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Microbial aspects related to edible insects and development of insect-based food products

Ph.D. Thesis

Andrea Roncolini

Tutor

Prof.ssa Francesca Clementi

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Al Signor D.,

a tutti quelli che saranno felici per la mia felicità.

Culodritto, dammi ancora la mano, anche se quello stringerla è solo un pretesto

per sentire quella tua fiducia totale che nessuno mi ha dato o mi ha mai chiesto

vola, vola tu, dov' io vorrei volare verso un mondo dove è ancora tutto da fare

e dove è ancora tutto, o quasi tutto, da sbagliare...

Francesco Guccini "Culodritto"

Preface

This Ph.D. thesis, titled 'Microbial aspects related to edible insects and development of insect-based food products', is submitted as a requirement for obtaining the Ph.D. Degree from the Ph.D. School of Agriculture, Food and Environmental Sciences (Università Politecnica delle Marche).

The work presented in this Ph.D. thesis has been carried out during the three-years research activities conducted by the author, Andrea Roncolini, at the Food Microbiology Lab of Department of Agriculture, Food and Environmental Sciences, Università Politecnica delle Marche (Ancona), under the supervision of Prof.ssa Francesca Clementi.

Andrea Roncolini
Ancona, November 2019

List of publications

Paper I

Osimani, A., Cardinali, F., Aquilanti, L., Garofalo, C., Roncolini, A., Milanović, V., Pasquini, M., Tavoletti, S., Clementi, F. 2017. Occurrence of transferable antibiotic resistances in commercialized ready-to-eat mealworms (*Tenebrio molitor* L.). International Journal of Food Microbiology, 263, 38-46.

Paper II

Osimani, A., Milanović, V., Garofalo, C., Cardinali, F., Roncolini, A., Sabbatini, R., De Filippis, F., Ercolini, D., Gabucci, C., Petruzzelli, A., Tonucci, F., Clementi, F., Aquilanti, L. 2018. Revealing the microbiota of marketed edible insects through PCR-DGGE, metagenomic sequencing and real-time PCR. International journal of food microbiology, 276, 54-62.

Paper III

Osimani, A., Milanović, V., Cardinali, F., Roncolini, A., Garofalo, C., Clementi, F., Pasquini, M., Mozzon, M., Foligni, R., Raffaelli, N., Zamporlini, F., Aquilanti, L. 2018. Bread enriched with cricket powder (*Acheta domesticus*): A technological, microbiological and nutritional evaluation. Innovative Food Science & Emerging Technologies.

Paper IV

Roncolini, A., Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., Sabbatini, R., Clementi, F. 2018. Il potenziale degli insetti edibili: aspetti microbiologici. Industrie Alimentari, 57, 3-14.

Paper V

Milanović, V., Osimani, A., Roncolini, A., Garofalo, C., Aquilanti, L., Pasquini, M., Tavoletti, S., Vignaroli, C., Canonico, L., Ciani, M., Clementi, F. (2018). Investigation of the dominant microbiota in ready-to-eat grasshoppers and mealworms and quantification of carbapenem resistance genes by qPCR. Frontiers in microbiology, 9.

Paper VI

Roncolini, A., Milanović, V., Cardinali, F., Osimani, A., Garofalo, C., Sabbatini, R., Clementi, F., Pasquini, M., Mozzon, M., Foligni, R., Raffaelli, N. Zamporlini, F., Minazzato, G., Trombetta, M. F., Van Buitenen, A., Van Campenhout, L., Aquilanti L. 2019. Protein fortification with mealworm (*Tenebrio molitor* L.) powder: Effect on textural, microbiological, nutritional and sensory features of bread. PloS one, 14(2), e0211747.

Paper VII

Milanović, V., Cardinali, F., Aquilanti, L., Garofalo, C., Roncolini, A., Sabbatini, R., Clementi, F., Osimani, A. 2019. A glimpse into the microbiota of marketed ready-to-eat crickets (*Acheta domesticus*). Indian Journal of Microbiology, 1-4.

Paper VIII

Roncolini, A., Cardinali, F., Aquilanti, L., Milanović, V., Garofalo, C., Sabbatini, R., Abaker, M. S., Pandolfi, M., Pasquini, M., Tavoletti, S., Clementi, F., Osimani, A. 2019. Investigating Antibiotic Resistance Genes in Marketed Ready-to-Eat Small Crickets (*Acheta domesticus*). Journal of Food Science.

Paper IX

Osimani, A., Milanović, V., Roncolini, A., Riolo, P., Ruschioni, S., Isidoro, N., Loreto, N., Franciosi, E., Tuohy, K., Olivotto, I., Zarantoniello, M., Cardinali, F., Garofalo, C., Aquilanti, L., Clementi, F. 2019. *Hermetia illucens* in diets for zebrafish (*Danio rerio*): A study of bacterial diversity by using PCR-DGGE and metagenomic sequencing. PloS one, 14(12).

Paper X

Roncolini, A., Milanović, V., Aquilanti, L., Cardinali, F., Garofalo, C., Sabbatini, R., Clementi, F., Pasquini, M., Mozzon, M., Foligni, R., Trombetta, M. F., Haouet, M. N., Altissimi, M. S., Di Bella, S., Piersanti, A., Griffoni, F., Reale, A., Niro, S., Osimani, A. 2020. Lesser mealworm (*Alphitobius diaperinus*) powder as a novel baking ingredient for manufacturing high-protein, mineral-dense snacks. Food Research International, 109031.

Other publications

Tazioli, A., Aquilanti, L., Clementi, F., Nanni, T., Palpacelli, S., Roncolini, A., Vivalda, P. (2017). Parameters of flow in porous alluvial aquifers evaluated by tracers. Flowpath.

Tazioli, A., Aquilanti, L., Clementi, F., Marcellini, M., Nanni, T., Palpacelli, S., Roncolini, A., Vivalda, P. M. (2019). Flow parameters in porous alluvial aquifers evaluated by multiple tracers. Rendiconti Online Societa Geologica Italiana, 47, 126-132. doi:10.3301/ROL.2019.23

Osimani, A., Ferrocino, I., Agnolucci, M., Cocolin, L., Giovannetti, M., Cristani, C., Palla, M., Milanović, V., Roncolini, A., Sabbatini, R., Garofalo C., Clementi F., Cardinali, F., Petruzzelli, A., Gabucci, C., Tonucci, F., Aquilanti, L. (2019). Unveiling hákarl: A study of the microbiota of the traditional Icelandic fermented fish. Food microbiology, 82, 560-572.

Garofalo, C., Ferrocino, I., Reale, A., Sabbatini, R., Alkić-Subašić, M., Boscaino, F., Roncolini, A., Cardinali, F., Aquilanti, L., Pasquini, M., Federica Trombetta, M. F., Tavoletti, S., Coppola, R., Cocolin, L., Blesić, M., Sarić, Z., Clementi, F., Osimani, A. Characterization of kefir grains from Bosnia and Herzegovina and their use in traditional vs backslopping methods for kefir production: a diversity of microbial dynamics and nutritional and volatilome profiles. *Under review*.

Abstract

FAO declared that human population is worldwide increasingly growing and in 2050 it will reach 9 billion people. The consequent increase in food demand, and in particular, in proteins will cause several negative effects on the environment due to the intensive animal farming. In this scenario, a potential solution is represented by edible insects. First of all, their rearing is characterized by a higher environmental sustainability than livestock. Moreover, they are a nutritious food especially in terms of essential amino acids, unsaturated fatty acids, minerals, vitamins and fibre.

In the European Union, insects were defined as "*novel food*" by the Regulation (EU) No 2015/2283. In the same year, EFSA requested further research to better assess microbiological and chemical risks related to edible insects.

In this context, the present PhD. thesis was aimed to investigate about edible insects microbiota to collect information about their feasibility as food. Furthermore, since they can act as reservoir of transferable antibiotic resistance genes which can even be carried by pathogens, part of the research was focused on the occurrence of such genes in edible insects samples. Briefly, results showed the presence of commensal, spoilage and potential pathogen agents and the occurrence of transferable resistance genes. Interestingly, data highlighted the influence of insect species, growth substrate, rearing and environmental conditions.

Finally, to overcome consumers scepticism generated by insects consumption as food, the development of insect-based food products where insects are invisible was assessed. To get information about the feasibility of these products, microbiological, technological and sensory analysis were performed.

In a few words, the presence of spore-forming bacteria was detected in each bread and in the rusks. Moreover a different insect powder effect on the technological parameters of bread and on the consumers appreciation was observed.

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1. Introduction

1.1 Background and aim

It was estimated that in 2050 world population will be of about 9 billion people (van Huis et al., 2013). This will cause an increase in food consumption which will almost double for protein sources (Mc Leod, 2011). As a consequence, intensive livestock farming will imply deforestation for feed crops and an increase in greenhouse gases emissions and water consumption (Chapagain and Hoeckstra, 2003; Steinfeld et al., 2006; Wirsenius et al., 2010).

In this context, it is indispensable to find an "eco-sustainable" protein source. A possible strategy could be the inclusion of edible insects as an alternative protein source in human and animal diet. Indeed their rearing ensure a high environmental sustainability if compared to livestock farming (Smil, 2002; Collavo et al., 2005; Oonincx et al., 2010; Oonincx and de Boer, 2012; Veldkamp et al., 2013). Moreover, they are characterized by a high nutritional quality in terms of essential amino acids, unsaturated fatty acids, minerals, vitamins and fibre (Rumpold and Schlüter, 2013; Belluco et al., 2015).

In Europe, insects were, only recently, considered for human and animal consumption. The European Regulation (EU) No 2015/2283 that entered into force on January 1, 2018 classified edible insects into the so-called "*novel foods*" category. Moreover, in the same year, European Food Safety Authority (EFSA) has issued an Opinion on the microbiological and chemical risk profile of insects as food and feed (EFSA, 2015), in which the need to have more scientific data in order to carry out a risk analysis on edible insects was underlined.

Regarding microbiological aspects, microbial agents that can potentially be transmitted via consumption of insects are mainly associated with their intrinsic flora (intestinal tract and other anatomical compartments) or related to extrinsic sources, such as the environment and the rearing (substrates and feed), handling, processing and preservation conditions (EFSA, 2015). Furthermore, as reported by Grabowski and Klein (2017), edible insects can be natural reservoirs of many bacterial species, including human pathogens (e.g., *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Campylobacter* spp., and *Salmonella* spp.), which can be present in the marketed products when processing is not hygienically carried out. Additionally, microorganisms, and especially pathogens, can carry resistance genes to commonly used antibiotics, whose dangerous effect on human health and world economy are well-known (ECDC, EFSA and EMA, 2015). Although in insect rearing antibiotics are used only as emergency treatment, it was demonstrated that insects intestinal tract can represent an in vivo model for the natural conjugation-mediated horizontal transfer of AR plasmids among bacteria (Tarapoulouzi et al., 2013; Eilenberg et al., 2015; Anacarso et al., 2016).

Entomophagy is actually worldwide traditionally practised. It is estimated that at least 2.5 billion people regularly eat insects. Indeed insects consumption as food is not a new concept in many parts of

the world where they represent an important element of human diet (van Huis, 2013). On the contrary, in many Western rich countries insects consumption still remains part of a niche food sector of novelty snacks. Regarding that, several studies conducted across Europe concluded that adopting insects as food was generally considered unrealistic and even unacceptable (Verbeke, 2015). For instance, in a study carried out in the Netherlands, de Boer et al. (2013) showed that among snacks based on environmentally-friendly proteins (hybrid meat, lentils, beans, seaweed and locusts) the locusts-based snack was preferred by only 4% of the study participants, whereas 79% of them defined it as the snack they would least like to taste.

To overcome consumers scepticism and disgust related to edible insects two different approaches can be followed (in combination or separately). First of all, education and communication programmes which highlight the potential of edible insects from an environmental, nutritional and social point of view to improve consumers awareness (van Huis, 2013). For example, it was proven that "Bug banquets", i.e. a combination of educational talk and a direct entomophagy experience where consumers are subjected to educational talk and tasting classes, could be effective in improving consumers acceptance related to edible insects (Wood and Looy, 2000; Looy and Wood 2006). A different approach to prevent the surprise and novelty of seeing insects on the plate might be represented by the development of insects-based food products where insects are present in an "invisible way" (Mlcek et al., 2014). In this case, the right selection of an adequate food in which include insects could be a successful strategy. In particular, in this PhD project, the choice has fallen on wheat bread for two main reasons: i) it is a staple food being one of the main carbohydrate sources in human daily diet worldwide; ii) it is usually prepared with refining wheat, characterized by a nutritional quality reduction in terms of protein, fiber, vitamins and minerals, which are present in considerable amounts in insects. Thus, a dual goal could be obtained: insects were included in an invisible way in a food product which could has been fortified in its nutritional qualities.

In the light of the above, the present PhD. thesis is intended to get more in depth information about edible insects microbiota to contribute to build a first useful database to know and prevent eventual microbiological hazards. Furthermore, in order to enhance consumers acceptance regarding insects as food, an investigation about the suitability of different insect powders in baked good production was carried out.

1.2 Outline of the thesis

The present PhD. thesis is mainly based on ten articles published, accepted or under review for publication by peer-reviewed journals.

A brief description of the outline of this thesis follows.

First of all an overview of the state-of-art of edible insects potential as food and a focus on their food safety aspects and consumers acceptance of edible insects as food is presented (Chapter 2).

In Chapter 3, methods ususally used in food microbiology are listed and described focusing mainly on those performed in the present PhD. project.

Chapters 4 and 5 illustrate the experimental obtained results. In particular, Chapter 4 describes results regarding edible insect microbiota characterization and about investigation on the occurrence of transferable resistance genes in edible insects samples; whereas, in Chapter 5, results about the development of insect-based food products are reported.

Chapter 6 contains a summary of the reached results and perspectives and ideas for future research. Finally, bibliographic references are listed in Chapter 7.

1.3 Compliance with ethical standards

All procedures involving animals were conducted in line with Italian legislation on experimental (84/94-A).

2. The potential of edible insects

The following paragraphs contain information about edible insects potential in terms of rearing environmental sustainability and nutritional properties, with a focus on European Regulation followed by the state-of-the-art of microbial aspects studies and the occurrence of transferable resistance genes related to edible insects. Finally, consumers acceptance about insects as food is described.

2.1 Potential of edible insects in environmental, nutritional and social concerns

"*Everyone has the right to a standard of living adequate for the health and well-being of himself and of his family, including food, clothing, housing and medical care and necessary social services, and the right to security in the event of unemployment, sickness, disability, widowhood, old age or other lack of livelihood in circumstances beyond his control.*" (Article 25 of the Universal Declaration of Human Rights, 10 December 1948). As declared by the General Assembly of United Nations, food must be guaranteed to every human worldwide. But what will happen if the human population will heavily grow in the next decades? In 2009, United Nations Population Division reported that it will rise up to 9.71 billion people in 2050 and 11 billion people at the end of the century. Thus will generate an increase in food consumption. In 2050 people to be fed will be 1.3 times more than in 2010 (United Nations Population Division, 2009). In particular, meat and meat based food production will be 58% greater than that recorded in 2010 (McLeod, 2011). The substantial growth of protein request will cause an increase mainly regarding traditional protein sources (livestock, swine, poultry) production and consequent negative effects on the environment (Tilman et al., 2002; Fiala, 2008). Indeed, traditional farming is characterized by high greenhouse gas emission and water consumption. Cattle farming is responsible of 18% of global greenhouse gas emission, while 1 kg of animal proteins means a total water consumption 5-20 times higher compared to the production of 1 kg of vegetable proteins (Chapagain and Hoeckstra, 2003; Steinfeld et al., 2006). This is even worse considering that in 2025, 1.8 billion people will live under drought condistions (FAO, 2012). Moreover, land use and deforestation intended for livestock production require around 80% of total agricultural land. Numbers are huge: 3.4 billion ha (26% of emerged lands) for pastures; 470 million ha of arable land for animal feed production. Finally, around 2.4 million ha of forest per year are removed for for pasture and around 0.5 million ha per year for feed crops (Steinfeld et al., 2006).

The heavy pressure of traditional protein sources production on the environment might be overcome, or at least reduced, with alternative and more sustainable (from an environmental point of view) protein sources. One of these could be represented by edible insects for humans and animals for three main reasons.

First of all, edible insects might be useful to reduce negative effects on the environment related to traditional farming: i) moderately low greenhouse gas and relatively little ammonia emission; ii) lower

water consumption; iii) deforestation for feed crops is not required for insects, whose abundance and distribution are influenced by habitat availability; iv) high conversion efficiency of growth substrate in high quality biomass; v) potential (according to regulations) use of food industry wastes as growth substrate (Smil, 2002; Collavo et al., 2005; Oonincx et al., 2010; Oonincx e de Boer, 2012; Veldkamp et al., 2013).

Further than environmental benefits, edible insects are characterized by good nutritional properties. Data collected in literature (Table 2.1) show several positive aspects: i) notable amount of energy, proteins and essential aminoacids; ii) high content of mono- and/or polyunsaturated fatty acids; iii) good source of fiber thanks to their content in chitin; iv) high levels of micronutrients, such as copper, iron, magnesium, manganese, phosphorous, selenium and zinc; v) high amount of vitamins, namely riboflavin, pantothenic acid, biotine and occasionally folic acid (Defoliart, 1992; Ekpo and Onigbinde, 2005; Elorduy et al., 1997; Finke and Oonincx 2014; Koide, 1998; Malaisse and Parent, 1980; Massieu et al., 1958; Massieu et al., 1959; Oliveira et al., 1976; Osimani et al., 2017c; Rumpold and Schlüter, 2013; Craig, 2010; Van Huys, 2013; Yi et al., 2013).

Table 2.1. *Nutritional composition [%] and energy content [kcal/100 g] of edible insects (based on dry matter).*

	Energy (Kcal/100 g)			Protein (%)			Fat (%)			Fiber (%)			NFE (%)			Ash (%)		
	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean	Min.	Max.	Mean
Blattodea	n.r.	n.r.	n.r.	43.90	65.60	57.30	27.30	34.20	29.90	3.00	8.44	5.31	0.78	10.09	4.53	2.48	3.33	2.94
Coleoptera	282.32	652.30	490.30	8.85	71.10	40.69	0.66	69.78	33.40	1.40	25.14	10.74	0.01	48.60	13.20	0.62	24.10	5.07
Diptera	216.94	552.40	409.78	35.87	63.99	49.48	11.89	35.87	22.75	9.75	16.20	13.56	1.25	8.21	6.01	5.16	25.95	10.31
Hemiptera	328.99	622.00	478.99	27.00	72.00	48.33	4.00	57.30	30.26	2.00	23.00	12.40	0.01	18.07	6.08	1.00	21.00	5.03
Hymenoptera	391.00	655.00	484.45	4.90	66.00	46.47	5.80	62.00	25.09	0.86	29.13	5.71	0.00	73.60	20.25	0.71	9.31	3.51
Isoptera	n.r.	n.r.	n.r.	20.40	65.62	35.34	21.35	46.10	32.74	2.70	7.85	5.06	1.13	43.30	22.84	1.90	11.26	5.88
Lepidoptera	293.00	776.85	508.89	13.17	74.34	45.38	5.25	77.17	27.66	0.12	29.00	6.60	1.00	66.60	18.76	0.63	11.51	4.51
Odonata	431.33		431.33	54.24	56.22	55.23	16.72	22.93	19.83	9.96	13.62	11.79	3.02	6.23	4.63	4.21	12.85	8.53
Orthoptera	361.46	566.00	426.25	6.25	77.13	61.32	2.40	53.05	13.41	1.01	22.08	9.55	0.00	85.30	12.98	0.34	9.36	3.85

n.r. not reported;
NFE, nitrogen-free extract, i.e., carbohydrates (NFE = 100% – (protein + crude fat + ash + crude fiber + moisture)).

The wide variablity showed within and among insect orders (Table 2.1) highlights the difficulty to obtain an accurate evaluation of edible insects nutritional properties since they are influenced by many factors, such as insect gender and species, life stage, environmental and rearing conditions (temperature, water availability, photoperiod, growth substrate), handling and processing procedures.

Finally, benefits of the adoption in human and animal diet regard even social and economic aspects. Indeed, insect rearing can be developed using high or low technologies, depending on investment capacity, providing opportunities even for the poorest section of society, for both urban and rural people (van Huis, 2013).

2.2 European regulation about edible insects

The precautionary principle is one of the fundamental aspects provided by European legislation on food safety. Regulation (CE) 178/2002, establishes principles and general requirements about food legislation, and in particular: i) insitutes the European Food Safety Authority (EFSA); ii) sets

procedures about food safety; iii) protects European Union consumers. Moreover, the "Hygiene Package", defined in the above cited Regulation, establishes the rules for food business operators and for public food safety authority in order to ensure the safety of food products imported and commercialized inside the EU, according to Regulation (CE) n. 852/2004 and Regulation (EU) 2017/625. Finally, Regulation (CE) n. 2073/2005 provides criteria for a few food related microorganisms, their toxins and metabolites in food and food processing.

Focusing on edible insects, in 2015 EFSA Scientific Committee issued an Opinion about risk profile related to production and consumption of insects as food and feed.

The Opinion noted the lack of knowledges about hazards related to edible insects consumption for humans and animals, highlighting the limited amount of sistematically collected data and concluded that "further research for better assessment of microbiological and chemical risks from insects as food and feed including studies on the occurrence of hazards when using particular substrates, like food waste and manure is recommended"(EFSA, 2015).

In the same year, Regulation (EU) n. 2015/2283, in force since 1st January 2018, was specifically edited to redefine the assessment and commercialization procedures of "*novel foods*", including insects and their parts, in Europe. Indeed, the "*novel foods*"category includes all those foods which were not extensively consumed before before March 1997 in the European Union.

Furthermore, EFSA Scientific panel of dietetic products, nutrition and allergies recently updated technical guidance and tools for preparing and submitting application for the commercialization of a novel food; this guidance was specifically published to help the producers to apply a correct request in order to demonstrate the safety of their foodstuffs. On the basis of the provided information, EFSA evaluate the safety of each food product for the consumption purposed by the producer.

2.3 Edible insects: food safety and focus on their microbiota

Although, insects have always been part of the human diet, not every insect species are food-safe.

Main hazards related to edible insects consumption derive from several factors: i) chemical contaminants, namely pesticides, toxins and heavy metals (Pb, Hg e Cd) which can cause irritation, acute and chronic intoxication and can have mutagenic or cancerogenic effects; ii) allergens, e. g. chitin; iii) biological agents such as mycotoxins, bacterial toxins or microbial pathogens (Devkota e Schmidt, 2000; Reese et al., 2007; van der Spiegel et al., 2013; Belluco et al., 2015).

Focusing on microbiological aspects related to edible insects, the state of the art at the beginning of this PhD. project is shown in Table 2.2.

