Effects of veterinary drugs on rearing and safety of black soldier fly (*Hermetia illucens*) larvae

E.F. Hoek-van den Hil*, M.G.M. van de Schans, G. Bor and H.J. van der Fels-Klerx

Wageningen Food Safety Research (WFSR), Wageningen University & Research, P.O. Box 230, 6700 AE Wageningen, the Netherlands; elise.hoek@wur.nl

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Abstract

Insect proteins are expected to be increasingly used for food and feed. Black soldier fly larvae (BSF, *Hermetia illucens*) can convert low quality organic substrates, such as manure, into protein-rich ingredients for food and feed. However, pig and chicken manure can contain residues from antibiotics and anti-parasitic drugs, resulting from treatments of the animals for diseases. This study aimed to evaluate the possible effects of veterinary drugs on black soldier fly larvae rearing, including growth and survival, as well as on the presence of residues in the larvae. The study focused on regularly found veterinary drugs in manure. Five-days old larvae were exposed to either 0.05 and 0.5 mg/kg flubendazole (FLUB), 0.05 and 0.5 mg/kg ivermectin (IVM), 0.5 and 5 mg/kg doxycycline (DOX), 0.5 and 5 mg/kg flumequine (FLUM) or 0.5 and 5 mg/kg sulfadiazine (SULF) for one week. The growth of larvae reared on substrate with IVM (0.5 mg/kg) was significantly lower than the control, while the survival of the larvae was not affected. The growth and survival of the larvae was not affected by the other treatments. Chemical analyses showed that concentrations of the veterinary drugs in the larvae, after exposure, were generally low. Only DOX concentrations in the larvae were high; these levels would exceed the European Commission maximum limit for DOX in meat products. Mass-balance calculations showed possible degradation or metabolism of veterinary drugs by the larvae, except for SULF. In conclusion, when using manure as substrate for BSF rearing, the possible presence of veterinary drugs in manure should be carefully controlled to ensure optimal insect growth and safety of the insect products.

Keywords: insects, antibiotics, antiparasitic drugs, manure, safety

1. Introduction

Edible insects are gaining more and more interest as they are expected to become an important alternative ingredient for food and feed with minimal use of natural resources (Foley *et al.*, 2011; Godfray *et al.*, 2010; Van Huis *et al.*, 2013; Veldkamp *et al.*, 2012). Edible insect larvae have the capacity to convert low quality organic resources into protein-rich ingredients for food and feed (Fasolin *et al.*, 2019). In particular, the black soldier fly (BSF, *Hermetia illucens*) is capable to grow efficient on low organic waste streams, like plant based side streams or manure (Bosch *et al.*, 2019; Miranda *et al.*, 2019, 2020; Oonincx *et al.*, 2015). BSF have high protein content and a good amino acid composition for feed (Bosch *et al.*, 2014). The environmental impact of BSF reared on residual streams is lower than BSF reared on conventional feed resources (Bosch *et al.*, 2019). Rearing insects on manure will make insect production more economic and competitive towards other animal proteins, and will increase the resource use efficiency and circularity of our food system. However, before bringing BSF to the market for feed and food use, their safety should be investigated. To date, very limited data related to the safety of insects reared on residual materials are available and, therefore, such data should be collected, among which data related to veterinary drugs (Charlton *et al.*, 2015; Fels-Klerx *et al.*, 2018; Meyer *et al.*, 2021). Manure may contain antibiotic and/or anti-parasitic drug residues as a result of treatment of the production animals, like fattening pigs, broilers and laying hens, with these

veterinary drugs against animal diseases (Massé, 2014). In previous research it was shown that 70 to 88% of the manure samples collected in the Netherlands contained antibiotics and/or anti-parasitic drugs (Berendsen *et al.*, 2015; Jansen *et al.*, 2019). These veterinary drug residues present in the manure could be taken up by the insect larvae hereby affecting the safety of the insect products. Also, antibiotic and anti-parasitic drugs may affect insect viability reducing insect growth and survival and thus insect rearing productivity (Gao *et al.*, 2019; Roeder *et al.*, 2010).

This study aimed to investigate effects of veterinary drugs on the survival and growth of black soldier flies (*H. illucens*) as well as on the possible transfer of veterinary drug residues from substrates into BSF larvae. To this end, experiments were performed in which larvae of the black soldier fly were grown on substrate spiked with different antibiotic and anti-parasitic drugs. Insect survival and growth, as well as the presence of the veterinary drugs in the harvested larvae were determined.

2. Materials and methods

Reference standards

Doxycycline (DOX, 97%), flubendazole (FLUB, 98%), flumequine (FLUM, 99%), ivermectin (IVM, 97%), selamectin, and sulfadiazine (SULF, 100%), were purchased at Sigma-Aldrich (St. Louis, MO, USA). Aminoflubendazole (FLUB-A, 100%), hydroxyflubendazole (FLUB-H, 100%) and flubendazole-d3 were purchased at Witega (Darmstadt, Germany). Natamycine (98%), sulfadiazine N4 acetyl (97%) and the internal standards doxycycline-d3, sulfadiazine-d4, flumequine-13C3 and ivermectin-d2 were purchased at Toronto Research Chemicals (Toronto, ON, Canada).

