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Short communication

Getting insight into the prevalence of antibiotic resistance genes in specimens of marketed edible insects

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ABSTRACT

This study was aimed at investigating the occurrence of 11 transferable antibiotic resistance (AR) genes [*erm*(A), *erm*(B), *erm*(C), *vanA*, *vanB*, *tet*(M), *tet*(O), *tet*(S), *tet*(K), *mecA*, *blaZ*] in 11 species of marketed edible insects (small crickets powder, small crickets, locusts, mealworm larvae, giant waterbugs, black ants, winged termite alates, rhino beetles, mole crickets, silkworm pupae, and black scorpions) in order to provide a first baseline for risk assessment. Among the AR genes under study, *tet*(K) occurred with the highest frequency, followed by *erm*(B), *tet*(S) and *blaZ*. A high variability was seen among the samples, in terms of occurrence of different AR determinants. Cluster Analysis and Principal Coordinates Analysis allowed the 11 samples to be grouped in two main clusters, one including all but one samples produced in Thailand and the other including those produced in the Netherlands.

1. Introduction

Over the last decades the extensive and improper use of antibiotics has led to an increase of resistances among pathogenic bacteria responsible for human infections (Clementi and Aquilanti, 2011). Despite the huge amount of scientific literature on this specific topic, a recent report of the European Centre for Disease Prevention and Control (ECDC), the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA) (ECDC, EFSA and EMA, 2015), while confirming in both humans and animals a positive association between consumption of antimicrobials and emergence of the corresponding resistance in bacteria on the one side, highlighted the need for further in depth due to data limitations and the complexity of the antimicrobial resistance phenomenon, on the other side. Food consumption undoubtedly represents one of the main routes for the entrance of antibiotic resistant bacteria and their genes into the human digestive tract (Aquilanti et al., 2007). Many factors in the food chain can influence the antibiotic resistance (AR) cycle; among these, the antimicrobial molecules used in animal breeding, microbial co-selection, mechanisms of fitness and persistence, host lifestyle, and food processing conditions (Wang et al., 2012).

Edible insects are one of the oldest foods of human history. To date, they constitute part of the traditional diets of at least 2 billion people in the world. In western countries they represent a new and atypical food, whose consumption is going to increase as they are rich in protein and good fats and they are high in calcium, iron and zinc (van Huis et al., 2013).

European legislation on food safety has not yet defined safety standards or guidelines on the consumption of edible insects, although, actually some new insect-based products are sold in the European market, as: bug larvae in the UK, mealworm burgers in the Netherlands, and vegetable spreads containing mealworms in Belgium. Furthermore, dried insect snacks can be even purchased worldwide via the internet.

To date, pest insects such as flies, cockroaches, bedbugs, beetles, and pollinators such as honeybees have been found to act as reservoir of AR genes (Zurek and Ghosha, 2014). In examples, Larson et al. (2008) studied the AR of *Enterococci* isolated from the red flour beetle, a pest insect, resulting positive for the occurrence of resistance to neomycin, tetracycline, erythromycin and vancomycin. Allen et al. (2009) reported the presence of AR determinants in midgut bacteria of gypsy moth larvae, whereas Lowe and Romney (2011) isolated vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* from human bedbugs. More recently, Tian et al. (2012) found mobile genes coding for resistance to tetracycline and oxytetracyline in bacteria isolated from the gut of honeybees, while Tetteh-Quarcoo et al. (2013) and Wannigama et al. (2014) found the presence of AR genes in fruit flies, oil fly larvae, and cockroaches.

To the best of the author's knowledge, to date no research studies have been conducted on the occurrence of transferable AR genes in marketed edible insects. Based on these premises, this study was aimed at investigating the occurrence of transferable AR genes in marketed edible insects in order to provide a first baseline for risk assessment.