Table 2.2. *Studies regarding edible insects microbiota characterization since 2000 to 2017.*

Country of origin	Species	Sample characteristics	Reference
Botswana	*Imbrasia belina*	Degutted insects, raw or boiled	Mpuchane et al., 2000
Botswana	*Imbrasia belina*	Raw or dried larvae and gut content	Sympania et al., 2000
Nigeria	*Bunaea alcinoe*	Whole raw larvae and gut content	Amadi et al., 2005
Nigeria	*Musca domestica*	Cuticle and gut content	Banjo et al., 2005
Nigeria	*Bunaea alcinoe*	Degutted insect, seasoned with black pepper and onion, pan-fried and wrapped in Gmelina arborea leaves	Braide et al., 2011
The Netherlands	*Tenebrio molitor* *Acheta domesticus*	Whole insects, raw, fried, roasted, boiled (different bioling times), or milled, subjected to lactic fermentation and stored at different temperatures	Klunder et al., 2012
Nigeria	*Rhynchophorus phoenicis*	Whole larvae, raw or cooked	Opara et al., 2012
Germany	*Tenebrio molitor* *Hermetia illucens*	Defatted insect powder	Bußler et al., 2016
Europe and Asia	*Acheta domesticus* *Locusta migratoria* *Omphisa fuscidentalis* *Alphitobius diaperinus* *Tenebrio molitor* *Hermetia illucens* *Musca domestica* *Oxya yezoensis* *Vespula flaviceps* *Bombyx mori*	Whole fried and spiced insects, whole dried insects, insects powder, insect extrudate, whole insects cooked in soya sauce and others (e.g. parfait)	Grabowsky and Klein 2016
The Netherlands and Thailandia	*Acheta domesticus* *Locusta migratoria* *Tenebrio molitor* *Belostomatidae* *Polyrhachis* *Termitoidae* *Hyboschema contractum* *Gryllotalpidae* *Bombyx mori* *Heterometrus longimanus*	Ehole insects, boiled dried and pan-fried	Milanović et al., 2016
The Netherlands and Belgium	*Acheta domesticus* *Locusta migratoria* *Tenebrio molitor*	Whole dried insects	Osimani et al., 2016
Belgium	*Tenebrio molitor* *Locusta migratoria migratorioides*	Raw milled insects	Stoops et al., 2016
The Netherlands	*Acheta domesticus* *Locusta migratoria* *Tenebrio molitor*	Whole insects and insects powder	Garofalo et al., 2017
Belgium	*Tenebrio molitor* *Alphitobius diaperinus*	Raw insects powder	Stoops et al., 2017
Belgium and the Netherlands	*Acheta domesticus* *Grylloides sigillatus* *Tenebrio molitor*	Whole raw larvae	Vandeveyer et al., 2017

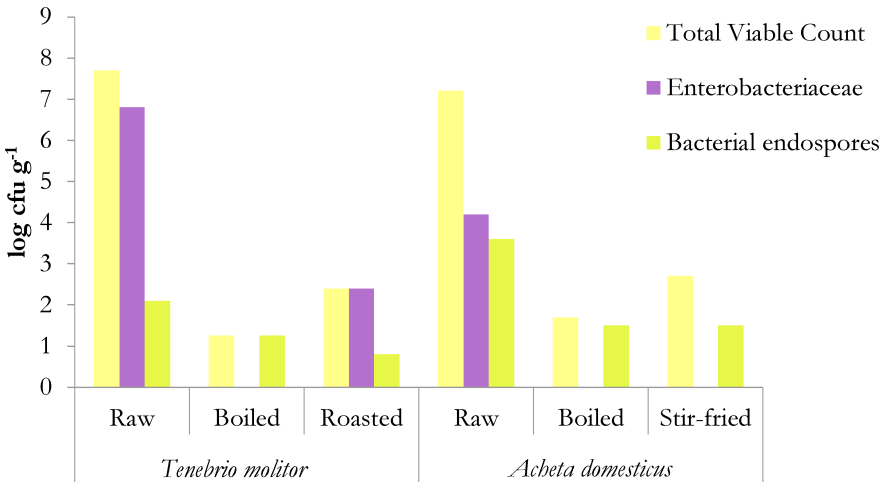
Most of the listed investigation were carried out to get information about bacterial communities and only a few concerned Fungi.

From a quantitative point of view, collected data reported a high variability regarding both raw and processed insects. In detail, raw insects showed total mesophilic aerobes viable counts ranging between 4 log UFC g^{-1} and 10 log UFC g^{-1} (Mpuchane et al., 2000; Amadi et al., 2005; Agabou e Alloui, 2010; Ali et al., 2010; Klunder et al., 2012; Henandez-Flores et al., 2015; Bußler et al., 2016; Stoops et al., 2016; Caparros Megido et al., 2017; Stoops et al., 2017; Vandeweyer et al., 2017a,b; Wynants et al., 2017); moreover Enterobacteriaceae, which are considered as hygiene indicators in food industry, generally resulted between <1 log UFC g^{-1} and 8 log UFC g^{-1} (Mpuchane et al., 2000; Klunder et al., 2012; Garofalo et al., 2017; Osimani et al., 2017b,c; Stoops 2017; Vandeweyer et al., 2017a); finally, spore-forming bacteria, which include, among others, microbial pathogens and food spoilage agents, ranged between <1 log UFC g^{-1} and 4 log UFC g^{-1} (Klunder et al, 2012; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2017c; Vandeweyer et al.,2017b; Stoops et al., 2017).

The reported variability can be due to several factors such as insect gender and species, life stage or environmental and rearing conditions (temperature, water availability, photoperiod, growth substrate), handling and processing procedures. For instance, the effectiveness of heat treatment during processing procedures was highlighted for all the above cited bacteria. Boiling or drying treatment substantially reduced total mesophilic aerobes and Enterobacteriaceae under 5 log UFC g^{-1} and 3 log UFC g^{-1}, respectively, whereas spore-forming bacteria resulted about 2 orders of magnitude lower than in raw samples. A good illustration of that can be found in the study carried out by Klunder et al. (2012) about the potential effect of heat treatment on Tenebrio molitor, *Acheta domesticus* and *Brachytrupus* sp. microbiota.

Results are reported in Figure 2.1.

Figure 2.1*. Assessment of different heat treatment effects on total mesophilic aerobes, Enterobacteriaceae and spore-forming bacteria related to T. molitor, A. domesticus e Brachytrupus sp..*



Source. Klunder et al., 2012.

Concerning the qualitative composition of microbiota related to edible insects, results obtained from studies reported in Table 2.2 show a high biodiversity among the different analysed samples due to the intrinsic insect characteristics, as well as the rearing, handling, processing and conservation procedures and as the performed analysis. Indeed, microbiota composition was defined using three different strategies: i) viable counts, microbial isolation and identification of the isolates through phenotypic characterization (Mpuchane et al., 2000; Sympania et al., 2000; Amadi et al., 2005; Banjo et al., 2005; Agabou and Alloui, 2010; Ali et al., 2010; Braide et al., 2011; Opara et al., 2012; Bußler et al., 2016; Grabowsky and Klein, 2016; Caparros Megido, 2017; de Oliveira et al., 2017; Vandeweyer et al., 2017a, b); ii) microbial isolation and identification via seuquencing of specific genes considered as phylogenetic markers (Klunder et al., 2012; Hernández-Flores et al., 2015); iii) microbial identification with culture-independent methods such as PCR-DGGE and Next-Generation Sequencing (NGS) (Jung et al., 2014; Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2017c; Wynants et al., 2017).

Focusing on latter studies, the already mentioned biodiversity might be referred to the insect species. In detail, Osimani et al. (2017c) and Garofalo et al. (2017a) performed PCR-DGGE analysis and pyrosequencing, respectively, to study the microbiota related to processed (boiled, dried and in case milled) insects: *A. domesticus* (cricket powder and dried cricket), *L. migratoria* and *T. molitor*: Briefly, regarding to *A. domesticus* microbiota, both studies reported the presence of spore-forming bacteria, namely *Bacillus cereus* and *Bacillus weihestephanenis* (potential pathogens) via PCR-DGGE and *Clostridium* sp. via pyrosequencing. It is noteworthy that *Clostridium* genus includes spoilage agent or pathogen (Soh et al., 1991). Concerning *L. migratoria*, species belonging to genus *Weissella* and to Enterobacteriaceae family prevalence was reported by PCR-DGGE as well as by pyrosequencing. This results were confirmed on raw locusts analyzed by the study of Stoops et al. (2017) which reported that the microbiota of this insect was characterized by the prevalence (88.5%) of lactic acid bacteria (*Weissella* sp., *Lactococcus* sp., *Enterococcus* sp. and Enterobacteriaceae). The similarity between microbiota of both raw and processed locusts suggests an intrinsic composition of the microbiota related to this insect species and not influenced by rearing or processing techniques. Finally, Enterobacteriaceae were reported also for *T. molitor* via PCR-DGGE and pyrosequencing. Interestingly, for this insect, pyrosequencing showed the prevalence of *Spiroplasma* sp. which was also reported by Jung et al. (2014) and Stoops et al. (2017). Species belonging to *Spiroplasma* sp. can be pathogen for insects (*Spiroplasma apis* and *Spiroplasma melliferum*), plants (*Spiroplasma citri* and *Spiroplasma kunkelii*) and vertebrates (Henning et al., 2006; Bastian et al., 2012; Nai et al., 2014; Zheng and Chen, 2014).

However, further research should be carried out to confirm this data.

Hazards related to edible insects micriobiota do not regard only its composition, but also the occurrence of transferable antibiotic resistance genes. Over the years, a huge amount, sometimes inappropriate, of antibiotics was used in zootechnics, aquaculture, agriculture and in the medical sector. This irresponsible behaviour caused a selective pressure on microbes which brought to the development of antibiotic resistance (AR). The resulting bacterial competitive advantage represents a serious issue for human helath (ECDC, EFSA, e EMA 2015). Indeed, if AR genes are transferred to human pathogens they can become difficult to eradicate resulting in higher mortality rates (Clementi e Aquilanti, 2011).

In this context, at the beginning of the present PhD. project, Milanović et al. (2016) and Osimani et al. (2017b) had already demonstrated the occurrence of transferable AR genes in the microbiota of different edible insetcts species, coming from Thailand and the Netherlands, or Belgium, Thailand and the Netherlands, respectively. In both studies, nested-PCR was perfomed to investigate about the presence of resistance genes to 11 antibiotics, namely macrolide-lincosamide-streptogramin B [*erm(A), erm(B), erm(C)*], vancomycin (*vanA and vanB*), tetracyclines [*tet(M), tet(O), tet(S), and tet(K)*], beta-lactams (*blaZ and mecA*) and aminoglycosides. In addition Osimani et al. (2017b) assessed the presence of *aac(6')-Ie e aph(2")-Ie*, which codify for resistance to aminoglycosides. In detail, most abundant genes in both studies were tetracycline and beta-lactams resistance genes. A further interesting data was the different diffusion of transferable AR genes depending on the country of origin. Thus suggesting a potential effect related to the provenance of the samples.

2.4 Edible insects and human consumption

Entomophagy, namely the practice of eating insect, is widespread mainly in Asia, Africa, South America and Australia. Edible insects can be eaten whole (raw or processed through drying, roasting, boiling or frying treatment), in powder or paste. In alternative they are also used as source for the extraction of proteins, fats or fiber (van Huis, 2013).

Many species are consumed worldwide and, among them, EFSA, in 2015, published a list of insects with the greatest potential as food and feed including *Musca domestica* (housefly), *Hermetia illucens* (black soldier fly), *T. molitor* (mealworm), *Zophobas atratus* (giant mealworm), *Alphitobus diaperinus* (lesser mealworm), *Galleria mellonella* (greater wax moth), *Achroia grisella* (lesser wax moth), *Bombyx mori* (domestic silkmoth), *A. domesticus* (house cricket), *Gryllodes sigillatus* (tropical house cricket), *Locusta migratora migratorioides* (African migratory locust), *Schistocerca Americana* (American grasshopper) (EFSA, 2015). However, in Europe entomophagy is perceived with scepticism and disgust and is associated with primitive behaviours (Kellert, 1993). In order to stimulate consumers acceptance towards edible insects, two different approaches could be adopted: i) education programs to underline edible insect potential for environment and health; ii) development of insect-based food products where insects presence is masked (Van Huis et al., 2013; Verbeke et al., 2014; Mlcek et al., 2014). Before the beginning of the present PhD. project, this latter approach was used in two different studies which revealed potential applications of edible insects as ingredients in food production. Stoops et al. (2017) developed a minced meat-like product using *T. molitor* or *A. diaperinus*. In more detail, the technological procedures set up and the assessment of the microbial dynamics occurring during the production and the conservation of the product were carried out. de Oliveira et al. (2017) exploited *Nauphoeta cinerea* (speckled cockroach) in bread-making. In particular, three flour blend formulations characterized by 5, 10 and 15% inclusion of *N. cinerea*, were used to produce three different bread loaves. Products were subjected to rheological and sensory analysis and the results were compared with those related to bread obtained by using only wheat flour. Furthermore, Bußler et al. (2016) investigating about the recovery and technological functionality of powders and proteins extracted from *T. molitor* and *H. illucens* demonstrated the insects availability in the production of ingredients for the development of protein enriched foods.

3. Overview on methods

A correct selection of the appropriate method to collect information about microbiological aspects related to edible insects is essential for an accurate risk analysis.

Food microbiology is a fast-moving branch of science.

Before the birth of polymerase chain reaction (PCR), microbiological investigations were carried out by growing microorganisms on syntethic media, however the achieved data were characterized by a low-value informative content, due to several limitations related to the variability of the microbial growth performance on artificially reproduced environments. Afterwards, the adoption of PCR-based methods was introduced in food microbial ecosystems studies, thus allowing to avoid the microbial cultivation limitations.Microbial identification and dynamics in food research are currently achieved by using a polyphasic approach, including culture-dependent and -independent methods.

In the next paragraphs, culture-dependent and -independent methods usually exploited in food microbiology are described, focusing on the techniques used in the present PhD. project.

3.1 Culture-dependent methods

Culture-dependent methods are based on microbial cultivation on specific culture media (e.g., total aerobic count on PCA medium, presumptive lactic acid bacteria count on MRS, Enterobacteriaceae on VRBGA). Through these methods, microbial identification can be achieved observing morphological characteristics of the isolates (colony morphology and colour further than cell morphology) or using phenotypic (physiological and biochemical) tecniques. Each cultivation parameter, such as oxygen tolerance, nutritional requirements, antibiotic susceptibility, incubation temperatures and/or times, and the pH of the medium, can be used to identify microorganisms in a specific food sample.

Threfore, culture-dependent methods results are directly and strictly influenced by microbial growth perfrormance. Thus resulating in pitfalls due to different factors: i) microorganisms are not always able to grow on synthetic media because of the difficulty to reproduce microbial natural habitat conditions or due to their physiological status (i.e., Viable But Not Cultivable (VBNC), stressed and/or injured cells); ii) it might be possible that less represented microbial cells are not revealed through traditional microbiological methods (Cocolin et al., 2013; Ercolini, 2004; Garofalo et al., 2017). In this regard, Hugenholtz et al. (1998) stated that "our knowledge of the extent and character of microbial diversity has been limited, however, by reliance on the study of cultivated microorganisms. It is estimated that >99% of microorganisms observable in nature typically are not cultivated by using standard techniques".

Moreover, classical plating methodologies sensitivity usually reaches group, family or genus level, even if an upgrade can be represented by the adoption of phenotyping (i.e. API galleries or other

methods such as the Phene PlateTM system) or genotyping (especially RAPD-PCR) (Olive and Bean, 1999; Giraffa and Neviani, 2000; Iversen et al., 2002; Lund et al., 2002).

In particular, in the present PhD. project, culture-dependent techniques were mostly used to have information about the microbial load occurring in edible insects under study.

3.2 Culture-independent methods

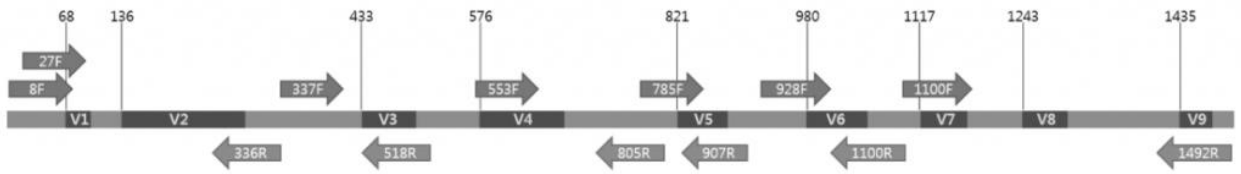
Culture-independent methods consist in the analysis of directly extracted nucleic acid (DNA or RNA) from a food sample, thus achieving a qualitative and quantitative picture of the occurring microbial community.

Although, in food microbiology, culture-independent methods were originally used to investigate about microbial dynamics in food fermentation and food spolilage assays, to date they are also exploited in food microbial ecology studies. Microorganisms present in a food ecosystem can be identified through genetic fringerprinting techniques which provide a profile about genetic diversity of a microbial community (Ercolini et al., 2004).

Since most of these methods are PCR-based, a fundamental parameter to take into account to achieve a proper profile of the microbial population in food ecosystems is the right selection of the DNA region to amplify. The "perfect DNA region" has two charachteristics: i) it has to be present in each member of the microbial group under study; ii) it has to include conserved regions, to allow primers design and annealing, and variable regions, which enable discrimination in microbial population (Cocolin et al., 2013).

Genes econding for ribosomal RNA (rRNA) satisfy "perfect DNA region" characteristics. Indeed, 16S rRNA and 26S rRNA sequences are often used for bacteria and yeast investigation in food microbiology research. In particular, as shown in Figure 3.1, 16S rDNA gene includes nine hypervariable regions flanked by conserved sequences (Neefs et al., 1993).

Figure 3.1. *16S rDNA gene structure.*

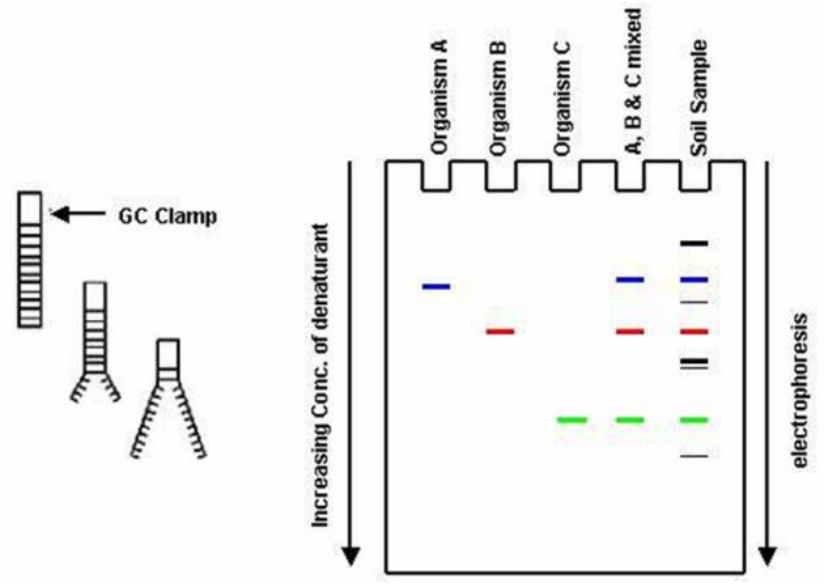


After the amplification, the objective is to discriminate among the occurring microorganisims. To this aim, Denaturing Gradient Gel Electrophoresis (DGGE) is one of the most commonly used. This technique enables the separation of PCR amplicons which are characterized by same lenght but different base pair sequences.

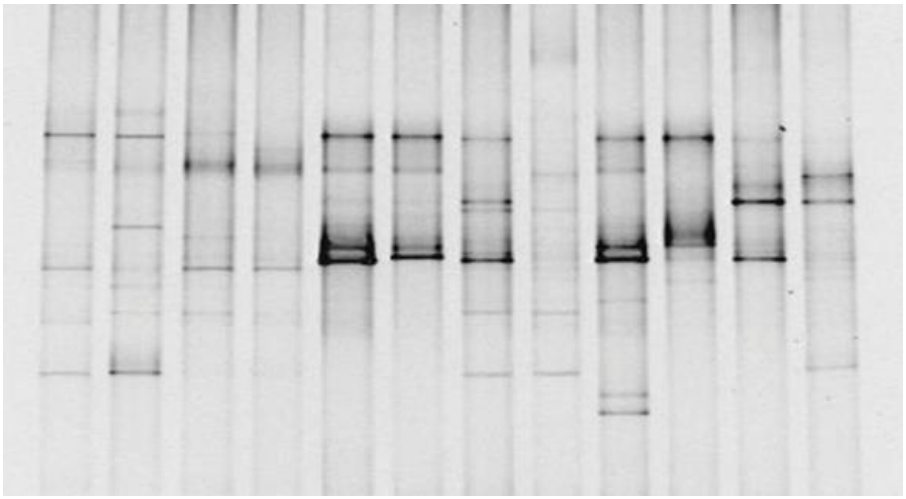
Figure 3.2 illustrates DGGE principles (panel a) and gel obtained after the electrophoresis step (panel b).

Figure 3.2.2*. DGGE denaturation principles (panel a) and DGGE resulting gel (panel b).*

a



b



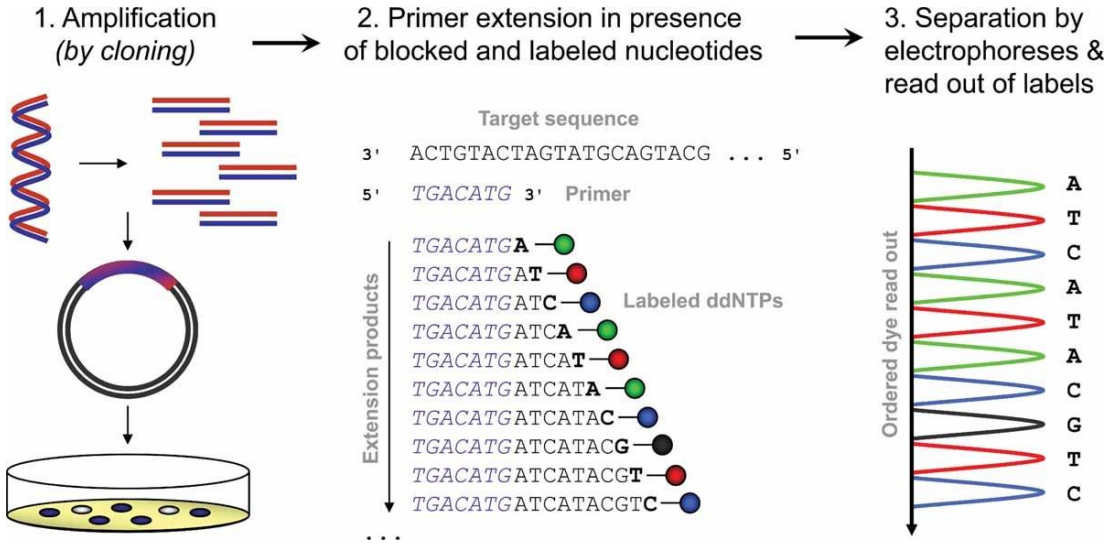
Through DGGE fragments separation can be obtained in a denaturating gradient gel thanks to their differential denaturation (melting) profile. The denaturing agents contained within an acrylamide gel are urea and formammide. In detail, to gain a linear denaturing gradient, two solutions (low and high denaturing gradient agents concentration) are prepared, mixed with the acrylamide gel and finally poured, using a gradient former, between two glass layers fitted in a specific casting. Therefore, during DGGE double-strand DNA fragments are subjected to an increasing denaturing environment, which cause their partial denaturation. Partial DNA fragments denaturation occurs in correspondence of the so called "melting domains", namely discrete regions characterized by specific melting temperature (Tm). The partial denaturation of melting domains reduces the mobility of the entire DNA fragment inside the gel until it stops (Myers et al., 1987). A complete denaturation is avoided by the presence of a GC clamp which is added to the PCR forward primer (Myers et al., 1985; Sheffield et al.,1989). A

costant temperature between 55 and 65°C is set for the electophoresis. After electrophoresis step, gel bands are cut and the DNA is left to elute in tubes containing water. Finally, DNAs are sequenced and the obtained sequences are compare to those present in specific databases.