Reagents

Acetonitrile (ACN), ammonium (25%), ammoniumacetate, citric acid monohydrate, di-sodium hydrogen phosphate, ethanol (EtOH), ethylenediaminetetraacetic acid (EDTA), formic acid (FA), and methanol (MeOH), and were purchased at Merck (Kenilworth, NJ, USA). Ammonium formate (97%) and dimethyl sulfoxide (DMSO), lead acetate trihydrate, trifluoric acetic acid (TFA), were purchased at Sigma-Aldrich. Bondesil-PSA (40 µM) was purchased at Agilent (Santa Clara, CA, USA).

McIlvain-EDTA buffer was prepared by adding 500 ml 0.1 M citric acid, 280 ml 0.2 M di-sodium hydrogen phosphate and 74.4 g sodium-EDTA to 1 l water into a 2 l volumetric flask. The pH was adjusted to 4.0 using citric acid solution or di-sodium hydrogen phosphate solution and the solution was diluted with water up to the mark. Stock solutions of reference standards and internal standards were prepared once at a concentration at 100 mg/l for flumequine-13C3, FLUB-A, FLUB-H, IVM, flubendazole-d3, ivermectin-d2, selamectin and at 1000 mg/l for the other compounds and stored at -80 °C. DOX, SULF, IVM, doxycycline-d3, sulfadiazine-d4, natamycine, ivermectin-d2 and selamectin were dissolved in MeOH. FLUM and flumequine-13C3 were dissolved in a solution of 2% 2 M ammonium hydroxide in MeOH. FLUB was dissolved in DMSO and FLUB-A, FLUB-H were dissolved in a solution of 5% FA, 45% water, 50% EtOH. For the addition of the compounds to the insect substrate solutions of DOX, SULF and FLUM were made at a concentration of 1000 mg/l and solutions of IVM and FLUB were made at a concentration of 100 mg/l. To prevent mould formation in the stability test a natamycine solution was made at a concentration of 2,000 mg/l. For the analysis a mixed solution of reference standards was made at a concentration of 10 mg/l in MeOH. The internal standard solution for the analysis of antibiotics was made at a concentration of 5 mg/l and the internal standard solution for the analysis of antiparasitic compounds was made at a concentration of 2.5 mg/l.

Selection of veterinary drugs

Based on literature, analytical data of WFSR, antibiotic usage data and expert opinions on usage, several regularly used antiparasitic drugs and antibiotics for broiler chickens and fattening pigs were selected (Berendsen *et al.*, 2015; Jansen *et al.*, 2019; SDa, 2020). In this selection, it was ensured that, compounds from different antibiotic and anti-parasitic drugs classes were included. Antibiotics from the subclasses tetracyclines, quinolones and sulfonamides were selected, being DOX, FLUM and SULF. Anti-parasitic drug from the subclasses benzimidazoles and avermectins were selected, being FLUB and IVM. For each of these substances, two concentrations were selected for use in the experimental treatments. For the substances DOX, FLUB and FLUM, the mean and maximum concentrations regularly found in chicken or pig manure in the Netherlands were used (Berendsen *et al.*, 2015; Jansen *et al.*, 2019; SDa, 2020). Only for SULF and IVM the high concentration was set at 10 times the mean concentrations found in manure (Berendsen *et al.*, 2015; Jansen *et al.*, 2019).

Treatments

Standard control substrate (commercial broiler feed) was spiked with veterinary drugs to obtain controlled and standard conditions with known veterinary drug concentrations. This setup makes it possible to draw strong conclusions on the influence of the specific veterinary drugs on insect growth and survival and possible veterinary drug accumulation in the insects. Commercial broiler feed was grinded at 1 mm to obtain homogeneous substrate. Reference standards were added to 50 g of water and this solution was added to 25 g of the ground feed. Reference standards were added to obtain a concentration of 0.5 and

5 mg/kg DOX (DOX-0.5 and DOX-5), FLUM (FLUM-0.5 and FLUM-5), and SULF (SULF-0.5 and SULF-5), and a concentration of 0.05 and 0.5 mg/kg FLUB (FLUB-0.05 and FLUB-0.5) and IVM (IVM-0.05 and IVM-0.5) in the substrates. Control treatments were prepared with the different solvents separately, DMSO, MeOH, ammonia methanol ($MeOH/NH_3$), and only by adding water (blanc). The substrate was made homogeneous and approximately 20 g was removed for the analysis of the antibiotics and anti-parasitic drugs concentrations in the substates. In this way, the exact starting concentrations were determined.

Insect experiment

Black soldier fly (*H. illucens*) larvae (5 days old) were reared on the substrate with spiked veterinary drugs for seven days. One hundred larvae (manually counted) were reared per insect breeding dish (100×40 mm with ventilation cap of 40 mm, Novolab NV, Geraardsbergen, Belgium), with three dishes (biological replicates) per treatment. In total, five different treatments with each two concentrations of veterinary drugs (10 experimental treatments), and four different solvent control treatments were included in the experiment (n=3 per treatment). Additionally, breeding dishes without larvae with substrate with the different veterinary drugs were added to the experiment, 10 experimental treatments, in the same conditions for seven days (n=2 per treatment), to check the stability of the veterinary drugs during the experimental conditions. Natamycine (final concentration in the substrate 40 mg/kg) was added to these additional dishes without larvae to prevent fungal growth. The insect breeding dishes were randomly distributed and placed in a climate chamber (28 °C, humidity of 70%, light regime of 12:12 h). After seven days, per dish, insects were taken out of the substrate with a tweezer, manually counted, washed with hand-warm tap water, gently dried with a paper tissue and weighted. Weights of the substrate at the start and at the end of the 7 days period were also determined per dish. All samples, including the harvested insects, substrate prior to the treatment and residual materials, all per dish, were stored at -20 °C for further analysis.

