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[Tomke Asendorf](#)*, [Christine Wind](#), [Andreas Vilcinskas](#)

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Article

Testing Analytical Methods for Antibiotic Detection in *Tenebrio molitor* Larvae: A Controlled Feeding Trial

Tomke Asendorf ^{1,*}, Christine Wind ² and Andreas Vilcinskas ^{3,4}

¹ State Institute for Chemical Analysis and Veterinary Diagnostics Freiburg, Am Moosweiher 2, 79108 Freiburg, Germany

² State Institute for Chemical Analysis and Veterinary Diagnostics Karlsruhe, Weißenburger Str. 3, 76187 Karlsruhe, Germany

³ Branch for Bioresources, Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Ohlebergsweg 12, 35392 Giessen, Germany

⁴ Institute for Insect Biotechnology, Justus Liebig University Giessen, Heinrich-Buff-Ring 26, 35392 Giessen, Germany

* Correspondence: tomke.asendorf@posteo.de

Abstract

Background: As edible insects gain importance as sustainable protein sources, their integration into the food system requires that they meet the same safety standards as conventional animal products. This includes systematic testing for residues of pharmacologically active substances, such as antibiotics. To enable such monitoring, validated analytical methods for insect matrices are essential—but currently lacking. This study evaluates whether LC-MS/MS methods already validated for conventional animal products are suitable for detecting antibiotics in edible insects. **Methods:** A feeding trial was conducted with *Tenebrio molitor* larvae using three antibiotics—tiamulin, chloramphenicol, and erythromycin—selected based on prior trace detections, known associations with insect use, or the presence of related resistance genes in insect-based products. The antibiotics were incorporated into feed, and their homogeneity and stability were confirmed. After seven days of feeding and a 24-hour fasting period, larvae were analyzed. **Results:** All three antibiotics were detected in the insects, although residue levels significantly declined after fasting. **Conclusion:** These results demonstrate the applicability of existing methods to insect matrices and highlight the need for further validation. The study provides a reproducible model for further feeding trials and discusses potential drivers of antibiotic misuse in insect farming as a foundation for developing and expanding testing methods to ensure the food safety of edible insects.

Keywords: edible insects; *Tenebrio molitor* larvae; antibiotic residues; LC-MS/MS analysis; feeding trial; food safety monitoring

1. Introduction

With the globally increasing demand for sustainable protein sources, edible insects are gaining growing attention from the scientific community, industry, and policymakers [1–3]. Insects for food and feed production offer numerous ecological advantages, such as high feed conversion efficiency, low greenhouse gas emissions, and the ability to be reared on organic waste streams [4–6]. As a potential alternative to conventional meat products, they are considered a promising solution for easing pressure on the environment and agricultural systems in the context of climate change and a growing global population [7–9].

At the same time, edible insects—as a novel food—face particular challenges regarding food safety and consumer acceptance [10]. Both aspects are essential and mutually reinforcing. Concerns

about the uncontrolled use of substances such as antibiotics have already been raised in public discourse [11].

Indeed, the use of antibiotics such as chloramphenicol has been documented in the context of insect rearing for scientific purposes—for instance, in studies on pest insect control or in the rearing of *Bombyx mori* for silk production [12,13]. The use of silkworm pupae as a by-product of silk production has already been proposed as a potential additional edible insect [14]. Chloramphenicol is banned for the use in food-producing animals under Regulation (EU) No 37/2010 Annex Table 2. Accordingly, reliable detection methods are essential to monitor its potential use in the rearing of edible insects.

Moreover, the planned use of substrates such as manure entails the risk that residues from conventional livestock farming—including antibiotics—could enter insect production systems and ultimately contaminate edible insect products or insect-based feed [15,16]. Conversely, the use of insect frass as fertilizer could pose a risk for the development of antibiotic resistance in soil microbiomes if antibiotics were to be used in insect farming without proper regulation [17–19]. Improper feeding practices, such as the use of by-products from the meat industry, could likewise lead to contamination with antibiotics or other pharmacologically active substances [20].

