AGRICULTURAL BIOLOGY,
2024, V. 59, Iss. 3, pp. 571-581
[SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA]

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Waste-to-profit technologies

UDC 638.45:636.087

doi: 10.15389/agrobiology.2024.3.571eng
doi: 10.15389/agrobiology.2024.3.571rus

INFLUENCE OF ANTIBIOTIC CEFTRIAXONE ON THE DEVELOPMENT OF THE YELLOW MEALWORM BEETLE (*Tenebrio molitor* L.)

E.V. MECHTAEVA ✉**, A.V. ZEROV, V.V. DZYUBENKO, P.N. SOROKOUMOV, M.V. NOVIKOVA, D.S. RYABUKHIN, A.Z. ZHURAVLEVA, V.Yu. SITNOV**

All-Russian Research Institute for Food Additives — Branch of Gorbatov Federal Research Center for Food Systems RAS, 55, Liteyny prospekt, St. Petersburg, 190000 Russia, e-mail mechtaeva.lisa@gmail.com (✉ corresponding author), zerovaleksei1995@gmail.com, torrid18@yandex.ru, sorokoumov_pavel@mail.ru, mariazakharova@bk.ru, rdms@bk.ru, aigul-zhuravleva@mail.ru, v.sitnov@fncps.ru

ORCID:
Mechtaeva E.V. orcid.org/0000-0001-7879-7994
Zerov A.V. orcid.org/0000-0003-4296-5225
Dzyubenko V.V. orcid.org/0000-0002-8145-122X
Sorokoumov P.N. orcid.org/0000-0002-8767-3720
Novikova M.V. orcid.org/0000-0002-4195-0649
Ryabukhin D.S. orcid.org/0000-0001-5345-0038
Zhuravleva A.Z. orcid.org/0009-0003-2683-269X
Sitnov V.Yu. orcid.org/0000-0003-1927-1997

The authors declare no conflict of interests
Acknowledgements:
This research was part of the research topics № FGUS-2024-0010 and № FGUS-2022-0018.
Final revision received October 23, 2023
Accepted November 13, 2023

