Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and the Netherlands

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Abstract: The occurrence of antibiotic resistance genes in foodstuffs involves a human health risk. Fresh edible insects present an emerging source of human food but they were not yet assessed in a quantitative way for antibiotic resistances as a matter of food safety. In this study, a real-time quantitative PCR assessment was optimised to detect and quantify relevant transferable antibiotic resistance genes [tet(O, K, M, S) and erm(B)] in edible insects. Subsequently, the technology was applied on 30 fresh insect samples, including two mealworm species and two cricket species from different production batches and rearing companies in Belgium and the Netherlands. The sampling periods and the post-harvest treatments applied were also taken into account. Results showed that mealworms contained, on average, higher numbers of tet(K), tet(M), and tet(S) genes than crickets, but tet(O) was almost uniquely present in crickets. The erm(B) gene was only detected in one mealworm sample and the tet(K) gene showed higher abundances in samples originating from the Netherlands than in samples from Belgium. A large difference in antibiotic resistance profile was revealed between mealworms and crickets, but not between different mealworm species or cricket species. Species-specific microbiomes and insect feed may have contributed to this distinction. Interestingly, important correlations between the presence of some tet genes and the microbiota previously encountered in the investigated edible insects were uncovered. While a geographical distribution was observed for the tet(K) gene, post-harvest treatments and sampling period were not shown to have a significant influence on the occurrence of the antibiotic resistance genes considered. In conclusion, insects may carry considerable amounts of antibiotic resistance genes, but the health risk in terms of antibiotic resistances is comparable to other food matrices.

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To the Chief Editor of
International Journal of Food Microbiology
Prof. Cocolin

Cover letter

Dear Prof. Cocolin,

Please find enclosed a copy of the manuscript entitled "**Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and The Netherlands**" (6706 words, excluding tables and figures, including references), intended for publication in *International Journal of Food Microbiology* as full research paper. The *International Journal of Food Microbiology* focusses on novel publications with a clear connection to the microbiology of foods, including food safety and public health. Therefore, we are convinced that our research paper brings relevant information to the international audience in food microbiology for several reasons:

1. This paper is the first that assesses the occurrence of transferable antibiotic resistance genes in the microbiome of **fresh** edible insects. Please note that this paper is a second extension (including the same samples) to our paper published in *International Journal of Food Microbiology*, which is why we believe this new manuscript is suitable for IJFM too. (Vandeweyer et al. (2017) Microbial counts of mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing companies and different production batches. Int. J. Food Microbiol. 242, 13–18.)

2. Previous research on antibiotic resistance genes in **processed** edible insects did not include quantification of the resistance genes. This research was executed using newly optimised real-time quantitative PCR protocols to simultaneously **detect and quantify** the resistance genes in edible insects. With this quantitative knowledge, we believe that our results can serve for a more detailed health risk assessment with regard to antibiotic resistances in foodstuffs.

3. Since insect-based food products are being developed and Novel Food dossiers are being composed, *in-depth* information covering food safety risks is an urgent need, as also requested by several European bodies (EMA, ECDC, EFSA, SCENIHR).

We hereby confirm that the work has not been published elsewhere nor it has been simultaneously submitted for publication elsewhere. There are no relevant competing interests (both financial and personal) that the Editor may consider relevant to the manuscript. Four referees are proposed i.e Katarzyna Ignasiak and Anthony Maxwell (John Innes Centre, UK), Laura Gasco (Università degli Studi di Torino, Italy) and Nils Grabowski (University of Veterinary Medicine Hannover, Germany). They are no members or former members of our organization, nor have they been associated with us.

We are looking forward to hear from you and we thank you in advance for the time and effort you expend to consider our work.

Yours sincerely,

Vesna Milanović

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***Highlights (for review)**

- Occurrence of antibiotic resistance genes in fresh edible insects was investigated
- A real-time PCR protocol was optimised to detect and quantify these genes
- Mealworms and crickets contained significantly different AR gene profiles
- *tet*(S) gene occurrence was found to be related to geographical sample distribution
- Presence of AR genes was linked to insect-specific microbiomes and insect feed

Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and the Netherlands

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Abstract

The occurrence of antibiotic resistance genes in foodstuffs involves a human health risk. Fresh edible insects present an emerging source of human food but they were not yet assessed in a quantitative way for antibiotic resistances as a matter of food safety. In this study, a real-time quantitative PCR assessment was optimised to detect and quantify relevant transferable antibiotic resistance genes [*tet*(O, K, M, S) and *erm*(B)] in edible insects. Subsequently, the technology was applied on 30 fresh insect samples, including two mealworm species and two cricket species from different production batches and rearing companies in Belgium and the Netherlands. The sampling periods and the post-harvest treatments applied were also taken into account. Results showed that mealworms contained, on average, higher numbers of *tet*(K), *tet*(M), and *tet*(S) genes than crickets, but *tet*(O) was almost uniquely present in crickets. The *erm*(B) gene was only detected in one mealworm sample and the *tet*(K) gene showed higher abundances in samples originating from the Netherlands than in samples from Belgium. A large difference in antibiotic resistance profile was revealed between mealworms and crickets, but not between different mealworm species or cricket species. Species-specific microbiomes and insect feed may have contributed to this distinction. Interestingly, important correlations between the presence of some *tet* genes and the microbiota previously encountered in the investigated edible insects were uncovered. While a geographical distribution was observed for the *tet*(K) gene, post-harvest treatments and sampling period were not shown to have a significant influence on the occurrence of the antibiotic resistance genes considered. In conclusion, insects may carry considerable amounts of antibiotic resistance genes, but the health risk in terms of antibiotic resistances is comparable to other food matrices.