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To this end, the bacterial DNA was extracted directly from some marketed edible insects (small crickets powder, small crickets, locusts, mealworm larvae, giant waterbugs, black ants, winged termite alates, rhino beetles, mole crickets, silkworm pupae, and black scorpions) (Fig. 1) and further screened with optimized PCR and nested-PCR assays for the occurrence of 11 genes [*erm*(A), *erm*(B), *erm*(C), *vanA*, *vanB*, *tet*(M), *tet*(O), *tet*(S), *tet*(K), *mecA*, *blaZ*] coding for resistance to antibiotics conventionally used in clinical practice.

2. Materials and methods

2.1. Collection of edible insects

Eleven species of edible insects (Table 1) were purchased via the internet from dealers located in The Netherlands (samples A–D) and Thailand (samples E–M), respectively. For each species, two samples of processed insects (boiled, dried and salted) and, hence, ready to be consumed, were bought. Insects were shipped in sealed plastic bag packages of variable weight, by international express currier and stored at ambient temperature until analyses.

2.2. Microbial counts

Five grams of each sample was weighed aseptically in sterile bags, diluted in 45 ml of peptone water (bacteriological peptone 1 g l^{-1}), homogenized in a Stomacher 400 Circulator apparatus (PBI, Milan, Italy) for 10 min at 260 rpm and serially ten-fold diluted in the abovementioned solution; aerobic colony counts were assessed by inclusion spreading in Standard Plate Count Agar (PCA, Oxoid, Basingstoke, UK), with aerobic incubation at 32 °C for 48 h (Osimani et al., 2011).

2.3. Reference strains

Eleven antibiotic-resistant strains, each of which carrying at least one or two AR genes of interest, were used as positive controls in the PCR and nested-PCR reactions (Table 1). *Enterococcus faecalis* JH2-2 (Jacob and Hobbs, 1974) was used as a negative control in all the PCR assays.

2.4. DNA extraction

The DNA from the 11 reference strains was extracted using the method proposed by Hynes et al. (1992) with some modifications as reported by Osimani et al. (2015). For each species of edible insects, the two samples were pooled and subjected to DNA extraction. The microbial DNA was extracted directly from insect samples using the PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA). Briefly, 1 mL of each homogenate (dilution 10^{-1}) used for microbial counts was centrifuged to produce a pellet that was processed in accordance with the kit manufacturer's instructions. The DNA quantity and purity of all the DNA extracts were assessed by optical readings at 260, 280 and 234 nm, respectively, using UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan).

2.5. PCR and nested PCR amplification of AR genes

DNA extracts obtained from each pool of samples were amplified in the PCR reactions targeting 11 genes codifying for resistance to tetracyclines [*tet*(M), *tet*(O), *tet*(S), *tet*(K)], macrolide–lincosamide–streptogramin B [*erm*(A), *erm*(B), *erm*(C)], vancomycin [*vanA*, *vanB*] and beta-lactams (*blaZ*, *mecA*). Only samples resulted negative after PCR reaction were subjected to further nested-PCR assay to increase amplification sensitivity.

Two microliters of the DNA extract (~10 ng of microbial DNA) or the PCR product was amplified by PCR and nested-PCR, respectively, in a total volume of 25 μl containing: 1× buffer [500 mM KCl and 100 mM Tris–HCl (pH 8.3)], 50 pmol of each primer, 2.5 or 3.0 mM $MgCl_2$, (3.0 mM $MgCl_2$ for the amplification of erm genes in both assays), 0.2 mM dNTPs (2.5 mM for the amplification of erm genes in both assays) and 0.75 U of Taq polymerase (AmpliTaq Gold, Applied Biosystem, Foster City, CA). Primer pair sequences, target genes, annealing temperatures (Ta), product sizes (bp) and references are listed in Tables 2 and 3 for PCR and nested-PCR, respectively. The primers designed for this study were based on the sequences published in GenBank (accession numbers in Tables 2 and 3) using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA). PCR thermal cycling conditions for different genes were optimized as follows; for *tet*(S) and *tet*(M): 1 min at 94 °C, 1 min at Ta, 2 min at 72 °C; for *tet*(O), *erm*(A), *erm*(B), *erm*(C): 1 min at 94 °C, 1 min at Ta, 1 min at 72 °C; for *tet*(K), *vanA*, *vanB*, *blaZ* and *mecA*: 30 s at 94 °C, 30 s at Ta, 30 s at 72 °C.