Abstract

Antibiotics can enter the soil with waste and affect insects in their natural habitat, moreover they are often found in feed for farm animals, which is also used for rearing insects for food and feed. In addition, the possibility of bioconversion of agricultural waste with larvae is currently being widely studied. Such waste may contain significant amounts of antibiotics, which affect the growth, development and microbiota of insects used to process it. Ceftriaxone is a third-generation cephalosporin antibiotic. The influence of cephalosporin antibiotics on insects is little studied, while it depends on both the insect species and the type of antibiotic. The yellow mealworm beetle is an ediable insect and a pest of grain products, most often found in the bins of flour warehouses, bakeries, mills, and feed. In this work, the effect of different concentrations of the ceftriaxone on the weight and survival rate of the *T. molitor* larvae, several parameters of larvae composition, as well as metamorphosis and the number of offspring was shown. The ability of *T. molitor* to consume the feed substrate or process waste containing ceftriaxone was demonstrated for the first time. The aim of this work was to study the effect of the antibiotic ceftriaxone on the growth, development and reproductive performance of the yellow mealworm. The experiments were carried out in the insectarium of the All-Russian Research Institute for Food Additives (St. Petersburg, Russia) from July to October 2022. Before the experiment, the insects were reared at 25±2 °C and relative humidity of 50±10 %. A diet for yellow mealworm were composed of wheat bran (16.0 g proteins, 4.0 g fats, 65,0 g carbohydrates per 100 g of the product) and carrot as a source of water. The effect of ceftriaxone (Biosintez, Russia) on insects at concentrations of 0, 0.1, 1, 10, and 100 mg/kg of feed (wheat bran) dry weight was studied. To obtain the feed with certain antibiotic concentration the following procedure was carried out: ceftriaxone solutions with required concentration were added to wheat bran in a 1:1 ratio and thoroughly mixed, then the feed was dried in a convection dryer UF110plus (Memmert GmbH & Co.KG, Germany) at 40 °C for 24 h. The experiment included three stages: the study of the growth rate of larvae, the calculation of the number of pupated and turned into adult insects, and the assessment of the number of *T. molitor* offspring. Feed conversion ratio (FCR) and efficiency of conversion of digested food (ECD) were calculated. FCR and ECD were calculated given wet feed (bran + carrots) and for bran weight only. The water content of the larvae was determined by drying a sample in the Vacuum Oven OV-12 (Jeio Tech, Korea) at 40 °C. To determine the ash content of the larvae, dried samples were placed in crucibles calcined to constant weight and burned in a SNOL 8.2/1100 muffle furnace (Umega Group, AB, Lithuania) using the following calcination regime: 60 min at 250 °C + 6 h at 550 °C. The fatty acid composition was determined by gas chromatography with mass spectrometry detection on a gas chromatograph Varian 450-GC GC (Varian Chromatography Systems, Netherlands) with a mass spectrometer detector Varian 240-MS (Varian, Inc., USA). Methylation and extraction of fatty acids from samples dried in the Vacuum Oven OV-12 at 40 °C was carried out using a 15 % solution of sulfuric acid in methanol and chloroform. The results of the study showed that the addition of ceftriaxone to the diet of the yellow mealworm beetles at a concentration of 100 mg/kg led to the acceleration in weight gain by the larvae (p = 0.029). No relationship was observed between *T. molitor* larval survival rate and antibiotic concentration (p > 0.05). In addition, no statistically significant differences were found between the fatty acid composition, feed conversion ratios, ash content and water content of the larvae fed on wheat bran with different ceftriaxone concentrations (p > 0.05). There was no relationship between the antibiotic content (0-100 mg/kg) and the productivity of the yellow mealworm, and no changes were found in the processes of pupation and transformation into imago when the antibiotic was added to the feed (p > 0.05). Therefore, *T. molitor* can be reared on a diet containing ceftriaxone while maintaining the vitality and basic characteristics of insects necessary for their further use. In addition, these results mean that ceftriaxone does not adversely affect the large mealworm beetles as a pest of grain crop products.

Keywords: *Tenebrio molitor*, yellow mealworm, antibiotics, ceftriaxone, larvae, imago, adults, oviposition

Antibiotics are used in agriculture to prevent and treat infectious diseases and to stimulate animal growth. Addition of antibiotics to drinking water and feed for farm animals leads to their accumulation in tissues [1], milk [2], feathers [1] and the emergence of antibiotic-resistant bacteria in the gastrointestinal tract, as well as in excrement [3]. Up to 90% of some antibiotics are eliminated from the body without being metabolized [4]. They enter the soil with manure and from there can be absorbed by plants [4]. Antibiotics also affect insects in their natural habitat and are often found in livestock feed used on food insect and feed insect farms. In addition, the possibility of bioconversion of manure and other waste from livestock farms using insect larvae is currently being in focus. Such waste may contain significant amounts of antibiotics (0.0004-1420.8 mg/kg) [4, 5], which, in turn, affect the growth, development and microbiota of insects used for their processing [6-8].

The mealworm (*Tenebrio molitor* L., Coleoptera: Tenebrionidae) is a very common pest of grain production, but also an edible insect, the consumption of which is permitted in a number of countries, including the European Union [9]. The mealworm is approx. 40% protein, 30% fat [10] which includes linoleic, oleic and palmitic acids [11], and aslo contains vitamins and minerals [12]. Much work has been devoted to the use of *T. molitor* larvae in feed for farm animals and in aquaculture [10, 13, 14]. The mealworm can feed on grain, flour and bran and often damages grain stocks by spreading into feed mills, degrading product quality. The possibility of using *T. molitor* for processing plant waste was also shown [13].