These uncertainties underscore the necessity of systematically monitoring insect-based food products for residues of pharmacologically active substances, as is already standard practice for other animal-derived foods. The restriction of antibiotic use in conventional livestock production is primarily based on concerns over the emergence and spread of antibiotic resistance [21]. Initial studies have already demonstrated that edible insects can serve as carriers of antibiotic-resistant bacteria [22–25], indicating that the use of antibiotics in insect farming could similarly contribute to the selection and proliferation of resistant strains [26].

As early as 2015, the European Food Safety Authority (EFSA) recommended monitoring edible insects for residues of pharmacologically active substances [15]. Since 2022 by EU law insects have to be included in official control plans. Testing for the pharmaceutical substances listed in the plan is therefore mandatory. Due to the currently low production volumes in Germany, the number of required samples remains limited: only two samples had to be analyzed in total across 2023 and 2024. However, this number is expected to increase in the coming years. By that time at the latest, validated and appropriate analytical methods for the reliable testing of insects must be available [27].

Many questions in this context remain unresolved. Are pharmacologically active substances used at all in insect farming? If so, which active compounds are relevant for commonly farmed edible insect species? Can existing analytical methods be applied, or is there a need to develop new approaches?

As a first attempt to address these questions, our institute screened 14 samples from online retailers in 2020 for various antibiotics using detection methods already validated for animal-based matrices. In total, 75 individual compounds were analyzed, including various tetracyclines, pleuromutilins, and amphenicols. Notably, tiamulin was detected in one of the samples [28].

With the present study, we approached the open questions by initially designing a feeding experiment to gain an idea of whether antibiotics administered to insects can be detected using the analytical methods already established in our laboratories. In this context, we administered the three antibiotics tiamulin, chloramphenicol, and erythromycin to mealworms (*Tenebrio molitor* larvae) and analyzed them using our existing methods. Finally, we discussed various motivations for the use of pharmacologically active substances in edible insect farming, aiming to identify potential entry points for future research.

2. Results

2.1. Impact of Antibiotics on Mealworm Weight

Tiamulin administration resulted in significantly lower weight in mealworms compared to the control group ($p = 0.023$, Mann-Whitney U test). In contrast, erythromycin and chloramphenicol also

resulted in lower weights compared to the control group, but these differences were not statistically significant ($p = 0.219$ and $p = 0.579$, respectively, Mann-Whitney U test). Moreover, while all other groups exhibited an increase in weight over the seven-day period, the group administered tiamulin experienced an average loss of 0.25 g.

Using GC-MS, we detected no residual methanol in the flour of the control groups. There was also no significant weight difference between mealworms in the methanol-treated and untreated control groups ($p = 0.544$, Mann-Whitney U test). The raw data can be found in the supplementary material S2.

2.2. Evaluation of Homogeneity and Stability of Antibiotic-Containing Feed Samples Using LC-MS/MS Analysis

The concentrations of all three antibiotics used exceeded the upper calibration range of the analytical method. Accordingly, the sample weight of the feed samples was adjusted, and the samples were diluted as follows: For the flour samples spiked with tiamulin, 0.5 g instead of 5 g was weighed per sample. Before solid phase extraction (SPE), a 1:20 dilution was performed. For the flour samples spiked with chloramphenicol, 0.1 g instead of 10 g was weighed per sample. We collected the residue using 2 ml instead of 0.5 ml (water/acetonitrile, 80/20, v/v), and diluted the extract 1:10 prior to the SPE measurement. For the flour samples spiked with erythromycin, 0.5 g instead of 5 g was weighed, and the extract was diluted 1:50 before SPE (0.2 ml extract + 9.8 ml McIlvaine buffer with Na-EDTA (pH 4)).

Due to these high concentrations and to establish an initial estimate of the detection limit, we reduced the amount of erythromycin in the feed by a factor of 1000 in a second feeding trial. Thus, the concentration in this case was 31.3 μg erythromycin per 100 g of flour. In this case, 1 g of the erythromycin-flour mixture was weighed for LC-MS/MS analysis.