Even though it allows to avoid the drawbacks related to classical microbiology techniques, PCR-DGGE can carry its own bias deriving from each step, from sampling to electophoresis. Further than sampling and sample handling, which can affect the microbial population in the food by increasing or reducing the number and the species to be detected, DNA extraction is the first source of pitfalls due to different reasons: i) species present in different amounts can influence the concentration of the DNA and its detectability; ii) a good DNA extraction is difficult to achieve in presence of a complex matrix; iii) natural cellular components, such as lipids, proteins and salts can act as polymerase inhibitors (Wilson, 1997; Ercolini et al., 2004). Moreover, it was demonstrated that PCR target selection is an important step, indeed, targeting different 16S variable regions may lead to different results in species composition of the same sample (Ercolini et al., 2003). Finally, also DGGE can represent a limiting factor: i) fragments can not be longer than 500 bp, thus resulting in the difficulty to achieve a reliable identification since relatively small 16S rDNA fragments are not always different within the same genus; ii) the 16S rDNA fragments might have identical melting behaviour, hence they comigrate in DGGE; iii) multiple copies of the 16S rDNA gene with sequence microheterogeneity can be present; iv) high microbial species detection limit ranging between 4 and 8 log cfu g^{-1} (Nübel et al., 1996; Dewettinck et al., 2001; Ogier et al., 2002; Fasoli et al., 2003; Temmerman et al., 2003; Ercolini et al., 2004).

In the last decades, even DNA sequencing techniques are playing a key role in food microbiota investigations. Sanger sequencing process is shown in Figure 3.3.

Figure 3.3. *Schematic representation of the Sanger sequencing process.*



Source: Kircher and Kelso, 2010.

DNA is fragmented and cloned into bacterial vectors and afterwards amplified. The obtained copies are used as template for reverse strand synthesis by randomly adding deoxy-nucleotides and fluorescence labelled dideoxy-nucleotides, thus creating differently extended molecules. Finally, resulting molecules are separated through capillary electrophoresis and the terminating ddNTPs is read out sequentially.

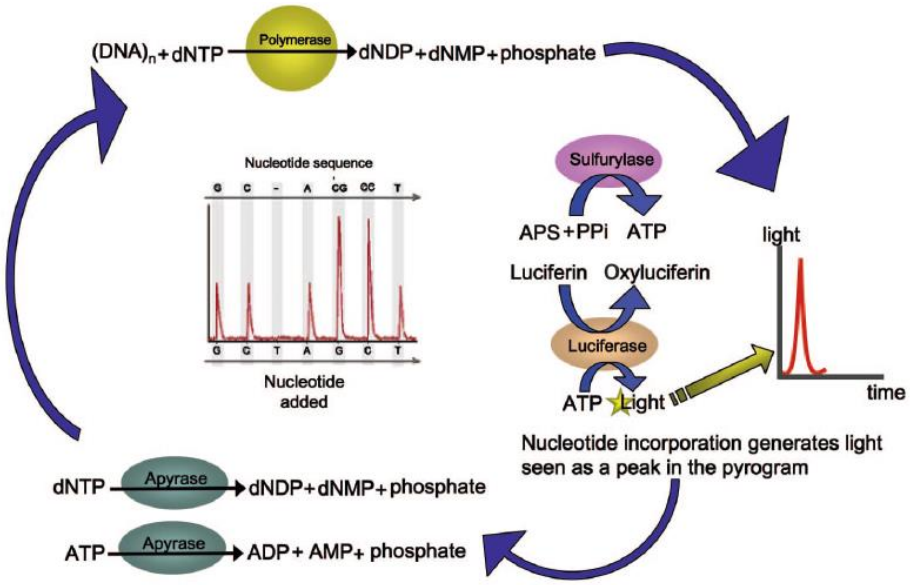
Those mostly used in taxonomic analysis of food microbiota are 16S rDNA sequencing, metagenomics and metatranscriptomics.

Recently, metagenomics showed its potential in in-depth microbial identification being able to reach the species/strain level. Metagenomics consists in the sequencing of the total amount of DNA included in a sample without any prior amplification (Cao et al., 2017). The obtained sequences are compared against database such as k-mer (Compeau et al., 2011), SILVA (Quast et al., 2013). Further than the microbial identification potential, metagenomics provides also concrete information about genes and genomes structure present in a food matrix (Cao et al., 2017). Among all metagenomics drawbacks, as it consists in a culture-independent DNA analysis, this technique can not distinguish between dead and alive microbial population (Ercolini, 2013). This limit can be overcome by using metatranscriptomics. Metatransciptomics is based on the seuqencing of the whole RNA present in a sample. Further than the discrimination of viable and not viable microbial population, it provides information about functional characterization of the different microorganisms.

To date, 16S rDNA sequencing represent one of the most important culture-independent methods in food microbial ecology research. It consists in sequencing of pre-amplified 16S rDNA regions. Obtained sequences are clustered into Operational Taxonomic Units (OTU) which are then queried against databases for the microbial identification.

This analysis can be perfromed by several platforms. Illumina is the NGS platform used for the analysis performed during the present PhD. project. Briefly, its chemistry, shown in Figure 3.4, consists in a sequencing by synthesis and it is made up by four enzimes (Ronaghi et al., 1996).

Figure3.2.4. *Schematic representation of pyrosequencing chemistry.*



Source: Petrosino et al., 2009.

DNA polymerase well-known activity generates inorganic pyrophosphate, which is used by sulfurilase to produce ATP. ATP just produced is used by luciferase to convert, causing the emission of light pulses; the amplitude of the signal is related to the number of nucleotides added, higher is the amplitude higher is the number.

NGS platforms in 16S rDNA sequencing carries with it several advantages in comparison to Sanger sequencing. First of all, Sanger sequencing results can show a lower biodiversity since it allows to sequence only a smaller proportion of amplicons, thus compromising the comprehensive description of the microbial community.

The introduction of NGS platforms in 16S rDNA sequencing additionally brought to a deeper identification bacterial members of the community by several orders of magnitude even at a much lower cost (Claesson et al., 2009). Moreover, pyrosequencing allowed individual samples indexing and encouraged multiplexing during each run (Hamady et al., 2008). This approach is also widely used beacause of the availability of free and easy bioinformatic tools designed for sequencing data analysis, such as QIIME (Quantitative Insights Into Microbial Ecology), mothur, and USEARCH (ultra-fast sequence analysis) (Schloss et al., 2009; Caporaso et al., 2010; Edgar, 2010).

At the same time, several challenges come from this approach: i) since short reads can be sequenced, bacterial classification often do not go beyond the genus level; ii) Gram-negative bacteria result underestimated if compared to those detected via transmission electron microscopy and Gram staining; iii) an unaccurate choice of the region to be amlified can cause issues, as already cite in this paragraph (Ercolini et al., 2003a; Claesson et al., 2010; Hugon et al., 2013).

Another extremely popular technique in food microbiology testing and microbial ecology studies is Quantitative PCR (qPCR or real-time PCR or real-time qPCR). Briefly, as described by Bokulich and Mills (2012), this PCR-based technique is characterized by a real-time tracking of the template amplification through a fluorescent reporter. A threshold is se in order to distinguish sample fluorescence and background noise and to determine, for each sample, the threshold cycle (Ct), namely the cycle at which sample fluorescence crosses the baseline threshold. Finally a standard curve is used for the quantification of unknown samples, plotting Ct and gene copy number or cell count.

Real-time qPCR is widespread used in order to quantify microbial populations in food, but can be also used to study about the occurrence of a given species or strain just by using specific primers, therefore not on the basis of a taxonomic identification but instead by investigating specific genes. For instance, qPCR can be used in beer research for the detection of lactic acid bacteria targeting the horA gene, which is responsible for the resistance to hop antimicrobial iso-α-acids (Haakensen et al., 2007). A further advantage related to qPCR is related to NGS technologies (Bokulich and Mills, 2012). Indeed, genes sequence information obtained via NGS technologies can be exploited to develop new target primers for the investigation of specific microorganisms in food.

In the present PhD. project, qPCR was used for both detection of microbial species of interest (cfr. paragraph 4.1) and occurrence of transferable antibiotic genes. This latter aim was gained also using nested-PCR. Nested-PCR is a modification of PCR that is characterized by an enhanced sensitivity and specificity. It foresees the use of two primer set and two successive PCR runs. The first primer set that is used in the first PCR run is designed to anneal to sequences upstream from the second primer set, used in the subsequent PCR run. Amplicons from the first PCR run are used as a template in the second PCR run. Hence, nested-PCR is well suited for the detection of very small amounts of target DNA sequences. This latter trait undoubtedly represents one of the main advantages of nested-PCR; when applied to the detection of transferable resistances, this technique allows small differences in AR gene abundance between different samples or ecosystems to be revealed. Limits of the detection of the PCR and nested-PCR protocols applied in the present study had preliminary been determined by Garofalo et al. (2007).

In the light of concepts expressed in the latter two paragraphs, during this PhD. project a polyphasic approach, constituted by culture-dependent and culture-independent methods, was adopted in order to have information about microbial load (viable counts), microbiota composition (PCR-DGGE, pyrosequencing and real-time qPCR) and occurrence of transferable resistance genes (real-time qPCR and nested-PCR). Indeed, no method can be a stand-alone tool in food microbial populations definition. A combined approach including different culture-dependent and -independent methods can allow to collect more accuarate information about the microbial aspects.

4. Characterization of the microbiota related to edible insects

In the present chapter, studies about microbiological aspects and transferable resistance genes occurrence related to edible insects are listed and described.

4.1 Revealing the microbiota of marketed edible insects through PCR-DGGE, metagenomic sequencing and real-time PCR (Paper II)

In 2015, EFSA Scientific Committee edited an opinion about hazards related to insects consumption as food and feed in which the importance of further research to assess microbiological and chemical risks related to edible insects was underlined (EFSA, 2015).

Regarding microbiological aspects, information about the safety of edible insects consumption is scarce when compared to the knowledge on common protein sources as dairy products, meat and eggs. From the literature, a great microbial diversity among insect species emerged, due to the intrinsic flora of insects or to differences in rearing conditions, handling, processing and preservation.

To date, the scientific community has mainly focused on the study of the microbiota of a few edible insect species (e.g., *Alphitobius diaperinus*, *Tenebrio molitor*, *Locusta migratoria*, and *Acheta domesticus*) that show potential applications in the food industry.

In this context, to collect information on a wider range of insects, the present study was aimed to identify the microbiota present in six species of processed edible insects produced in Thailand and marketed worldwide via the internet, namely, giant water bugs (*Belostoma lutarium*), black ants (*Polyrhachis*), winged termite alates (*Termitoidae*), rhino beetles (*Hyboschema contractum*), mole crickets (*Gryllotalpidae*), and silkworm pupae (*Bombyx mori*).

The study was performed by using culture-independent analysis and microbial identification by molecular methods, and in particular by Polymerase Chain Reaction Denaturing Gradient Gel Electophoresis (PCR-DGGE) and metagenomic sequencing. Indeed, as already highlithed by Ercolini and Cocolin (2014) the integration of PCR-DGGE and metagenomic sequencing provides a more accurate overview of the microbial composition and relative abundances of the detected microbial species or genera.

4.1.1 Materials and methods

Collection of samples

Six species of edible insects ready to be consumed, namely, giant water bugs (*Belostoma lutarium*), black ants (*Polyrhachis*), winged termite alates (*Termitoidae*), rhino beetles (*Hyboschema contractum*), mole crickets (Gryllotalpidae), and silkworm pupae (*Bombyx mori*) were purchased via the internet from a dealer located in Thailand. For each species, two samples of boiled, dried and salted insects were purchased. Insects were delivered at ambient temperature by the producer in sealed plastic bags of variable weight. Afterwards, samples were stored at room temperature until analyses.

DNA extraction and 16S rRNA gene amplification

Microbial DNA was extracted from the insect samples using the PowerFood Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) (Milanović et al., 2016). The assessment of DNA template quantity and purity was carried out by optical readings at 260, 280 and 234 nm using a UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Duplicate DNA extracts for each insect species were pooled and 100 ng of each pooled DNA extract was amplified through PCR in a 50-µL reaction volume using universal primers for eubacteria P27f (5-GAG AGT TTG ATC CTG GCT CAG-3) and P1495r (5-CTA CGG CTA CCT TGT TAC GA-3). The 16S rRNA gene amplicons were purified using the GFXTM PCR DNA and Gel Band Purification Kit (EuroClone), according to the manufacturer's instructions. The purified amplicons were used for both PCR-DGGE analysis and metagenomic sequencing.

PCR-DGGE analysis

The purified 16S rRNA gene amplicons were further amplified using primers 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') spanning the V3 region of the 16S rRNA gene. A GC clamp was attached to the 338f primer as proposed by Ampe et al. (1999). The reaction mixtures and amplification conditions were previously described by Osimani et al. (2015). DGGE analysis was performed with a vertical DCode electrophoresis system (Bio-Rad Laboratories), as already described by Garofalo et al. (2015). Selected DGGE bands were excised by gel cutting tips, re-suspended in sterile water, and incubated overnight under refrigeration (4 °C) to allow DNA to diffuse out of the gel cuts (Garofalo et al., 2008). Eluted DNA was subjected to PCR amplification under the conditions described above and using a forward primer without a GC clamp. Amplicons were then sent to Genewiz (Hope End, Takeley, United Kingdom) for purification and sequencing (Milanović et al., 2017). The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih. gov/) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

Metagenomic sequencing

The V3-V4 region of the 16S rRNA gene was amplified using recently described primers and conditions (Berni Canani et al., 2017). Library multiplexing, pooling and sequencing were carried out following the Illumina 16S Metagenomic Sequencing Library Preparation protocol on a MiSeq platform and using the MiSeq Reagent kit v2, leading to 2 × 250 bp paired-end reads.

Bioinformatics and statistical analysis

Sequence filtering and processing was carried out as recently reported (Berni Canani et al., 2017). Briefly, forward and reverse reads were joined using FLASH (Magoc and Salzberg, 2011). Joined reads were quality trimmed (Phred score <20), and short reads (<250 bp) were discarded using PRINSEQ

(Schmieder and Edwards, 2011). High quality reads were then imported into QIIME (Caporaso et al., 2010). OTUs were chosen through a de novo approach and the uclust algorithm, and taxonomic assignment was obtained using the RDP classifier and the Greengenes database (McDonald et al., 2012). To avoid biases due to the different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample.

Real-time PCR analyses

All real-time PCR assays were performed with a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany) as described in the following paragraphs.

Detection of Coxiella burnetii

The presence of *Coxiella burnetii* was assessed by real-time PCR assay using an Adiavet™ Coxiella RealTime kit (Bio-X Diagnostics, Rochefort, Belgium) for amplification of the template DNA, as previously described by Guidi et al. (2017). The sequence targeted by the assay was the *C. burnetii*-specific insertion sequence IS1111.

Detection of Shiga-toxin E. coli (STEC)

Detection of STEC was carried out as previously described by Petruzzelli et al. (2013), according to the ISO/TS 13136:2012 standard method. This method targets the virulence genes *stx1*, *stx2* and *eae* and serogroup-specific genes for O157, O145, O111, O26 and O103. Amplification was performed using the QuantiFast Pathogen PCR+IC kit (Qiagen, Hilden, Germany) in combination with the primer set and 5'- FAM-labelled probes reported in Table 4.1.1.

Table 4.1 1*. Nucleotide sequences of primers and probes employed in real-time PCR assay for the detection of Shiga-toxin E. coli stx1, stx2 and eae are genes encoding STEC pathogenicity factors.*

Target gene	5'-3' nucleotide sequence of forward (Fw) and reverse (Rev) primers and probesa	Amplicon size (bp)	Sequence localization (nt)	References
stx1	Fw- TTTGTYACTGTSACAGCWGAAGCYTTACG Rev- CCCCAGTTCARWGTRAGRTCMACRTC Probe- CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	878–906 983–1008 941–971	Perelle et al., 2004
stx2	Fw- TTTGTYACTGTSACAGCWGAAGCYTTACG Rev- CCCCAGTTCARWGTRAGRTCMACRTC Probe- TCGTCAGGCACTGTCTGAAACTGCTCC	128	785–813 785–813 838–864	Perelle et al., 2004
eae	Fw- CATTGATCAGGATTTTTCTGGTGATA Rev- CTCATGCGGAAATAGCCGTTA Probe- ATAGTCTCGCCAGTATTCGCCACCAATACC	102	899–924 1000–979 966–936	MøllerNielsen and Thorup Andersen, 2003
rfbE (O157)	Fw- TTTCACACTTATTGGATGGTCTCAA Rev- CGATGAGTTTATCTGCAAGGTGAT Probe- AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88	348–372 412–435 381–410	Perelle et al., 2004
wbdI (O111)	Fw- CGAGGCAACACATTATATAGTGCTTT Rev- TTTTTGAATAGTTATGAACATCTTGTTTAGC Probe- TTGAATCTCCCAGATGATCAACATCGTGAA	146	3464–3489 3579–3609 3519–3548	Perelle et al., 2004
wzx (O26)	Fw- CGCGACGGCAGAGAAAATT Rev- AGCAGGCTTTTATATTCTCCAACTTT Probe- CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135	5648–5666 5757–5782 5692–5724	Perelle et al., 2004
ihp1 (O145)	Fw- CGATAATATTTACCCCACCAGTACAG Rev- GCCGCCGCAATGCTT Probe- CCGCCATTCAGAATGCACACAATATCG	132	1383–1408 1500–1514 1472–1498	Perelle et al., 2004
wzx (O103)	Fw- CAAGGTGATTACGAAAATGCATGT Rev- GAAAAAAGCACCCCCGTACTTAT Probe- CATAGCCTGTTGTTTTAT	99	4299–4323 4397–4375 4356–4373	Perelle et al., 2005

[a] *Y=C or T; S=C or G; W=A or T; R=A or G; M=A or C*

Detection of Pseudomonas aeruginosa

Detection of *P. aeruginosa* was carried out as previously described by Amagliani et al. (2013). Briefly, the QuantiFast Pathogen PCR+IC kit (Qiagen) was used in combination with the primers ecfXF–ecfXR. A dual-labelled probe ecf-X-TM with 5'-FAM instead of Yakima Yellow was used as the reporter fluorophore.

4.1.2 Results

PCR-DGGE analysis

The results of PCR-DGGE analysis (Table 4.1.2) highlighted the dominance of spore-forming bacteria from the genera *Geobacillus*, *Bacillus*, *Paenibacillus* and *Clostridium*. Moreover, the genera *Corynebacterium*, *Pseudomonas* and *Vibrio* were also detected.

Table 4.1.2. *Sequencing results of the bands excised from the bacterial denaturing gradient gel electrophoresis (DGGE) gels.*

Samples	Closest relative	% Ident.[a]	Acc. No.[b]
Silkworm pupae	*Geobacillus* spp.	100	DQ119662
	Corynebacterium spp.	100	KU133332
	Bacillus cereus	97	GQ495095
	Bacillus lehensis	97	KR140380
	Paenibacillus apiarius	97	NR_114809
Rhino beetles	*Bacillus simplex*	97	KC595864
	Propionivibrio limicola	76	NR_025455
	Corynebacterium mustelae	84	NR_116621
	Alloiococcus otitis	74	NR_113771
	Herbaspirillum lusitanum	79	NR_028859
Rhino beetles	*Bacillus simplex*	97	KC595864
	Propionivibrio limicola	76	NR_025455
	Corynebacterium mustelae	84	NR_116621
	Alloiococcus otitis	74	NR_113771
	Herbaspirillum lusitanum	79	NR_028859
Black ants	*Pseudomonas indica*	97	NR_028801
	Lysobacter xinjiangensis	82	NR_116465
	Acinetobacter gerneri	94	NR_028852
	Pseudomonas taeanensis	97	NR_116651
	Pseudomonas aestusnigri	87	NR_126210
	Pseudomonas spp.	97	KX270014
	Pseudomonas spp.	97	AB812783
	Gamma proteobacterium	89	HQ663267
	Pseudomonas benzenivorans	97	NR_116904
	Pseudomonas spp.	97	KX270014
Mole crickets	*Bacillus cytotoxicus*	97	NR_074914
	Bacillus cytotoxicus	98	NR_074914
Winged termite alates	*Bacillus cytotoxicus*	98	NR_074914
Giant waterbugs	*Vibrio hangzhouensis*	97	NR_044396
	Vibrio diazotrophicus	97	NR_026123
	Clostridium sordellii	100	NR_112173
	Clostridium hiranonis	97	NR_028611

[a] *Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database;*

[b] *Accession number of the sequence of the closest relative found by a BLAST search.*

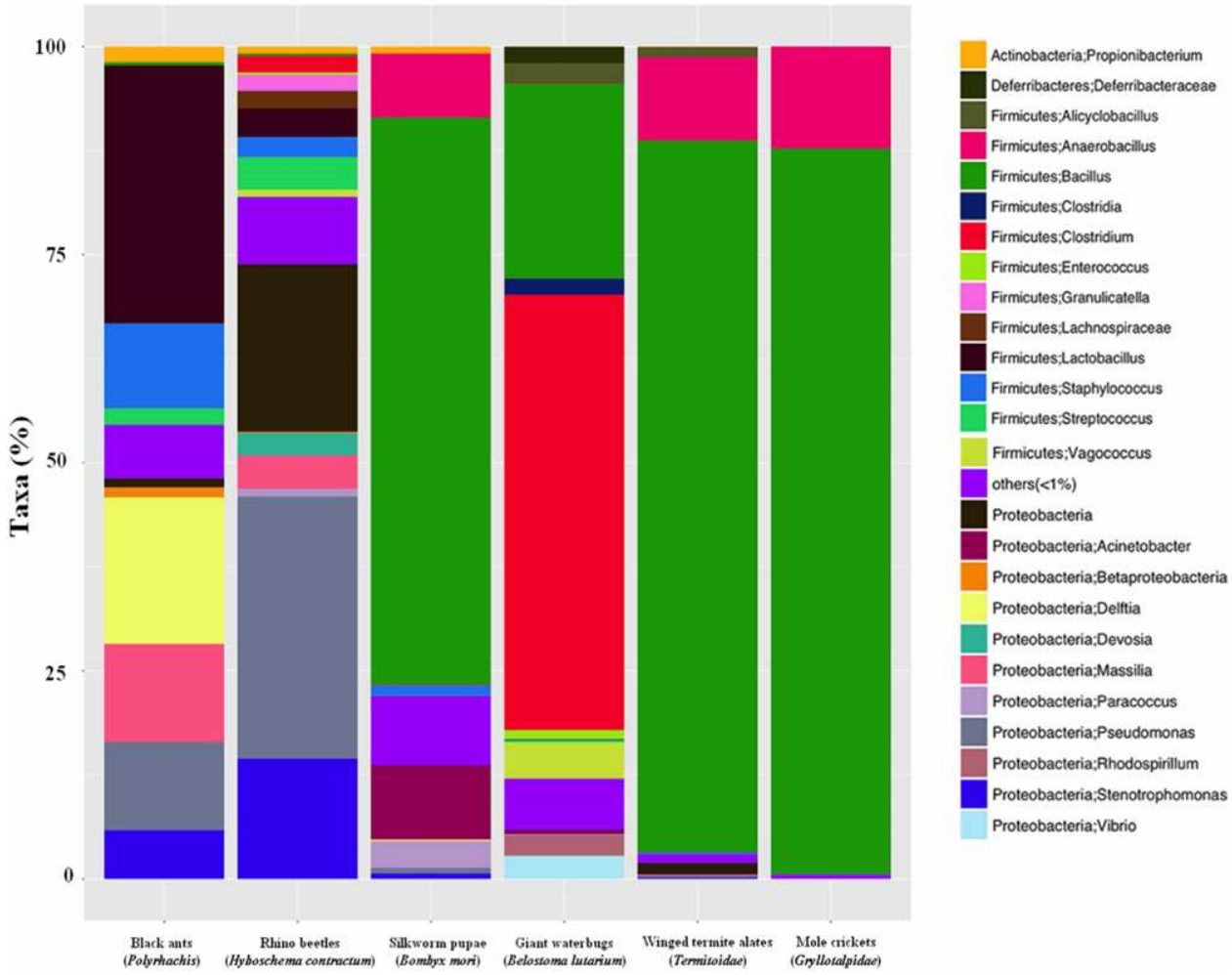
Specifically, the microbiota of silkworm pupae were characterized by the presence of the closest relatives to *Geobacillus* spp., *Bacillus cereus*, *Bacillus lehensis*, *Paenibacillus apiaries* and *Corynebacterium* spp. For rhino beetles, the closest relatives to *Bacillus simplex* were detected with a sequence identity higher

than 97%, while the closest relatives to *Propionivibrio limicola*, *Corynebacterium mustelae*, *Alloiococcus otitis*, and *Herbaspirillum lusitanum* were also found, albeit with a sequence identity lower than 97%. The microbiota of black ants was dominated by *Pseudomonas* spp., including *Pseudomonas indica*, *Pseudomonas benzenivorans*, and *Pseudomonas taeanensis*. *Lysobacter xinjiangensis*, *Acinetobacter gerneri*, *Pseudomonas aestusnigri*, and *Gamma proteobacterium* were also detected with a sequence identity lower than 97%. Mole crickets and winged termite alates were dominated by the closest relatives to *Bacillus cytotoxicus*. Finally, the microbiota of giant water bugs was characterized by *Clostridium sordellii*, *Clostridium hiranonis*, *Vibrio hangzhouensis*, and *Vibrio diazotrophicus*.