Few studies have been devoted to the effect of cephalosporin antibiotics on insects, antibiotics from the tetracycline, sulfonamide, and quinolone groups are more often considered, although the degree of influence depends on both the insect species and the type of antibiotic [15, 16]. Ceftriaxone is a third-generation cephalosporin antibiotic [2] that is primarily used in humans but is also effective in animals.

This work shows for the first time the effect of different concentrations of the antibiotic ceftriaxone in the feed substrate on the weight and survival of *T. molitor* larvae, some parameters of the larvae chemical composition, metamorphosis and the number of offspring. For the first time, the ability of *T. molitor* to consume feed substrate or process waste containing ceftriaxone has been established.

The purpose of the work was to evaluate the effect of the antibiotic ceftriaxone on the growth, development and reproduction of the mealworm.

Maerial and methods. The experiments were carried out in the insectarium of the All-Russian Research Institute of Food Additives from July to October 2022. Mealworm larvae were taken from the stock colony of insects kept in the insectarium. Before the experiment, insects were raised at a temperature of 25±2 °C and a relative air humidity of 50±10%. Wheat bran was used for feeding (nutrition value per 100 g of product: proteins 16.0 g, fats 4.0 g, carbohydrates

65.0 g; GOST 7169-2017. Moscow, 2018) with the addition of carrots as a source of moisture.

The concentrations of the antibiotic ceftriaxone (Biosintez, Russia) chosen for the study were 0; 0.1; 1; 10 and 100 mg/kg dry weight of feed. To obtain feed with a certain antibiotic content, aqueous solutions of ceftriaxone of the required concentration were prepared, added to wheat bran in a 1:1 ratio with stirring, then the bran was dried in a UF110plus convection oven (Memmert GmbH & Co.KG, Germany) at 40 °C for 1 day with occasional stirring.

The experiment was performed in three stages: studying the growth rate of larvae, counting insects that pupated and turned into adults, and assessing the oviparous activity of T. molitor.

At the first stage of the experiment, 200-250 larvae of *T. molitor* at the age of 5-7 weeks (the exact number and weight of insects in each sample were known) were placed in 200 ml plastic containers containing 40.00 g of dry feed with various concentrations of antibiotic. The containers were kept in an IN75plus thermostat (Memmert GmbH & Co. KG, Germany) at 25±1 °C and 60±5% humidity for 4 weeks. Wet feed (carrots) was given twice a week, 1.50 g in the first 2 weeks of the experiment, 3.00 g in the second 2 weeks. For each antibiotic concentration, experiments were carried out in 6 biological replicates. One and two weeks after the start of the experiment, two containers for each concentration of ceftriaxone were removed from the thermostat, the larvae were separated from the feed, counted and weighed, and then placed back. After 4 weeks, when the first pupae began to appear, all containers were removed from the thermostat. Excrements, feed and insects were separated by sifting on sieves with different hole diameters (0.25; 0.5; 1; 3 mm), all components were weighed separately on a GR-200 analytical balance (AND Co., LTD, Japan). The larvae were counted and 100 individuals were returned to containers for the second stage of the experiment (3 replicates out of 6), the remaining larvae were frozen at –20 °C.

At the second stage, 100 insects left after the first stage were cultured under similar conditions for 6 weeks, 10.00 g of feed was added to each container, 1.50 g of carrots were given twice a week. For each antibiotic concentration, biological replicates were 3-fold. Every 2 weeks, all insects were removed from the thermostat, separated from the feed, divided by developmental stages (larvae, pupae, adults), counted and weighed. After 6 weeks, insects were removed from containers and frozen. Excrement and eggs that were laid by the beetles from the moment of their appearance until the end of the second stage of the study remained in the containers.