Nested-PCR cycling condition for *tet*(S), *tet*(M), *tet*(O), *tet*(K), *erm*(B), *erm*(C), *vanA*, *vanB*, *blaZ* and *mecA* genes was as follows: 30 s at 94 °C, 30 s at Ta, 30 s at 72 °C; for *erm*(A): 1 min at 94 °C, 1 min at Ta, 1 min at 72 °C. PCR assays (35 amplification cycles) included heating at 95 °C for 10 min and final extension at 72 °C for 7 min. Positive (Table 1) and negative (*E. faecalis* JH2-2) controls were used in each PCR assay together with a blank (PCR mixture added with water instead

Fig. 1. Edible insect samples subjected to viable counting and PCR and nested-PCR amplification of antibiotic resistance genes. A: small crickets powder (*Acheta domesticus*); B: small crickets (*Acheta domesticus*); C: locusts (*Locusta migratoria*); D: mealworm larvae (*Tenebrio molitor*); E: giant waterbugs (*Belostomatidae*); F: black ants (*Polyrhachis*); G: winged termite alates (*Termitoidae*); H: rhino beetles (*Hyboschema contractum*); I: mole crickets (*Gryllotalpidae*); L: silkworm pupae (*Bombyx mori*); M: black scorpions (*Heterometrus longimanus*).

Table 1
Edible insects microbial counts and results of PCR and nested-PCR amplification of AR genes.

#	Samples	Aerobic colony counts (cfu/g)	Assays	*erm*(A)[a]	*erm*(B)[b]	*erm*(C)[c]	*vanA*[d]	*vanB*[e]	*tet*(M)[f]	*tet*(O)[g]	*tet*(S)[h]	*tet*(K)[i]	*mecA*[l]	*blaZ*[m]	% of positivity for AR genes
A	Small crickets powder	$(8.2 \pm 0.5) \times 10^4$	PCR	−	−	−	−	−	−	−	−	−	−	−	
	(*Acheta domesticus*)		n-PCR	−	−	−	−	−	−	−	−	−	−	−	0
B	Small crickets	$(1.4 \pm 0.2) \times 10^4$	PCR	−	−	−	−	−	+	−	−	+	−	−	
	(*Acheta domesticus*)		n-PCR	−	−	−	−	−	n.d.	+	−	n.d.	−	−	27.3
C	Locusts	$(1.9 \pm 1.1) \times 10^4$	PCR	−	−	−	−	−	−	−	−	+	−	−	
	(*Locusta migratoria*)		n-PCR	−	−	−	−	−	−	−	−	n.d.	−	−	9.1
D	Mealworm larvae	$(5.9 \pm 0.2) \times 10^4$	PCR	−	−	−	−	−	+	−	+	+	−	−	
	(*Tenebrio molitor*)		n-PCR	−	−	−	−	−	n.d.	−	n.d.	n.d.	−	−	27.3
E	Giant waterbugs	$(2.2 \pm 0.7) \times 10^2$	PCR	−	−	−	−	−	−	−	−	−	−	−	
	(*Belostomatidae*)		n-PCR	−	+	−	−	−	−	−	−	+	−	+	27.3
F	Black ants	$<10^1$	PCR	−	−	−	−	−	−	−	−	+	−	−	
	(*Polyrhachis*)		n-PCR	−	+	+	−	−	−	−	−	n.d.	−	+	36.4
G	Winged termite alates	$(4.1 \pm 0.3) \times 10^6$	PCR	−	−	−	−	−	−	−	−	−	−	−	
	(*Termitoidae*)		n-PCR	−	+	+	−	−	−	−	+	+	−	+	45.4
H	Rhino beetles	$(4.2 \pm 0.5) \times 10^3$	PCR	−	−	−	−	−	−	−	−	−	−	−	
	(*Hyboschema contractum*)		n-PCR	−	+	−	−	−	−	−	+	+	−	+	36.4
I	Mole crickets	$(9.2 \pm 0.1) \times 10^6$	PCR	−	−	−	−	−	−	−	+	+	−	−	
	(*Gryllotalpidae*)		n-PCR	−	+	−	−	−	−	−	n.d.	n.d.	−	+	36.4
L	Silkworm pupae	$(3.0 \pm 1.0) \times 10^2$	PCR	−	−	−	−	−	−	−	−	−	−	−	
	(*Bombyx mori*)		n-PCR	−	−	−	−	−	−	−	+	+	−	+	27.3
M	Black scorpions	$(1.2 \pm 0.9) \times 10^4$	PCR	−	−	−	−	−	−	−	+	+	−	−	
	(*Heterometrus longimanus*)		n-PCR	−	−	−	−	−	−	−	n.d.	n.d.	−	−	18.2
	% of positive samples for each determinant			0	45.4	18.2	0	0	18.2	9.1	54.5	90.9	0	54.5	

