



UNIVERSITÀ POLITECNICA DELLE MARCHE
Repository ISTITUZIONALE

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

This is a pre print version of the following article:

Original

Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and the Netherlands / Dries, Vandeweyer; Milanovic, Vesna; Garofalo, Cristiana; Osimani, Andrea; Clementi, Francesca; Leen Van Campenhout, ; Aquilanti, Lucia. - In: INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY. - ISSN 0168-1605. - ELETTRONICO. - 290:(2019), pp. 288-295. [10.1016/j.ijfoodmicro.2018.10.027]

Availability:

This version is available at: 11566/261666 since: 2022-06-01T17:15:07Z

Publisher:

Published

DOI:10.1016/j.ijfoodmicro.2018.10.027

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. The use of copyrighted works requires the consent of the rights' holder (author or publisher). Works made available under a Creative Commons license or a Publisher's custom-made license can be used according to the terms and conditions contained therein. See editor's website for further information and terms and conditions.

This item was downloaded from IRIS Università Politecnica delle Marche (<https://iris.univpm.it>). When citing, please refer to the published version.

(Article begins on next page)

Manuscript Number:

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

Article Type: Full Length Article

Keywords: edible insects; mealworms; crickets; antibiotic resistance; real-time PCR.

Corresponding Author: Dr. vesna milanovic,

Corresponding Author's Institution:

First Author: Dries Vandeweyer

Order of Authors: Dries Vandeweyer; vesna milanovic; Cristiana Garofalo; Andrea Osimani; Francesca Clementi; Leen Van Campenhout; Lucia Aquilanti

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.

Suggested Reviewers: Katarzyna Ignasiak
katarzyna.ignasiak@jic.ac.uk

Tony Maxwell

tony.maxwell@jic.ac.uk

Laura Gasco
laura.gasco@unito.it

Nils Th. Grabowski
Nils.Grabowski@tiho-hannover.de

Ancona 18.06.2018

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 *Grylodes 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

Dipartimento di Scienze Agrarie, Alimentari ed Ambientali
Università Politecnica delle Marche
Via Brecce Bianche, 60131 Ancona, Italy

- 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

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

3 Dries Vandeweyer^a, Vesna Milanović^{b,*}, Cristiana Garofalo^b, Andrea Osimani^b, Francesca Clementi^b, Leen Van
4 Campenhout^a & Lucia Aquilanti^b

5

6 ^a *KU Leuven, Department of Microbial and Molecular Systems (M²S), Lab4Food, Geel Campus, 2440 Geel,*
7 *Belgium*

8 ^b *Università Politecnica delle Marche, Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, via Brecce*
9 *Bianche,, 60131 Ancona, Italy*

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28 * *Corresponding author:* dr. Vesna Milanović, Università Politecnica delle Marche, Via Brecce Bianche, 60131

29 Ancona, Italia. E-mail: v.milanovic@univpm.it.

30 **Abstract**

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

49

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

51

52

53

54

55

56

57

58

59 **1 Introduction**

60 Since the recent introduction of edible insects in several European countries, the microbial quality and
61 safety of insects used for human consumption was repeatedly investigated (Garofalo et al., 2017; Klunder et al.,
62 2012; Osimani et al., 2017c; Rumpold and Schlüter, 2013; Stoops et al., 2017, 2016; van der Spiegel et al., 2013;
63 Vandeweyer et al., 2018, 2017a, 2017b; Wynants et al., 2018), as recommended by different scientific opinions
64 and advices (ANSES, 2015; EFSA Scientific Committee, 2015; NVWA, 2014; SHC and FASFC, 2014). The
65 new European Novel Food regulation (EU 2015/2283), which took effect in January 2018, has evoked an
66 increase in edible insect research as well.