At the third stage, 40.00 g of crushed wheat bran without antibiotic was added to containers with eggs of mealworm, laid at the second stage, and the egg were incubated for 1 month in a thermostat at 25 °C. Carrots were given out twice a week in abundance. At the end of the experiment, all larvae (second generation) were separated from the feed substrate, counted and weighed. Progeny were quantified by dividing the total number of 2nd generation larvae by the number of adults that were in the container from the time they emerged until the end of the second phase of the study, and by the total number of weeks that the adults spent in the containers.

The feed conversion ratio (FCR) was calculated by the formula:

$$FCR = (m_{feed\ initial} - m_{feed\ final})/(m_{larvae\ final} - m_{larvae\ initial}),$$

where $m_{feed\ initial}$ is initial mass of feed added to the container, g; $m_{feed\ final}$ is the mass of feed remaining after the end of the experiment, g; $m_{larvae\ final}$ is total mass of larvae after the end of the experiment, g; $m_{larvae\ initial}$ is initial mass of larvae placed in a container, g.

The efficiency of conversion of digested food (ECD) was calculated as

$$ECD = (m_{larvae\ final\ dry} - m_{larvae\ initial\ dry})/(m_{feed\ initial\ dry} - m_{feed\ final\ dry} - m_{excrements\ final\ dry}),$$

where $m_{larvae\ final\ dry}$ is final dry weight of larvae, g; $m_{larvae\ initial\ dry}$ is initial dry weight of larvae, g; $m_{feed\ initial\ dry}$ is initial dry weight of feed, g; $m_{feed\ final\ dry}$ is final dry weight of feed, g; $m_{excrements\ final\ dry}$is dry weight of excrement at the end of the experiment, g.

FCR and ECD were calculated with and without accounting wet feed (carrots), i.e., by weight of bran only.

The water content in the body of the larvae was determined by drying a sample of insects on pre-weighed aluminum cups in a Vacuum Oven OV-12 vacuum drying oven (Jeio Tech, Korea) at 40 °C to constant weight, and was calculated using the following formula:

$$\text{Water content} = (m_{sample\ before\ drying} - m_{sample\ after\ drying})/m_{sample\ before\ drying} \times 100\%.$$

To determine the ash content of the larvae, dried samples were placed in crucibles heated to constant weight and burned in a muffle furnace SNOL 8.2/1100 (Umega Group, AB, Lithuania) for 60 min at 250 °C + 6 h at 550 °C. Ash content was calculated using the equation:

$$\text{Ash content} = m_{sample\ after\ combustion}/m_{sample\ before\ combustion} \times 100\%.$$

Calculation of feed conversion coefficients and conversion of digested feed, water and ash content assessments were carried out after 4-week incubation (first stage) in 6 biological replicates, for ash content in 4 replicates).

The fatty acid composition was determined by gas chromatography with mass spectrometric detection on a Varian 450-GC gas chromatograph (Varian Chromatography Systems, the Netherlands) with a Varian 240-MS mass spectrometric detector (Varian, Inc., USA). The following materials and equipment were used: capillary column WCOT fused silica 50 m×0.25 mm ID Coating CP-WAX 58 (FFAP)-CB DF = 0.2 (Varian, Inc., USA), helium grade 6.0 (Tekhnologiya LLC, Russia), sulfuric acid (reagent grade, Shchekinoazot LLC, Russia), methanol (special gade for gradient HPLC, TD HIMMED, Russia), chloroform (high purity grade, JSC EKOS1, Russia), deionized water, standards for fatty acid methyl esters CRM18918 F.A.M.E. Mix, C8-C24 (Sigma-Aldrich, USA).

Fatty acids from samples dried in a vacuum drying oven Vacuum Oven OV-12 at 40 °C were methylate and extracted with a 15% solution of sulfuric acid in methanol and chloroform. *T. molitor* larvae after 4 weeks of incubation were used for analysis; the experiment was carried out in 6 biological replicates.