Key words: edible insects, mealworms, crickets, antibiotic resistance, real-time PCR

1 Introduction

Since the recent introduction of edible insects in several European countries, the microbial quality and safety of insects used for human consumption was repeatedly investigated (Garofalo et al., 2017; Klunder et al., 2012; Osimani et al., 2017c; Rumpold and Schlüter, 2013; Stoops et al., 2017, 2016; van der Spiegel et al., 2013; Vandeweyer et al., 2018, 2017a, 2017b; Wynants et al., 2018), as recommended by different scientific opinions and advices (ANSES, 2015; EFSA Scientific Committee, 2015; NVWA, 2014; SHC and FASFC, 2014). The new European Novel Food regulation (EU 2015/2283), which took effect in January 2018, has evoked an increase in edible insect research as well.

As recently reviewed by Dobermann et al. (2017), the main challenges of mass rearing of edible insects include the bacterial contamination of the end products, e.g. high counts of spore-forming bacteria, total mesophilic aerobes, and Enterobacteriaceae, and the potential occurrence of human pathogens as well as the risks of antibiotic usage in such mass rearing. Concerning this latter aspect, the use and misuse of antibiotics are known to have a major effect on the prevalence of antibiotic resistant microorganisms, for example in primary production, food, feed, and the environment (Clementi and Aquilanti, 2011; Verraes et al., 2013). Hence, for 2016-2020 the European Medicine Agency (EMA) expressed a joint opinion with the European Food Safety Authority (EFSA) on measures to reduce the use of antimicrobial agents in animal husbandry (also known as the 'RONAFA' opinion) (EMA and EFSA, 2017). Even earlier reports published jointly by EMA and European bodies including the European Centre for Disease Prevention and Control (ECDC), EFSA, and the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) have emphasised the need for the prudent use of antibiotics in animals (ECDC et al., 2009). Concerning edible insects, no data are currently available about the use of antibiotics in mass rearing and the occurrence of antibiotic resistances (ARs) in edible insects is limitedly assessed (Milanović et al., 2016; Osimani et al., 2017a, 2017b), despite the recommendations posed in the EFSA opinion (EFSA Scientific Committee, 2015) and two joint reports of the ECDC, EFSA, and EMA (ECDC et al., 2017, 2015).

Antibiotic resistances may pose a risk in animal and human health, since they are easily transferred through horizontal gene transfer between microorganisms, including pathogens (Gogarten et al., 2009; Verraes et al., 2013). For edible insects, which typically contain high microbial counts (Vandeweyer et al., 2017a), the transfer of such ARs can establish important food safety risks. Food, especially that of animal origin, is an important vehicle in the transfer of antibiotic resistance genes into the human digestive tract and its associated

microbiome (Verraes et al., 2013). Because edible insects are generally used as a whole in food products and because starvation has shown not to alter the microbiome, at least in mealworms (Wynants et al., 2017), the complete microbial community (i.e. also intestinal) is included in the foodstuffs.

Edible insects are typically processed prior to consumption (Fombong et al., 2017; van Huis et al., 2013; Vandeweyer et al., 2017c). To lower microbial counts, many processing steps involve a heat treatment causing a number of lesions in microbial cells. These include membrane damage, loss of nutrients and ions, ribosome aggregation, and even DNA strand breaks (Mañas and Pagán, 2005). However, less vulnerable microorganisms (e.g. bacterial spores (Vandeweyer et al., 2017c)) and their AR genes may survive the minimal heating treatments frequently applied for insects or even be triggered in AR transfer (Verraes et al., 2013). Processing may also cross-contaminate insects with ARs initially not carried by their associated microbiota (Verraes et al., 2013). Recently, a few studies have been performed to investigate the occurrence and distribution of transferable AR genes in processed, ready-to-eat insects available on the European market (Milanović et al., 2016; Osimani et al., 2017b, 2017a), but so far, fresh insects, i.e. living insects at the end of their rearing cycle collected from industrial rearing facilities, have not been subjected to AR assessment, yet. In all former studies, a number of genes inducing resistance against classes of antibiotics commonly used in both human and animal therapy (e.g. tetracyclines and macrolides) were detected by qualitative nested PCR. According to Penders et al. (2013), three different metagenomic approaches are currently applied to study the AR pool: PCR-based metagenomics, functional metagenomics, and sequence-based metagenomics. Although nested PCR assays are characterised by an extremely high sensitivity for detection of target AR genes (Milanović et al., 2016; Osimani et al., 2017b, 2017a), this technique does not allow an effective quantification of the amount of gene copies occurring in a given sample. To date, real-time PCR (qPCR) techniques have been applied in a number of food matrices to detect and quantify both tetracycline and erythromycin resistance genes (Flórez et al., 2014). However, to the authors' knowledge, no qPCR assays have been used or optimised for the analysis of transferable ARs in edible insects, yet.

Based on these premises, the present study was aimed at detecting and quantifying a set of transferable tetracycline and erythromycin resistance genes in freshly reared edible insects to be used as an ingredient for insect food production. To this end, fresh mealworms and crickets collected from different rearing facilities in Belgium and the Netherlands and from different rearing cycles per facility were analysed. After DNA extraction,

all samples were screened by qPCR for tetracycline *tet*(K), *tet*(O), *tet*(M), *tet*(S) and erythromycin *erm(*B) resistance genes previously found in edible insects (Milanović et al., 2016; Osimani et al., 2017b, 2017a).