67 As recently reviewed by Dobermann et al. (2017), the main challenges of mass rearing of edible insects
68 include the bacterial contamination of the end products, e.g. high counts of spore-forming bacteria, total
69 mesophilic aerobes, and Enterobacteriaceae, and the potential occurrence of human pathogens as well as the
70 risks of antibiotic usage in such mass rearing. Concerning this latter aspect, the use and misuse of antibiotics are
71 known to have a major effect on the prevalence of antibiotic resistant microorganisms, for example in primary
72 production, food, feed, and the environment (Clementi and Aquilanti, 2011; Verraes et al., 2013). Hence, for
73 2016-2020 the European Medicine Agency (EMA) expressed a joint opinion with the European Food Safety
74 Authority (EFSA) on measures to reduce the use of antimicrobial agents in animal husbandry (also known as the
75 'RONAFA' opinion) (EMA and EFSA, 2017). Even earlier reports published jointly by EMA and European
76 bodies including the European Centre for Disease Prevention and Control (ECDC), EFSA, and the European
77 Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) have
78 emphasised the need for the prudent use of antibiotics in animals (ECDC et al., 2009). Concerning edible insects,
79 no data are currently available about the use of antibiotics in mass rearing and the occurrence of antibiotic
80 resistances (ARs) in edible insects is limitedly assessed (Milanović et al., 2016; Osimani et al., 2017a, 2017b),
81 despite the recommendations posed in the EFSA opinion (EFSA Scientific Committee, 2015) and two joint
82 reports of the ECDC, EFSA, and EMA (ECDC et al., 2017, 2015).

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

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

91 Edible insects are typically processed prior to consumption (Fombong et al., 2017; van Huis et al.,
92 2013; Vandeweyer et al., 2017c). To lower microbial counts, many processing steps involve a heat treatment
93 causing a number of lesions in microbial cells. These include membrane damage, loss of nutrients and ions,
94 ribosome aggregation, and even DNA strand breaks (Mañas and Pagán, 2005). However, less vulnerable
95 microorganisms (e.g. bacterial spores (Vandeweyer et al., 2017c)) and their AR genes may survive the minimal
96 heating treatments frequently applied for insects or even be triggered in AR transfer (Verraes et al., 2013).
97 Processing may also cross-contaminate insects with ARs initially not carried by their associated microbiota
98 (Verraes et al., 2013). Recently, a few studies have been performed to investigate the occurrence and distribution
99 of transferable AR genes in processed, ready-to-eat insects available on the European market (Milanović et al.,
100 2016; Osimani et al., 2017b, 2017a), but so far, fresh insects, i.e. living insects at the end of their rearing cycle
101 collected from industrial rearing facilities, have not been subjected to AR assessment, yet. In all former studies, a
102 number of genes inducing resistance against classes of antibiotics commonly used in both human and animal
103 therapy (e.g. tetracyclines and macrolides) were detected by qualitative nested PCR. According to Penders et al.
104 (2013), three different metagenomic approaches are currently applied to study the AR pool: PCR-based
105 metagenomics, functional metagenomics, and sequence-based metagenomics. Although nested PCR assays are
106 characterised by an extremely high sensitivity for detection of target AR genes (Milanović et al., 2016; Osimani
107 et al., 2017b, 2017a), this technique does not allow an effective quantification of the amount of gene copies
108 occurring in a given sample. To date, real-time PCR (qPCR) techniques have been applied in a number of food
109 matrices to detect and quantify both tetracycline and erythromycin resistance genes (Flórez et al., 2014).
110 However, to the authors' knowledge, no qPCR assays have been used or optimised for the analysis of
111 transferable ARs in edible insects, yet.

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

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

118

119 **2 Materials and methods**

120 *2.1 Fresh insect sampling*

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

132

133 *2.2 Reference strains*

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

137

138 *2.3 Sample preparation and DNA extraction*

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

145 using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The concentration of the
146 extracted DNA was standardised to 2 ng/µl for all the samples. To check the effectiveness of bacterial DNA
147 extraction, the DNA suspensions were amplified by end-point PCR using the universal prokaryotic primers
148 338F-518R, as previously described (Osimani et al., 2017b). DNA from the reference strains was extracted
149 following the procedure previously detailed by Osimani et al. (2015).

150

151 *2.4 Construction of qPCR standards*

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

165

166 *2.5 Real Time qPCR quantification*

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

174 The qPCR conditions for the genes *tet(O)*, *tet(M)*, and *tet(K)* included an initial denaturation step of 5
175 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
176 *erm(B)*, qPCR conditions were as described by Flórez et al. (2014), with a slight modification in the last step
177 (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
178 curve step with temperature gradually increasing from 60 °C to 95 °C by 0.4 °C/s.

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

190

191 *2.6 Statistical analyses*

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

199

200 **3 Results and discussion**

201

202 *3.1 Accuracy of qPCR assessments*

203 Standard curves created for each AR gene qPCR assessment showed R²-values of 0.99 and efficiencies
204 between 0.95 and 1.05. Detection limits, defined as the lowest gene copy number per reaction in which the
205 linearity was maintained, were in order of 10¹ for *tet(K)* gene and 10² for *tet(M)*, *tet(S)*, *tet(O)* and *erm(B)* genes,
206 respectively. qPCR assessments were therefore considered reliable, efficient and sensitive.