Following Herrman et al., we assessed the homogeneity of the antibiotics in the flour by calculating the coefficient of variation (CV) of the samples (Table 1) [29].

Table 1. Homogeneity of antibiotics in feed.

	Tiamuli n	Chloramphenico l	Erythromyci n	Erythromycin/100 0
average $\bar{X}$ [mg/kg]	325.9	119	185.8	0.3
SD [mg/kg]	43.4	52	36.2	0.03
coefficient of variation (CV)				
[%]	13.3	43.7	19.5	11.5

The highest CV, and thus the lowest homogeneity, was observed in the flour spiked with chloramphenicol, with a coefficient of variation of 43.7%. The measured concentration of chloramphenicol in the flour was 119 ± 52 mg/kg. The coefficient of variation for the flour spiked with erythromycin was 19.5%, with a measured concentration of 185.8 ± 36.2 mg/kg. In the case of tiamulin and the 1:1000 diluted erythromycin, we achieved a coefficient of variation below 15%. For tiamulin, the CV was 13.3%, with a measured concentration of 325.9 ± 43.4 mg/kg in the flour. The coefficient of variation for the 1:1000 diluted erythromycin was 11.5%, with a measured concentration of 0.3 ± 0.03 mg/kg.

Furthermore, the antibiotics remained stable throughout the experiment under the given experimental conditions (Figure 1). In the control feed group, none of the administered antibiotics were detected.

2.3. Detection and Quantification of Antibiotics in Mealworms Using LC-MS/MS Analysis

We were able to detect and quantify all three antibiotics in the mealworms after 7 days of feeding and an additional 24-hour feed withdrawal period, except for the 1:1000 diluted erythromycin. In this

group only traces of erythromycin below the limit of quantification were detected immediately after the 7-day feeding period. After 24 hours of feed withdrawal, it was no longer detectable. All control groups remained blank.

In the case of chloramphenicol and undiluted erythromycin, the measured concentrations exceeded the calibration curve. Therefore, a 1:100 dilution of the final extracts (10 µl extract + 990 µl water/acetonitrile) was performed for quantification. Since this dilution was still insufficient for erythromycin quantification, the already 1:100 diluted extract was further diluted 1:5.