2 Materials and methods

2.1 Fresh insect sampling

A total of 30 fresh insect samples were obtained from 9 rearing companies located in Belgium and the Netherlands (Table 1). Samples were taken at rearing stages used for consumption (except for one cricket sample taken at nymph stage: BCR 1.4a). Insect species investigated included mealworms (*T. molitor*, 17 samples), lesser mealworms (*Alphitobius diaperinus*; 3 samples), house crickets (*Acheta domesticus*; 5 samples), and tropical house crickets (*Gryllodes sigillatus*, also banded cricket; 5 samples). Most rearing companies were sampled several times, thus investigating different production cycles (batches) from the same facility. All insects were reared according to company-specific optimised protocols, which were only partly revealed. Important rearing details and post-harvest handlings are detailed in Table 1. Samples from the same batch which were slightly different (e.g. post-harvest treatment) were given a different letter in the sample code. After transportation from the rearing facility to the laboratory, insects were frozen (-21 °C) until DNA extraction, to preserve the DNA.

2.2 Reference strains

Five reference strains, each carrying one of the AR genes under study, were used for the construction of qPCR standards and as positive controls in the qPCR runs (Table 2). The strain *Enterococcus faecalis* JH2-2 (Jacob and Hobbs, 1974) was used as a negative control.

2.3 Sample preparation and DNA extraction

Five grams of each thawed (ambient temperature, 1 hour) insect sample were aseptically crushed and homogenised in 45 mL of sterile peptone water (peptone, 1 g/l) for 2 minutes at 260 rpm using a Stomacher 400 Circulator (PBI, Milan, Italy). Subsequently, 1.5 ml of each homogenate was centrifuged at 16,000 g for 5 minutes to produce a pellet containing the bacterial cells. Total bacterial DNA was extracted from 0.2 grams of each pellet using PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The quantity and the purity of the extracted DNA were determined

using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The concentration of the extracted DNA was standardised to 2 ng/µl for all the samples. To check the effectiveness of bacterial DNA extraction, the DNA suspensions were amplified by end-point PCR using the universal prokaryotic primers 338F-518R, as previously described (Osimani et al., 2017b). DNA from the reference strains was extracted following the procedure previously detailed by Osimani et al. (2015).

2.4 Construction of qPCR standards

The DNA extracted from the reference strains carrying the AR genes under study were used for the creation of qPCR standard curves. The *erm*(B) and *tet*(O) gene amplicons were obtained by end-point PCR (MyCycler, Bio-Rad Laboratories, Hercules, CA, USA) using Sibenzyme Taq DNA polymerase (Novosibirsk, Russia). Primers and cycling conditions were used as previously described by Milanović et al. (2017) and Flórez et al. (2014), respectively. The *tet*(K), *tet*(M), and *tet*(S) gene amplicons were obtained by qPCR (Mastercycler® ep realplex, Eppendorf, Hamburg, Germany) using qPCR primers and conditions described by Flórez et al. (2014). Obtained PCR products were checked for the correct size by electrophoresis on a 1.5% agarose gel and purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK), following the manufacturer's instruction. The quantity and purity of the purified PCR products were determined (Nanodrop ND 1000, Thermo Fisher Scientific) and the gene copy number for each AR gene under study was calculated based on the size and mass of the amplicons using an online calculator (www.idtdna.com). For the creation of the qPCR standard curves, tenfold serial dilutions of the purified amplicons of each AR gene were prepared.

2.5 Real Time qPCR quantification

Bacterial DNA extracted from the insect samples was screened by qPCR for the absolute quantification of the gene *erm*(B), coding for resistance to erythromycin, and the genes *tet*(O), *tet*(M), *tet*(S), and *tet*(K), coding for resistance to tetracyclines. The qPCR reactions were performed using the Mastercycler® ep realplex (Eppendorf) with the qPCR primers described by Flórez et al. (2014). Four µl (8 ng) of the extracted DNA was amplified in a total volume of 10 µl including 5 µl of QPCR Green Master Mix LRox 2X (Biotechrabbit GmbH, Hennigsdorf, Germany) and 900 nM of the forward and reverse primer. In each assay, the opportune positive and negative controls were run together with a blank (molecular grade water instead of DNA).

The qPCR conditions for the genes *tet*(O), *tet*(M), and *tet*(K) included an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. For the amplification of *tet*(S) and *erm*(B), qPCR conditions were as described by Flórez et al. (2014), with a slight modification in the last step (60.5 °C for 45 s instead of 60 °C for 1 min) for the amplification of *erm*(B). All cycles were followed by a melt curve step with temperature gradually increasing from 60 °C to 95 °C by 0.4 °C/s.

DNA extracts from the insect samples were run along with the tenfold dilutions of the standards for each AR gene under study prepared as described above. The absolute gene copy number per reaction was calculated using the slope of the obtained standard curves for each sample and each target AR gene. All reactions were performed in duplicate. Per sample, results from the duplicate analyses and from Nanodrop measurements were used to calculate a mean copy number per gram of insect. The Mastercycler® ep realplex software was used for the baseline and threshold calculation. To check for the amplification specificity, melting temperature analysis was performed and the expected size of the PCR products was checked on 1.5 % agarose gel. Amplicons from randomly selected positive insect samples were sent to Beckman Coulter Genomics (London, UK) for purification and sequencing. Online similarity searches in the GenBank database were performed by BLAST analysis. All sequences analysed had a $\geq$ 97% similarity with the expected antibiotic resistance gene, definitely confirming the specificity of the primer set used for the qPCR runs.