207

208 3.2 Quantitative detection of antibiotic resistance genes in insect samples

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

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

232 et al., 2017a, 2017b). Since the microbiome is known to be specific for different edible insects (Garofalo et al.,
233 2017; Osimani et al., 2017c; Stoops et al., 2016; Vandeweyer et al., 2017b), AR genes may concomitantly be
234 present or absent in different insects, depending on the microbiota composition of these insects. Previously,
235 *tet(O)* has been detected in streptococci and campylobacteria (Chopra and Roberts, 2001), two microbial groups
236 that were already recovered from cricket samples, though at very low levels (Vandeweyer et al., 2017b). The
237 high copy numbers of *tet(O)* detected in the cricket samples analysed in this study suggest that also
238 microorganisms other than streptococci and campylobacteria might carry this determinant. In this regard, a
239 number of previous studies have clearly indicated that the microbial compositions of crickets and mealworms are
240 influenced largely by their diet (Colman et al., 2012; Wynants et al., 2018; Yun et al., 2014), thus explaining
241 potential differences occurring in the distribution of specific AR genes as well.

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

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

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

264 Regarding *tet(M)*, an ubiquitous occurrence of this determinant was revealed by qPCR analysis.
265 Although it was detected in all samples, mealworms contained, on average, a statistically ($p = 0.042$) higher
266 copy number of *tet(M)* than crickets. When comparing different batches produced by the same company, the
267 highest variety in *tet(M)* copies between samples was found for company 9 (*G. sigillatus*). Besides the type of
268 insect species and production batches, other potentially influencing factors considered, such as the geographical
269 origin or the type of post-harvest treatment, were not found to exert a significant influence on the distribution of
270 *tet(M)* among the samples analysed. Our results agree well with other studies (Milanović et al., 2016; Osimani et
271 al., 2017b), where *tet(M)* was frequently detected in various specimens of marketed edible insects, including
272 processed mealworms and crickets. Overall, these findings suggest a wide distribution of *tet(M)* in the
273 microbiome of edible insects. Its frequent detection as well as its occasionally high copy numbers (up to $4.52 \times$
274 10^7) might be attributed to the fact that *tet(M)* is typically located on conjugative transposons (e.g. Tn916 -
275 Tn1545 family) and can therefore easily be transferred from one bacterial species to another (Doherty et al.,
276 2000). Accordingly, *tet(M)* has been detected in numerous food matrices, including dairy (Flórez et al., 2014)
277 and meat products (Hölzel et al., 2011). Interestingly, in the present study, two samples (MW 1.1 and MW 1.2)
278 did not carry any other AR gene than *tet(M)*.

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

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

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

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

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

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

335

336 **4 Conclusions**

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

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

353

354 **Acknowledgements**

355 Funding: this research was supported by Flanders Innovation & Entrepreneurship (VLAIO) [Project
356 141129] and partly by the Università Politecnica delle Marche, Ancona, Italy, within the project “Edible insects:
357 new frontiers in food - FOODIN”.

358

359 **References**

360 ANSES, 2015. Opinion of the French Agency for Food, Environmental and Occupational Health & Safety on
361 “the use of insects as food and feed and the review of scientific knowledge on the health risks related to the
362 consumption of insects.” Maisons-Alfort, France.

363 Barbeyrac, B. de, Dutilh, B., Quentin, C., Renaudin, H., Bébéar, C., 1991. Susceptibility of *Bacteroides*
364 *ureolyticus* to antimicrobial agents and identification of a tetracycline resistance determinant related to
365 *tetM*. J. Antimicrob. Chemother. 27, 721–731. doi:10.1093/jac/27.6.721

366 Cai, M., Hu, R., Ma, S., Tomberlin, J.K., Yu, C., Zhang, K., Li, W., Li, Q., Yu, Z., Zhang, J., 2018. The
367 degradation of tetracycline by black soldier fly (Diptera: Stratiomyidae) larvae with intestinal
368 microorganisms, in: Insects to Feed the World. Journal of Insects as Food and Feed 4, Supplement 1,
369 Wuhan, Hubei, China, p. 22.