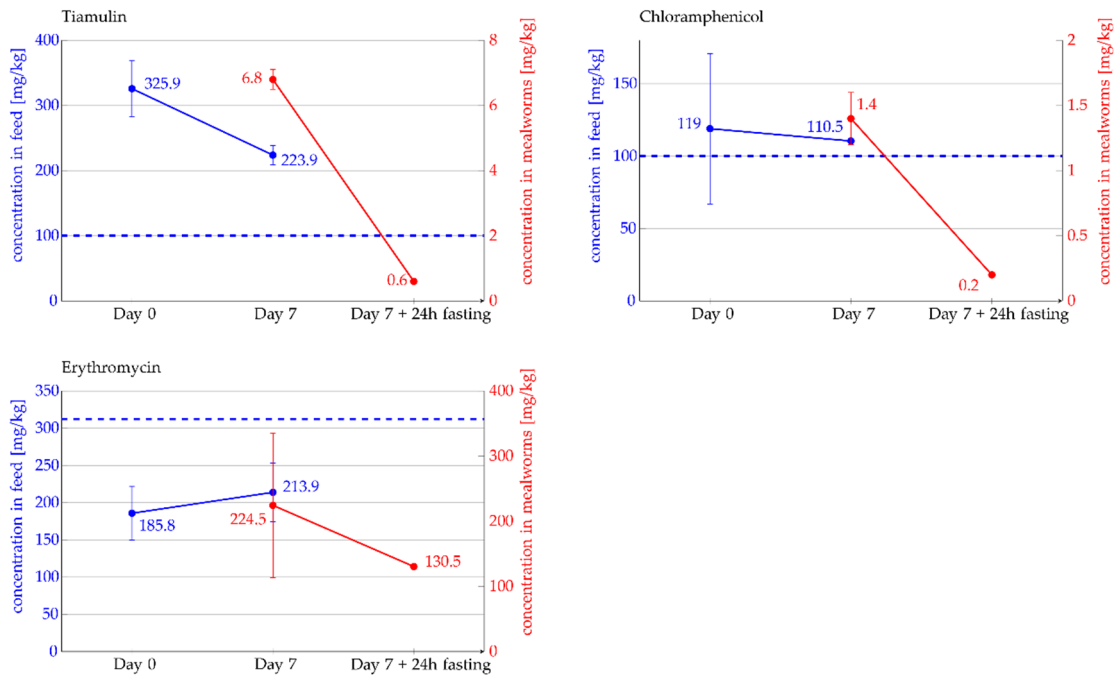


Figure 1. Concentration of tiamulin, chloramphenicol, and erythromycin (undiluted) measured in feed (left y-axis, blue) and in mealworms (right y-axis, red). Error bars indicate standard deviation (SD). The blue, dashed horizontal line represent the target feed concentration (left y-axis).

Figure 1 presents the targeted and actual measured concentration of tiamulin, chloramphenicol, and erythromycin (undiluted) in the feed, as well as the final concentration detected in the mealworms. In the case of tiamulin the concentration detected in mealworms was 6.8 ± 0.3 mg/kg immediately after the 7-day feeding period and decreased to 0.6 ± 0.03 mg/kg after 24 hours of feed withdrawal. This corresponds to a reduction of approximately 90% after 24 hours. As previously described, chloramphenicol exhibited the highest inhomogeneity in the feed, which is evident in Figure 1 from the error indicators at Day 0. In mealworms, we detected 1.4 ± 0.2 mg/kg of chloramphenicol after the 7-day feeding period. After 24 hours of feed withdrawal, the concentration decreased to 0.2 ± 0.002 mg/kg. This means that approximately 85% of the initially detected chloramphenicol was no longer quantifiable after the fasting period. The measured concentration of erythromycin in mealworms was 224.5 ± 111 mg/kg after 7 days of feeding and decreased to 130.5 ± 0.7 mg/kg after 24 hours of feed withdrawal (Figure 1). Due to an outlier in the measurement at Day 7, the results are less clear for erythromycin. The reduction after 24 hours of fasting ranged between 10% and 57%. However, overall, the detectable amount of erythromycin in mealworms also decreased.

3. Discussion

The present study represents an initial attempt to investigate whether the analytical methods currently established in our laboratories for other matrices are suitable for detecting antibiotics in edible insects.

To generate samples that realistically reflect potential market scenarios, we designed a feeding trial using antibiotics for which (i) we have previously detected trace amounts in insects [28], (ii) documented feeding practices involving other insect species exist, such as *Bombyx mori* [13], or (iii) antibiotic resistance genes have been detected in commercially available edible insect products, suggesting a possible prior exposure [30]. Selection was further guided by the availability of validated detection methods in our laboratory.

The results of our trial demonstrate that the applied methods are capable of detecting tiamulin, chloramphenicol, and erythromycin in *Tenebrio molitor* larvae. Based on these findings, the next step will be to validate the employed methods for the insect matrix and subsequently integrate them into routine surveillance procedures.

Subsequently, we, as a state analytical institute, can expand our methodological catalogue to include additional insect-specific substances. The experimental design presented here may serve as a template for the generation of test samples. It would be advisable to include different insect species as well as various processing stages. The homogeneity of the administered antibiotics could be improved by mixing the meal in a laboratory mill after the drying phase rather than grinding it manually with a mortar.

To determine which specific substances we need to anticipate in regulatory monitoring, it is essential to examine which pharmacologically active compounds might be used in insect farming. Insight can be gained by exploring the underlying reasons and motivations for the application of such substances in insects.