2.6 Statistical analyses

To investigate statistical differences among insect species, samples and rearing facilities, as well as influences of graphical distribution, sampling period and post-harvest treatments, one-way ANOVA with Tukey's post hoc test was performed for all AR genes. In case of unequal variances, Welch's ANOVA with Games-Howell post hoc test was used instead. All tests were performed with SPSS Statistics 23 (IBM, New York, NY, USA) and considered a 0.05 significance level. Finally, nonmetric multidimensional scaling (NMDS) was performed on the total AR gene composition of all samples using the R-package (R Development Core Team, 2013) "Vegan" (v.2.43) in RStudio (v1.1.442).

3 Results and discussion

3.1 Accuracy of qPCR assessments

Standard curves created for each AR gene qPCR assessment showed R^2-values of 0.99 and efficiencies between 0.95 and 1.05. Detection limits, defined as the lowest gene copy number per reaction in which the linearity was maintained, were in order of 10^1 for *tet*(K) gene and 10^2 for *tet*(M), *tet*(S), *tet*(O) and *erm*(B) genes, respectively. qPCR assessments were therefore considered reliable, efficient and sensitive.

3.2 Quantitative detection of antibiotic resistance genes in insect samples

All qPCR assessments, each detecting and quantifying one target AR gene, were applied for all 30 samples investigated. The results, expressed as gene copy number per gram of insect sample, are shown in Table 3. *Tet* genes were present in several samples with mean quantities ranging between 3.31×10^4 and 2.10×10^8 gene copies per gram of insect. Fresh edible insects have been reported to harbour up to 8 or 9 log cfu/g microorganisms (Vandeweyer et al., 2017a), thus suggesting that a large fraction of the microorganisms occurring in some samples carried at least one AR gene under study. More specific, *tet*(O), *tet*(K), *tet*(M) and *tet*(S) genes were encountered in 37%, 40%, 100% and 70% of the analysed samples, respectively. The *tet* genes investigated in this study are typically (but not uniquely) associated with gram positive bacteria (Chopra and Roberts, 2001). Therefore, the recovered *tet* genes may have been particularly carried by gram positive members of the edible insect microbiota, such as lactic acid bacteria (LAB), which have previously been found in large quantities (up to 8 log cfu/g) (Vandeweyer et al., 2017a). Indeed, it was suggested that LAB play an important role in the preservation and transfer of AR genes in foodstuffs and the animal gastrointestinal tract (Clementi and Aquilanti, 2011). Yet, especially the *tet*(M) gene is also occasionally encountered in gram negative bacteria, e.g. in members of the genus *Bacteroides* (Barbeyrac et al., 1991; Chopra and Roberts, 2001), which are known to be abundantly present in fresh edible crickets (Vandeweyer et al., 2018, 2017b). Interestingly, only one sample (MW 4.2b) contained a detectable number of *erm*(B) gene copies, coding for erythromycin (macrolide) resistance. The *erm*(B) gene is mostly associated with streptococci and enterococci (Leclercq, 2002) and may often be detected in combination with *tet*(M) because of their possible co-occurrence on the same transposon (Chopra and Roberts, 2001). Nevertheless, the absence of the *erm*(B) gene in most samples investigated here, suggests there was no co-occurrence with *tet*(M) in the insect microbiota associated with the samples.

Except for one mealworm sample (MW 4.2b), *tet*(O) was exclusively found in cricket samples at levels up to 4.24×10^7 gene copies. This finding agrees well with previous research, where *tet*(O) was detected in samples of processed edible crickets (*A. domesticus*), but rarely in other insects (Milanović et al., 2016; Osimani

et al., 2017a, 2017b). Since the microbiome is known to be specific for different edible insects (Garofalo et al., 2017; Osimani et al., 2017c; Stoops et al., 2016; Vandeweyer et al., 2017b), AR genes may concomitantly be present or absent in different insects, depending on the microbiota composition of these insects. Previously, *tet*(O) has been detected in streptococci and campylobacteria (Chopra and Roberts, 2001), two microbial groups that were already recovered from cricket samples, though at very low levels (Vandeweyer et al., 2017b). The high copy numbers of *tet*(O) detected in the cricket samples analysed in this study suggest that also microorganisms other than streptococci and campylobacteria might carry this determinant. In this regard, a number of previous studies have clearly indicated that the microbial compositions of crickets and mealworms are influenced largely by their diet (Colman et al., 2012; Wynants et al., 2018; Yun et al., 2014), thus explaining potential differences occurring in the distribution of specific AR genes as well.

In contrast to *tet*(O), the detection of *tet*(K) was more widespread among samples, although significantly ($p = 0.043$) higher average copy numbers were observed for mealworms compared to crickets. Significant differences were also seen between rearing companies, with rearer 3 producing mealworms with significantly the highest numbers of *tet*(K) copies ($p = 0.000$). Also between different batches from a single rearer, significant differences were observed (Table 3). In previous investigations on processed edible insects, *tet*(K) has frequently been detected as well (Milanović et al., 2016; Osimani et al., 2017b, 2017a), thus suggesting a wide distribution of *tet*(K) in edible insects. This might be explained by its location on small transferable plasmids that can easily integrate in the chromosome of different gram-positive bacteria (Chopra and Roberts, 2001). *Tet*(K) has been detected in numerous genera, including *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Chopra and Roberts, 2001), all of which have already been found in various edible insect species (Vandeweyer et al., 2018, 2017b; Wynants et al., 2018).