Bacterial reference strains used as positive control: [a]*Staphylococcus aureus* M.P. (1); [b]*Enterococcus hirae* Api 2.16 (1); [c]*Staphylococcus* spp. SE12 (2); [d]*Enterococcus faecium* PF3U (2); [e]*Enterococcus faecalis* ATCC 51299 (3); [f]*Lactobacillus casei/paracasei* ILC2279 (2); [g]*Streptococcus pyogenes* 7008 (1); [h]*Enterococcus italicus* 1102 (2); [i]*Staphylococcus aureus* COL. (1); [l]*Staphylococcus aureus* 27R (2); [m]*Staphylococcus aureus* ATCC 2921 (3).
(1) Collection of the Department of Life and Environmental Sciences (DiSVA), Polytechnic University of Marche, Italy.
(2) Collection of the Department of Agricultural, Food and Environmental Sciences (D3A), Polytechnic University of Marche, Italy.
(3) ATCC, American Type Culture Collection.
cfu: colony forming units; PCR: positive after PCR; n-PCR: positive after PCR and nested PCR; n.d.: not determined.

of DNA). All assays were carried out in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, USA).

Five microliters of each PCR product was analyzed by electrophoresis in 1.5% (w/v) agarose gel (Conda pronadisa, Spain) in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) containing 0.5 μg/ml ethidium bromide at 75 V for 45 min, using 100 bp DNA Ladder (SibEnzyme Ltd., Academtown, Russia) as a molecular weight standard. Gels were visualized under UV light and photographed with the Complete Photo XT101 system (Explera, Jesi, Italy).

2.6. Statistical analysis

Relative frequency of each AR gene was determined among the 11 insect species and within each species. The Cluster Analysis was carried out using the Jaccard Similarity Index Matrix; then a Principal Coordinates Analysis was performed after double centering the similarity matrix using NTSYS (Applied Biostatistics Inc., NY, USA). Results were graphically represented using two or three principal coordinates; clusters were identified based on the results of cluster analysis.

Table 2
PCR primers used in this study.

Target gene	Primer sequence (5′-3′)	T_a (°C)	Product size (bp)	Reference/accession number[a]
tet(M)	1-ACCCGTATACTATTTCATGCACT 2-CCTTCCATAACCGCATTTTG	48	1115	Garofalo et al. (2007)
tet(O)	1-AACTTAGGCATTCTGGCTCAC 2-TCCCACTGTTCCATATGCTCA	62	519	Olsvik et al. (1995)
tet(S)	1-TTCCTTTGGGTAGTGGCAT[b] 2-ACAACGGGCTGGAATTTCAC[b]	60	402	AM039486
tet(K)	1-TCGATAGGAACAGCAGTA 2-CAGCAGATCCTACTCCTT	55	169	Ng et al. (2001)
erm(A)	1-CAGGAAAAGGACATTTTACCAA 2-CTTCGATAGTTTATTAATATTAGT	50	572	Garofalo et al. (2007)/ Sutcliffe et al. (1996)
erm(B)	1-GAAAAGGTACTCAACCAAATA 2-AGTAACGGTACTTAAATTGTTTAC	54	639	Sutcliffe et al. (1996)
erm(C)	1-TCAAAACATAATATAGATAAA 2-GCTAATATTGTTTAAATCGTCAAT	50	642	Sutcliffe et al. (1996)
vanA	1-GGGAAAACGACAATTGC 2-GTACAATGCGGCCGTTA	58	732	Dutka-Malen et al. (1995)
vanB	1-ATGGGAAGCCGACAGTC 2-GATTTCGTTCCTCGACC	58	635	Dutka-Malen et al. (1995)
mecA	1-GGGATCATAGCGTCATTATTG 2-AGTTCTGCAGTACCGGATTTGC	58	1429	Murakami et al. (1991)/ Predari et al. (1991)
blaZ	1-ACTTCAACACCTGCTGCTTTC 2-TAGGTTCAGATTGGCCCTTAG	58	240	Garofalo et al. (2007)/ Martineau et al. (2000)

