered to larvae to replenish moisture (Ichikawa & Kurauchi 2009).

Collection of hemolymph from Z. *atratus* larvae and separation of plasma. Live 12th age larvae were selected for the experiment. Since the synthesis of anti-microbial peptides is primarily of inducible nature, to collect hemolymph, a needle was used to pierce the upper part of larva belly or the second pair of legs were torn off, and the transparent liquid was collected using an autosampler by pushing on the abdominal cavity. Hemocytes were removed by centrifuging for 10 minutes at 900 g and 4°C in a 5804 R benchtop centrifuge (Eppendorf, Germany).

Plasma ultrafiltration. A fraction below 30 kDa that was used in antibacterial tests was obtained from plasma using Microcon filters with a nominal molecular weight cut-off of 30 kDa.

Cultivation of microorganisms. Conditionally pathogenic gram-negative bacteria *E. coli* ATCC 25922 and gram-positive bacteria *B. subtilis* ATCC 6633=DSM 347 were used as test microorganisms. These strains are used to control the efficiency of antibacterial drugs in production. For the experiment, microorganisms were cultivated in LB (Lisogeny broth) liquid medium (g/l): peptone 5 g; yeast extract 1 g; NaCl 5 g; 1M NaOH 2 mL; pH = 7.0, for one day with continuous planetary stirring at 37°C.

Disk diffusion test (DDT). Bacteria of the strains under study from the liquid medium were seeded using a swab probe on the surface of agar medium in Petri dishes. Plasma samples were sterilized in vacuum using a MillexR-MP syringe filter (Merck KGaA, Germany) with a pore size of 0.22 μm. Sterile paper disks were impregnated by plasma samples and placed in a moistened condition onto the freshly plated bacteria. The seeded dishes were thermostated at 37°C for one day.

The antibacterial activity was measured after thermostating the dishes according to the size of limited growth zones (presence of single pinpoint colonies or no colonies) around the disks impregnated by plasma samples.

Photometric bacteriostatic test (PBT). The optical density of one day-old suspensions of bacterial cultures was measured in plastic trays with an optical path length of 10 mm using the NanoPhotometer Pearl UV/Vis spectrophotometer (Implen, Germany). Then they were diluted in the growth medium thereof to A600 = 0.05 (optical density at the wave length of 600 nm). Eight wells of a flat-bottom 96-well plate per single type of bacteria were used for each type of control and experimental samples.

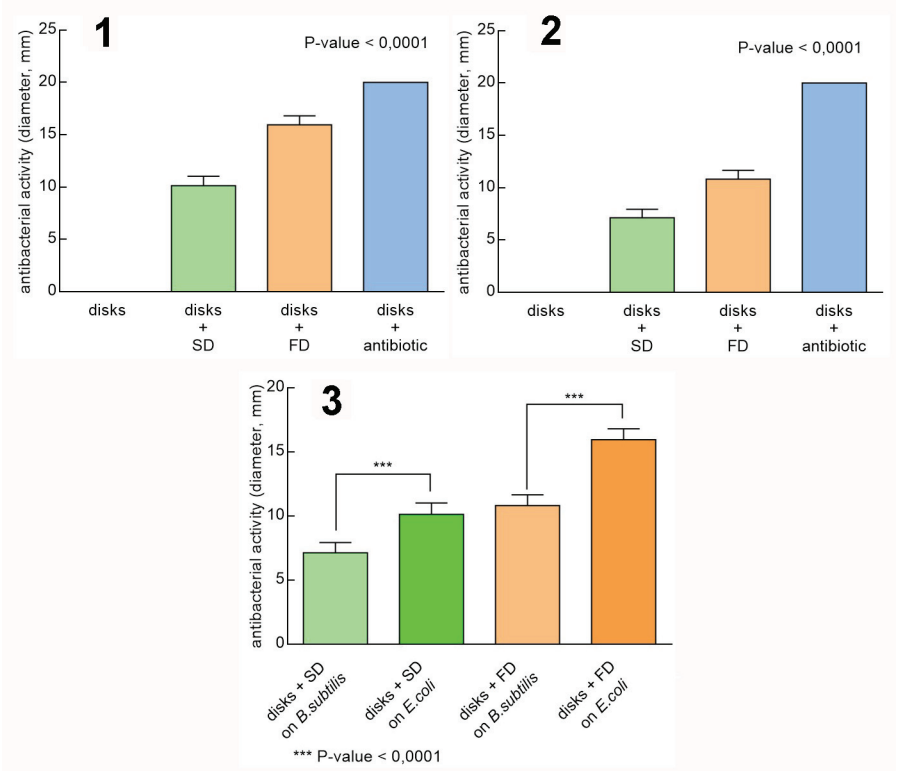
The optical density of bacteria was measured using the Cytation 5 imaging reader with Gen 5 software (BioTek Instruments, USA) in Endpoint mode at the wavelength of 600 nm for one day at 30-minute intervals at the incubation temperature of 37°C while stirring by mechanized agitation before each measurement.