Antibiotic analyses

Antibiotics were analysed with an validated and optimised LC-MS/MS method for antibiotic analysis. Details about this analysis will be described below. Sample preparation was performed according to Jansen *et al.* (2019) with slight modifications. Of each sample 2 g was weighed into 50 ml polypropylene tubes (Greiner Bio-One, Alphen aan de Rijn, the Netherlands). Internal standard solutions were added to every sample. The aliquots were shaken for 5 s on a vortex mixer and then left at room temperature during 20 min. Hereafter 4 ml of a freshly prepared 0.125% TFA in ACN solution was added and samples were shaken thoroughly by hand. Subsequently 4 ml of McIlvain-EDTA buffer was added. For the analyses of the larvae an ultra-torex was used (1 min, 3,300×*g*) to optimise the extraction recovery. This was not needed for the substrate and residual material samples. The samples were shaken head-over-head (Heidolph REAX-2, Schwabach, Germany) during 15 min. In order to further precipitate proteins and thus to prevent clogging of the Solid Phase Extraction (SPE) cartridge, 2 ml lead acetate solution (200 g/l) was added and samples were vigorously shaken by hand. Thereafter centrifuged (Biofuge Stratos centrifuge, Heraeus instruments, Hanau, Germany) for 10 min at 3,500×*g*. The supernatant was entirely decanted into a 12 ml glass tube. The ACN was evaporated at 40 °C under a gentle nitrogen flow (TurboVap LV Evaporator Zymark, Hopkinton, MA,USA), in order to remove the organic solvent to allow sufficient retention of even the most polar compounds on the SPE cartridge. The extracts were transferred to 50 ml polypropylene tubes and diluted by adding 13 ml of 0.2 M EDTA solution before SPE. A reversed-phase polymeric SPE cartridge 200 mg, 6 ml (Strata-X, Phenomenex, Torrance, CA, USA) was subsequently conditioned with 5 ml of MeOH and 5 ml of McIlvain-EDTA buffer. The entire extract was transferred onto the cartridge, which was washed with 5 ml of water and dried by applying vacuum for 1 min. The residues were eluted into a 12 ml glass tube using 5 ml of MeOH which was then evaporated until dry (40 °C, N2). Residues were reconstituted in 100 µl MeOH by vortex mixing and diluted with 400 µl of water. Due to the high concentration of veterinary drugs in the substrate and residual material, evaporation of the eluate was not necessary. The eluate was immediately diluted with water. The final extracts were centrifuged for 10 min at 2,500×*g* and transferred into UHPLC vials. The samples were analysed immediately or stored at -20 °C and analysed at a later point.

Chemical analysis of the substrate, larvae and residual material samples was performed according to Jansen *et al.* (2019) with slight modifications. In short, LC-MS/MS analysis was carried out using an AcquityUPLC system, coupled to an AB Sciex Q-trap 6500 mass spectrometer (Sciex Framingham, MA, USA). Chromatographic separation was done using a Kinetex C18 2.1×100 mm 1.7 µm analytical column (Phenomenex, Torrance, CA, USA), placed in a column oven operating at 40 °C. Both liquid chromatography and mass spectrometry settings, including ion transitions, were used as described by Berendsen *et al.* (2015). The mobile phases used were 2 mM ammonium formate and 0.016% FA in water (Solvent A) and 2 mM ammonium formate and 0.016% FA in MeOH (Solvent B). Operating at a flow rate of 0.3 ml/min, the used gradient was: 0-0.5 min, 1% B, 0.5-5.0 min, a linear increase to 100% B with a final hold of 1.0 min and an equilibration time of 3.5 min. The injection volume was 10 µl or 3 µl for high concentrations. Transitions of additional compounds are presented in Appendix A. Data processing was done

using MultiQuant 3.0.2 software (Sciex). The response of the compounds was corrected using the corresponding isotopically labelled internal standards. A matrix calibration curve consisting of aliquots (2 g) of a blank material (insects, substrate or residual material) were spiked at a relevant levels. For insects the range was 0-1 mg/kg, for substrate 0-10 mg/kg, and for residual material 0-50 mg/kg. The samples were prepared according to the sample preparation described above. The limit of detection varied from 0.5 µg/kg up to 10 µg/kg depending on the matrix.

Anti-parasitic analyses

Antiparasitic compounds were also analysed with an validated and optimised LC-MS/MS method for anti-parasitics. Details about this analysis will be described below. Of each sample 2 g sample was weighed into 50 ml polypropylene tubes (Greiner Bio-One). Internal standard solutions were added. The aliquots were shaken for 5 s on a vortex mixer and then left at room temperature during 20 min. Hereafter 5 ml of ACN was added and samples were shaken thoroughly by hand. For the analyses of the larvae an ultra-torex was used (1 min, 3,300×*g*) to optimise the extraction recovery. This was not needed for the substrate and residual material samples. The samples were shaken head-over-head (Heidolph REAX-2) during 15 min. Samples were centrifuged (Biofuge Stratos centrifuge) for 10 min at 3,500×*g*. The supernatant was entirely decanted into a 12 ml tube containing 200 mg 'primary secondary amine'. The aliquots were shaken head-over-head for 5 minutes and centrifuged (10 min, 3,500×*g*). The supernatant was entirely decanted into a 12 ml glass tube which was then evaporated until dry (55 °C, N2). Residues were reconstituted in 200 µl 50% MeOH by vortex mixing. The final extracts were transferred to UHPLC vials and analysed immediately or stored at -20 °C and analysed at a later point.