Metagenomic sequencing

After quality filtering, an average of 116,524 high quality reads/sample (± 15,905) was obtained. All samples showed high diversity, ranging from approximately 3600 (in black ants) to 8600 (in giant water bugs) OTUs. Figure 4.1.1 reports the relative abundances of the most abundant bacterial taxa in the edible insect samples analyzed.

Figure 4.1.1. *Relative abundance (%) of the most abundant microbial taxa in edible insect samples.*



The OTUs with relative abundance below 1% are grouped together in "others".

The microbiota of winged termites, mole crickets and silkworm pupae were dominated by Firmicutes, with abundances of >80% *Bacillus* and 10% *Anaerobacillus*. Firmicutes was also the main phylum in giant water bugs, where *Clostridium* represented the dominant genus (approximately 52%).

In contrast, black ants and rhino beetles showed higher abundances of *Proteobacteria*, and in particular of the genera *Stenotrophomonas* (14%) and *Pseudomonas* (31%) in rhino beetles and *Stenotrophomonas* (6%), *Pseudomonas* (11%), *Massilia* (12%) and *Delftia* (18%) in black ants. In addition, these samples also showed a higher diversity of Firmicutes genera, including *Lactobacillus*, *Staphylococcus*, *Streptococcus* and *Vagococcus*.

Real-time PCR analyses

Real-time PCR assays yielded negative results for all of the monitored pathogens in the analyzed samples.

Regarding the detection of *C. burnetii*, the cycle threshold was manually positioned at the take-off point of the positive control's amplification curve. Fluorescence plots reported no amplification signal for all of the template DNA from the samples analyzed (data not shown).

Concerning Shiga-toxin *E. coli* (STEC), the first step of detection consisted of the amplification of the *stx1* and *stx2* gene sequences coding for the Shiga-like toxins responsible for the pathogenicity of this *E. coli* group. The reactions were carried out in monoplex since both probes were labelled with the same fluorophore, FAM. Since no positive signal was recorded for the *stx1-stx2* genes from any of the samples (data not shown), no further identification of *eae* or serogroup-associated genes was needed according to the ISO 13136/2012 standard method.

For *P. aeruginosa*, the template for the positive amplification control was obtained from the *P. aeruginosa* ATCC 27853 reference culture. Whole extracted DNA was concentrated to 12.2 µg/ml (A260/280 = 1.30; A260/230 = 0.62).

Positive control reactions were prepared by adding 5 µl of template suspension to the reaction mix, corresponding to 61 ng of DNA. The rel-time PCR analysis yielded a negative result for all of the investigated samples, and therefore, no further tests were performed.

4.1.3 Discussion

Based on the results of both PCR-DGGE and metagenomic analysis, the microbiota of the analyzed edible insects was characterized by the presence of both commensal, spoilage and potentially pathogenic bacteria.

Specifically, in accordance with previous studies carried out with culture-independent techniques, the microbiota of some of the analyzed samples were largely dominated by spore-forming bacteria from the genera *Bacillus*, *Anaerobacillus*, *Clostridium*, *Paenibacillus* and *Geobacillus* (Osimani et al., 2018a; Stoops et al., 2016; Vandeweyer et al., 2017a; Vandeweyer et al., 2017b). Indeed, regarding the occurrence of *Bacillus*, as observed in winged termites, mole crickets, and silkworm pupae by both PCR-DGGE and metagenomic analysis and in rhino beetles by PCR-DGGE, species belonging to this bacterial genus have already been described in hymenopterans, ants, apid bees, honey bees, beetles, scarab beetles, butterflies, flies, crickets and mealworms (Garofalo et al., 2017a; Grabowski and Klein, 2017). Moreover, *Bacillus* is a symbiont in the termite gut that contributes to the degradation of lignocellulosic material (Cibichakravarthy et al., 2017). It is noteworthy that the genus *Bacillus* comprises potentially pathogenic species, including *Bacillus cereus*. This spore-forming bacterium is mostly associated with soil, where it comprises approximately 10% of the microbiota.

The *B. cereus* group includes seven species that are genetically related: *Bacillus anthracis*, *B. cereus* sensu stricto, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*. The seven species included in the *B. cereus* group are known as *B. cereus* sensu lato. In foods, *B. cereus* sensu stricto represents a public health threat for its ability to produce two gastro-intestinal syndromes causing vomiting or diarrhea (Osimani et al., 2018b; Petruzzelli et al., 2018).

Clostridium was detected by both PCR-DGGE and metagenomic analysis in the samples of giant water bugs, where it represented the dominant genus. Moreover, it was also detected through metagenomic analysis in winged termite alates, silkworm pupae and mole crickets, where it constituted a minor fraction of the microbiota. The presence of *Clostridium* species has already been reported in edible insects such as *Schistocerca gregaria* (desert locust), *T. molitor* (yellow meal beetle), *H. illucens* (black soldier fly), and *A. domesticus* (small cricket) (Garofalo et al., 2017a; Osimani et al., 2017a; Schlüter et al., 2017). The genus *Clostridium* encompasses pathogenic species such as *Clostridium botulinum*, *Clostridium perfringens* and *Clostridium difficile* that are responsible for different illnesses, which can lead to serious repercussions for consumers. In particular, *Clostridium sordellii*, which was detected by PCR-DGGE in giant water bugs, is able to produce the so-called *C. sordellii* lethal toxin (TcsL) and *C. sordellii* hemorrhagic toxin (TcsH), belonging to the large clostridial glucosylating toxin (LCGT) family (Popoff, 2017). The spore-forming ability of such bacteria is insidious in the food industry due to their extreme resistance to heat treatment.

The presence of *Anaerobacillus* was detected only by metagenomic analysis in winged termites, mole crickets and silkworm pupae. To our knowledge this is the first report of the presence of this genus in insects. The presence of *Anaerobacillus* has previously been reported in extreme environments such as soda lakes and aged indigo fermentation vats (Okamoto et al., 2017; Sorokin et al., 2015).

Paenibacillus apiarius was detected in silkworm pupae only via PCR- DGGE. Grabowski and Klein (2017) reported the presence of species belonging to this genus of spore-forming entomopathogenic bacteria in honeybees, scarab beetles and flies. In foods, paenibacilli are the causative agents of the spoilage of dairy products, non-pasteurized chilled ready-to-eat meals, pasteurized vegetable purées, and raw starchy foods (Helmond et al., 2017). It is noteworthy that, at the present time, there is a paucity of information on the characteristics of these spore formers in foods (Helmond et al., 2017).

Geobacillus spp. was only detected by PCR-DGGE in silkworm pupae, and its presence was previously reported in caterpillars as a pathogenic species (Grabowski and Klein, 2017).

As highlighted by Vandeweyer et al. (2017a), spore-forming bacteria can easily withstand the process treatments of edible insects, thus representing a challenge for the food industry. Hence, good manufacturing practices (e.g., effective heating steps) are pivotal to assure the safe consumption of insects as food or food ingredients. It is noteworthy that, at the present time, the majority of the insects sold on the market have previously been subjected to blanching, boiling or roasting steps, as well as (freeze) drying treatment (Vandeweyer et al., 2017b).

PCR-DGGE also allowed for the detection of *Corynebacterium* spp. in silkworm pupae, whose presence was already reported by Grabowski and Klein (2017) in the same insect species. Members of the genus *Massilia* were found at relatively high abundance in black ants via metagenomic analysis. Species belonging to this genus are mainly associated with the soil environment (Moquin et al., 2012). Moreover, Adrangi et al. (2010) reported endochitinase capabilities of *Massilia timonae*. This suggests a

possible symbiotic activity in the guts of ants for the digestion of fungal cell walls where chitin represents a major component (Epps and Penick, 2017). To our knowledge, no previous reports on the occurrence of *Massilia* in insects are available. In the food environment, the genus *Massilia* was recently associated with the microbiota of fruits such as apples and black-currants (Vepštaitė-Monstavičė et al., 2018).

Delftia was detected through metagenomic analysis with high abundance in black ants, and OTUs belonging to this genus were previously found by Mereghetti et al. (2017) in the microbiota of the Indian meal moth, *Plodia interpunctella*, which is considered one of the major pest commodities worldwide. *Delftia* species have also been found to be associated with opportunistic infections, both in immuno-compromised and immune-competent patients, and exhibits remarkable resistance to aminoglycosides and polymyxin (Camargo et al., 2014).

Stenotrophomonas was discovered through metagenomic analysis in black ants and rhino beetles. Species belonging to this bacterial genus were previously described by Grabowski and Klein (2017) in beetles, scarab beetles, butterflies and crambid butterflies. Interestingly, as recently reported by Deng et al. (2015), *Stenotrophomonas* spp. showed degrading capability for organophosphorus insecticides, thus suggesting its potential role in host resistance to insecticides. Moreover, an increase of *Stenotrophomonas* in the microbiota of the western corn rootworm *Diabrotica virgifera* was observed in response to pest control crop rotation (Vavre and Kremer, 2014). Among the *Stenotrophomonas* species, *Stenotrophomonas maltophilia* showed antagonistic activity towards fungal phytopathogens such as *Fusarium*, *Rhizoctonia* and *Alternaria* (Jankiewicz et al., 2012). Interestingly, Fukuda et al. (2017) recently reported that *S. maltophilia* cells ingested and hosted by houseflies (*Musca domestica*) can facilitate the spread of antimicrobial- resistant strains from environmental sources to humans, thus representing a public health concern in hospital settings.

Members of the genus *Pseudomonas* were detected by both PCR-DGGE and metagenomic analysis in black ants and only by metagenomics in rhino beetles. *Pseudomonas* has already been previously detected in both pest and edible insects such as cockroaches, acridid grasshoppers, lygaeid bugs, mealworms, butterflies, flies, centipedes, small crickets, grasshoppers and mealworm larvae (Garofalo et al., 2017a; Grabowski and Klein, 2017; Stoops et al., 2016; Vandeweyer et al., 2017a; Wynants et al., 2017; Wynants et al., 2018). As reported by Anderson and Kim (2018), *Pseudomonas* species (e.g., *Pseudomonas chlororaphis* or *P. aeruginosa*) can exert biopesticidal activity through the production of reactive oxygen species (ROS) elicitors, phenazines, pyoverdine-type iron-chelating siderophores, and volatile hydrogen cyanide (HCN), which are active against some insect species. Members of the *Pseudomonas* genus are well adapted to different environments such as soil, water, plants, animal tissues, and foods. *Pseudomonas* species are well-known food spoilers. Moreover, enzymes (proteases) produced by *Pseudomonas* species are heat-resistant and can maintain their activity after heat treatment processes used to eradicate microorganisms in food matrices (Caldera et al., 2016).

As shown by metagenomic analysis, black ants and rhino beetles were also characterized by the presence of the genera *Lactobacillus*, *Staphylococcus*, *Streptococcus* and *Vagococcus*. Vannette et al. (2017) reported the association of ants with some microbial taxa, including *Lactobacillus* species. Moreover, Olofsson et al. (2014) isolated novel species of *Lactobacillus* from the honey stomach of the honeybee *Apis mellifera*, thus confirming the natural occurrence of this genus in insects.

Vagococcus was previously detected in the gut microbiota of the insect Drosophila (Kim et al., 2017) and in mosquitos, with potential anti-viral activity through the production of secondary metabolites (Dennison et al., 2014).

The presence of *Staphylococcus* species was reported by Grabowski and Klein (2017) in the same insect species and in cockroaches, beetles, butterflies, silkworms and flies.

The presence of *Vibrio* in giant water bugs was ascertained by both PCR-DGGE and metagenomic analysis (although with low relative abundance). To the authors' knowledge, *Vibrio* has been rarely found in edible insects, although recent studies have reported the occurrence of *Vibrio* species in edible mealworms (Osimani et al., 2017c; Wynants et al., 2017). *Vibrios* are Gram-negative rod-shaped bacteria and include major pathogenic species with significant impacts on public health. This genus includes opportunistic species for both invertebrates and humans. Taxa that have been repeatedly involved in disease outbreaks include *Vibrio cholerae*, Vibrio parahaemolyticus and Vibrio vulnificus, whereas Vibrio alginolyticus, Vibrio harveyi, *Vibrio fluvialis*, *Vibrio furnissii*, *Vibrio metschnikovii*, and *Vibrio mimicus* generally represent lower- risk species (Austin, 2010).

From these data, it is interesting to note that black ants and rhino beetles exhibited similar microbiota, as well as mole crickets, winged termites alates, silkworm pupae and giant water bugs. Despite the different taxonomy of these insect species, this phenomenon may be due to the intrinsic microbiota of each species or to the rearing and processing conditions that influenced the specific microbiota of these insect samples. However, further studies are necessary to elucidate this aspect.

The major limitation of culture dependent methods is the under-estimation of the microbial community present in the studied matrices as it is estimated that only 1% of all microorganisms are cultivable. Moreover, the cells could be in viable but not culturable (VBNC) state (Oliver, 1993) or masked on the plates if present in minor concentration which can lead to false negative results (Cocolin et al., 2013). This latter aspect is of great concern, as VBNC food-born pathogens could emerge from this state in human organism and cause disease. On the other hand, the major advantage of these methods, which was not the aim of the present study, is the possibility to obtain the isolates that could be used in further studies. The culture-independent methods, especially their combination, as applied in the present study (DGGE, metagenomic sequencing and real-time PCR) can overcome the above mentioned disadvantages for their ability to identify a large portion of bacterial diversity. For these reasons, culture-independent methods are expected to continue to be used more frequently in many fields, including novel food microbiology.

Concerning the combination of the two different molecular techniques used within this study, it is worth noting that PCR-DGGE and metagenomic analyses were mainly concordant in terms of the overall biodiversity detected, and few discrepancies were found. Specifically, PCR-DGGE failed to detect some species, possibly due to the presence of several biases, such as the preferential DNA amplification of some DNA templates rather than others during PCR, the phenomenon of co-migration of the DGGE bands on the gel and the possible presence of faint bands on gel images that may be difficult to detect by naked eye, thus leading to an underestimation of bacterial diversity (Ercolini, 2004; Garofalo et al., 2017b; Milanović et al., 2017; Osimani et al., 2015). Furthermore, PCR-DGGE has been reported to only be capable of detecting dominant species with a concentration higher than 3 log colony forming units per milliliter or per gram (Cocolin et al., 2013; Garofalo et al., 2017b). In contrast, thanks to the higher efficiency and sensitivity of metagenomics, it was possible to

detect even low-abundance taxa. However, thanks to PCR-DGGE, in some cases, the identification of single bacterial taxa (e.g., within the genera *Bacillus* and *Pseudomonas*) was possible, thus integrating the results obtained by metagenomics, as previously found by Cardinali et al. (2017). The observed differences may also be due to the different specificity of the PCR protocols used (different primers and different hypervariable regions of the 16S rRNA gene targeted) and/or to the different databases used for sequence identification. The bacterial genera are reported irrespective of their source and method of detection. Regarding DGGE results, only the closest relatives with sequence identity ≥97% are reported, and for metagenomic sequencing results, only the most relevant detected genera are reported.

The absence of bacterial DNA belonging to the family Enterobacteriaceae was surprising. Indeed, based on the available literature, Enterobacteriaceae generally constitute a major fraction of the natural microbiota of edible insects (Garofalo et al., 2017a; Osimani et al., 2017a; Stoops et al., 2016; Vandeweyer et al., 2017a; Vandeweyer et al., 2017b; Wynants et al., 2017). Further investigations are needed to clarify this interesting finding. Enterobacteriaceae are also considered as hygiene indicators and show highly variable responses to heat treatments (Petruzzelli et al., 2016). The data obtained in the present study regarding the absence of Enterobacteriaceae suggest that good manufacturing practices were correctly implemented during food production, and that a degutting step may have also been carried out.

Concerning the apparent absence of "major" foodborne pathogens such as *Salmonella* and *Listeria monocytogenes*, the results obtained in the present study are in accordance with those reported by the Opinion of the French Agency for Food, Environmental and Occupational Health and Safety on "the Use of Insects as Food and Feed and the Review of Scientific Knowledge on the Health Risks Related to the Consumption of Insects" (ANSES Opinion, 2014), which reported a lack of positive results for these pathogens in different categories of processed insects.

To the best of authors' knowledge, this study represents the second application of real-time PCR on edible insects. Due to its high sensitivity, Real-time PCR represents a good alternative to conventional PCR and has a wide application for diagnostic and food testing. Such a technology can provide either qualitative (presence or absence of the target DNA) or quantitative outcomes (Navarro et al., 2015). In the present study, real-time PCR was used for the detection of *C. burnetii*, Shiga toxin-producing *E. coli* (STEC), and *P. aeruginosa*.

Although none of the monitored pathogens were found in the analyzed samples, it is known that *C. burnetii* has previously been detected in beetles, ticks and fleas (Psaroulaki et al., 2014; Rehácek, 1979). In humans, *C. burnetii* is the causative agent of Q fever, which can produce flu-like disease, endocarditis, chronic fatigue syndrome and repeated abortions (Arricau-Bouvery and Rodolakis, 2005; OIE, 2015; Van den Brom et al., 2015).

There is currently a lack of scientific knowledge on the occurrence of STEC in insects. As reported by Kaper et al. (2004), the STEC serotype O157:H7 in humans is the causative agent of bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). Indeed, Directive 2003/99/EC of the European Parliament and of the Council (Directive 2003/99/EC) included STEC infection in the list of communicable diseases to be monitored for and controlled in the EU.

Finally, *P. aeruginosa* is an opportunistic human pathogen that has already been found in different species of either pest or edible insects such as cockroaches, grasshoppers, lygaeid bugs, butterflies, and flies (Grabowski and Klein, 2017).

Recent studies on edible mealworms reported high counts of members of the genus *Pseudomonas* in fresh samples (Vandeweyer et al., 2017a; Wynants et al., 2017), thus highlighting the importance of good manufacturing practices (e.g. heat treatment) during insect processing to protect consumer health.

Table 4.1.3 summarizes the most relevant bacterial genera with a potential role in foodborne illnesses or food spoilage detected in the samples under study.

Table 4.1.3. *Most relevant bacterial genera with a potential role in foodborne illnesses or food spoilage detected in the samples under study.*

Detected bacterial genera	Potential role in foodstuffs	Reference
Bacillus	Pathogen or spoilage	André et al., 2017; Stiles et al., 2014
Clostridium	Pathogen or spoilage	André et al., 2017; Stiles et al., 2014
Delftia	Pathogen	Camargo et al., 2014
Geobacillus	Spoilage	André et al., 2017
Paenibacillus	Spoilage	Helmond et al., 2017
Pseudomonas	Pathogen or spoilage	Scales et al., 2014
Stenotrophomonas	Pathogen	Amoli et al., 2017
Vibrio	Pathogen	Austin, 2010

The bacterial genera are reported irrespective of their source and method of detection;
Regarding DGGE results, only the closest relatives with sequence identity ≥97% are reported;
Regarding metagenomic sequencing results, only the most relevant detected genera are reported.

4.1.4 Conclusions

The demand for edible insects in European countries is on the rise and the safety of consumption of this novel food must always be guaranteed. Regarding the studied samples, of note were the massive abundance of spore-forming bacteria mainly represented by the genera *Bacillus* and *Clostridium* in winged termites, mole crickets, silkworm pupae and in rhino beetles, the presence of members of the genus *Pseudomonas* in black ants and in rhino beetles and the presence of *Vibrio* spp. in giant water bugs. The absence of *Coxiella burnetii*, Shiga toxin-producing *E. coli* (STEC), and *Pseudomonas aeruginosa* in all samples was noteworthy. The results of this study confirm the importance of combining different molecular techniques to characterize the biodiversity of complex ecosystems such as edible insects. The presence of DNA ascribed to bacteria with potential pathogenic activity in humans suggests the need for careful application of good manufacturing practices during insect processing. To the best of the authors' knowledge, this is the first study on the microbiota of giant water bugs, black ants, winged termite alates, rhino beetles, mole crickets, and silkworm pupae sold in the market, and therefore this study provides a first database for understanding the eventual microbiological risks associated with the consumption of such insects. Future studies involving insect purchased from different producers are also needed to: i) evaluate the differences induced by rearing conditions and processing; ii) ensure that insect-based foods comply with the relevant microbiological criteria set out by Regulation (EC) 2073/, 2005 on microbiological criteria for foodstuffs (as amended by Regulation (EC) 1441/, 2007).