Statistical analysis. The Shapiro–Wilk test was used to check the data for normal distribution, and non-parametric methods was chose as a result. The dispersion analysis of diet effects on the antibacterial activity of larval hemolymph was carried out using the Kruskal–Wallis rank test. To evaluate the differences between two independent samplings, the Mann–Whitney U test was used. The number of repetitions for each sample: 16 in DDT, 49 in PBT. The analysis was conducted using a software package for statistical analysis Statistica 10 (StatSoft, USA) and GraphPad Prism 6 (GraphPad Holdings, USA).

RESULTS AND DISCUSSION

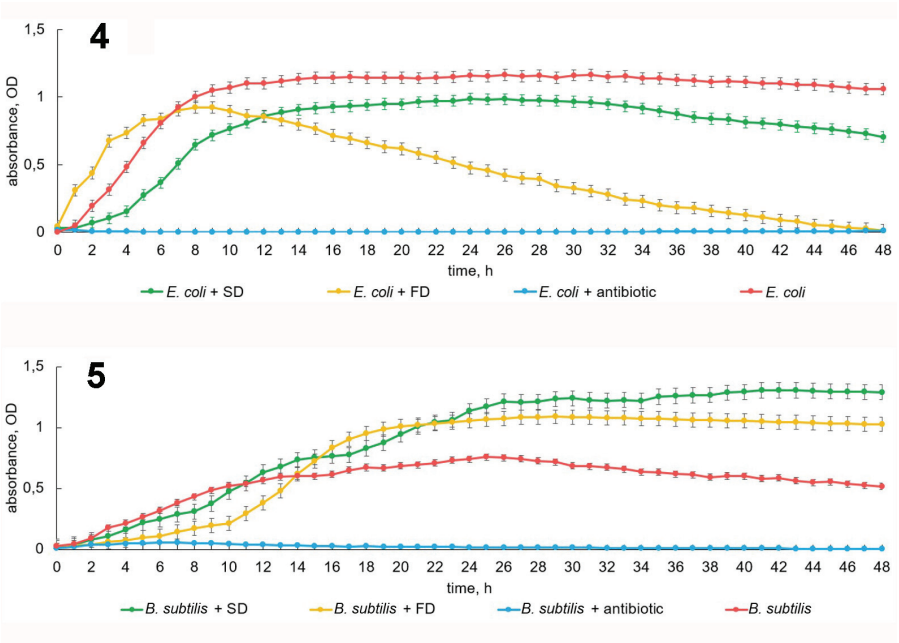
The works were intended to study the diet effects in laboratory conditions on the antibacterial activity of *Zophobas atratus* larval hemolymph. It was shown that larvae of Cerambycidae (*Callipogon relictus* Semenov, 1899) grown individually with an artificial fungi-based diet in laboratory conditions have high survivability (almost 100%) and a reduced period of development unlike other larvae that were cultivated on fodder plant sawdust (Yi *et al*., 2017).

In this study, the antibacterial activity of hemolymph is viewed as an indicator of the immune status of larvae. Comparisons of antibacterial activity of the hemolymph of larvae grown with various diets were done using two antibacterial tests: disk diffusion and photometric.



Figs 1–3. Results of disk-diffusive test. Effects of artificial fungi-based and standard diets on antibacterial activity of *Z. atratus* larval plasma represented here as the diameter (mm) of the growth suppression zone of gram-negative *E. coli* ATCC 25922 (1) and gram-positive *B. subtilis* ATCC 6633=DSM 347 (2) bacteria. Comparison of diet effects (3). The values represent average value ± standard deviation. Negative control is sterile disks, positive control is disks impregnated with antibiotics (cefotaxime at 0.1 mg/mL).