370 Charpentier, E., Gerbaud, G., Courvalin, P., 1994. Presence of the *Listeria* Tetracycline Resistance Gene *tet(S)*
371 in *Enterococcus faecalis*. Antimicrob. Agents Chemother. 38, 2330–2335. doi:10.1128/AAC.38.10.2330

372 Chopra, I., Roberts, M., 2001. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and
373 Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. 65, 232–260.

374 doi:10.1128/MMBR.65.2.232-260.2001

375 Clementi, F., Aquilanti, L., 2011. Recent investigations and updated criteria for the assessment of antibiotic

376 resistance in food lactic acid bacteria. *Anaerobe* 17, 394–398.
377 doi:<https://doi.org/10.1016/j.anaerobe.2011.03.021>

378 Colman, D.R., Toolson, E.C., Takacs-Vesbach, C.D., 2012. Do diet and taxonomy influence insect gut bacterial
379 communities? *Mol. Ecol.* 21, 5124–5137. doi:10.1111/j.1365-294X.2012.05752.x

380 Dobermann, D., Swift, J.A., Field, L.M., 2017. Opportunities and hurdles of edible insects for food and feed.
381 *Nutr. Bull.* 42, 293–308. doi:10.1111/nbu.12291

382 Doherty, N., Trzcinski, K., Pickerill, P., Zawadzki, P., Dowson, C.G., 2000. Genetic diversity of the *tet(M)* gene
383 in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* 44,
384 2979–2984. doi:10.1128/AAC.44.11.2979-2984.2000

385 ECDC, EFSA, EMA, 2017. ECDC/EFSA/EMA second joint report on the integrated analysis of the
386 consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans
387 and food-producing animals. *EFSA J.* 15. doi:10.2903/j.efsa.2017.4872

388 ECDC, EFSA, EMA, 2015. ECDC/EFSA/EMA first joint report on the integrated analysis of the consumption of
389 antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-
390 producing animals. *EFSA J.* 13, 1–114. doi:10.2903/j.efsa.2015.4006

391 ECDC, EFSA, EMA, SCENIHR, 2009. Joint Opinion on antimicrobial resistance (AMR) focused on zoonotic
392 infections. *EFSA J.* 7, 1372. doi:10.2903/j.efsa.2009.1372

393 EFSA Scientific Committee, 2015. Risk profile related to production and consumption of insects as food and
394 feed. *EFSA J.* 2015 13, 60 pp. doi:10.2903/j.efsa.2015.4257

395 EMA, EFSA, 2017. EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use
396 antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety
397 (RONAFA). *EFSA J.* 15, 245. doi:10.2903/j.efsa.2017.4666

398 Flórez, A.B., Alegría, Á., Rossi, F., Delgado, S., Felis, G.E., Torriani, S., Mayo, B., 2014. Molecular
399 Identification and Quantification of Tetracycline and Erythromycin Resistance Genes in Spanish and
400 Italian Retail Cheeses. *Biomed Res. Int.* 2014, 746859. doi:10.1155/2014/746859

401 Fombong, F.T., Van Der Borght, M., Vanden Broeck, J., 2017. Influence of Freeze-Drying and Oven-Drying
402 Post Blanching on the Nutrient Composition of the Edible Insect *Ruspolia differens*. *Insects* 8, 102–116.
403 doi:10.3390/insects8030102

404 Garofalo, C., Osimani, A., Milanović, V., Taccari, M., Cardinali, F., Aquilanti, L., Riolo, P., Ruschioni, S.,

405 Isidoro, N., Clementi, F., 2017. The microbiota of marketed processed edible insects as revealed by high-
406 throughput sequencing. *Food Microbiol.* 62, 15–22. doi:10.1016/j.fm.2016.09.012

407 Gevers, D., Masco, L., Baert, L., Huys, G., Debevere, J., Swings, J., 2003. Prevalence and Diversity of
408 Tetracycline Resistant Lactic Acid Bacteria and their tet Genes Along the Process Line of Fermented Dry
409 Sausages. *Syst. Appl. Microbiol.* 26, 277–283. doi:10.1078/072320203322346137

410 Giaccone, V., 2005. Hygiene and Health Features of “Minilivestock,” in: Paoletti, M.G. (Ed.), *Ecological*
411 *Implications of Minilivestock: Potential of Insects, Rodents, Frogs and Snails.* Science Publishers, Inc.,
412 Enfield, NH, USA, pp. 579–598.