4.2 *Hermetia illucens* in diets for zebrafish (*Danio rerio*): A study on the bacterial diversity through PCR-DGGE and metagenomic sequencing (Paper IX)

In the next decades, several negative effects related to human population growth and consequent intensive animal farming will occur on the environment (van Huis et al., 2013). Therefore, an eco-sustainable protein source is needed.

In this scenario, an alternative protein source could be represented by edible insects. Indeed, further than their well-known high nutritional properties, insect farming is characterized by several benefits on the environment than traditional protein sources: i) lower green house gases emission; ii) lower water consumption; iii) no required land clearing; iv) high efficiency in converting substrate in protein biomass; v) within the legislations, they are able to grow on substrates deriving from food industry by-products, thus adding value to these organic matrices (Smil, 2002; Collavo et al., 2005; Oonincx et al., 2010; Oonincx & de Boer, 2012; Van Huis et al., 2013; Veldkamp et al., 2013).

In 2015 the European Food Safety Authority (EFSA) delivered a Scientific Opinion on the risk profile associated with the use of insects as food for humans and animals (EFSA, 2015). Moreover, the European Commission, adopting Regulation No. 2017/893 of 24 May 2017, enabled the use of insects as processed animal proteins (PAPs) in aquaculture. Moreover, it was demonstrated that *H. illucens*, even grown on organic substrate, could partially or fully substitute fishmeal in fish diets (St-Hilaire et al., 2007a; Lock et al., 2014). Indeed, *H.illucens* ability in converting organic substrates in protein biomass is well-known (Diener et al., 2011).

In order to assess the exploitability of *H. illucens* in aquaculture under a feed safety point of view, the bacterial population composition during a 6-months zebrafish (*Danio rerio*) feeding trial was studied. To this end, zebrafish was fed with *H. illucens* grown on coffee silverskin – coffee roasting by-product characterized by high nutritional qualities (Borrelli et al., 2004; Costa et al., 2017; Mesias et al., 2014) – and vegetable substrate used as control. In particular, a polyphasic approach based on viable counting, PCR-DGGE and metagenomic 16S rRNA gene amplicon target sequencing (Illumina sequencing) was applied.

4.2.1 Materials and methods

Insects rearing

Two different *H. illucens* groups (in total 8000 larvae) obtained from a local company (Smart Bugs, Ponzano Veneto, Italy) were tested as zebrafish feed: the first group (HC) was reared on coffee by-products (C) (coffe silverskin obtained from roasting process at Saccaria Caffè SRL, Marina di Montemarciano, Italy) according to Vargas et al. (2018); the second one (HS), was reared on a vegetable substrate (S) composed of corn meal and fruit, and vegetable mixture (50:50). The experiments were performed in triplicate for each rearing condition. Both larval groups were collected at the prepupal stage, freeze dried, and processed to obtain a full-fat prepupae meal (Vargas et al., 2018). Before the feeding trial HC, HS, C, S and frass samples (excrement from larvae mixed with substrate residues) (HCF from HC or HSF from HS) were analyzed as described below.

Zebrafish rearing

Danio rerio feed with either 100% HC or 100% HS were produced as already reported by Zarantoniello et al. (2019). *D. rerio* were spawned and maintained under the rearing conditions already reported by (Zarantoniello et al., 2019). Embryos were then collected, counted under a stereomicroscope (Leica Wild M3B, Leica Microsystems, Nussloch, Germany) and randomly divided in two experimental groups (in triplicate) according to the two test diets (Zarantoniello et al., 2019). Fish (1500 larvae per dietary treatment; 3 x 20L tanks containing 500 larvae each) were daily fed (2 % body weight) the two different insect meals (HC; HS) for 6 months according to Vargas et al. (2018) and Zarantoniello et al. (2019). At the end of the experimental period fish were euthanized with an excess of anesthetic (Zarantoniello et al., 2018), and the microbiota of ZHC (gut content of zebrafish fed HC) and ZHS (gut content of zebrafish fed HS) were analyzed.

Microbiological analyses

Microbiological enumeration was carried out on samples C, HC, HCF, S, HS and HSF. Ten grams of each sample were aseptically diluted in 90 mL of sterile peptone water (bacteriological peptone 1 g/L) and homogenized in a Stomacher 400 Circulator apparatus (VWR International PBI, Milan, Italy). Homogenate ten-fold dilutions were inoculated on specific solid media for the enumeration of the following microorganisms: total mesophilic aerobes counted in accordance with UNI EN ISO 4833:2004 standard method; Enterobacteriaceae counted in accordance with ISO 21528-2:2004 standard method; for the enumeration of bacterial spores, the homogenized samples were subjected to thermal treatment at 80 °C for 15 min. followed cooling in iced water, to inactivate the vegetative cells (Osimani et al. 2017c) and then grown in Standard Plate Count Agar (Oxoid) incubated at 30 °C for 48 h. Presumptive lactic acid bacteria counts were carried out on MRS agar (Oxoid) added with cycloheximide (250 mg/L), incubated at 37°C for 72 h under anaerobiosis.

The presence of *Listeria monocytogenes* and *Salmonella* spp. was assessed in accordance with AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively.

Analyses were carried out in duplicate. The results were subjected to ANOVA using the software JMP v.11.0.0 (SAS Institute Inc., Cary, NC) and expressed as mean of log colony forming units (cfu) per gram ± standard deviation of triplicate biological independent experiments.

Molecular analyses

In order to have an insight into the microbial species occurring in the analyzed samples, C, HC, HCF, ZHC, S, HS, HSF and ZHS were subjected to culture-independent analyses via PCR-DGGE and Illumina sequencing. To this aim, DNA extraction from samples was carried out as already described by Garofalo et al. (2008). DNA extracts were standardized to 25 ng/μL and equal portions derived from the biological replicates of each sample were pooled together and vortexed vigorously.

PCR-DGGE analysis was performed as reported by Garofalo et al. (2008) through the PCR amplification of the V3 region of the 16S rRNA gene using universal prokaryotic primers 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') added with GC clamp and 518r (5'-ATT ACC GCG GCT GCT GG-3') (Ampe et al., 1999). After the DGGE, bands were excised, re-amplified and amplicons were sent for sequencing to Genewiz (Leipzig, Germany). The output sequence in FASTA format were compared with those deposited in the GenBank DNA database using the basic BLAST search tools (Altschul et al., 1990).

Samples were further subjected to Illumina analysis. In more detail, a 464-nucleotide sequence of the bacterial V3-V4 region (Baker et al., 2003; Claesson et al., 2010) of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified and processed as already described by Osimani et al. (2018a).

Unique barcodes were attached before the forward primers; the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter). The DNA concentration of amplicons was determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen). The generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2) (Caporaso et al., 2010). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price et al, 2009). Alpha and beta diversity metrics were calculated using the core-diversity plugin within QIIME2 and emperor (Vazquez-Baeza et al., 2013). Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 97% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables.

The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA546499.

4.2.2 Results and discussion

As reported in Table 4.2.1, except for HCF, the samples C, and HC were characterized by high counts for all the microbial groups assessed.

Table 4.2.1. *Viable counts carried on the analyzed samples.*

Samples	Total mesophilic aerobes	Bacterial spores	Lactic acid bacteria	Enterobacteriaceae
	log cfu g^{-1}			
Rearing chain 1				
C	8.13±0.03 ^{a}	7.45±0.02 ^{b}	7.42±0.01 ^{a}	5.35±0.06 ^{a}
HC	6.08±0.00 ^{c}	5.92±0.01 ^{c}	3.89±0.10 ^{c}	3.30±0.02 ^{b}
HCF	7.77±0.03 ^{b}	7.71±0.04 ^{a}	6.29±0.08 ^{b}	<1 ^{c}
Rearing chain 2				
S	5.83±0.04 ^{c}	2.53±0.04 ^{c}	3.93±0.04 ^{c}	4.18±0.08 ^{c}
HS	7.13±0.03 ^{b}	3.36±0.08 ^{b}	7.00±0.00 ^{b}	6.90±0.01 ^{b}
HSF	10.50±0.06 ^{a}	4.14±0.01 ^{a}	7.46±0.01 ^{a}	7.48±0.01 ^{a}

Results are expressed as log colony forming units g^{-1}.
For microbial counts, means ± standard deviations of triplicate independent experiments are shown;
For each rearing chain, within each column, means with different superscript letters are significantly different ($P < 0.05$);
C, coffee by-products; HC, H. illucens reared on coffee by-product; HCF, frass of H. illucens reared on coffee by-product; S, commercial substrate, HS, H. illucens reared on commercial substrate; HSF, frass of H. illucens reared on commercial substrate.

Of note, Enterobacteriaceae counts in samples HC and HCF were lower than in samples C, thus reflecting the already known inhibitory effect of *H. illucens* larvae on Enterobacteriaceae (Lalander et al., 2013). Surprisingly, the inhibitory effect was not observed in HS and HSF showing Enterobacteriaceae loads higher than in S samples.

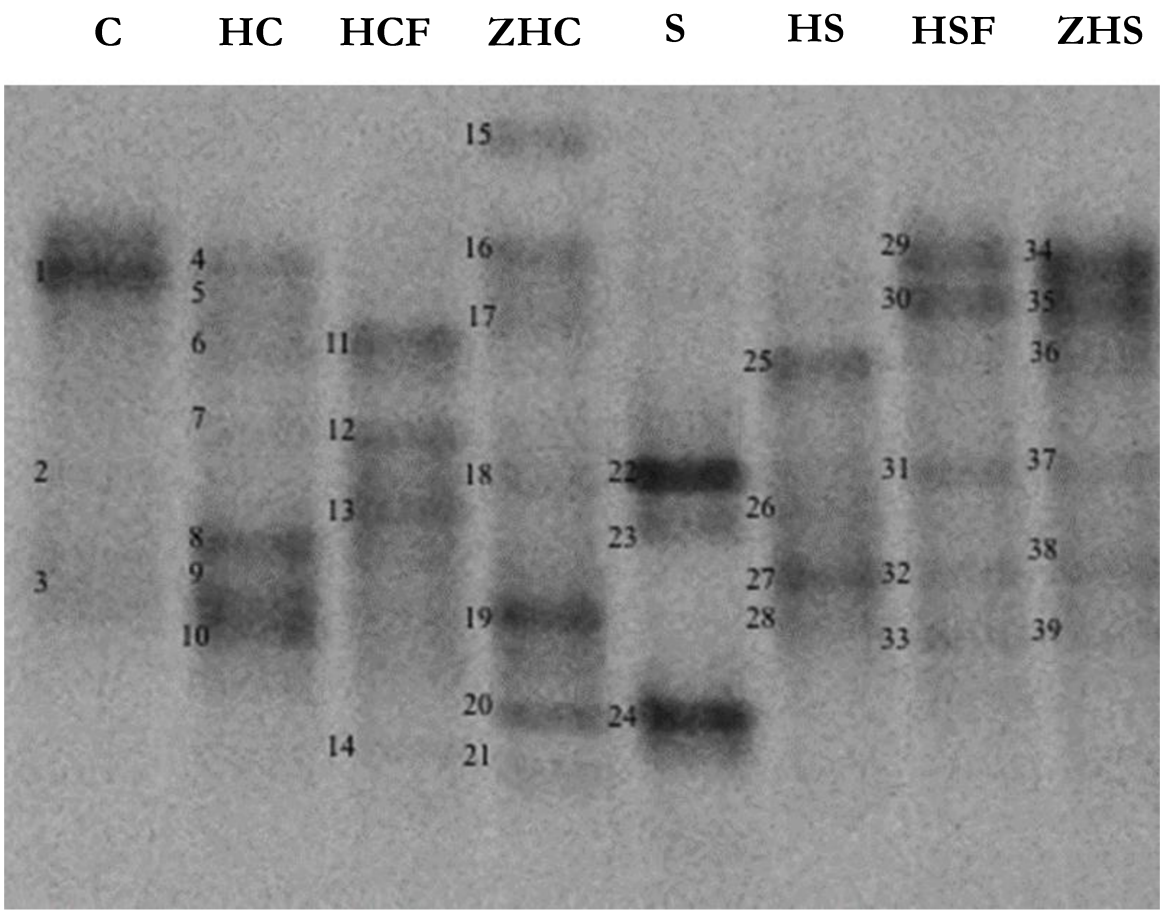
The data on mesophilic aerobes, bacterial spores, and LAB overall collected were in accordance with those reported by Wynants et al. (2019) that observed a wide variability based on the substrate, the rearing methods and the timing of larvae harvest. In frass samples, viable counts were generally higher likely due to the accumulation effect during the rearing period (Osimani et al., 2018a).

Finally, no *Listeria monocytogenes* or *Salmonella* spp. were detected in any of the analyzed sample.

The data on the microbiota overall collected highlighted very complex populations in almost all the samples where, thanks to the combination of PCR-DGGE and Illumina sequencing, major and minor taxa were detected.

Regarding PCR-DGGE analysis, Figure 4.2.1 shows the DGGE profiles, whereas the closest relatives, the percent identities, and the accession numbers of the sequences obtained are shown in Table 4.2.2.

Figure 4.2.1*. PCR-DGGE profile of bacterial DNA extracted directly from the analyzed samples.*



C, coffee by-products; HC, H. illucens reared on coffee by-product; HCF, frass of H. illucens reared on coffee by-product; S, commercial substrate, HS, H. illucens reared on commercial substrate; HSF, frass of H. illucens reared on commercial substrate; ZHC, gut content of zebrafish fed HC; ZHS, gut content of zebrafish fed HS.

Table 4.2.2. *Sequencing results of the bands excised from the DGGE gel obtained from the amplified fragments of bacterial DNA extracted directly from the analyzed samples.*

Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
C	1	*Desulfomonile limimaris*	99	NR_025079
	2	*Agromyces cerinus*	97	NR_036873
	3	*Aliihoeflea* sp.	97	JN210898
HC	4	*Streptomyces* sp.	97	MK702081
	5	*Lysobacter rhizophilus*	97	NR_152715
	6	*Streptomyces* sp.	95	MK702081
	7	*Corynebacterium jeikeium*	97	NR_118863
	8	*Kytococcus sedentarius*	97	NR_074714
	9	*Roseomonas oryzae*	98	NR_137403
	10	*Deferribacter desulfuricans*	97	NR_075025
HCF	11	*Hymenobacter coalescens*	97	NR_151971
	12	*Massilia alkalitolerans*	97	NR_043094
	13	*Blastochloris viridis*	98	NR_041712
	14	*Blastochloris viridis*	97	NR_041712
ZHC	15	*Mobilicoccus pelagius*	98	NR_113143
	16	*Pseudonocardia xishanensis*	98	NR_108411
	17	*Saccharopolyspora* sp.	96	MH777902
	18	*Corynebacterium* sp.	97	MH400695
	19	*Phycicoccus endophyticus*	97	NR_148775
	20	*Saccharomonospora* sp.	97	KP639601
	21	*Corynebacterium halotolerans*	97	NR_102500
S	22	*Phycicoccus endophyticus*	97	NR_148775
	23	Failed	-	-
	24	*Saccharomonospora* sp	96	KP639601
HS	25	*Lysobacter* sp.	98	MG237861
	26	*Rhodocyclus purpureus*	97	NR_044679
	27	*Aliihoeflea* sp.	97	JN210898
	28	*Lysobacter* sp.	97	MG237861
HSF	29	*Pseudomonas* sp.	98	KX079778
	30	*Bacillus* sp.	97	KX681796
	31	*Phycicoccus endophyticus*	97	NR_148775
	32	*Janibacter* sp.	97	DQ268766
	33	*Microbacterium* sp.	98	JN935776
ZHS	34	*Shewanella* sp.	97	MF155923
	35	*Bacillus* sp.	98	KX785165
	36	*Lysobacter* sp.	97	MG237861
	37	*Phycicoccus endophyticus*	97	NR_148775
	38	*Shewanella* sp.	97	MF155923
	39	*Microbacterium sp.*	98	JN935776

[a] *Bands are numbered as indicated in Figure 4.2.1;*

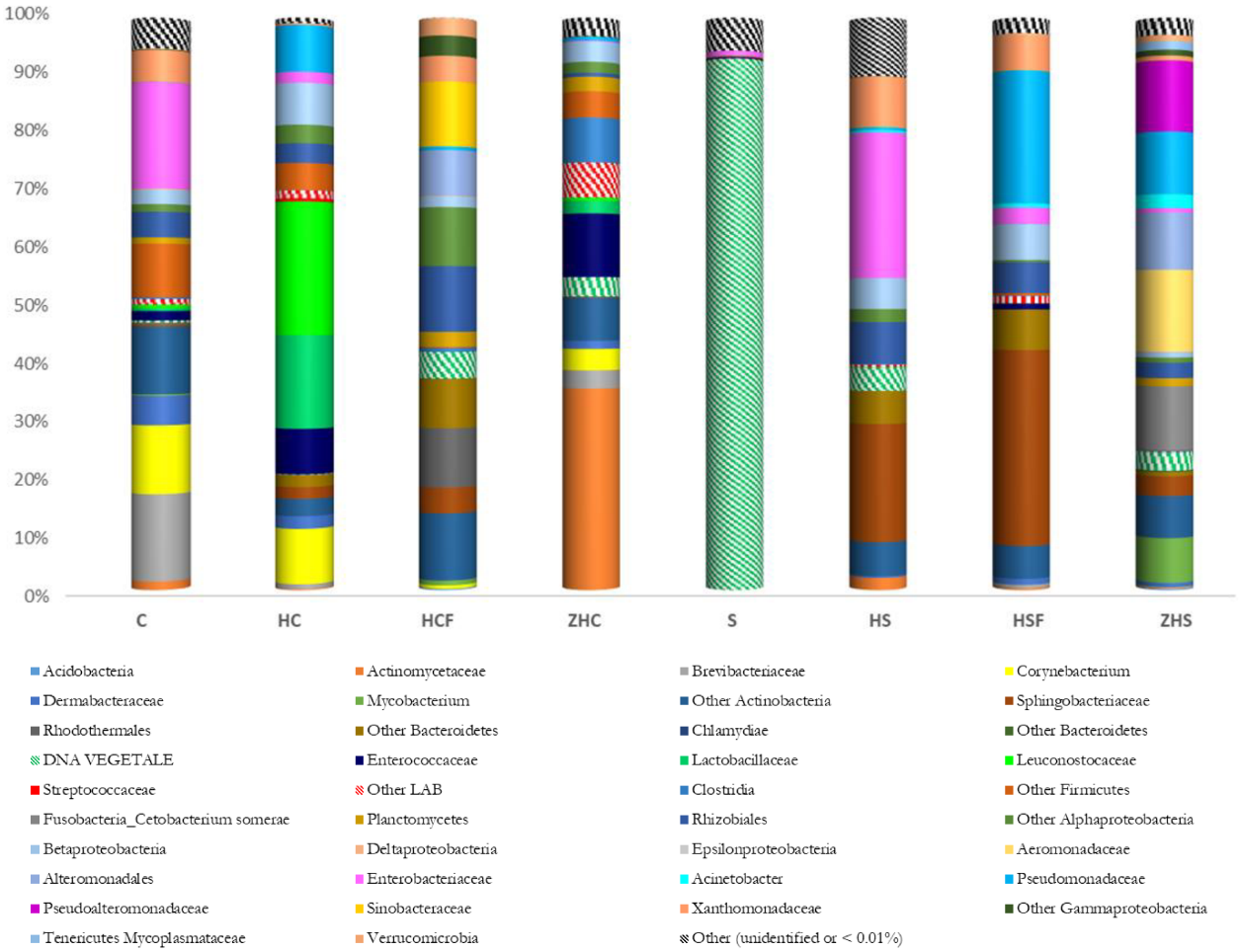
[b] *Percentage of identical nucleotides in the sequence obtained from the isolates and the sequence of the closest relative found in the GenBank database;*

[c] *Accession number of the sequence of the closest relative found by BLAST search;*

C, coffee by-products; HC, H. illucens reared on coffee by-product; HCF, frass of H. illucens reared on coffee by-product; S, commercial substrate, HS, H. illucens reared on commercial substrate; HSF, frass of H. illucens reared on commercial substrate; ZHC, gut content of zebrafish fed HC; ZHS, gut content of zebrafish fed HS.

Moreover, the results of Illumina sequencing are illsutrated in Figure 4.2.2, whereas Table 4.2.3 reports number of sequences analyzed (N reads), diversity richness (Chao 1), observed OTUs (OTUs), estimated sample coverage for 16S (Coverage) and diversity index (Shannon) for samples from the two rearing cycles.

Figure 4.2.2. *Results of Illumina sequencing of DNA extracted directly from the analyzed samples.*



C, coffee by-products; HC, H. illucens reared on coffee by-product; HCF, frass of H. illucens reared on coffee by-product; S, commercial substrate, HS, H. illucens reared on commercial substrate; HSF, frass of H. illucens reared on commercial substrate; ZHC, gut content of zebrafish fed HC; ZHS, gut content of zebrafish fed HS.

Table 4.2.3. *Number of sequences analyzed (N reads), diversity richness (Chao 1), observed OTUs (OTUs), estimated sample coverage for 16S (Coverage) and diversity index (Shannon) for samples from the two rearing cycles.*

Sample	N reads	Chao 1	OTUs	Coverage	Shannon
C	60,235	447	417	99.95	8,059
HC	65,288	1,164	931	99.77	8.774
HCF	60,530	614	565	99,92	8.361
ZHC	44,728	347	346	99,97	7.587
S	50,489	460	442	99,95	7.090
HS	49,096	1,113	914	99,65	8.857
HSF	63,101	1,430	1,151	99,79	9.079
ZHS	60,348	856	761	99,82	8.322

C, coffee by-products; HC, H. illucens reared on coffee by-product; HCF, frass of H. illucens reared on coffee by-product; S, commercial substrate, HS, H. illucens reared on commercial substrate; HSF, frass of H. illucens reared on commercial substrate; ZHC, gut content of zebrafish fed HC; ZHS, gut content of zebrafish fed HS.

In more detail, the DNA extracted from the samples successfully amplified the bacterial V3-V4 16S rRNA gene region. After splitting and quality trimming the raw data, 453,815 reads remained for subsequent analysis. After alignment, the remaining Operational Taxonomic Units (OTUs) were clustered at a 3% distance. To analyze the bacterial community richness in samples, the number of

OTUs, the Coverage estimator, the diversity Shannon index and the Chao1 richness estimator were determined using QIIME at 97% similarity levels (Table 4.2.3).

The Good's estimator of coverage was always over 99% for all the samples, which indicated that most of the bacterial phylotypes were detected. Based on the Shannon and Chao indices, pooled samples HC, HS and HSF showed the highest richness and evenness of the bacterial communities while pooled samples C, ZHC and S the lowest.