LC-MS/MS analysis was carried out using an AcquityUPLC system, coupled to an AB Sciex Q-trap 6500 mass spectrometer. Chromatographic separation was done using a Acquity UPLC HSS T3 2.1×100 mm 1.8 µm analytical column (Waters, Milford, MA, USA), placed in a column oven operating at 40 °C. The mobile phases used were 2 mM ammonium formate and 0.016% FA in water (Solvent A) and 2 mM ammonium formate and 0.016% FA in MeOH (Solvent B). Operating at a flow rate of 0.4 ml/min, the used gradient was: 0-1.0 min, 5% B, 1.0-6.0 min, a linear increase to 100% B with a final hold of 4.5 min and an equilibration time of 1.5 min. The injection volume was 5 or 2 µl for high concentrations. The mass spectrometer was operated in positive electrospray ionisation mode. The operating parameters were; curtain gas flow 35, nebulising gas flow 60 (N_2), heater gas flow 60, source temperature 500 °C and ion spray voltage 5,500 V. The precursor ions were fragmented to product ions, using collision induced dissociation (N_2). Transitions of the compounds are presented in Table S1.

Data processing was done using MultiQuant 3.0.2 software (Sciex). The response of the compounds was corrected using the corresponding isotopically labelled internal standards. Since no labelled internal standards were available in house, Flubendazole-d_3 was used for FLUB-A and FLUB-H. A matrix calibration curve consisting of nine aliquots (2 g) of a blank material (insects, substrate or residual material) were spiked at a relevant level. For insects and substrate the range was 0-1 mg/kg and for residual material 0-5 mg/kg. The samples were prepared according to the sample preparation described above. The limit of detection varied from 0.1 up to 5 µg/kg depending on the matrix.

Statistical analysis

For statistical analysis GraphPad Prism 5 (version 5.02, GraphPad Software, Inc., San Diego, CA, United States) was used. One-way analysis of variance (ANOVA) followed by a Dunnett's Multiple Comparison Tests were used to compare treatments with the respective solvent controls. Samples with a concentration below the respective limit of detection for the particular compound were considered as zero when calculating means.

3. Results

Spiking of the substrates

Intended and measured concentrations of the spiking of the veterinary drugs into the substrate were analysed to check if the spiking was successful, results are presented in Table 1. The measured concentrations relative to the intended spiked concentrations in the treatments varied between 57-103%. For DOX, FLUM and FLUB the measured concentrations were between 89-103%. Deviations were possibly possible due to the low solubility of some of the substances in water or due to inhomogeneous stock solutions. The standard deviations showed that the variation between the three replicates per treatment was low for all veterinary drugs; the coefficient of variation was ≤5%. Therefore, it was possible to use and trust the data by using the measured veterinary drug concentrations.

Survival and growth of the larvae

First possible effects of the veterinary drugs on the survival and growth of the larvae was studied. The survival rate of the larvae was not affected by the veterinary drug treatments, the survival was minimal 92% for all treatments (Figure 1). The survival of the larvae was not significantly different between the different treatments. At the start of the experiment the mean weight of the larvae per treatment varied between 3.9 and 4.4 mg. At the end of the experiment the mean weight of the larvae varied between 106 and 120 mg, except for IVM-0.5, the mean weight of these larvae was 12 mg (n=3). The growth of

Table 1. Concentrations of veterinary drugs in spiked substrates.

Treatment[1]	Abbreviation	Concentration of veterinary drugs (µg/kg)		Percentage of intended concentration (%)
		Mean	SD	-
Blanc	-	<LOD	-	-
Control DMSO	-	<LOD	-	-
Control MeOH	-	<LOD	-	-
Control MeOH/NH_3	-	<LOD	-	
Doxycycline (500 µg/kg)	DOX-0.5	450	5.8	89%
Doxycycline (5,000 µg/kg)	DOX-5	4,800	150	97%
Flumequine (500 µg/kg)	FLUM-0.5	490	0.2	98%
Flumequine (5,000 µg/kg)	FLUM-5	5,100	120	103%
Sulfadiazine (500 µg/kg)	SULF-0.5	320	5.8	65%
Sulfadiazine (5,000 µg/kg	SULF-5	3,300	170	66%
Flubendazole (50 µg/kg)	FLUB-0.05	45	2.0	90%
Flubendazole (500 µg/kg	FLUB-0.5	460	10	92%
Ivermectin (50 µg/kg)	IVM-0.05	28	0.6	57%
Ivermectin (500 µg/kg)	IVM-0.5	400	20	80%

[1] DMSO = dimethyl sulfoxide; MeOH = methanol; MeOH/NH_3 = ammonia methanol.

the BSF larvae reared on the substrate spiked with IVM-0.5 was significantly lower than the control (P<0.0001). The weight of the larvae reared on substrate with IVM-0.5 for one week was 3 times higher compared to the starting weight. For all other treatments the mean weight of the larvae was 25-30 times higher than the starting weight of the larvae. The growth of the larvae of the other treatments (all except for IVM-0.5) was not significantly different from the control (P>0.05, Figure 1).