Analogous to their use in pig production, these compounds could potentially be employed as growth promoters [31]. While no increase in body weight was observed, Hirose et al. reported positive effects of streptomycin on the development and lifespan of *Nezara viridula* [32]. All three antibiotics examined in our study tended to result in a decrease in body weight. Negative effects from the prophylactic administration of pharmacologically active compounds were also observed by Dickel et al. in *Parasemia plantaginis* larvae treated with fumagillin, including reduced oviposition and suppressed immune competence [33]. Webster et al. similarly reported a reduction in brood rearing in *Apis mellifera* following fumagillin treatment [34]. Cappellozza et al. found that the administration of chloramphenicol did not prevent the proliferation of *Enterococcus mundtii* and may even have promoted it by eliminating competing microorganisms [35]. Furthermore, it is possible that pharmacologically active substances reduce feed palatability, thereby indirectly counteracting weight gain. This could be investigated in analogy to Webster's experiment, in which no effect of fumagillin on syrup intake in honeybees was observed [34]. In folivorous insects such as *Locusta migratoria*, the application of the leaf disc test—used by Holopainen et al. to evaluate the palatability of herbicide-treated maize for *Carausius morosus*—could be considered [36,37]. To assess whether and which substances might be misused as growth promoters in insect farming, these compounds would need to be examined with regard to the same mechanisms known to enhance growth in pigs and poultry. These include, for example, effects on the gut microbiota [38] or non-antibiotic, anti-inflammatory actions [39].

Another motivation for the use of pharmacologically active substances in insect farming may be their prophylactic application to prevent recurrent infections. Relevant insights into which infections are of particular concern in insect rearing are provided by studies such as those by Eilenberg et al., and Gałęcki et al. In 2015, Eilenberg et al. provided an initial overview of diseases in insect farming based on a literature review and a small-scale survey of insect producers [40]. This overview was updated in 2021 by Maciel-Vergara et al., who complemented it with an analysis of potential triggers associated with the identified diseases [41]. In addition, Gałęcki and Sokół discuss parasitic organisms relevant to insect farming, such as *Nosema* spp. and gregarines [42].

Beyond prophylaxis against insect diseases during rearing, reducing the microbial contamination of the final product could also be a driving factor for the use of antibiotics.

An initial risk assessment identifying human-pathogenic microorganisms potentially relevant in edible insect products was conducted by Klunder et al. They examined mealworms and crickets

directly from rearing facilities and identified spore-forming bacteria as potential hazards [43]. A more comprehensive risk assessment was published by the EFSA in 2015 [15]. Additionally, Garofalo et al. provided an extensive literature review on the microbiota of insects covering the period from 2000 to 2019 [44]. As part of the present study, the generated samples were also subjected to microbiological analysis; however, no significant reduction in pathogenic microorganisms was observed in the antibiotic-treated groups compared to the control group (data not published). For this purpose, alternative decontamination methods—such as UV irradiation—may be more effective. The most recent approval of a mealworm product already includes this treatment, even though it is not directly used for microbial reduction in this case (Commission implementing regulation (EU) 2025/89).

The marked decrease in the concentrations of all three administered antibiotics in *Tenebrio molitor* following the 24-hour feed withdrawal period warrants further attention. This feed withdrawal has been mandated in all implementing regulations to date for the various insect products already approved under the Regulation (EU) 2015/2283 (e.g., for *Tenebrio molitor* larvae: Regulation (EU) 2022/169). It is essential that we are able to detect antibiotic residues even after this 24-hour withdrawal period. Since measurements of the feed over the course of the trial showed that the antibiotics remained chemically stable, the observed reduction in the insects must result from insect-internal processes. One possible explanation is that the mealworms excrete large amounts of the antibiotics unchanged. In this context, an analysis of mealworm feces would be of particular interest. Another possibility is that the antibiotics are metabolized within the insect. It is therefore necessary to determine which metabolites are formed. While the pharmacokinetics of the antibiotics used in this study have already been investigated in other livestock species [45–49], no corresponding studies are currently available for edible insects.