[a] Reference or GenBank accession number of sequence taken into account for the primer design.
[b] Primer designed for this study.

Table 3
Nested-PCR primers used in this study.

Target gene	Primer sequence (5′-3′)	T_a (°C)	Product size (bp)	Reference/accession number[a]
tet(M)	3-CTTAGGAAAATGGGGATTCC 4-GCGGTGATACAGATAAACC	50	1009	Garofalo et al. (2007)
tet(O)	3-TACCAGTGGTGCAATTGCAGA 4-TTATATGGGGATGCTGCCCAA	58	419	Garofalo et al. (2007)
tet(S)	3-CGCTATGGGTGTGAACAAGG[b] 4-GGAAATCTGCTGGCGTACTG[b]	64	106	AM039486
tet(K)	3-GAACAGCAGTATATGGAA 4-AAAAAGTGATTGTGACCA	50	118	Garofalo et al. (2007)
erm(A)	1-CAGGAAAAGGACATTTTACCAA 3-CTATAGAAATTGATGGAGGCTTA	58	518	Garofalo et al. (2007)
erm(B)	3-CAATTCCCTAACAAACAGAGG 2-AGTAACGGTACTTAAATTGTTTAC	60	420	Garofalo et al. (2007)/ Sutcliffe et al. (1996)
erm(C)	3-GTAATTTCGTAACTGCCATT 4-GCATGTTTTAAGGAATTGTT	52	502	Garofalo et al. (2007)
vanA	3-GTAGGCTGCGATATTCAAAGC 4-CGATTCAATTGCGTAGTCCAAT	58	231	Bell et al. (1998)
vanB	3-GGTGCGATACAGGGTCTGTT 4-GGAATGTCTGCTGGAACGAT	58	479	Garofalo et al. (2007)
mecA	3-AAAATCGATGGTAAAGGTTGGC 2-AGTTCTGCAGTACCGGATTTGC	55	533	Murakami et al. (1991)
blaZ	1-ACTTCAACACCTGCTGCTTTC 4-TGACCACTTTTATCAGCAACC	58	173	Martineau et al. (2000)

[a] Reference or GenBank accession number of sequence taken into account for the primer design.
[b] Primer designed for this study.

3. Results

Microbial counts of total mesophilic aerobes showed a wide variability among samples, with colony forming units (cfu)/g values ranging from 10 to 10^7 (Table 1).

The DNA extracted directly from the insects was first screened by PCR and, in case of negative result, by nested-PCR with internal primers. In some cases, targeted genes, as *erm*(B), *erm*(C) and *blaZ* could be amplified only by nested-PCR, whereas for other genes, namely *tet*(M), *tet*(S) and *tet*(K), the majority of the samples were already positive in the first PCR assay. Globally, the 11 AR genes under study occurred with frequencies varying from 9.1 to 90.9% (Table 1). Some of them were highly prevalent, as *tet*(K), which occurred with the highest frequency, followed by *erm*(B), *tet*(S) and *blaZ*, whereas others, namely *erm*(A), *vanA*, *vanB* and *mecA* were not detected at all.