Veterinary drugs in the larvae

The concentrations of the individual veterinary drugs were also analysed in the larvae and the residual materials, to conclude on possible presence of residues of these veterinary drugs in the larvae. For DOX-0.5 and DOX-5 treatments, the mean DOX concentrations in larvae were 203±25 and 1,800±58 µg/kg, respectively (Figure 2A). Concentrations of these compounds in the larvae were 46 and 37% of the

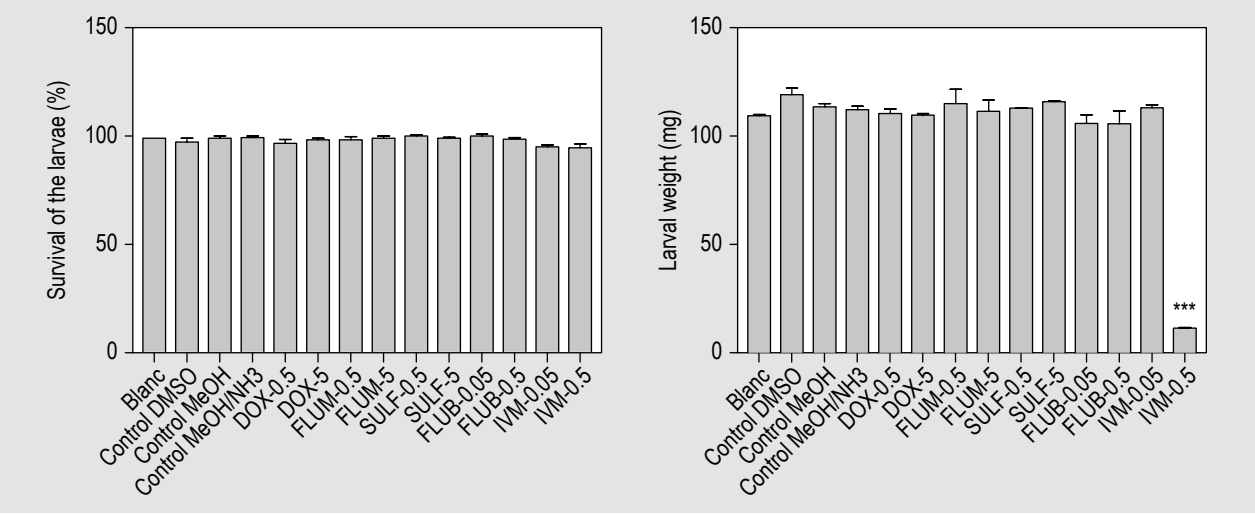

Figure 1. Survival rate and individual larval weight of the BSF larvae during the experiment of seven days. The larvae were reared on substrate spiked with different veterinary drugs. Results are presented as mean ± SEM, * indicates P<0.0001.**