It is noteworthy that, only for pooled samples S, vegetable DNA and a few saprophytic microbial species were evidenced; these data are in accordance with those recently reported by Osimani et al. (2018a) for wheatmeal used as insect feed. Pooled samples C revealed the dominance of Enterobacteriaceae, Brevibacteriaceae, *Corynebacterium*, Xanthomonadaceae and *Rhizobiales* (among this latter family, *Aliihoeflea* sp. was also detected via PCR-DGGE). The latter two taxa showed an increased percentage of relative abundance in pooled samples HCF. Pooled samples HC were characterized by the dominance of Leuconostoccaceae, Lactobacillaceae and Enterococcaceae that were minority in C, thus suggesting a positive selective pressure for these taxa in the insect gut. As reported by Costa et al. (2018), chlorogenic acid and caffeic acids represent important compounds in coffee by-products (silverskin) with potentially inhibitory effect on bacterial growth. Interestingly, different authors (Filannino et al., 2015; Fritsch et al., 2016; Parkar et al., 2008) reported that such compounds may also stimulate the growth of lactic acid bacteria (e.g. *Lactobacillus collinoides*, *Lactobacillus brevis*, etc), thus likely explaining the massive presence of Lactobacillaceae or Leuconostoccaceae in samples HC. It is noteworthy that lactic acid bacteria have widely been detected in different edible insect species with potential symbiotic or probiotic activities (Osimani et al., 2018c).

Among Actinobacteria detected in HC, PCR-DGGE allowed the presence of *Streptomyces*, *Corynebacterium*, and *Kytococcus* to be discovered. It is noteworthy that among *Streptomyces*, the species *Streptomyces griseus* and *Streptomyces coelicoloris* are chitinolytic bacteria that can improve chitin digestibility in insect-based feeds (Berini et al., 2018; Kroeckel et al., 2012). *Corynebacterium* has very recently been found in the gut of *H. illucens*, with functions in hydrogen metabolism, nitrogen cycle, and sulphur compound metabolism (Jiang et al., 2019). *Kytococcus* comprises Gram positive species of environmental origin (Kämpfer et al., 2009); although found as minority species, to the authors' knowledge this is the first report of *Kytococcus* in the microbiota of edible insects.

As already described for viable counts, a reduction of Enterobacteriaceae relative abundances in both HC and HCF was observed. It is likely that the presence of bioactive compounds from coffee by-product, as chlorogenic or caffeic acids, could have modulated the growth of such a bacterial family. Indeed, Zhang et al. (2018) recently reported a decrease in the population of *Escherichia coli* in the colon of pigs fed diets supplemented with chlorogenic acid. Moreover, De Smet et al. (2018) reported that the effectiveness of pathogen reduction by *H. illucens* can be affected by the composition of the substrate, thus supporting the results obtained in the present study.

HCF pooled samples were also characterized by the presence of a rich microbiota mainly composed by Alteromonadales, Rhizobiales, Bacteriodetes, Rhodothermales, and Sphingobacteriaceae. To the authors' knowledge the microbiota of *H. illucens* frass is actually poorly investigate, hence, no further comparison is possible. It is noteworthy that, as reported by Mitchell and Hanks (2009), frass can represent a vehicle for transmission of bacterial plant-pathogens as well as a rich culturing media for saprophitic microorganisms. Moreover, the frass microbiota can synthesize chemical compounds that can be perceived by other insects, thus affecting the oviposition of some species (Anbutsu and Togashi, 2002). Finally, insect frass used as fertilizer can also represent a source of safety risks for the

consumers due to the possible presence of human pathogens (Yang et al., 2014), hence, a careful microbiological assessment of such a by-product must always be included in risk assessment.

In HS pooled samples the dominance of Enterobacteriaceae, Sphingobacteriaceae, Rhizobiales (among which closest relatives to Aliihoeflea) and Xanthomonadaceae (among which closest relatives to Lysobacter) was highlighted.

Sphingobacteriaceae have already been found in the microbiota of crambid butterflies, whereas the occurrence of species belonging to Xanthomonadaceae has already been reported in butterflies, beetles and crickets, thus confirming the association of these two bacterial families with the microbiota of insects (Grabowski and Klein, 2017; Milanović et al., 2019). Finally, Rhizobiales have recently been detected in the microbiota of fleas by Nziza et al. (2019). Members of Rhizobiales (e.g. *Bartonella*) can represent facultative intracellular parasites able to cause human or wild and domestic animals' infections (Breitschwerdt et al., 2010).

In HS pooled samples, a low occurrence of Pseudomonadaceae was also observed, such a bacterial family was in turn massively present in HSF pooled samples, together with Sphingobacteriaceae and Enterobacteriaceae.

The microbiota of insects can strongly be influenced by feeding substrates and vertical transmission from mother to offspring (Osimani et al., 2018a). In the present study, coffee by-products could have exerted a selective pressure on the microbiota of HC due to the presence of bioactive compounds as chlorogenic acid and caffeine (Fernandez-Gomez et al., 2016), thus possibly explaining the differences in microbial composition between HC and HS.

The results obtained in the present study are in accordance with those very recently reported by Bruno et al. (2019) which demonstrated that feed substrate can affect *H. illucens* midgut microbiota composition. Zebrafish gut samples (ZHC or ZHS), originated from the two feeding trials, were characterized by specific microbial patterns in which Actinobacteria and Alteromonadales were always detected, irrespective of the diet used.

Among Actinobacteria, PCR-DGGE highlighted the presence of closest relatives to *Mobilicoccus*, *Pseudonocardia*, *Saccharopolyspora*, *Corynebacterium*, and *Phycicoccus* to be discovered in ZHC pooled samples. Moreover, among Alteromonadales, closest relatives to *Shewanella* were detected by PCR-DGGE in ZHS pooled samples, together with Actinobacteria genera (*Phycicoccus* and *Microbacterium*).

Actinobacteria have already been detected in the gut microbiota of zebrafish, thus constituting one of the major microbial taxa involved in the function of the intestinal barrier of the fish and playing an essential role in the synthesis of antibiotics against fish pathogens (Qiao et al., 2019). Hence, the detection of Actinobacteria in both ZHC and ZHS pooled samples witnesses a good health status of their gut systems.

High occurrence of Alteromonadales has been associated with good fish health in recirculating aquaculture systems (Xue et al., 2017). Moreover, the presence of *Shewanella* detected in the present study is in accordance with Rimoldi et al. (2019) that described such a bacterial genus as part of the core microbiota detected in the gut of rainbow trout (Oncorhynchus mykiss) fed *H. illucens*-based diet.

Interestingly, the presence of Enterobacteriaceae in fish guts was higher in ZHS samples respect to ZHC in terms of lower abundance, thus suggesting a different contribution of the two feeding substrates (HC or HS) in the modulation of Enterobacteriaceae in the fish gut. Moreover, fish fed with HC were characterized by the massive presence of Actinomycetaceae which, as recently reported

by Nurul et al. (2019), can establish a symbiotic association in fish with benefits between the bacteria and the host.

Finally, a higher relative abundance of *Clostridia* was detected in ZHC respect to ZHS. This finding could likely be explained by the stimulating activity of chlorogenic acid and caffeine, contained in zebrafish diet (HC), on such a bacterial taxon (Mills et al., 2015).

4.2.3 Conclusions

Overall, collected data suggested that the microbiota of *H. illucens* could have been influenced by the feeding substrates. Similarly, zebrafish gut microbiota could have been influenced by: i) the microbiota of *H. illucens*-based feed and ii) eventually present insect-deriving bioactive compounds contained in the experimental diets. Indeed, as recently reported by Bruni et al. (2018) the fish gut microbial community is plastic and can be influenced by insect-based feed. Moreover, the results of both DGGE and Illumina sequencing showed the presence of a core microbiota constantly associated with zebrafish gut, irrespective of the diet.

As highlighted by Huyben et al. (2019), a high bacterial diversity can lead to an improved health gut of fish. Indeed, a rich microbial community could increase host resistance towards pathogen invasion and intestinal infection. Hence, the low microbial richness showed by ZHC should be carefully considered when diets including HC are suggested.

Further research is needed to understand the interactions among *H. illucens* microbiota, *H. illucens* bioactive compounds (i.e., chitin, polyphenols, etc.) and the zebrafish gut microbiota.

Moreover, data collected on insect frass can be useful to widen the knowledge on such a poorly investigated insect rearing by-product for its further use.

4.3 Investigating about the dominant microbiot and the antibiotic resistance genes occurring in marketed ready-to-eat small crickets (*Acheta domesticus*) (Papers VII and VIII)

Edible insects introduction in the European food market has only recently been authorized with Regulation (EU) No. 2015/2283. In the same year, the European Food Safety Authority (EFSA) produced a scientific opinion on the risk profile associated with the use of insects as food and feed in which highlighted the lack of scientific studies on the potential chemical and microbiological hazards associated with edible insects consumption. The currently available information highlighted that, besides their high nutritional value, edible insects can also be a source of chemical and biological contaminants that must be carefully assessed and mitigated before consumption. Indeed, insects can be vectors of several microbial species harbored by both their gut and external cuticle (EFSA, 2015). Together with the risk related to the presence of living cells or spores of potential human pathogens, the bacterial microbiota associated with edible insects can also be a source of transferable antibiotic

resistance genes that can be introduced into the human body at the time of consumption. In addition, it is already well-known that antibiotic resistance (AR) is considered a major menace to human health and food safety. As reported by Bengtsson-Palme (2017), the food supply chain can join resistant bacteria or their genes with humans, and as such the occurrence of food-borne transferable resistances must be included in the risk analysis of food production.

Based on these considerations, the present investigation was aimed at a dual goal regarding ready-to-eat small crickets (*A. domesticus*) already marketed in the European Union (EU): i) getting an insight into the bacterial biotaof this insect; ii) evaluating the occurrence of 12 selected transferable genes conferring resistance to tetracyclines, macrolide-lincosamide-streptogramin B (MLSB), vancomycin, beta-lactams, and aminoglycosides. Concerning the latter point, the genes under study were selected among those having the highest risk levels, based on the likelihood of their introduction into human pathogens, and the relative consequences of such an event on human health (Martinez, Coque, & Baquero, 2015). In fact, these genes are widespread among both commensal bacteria (Aarts & Margolles, 2015) and human pathogens typically found in food-producing animals, aquaculture, fruits, vegetables (Rolain, 2013), and even edible insects (Milanovic´ et al., 2016; Osimani et al., 2017b, 2017c, 2018d; Vandeweyer et al., 2019).

In more detail, the present study was carried out by performing viable counting and PCR-DGGE for the bacterial biota composititon assessment and optimized polymerase chain reaction (PCR) and nested-PCR assays for the qualitative detection of the selected AR genes.

4.3.1 Materials and Methods

Purchase of edible insects

Thirty-two samples of processed (boiled and dried) ready-to-eat crickets produced by four producers each located in an European country, being Austria, Belgium, France, and the Netherlands, were purchased from internet providers and shipped to our laboratory at ambient temperature. EU countries for collection of the crickets were selected among those that are most active in insect rearing, counting the highest number of start-ups, and companies producing insects for human consumption. To date, producers located in Belgium and the Netherlands have already been involved in research studies aimed at assessing the microbiological traits and hazards of small crickets (Garofalo et al., 2017a; Milanović et al., 2016; Osimani et al., 2017a; Vandeweyee et al., 2017a; Vandeweyer et al., 2017b; Vandeweyer et al., 2018). To the authors' knowledge, no producers from France and Austria have ever been involved in similar investigations. From each producer, two different batches of the crickets were bought in October 2017 (Batch 1) and April 2018 (Batch 2), respectively, each including four samples (replicates). After arrival, the edible crickets were stored at room temperature in their original sealed plastic bags and analyzed within 2 days. The samples were labeled as shown in Table 4.3.3.

Water activity measurements

To assess the microbial stability of the samples, the water activity (a_w) was measured in accordance with the ISO 21807:2004 standard method using the Aqualab 4TE apparatus (Meter Group, Pullman, WA, USA). All measurements were carried out in duplicate.

Microbial enumeration

Ten grams (about 140 to 150 cricket units) of each sample was weighted aseptically in sterile bags, crushed with a mortar, added with 90 mL of sterile peptone water (bacteriological peptone, 1 g L^{-1}) and homogenized for 10 min at 260 rpm in a Stomacher 400 Circulator apparatus (VWR Intl., Milan, Italy). The resulting homogenates (dilution 10^{-1}) and further 10-fold serial dilutions of the samples were used for microbial enumeration.

The viable counts of total mesophilic aerobes were performed using the pour plate method in standard plate count agar (Oxoid, Basingstoke, UK), incubated for 48 h at 30 °C. Lactic acid bacteria were enumerated on MRS agar (Oxoid) supplemented with cycloheximide after incubation at 30 °C for 48 h under anaerobiosis (Osimani et al., 2009). Enterobacteriaceae were enumerated in accordance with the ISO 21528-2:2004 standard method. The enumeration of Pseudomonadaceae was performed as described by Garofalo et al. (2017b) using Pseudomonas Agar Base (PAB) medium incubated for 24 to 48 h under aerobic conditions. For the enumeration of spore-forming bacteria, the prepared homogenates of each sample were heat-shocked for 15 min at 80 °C to inactivate the vegetative cells, and then immediately cooled on ice, serially diluted in sterile peptone water (peptone, 1 g L^{-1}), spread onto standard plate count agar and incubated as described above (Milanović et al., 2017). The enumeration of *Listeria monocytogenes* was carried out in accordance with the UNI EN ISO 11290-2:2017 standard method. The results were compared with food safety limits established by Regulation (EC) No. 2073/2005 on the microbiological criteria for foodstuffs, as amended by Regulation (EC) No. 1441/2007. These regulations set a limit of 2 log cfu (colony forming units) of *L. monocytogenes* per gram in ready-to-eat foods unable to support the growth of the pathogen, other than those intended for infants or for special medical purposes. The above-mentioned limit is intended for products with a pH ≤ 4.4 or $a_w \leq 0.92$, or for products with a pH ≤ 5.0 and $a_w \leq 0.94$ placed on the market during their shelf-life. The results of viable counts were expressed as the means of log cfu per gram of sample ± standard deviations calculated from two technical replicates for each sample.

Bacterial DNA extraction

A total of 1.5 mL of each sample homogenate (10^{-1} dilution) was centrifuged for 5 min at 16,000 × g to obtain cell pellets used for the extraction of total microbial DNA with the PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). A Nanodrop ND 1000 (Thermo Fisher Scientific, Wilm- ington, DE, USA) was used to quantify the extracted DNA and determine purity. The extracted DNA was subsequently standardized to 25 ng/μL for further analysis. To check for the presence of bacterial DNA, 2 μL (approximately 50 ng) of the extracted DNA was amplified using a My Cycler Thermal Cycler (BioRad Laborato- ries, Hercules, CA, USA) with the universal prokaryotic primers, 27F and 1495R (Aquilanti et al., 2004; Weisburg et al., 1991). The resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels and photographed using the Complete Photo XT101 system (Explera, Jesi, Italy) after exposure to UV light.

PCR-DGGE analysis

Equal portions of DNA extracts (standardized to 25 ng μL^{-1}) obtained from the 4 cricket samples derived from the same producer and production batch were pooled together and vortexed vigorously. Eight final pooled DNA samples were obtained and labeled as follows: AU-B1 and AU-B2 (DNA pools of the samples from the two batches from Austria); BL-B1 and BL-B2 (DNA pools of the samples from the two batches from Belgium); FR-B1 and FR-B2 (DNA pools of the samples from

the two batches from France); NL-B1 and NL-B2 (DNA pools of the samples from the two batches from the Netherlands). PCR–DGGE analysis was performed as follows: one hundred nanograms of each DNA extract was amplified through PCR in a 50 µL reaction volume with the primer pair 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') and 518r (5-ATT ACC GCG GCT GCTGG-3') for the amplification of the V3 region of the 16S rRNA gene. AGC clamp was attached to the 338f primer as proposed by Ampe et al.(1999). The reaction mixtures and amplification conditions were already described by Osimani et al. (2015). Amplicons of duplicate samples for each sampled batch were pooled in equal proportions, following the procedure proposed by Ezeokoli et al. (2016). DGGE analysis was carried out as already described by Garofalo et al. (2015). Sequencing of the DGGE bands and sequence analysis were carried out as previously described by Osimani et al. (2015). The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).

PCR and nested-PCR amplification of AR determinants

The bacterial DNA extracted directly from 32 cricket samples was subjected to a PCR screening for the presence of 12 AR genes conferring resistance to tetracyclines [*tet(M)*, *tet(O)*, *tet(K)*, *tet(S)*], MLSB [*erm(A)*, *erm(B)*, *erm(C)*], vancomycin (*vanA*, *vanB*), beta-lactams (*mecA*, *blaZ*), and aminoglycosides [*aac(6')-Ie aph(2")- Ia*, hereafter referred to as *aac-aph*].

To enhance the sensitivity, a nested-PCR method was used to screen the samples that were negative after the first round of PCR. The reference strains used as positive controls for each gene were as follows: *Staphylococcus aureus* M.P., *Enterococcus hirae* Api 2.16, and *Staphylococcus* spp. SE12 for the *erm(A)*, *erm(B)*, and *erm(C)* genes, respectively; *Enterococcus faecium* PF3U and *Enterococcus faecalis* ATCC 51299 for the *vanA* and *vanB* genes, respectively; *Lactobacillus casei/paracasei* ILC2279, *Streptococcus pyogenes* 7008, *Enterococcus italicus* 1102, and *S. aureus* COL. for the *tet(M)*, *tet(O)*, *tet(S)*, and *tet(K)* genes, respectively; *S. aureus* 27R for the gene *mecA*; *S. aureus* ATCC 2921 for the *blaZ* gene, and *E. faecium* M48 for the *aac-aph* gene. The reference strains were obtained from American Type Culture Collection (*vanA* and *blaZ* genes), the Dept. of Agricultural, Food and Environmental Sciences (D3A) collection [*erm(C)*, *vanA*, *tet(M)*, *tet(S)*, and *mecA* genes] and the Dept. of Life and Environmental Sciences (DiSVA) collection [*erm(A)*, *erm(B)*, *tet(O)*, *tet(K)*, and *aac-aph* genes], both from the Univ. Politecnica delle Marche, Italy. *E. faecalis* JH2-2 (Jacob & Hobbs, 1974) was used as a negative control. PCR and nested-PCR assays details are reported in Table 4.3.1 and 4.3.2 respectively.

The amplification products were analyzed by electrophoresis as described above and the expected sizes of the amplicons were checked by comparing them with a 100 bp DNA molecular weight marker (HyperLadderTM 100 bp, Bioline, UK). In addition, to check for the effective amplification of AR genes, arbitrarily selected positive samples for each gene were sent to Genewiz (Takeley, UK) for purification and sequencing. The obtained sequences were analyzed by BLAST (Basic Local Alignment Search Tool) available from NCBI (Natl. Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov). The results of the PCR/nested-PCR screening for the presence of target AR genes were expressed as + (positive) or – (negative) for each of 32 analyzed cricket samples.

Table 4.3.1. *Primers used in the PCR assays targeting the 12 resistance genes of interest, and the corresponding annealing temperature (Ta), product size, reaction mixture, PCR program and references.*

AR gene	Primer sequence (5'-3')	Ta (°C)	Product size (bp)	Reaction mixture	PCR program	Reference
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-CAATACGAGAGCCGGGTTTC 2-ACAACGGGCTGGAATTTCAC	60	382	□	○	Milanović et al. (2017)
tet(K)	1-TCGATAGGAACAGCAGTA 2-CAGCAGATCCTACTCCTT	55	169	□	○○○	Ng et al. (2001)
erm(A)	1-CAGGAAAAGGACATTTTACCAA 2-CTTCGATAGTTTATTAATATTAGT	50	572	□□	○○	Garofalo et al. (2007)
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	□	○○○	Predari et al. (1991) Murakami et al. (1991)
blaZ	1-ACTTCAACACCTGCTGCTTTC 2-TAGGTTCAGATTGGCCCTTAG	58	240	□	○○○	Martineau et al. (2000) Garofalo et al. (2007)
aac-aph	1-GAGCAATAAGGGCATACCAAAAATC 2-CCGTGCATTTGTCTTAAAAAACTGG	58	505	□	○○○	Kao et al. (2000)

□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 0.2 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
□□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 3.0 mM MgCl2, 0.25 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 1 min., Ta 1 min, 72°C, 2 min. Final extension 72°C, 7 min.;
○○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 1 min., Ta 1 min., 72°C, 1 min. Final extension 72°C, 7 min.;
○○○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 30 sec., Ta 30 sec., 72°C, 30 sec. Final extension 72°C, 7 min.

Table 4.3.2. *Primers used in the nested-PCR assays targeting the 12 resistance genes of interest, and the corresponding annealing temperature (Ta), product size, reaction mixture, PCR program and references.*

Target gene	Primer sequence (5'-3')	Ta (°C)	Product size (bp)	Reaction mixture	PCR program	Reference
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 4-GGAAATCTGCTGGCGTACTG	64	106	□	○	Milanović et al. (2017)
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)
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. (2001)
aac-aph	1-GAGCAATAAGGGCATACCAAAAATC 4-GCCACACTATCATAACCACT	62	220	□	○	Garofalo et al. (2007)

□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 2.5 mM MgCl2, 0.2 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
□□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 3.0 mM MgCl2, 0.25 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 30 sec., Ta 30 sec., 72°C, 30 sec. Final extension 72°C, 7 min.;
○○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 1 min., Ta 1 min., 72°C, 1 min. Final extension 72°C, 7 min.;
A blank control containing only molecular biology grade water added to the PCR mix (Sigma-Aldrich, St. Louis, MO, USA) was used in each PCR reaction to check for contaminants.

Statistical analysis

The results of the viable counts were expressed as the mean of the log cfu per gram of sample ± standard deviation. Due to the very low frequency of samples showing viable counts of lactic acid bacteria, Enterobacteriaceae and Pseudomonadaceae <1 log cfu g^{-1}, data for these microbiological parameters were not subjected to statistical analyses.

Viable counts of the total mesophilic aerobes and spore-forming bacteria were analyzed using a fixed model two-way balanced ANOVA, with Producers (P) and Batches (B) as the main factors and a Producer × Batches (P × B) interaction. The "among samples within (P × B) mean square" was the error term used to carry out F-tests for mean squares of main effects and interaction effects, whereas the "among viable counts replicates within samples" was the sampling error. Multiple comparisons among means were carried out using the Protected Least Significant Difference (LSD) test (Steele & Torrie, 1960). JMP11 Software was used for the statistical data analysis.

Relative percentages of positivity for the 12 antibiotic resistance determinants were calculated for each producer as the number of PCR-positive or nested-PCR-positive samples divided by the total number of samples assayed (=8).

A multivariate principal component analysis (PCA) was applied to investigate the variability in AR genes among producers and/or batches. For this purpose, AR genes that had never been detected by either PCR or nested-PCR were excluded from the PCA. The average frequency of positive samples was calculated for each batch of each producer.