For all studied parameters, mean values (M) and confidence intervals (Δ) were calculated at a significance level of $\alpha = 0.05$ using Microsoft Office Excel 2016 software. The statistical significance of the difference between parameters was assessed by one-way analysis of variance (ANOVA) in RStudio software. Differences were considered significant at $p < 0.05$. In this case, a pairwise comparison of the difference between the average values obtained in each experiment was also carried out using Tukey's HSD test (honestly significant differences, test results are indicated using letter indices (a, b).

Results. The antibiotic ceftriaxone added to the feed substrate of *T. molitor* at 100 mg/kg of feed slightly accelerated weight gain by larvae ($p = 0.029$) (Fig. 1). There are no data on the mass of larvae at week 10 of the experiment, since by this moment all the larvae had pupated.

Antibiotics used in livestock production enhance the growth of farm animals [17]. For insects, a decrease in the mass of individuals fed substrates containing antibiotics is more common, or there is no effect. For example, in the work of R.A. Sherman et al. [15] showed that high concentrations of gentamicin led to a decrease in the weight of *Phaenicia sericata* larvae compared to control group, while ampicillin, cefazolin, ceftizoxime, clindamycin, mezlocillin, and vancomycin had no significant effect. The weight of *Musca domestica* larvae decreased when amoxicillin, gentamicin, and levofloxacin were added to the diet [6]. However, there is evidence showing the positive effect of antibacterial drugs on insect growth. For example, the weight of *Bombyx mori* silkworm larvae increased with increasing concentrations of norfloxacin added to the feed, 50 and 100 ppm concentrations were studied) [18].

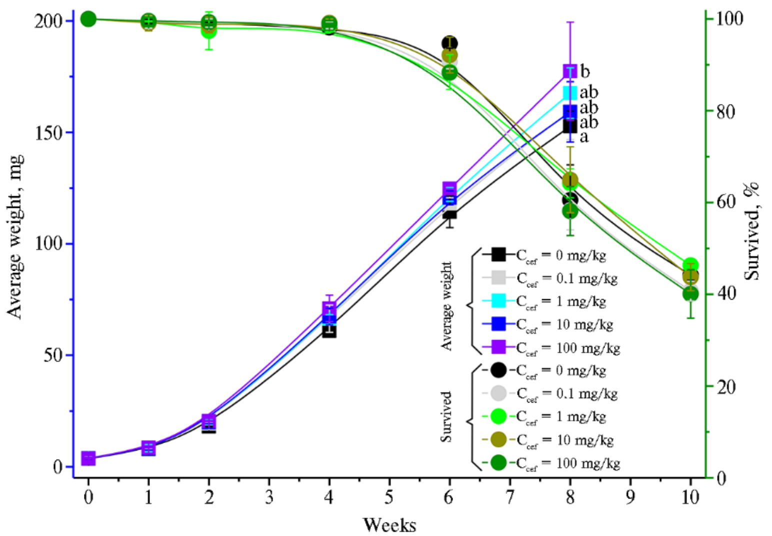

Fig. 1. Survival and average weight of *Tenebrio molitor* larvae cultured on substrates with different concentrations of ceftriaxone (C_{cef}) (*n* = 6, *M*±Δ). Letter indices (a, b) indicate the results of the Tukey test (the same letter for insignificant difference, different letter for significant difference).

The accelerated increase in weight in mealworm larvae when ceftriaxone is added to the feed substrate may be due to the suppression of unfavorable microbiota [19]. For example, D. PreuЯer et al. [20] associate the increased survival of *Calliphora vomitoria* larvae when fed with a substrate containing ceftriaxone and levofloxacin at concentrations of 0.89-28.57 mg/kg with the suppression or slowdown of the development of orgaisms pathogenic for the larvae. In our study, there was no relationship between the survival of *T. molitor* larvae and the antibiotic concentration ($p > 0.05$) (see Fig. 1). The decrease in the survival rate of the *T. molitor* large after 6 weeks of the experiment was caused by the onset of the pupation process which is associated with an increase in insect mortality and a significant increase in cannibalism [21].