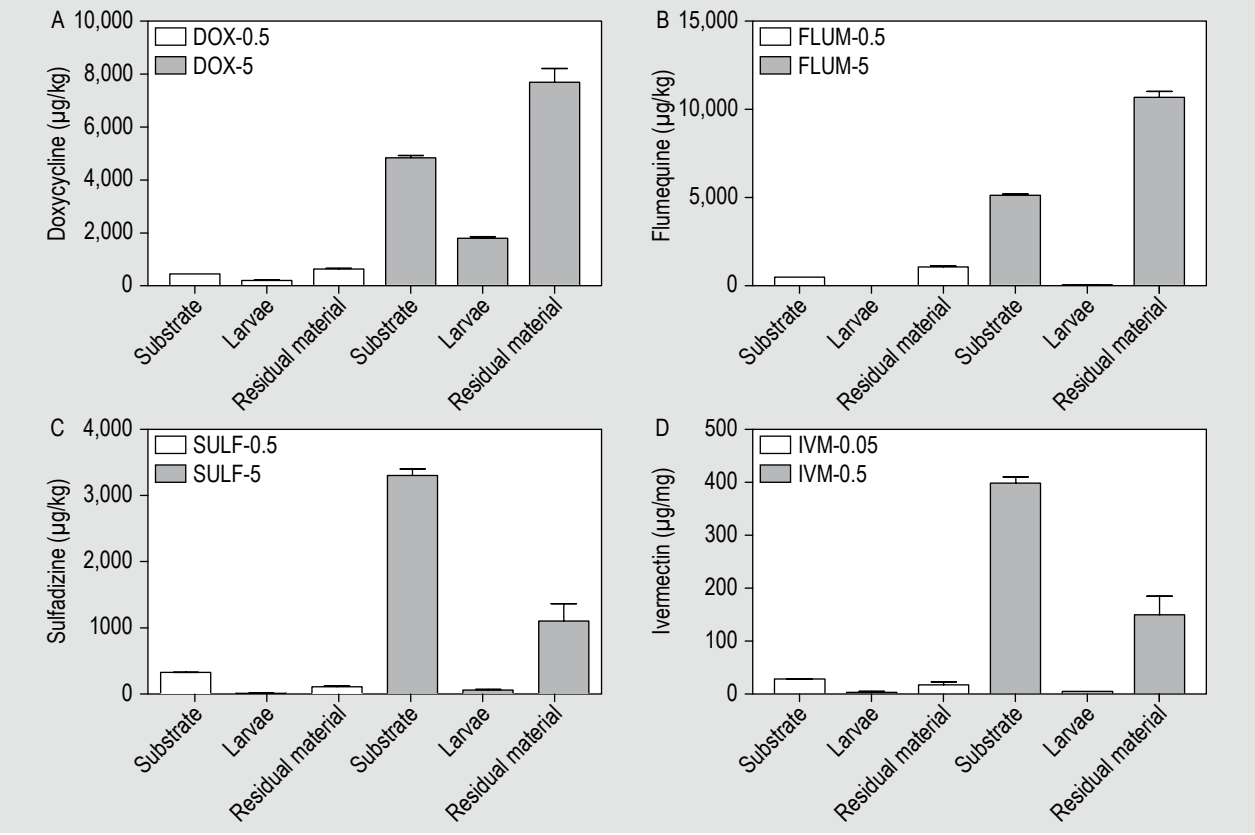

Figure 2. (A) doxycycline (DOX), (B) flumequine (FLUM), (C) sulfadiazine (SULF), and (D) ivermectin (IVM) concentrations in substrate, larvae and residual material during rearing on different substrates with 0.05, 0.5 or 5 mg/kg of the individual veterinary drugs for 7 days. LOD: 5 µg/kg for DOX, 0.5 µg/kg for FLUM, 2 µg/kg for SULF, and 5 µg/kg for IVM. Results are presented as mean ± SEM.

concentrations in the substrate. DOX concentrations in control substrate, larvae and residual material were <LOD of 5 µg/kg.

Mean FLUM concentrations found in larvae reared on FLUM-0.5 and FLUM-5 were 7.2±1.7 and 66±13 µg/kg, respectively (Figure 2B). Concentrations in the larvae were, respectively, 1.5 and 1.3% of the concentrations found in the substrate. FLUM concentrations in control substrate, larvae and residual material were <LOD of 0.5 µg/kg.

Mean SULF concentrations found in larvae reared on SULF-0.5 and SULF-5 were 9.4±3.2 and 61±19 µg/kg, respectively (Figure 2C). Concentrations in the larvae were, respectively, 2.9 and 1.8% of the concentrations found in the substrate. The metabolite N-acetyl sulfadizine was not found in the larvae. SULF concentrations in control substrate, larvae and residual material were <LOD of 2 µg/kg.

Mean IVM concentrations found in the larvae after 7 days were approximately 5 µg/kg for rearing on IVM-0.05 and IVM-0.5, but for one treatment related to larvae reared on IVM-0.05 the concentration of the larvae sample was <LOD of 5 µg/kg. The IVM concentrations in the larvae were 18 and 1.3% of the measured concentrations in the substrates at the start of the experiment (Figure 2D). IVM concentrations in control substrate, larvae and residual material were <LOD of 5 µg/kg.

FLUB and the metabolites FLUB-OH and FLUB-NH_2 were determined in larvae reared on FLUB. The mean concentrations found in larvae reared on FLUB-0.5 were 2.0±0.34, 0.45±0.03 and 3.8±0.9 µg/kg, respectively, for FLUB, FLUB-OH and FLUB-NH_2. Concentrations of FLUB and the FLUB metabolites in larvae reared on FLUB-0.05 were at or below LOD. The concentrations in the larvae reared on FLUB-0.5 were 0.07, 0.1 and 0.8% of the FLUB concentrations found in the substrate at the start of the experiment, respectively for FLUB, FLUB-OH and FLUB-NH_2, the total FLUB concentration (FLUB + FLUB metabolites) in the larvae, reared on FLUB-0.5, is in total 1% of the concentrations in the start substrate (Figure 3).In the control larvae no veterinary drugs were found, all tested compounds were <LOD.

Overall, the concentrations found in the larvae for all tested veterinary drugs were lower than the concentration in the substrate, percentages of concentrations in the larvae relative to the measured start substrates were between 1 and 46%. So, results showed that there was no accumulation of the tested veterinary drugs in the larvae.