413 Gogarten, M.B., Gogarten, J.P., Olendzenski, L.C. (Eds.), 2009. Horizontal Gene Transfer, *Methods in*
414 *Molecular Biology.* Humana Press, Totowa, NJ. doi:10.1007/978-1-60327-853-9

415 Grabowski, N.T., Klein, G., 2016. Microbiology of processed edible insect products – Results of a preliminary
416 survey. *Int. J. Food Microbiol.* 243, 103–107. doi:10.1016/j.ijfoodmicro.2016.11.005

417 Hölzel, C.S., Huther, S.K., Schwaiger, K., Kämpf, P., Bauer, J., 2011. Quantity of the Tetracycline Resistance
418 Gene *tet(M)* Differs Substantially between Meat at Slaughterhouses and at Retail. *J. Food Sci.* 76, M318–
419 M323. doi:10.1111/j.1750-3841.2011.02239.x

420 Ishihara, K., Nakajima, K., Kishimoto, S., Atarashi, F., Muramatsu, Y., Hotta, A., Ishii, S., Takeda, Y., Kikuchi,
421 M., Tamura, Y., 2013. Distribution of antimicrobial-resistant lactic acid bacteria in natural cheese in Japan.
422 *Microbiol. Immunol.* 57, 684–691. doi:10.1111/1348-0421.12090

423 Jacob, A.E., Hobbs, S.J., 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in
424 *Streptococcus faecalis* var. *zymogenes*. *J. Bacteriol.* 117, 360–72.

425 Kim, S.-R., Nonaka, L., Suzuki, S., 2004. Occurrence of tetracycline resistance genes *tet(M)* and *tet(S)* in
426 bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.* 237, 147–156.
427 doi:10.1016/j.femsle.2004.06.026

428 Klunder, H.C., Wolkers-Rooijackers, J., Korpela, J.M., Nout, M.J.R., 2012. Microbiological aspects of
429 processing and storage of edible insects. *Food Control* 26, 628–631. doi:10.1016/j.foodcont.2012.02.013

430 Leclercq, R., 2002. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance
431 Elements and Their Clinical Implications. *Clin. Infect. Dis.* 34, 482–492. doi:10.1086/324626

432 Mañas, P., Pagán, R., 2005. Microbial inactivation by new technologies of food preservation. *J. Appl. Microbiol.*
433 98, 1387–1399. doi:10.1111/j.1365-2672.2005.02561.x

434 Manuzon, M.Y., Hanna, S.E., Luo, H., Yu, Z., Harper, W.J., Wang, H.H., 2007. Quantitative Assessment of the
435 Tetracycline Resistance Gene Pool in Cheese Samples by Real-Time TaqMan PCR. *Appl. Environ.*
436 *Microbiol.* 73, 1676–1677. doi:10.1128/AEM.01994-06

437 Milanović, V., Osimani, A., Aquilanti, L., Tavoletti, S., Garofalo, C., Polverigiani, S., Litta-Mulondo, A.,
438 Cocolin, L., Ferrocino, I., Di Cagno, R., Turrone, S., Lazzi, C., Pellegrini, N., Clementi, F., 2017.
439 Occurrence of antibiotic resistance genes in the fecal DNA of healthy omnivores, ovo-lacto vegetarians
440 and vegans. *Mol. Nutr. Food Res.* 61, 1601098. doi:10.1002/mnfr.201601098

441 Milanović, V., Osimani, A., Pasquini, M., Aquilanti, L., Garofalo, C., Taccari, M., Cardinali, F., Riolo, P.,
442 Clementi, F., 2016. Getting insight into the prevalence of antibiotic resistance genes in specimens of
443 marketed edible insects. *Int. J. Food Microbiol.* 227, 22–28. doi:10.1016/j.ijfoodmicro.2016.03.018

444 NVWA, 2014. Advisory report on the risks associated with the consumption of mass-reared insects. Utrecht, The
445 Netherlands.