As far as the different species of edible insects analyzed are considered, a high variability was seen in terms of occurrence of AR determinants. More specifically, the cricket powder (sample A) was negative for all the 11 determinants, whereas the winged termite alates (G) carried five AR genes, followed by black ants (sample F), rhino beetles (sample H) and mole crickets (sample I), which carried four AR genes, each.

Cluster Analysis clearly separated sample A (small cricket powder) from all the others; the remaining samples were grouped in two main clusters: one cluster included small crickets, locusts, mealworm larvae, and black scorpion (samples B, C, D, and M, respectively), whereas the second cluster comprised of giant waterbugs, black ants, winged termite alates, rhino beetles, mole crickets, and silkworm pupae (samples E, F, G, H, I, and L, respectively). In particular, rhino beetles and mole crickets showed an identical AR gene pattern (Fig. 2). Tridimensional representation of Principal Coordinates (Fig. 3) better reflected the relationships among the eleven samples; in fact, within each cluster (1 and 2) the sub-clustering structure clearly reflects the Cluster Analysis. In particular, within cluster 2 mealworm larvae (sample D) and black scorpion (sample M) were clearly separated from small crickets (sample B) and locusts (sample C) by the third principal component. In cluster 1 the

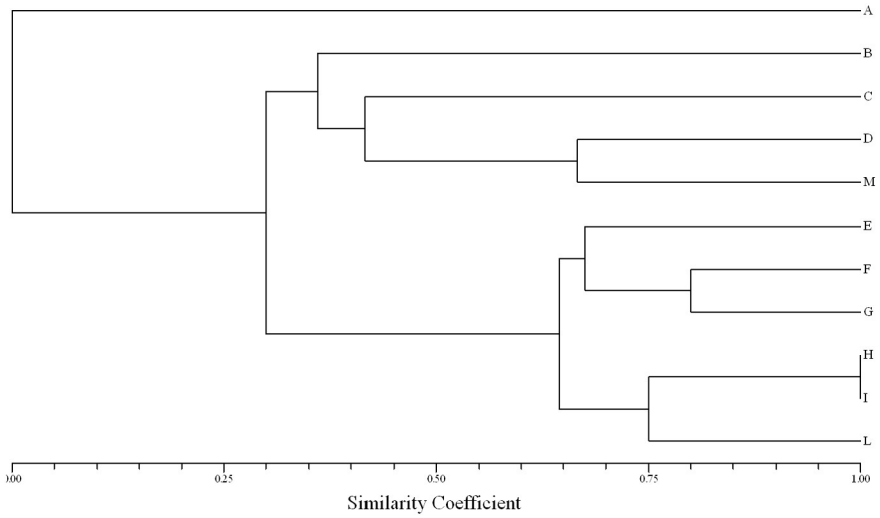

Fig. 2. Dendrogram resulting from Cluster Analysis. Letters refer to the samples listed in Table 1.

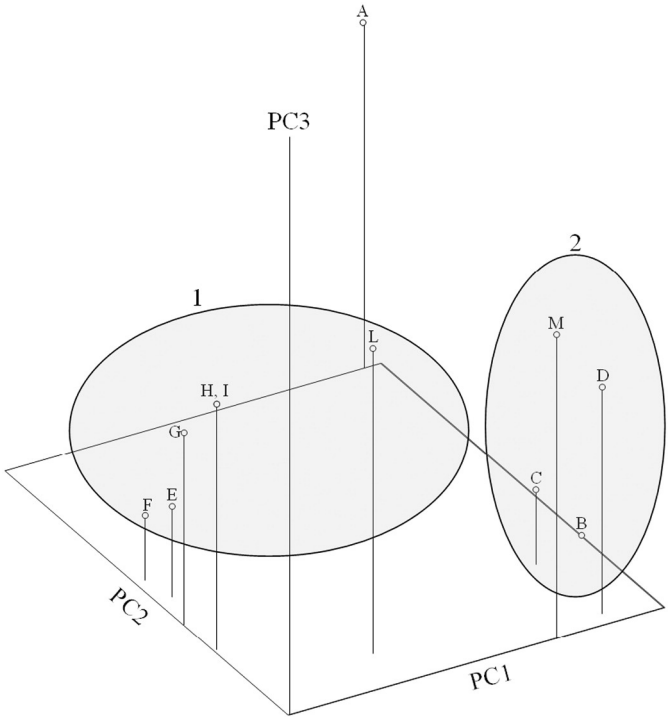

Fig. 3. Results from Principal Coordinates Analysis showing the two clusters (1 and 2) identified by the Cluster Analysis. Letters refer to the samples listed in Table 1.