The DDT was used to measure the diameter of growth suppression zones. The resulting data were used to build histograms (Figs 1–3) that provide for the following conclusions: plasma samples of larvae grown with FD have a prominent antibacterial activity as compared to plasma samples of larvae grown with SD with a confidence level below 0.0001 (P-value < 0.0001). All plasma samples much better inhibit the growth of gamma-negative *E. coli* ATCC 25922 than gram-positive *B. subtilis* ATCC 6633=DSM 347 bacteria (3). The test showed the presence of antibacterial activity of hemolymph and correlation thereof with the feed composition. The PBT was used to study the antimicrobial effect of hymolymph over the growth cycle of bacteria.

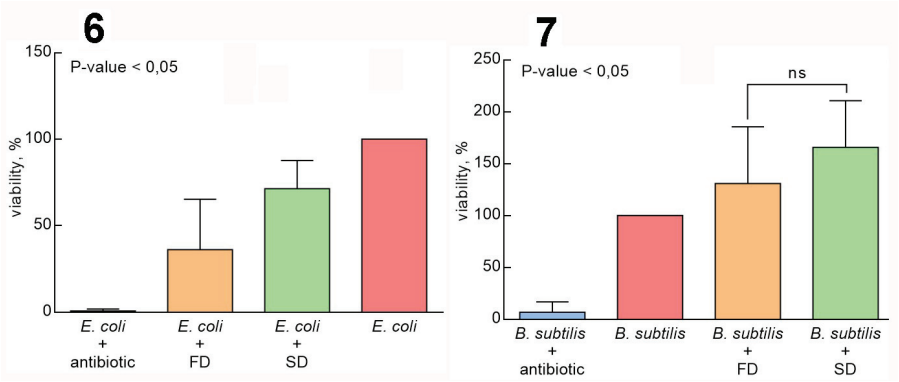


Figs 4, 5. Results of photometric bacterial test. Dynamics of effects of artificial and standard diets on antibacterial activity of hemolymph fraction of *Z. atratus* larvae below 30 kDa within 48 hours represented here as the optical density of gram-negative *E. coli* ATCC 25922 (4) and gram-positive *B. subtilis* ATCC 6633=DSM 347 (5) bacteria at the wavelength of 600 nm. The lower the optical density is, the lower the amount of bacteria is in the suspension. The values represent average value ± standard deviation. Negative control is the suspension of bacteria, positive control is the suspension of bacteria with antibiotics (cefotaxime at 0.1 mg/mL).

The photometric test measured the optical density for each hour of incubation of bacterial cultures with samples. This resulted in growth dynamics charts (Figs 4, 5). As charts show, the hemolymph fraction below 30 kDa exhibited various bacteriostatic properties towards the bacteria strains used.

For gram-negative bacteria *E. coli* ATCC 25922, the chart had growth lines with samples below the normal culture growth. The culture started to grow within the first hour of incubation in the additive-free medium (negative control) and within 4 hours in the presence of an SD sample. In this case, the growth curves were similar and almost parallel but with a lower rate as compared to the control until the end of the experiment. Both in case of control and an FD sample, the optical density of the suspension increased abruptly and nearly synchronously starting with the first hour until the 5th hour. In the control, growth continued until 9 hours, after which the curve reached a plateau. In the presence of an FD sample in the suspension, growth of the culture stopped from 5th to 12th hour, followed by a gradual decline, and approached the positive control (bacteria with antibiotics) by the end of the second day.

For gram-positive bacteria *B. subtilis* ATCC 6633=DSM 347, the chart (Fig. 5) was different as compared to the previously described case (Fig. 4). The samples showed activity only within the first 12 hours of incubation and then served only as a growth-stimulating nutrient substrate. The FD sample showed a longer activity than SD samples, inhibiting growth of the culture by two more hours (8th to 10th), followed by an abrupt exponential growth.



Figs 6, 7. Results of photometric bacterial test. Viability of gram-negative *E. coli* ATCC 25922 (6) and gram-positive *B. subtilis* ATCC 6633=DSM 347 (7) bacteria. The values represent average value ± standard deviation. Negative control is the suspension of bacteria, positive control is the suspension of bacteria with antibiotics (cefotaxime at 0.1 mg/mL).

Similar results are given in 48-hours viability histograms (Figs 6, 7). As to *E. coli* ATCC 25922, the FD samples decreased the amount of bacteria almost by half (52%) as compared to the negative control, and the SD sample decreased it only by 35%. As to *B. subtilis* ATCC 6633=DSM 347, the results are opposite. Both samples increased the amount of bacteria: FD by 10%, SD by 50%.