446 Osimani, A., Cardinali, F., Aquilanti, L., Garofalo, C., Roncolini, A., Milanović, V., Pasquini, M., Tavoletti, S.,
447 Clementi, F., 2017a. Occurrence of transferable antibiotic resistances in commercialized ready-to-eat
448 mealworms (*Tenebrio molitor* L.). *Int. J. Food Microbiol.* 263, 38–46.
449 doi:10.1016/j.ijfoodmicro.2017.10.009

450 Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., Cardinali, F., Taccari, M., Pasquini, M., Tavoletti, S.,
451 Clementi, F., 2017b. Transferable Antibiotic Resistances in Marketed Edible Grasshoppers (*Locusta*
452 *migratoria migratorioides*). *J. Food Sci.* 82, 1184–1192. doi:10.1111/1750-3841.13700

453 Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., Clementi, F., 2015. Unpasteurised commercial boza as a
454 source of microbial diversity. *Int. J. Food Microbiol.* 194, 62–70. doi:10.1016/j.ijfoodmicro.2014.11.011

455 Osimani, A., Garofalo, C., Milanović, V., Taccari, M., Cardinali, F., Aquilanti, L., Pasquini, M., Mozzon, M.,
456 Raffaelli, N., Ruschioni, S., Riolo, P., Isidoro, N., Clementi, F., 2017c. Insight into the proximate
457 composition and microbial diversity of edible insects marketed in the European Union. *Eur. Food Res.*
458 *Technol.* 243, 1157–1171. doi:10.1007/s00217-016-2828-4

459 Osimani, A., Milanović, V., Cardinali, F., Garofalo, C., Clementi, F., Pasquini, M., Riolo, P., Ruschioni, S.,
460 Isidoro, N., Loreto, N., Franciosi, E., Tuohy, K., Petruzzelli, A., Fogliani, M., Gabucci, C., Tonucci, F.,
461 Aquilanti, L., 2018a. The bacterial biota of laboratory-reared edible mealworms (*Tenebrio molitor* L.):
462 From feed to frass. *Int. J. Food Microbiol.* 272, 49–60. doi:10.1016/j.ijfoodmicro.2018.03.001

463 Osimani, A., Milanović, V., Garofalo, C., Cardinali, F., Roncolini, A., Sabbatini, R., De Filippis, F., Ercolini, D.,
464 Gabucci, C., Petruzzelli, A., Tonucci, F., Clementi, F., Aquilanti, L., 2018b. Revealing the microbiota of
465 marketed edible insects through PCR-DGGE, metagenomic sequencing and real-time PCR. *Int. J. Food*
466 *Microbiol.* 276, 54–62. doi:10.1016/j.ijfoodmicro.2018.04.013

467 Penders, J., Stobberingh, E.E., Savelkoul, P.H.M., Wolffs, P.F.G., 2013. The human microbiome as a reservoir
468 of antimicrobial resistance. *Front. Microbiol.* 4, 87. doi:10.3389/fmicb.2013.00087

469 R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for
470 Statistical Computing, Vienna, Austria.

471 Rumpold, B.A., Schlüter, O.K., 2013. Nutritional composition and safety aspects of edible insects. *Mol Nutr*
472 *Food Res* 57, 802–823. doi:10.1002/mnfr.201200735

473 SHC, FASFC, 2014. Food safety aspects of insects intended for human consumption (Sci Com dossier 2014/04;
474 SHC dossier n° 9160). Brussels, Belgium.

475 Stoops, J., Crauwels, S., Waud, M., Claes, J., Lievens, B., Van Campenhout, L., 2016. Microbial community
476 assessment of mealworm larvae (*Tenebrio molitor*) and grasshoppers (*Locusta migratoria migratorioides*)
477 sold for human consumption. *Food Microbiol.* 53, Part B, 122–127. doi:10.1016/j.fm.2015.09.010

478 Stoops, J., Vandeweyer, D., Crauwels, S., Verreth, C., Boeckx, H., Van Der Borgh, M., Claes, J., Lievens, B.,
479 Van Campenhout, L., 2017. Minced meat-like products from mealworm larvae (*Tenebrio molitor* and
480 *Alphitobius diaperinus*): Microbial dynamics during production and storage. *Innov. Food Sci. Emerg.*
481 *Technol.* 41, 1–9. doi:10.1016/j.ifset.2017.02.001

482 van der Spiegel, M., Noordam, M.Y., van der Fels-Klerx, H.J., 2013. Safety of Novel Protein Sources (Insects,
483 Microalgae, Seaweed, Duckweed, and Rapeseed) and Legislative Aspects for Their Application in Food
484 and Feed Production. *Compr. Rev. Food Sci. Food Saf.* 12, 662–678. doi:http://dx.doi.org/10.1111/1541-
485 4337.12032

486 van Huis, A., Van Itterbeeck, J., Klunder, H.C., Mertens, E., Halloran, A., Muir, G., Vantomme, P., 2013. Edible
487 insects: Future prospects for food and feed security. FAO (Food and Agricultural Organization of the
488 United Nations), Rome.