sub-cluster formed by giant waterbugs (sample E), black ants (sample F) and winged termite alates (sample G) was identified mainly by the first and the second principal coordinates. The samples rhino beetles (sample H) and mole crickets (sample I) clearly overlapped, whereas silkworm pupae (sample L) was clearly distinguished from all the others mainly by the first and second principal coordinates. As expected, small cricket powder (sample A) was clearly positioned far from the two main clusters.

4. Discussion

As viable counts performed onto the eleven samples of edible insects are comparatively evaluated with those performed by other authors onto different insects such as crickets, mealworm larvae, and grasshoppers the following considerations can be made.

Most of the samples analyzed in this study were characterized by a load of total mesophilic aerobes comprised between 10^4 and 10^7 cfu/g, which is comparable to that previously reported for the same microbial group by other authors and comprised between 10^5 and 10^7 cfu/g (Ali et al., 2010; Giaccone, 2005; Klunder et al., 2012). Generally, insects have a so broad taxonomic, ecological, morphological and even physiological diversity, that it is difficult to generalize about aspects pertaining to their intestinal microbiota. Accordingly, for some insects, as f.i termites, the microorganisms colonizing the digestive tract are essential for food digestion and production of nutrients, whereas some other insects, as plant sap-feeding insects, have scarce or no gut microflora (Engel and Moran, 2013). From the available literature, numerous microorganisms, including lactic acid and spore-forming bacteria, *Enterobacteriaceae*, staphylococci, *Acinetobacter*, and *Pseudomonas* have been reported to colonize or live in association with insects (Broderick et al., 2004; Leroy et al., 2011; Mason et al., 2011; Stavrinides et al., 2009, 2010). For most of these bacteria, an involvement in the spread of transferable AR has already been documented though in different food matrices. As concerns the edible insects analyzed in this study, the high microbial loads found in almost all the samples suggest the survival of spore-forming bacteria during processing, including boiling and/or the occurrence of post-processing cross-contaminations. Furthermore, it is not possible to exclude that the microorganisms present in the samples under study derive, at least in part, from the contamination during transformation.

The food chain represents one of the main routes for the development, persistence and spreading of antibiotic resistant microorganisms and their genes into the human gut (Wang et al., 2012).

To date, there is a lack of data on the occurrence and spread of AR genes in edible insects, including those which codify for resistance to antibiotics routinely used in clinical practice. To the best of the author's knowledge, this is the first report on the screening of marketed edible insects for the occurrence of transferable AR genes. The results of the present study are in line with the highest frequency of *tet*(K) and *erm*(B) in specimens of raw meat, as reviewed by Wang et al. (2012). Genes conferring resistance to erythromycin and tetracycline were also found in retail cheese products, fresh produce and seafood (Wang et al., 2012), the latter genes being also retrieved in ready-to-eat food (Li and Wang, 2010).

Most published reports on AR have been focussed on the occurrence of AR genes in bacteria isolated from humans and food-producing animals. Very recently, ECDC, EFSA and EMA (2015) have jointly established the association between the use of some antimicrobials in humans and food-producing animals and the spread of AR, on the basis of data collected in the years 2011 and 2012 by EU monitoring networks; these three independent European Agencies also pointed out the many factors that can contribute to enhance the phenomenon of AR, namely co-resistance, human travels, import and trade of food, and trade of live animals, both between and within countries (ECDC, EFSA and EMA, 2015). From data collected in the two-year period considered it emerges that macrolides, including erythromycin, are mostly used for the treatment of infections caused by *Campylobacter* spp. (gastroenteritis) and other Gram-positive bacteria and respiratory infections suspected to be caused by *Mycoplasma pneumoniae*. Macrolides, used in both hospitals and the community, are considered by WHO (2011) as critically important antimicrobials with the highest priority for human medicine (ECDC, EFSA and EMA, 2015).