Concerning their geographical origin, an unequal distribution of *tet*(K) was observed among samples. Indeed, only 13% of the Belgian samples (2 out of 16) harboured *tet*(K), while 71% of the samples collected in the Netherlands (10 out of 14) were positive for this gene. Statistical analysis confirmed this evidence, and also revealed a significant difference ($p = 0.023$) in the number of *tet*(K) gene copies between Dutch and Belgian samples. Overall, these findings suggest that the occurrence of *tet*(K) and even its relative abundance might be geographically determined for freshly reared insects. A previous study investigating the occurrence of transferable antibiotic resistances in processed mealworms by nested PCR did not report any significant difference between Belgian and Dutch samples, all being positive for *tet*(K) (Osimani et al., 2017a). However,

most of those Belgian samples were positive only after the second set of PCR runs, whereas in 60% of the Dutch samples, *tet*(K) had already been amplified after the first set of PCR runs, thus suggesting a different abundance of the target sequence.

Regarding *tet*(M), an ubiquitous occurrence of this determinant was revealed by qPCR analysis. Although it was detected in all samples, mealworms contained, on average, a statistically ($p = 0.042$) higher copy number of *tet*(M) than crickets. When comparing different batches produced by the same company, the highest variety in *tet*(M) copies between samples was found for company 9 (*G. sigillatus*). Besides the type of insect species and production batches, other potentially influencing factors considered, such as the geographical origin or the type of post-harvest treatment, were not found to exert a significant influence on the distribution of *tet*(M) among the samples analysed. Our results agree well with other studies (Milanović et al., 2016; Osimani et al., 2017b), where *tet*(M) was frequently detected in various specimens of marketed edible insects, including processed mealworms and crickets. Overall, these findings suggest a wide distribution of *tet*(M) in the microbiome of edible insects. Its frequent detection as well as its occasionally high copy numbers (up to 4.52×10^7) might be attributed to the fact that *tet*(M) is typically located on conjugative transposons (e.g. Tn916 - Tn1545 family) and can therefore easily be transferred from one bacterial species to another (Doherty et al., 2000). Accordingly, *tet*(M) has been detected in numerous food matrices, including dairy (Flórez et al., 2014) and meat products (Hölzel et al., 2011). Interestingly, in the present study, two samples (MW 1.1 and MW 1.2) did not carry any other AR gene than *tet*(M).

Concerning *tet*(S), a lower occurrence was observed in comparison with *tet*(M), with 70% (21 out of 30) of the samples found to be positive. Again, the presence and copy numbers of *tet*(S) were significantly higher ($p = 0.000$) in mealworms than in crickets. *Tet*(S) was first discovered in *Listeria monocytogenes* (Charpentier et al., 1994), but, to the authors' knowledge, *L. monocytogenes* has never been detected in either industrially (Giaccone, 2005; Grabowski and Klein, 2016; Milanović et al., 2016; Osimani et al., 2018b, 2017c, Vandeweyer et al., 2018, 2017a; Wynants et al., 2018) or laboratory reared edible insects (Osimani et al., 2018a). However, Charpentier et al. (1994) reported the transfer of *tet*(S) from *Listeria* to *Enterococcus*, a genus whose representatives have been detected in previous studies (Vandeweyer et al., 2017b; Wynants et al., 2018) in most of the samples analysed here. If *tet*(S) was effectively carried by enterococci, its higher detection frequency in mealworms might be explained by the higher relative abundance of *Enterococcus* spp. in mealworms compared to crickets, as revealed by Next Generation Sequencing (Vandeweyer et al., 2017b). In addition to *Listeria* and

Enterococcus spp., *tet*(S) has mainly been detected in *Lactococcus* (Ishihara et al., 2013; Kim et al., 2004) and *Streptococcus* (Gevers et al., 2003). Since these genera were encountered in numerous mealworm and cricket samples already (Vandeweyer et al., 2017b; Wynants et al., 2018), they might as well have introduced the *tet*(S) gene in the samples investigated if they indeed carried the *tet*(S) determinant. Significantly different gene copy numbers were also found among different insect batches collected from the same rearing company, with rearer 2 showing the highest *tet*(S) copy numbers, up to 2.10×10^8. Our findings agree well with previous research (Milanović et al., 2016; Osimani et al., 2017b, 2017a), revealing a widespread occurrence of *tet*(S), with more than 50% of the samples found to be positive by nested PCR.

Figure 1 summarises the differences emerged among the samples analysed in terms of detected AR genes. Distances between different points are a measure of the dissimilarity between different samples. A clear distinction between mealworm (green) and cricket (blue) samples is shown, as a consequence of the differences in the occurrence and relative abundance of *tet*(O), *tet*(M), *tet*(K) and *tet*(S) in mealworms and crickets. Generally, mealworms contained higher copy numbers of *tet*(K), *tet*(M) and *tet*(S) than crickets, and contrary to these latter samples, only occasionally harbour a detectable level of *tet*(O). In Figure 1, the grouping of different rearing batches is also shown. Overall, batches from the same company are (at least moderately) clustered together, with the exception of MW 1.3 which differed from both other batches produced by rearing company 1 (MW 1.1 and MW 1.2) for the presence of *tet*(S). Also for company 4, one sample (MW 4.2b) differs greatly from the other six for the occurrence of four out of five AR genes investigated. According to the statistical analyses, sampling period (autumn/winter vs. spring/summer) and post-harvest treatment had no influence on the occurrence and abundance of the AR genes analysed. Furthermore, differences were seen between mealworms and crickets, but not among different species within the same order.