Faster weight gain by larvae under the influence of ceftriaxone can be considered as a positive effect in the case of waste processing using *T. molitor*, but this is a negative factor if we consider the mealworm as a pest. Thus, the presence of ceftriaxone in feed invased with *T. molitor* larvae will accelerate the development of the insect and, consequently, spoilage of products.

The antibiotic is primarily active against the microbial community. Although the interaction of microbiota with insects has not been described in detail, its role in their life is highly valued [22]. Based on this, we can assume the effect of the antibiotic on the biochemical composition of the insect's body. In our experiьуте, adding ceftriaxone to the substrate at 0-100 mg/kg of feed had no effect on the fatty acid composition of mealworm. There were no statistically significant differences between the content of the analyzed fatty acids in larvae fed different concentrations of ceftriaxone ($p > 0.05$), with the exception of linoleic acid ($C_{18:2}$). The amount of linoleic acid decreased slightly with increasing antibiotic concentration according to ANOVA ($p = 0.008$), but Tukey's test showed no significant differences (Fig. 2).

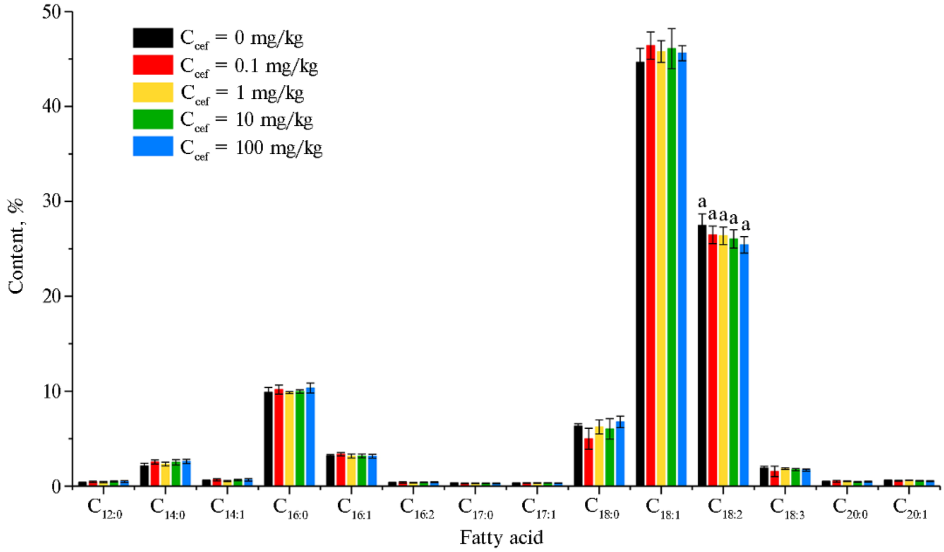

Fig. 2. Fatty acid composition of *Tenebrio molitor* larvae after 4-week culture on substrates with different concentrations of ceftriaxone (C_{cef}) (*n* = 6, *M*±Δ). Letter indices (a) indicate the results of the Tukey test (the same letters mean insignificant difference).

It should be noted that the effect of adding antibiotics to the feed substrate on the composition of insects is currently practically unstudied. According to L. Goane et al. [23], the addition of ciprofloxacin and piperacillin (10 and 200 μg/ml) led to a decrease in the protein and fat content in *Anastrepha fraterculus* adults and did not affect the carbohydrate content. Feed supplementation with roxithromycin, trimethoprim and pesticides had no effect on the fat content of *Hermetia illucens* larvae [7].

We did not find a relationship of the concentration of ceftriaxone in feed with ash content of insects, feed conversion rate, and water content in the body of mealworm larvae ($p > 0.05$) (Table 1).