489 Vandeweyer, D., Crauwels, S., Lievens, B., Van Campenhout, L., 2017a. Microbial counts of mealworm larvae
490 (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing
491 companies and different production batches. *Int. J. Food Microbiol.* 242, 13–18.

492 doi:10.1016/j.ijfoodmicro.2016.11.007

493 Vandeweyer, D., Crauwels, S., Lievens, B., Van Campenhout, L., 2017b. Metagenetic analysis of the bacterial
494 communities of edible insects from diverse production cycles at industrial rearing companies. *Int. J. Food*
495 *Microbiol.* 261, 11–18. doi:10.1016/j.ijfoodmicro.2017.08.018

496 Vandeweyer, D., Lenaerts, S., Callens, A., Van Campenhout, L., 2017c. Effect of blanching followed by
497 refrigerated storage or industrial microwave drying on the microbial load of yellow mealworm larvae
498 (*Tenebrio molitor*). *Food Control* 71, 311–314. doi:10.1016/j.foodcont.2016.07.011

499 Vandeweyer, D., Wynants, E., Crauwels, S., Verreth, C., Viaene, N., Claes, J., Lievens, B., Van Campenhout,
500 L., 2018. Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House
501 Crickets (*Gryllobates sigillatus*) for Human Consumption. *Appl. Environ. Microbiol.* 84, AEM.00255-18.
502 doi:10.1128/AEM.00255-18

503 Verraes, C., Van Boxstael, S., Van Meervenue, E., Van Coillie, E., Butaye, P., Catry, B., de Schaezen, M.A.,
504 Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J., Herman,
505 L., 2013. Antimicrobial Resistance in the Food Chain: A review. *Int. J. Environ. Res. Public Health* 10,
506 2643–2669. doi:10.3390/ijerph10072643

507 Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., Bruyninckx, L., Van Campenhout, L.,
508 2017. Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community
509 composition of mealworm larvae (*Tenebrio molitor*). *Innov. Food Sci. Emerg. Technol.* 42, 8–15.
510 doi:10.1016/j.ifset.2017.06.004

511 Wynants, E., Crauwels, S., Verreth, C., Gianotten, N., Lievens, B., Claes, J., Van Campenhout, L., 2018.
512 Microbial dynamics during production of lesser mealworms (*Alphitobius diaperinus*) for human
513 consumption at industrial scale. *Food Microbiol.* 70, 181–191. doi:10.1016/j.fm.2017.09.012

514 Yun, J.-H., Roh, S.W., Whon, T.W., Jung, M.-J., Kim, M.-S., Park, D.-S., Yoon, C., Nam, Y.-D., Kim, Y.-J.,
515 Choi, J.-H., Kim, J.-Y., Shin, N.-R., Kim, S.-H., Lee, W.-J., Bae, J.-W., 2014. Insect gut bacterial diversity
516 determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Appl. Environ.*
517 *Microbiol.* 80, 5254–64. doi:10.1128/AEM.01226-14