The data collected in this study suggest that edible insects can effectively harbour transferable AR genes that might be mobilised at any stage of the food chain, from rearing up until processing and even consumption. In this regard, the quantification of transferable AR genes in edible insects can contribute to a better evaluation of the health risks associated with the consumption of this novel food, since a higher AR gene copy number is intrinsically associated with a higher risk. Compared to other food matrices, the resistance gene quantity carried by edible insects varies within the same range. For example, the *tet*(M) gene was reported to be present almost up to 7 and 8 log copies/cm² chicken and pork meat, respectively (Hölzel et al., 2011). Likewise, for cheeses, the

tet(S) gene was observed ranging from 4.5 up to 8 log copies/g (Manuzon et al., 2007). In this regard, edible insects pose a similar risk as other food matrices in terms of antibiotic resistance genes carried.

As elucidated by Vandeweyer et al. (2018) and Wynants et al. (2018), the microbiome of edible insects and their feed is closely correlated, thus suggesting that feed used for insect rearing might represent a source of AR microorganisms and/or AR genes. While the use of antibiotics as growth promoters in animal nutrition is strictly prohibited in Europe (Regulation (EC) N° 1831/2003), their therapeutic use in case of emergencies is allowed. To the authors' knowledge, no antibiotics have been administered to the insects analysed in this study. This suggests a role of other factors in the distribution and occurrence of the detected resistances. A possible influencing factor may be the contamination of feed and/or rearing environments with resistant microbes and their genes. Also the selective pressure exerted by both the occurrence of antibiotic residues in feed and water provided to insects and the use of chemical agents for surface cleaning and disinfection may be of influence. Since only freshly reared insects were analysed in this study, no mitigation strategies to reduce the occurrence and relative abundance of AR genes in edible insects, such as starvation, heat treatment, drying, etc. were investigated. Hence, further research to unravel the fate of AR genes in insects during further processing into food is necessary. While AR genes in edible insects can pose a health risk, it was also noted by Cai et al. (2018) that insects and their intestinal microbiota may play a role in the degradation of e.g. tetracyclines. This includes an interesting path for future research as well.

4 Conclusions

The occurrence of transferable antibiotic resistance genes in food products may pose a risk for human health. Insects, considered as an emerging source of proteins in Western countries, are currently intensively being investigated for their food safety. This study provides quantitative data on the presence of a selected pool of transferable AR genes in 30 samples of freshly reared mealworms and crickets from different industrial rearers. As a whole, genes conferring resistance to tetracyclines were detected with a high frequency, ranging from 37% up to 100% of the samples, for *tet*(O) and *tet*(M), respectively. A significantly different distribution of these genes was seen in fresh mealworms compared to crickets, with mealworms harbouring a higher copy number of *tet*(K), *tet*(M), and *tet*(S), while *tet*(O) occurred exclusively in crickets. Based on the results collected in this study as well as in a previous one on the same samples, these differences might be ascribed to differences in the microbial composition and the feed source of the insects analysed. Also, clear correlations between sample

microbiota previously reported and the occurrence of certain genes known to be carried by specific genera were observed. Moreover, a geographical distribution seems to exist for *tet*(K), with a significantly higher occurrence in samples from the Netherlands than from Belgium. A remarkably lower occurrence of *erm*(B) was observed, with only one mealworm sample found to be positive by qPCR. Further research is needed to elucidate the sources of these AR genes during the rearing of the insects as well as their distribution during and after processing into foodstuffs.

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List of figure captions

Figure 1 Non-metric multidimensional scaling (NMDS) ordination composed of all antibiotic resistance gene data for all 30 samples investigated (stress value of 0.146). Mealworm samples are represented by a green colour (light green: yellow mealworms, dark green: lesser mealworms) and cricket samples by a blue colour (dark blue: house crickets, light blue: banded crickets). The distance between different points on the plot reflects their similarity level: the more similar the AR gene composition, the smaller the distance between the points. The plot was constructed based on the AR gene copy number per gram of insect for all five genes assessed.**ables**

Table 1

Table 1. Sample information.