1. Feed conversion coefficients, water content and ash content of *Tenebrio molitor* larvae after 4-week culture on substrates with different concentrations of ceftriaxone (C_{cef}) (*M*±Δ)

C_{cef}, mg/kg	Water contnt, %	Ash content, %	FCR (bran)	FCR (bran + carrot)	ECD (bran)	ECD (bran + carrot)
0	60.5±1.8	3.8±0.2	2.6±0.1	3.7±0.1	0.39±0.03	0.34±0.02
0.1	58.6±3.2	3.7±0.3	2.8±0.2	3.9±0.2	0.38±0.10	0.31±0.08
1	58.8±2.0	3.4±0.3	2.7±0.2	3.8±0.2	0.37±0.01	0.33±0.01
10	59.6±2.9	3.5±0.1	2.7±0.2	3.7±0.3	0.35±0.02	0.32±0.02
100	59.4±3.2	3.6±0.2	2.6±0.2	3.6±0.2	0.38±0.02	0.34±0.02

Note. ECD — efficiency of conversion of digested food, FCR — feed conversion ratio.
Calculation of ECD and FCR and determination of water content was carried out based on the results of 6 biological replicates (*n* = 6), assessment of ash content based on the results of 4 biological replicates (*n* = 4). No significant differences between values were found ($p > 0.05$).

We also investigated the dependence of the *T. molitor* pupation and transformation into adults on the concentration of ceftriaxone in the feed substrate. The results showed that the metamorphosis of *T. molitor* was not affected by ceftriaxone at a concentration of 0.1-100 mg/kg of feed (Fig. 3). According to the literature data, antibiotics can negatively affect the pupation of insects, slowing down the process or reducing the number of pupae and hatched adults. For example, a mixture of sulfonamides at 10 mg/kg reduced the number of pupae and adults of the black soldier fly [8)] D. PreuЯer et al. [20] showed that a mixture of ceftriaxone with levofloxacin at concentrations of 28.57 and 3.57 mg/kg led to a delay in the pupation of *C. vomitoria*. However, A.C.P. Ferraz et al. [24] demonstrated that gentamicin at concentrations of 4.44; 13.33 and 66.66 mg/ml does not affect pupation and hatching of *Chrysomya putoria* adults.

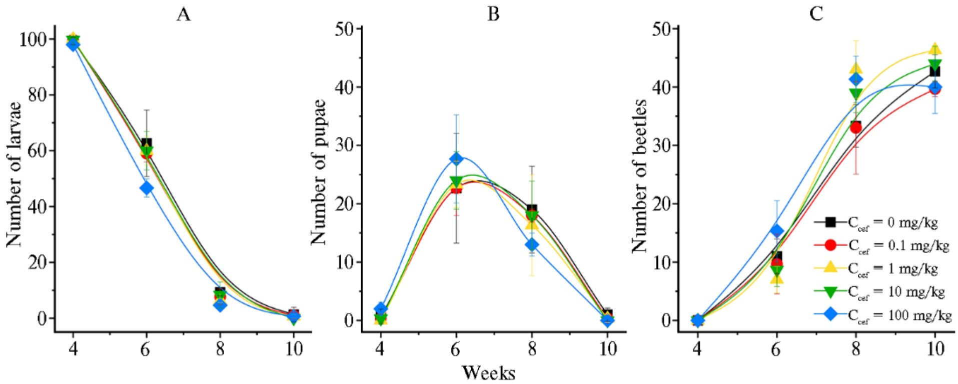

Fig. 3. Number of larvae (A), **pupae** (B) **and imagos** (C) **of *Tenebrio molitor* cultured on substrated with different concentrations of ceftriaxone** (C_{cef}) ($n = 3$, $M \pm \Delta$).

The number and average weight of larvae of the 2nd generation of the mealworm also did not depend on the addition of an antibiotic to the feed substrate ($p > 0.05$) (Table 2). The wide range in the average weight of larvae was caused by a significant difference in the number of larvae of the 2nd generation in the biological replicates of the experiment and, as a result. by the unequal amount and different availability of feed for each individual during growth. The difference in the number of larvae was probably due to the unequal ratio of male and female individuals in the replicates.