Findings from such research could serve as a scientific basis for policymakers in defining maximum residue limits (MRLs) in insects. As of now, there is a complete lack of specific legal regulations concerning the use of antibiotics in insect farming. As outlined in the introduction, no veterinary drugs are currently approved for use in the production of edible insects, and, accordingly, no MRLs have been established for insects (cf. Table 1 of Regulation (EU) No 37/2010). The use of antibiotics in edible insects is currently only described under the cascade principle, as outlined in Article 113 of Regulation (EU) 2019/6. The permissible MRLs in this context can be derived according to Implementing Regulation (EU) 2018/470, which results in applying the lowest specified MRLs to insects. For tiamulin, the MRLs are set at 100 µg/kg (target tissue: muscle in pigs, chickens, and turkeys), and for erythromycin, at 40 µg/kg (target tissue: milk). The use of chloramphenicol is entirely prohibited, as outlined in Table 2 of Regulation (EU) No 37/2010.

Conventional meat consists primarily of muscle, while insect products include the whole animal and are tested accordingly. Therefore, it is imperative to determine whether the MRLs could potentially be aligned with liver values observed in other species. Notably, in pigs, rabbits, and poultry, the highest concentrations of tiamulin are found in the liver [50]. Similarly, it can be hypothesized that the highest antibiotic residues might accumulate in the fat bodies of insects [51,52].

The prohibition of antibiotics as growth promoters in livestock farming was primarily implemented to counteract the emergence and spread of antibiotic-resistant pathogens [21]. Numerous publications have already identified resistance genes in insects and insect-derived products, suggesting that the administration of antibiotics in insects, as observed in conventional livestock, could similarly promote the selection and proliferation of antibiotic-resistant pathogens [30,53–56].

4. Materials and Methods

4.1. Antibiotics

We aimed to select antibiotics with documented associations to insects in the literature or based on our findings in routine testing, and for which a detection method exists in our laboratory. At the same time, we sought to include diverse antibiotics from different active substance groups with

various spectra of activity. For our project, we opted to use pure antibiotic standards rather than complete pharmaceutical formulations. This choice was made because the standards are more readily available and free from unpredictable additives.

The antibiotics used were: Tiamulin (Sigma-Aldrich, USA), chloramphenicol (Sigma-Aldrich, USA), and erythromycin (Sigma-Aldrich, USA).

Tiamulin was previously detected in an insect product in a study conducted at our institute [28]. It is a semi-synthetic derivative of the naturally occurring antibiotic pleuromutilin and is effective against the following pathogens: *Brachyspira hyodysenteriae*, mycoplasma, streptococci, staphylococci, pasteurella, treponema, leptospira, and arcanobacteria [57]. Chloramphenicol has been used in silkworm breeding for some time [13,35]. Its use in edible insect production could therefore be conceivable. Chloramphenicol belongs to the amphenicol group and is effective against a broad spectrum of bacterial infections but is now used only as a reserve antibiotic due to severe side effects. Erythromycin was chosen due to its relevance in antibiotic resistance. Osimani et al. detected erythromycin resistance genes in ready-to-eat edible insects [55]. This could suggest prior exposure to the antibiotic or the widespread presence of resistance genes [58]. Both scenarios highlight the relevance of erythromycin as a target to our analysis, as its presence could further promote the proliferation of resistant bacteria within edible insects. Erythromycin is part of the macrolide antibiotic group and is used, among other applications, to treat various respiratory infections [59].

Edible insects are not explicitly mentioned in Table 1 of Regulation (EU) No 37/2010. Therefore, no veterinary medicines are approved for edible insects. However, some pharmacologically active substances are approved for all food-producing animal species (e.g., erythromycin). Prescribing veterinary drugs for insects is therefore only permissible under the cascade described in Article 113 of Regulation (EU) 2019/6 to prevent undue suffering. For antibiotics in particular, Article 107 of this regulation applies. The use of antibiotics solely for prophylaxis or growth promotion is not permitted. Commission implementing regulation (EU) 2022/1255 does not list any of the antibiotics used. Accordingly, Article 113 of Regulation 2019/6 may be applied. The antibiotics erythromycin and tiamulin used in our experiments may, therefore, be administered to insects in emergencies. Chloramphenicol, however, is prohibited under Table 2 of Regulation (EU) No 37/2010 and may not be used.