Sample ID	Rearing company	Batch	Sampling period	Country	Insect species	Post-harvest treatment	Remarks
MW 1.1	1	1	March 2015	Belgium	*T. molitor*[1]	24 h starving	During starving, carrots were provided
MW 1.2	1	2	May 2015	Belgium	*T. molitor*	48 h starving	
MW 1.3	1	3	September 2015	Belgium	*T. molitor*	48 h starving	
MW 2.1	2	1	March 2015	The Netherlands	*T. molitor*	None	
MW 2.2	2	2	June 2015	The Netherlands	*T. molitor*	None	
MW 2.3	2	3	October 2015	The Netherlands	*T. molitor*	12 h starving	
MW 3.1	3	1	May 2015	The Netherlands	*T. molitor*	96 h starving	
MW 3.2	3	2	July 2015	The Netherlands	*T. molitor*	96 h starving	
MW 3.3	3	3	November 2015	The Netherlands	*T. molitor*	96 h starving	Refrigerated starving
MW 4.1a	4	1	February 2016	Belgium	*T. molitor*	48 h starving	
MW 4.1b	4	1	February 2016	Belgium	*T. molitor*	None	
MW 4.2a	4	2	February 2016	Belgium	*T. molitor*	None	
MW 4.2b	4	2	February 2016	Belgium	*T. molitor*	48h starving	Refrigerated starving
MW 4.3a	4	3	March 2016	Belgium	*T. molitor*	None	
MW 4.3b	4	3	March 2016	Belgium	*T. molitor*	48 h starving	Refrigerated starving
MW 4.4	4	4	August 2017	Belgium	*T. molitor*	None	
MW 5.1	5	1	February 2017	Belgium	*T. molitor*	None	
LMW 1.1a	6	1	July 2016	The Netherlands	*A. diaperinus*[2]	None	
LMW 1.1b	6	1	July 2016	The Netherlands	*A. diaperinus*	24 h starving	
LMW 1.2	6	2	July 2016	The Netherlands	*A. diaperinus*	None	
HCR 1.2	7	2	June 2015	The Netherlands	*A. domesticus*[3]	12 h starving	Refrigerated starving
HCR 1.3	7	3	September 2015	The Netherlands	*A. domesticus*	12 h starving	Refrigerated starving
HCR 2.1	8	1	April 2015	The Netherlands	*A. domesticus*	None	
HCR 2.2	8	2	July 2015	The Netherlands	*A. domesticus*	None	
HCR 2.3	8	3	October 2015	The Netherlands	*A. domesticus*	12 h starving	
BCR 1.1	9	1	August 2015	Belgium	*G. sigillatus*[4]	12 h starving	
BCR 1.2	9	2	October 2015	Belgium	*G. sigillatus*	12 h starving	Refrigerated starving
BCR 1.3	9	3	December 2015	Belgium	*G. sigillatus*	None	

BCR 1.4a	9	4	November 2016	Belgium	*G. sigillatus*	None	Nymph stage (26 days old)
BCR 1.4b	9	4	November 2016	Belgium	*G. sigillatus*	None	Adult stage

[1]*T.: Tenebrio;* [2]*A.: Alphitobius;* [3]*A.: Acheta;* [4]*G.: Gryllodes*

Table 2

Table 2. Bacterial reference strains used as positive controls in qPCR runs.

Strain	Antibiotic resistance gene	Source
Enterococcus faecalis TO37a	*erm*(B)	Department collection D3A[1]
Enterococcus faecalis TO15a	*tet*(M)	Department collection D3A
Enterococcus italicus 1102	*tet*(S)	Department collection D3A
Streptococcus pyogenes 7008	*tet*(O)	Department collection DiSVA[2]
Staphylococcus aureus COL	*tet*(K)	Department collection DiSVA

[1] Culture Collection of the Department of Agricultural, Food and Environmental Sciences (D3A), Università Politecnica delle Marche, Ancona, Italy;

[2] Culture Collection of the Department of Life and Environmental Sciences (DiSVA), Università Politecnica delle Marche, Ancona, Italy.

Table 3

Table 3. Mean antibiotic resistance gene copy number per g insect sample.[$]