2. Number and average weight of the 2nd generation larvae of *Tenebrio molitor* cultured on substrates with different concentrations of ceftriaxone (C_{cef}) ($n = 3$, $M \pm \Delta$)

C_{cef}, mg/kg	Larvae of the 2nd generation	
	number per 1 beetle per week	average weight, mg
0	1.9±0.4	12.6±0.3
0.1	1.5±0.4	18.2±5.2
1	1.9±0.8	16.3±8.4
10	1.9±0.3	11.9±3.4
100	2.5±0.5	9.6±0.9

N o t e. No significant differences between values were found ($p > 0.05$).

The publications more often describes the negative effect of antibiotics on the egg-laying activity of insects. For example, L. Goane et al. [23] showed that ciprofloxacin (10 µg/ml) and piperacillin (200 µg/ml) reduced the number of eggs laid by *A. fraterculus* females. However, in the same, as well as in some other works [25], the authors indicate that the effect of the antibiotic on the oviposition in insects depends on the nutritional value of the feed substrate and, in the presence of a full set of amino acids necessary for the insect, may be absent. Therefore, negative impacts may occur when *T. molitor* is used to process wastes of low nutritional value. However, in the case of a highly nutritious substrate containing an antibiotic, for example, feed, grain or other grain and crop products, the pest of which can be *T. molitor*, its reproduction will not slow down.

Mealworm larvae contain significant amounts of protein, and their use as an alternative source of protein in food [26, 27] and livestock feed [28, 29)] production is being explored. To effectively grow *T. molitor* for these purposes on an industrial scale, it is necessary to determine the effect of all feed components on the insect growth and development. Many farm animal feeds contain antibiotics, so it is important to understand the likely effects of different antibiotics on the mealworm. For example, it has been shown [30] that an antibiotic produced by *Trichoderma harzianum* and added to the feed substrate of *T. molitor* in an amount of 1000 mg/kg leads to the death of 80% of insects after 17 days.

The results of our study showed that ceftriaxone at concentrations of 0.1-100 mg/kg of feed does not have a negative effect on the development of the large mealy beetle. However, the literature reports that it appears only with a significant increase in the amount of antibiotic. For example, gentamicin at concentrations of 4, 40, 400 μg/ml did not affect the weight of *Phaenicia sericata* larvae, but at a concentration of 4000 μg/ml, the weight and survival of larvae, as well as the proportion of individuals that turned into adults, decreased significantly [15]. The antibiotics oxytetracycline and tylosin had a negative effect on the survival and reproductive capacity of three representatives of soil fauna (*Folsomia fumetaria*, *Enchytraeus crypticus* and *Aporrectodea caliginosa*) only at concentrations above 3000 mg/kg [16].

Thus, the antibiotic ceftriaxone at concentrations of 0.1-100 mg/kg of feed did not have a negative effect on the growth, development and productivity of *Tenebrio molitor*. We did not find a relationship between the survival of *T. molitor* larvae and antibiotic concentration. No changes were found in the pupation and transformation into adults when adding an antibiotic to the feed substrate. The feed conversion ratio, water content in the body of the larvae, ash content and fatty acid composition of *T. molitor* larvae after 4 weeks of culture also did not depend on the concentration of the antibiotic in the substrate. At a 100 mg/kg cefriaxone concentration, there was an increase in the average weight of the larvae. Therefore, for growing mealworms, you can use feed containing ceftriaxone in concentrations up to 100 mg/kg. *T. molitor* is able to process waste that contains this antibiotic, remaining viable with basic parameters necessary for its further use, i.e., weight, survival rate, the chemical composition, ability to reproduce. Also, these results mean that ceftriaxone does not have a negative effect on the mealworm as a pest of grain and crop products.

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