The opposite effect on gram-positive and gram-negative bacteria can be explained by a specific feature of cellular walls. It is thicker in gram-positive bacteria (20 to 60 nm), homogeneous with a higher, as compared to gram-negative bacteria, concentration of murein (up to 40x). It should be noted that in some cases the humoral

immunity of insects is somewhat para-specific: depending on the nature of pathogen, AMPs of different families are synthesized (Hancock *et al*., 1999), active against specific strains and bacterial groups.

However, from a practical point of view, the search of compounds active against gram-negative bacteria is much more important. Due to the multilayer cellular wall and the presence of an organic external membrane consisting of proteins, phosphotides and lipopolyssacharides, gram-negative bacteria have a higher non-specific antibiotic resistance.

The high antibacterial activity of the hemolymph of larvae grown with FD shows that the diet is an important factor of immune activity and mediates its link with physiological functions. This result correlates with those shown for Egyptian cotton budworm (*Spodoptera littoralis*) and can be related to the shared mechanisms of adaptation to the habitat in various groups of insects (Lee *et al*., 2008).

Particular mechanisms of the obtained results can be interpreted in different ways. First of all, humoral mechanisms of immune response in insects include activation of proteolytic cascades in plasma, dominated by the synthesis of antimicrobial peptides (Briggs, 1958; Glupov, 2009). Surface structures of the pathogen act as a powerful trigger of immune response. It is suggested that AMP biocide activity is defined by protonated aminogroups whose positive charge causes the binding of the biopolymer with anion components of surface structures in microorganism cells (lipopolyssacharide of gram-negative bacteria) by means of electrostatic interaction (Hancock, 1995; Zasloff, 2002). In view of these facts, it is possible that some microorganisms that triggered the immune response observed in the tests made their way to the insect organism together with the diet.

Secondly, in addition to pathogenic exogenous microorganisms, there is conclusive evidence that various non-pathogenic microorganisms can consistently or temporarily inhabit the hemolymph in various insects (Jaenike *et al*., 2010; Xie *et al*., 2010; Xie *et al*., 2013; Hamilton *et al*., 2015; Paredes *et al*., 2016; Ballinger & Perlman, 2017; Blow *et al*., 2019). The hemolymph is the primary way of translocation of nutrients within the insect body, as well as the primary place of storage of nutrients. Therefore, the supply of nutrients being part of the diet may act as a substrate for the development of microorganisms that are part of the hemolymph microbiome. To prove this hypothesis, microbiome studies are required.

The third suggestion of a high antibacterial activity of larvae hemolymph with FD is based on the hypothesis: proteins maintain or activate the immune system (Lee *et al*., 2006), and improved nutrition leads to fat accumulation and intensive development of the fat body that is the primary source of plasmatic proteins (Palli & Locke, 1988; Hoffmann & Hoffmann, 1990; Hoffmann & Reicchart, 2002). In accordance with this hypothesis, it may come as no surprise that those larvae that were kept on the standard diet had lower internal reserves of protein and, therefore, lower efficiency of the pathogen resistance mechanisms. High metabolic expenditures for processing the standard diet consisting mainly of cellulose lead to limited energy reserves (Karowe & Martin, 1989).

Currently, there are many papers studying the effects of diets on the immunity of various groups of insects. Various approaches were used: decreasing the amount of food (Siva-Jothy & Thompson, 2002), controlling the amount of specific nutrients in the diet (Stoehr, 2007), balancing primary microelements (Lee *et al*., 2006), and adding various vegetable materials (Ojala *et al*., 2005; Kapari *et al*., 2006; Klemola *et al*., 2007). So, the quality of feed defines the physiological mechanisms of insects in infection resistance. Considering the immune competence for detritophagous animals, this paper is fundamental for understanding the links between feed and organismal functions in saproxylic beetles and is the first report on immune status regulation in insects of this group.

CONCLUSION

First data are obtained on the antibacterial activity of the hemolymph of *Z. atratus* larvae cultivated in laboratory conditions for various diets and feed compositions. Larvae grown on a fungi-based diet corresponding to trophical preferences of beetles in natural conditions have a hemolymph with antibacterial activity against gram-negative bacteria. These data are confirmed by DDT and PBT. We found that the hemolymph fraction below 30 kDa inhibits the growth of gram-negative bacteria *E. coli* ATCC 25922, but has no activity against gram-positive bacteria *B. subtilis* ATCC 6633=DSM 347.

Further studies and search for optimal conditions for mass production of insects will provide not only new sources of proteins for fodders for agricultural animals, but also antibacterial compounds of a wide spectrum of activity, and will help develop lines of saproxylic larvae with an improved immune system and high level of AMP expression.

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