The PCA was carried out using the correlation matrix among AR gene frequencies (NTSYS 2.02i software, Applied Biostatistics Inc., NY, USA). Eigenvalues and eigenvectors were obtained and the most important principal components (PCs) were identified based on the cumulative amount of total variance explained. The relative importance of each AR gene in each PC was determined based on the absolute value of eigenvector coefficients (Rencher, 1995).

4.3.2 Results and Discussion

In the present study, 32 samples of ready-to-eat crickets (*A. domesticus*) purchased from four EU producers, located in Austria, Belgium, France, and the Netherlands, and two production batches from each producer were subjected to viable counts of different microbial groups, PCR-DGGE and further screened for the occurrence of 12 selected genes coding for widely spread transferable ARs.

The a_w measurements showed values between 0.49 ± 0.01 (from samples produced in the Netherlands) and 0.52 ± 0.03 (from samples produced in Austria), thus assuring the microbiological stability of samples during their shelf-life. Indeed, at a_w values <0.90, the bacterial growth is generally inhibited, although at aw values <0.70 some yeasts and molds may still grow. At a_w values <0.60, a food can be classified as dehydrated, which is generally stable in terms of microbial growth (Labuza, 1980).

The a_w values measured in all the samples were useful to attest that the ready-to-eat crickets were also unable to support the growth of *L. monocytogenes*, for which viable counts were always <2 log cfu g^{-1}. Hence, all the analyzed samples were shown to comply with the food safety reference limits set by Regulation (EC) No. 2073/2005, as amended by Regulation (EC) No. 1441/2007.

The results of viable counts are reported in Table 4.3.3.

For all the samples analyzed, viable counts of Pseudomonadaceae <1 log cfu g^{-1} were observed; a similar trend was seen for Enterobacteriaceae, with a few exceptions concerning the samples collected from the second batch of crickets from Austria, France, and the Netherlands, where viable counts were slightly above 1 log cfu g^{-1}.

The viable counts of total mesophilic aerobes ranged between 1.99 ± 0.12 and 6.35 ± 0.00 log cfu g^{-1} in samples from France, and Austria, respectively, whereas those of spore-forming bacteria were between <1 and 5.63 ± 0.10 log cfu g^{-1} in samples from the same two countries, respectively.

Table 4.3.3. *Mean counts (± standard deviation) of total mesophilic aerobes, spore-forming bacteria, lactic acid bacteria, Enterobacteriaceae, and Pseudomonadaceae in samples of edible crickets produced in Austria (AU1-AU8), Belgium (BL1-BL8), France (FR1-FR8), and the Netherlands (NL1-NL8).*

Samples	Total mesophilic aerobes	Spore-forming bacteria	Lactic acid bacteria	Enterobacteriaceae	Pseudomonadaceae
	log cfu g^{-1}				
AU1-B1	5.01±0.02	2.66±0.26	<1	<1	<1
AU2-B1	4.99±0.08	2.50±0.28	<1	<1	<1
AU3-B1	4.93±0.01	2.24±0.34	<1	<1	<1
AU4-B1	5.14±0.01	4.60±0.12	<1	<1	<1
AU5-B2	4.29±0.02	2.38±0.11	<1	1.35±0.49	<1
AU6-B2	6.35±0.0	5.63±0.10	<1	0.5±0.71	<1
AU7-B2	4.33±0.0	2.31±0.05	<1	1.39±0.12	<1
AU8-B2	5.58±0.05	4.97±0.04	<1	<1	<1
BL1-B1	2.77±0.15	1.87±0.12	<1	<1	<1
BL2-B1	3.62±0.33	1.39±0.12	<1	<1	<1
BL3-B1	2.07±0.10	<1	<1	<1	<1
BL4-B1	3.93±0.04	2.18±0.10	<1	<1	<1
BL5-B2	3.90±0.08	3.25±0.00	<1	<1	<1
BL6-B2	3.69±0.02	2.20±0.23	0.5±0.71	<1	<1
BL7-B2	3.09±0.12	2.56±0.00	<1	<1	<1
BL8-B2	4.09±0.02	2.65±0.00	<1	<1	<1
FR1-B1	2.94±0.02	1.39±0.12	<1	<1	<1
FR2-B1	3.40±0.04	1.77±0.10	<1	<1	<1
FR3-B1	1.99±0.12	<1	<1	<1	<1
FR4-B1	2.85±0.01	1.15±0.21	<1	<1	<1
FR5-B2	3.85±0.01	2.15±0.21	3.24±0.11	<1	<1
FR6-B2	5.07±0.02	0.50±0.71	2.95±0.09	1.30±0.43	<1
FR7-B2	5.54±0.11	1.65±0.07	2.48±0.03	<1	<1
FR8-B2	5.82±0.01	<1	5.75±0.02	1.35±0.49	<1
NL1-B1	4.28±0.06	3.69±0.12	<1	<1	<1
NL2-B1	4.87±0.09	2.77±0.03	<1	<1	<1
NL3-B1	4.86±0.8	3.47±0.06	<1	<1	<1
NL4-B1	4.92±0.15	2.93±0.02	<1	<1	<1
NL5-B2	3.29±0.02	2.13±0.07	1.30±0.43	<1	<1
NL6-B2	2.67±0.03	1.81±0.05	<1	0.74±1.04	<1
NL7-B2	5.14±0.00	4.20±0.05	<1	<1	<1
NL8-B2	2.97±0.11	1.90±0.00	<1	1.50±0.28	<1

Counts are expressed as log colony forming units (cfu) g^{-1};

** For total mesophilic aerobes and spore-forming bacteria, within each producer, multiple comparisons among means were carried out through the Tukey's HSD test; within each producer means followed by different letters are significantly different ($P < 0.05$).*

Regarding spore-forming bacteria, many authors have previously reported the stable occurrence of these microorganisms in edible insects (Garofalo et al., 2017a; Klunder, Wolkers- Rooijackers et al., 2012; Osimani et al., 2017a, 2017b, 2017c; Stoops et al., 2016; Vandeweyer et al., 2017b, 2018; Wynants et al., 2018). Indeed, spore-forming bacteria are able to produce endospores that are characterized by heat resistance, allowing them to survive processing treatments. This peculiar feature constitutes a potential risk for the health of consumers when spores are produced by spore-forming pathogens (for example, *Bacillus cereus*) (Osimaniet al., 2018b). Although no safety limits for spore-forming bacteria in ready-to-eat foods have been set in the EU, a load of 2 log cfu g^{-1} of *B. cereus* viable cells is generally accepted as a safety threshold by the national guidelines of European Member States and the scientific literature (Osimani et al., 2018c). In addition, it has been acknowledged that *B. cereus* counts between 5 and 6 log cfu g^{-1} can lead to intoxication (FSAI, 2016). In the present study, the mean value of spore-forming bacteria (5.63 ± 0.10 log cfu g^{-1}) found in the processed boiled and dried

crickets suggests that more in-depth studies on the occurrence of pathogenic species in this food matrix should be carried out. At this regard, species of *Bacillus* have been shown to be among the most resistant to food drying, together with *Clostridium botulinum* and *Clostridium perfringens* (Fogele et al., 2018).

To the authors' knowledge, no previous data from the scientific literature are available on the viable counts of Pseudomonadaceae in either crickets or other edible insects, hence no further comparison with other edible insects is possible. The enumeration of Pseudomonadaceae could provide useful information on the quality and safety of foods, because this bacterial family encompasses well-known spoilage (for example, *Pseudomonas fluorescens* and *Pseudomonas fragi*) and pathogenic species (for example, *Pseudomonas aeruginosa*).

Regarding Enterobacteriaceae, the sporadic occurrence of viable counts above 1 log cfu g^{-1} was in accordance with values previously detected in ready-to-eat mealworms and grasshoppers commercialized in the EU (Osimani et al., 2017a, 2017b).

As far as lactic acid bacteria are concerned, a few samples showed viable counts between 0.50 and 5.75 log cfu g^{-1}, whereas most of the samples had viable counts that were <1 log cfu g^{-1}. The average load of this microbial group was similar to that reported by Osimani et al. (2017b, 2017c) for the same microorganisms in ready-to-eat mealworms and grasshoppers marketed in the EU.

Due to the very low frequency of samples with viable counts of lactic acid bacteria, Enterobacteriaceae and Pseudomonadaceae that were <1 log cfu g^{-1}, no statistical analyses were performed for these microbial groups. ANOVA results for the total mesophilic aerobes and spore-forming bacteria are shown in Table 4.3.4, and multiple comparisons among means are reported in Table 4.3.5.

Due to the significant interaction in the fixed model ANOVA, multiple comparisons among the means of each "Producer × Batch" combination were analyzed to reveal significant differences among the batches supplied by each producer. As a result, both of the batches of crickets from Austria showed the highest mean total mesophilic aerobes values, whereas the two batches from Belgium showed the lowest mean values. By contrast, samples from the Netherlands were characterized by higher and lower total mesophilic aerobes mean values in batches 1 and 2, respectively, whereas the opposite trend was observed for the samples from France.

Table 4.3.4. *ANOVA results for total mesophilic aerobes and spore-forming bacteria.*

	Total mesophilic aerobes				**Spore-forming bacteria**			
Source of variation	**df**	**MS**	**F**		**df**	**MS**	**F**	
Producer	3	7.863	7.26	**	3	16.672	8.01	***
Batch	1	3.128	2.89	ns	1	2.013	0.97	ns
Producer x Batch	3	8.297	7.66	***	3	3.154	1.52	ns
Experimental error	24	1.084	130.52	***	24	2.081	63.36	***
Sampling error	32	0.008			32	0.033		

***significant P < 0.001;*

****significant P < 0.0001.*

Table 4.3.5. *Results of multiple comparisons among the means of Producer × Batches interactions (a) and the means among Producers (b) for total mesophilic aerobes and spore-forming bacteria variables (Protected LSD test).*

a

Producer x Batch Interaction					
Total mesophilic aerobes			**Spore forming bacteria**		
	Mean*			Mean*	
AU-B2	5.13	a	AU-B2	3.82	a
FR-B2	5.07	a	NL-B1	3.21	a
AU-B1	5.02	a	AU-B1	3.00	a
NL-B1	4.73	ab	BL-B2	2.67	ab
BL-B2	3.69	bc	NL-B2	2.51	abc
NL-B2	3.52	c	BL-B1	1.36	bc
BL-B1	3.10	c	FR-B1	1.08	c
FR-B1	2.80	c	FR-B2	1.08	c

b

Producer					
Total mesophilic aerobes			**Spore forming bacteria**		
	Mean*			Mean*	
AU	5.08	a	AU	3.41	a
NL	4.12	ab	NL	2.86	ab
FR	3.93	b	BL	2.01	bc
BL	3.40	b	FR	1.08	c

**Within each column, means followed by different letters are significantly different (P < 0.05).*

Overall, the two batches from Austria and Belgium had relatively comparable loads of total mesophilic aerobes, with Austria showing a significantly higher load of this microbial group than Belgium (Table 4.3.5). By contrast, batches 1 and 2 from France and the Netherlands had significantly different counts of total mesophilic aerobes (Table 4.3.5).

The "Producer × Batch" mean square was not significant whereas a significant "among Producers" mean square was found (Table 4.3.4).

In more detail, the producer from Austria showed a significantly higher spore-forming bacteria mean than producers from Belgium and France, whereas the producer from the Netherlands had an intermediate mean number of spore-forming bacteria (Table 4.3.5). Even though the "Producer × Batch" interaction was not significant, multiple comparisons among the means revealed that the trend of spore-forming bacteria between batches 1 and 2 from Austria, Belgium, and the Netherlands was similar to the trend observed for total mesophilic aerobes.

By contrast, samples from France had a lower number of spore-forming bacteria in both batches 1 and 2; however, batch 2 showed a significantly higher number of spore-forming bacteria than batch 1. Therefore, comparing the results of the two variables analyzed (total mesophilic aerobes and spore-forming bacteria), while the results from the products from France differed from Austria, Belgium, and the Netherlands, a similar trend among total mesophilic aerobes and spore-forming bacteria counts was seen in these countries.

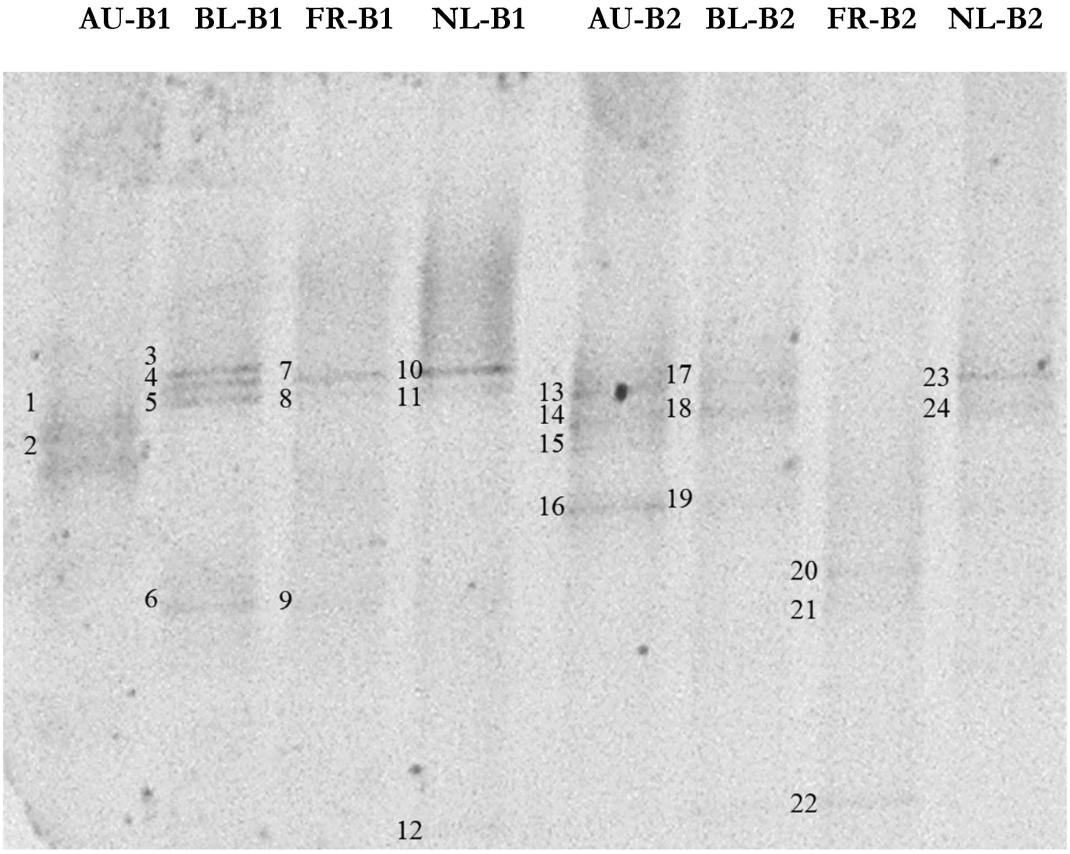
PCR–DGGE has been extensively applied in food and environmental microbiology representing a valid molecular tool for the exploration of the food microbial communities (Garofalo et al., 2017b). The results of PCR–DGGE analysis are shown in Figure 4.3.1 and Table 4.3.6.

Samples from Austria were found to be most closely related to *Hespellia* (in pooled samples from batch 1), *Ruminococcus* and *Clostridium* (both in pooled samples from batch 2), with a sequence identity

from 97 to 100%. By contrast, the samples from Belgium were found to be most closely related to *Lysobacter*, *Staphylococcus* (both in pooled samples from batch 1) and *Clostridium* (in pooled samples from batch 2), with sequence identities between 97 and 99%. Moreover, pooled samples from Batch 1 of the crickets produced in France were found to be most closely related to *Staphylococcus* and *Pseudomonas*, whereas pooled samples from Batch 2 were characterized by *Pseudomonas* and *Hydrogenophilus*, with sequence identities from 97 to 100%. Finally, regarding the pooled samples produced in the Netherlands, batch 1 was characterized by the presence of the *Staphylococcus* and *Hydrogenophilus*, whereas batch 2 by the presence of the *Clostridium* and *Ruminococcus*, all with a sequence identities between 97 and 100%.

Regarding the species detected in the pooled samples, some considerations can be made. *Clostridium* was detected in the Austrian, Belgian and the Dutch samples. This spore-forming bacterial genus has previously been reported in ready-to-eat small crickets and cricket powder analyzed by both PCR–DGGE and metagenomic sequencing, as well as in other edible insects such as giant water bugs, mealworms, desert locusts, and black soldier flies (Schlüter et al., 2017; Winants et al., 2018 Garofalo et al., 2019). In foodstuffs, pathogenic *Clostridium* species like *Clostridium botulinum*, *Clostridium difficile* and *Clostridium perfringens*, represent a health threat for consumers because they are able to resist heat treatments and produce toxins (Popof, 2017).

Figure 4.3.1. *DGGE profiles of the bacterial DNA obtained from insect samples and amplified with primer pair U968GCL1401R. The labeled DGGE bands were sequenced.*



AU, *Austria;* BL, *Belgium;* FR, *France;* NL, *the Netherlands;* B1, *Batch 1;* B2, *Batch 2.*

Table 4.3.6. *The results of thesequencing of the bands excised from the DGGE gel.*

Sample	Band	Closest relative	% Identity[a]	Acc. no[b]	Acc. no[c]
AU B1	1	*Hespellia porcina*	100	NR_025206	MH891537
	2	*Hespellia porcina*	96	NR_025206	MH891538
BL B1	3	*Staphylococcus capitis*	99	MH158289	MH891539
	4	*Staphylococcus* sp.	99	MF948900	MH891542
	5	*Staphylococcus capitis*	99	MH158289	MH891540
	6	*Lysobacter* sp.	98	MG198704	MH891541
FR B1	7	*Staphylococcus* sp.	99	MF948900	MH891542
	8	Failed	–	–	–
	9	*Pseudomonas* sp.	100	HM626451	MH891543
NL B1	10	*Staphylococcus* sp.	97	MH817399	MH891544
	11	Failed	–	–	–
	12	*Hydrogenophilus hirschii*	100	NR_104788	MH891545
AU B2	13	*Ruminococcus gauvreauii*	100	NR_044265	MH891546
	14	*Clostridium* sp.	97	AF443594	MH891547
	15	Failed	–	–	–
	16	*Clostridium* sp.	95	AF443594	MH891548
BL B2	17	*Clostridium* sp.	97	LC341577	n.d.
	18	*Clostridium* sp.	97	AF443594	MH891549
	19	Failed	–	–	–
FR B2	20	Failed	–	–	–
	21	*Pseudomonas* sp.	100	HM626451	MH891550
	22	*Hydrogenophilus hirschii*	99	MF595849	MH891551
NL B2	23	*Ruminococcus gauvreauii*	98	NR_044265	MH891552
	24	*Clostridium* sp.	98	AF443594	MH891553

n.d., sequences not deposited in the GenBank database (\200 bp); AU, Austria; BL, Belgium; FR, France, NL, the Netherlands; B1, Batch 1; B2, Batch 2;

[a]*Percent of similarity between the sequences obtained from the PCR–DGGE analysis and the sequences deposited in the GenBank database;*

[b]*Accession number of the sequences found by a BLAST search;*

[c]*GenBank accession number of the deposited sequences.*

The detection of *Staphylococcus* at both the species and genus levels in the batch 1 pooled samples from Belgium, France and the Netherlands agrees well with its previous detection in a number of edible insects including cockroaches, ants, rhino beetles, beetles, butterflies, silkworms, mealworms and flies (Garofalo et al., 2019). The genus *Staphylococcus* encompasses both saprophytic species and species with pathogenic potential.

Pseudomonas was detected only in the pooled samples from France. To date, this bacterial genus has been found in cricket-based products through metagenomic sequencing, as well as in different insect species, such as grasshoppers, cockroaches, flies, rhino beetles, lygaeid bugs, butterflies, and mealworms (Garofalo et al., 2019). Interestingly, Osimani et al. (2018a)has recently detected the DNA of *Pseudomonas aeruginosa* in the frass of laboratory-reared mealworms, thus suggesting the possible occurrence of this pathogenic species in edible insects. Concerning *Hespellia porcina*, to the authors' knowledge, this is the first detection of this organism in insects and more specifically, in crickets produced in Austria. First isolated from swine manure storage pits, it has been more recently detected in the cecal microbiota of chicken as a commensal microorganism, suggesting the possible occurrence of this microorganism in the insect gut (Whitehead et al., 2004; Stanley et al., 2013). No reports on the occurrence of *H. porcina* in foodstuff are currently available in the scientific literature for a further comparison of the data. To the authors' knowledge, the presence of *Ruminococcus gauvreauii*, detected in both the pooled samples from Austria (batch 2) and the Netherlands (batch 2), has never been reported in edible insects, although Garofalo et al. (2017b) highlighted the presence of

Ruminococcaceae in samples of ready-to-eat small crickets and cricket powder by using Next Generation Sequencing (NGS). Very recently, the detection of *Ruminococcus* in the gut microbiota of chicken that were fed edible insect-based feed was also observed (Biasato et al., 2018).

The results of the PCR-based screening of the target AR genes are shown in Table 4.3.7.

Table 4.3.7. *Results of PCR amplification of AR determinants in samples of ready-to-eat edible crickets produced in Austria (AU1-AU8), Belgium (BL1-BL2), France (FR1-FR2), and the Netherlands (NL1-NL2).*

Samples	**Assays**	**Antibiotic resistance determinants**											
		erm(A)	*erm(B)*	*erm(C)*	*vanA*	*vanB*	*tet(M)*	*tet(O)*	*tet(S)*	*tet(K)*	*mecA*	*blaZ*	*aac-aph*
AU1-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	-	-	-	-	n.d.	n.d.	-	+	-	-	+
AU2-B1	PCR	-	-	-	-	-	+	-	-	-	-	-	-
	n-PCR	-	-	+	-	-	n.d.	+	+	+	-	-	-
AU3-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	+	-	-	-	n.d.	n.d.	-	+	-	-	-
AU4-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	+	-	-	-	n.d.	n.d.	+	+	-	-	-
AU5-B2	PCR	-	-	-	-	-	+	-	-	-	-	-	-
	n-PCR	-	-	+	-	-	n.d.	+	+	+	-	-	-
AU6-B2	PCR	-	+	-	-	-	+	-	-	-	-	-	-
	n-PCR	-	n.d.	-	-	-	n.d.	+	+	+	-	-	-
AU7-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	-	-	-	-	-
AU8-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	nPCR	-	-	-	-	-	-	+	-	+	-	+	-
AU % of positivity for each determinant		0	37.5	25	0	0	75	87.5	50	87.5	0	12.5	12.5
BL1-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	nPCR	-	-	-	-	-	n.d.	n.d.	+	+	-	-	+
BL2-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	nPCR	-	-	-	-	-	n.d.	n.d.	+	-	-	-	-
BL3-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	-	-	-	-	n.d.	n.d.	+	-	-	-	-
BL4-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	+	-	-	-	n.d.	n.d.	+	-	-	-	-
BL5-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	+	-	-	+	-	+	-
BL6-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	-	-	-	+	-
BL7-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	+	-	+	-	-	-
BL8-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	+	-	-	-	+	-
BL % of positivity for each determinant		0	37.5	0	0	0	50	75	50	37.5	0	37.5	12.5

Samples	Assays	Antibiotic resistance determinants											
		erm(A)	*erm(B)*	*erm(C)*	*vanA*	*vanB*	*tet(M)*	*tet(O)*	*tet(S)*	*tet(K)*	*mecA*	*blaZ*	*aac-aph*
FR1-B1	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	nPCR	-	+	+	-	-	-	-	+	-	-	-	-
FR2-B1	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	nPCR	-	+	+	-	-	+	-	+	-	-	-	-
FR3-B1	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	+	-	+	-	-	-	-
FR4-B1	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	+	-	-	-	-
FR5-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	+	+	+	-	-	+	-
FR6-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	-	-	-	-	-
FR7-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	+	+	-	-	-
FR8-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	-	-	-	+	-
FR % of positivity for each determinant		0	50	25	0	0	37.5	12.5	75	12.5	0	25	0
NL1-B1	PCR	-	-	-	-	-	+	-	-	-	-	-	-
	nPCR	-	-	-	-	-	n.d.	+	+	-	-	-	-
NL2-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	nPCR	-	-	-	-	-	n.d.	n.d.	+	-	-	-	-
NL3-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	+	-	-	-	n.d.	n.d.	+	-	-	-	-
NL4-B1	PCR	-	-	-	-	-	+	+	-	-	-	-	-
	n-PCR	-	-	-	-	-	n.d.	n.d.	+	-	-	-	-
NL5-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	+	+	-	-	-	-	-
NL6-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	+	+	-	-	-	+	-
NL7-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	-	-	-	-	-
NL8-B2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	+	-	-	-	-
NL % positivity for each determinant		0	12.5	0	0	0	75	75	62.5	0	0	12.5	0
Overall % of positivity for each determinant		0	34.4	12.5	0	0	62.5	62.5	59.4	34.4	0	21.9	6.25

PCR: positive after PCR; n-PCR: positive after PCR and nested PCR; n.d. not determined.