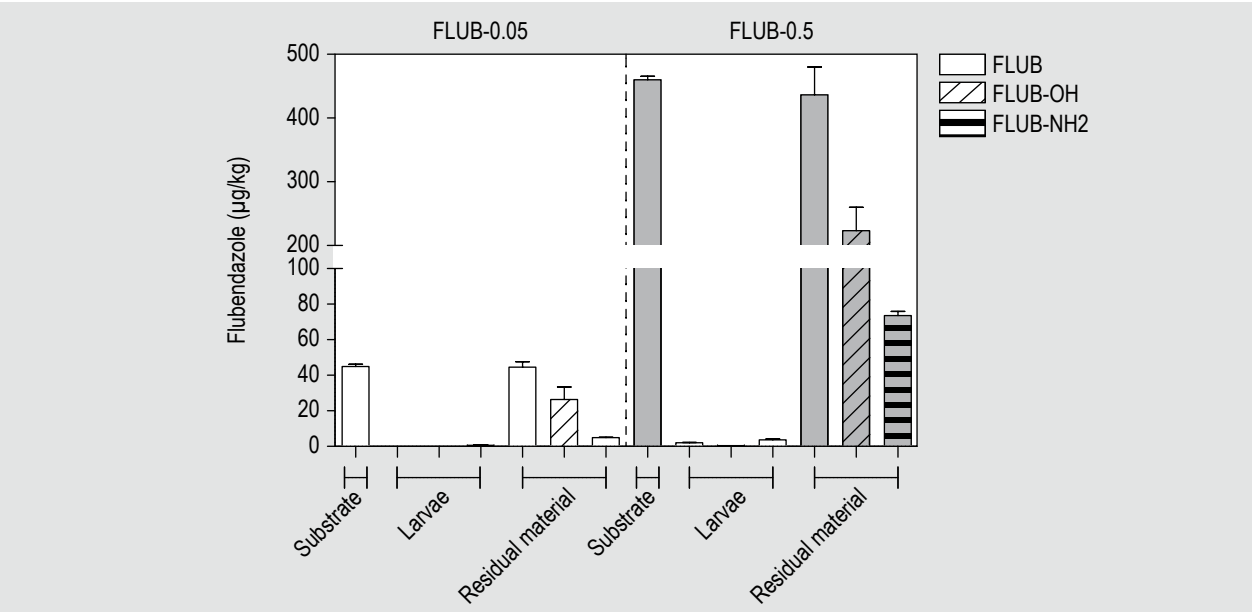

Figure 3. Flubendazole (FLUB), flubendazole-OH (FLUB-OH) and flubedazole-NH_2 (FLUB-NH_2) concentrations in substrate, larvae and residual material during rearing on substrates with 50 and 500 µg/kg flubendazole for 7 days. LOD: 0.5 µg/kg for FLUB, 0.2 µg/kg for FLUB-OH, 1 µg/kg for FLUB-NH_2. Results are presented as mean ± SEM.

Mass balance

A mass balance was calculated to investigate how much of the original absolute amount of veterinary drugs spiked into the substrate was found back in the insects and residual material; this amount was expressed as percentage of the original (measured) amount in the substrate. The amount of substrate at the start of the experiment was 54 g per treatment, while the residual material was between 8.5-11 g for all treatments, except for IVM-0.5 for which it was 23 g. The mass-balance calculations showed that – for all veterinary drugs – <40% of the initial amount of added veterinary drugs was found back (in larvae and residual material), as shown in the grey columns in Figure 4. Part of the 'missing amounts' may be due to natural breakdown of the compounds in this experimental set-up. This breakdown was also seen during the stability experiment in boxes without larvae. The percentage of natural breakdown of the compounds is shown in the white columns in Figure 4.

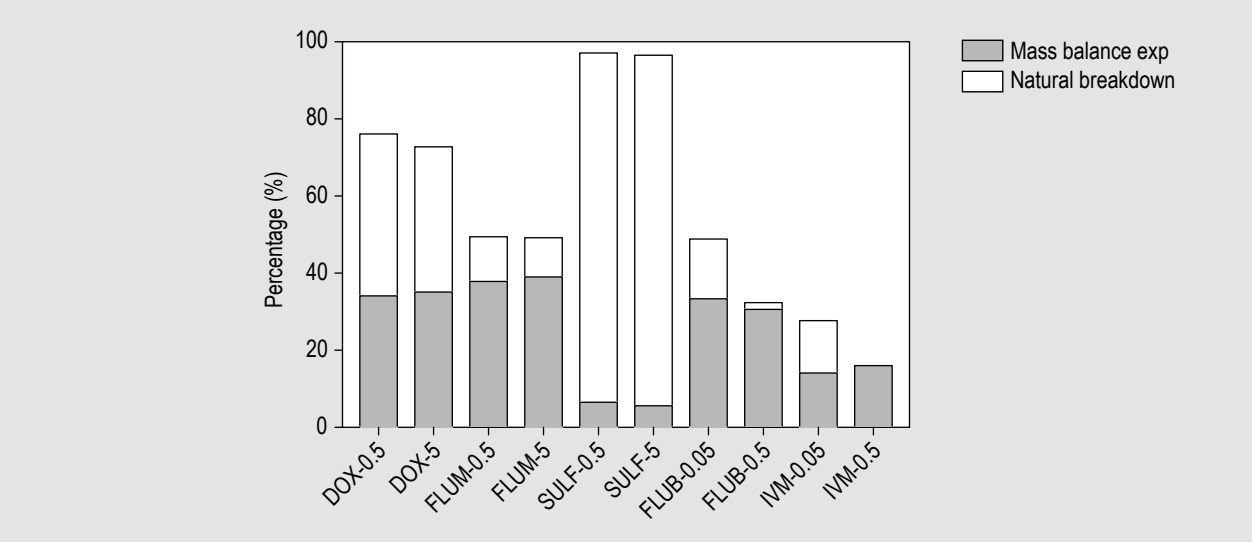

Figure 4. Mean mass balance of the amount of veterinary drugs found back in the insects and residual materials compared to the original amount of veterinary drugs measured in the substrate in percentages (grey columns). Mean percentage of natural breakdown during the experimental set-up of the veterinary drugs in the substrates is shown in white columns.