4.2. Supplementing Feed with Antibiotics

The tiamulin dose was 10 mg per 100 g of whole wheat flour (dmBio, Karlsruhe, Germany). In the absence of indications from the literature, the dose recommendation of the European Commission from Annex I of Article 35 of Directive 2001/82/EC (EMEA/V/A/137) was used as a guide. Based on Cappellozza et al., 10 mg chloramphenicol per 100 g flour was used [13]. For erythromycin, a concentration of 31.3 mg erythromycin per 100 g flour was employed. This was derived from the publication by Alippi et al. and adjusted to the weight of the mealworms determined in preliminary tests [60].

All antibiotics were initially dissolved in methanol (Merck KGaA, Germany) at a concentration of 1 mg/ml. The dissolved antibiotics were then combined with whole wheat flour in a 2:1 ratio as follows: For tiamulin and chloramphenicol we added 20 ml of the 1 mg/ml chloramphenicol resp. tiamulin solution to 10 g flour. Following the evaporation of the methanol, the flour mixtures were homogenised with a mortar and mixed with 190 g flour, which adds up to 200 g spiked flour each. For erythromycin we added 62.5 ml erythromycin solution (1 mg/ml) to 31.3 g flour. After evaporation, we added 168.7 g flour, which adds up to 200 g flour.

To ensure that the solvent methanol had no effect on the mealworms, we analyzed the control group flour (with and without methanol treatment) using headspace GC-MS (Thermo Trace 1310 GC, ThermoFisher MA, USA, detection limit: 50 mg/kg).

4.3. Feeding Experiment

Mealworms (larvae of the mealworm beetle *Tenebrio molitor*) were obtained from diemehlwurmfarm (Gronau, Germany) and were reared at the State Institute for Chemical Analysis and Veterinary Diagnostics Freiburg. A total of eight boxes, each measuring 15 x 11.5 x 7 cm, were used. Each box was filled with 10 g of mealworm and 10 g of whole wheat flour. Two boxes were filled with flour mixed with tiamulin, chloramphenicol, or erythromycin, respectively. Four boxes served as control groups. In two of them, the mealworms were fed only with flour, while in the other two, the flour was supplemented with methanol, which was then allowed to evaporate, mirroring the antibiotic treatment conditions. To assess the potential impact of antibiotics on the animal's weight, we weighed the mealworms before and after seven days of antibiotic treatment. To obtain a representative average of weight development, we randomly selected and weighed 10 batches of 10 mealworms per group. All larvae were incubated at 27°C and 60–70% humidity in an incubator (Binder, Germany) for seven days. After this period, one box of each substrate type was frozen at -20°C. The remaining boxes were sieved to extract the larvae from the substrate, and the larvae were fasted in the empty boxes for another 24 h, and then frozen at -20°C. All larvae were kept at -20°C for at least 24 hours before processing to ensure all larvae were killed. The four boxes frozen after seven days were sieved to separate larvae from the substrate, as with all other boxes. The larvae were rinsed under running tap water, dried on paper towels, and homogenized in an IKA Tube Mill 100 control (IKA, Germany). Homogenates prepared from the tiamulin, chloramphenicol, erythromycin, and control groups after 0 and 24 h fasting were stored at -20 °C.

4.4. Evaluation of Homogeneity and Stability of Antibiotic-Containing Feed Samples Using LC-MS/MS Analysis

To assess the homogeneity of the antibiotic-feed mixtures, we analyzed five samples of each mixture immediately after preparation. To evaluate the stability of the antibiotics contained in the feed, we analyzed residual feed samples at the end of the feeding trial. Specifically, we examined the sieved feed residues from each experimental group, including tiamulin, chloramphenicol, and erythromycin, collected from both the 0-hour fasting group and the 24-hour fasting group. The feed analysis was conducted following the method 14.1.5 of the Association of German Agricultural Analytic and Research Institutes (Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten e.V. (VDLUFA e. V.)) [61]. The analyses were performed at the department of pharmaceutical residues of the State Institute for Chemical Analysis and Veterinary Diagnostics Karlsruhe, Germany. The only deviation from the standardized method was the use of a drying temperature of 40°C during sample concentration.