518

519 **List of figure captions**

520

521 **Figure 1** Non-metric multidimensional scaling (NMDS) ordination composed of all antibiotic resistance gene
522 data for all 30 samples investigated (stress value of 0.146). Mealworm samples are represented by a green colour
523 (light green: yellow mealworms, dark green: lesser mealworms) and cricket samples by a blue colour (dark blue:
524 house crickets, light blue: banded crickets). The distance between different points on the plot reflects their
525 similarity level: the more similar the AR gene composition, the smaller the distance between the points. The plot
526 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	<i>T. molitor</i> ¹	24 h starving	During starving, carrots were provided
MW 1.2	1	2	May 2015	Belgium	<i>T. molitor</i>	48 h starving	
MW 1.3	1	3	September 2015	Belgium	<i>T. molitor</i>	48 h starving	
MW 2.1	2	1	March 2015	The Netherlands	<i>T. molitor</i>	None	
MW 2.2	2	2	June 2015	The Netherlands	<i>T. molitor</i>	None	
MW 2.3	2	3	October 2015	The Netherlands	<i>T. molitor</i>	12 h starving	
MW 3.1	3	1	May 2015	The Netherlands	<i>T. molitor</i>	96 h starving	Refrigerated starving
MW 3.2	3	2	July 2015	The Netherlands	<i>T. molitor</i>	96 h starving	
MW 3.3	3	3	November 2015	The Netherlands	<i>T. molitor</i>	96 h starving	
MW 4.1a	4	1	February 2016	Belgium	<i>T. molitor</i>	48 h starving	
MW 4.1b	4	1	February 2016	Belgium	<i>T. molitor</i>	None	
MW 4.2a	4	2	February 2016	Belgium	<i>T. molitor</i>	None	
MW 4.2b	4	2	February 2016	Belgium	<i>T. molitor</i>	48h starving	Refrigerated starving
MW 4.3a	4	3	March 2016	Belgium	<i>T. molitor</i>	None	Refrigerated starving
MW 4.3b	4	3	March 2016	Belgium	<i>T. molitor</i>	48 h starving	
MW 4.4	4	4	August 2017	Belgium	<i>T. molitor</i>	None	
MW 5.1	5	1	February 2017	Belgium	<i>T. molitor</i>	None	
LMW 1.1a	6	1	July 2016	The Netherlands	<i>A. diaperinus</i> ²	None	
LMW 1.1b	6	1	July 2016	The Netherlands	<i>A. diaperinus</i>	24 h starving	
LMW 1.2	6	2	July 2016	The Netherlands	<i>A. diaperinus</i>	None	
HCR 1.2	7	2	June 2015	The Netherlands	<i>A. domesticus</i> ³	12 h starving	Refrigerated starving
HCR 1.3	7	3	September 2015	The Netherlands	<i>A. domesticus</i>	12 h starving	Refrigerated starving
HCR 2.1	8	1	April 2015	The Netherlands	<i>A. domesticus</i>	None	
HCR 2.2	8	2	July 2015	The Netherlands	<i>A. domesticus</i>	None	
HCR 2.3	8	3	October 2015	The Netherlands	<i>A. domesticus</i>	12 h starving	
BCR 1.1	9	1	August 2015	Belgium	<i>G. sigillatus</i> ⁴	12 h starving	Refrigerated starving
BCR 1.2	9	2	October 2015	Belgium	<i>G. sigillatus</i>	12 h starving	
BCR 1.3	9	3	December 2015	Belgium	<i>G. sigillatus</i>	None	

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

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

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

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

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

Sample code	Mean gene copy number per g insect						
	<i>tet(O)</i>	<i>tet(K)</i>	<i>tet(M)</i>	<i>tet(S)</i>			<i>erm(B)</i>
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	$\pm 4.50 \times 10^3$ ^a	4.07×10^4	$\pm 1.12 \times 10^3$ ^a	7.88×10^5	$\pm 6.27 \times 10^3$ ^a	N.D.	N.D.
HCR 2.2	7.15×10^6	$\pm 1.50 \times 10^5$ ^a	N.D.		3.89×10^5	$\pm 3.46 \times 10^4$ ^a	N.D.	N.D.
HCR 2.3	4.24×10^7	$\pm 4.88 \times 10^6$ ^a	2.76×10^5	$\pm 6.46 \times 10^4$ ^b	3.48×10^6	$\pm 4.98 \times 10^4$ ^b	N.D.	N.D.
BCR 3.1	2.63×10^6	$\pm 1.39 \times 10^5$ ^a	N.D.		1.54×10^5	$\pm 1.10 \times 10^4$ ^a	N.D.	N.D.
BCR 3.2	1.01×10^7	$\pm 4.21 \times 10^4$ ^b	N.D.		1.71×10^6	$\pm 4.00 \times 10^3$ ^b	5.37×10^5	$\pm 3.34 \times 10^4$ ^a
BCR 3.3	4.28×10^6	$\pm 4.17 \times 10^5$ ^{a,b,c}	N.D.		4.09×10^5	$\pm 1.25 \times 10^4$ ^c	6.06×10^5	$\pm 1.03 \times 10^5$ ^a
BCR 3.4a	2.03×10^5	$\pm 7.20 \times 10^3$ ^c	N.D.		6.43×10^4	$\pm 1.26 \times 10^3$ ^{a,c}	N.D.	N.D.
BCR 3.4b	5.70×10^5	$\pm 7.99 \times 10^4$ ^c	4.57×10^4	$\pm 9.12 \times 10^3$	5.66×10^5	$\pm 5.96 \times 10^4$ ^{a,c}	N.D.	N.D.

² \$Data are the mean values of two qPCR assessments \pm standard deviation. ^{a,b,c}Means per rearing batch from the same rearing company with the same superscript within the same columns

³ do not differ significantly ($p > 0.05$).

⁴ +N.D. = not detected.

Fig. 1