4. Discussion

Veterinary drugs could be present in potential substrates for BSF rearing, therefore, the effects on growth and survival of the larvae as well as on possible accumulation and uptake of veterinary drugs in the larvae have been studied. Results showed that the growth and the survival of the larvae were not affected by presence of DOX, FLUM, SULF, FLUB, and IVM-0.05 in the substrates. However, the growth of the larvae reared on substrate with IVM-0.5 was significantly lower than the control, though the total survival of the larvae was not affected. To date, limited data on the effects of veterinary drugs on growth and survival of BSF larvae have been published. Authors have indicated that in case of using veterinary drugs to prevent infections during insect rearing an optimal balance should be found to obtain no negative effects on growth and survival of the insects (Roeder *et al.*, 2010).

Furthermore, it has been shown that the growth of BSF larvae could be affected by certain veterinary drugs, the antiparasitic drug, moxidectin had a negative effect on the growth of BSF larvae. The concentrations moxidectin in this study were in the range as found in cattle faeces (Alyokhin *et al.*, 2019). The presence of the antibiotic sulfonamide in the substrate of 10 mg/kg, resulting in 30% survival of the BSF larvae (Gao *et al.*, 2019). In this study of Gao *et al.* (2019) the sulfonamide, SULF, was detected in the prepupae, while sulfamonomethoxine, sulfamethoxazole, and sulfamethazine were not detected in the larvae. In the present study, SULF was also detected in the larvae, while the survival was not affected by SULF, possibly due to the lower concentration (5 mg/kg) of SULF in the substrate as compared to the study of Gao *et al.* (2019b).

In this study, the initial amount of doxycycline in the substrate was reduced by 56% and sulfadiazine was reduced by 72% after 7 days by the BSF larvae. In some studies, it has been observed that BSF larvae can reduce pharmaceuticals in the environment. Degradation of tetracycline by BSF larvae (Cai *et al.*, 2018) and the coccidiostat and feed additive monensin by *M. domestica* larvae due to intestinal microbiota in the larvae has been shown (Li *et al.*, 2019). Furthermore, concentrations of the pharmaceuticals carbamazepine, roxithromycin, and trimethoprim were reduced by BSF larvae in compost substrate (Lalander *et al.*, 2016). Another study showed biodegradation of antibiotics, including tetracyclines, sulfonamides, and fluoroquinolones, as present in swine and chicken manure by *M. domestica* larvae (Zhang *et al.*, 2014). These authors showed that insect larvae could be able to degrade or metabolise veterinary drugs which can be present in the substrate, however, natural breakdown due to other factors, like the experimental set-up, were not studied. Degradation or metabolism of veterinary drugs is also apparent from the results of the mass balance calculations for the tested veterinary drugs DOX, FLUM, FLUB, and IVM in this study, which were <100%. However, the mass balance of sulfadiazine was almost 100% taken into account the natural breakdown of this compound, which implies no metabolism or degradation of SULF by BSF larvae in this study. This is confirmed by the fact that the metabolite N-acetyl sulfadizine was not found in the larvae or substrate after seven days of exposure to SULF in the present study. In the present study, FLUB was partly metabolised by the larvae into FLUB-OH and FLUB-NH_2, however taken into account the formed metabolites and their stability in the mass balance calculation, only up to 50% was found. Therefore, besides SULF, for all other veterinary drugs it is recommended to study possible formed breakdown products and to investigate if these breakdown products are still biological active.

As no maximum residue limits (MRLs) are available for the presence of veterinary drugs in insects used as feed or food ingredients, the detected concentrations were compared with the most relevant MRL for food producing animals (Regulation (EU) 37/2010). DOX concentrations found in larvae were high (200-1,800 µg/kg) and these concentrations exceed the MRL for DOX in bovine or poultry muscle of 100 µg/kg. This is concerning, because concentrations spiked to the substrate were in the range of DOX levels regularly found in chicken or pig manure (Berendsen *et al.*, 2015; Jansen *et al.*, 2019). For the other studied veterinary drugs the most relevant MRLs for comparison were not exceeded; these included the MRL of FLUB of 50 ug/kg for muscle of poultry and porcine, the MRL of FLUM of 200 ug/kg for muscle of bovine, ovine, caprine and porcine, the MRL of IVM of 100 ug/kg for liver and fat of mammalian food producing species, the MRL of the sum of all substances belonging to the sulfonamides of 100 ug/kg for all food producing species (Regulation (EU) No 37/2010). This was also observed in a range of insect larvae, including BSF, from diverse production methods and geographical locations, where no veterinary drugs were detected in the larvae, besides nicarbazin in one *M. domestica* sample (Charlton *et al.*, 2015).

In conclusion, this study found high DOX concentrations in the larvae, while concentrations of other investigated veterinary drugs in the larvae were relatively low. It is possible that breakdown products are formed which could end up in the larvae, these breakdown products could be bioactive. Therefore, possible breakdown of veterinary drugs by insect larvae should be further studied. Results of this study are promising since veterinary drugs residues that may be present in manure did not affect the survival of BSF larvae reared on the spiked substrates. Growth of the larvae was only decreased for the IVM-0.5 treatment, however, this concentration is about 10× the concentration regularly found in manure pits. Other veterinary drugs studied did not show a decrease in larval growth. Overall, this study showed that the presence of veterinary drugs in

manure should be determined prior to selecting manure types, which could possibly be used as substrate for insect rearing, so as to ensure optimal insect growth and safety of insect products.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2021.0122.

Table S1. Additional transitions and parameters for the antibiotic and anti-parasitic analyses that were not included in a previous study (Berendsen *et al*., 2015).

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Conflict of interest

The authors declare no conflict of interest.

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