Tetracyclines have been used extensively for decades to treat bacterial infections in humans and in animals, being also exploited as growth promoters in animal feeds. Resistance to tetracyclines can be due to ribosomal protection, enzymatic inactivation, or drug efflux, but among the about 40 known classes of tetracycline resistance determinants, the majority are responsible for the latter resistance mechanism (Thompson et al., 2007).

Beta-lactams target transpeptidase enzymes that synthesize the bacterial cell wall; this family of antibiotics is currently the most widely utilized. Bacterial resistance to β-lactams can be attained by three mechanisms: the production of β-lactam-hydrolyzing β-lactamase enzymes, the synthesis of β-lactam-insensitive cell wall transpeptidases, which is the major cause of resistance in many pathogens among which some *Staphylococcus* and *Streptococcus* species, and the active expulsion of β-lactam molecules from Gram-negative cell wall, which is a major cause of resistance in *Pseudomonas* species and other pathogenic Gram-negative microorganisms (Wilke et al., 2005). In the year 2011, the EFSA published a Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases (ESBL) and/or AmpC β-lactamases (AmpC) in food and food-producing animals. In particular, the EFSA Panel concluded that ESBLs may be defined as plasmid-encoded enzymes found in the *Enterobacteriaceae*, frequently in *Escherichia coli* and *Klebsiella pneumoniae*, that confer resistance to a variety of β-lactam antibiotics, including penicillins, 2nd-, 3rd- and 4th-generation cephalosporins and monobactams, but usually not the carbapenems or the cephamycins. The EFSA Panel highlighted the importance of measures for increasing farm biosecurity and controls on animal trade, and by improving hygiene throughout the food chain, and implementing other general controls for food-borne pathogens (EFSA, 2011).

Interestingly, the results from Principal Coordinates Analysis identified by the Cluster Analysis (Fig. 2) seem to suggest a separation of the samples on the basis of their producer. In more detail, samples purchased in Thailand belonged to cluster 1, whereas, except for sample M, those produced in The Netherlands were included in cluster 2. Unfortunately no information on either breeding or processing techniques in use by each producer is available; if AR determinants are considered as environmental pollutants (Martinez, 2009), it can be hypothesized that each production plant is more or less contaminated by specific AR genes. The grouping of samples by producer might be also explained by differences in the use of biocides for sanitization. Indeed, as reported by McBain et al. (2002), AR can be positively correlated with the inappropriate use or abuse of sanitizer compounds as triclosan, quaternary ammonium compounds, chlorhexidine and trisodium phosphate, which are routinely utilized by food industries. In Europe, and hence in The Netherlands, where samples A, B, C and D were produced, the use of disinfectants is set by Regulation (EC) No. 528/2012 (Lavilla Lerma et al., 2015), whereas in Thailand, where samples E, F, G, H, I, L, and M were produced, is in force The Hazardous Substance Act B.E.2556 of the Thailand Food and Drug Administration (2013). The different legislation and hence the use of sanitizer might explain the establishment of a different selective pressure on the microorganisms carried by the edible insects produced and commercialized in the two countries, which in turn might have determined the different positioning of samples in clusters.

It remains still unclear the results obtained onto the cricket powder (A) which, though produced in the same production site of samples B, C, and D, did not carry any of the eleven AR genes considered, being positioned out of all the clusters.

5. Conclusion

The results of the present study represent the first attempt to define the incidence of insect consumption on the spread of AR genes to the human microbioma. In order to improve the implementation of effective mitigation strategies, studies on commensal bacteria must be carried out to establish the main species involved in AR dissemination in different microbial ecosystems (Wang and Schaffner, 2012). This study, albeit carried out on a limited number of samples, sheds a first light on the AR phenomenon related to a food product that, in future, could become part of the diet of the people of European countries, representing both a source of risk and a new resource from the great potential, that requires in-depth studies.

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