Sample code	Mean gene copy number per g insect				
	***tet*(O)**	***tet*(K)**	***tet*(M)**	***tet*(S)**	***erm*(B)**
MW 1.1	N.D.[+]	N.D.	$5.58 \times 10^{5} \pm 3.84 \times 10^{4}$ [a]	N.D.	N.D.
MW 1.2	N.D.	N.D.	$1.48 \times 10^{5} \pm 5.41 \times 10^{3}$ [a]	N.D.	N.D.
MW 1.3	N.D.	N.D.	$6.91 \times 10^{5} \pm 2.95 \times 10^{5}$ [a]	$9.97 \times 10^{7} \pm 3.83 \times 10^{7}$	N.D.
MW 2.1	N.D.	$5.76 \times 10^{5} \pm 8.23 \times 10^{4}$ [a]	$1.29 \times 10^{5} \pm 4.80 \times 10^{3}$ [a]	$2.10 \times 10^{8} \pm 1.39 \times 10^{7}$ [a]	N.D.
MW 2.2	N.D.	$3.31 \times 10^{4} \pm 9.82 \times 10^{2}$ [a]	$6.58 \times 10^{4} \pm 1.60 \times 10^{4}$ [a]	$1.99 \times 10^{6} \pm 1.60 \times 10^{5}$ [b]	N.D.
MW 2.3	N.D.	$4.02 \times 10^{5} \pm 1.37 \times 10^{5}$ [a]	$1.06 \times 10^{5} \pm 2.71 \times 10^{4}$ [a]	$6.31 \times 10^{7} \pm 2.02 \times 10^{7}$ [c]	N.D.
MW 3.1	N.D.	$2.44 \times 10^{6} \pm 2.42 \times 10^{4}$ [a]	$2.07 \times 10^{5} \pm 1.30 \times 10^{4}$ [a]	$3.60 \times 10^{6} \pm 2.31 \times 10^{4}$ [a]	N.D.
MW 3.2	N.D.	$9.36 \times 10^{4} \pm 1.29 \times 10^{4}$ [b]	$2.29 \times 10^{5} \pm 1.25 \times 10^{4}$ [a]	$2.40 \times 10^{6} \pm 1.03 \times 10^{4}$ [b]	N.D.
MW 3.3	N.D.	$5.14 \times 10^{6} \pm 3.94 \times 10^{4}$ [c]	$2.58 \times 10^{6} \pm 1.17 \times 10^{5}$ [b]	$1.15 \times 10^{7} \pm 8.88 \times 10^{5}$ [a,b]	N.D.
MW 4.1a	N.D.	N.D.	$1.00 \times 10^{5} \pm 5.62 \times 10^{3}$ [a]	$3.80 \times 10^{5} \pm 1.47 \times 10^{5}$ [a,b]	N.D.
MW 4.1b	N.D.	N.D.	$2.77 \times 10^{6} \pm 4.13 \times 10^{4}$ [a]	$6.34 \times 10^{7} \pm 2.15 \times 10^{6}$ [a,b]	N.D.
MW 4.2a	N.D.	N.D.	$2.45 \times 10^{6} \pm 9.13 \times 10^{4}$ [a]	$3.72 \times 10^{7} \pm 4.09 \times 10^{6}$ [a]	N.D.
MW 4.2b	$5.07 \times 10^{5} \pm 5.52 \times 10^{4}$	N.D.	$4.52 \times 10^{7} \pm 2.02 \times 10^{6}$ [a]	$4.12 \times 10^{7} \pm 6.73 \times 10^{5}$ [a]	$3.18 \times 10^{5} \pm 8.42 \times 10^{3}$
MW 4.3a	N.D.	N.D.	$4.66 \times 10^{4} \pm 5.62 \times 10^{3}$ [a]	$5.15 \times 10^{7} \pm 1.14 \times 10^{6}$ [a,b]	N.D.
MW 4.3b	N.D.	N.D.	$1.59 \times 10^{7} \pm 2.65 \times 10^{5}$ [a]	$6.92 \times 10^{5} \pm 4.05 \times 10^{5}$ [a,b]	N.D.
MW 4.4	N.D.	$2.78 \times 10^{4} \pm 4.53 \times 10^{3}$	$4.75 \times 10^{6} \pm 3.38 \times 10^{5}$ [a]	$7.86 \times 10^{5} \pm 6.97 \times 10^{4}$ [b]	N.D.
MW 5.1	N.D.	N.D.	$3.47 \times 10^{4} \pm 3.26 \times 10^{3}$	$1.12 \times 10^{6} \pm 3.76 \times 10^{5}$	N.D.
LMW 1.1a	N.D.	$3.03 \times 10^{4} \pm 1.70 \times 10^{3}$ [a]	$2.71 \times 10^{6} \pm 1.46 \times 10^{5}$ [a]	$9.15 \times 10^{6} \pm 8.64 \times 10^{5}$ [a]	N.D.
LMW 1.1b	N.D.	N.D.	$8.95 \times 10^{5} \pm 3.00 \times 10^{4}$ [a]	$5.52 \times 10^{6} \pm 4.92 \times 10^{5}$ [a]	N.D.
LMW 1.2	N.D.	$8.85 \times 10^{4} \pm 2.03 \times 10^{4}$ [b]	$7.96 \times 10^{6} \pm 9.65 \times 10^{5}$ [b]	$2.95 \times 10^{7} \pm 4.23 \times 10^{6}$ [b]	N.D.
HCR 1.2	$1.94 \times 10^{6} \pm 3.54 \times 10^{4}$ [a]	N.D.	$3.97 \times 10^{5} \pm 1.29 \times 10^{4}$ [a]	N.D.	N.D.
HCR 1.3	$6.99 \times 10^{6} \pm 4.10 \times 10^{5}$ [b]	N.D.	$1.53 \times 10^{6} \pm 9.53 \times 10^{4}$ [b]	$8.44 \times 10^{5} \pm 2.95 \times 10^{4}$	N.D.

HCR 2.1	7.06×10^{6}	±	4.50×10^{3} [a]	4.07×10^{4}	±	1.12×10^{3} [a]	7.88×10^{5}	±	6.27×10^{3} [a]	N.D.			N.D.
HCR 2.2	7.15×10^{6}	±	1.50×10^{5} [a]	N.D.			3.89×10^{5}	±	3.46×10^{4} [a]	N.D.			N.D.
HCR 2.3	4.24×10^{7}	±	4.88×10^{6} [a]	2.76×10^{5}	±	6.46×10^{4} [b]	3.48×10^{6}	±	4.98×10^{4} [b]	N.D.			N.D.
BCR 3.1	2.63×10^{6}	±	1.39×10^{5} [a]	N.D.			1.54×10^{5}	±	1.10×10^{4} [a]	N.D.			N.D.
BCR 3.2	1.01×10^{7}	±	4.21×10^{4} [b]	N.D.			1.71×10^{6}	±	4.00×10^{3} [b]	5.37×10^{5}	±	3.34×10^{4} [a]	N.D.
BCR 3.3	4.28×10^{6}	±	4.17×10^{5} [a,b,c]	N.D.			4.09×10^{5}	±	1.25×10^{4} [c]	6.06×10^{5}	±	1.03×10^{5} [a]	N.D.
BCR 3.4a	2.03×10^{5}	±	7.20×10^{3} [c]	N.D.			6.43×10^{4}	±	1.26×10^{3} [a,c]	N.D.			N.D.
BCR 3.4b	5.70×10^{5}	±	7.99×10^{4} [c]	4.57×10^{4}	±	9.12×10^{3}	5.66×10^{5}	±	5.96×10^{4} [a,c]	N.D.			N.D.

[$]Data are the mean values of two qPCR assessments ± standard deviation. [a,b,c]Means per rearing batch from the same rearing company with the same superscript within the same columns do not differ significantly ($p > 0.05$).

[+]N.D. = not detected.

Figure 1

Fig. 1