Samples were extracted using McIlvaine buffer with Na-EDTA (pH 4) and purified via SPE using Oasis HLB 6cc 200 mg cartridges (Waters, USA). The analysis was conducted using an Agilent 6460 Triple Quadrupole LC/MS system (Agilent, USA). Data evaluation was performed using the Agilent MassHunter 10 software (Agilent, USA).

4.5. Detection and Quantification of Antibiotics in Mealworms Using LC-MS/MS Analysis

The analyses were performed at the department of pharmaceutical residues at the State Institute for Chemical Analysis and Veterinary Diagnostics Karlsruhe, Germany. The method used is validated according to the Commission Implementing Regulation (EU) 2021/808 for various foodstuffs of animal origin and is used in routine testing for antibiotic residues. The adaption of this method for insects is not part of the present study. Therefore, only a brief summary is provided here (for detailed information see supplementary material S1). Homogenized and pre-weighed samples were thawed. After adding 10 mL McIlvaine buffer with Na-EDTA (pH 4), the samples were vortexed for approximately 1 min, shaken for 10 min, sonicated for 5 min, and centrifuged at 3148 g for 10 min. The supernatant was filtered through a folded filter and collected in a 50 mL centrifuge tube. The extraction was repeated with 5 mL McIlvaine buffer with Na-EDTA (pH 4), and the filter was rinsed

with an additional 2 mL McIlvaine buffer with Na-EDTA (pH 4). The entire extract was then subjected to SPE. An OASIS HLB SPE cartridge (6 mL, 200 mg) was conditioned with 6 mL methanol and 6 mL deionized water. The extraction solution was loaded onto the cartridge, washed with 6 mL ultrapure water/methanol (95/5, v/v), and dried under vacuum for 10 min. Elution was performed with 6 mL methanol into a centrifuge tube. The eluate was evaporated to dryness under a nitrogen stream at 40°C. The dry residue was reconstituted in 1 mL ultrapure water/acetonitrile (90/10, v/v) using vortex mixing and ultrasonication. After centrifugation at approximately 15000 rpm, the solution was transferred to an HPLC vial for LC-MS analysis or stored frozen until measurement. The identification of target analytes in the samples was based on mass spectrometric data, specifically the detection of at least two MS² fragment ions, and retention time comparison with corresponding standards. Each sample was initially processed and analyzed once. If a target analyte was detected, its concentration was determined using a matrix calibration curve established with control samples. In cases of critical initial findings, multiple additional sample preparations were performed and analyzed. To generate the matrix calibration curve, blank samples (e.g., five replicates) were fortified with different concentrations of the analyte, processed, and analyzed. The final result was obtained by averaging the individual measurements.

5. Conclusions

In summary, the findings of this study highlight the feasibility of detecting antibiotics in edible insects using established analytical methods. The experimental design provides a valuable foundation for future feeding trials, which will be necessary to expand the methodological repertoire to include insect-relevant substances, as well as to analyze the pharmacokinetics and pharmacodynamics in insects.

Such efforts will contribute to a robust scientific basis for developing maximum residue limits, establishing appropriate detection methods, and safeguarding public health in the context of edible insect production.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Supplementing material S1: Test procedure for the determination of antibiotics in animal-origin foods (excluding honey) using LC-MS/MS; Supplementing material S2: Weight development of *Tenebrio molitor* larvae after antibiotic administration.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

EFSA	European Food Safety Authority
SPE	Solid Phase Extraction
CV	Coefficient of Variation
MRL	Maximum Residue Limit

VDLUFA Association of German Agricultural Analytic and Research Institutes (Verband deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten e.V.)

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