The use of PCR-based assays for the detection of AR genes has recently been reviewed by Penders, et al. (2013). In their mini-review, the authors suggested the high suitability of PCR-based approaches for detection and/or quantification of AR genes. In particular, nested-PCR is

characterized by a high sensitivity of small amounts of target DNA sequences, thus representing an important tool for the detection of very small differences in AR genes abundance. Limits of the detection of the PCR and nested-PCR protocols applied in the present study had been preliminary determined by Garofalo et al. (2007). This allowed a quantification of targeted genes in the processed edible insects to be determined, with gene copy numbers g^{-1} of sample ranging from 10^5 [*erm(B)*, *erm(C)*, and *blaZ*] to 107 [*tet(M)*, *tet(O)*, *tet(K)*, *tet(S)*, *erm(A)*, *mecA*, *vanA*, and *vanB*] and from 10^2 [*erm(B)*, *erm(C)*, and *blaZ*] to 10^4 [*tet(M)*, *tet(O)*, *tet(K)*, *tet(S)*, *erm(A)*, *mecA*, *vanA*, and *vanB*] after the first and second round of PCRs, respectively.

As expected, for those genes with a PCR detection limit of 10^7 gene copy number g^{-1} of sample [*erm(C)*, *vanA*, *tet(O)*, *tet(S)*, *tet(K)*, and *mecA*], an increase in the number of positive samples was seen after the second round of PCRs.

As very recently elucidated by Manaia (2017), a low abundance of a given AR gene does not linearly imply a mitigation of the risk for its horizontal transfer to potential pathogens. Hence, as stated by the same author even AR genes that are below or slightly above the limits of detection and quantification can represent a significant risk for human health.

Genes conferring resistances to tetracycline were the most prevalent, being mainly detected already after the first round of PCRs, thus suggesting the occurrence of gene copies g^{-1} of sample $\geq 10^5$ [*tet(M)*], 107 [*tet(O)*], and 10^8 [*tet(K)*], respectively. After the second round of PCRs, both *tet(M)* and *tet(O)* were detected in 62.5% of the samples, thus indicating the occurrence of these genes at a level $\geq 10^0$ and 10^4 gene copies g^{-1} of sample, respectively. More specifically, for *tet(M)*, the percent of positive samples ranged from 37.5% (samples from France) to 75.0% (samples from Austria). Similarly, for *tet(O)*, samples from France showed a low percentage of positive samples (12.5%), whereas 87.5% of the samples from Austria were positive after the second round of PCRs. A high percent of positive samples (59.4%) was also observed for *tet(S)*, with samples from France and the Netherlands showing frequencies of 75% and 62.5%, respectively. Among the analyzed samples, *tet(K)* was detected with the lowest frequency (34.4%), although 87.5% of the samples produced in Austria were positive for this gene. On the contrary, no crickets produced in the Netherlands were found to be positive for the *tet(K)* gene.

Tetracyclines are a class of broad-spectrum natural or semisynthetic antibiotics that are active against both Gram-positive and Gram-negative bacteria. Molecules belonging to this antibiotic class possess a common hydronaphthacene core containing four fused rings. This functional group influences the permeability of these molecules across biological membranes, thus mediating the absorption of the antibiotic by bacterial cells (Pérez-Rodríguezet al., 2018). Resistance to tetracycline is generally mediated by the Tn916 conjugative transposon family, as well as other transposons (for example, Tn5253, Tn5385, ICESp2905) (Marosevic et al., 2017). These mobile elements code for proteins involved in efflux pump systems, ribosomal protection, and enzymatic inactivation of tetracyclines (Marosevic et al., 2017).

The high frequencies of *tet* genes found in the present study are in agreement with recent studies investigating the occurrence of these AR genes in different species of edible insects (Milanović et al., 2016; Osimani et al., 2017b, 2017c), with a few differences. Indeed, in the present study *tet(O)* was found most prominently, whereas in the cited studies this AR determinant was detected from low to very low frequencies. By contrast, in the studies of Milanović et al. (2016) and Osimani et al. (2017b),

(2017c), *tet(K)* was the most detected AR gene, whereas in the present research, it was detected with the lowest frequency among the tet genes.

To date, a number of authors (Larson et al., 2008; Levy & Marshall, 2013; Pai, 2013) have previously demonstrated the presence of tetracycline resistance genes in different insect species (for example, feed mill insects, cockroaches, and honeybees), thus contributing to our understanding of the role of insects in the spread of these genes in both the environment and the food chain. Regarding the *tet* genes targeted in the present study and broadly distributed among all the analyzed samples, the detection levels were in accordance with those previously reported in the EU summary report by the EFSA and European Centre for Disease Prevention and Control (ECDC) on AR in zoonotic and indicator bacteria from humans, animals, and food (EFSA and ECDC, 2016).

Regarding the genes that confer resistance to erythromycin, 34.4% of the samples were positive for *erm(B)*, always after the second round of PCRs, except for sample AU6-B2, thus suggesting the occurrence of a gene copy number g^{-1} of sample comprised between 10 (limit of detection of nested-PCR) and 10^5 (limit of detection of PCR for the same AR gene). Moreover, the percent of positive samples ranged from 12.5% in samples produced in the Netherlands to 50.0% in samples produced in France. Austria and Belgium showed an intermediate number of positive samples. Only samples from France and Austria were positive for *erm(C)*, with a positive frequency of 25.0%, again always after the second round of PCRs, thus revealing a gene copy number g^{-1} of sample comprised between 10 and 10^5. Finally, no samples were positive for *erm(A)* after the second round of PCRs, characterized by a limit of detection of 10^3 gene copy number g^{-1} of processed sample.

MLSB antibiotics are commonly used to treat salmonellosis, shigellosis, and sexually transmitted diseases. This class of antibiotics is able to inhibit the peptidyl transferase reaction (lincosamides and streptogramin A) or peptide elongation (erythromycin) and blocks the ribosomal exit of the nascent peptide chains (streptogramin B) (Cocito et al., 1997; Marosevic et al., 2017; Poulsen, Kofoed, & Vester, 2000). To date, *erm* (erythromycin rRNA methylase) determinants represent a threat to human health because they confer cross-resistance to macrolides, lincosamides, and streptogramin B (Marosevic et al., 2017). It is noteworthy that some transposons (for example, Tn1545 and Tn917) involved in the transfer of erm genes can simultaneously carry tet determinants (Marosevic et al., 2017), leading to the occurrence of multiresistance.

In the present study, a relatively low occurrence of *erm* genes was found when compared to other food sources of animal origin (Garofalo et al., 2007). Very recently, *erm(A)*, *erm(B)*, and *erm(C)* have been detected, although with low frequencies, in commercialized mealworms (Osimani et al., 2017c), whereas only *erm(B)* and *erm(C)* were detected in marketed grasshoppers (Osimani et al., 2017b). As reported by the European Medicines Agency (EMA, 2010), resistance to macrolides and lincosamides first appeared among staphylococci and streptococci isolated from pigs and cattle, respectively. Moreover, a high prevalence of resistance to these antibiotics occurs in enterococci. Overall, the low number of samples positive for erm genes in the present study reflects what has been reported for this class of AR genes by the EFSA and the ECDC (EFSA and ECDC, 2014) in food producing animals in Europe.

Regarding beta-lactams, 21.9% of the samples were positive for the *blaZ* gene always after the second round of PCRs (limit of detection of 10^0 gene copy number g^{-1} of sample) with frequencies comprised between 12.5% (in samples from Austria and the Netherlands) and 37.5% (in samples from

Belgium). No samples were positive for *mecA*, after neither the first (limit of detection of 10^5 gene copy number g^{-1} of sample) or second (limit of detection of 10^2 gene copy number g^{-1} of sample) round of PCRs.

The percent of samples positive for both *blaZ* and *mecA* were in accordance with previous studies of commercialized edible insects carried out by Milanovic´et al. (2016) and Osimani et al. (2017b, 2017c). A generally low occurrence of *mecA* emerged from all these studies, with no detection at all in commercialized grasshoppers (Osimani et al., 2017b), which are included in the same taxonomic order as crickets (Orthoptera). To the authors' knowledge, no other studies on the occurrence of beta-lactamase genes in both edible insects and insect pests are currently available in the scientific literature for a further comparison of the data.

Beta-lactams are broad spectrum antibiotics that primarily act as inhibitors of transpeptidases (specialized acyl serine transferases), thus compromising the synthesis of the bacterial cell wall (van Bambeke et al., 2017). The production of beta-lactamases is mainly encoded by the *blaZ* gene (Pinho, 2008), while the *mecA* gene is responsible for methicillin resistance, coding for penicillin-binding protein (PBP) 2A (Wielders, Fluit, Brisse, Verhoef, & Schmitz, 2002).

The emergence and spread of genes coding for the extended-spectrum beta-lactamases in Enterobacteriaceae (ESBL-E) is becoming a public health threat. The ECDC recently issued a systematic review of the effectiveness of infection control measures to prevent the transmission of ESBL-E through cross-border transfer between patients (ECDC, 2014). As reported by the ECDC (2014), while some European countries have addressed the spread of ESBL-E with guidelines, strategies, or national/local task forces, only a few official guidelines or guidance documents relating to infection control measures for ESBL-E have been published.

With respect to *aac-aph*, a low percentage of positive samples (6.3%) was observed in the present study, again always after the second round of PCR, thus revealing the occurrence of a gene copy number g-1 of sample comprised between 10^2 (limit of detection of the second round of PCRs) and 10^8 (limit of detection of the second round of PCRs). Only samples from Austria and Belgium were positive for this AR determinant (12.5%), whereas no positive samples were found in products from France or the Netherlands. This low occurrence of *aac-aph* is in accordance with the data reported by Osimani et al. (2017b, 2017c) in ready-to-eat grasshoppers and mealworms.

To the authors' knowledge, there is a paucity of data reporting the occurrence of genes conferring resistance to aminoglycosides in edible insects, thus making a further comparison of the results difficult. Larson et al. (2008) have previously isolated streptomycin and neomycin-resistant strains of Aerococcus viridans and Enterococcus (*Enterococcus faecium*; *Enterococcus gallinarum*) from stored-products (*Tribolium castaneum*); their study was the first report to shed light on the contribution of these microorganisms to the spread of aminoglycoside resistance genes in edible insects.

Aminoglycosides are a broad-spectrum class of antibiotics commonly used to treat human infections caused by Gram-negative pathogens such as *Pseudomonas*, *Acinetobacter*, and *Enterobacter* (EMA, 2017a; Germovsek, Barker, & Sharland, 2017). Moreover, these antibiotics are used against a wide range of infections in food-producing animals and in companion animal species (EMA, 2017a). The mechanisms of resistance to aminoglycosides are complex, including efflux pumps, enzymatic drug, or target-site modifications, and vary depending on both the molecule involved and the bacterial

species (EMA, 2017a). As recently highlighted by the ECDC Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) (ECDC, 2017), *Escherichia coli* and *Klebsiella pneumoniae* show an alarming increase in resistance to aminoglycosides, thus raising concerns for the future effectiveness of clinical therapies exploiting these antimicrobials.

Finally, among the 32 analyzed samples, *vanA* and *vanB* were never detected by either PCR (limit of detection of 10^6 and 10^5 gene copy number g^{-1} of sample, respectively) or nested-PCR (limit of detection of 10^0 and 10^1 gene copy number g^{-1} of sample, respectively). This result is in accordance with what has been reported by other authors who analyzed both edible insects (Milanović et al., 2016; Osimani et al., 2017b) and insect pests (Pai et al., 2005). Of note, Osimani et al. (2017c) detected both *vanA* and *vanB* genes in samples of commercialized edible mealworms using the same approach. Vancomycin is an antibiotic used for serious infections, such as those in the lining of the gut caused by Clostridium difficile and methicillin-resistant *S. aureus* (EMA, 2017b). In the ECDC Summary of the Latest Data on Antibiotic Resistance in the European Union (ECDC, 2016) a worrying increase in vancomycin-resistant *E. faecium* strains was also reported.

It is noteworthy that AR gene transmission may also occur through environmental contamination (for example, manure to water to humans) (Hudson et al., 2017). It has been reported that both water and fertilizer could have an impact in the spread of resistance genes and resistant bacteria (Kang et al., 2016; Vaz-Moreira et al., 2014). Therefore, the water used in the rearing of insects or the fertilizers used in the production of insect feed could have affected the diffusion of the detected resistance genes in the analyzed crickets. Moreover, sanitation operations applied in food processing environments could have led to the coselection of antibiotic resistant bacteria, according to what recently elucidated by Hudson et al. (2017).

Since *erm(A)*, *vanA*, *vanB*, and *mecA* were not detected in this study, they were not included in the PCA analysis.

Eigenvalues and cumulative percentage variance explained by each PC and eigenvectors of the three most important principal components identified are reported in Table 4.3.8 (panel a and b resepctively). The statistical analysis showed that three PCs with eigenvalues higher than 1 explained 89.5% of the total variance. Figure 4.3.2 identifies two main clusters, where Cluster 1 included three of the four batch1 units (AU-B1, BL-B1, and NL-B1) and Cluster 2 included all four batch 2 units (AU-B2, BL-B2, FR-B2, and NL-B2). Sample FR-B1 was positioned away from all the clusters.

Table 4.3.8. *Results of PCA analysis based on AR gene frequencies:eigenvalues and percentage of variance explained (panel a); eigenvectors of the three most important principal components identified (panel b).*

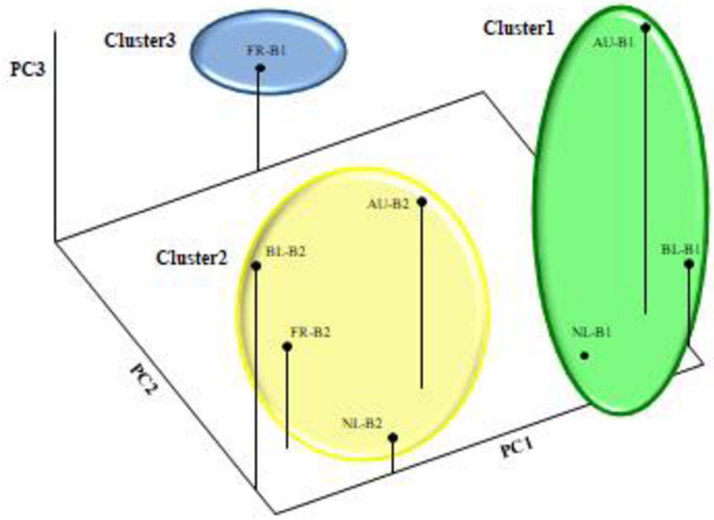
a

Eigenvalues	Variance percent	Variance cumulative
3.34	41.78	41.78
2.18	27.29	69.07
1.63	20.39	89.46
0.41	5.16	94.62
0.28	3.46	98.08
0.14	1.73	99.81
0.02	0.19	100.00
0.00	0.00	100.00

b

Eigenvectors			
AR Genes	PC1	PC2	PC3
erm(B)	0.0472	0.7787	0.4640
erm(C)	0.1629	0.8742	0.3279
tet(M)	0.9699	-0.1361	-0.0873
tet(O)	0.7155	-0.6237	0.1279
tet(S)	0.6854	0.4567	-0.4765
tet(K)	0.1823	-0.2211	0.9226
blaZ	-0.8735	-0.3250	0.2484
aac-aph	0.7713	-0.2043	0.3801

Figure 4.3.2. *Results of Principal Component Analysis of AR determinants showing the variation characterizing the four producers based on AR determinant frequencies.*



AU, Austria; BL, Belgium; FR, France; NL, the Netherlands.

In more detail, PC1 encompassed most of the variation among the Producer-Batch units, explaining 41.8% of the variance. PC1 eigenvector coefficients (Figure 4.3.8, panel b) revealed that *tet(M)*, *tet(O)*, *tet(S)*, *aac-aph*, and *blaZ* were the most important AR genes, as they were characterized by high coefficients (in absolute values). In more detail, PC1 differentiated Cluster 1 from Cluster 2 and FR-B1, with Cluster 1 being characterized by high frequencies of *tet(M)*, *tet(O)*, *tet(S)*, and *aac-aph* (positive coefficients) and a low frequency of *blaZ* (negative coefficient). PC2 explained 27.3% of the variance and separated FR-B1 from all the other samples due to its higher frequency of *erm(B)* and *erm(C)* (positive coefficients). By contrast, PC3, which explained 20.4% of the variance, identified variability within Clusters 1 and 2, mainly due to *tet(K)* (Figure 4.3.2, panel b and c).

Overall, the PCA analysis based on the AR gene frequencies differentiated samples of batch 2 from those of batch 1 providing evidence for a difference between the producer from France and all the other producers in the batch 1 samples. These results are summarized in Table 4.3.9, where the "Producer by Batches" samples were ordered based on the PCA groupings.

An indication of a high variation in the risk associated with the occurrence of AR genes in edible insects among different producers and even production batches from the same producer emerges

from the PCA analysis.

Table 4.3.9. *Producer by batches samples ordered based on PCA grouping.*

Cluster	PCA Units	PC1 *tet(M)*	PC1 *tet(O)*	PC1 *tet(S)*	PC1 *aac-aph*	PC1 *blaZ*	PC2 *erm(B)*	PC2 *erm(C)*	PC3 *tet(K)*
1	AU-B1	1.00	1.00	0.50	0.25	0.00	0.50	0.25	1.00
1	BL-B1	1.00	1.00	1.00	0.25	0.00	0.25	0.00	0.25
1	NL-B1	1.00	1.00	1.00	0.00	0.00	0.25	0.00	0.00
2	AU-B2	0.50	0.75	0.50	0.00	0.25	0.25	0.25	0.75
2	BL-B2	0.25	0.50	0.00	0.00	0.75	0.50	0.00	0.50
2	FR-B2	0.25	0.25	0.50	0.00	0.50	0.25	0.00	0.25
2	NL-B2	0.50	0.50	0.25	0.00	0.25	0.00	0.00	0.00
3	FR-B1	0.50	0.00	1.00	0.00	0.00	0.75	0.50	0.00

As reported by the World Health Organization (2018), the increase in AR is favored by the misuse and overuse of antibiotics, hence, measures to reduce the impact and to limit the spread of AR are constantly implemented by governments and international Public Health Institutions. In October 2017, the EMA published (EMA, 2017a), with the help of its Committee for Medicinal Products for Veterinary Use (CVMP) and its Antimicrobials Working Party (AWP), the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project report (EMA, 2017a), which presented data on the sales of veterinary antimicrobial agents from 30 European countries in 2015.

Given the lack of information on either breeding or processing techniques of the analyzed insects, a number of hypotheses can be set forth as an explanation for the occurrence of some of the AR genes under study. As reported in the EFSA opinion (EFSA, 2015), although the use of antibiotics in insect rearing is not a common practice, antimicrobials can sometimes be used at industrial rearing facilities as an emergency therapy against bacteriosis or mycosis.

To date, chlortetracycline and streptomycin have been used in laboratory-reared honey bees and Nezara viridula, respectively (Hirose, Panizzi, & Cattelan, 2006; Peng, Mussen, Fong, Mon- tague, & Tyler, 1992). More recently, the use of chloramphenicol for the treatment of silkworms reared in mass has been reported (Cappellozza et al., 2011). In addition, as early as 1996, the presence of naturally occurring antibiotics in insect larvae (*Lucilia sericata*) has been documented (Sherman & Wyle, 1996), whereas in 2008 Sheppard, Newton, and Burtle detected the occurrence of antibiotic substances in larvae of Hermetia illucens. All these evidences suggest a possible selective pressure of either deliberately used or naturally occurring antibiotics on the microbiota of insects analyzed in the present study.

4.3.3 Conclusions

It is noteworthy that, for biological threats, the specific production procedures, the type of the substrate, the harvest period, the type of the insect and their developmental phase could exert an impact on the occurrence of microbial species. Furthermore, the vertical transmission of the microorganisms can be due to the evacuation of the bacteria during egg laying or by the contamination of the egg with symbionts defaecated from the mother's gut to offspring, thus affecting the microbial community of the insects (Osimani et al., 2018a). When insects are intended for commercialization, further processing could also alter the presence of the occurring microbial species.

Hence, to assure a safe product, the need for a full standardization of production technologies, including feed supply as well as rearing and processing practices, is recommended. Furthermore, as reported by Mc Nulty et al. (2016), the science-based monitoring of AR represents a pivotal source of information for the implementation of mitigation strategies and to assure food safety. Hence, any novel information on AR to antimicrobials conventionally used in clinical practice is of importance. Regarding edible insects, as studies of AR are expanded, new aspects of this topic will come to light. If the results of Milanović et al. (2016) and Osimani et al. (2017b, 2017c) provided evidence for the contribution of different EU producers of edible insects to the occurrence and distribution of AR determinants, the present study furthers this line of investigation by focusing on the effects of different production batches. At this regard, an intrabatch variation was seen in the occurrence and distribution of transferable resistances. This evidence, coupled with the observed differences in the viable counts of different batches of small crickets, suggests a low standardization of the production processes. In EU, production cycles at insect rearing facilities are still highly variable, and insect-based foods are mainly produced in a batchwise manner, as recently elucidated by Vandeweyer et al. (2017b); thus, microbiota, including antibiotic resistant microorganisms, can vary drastically over time.

Based on the overall results of this study, a need for a full standardization of production technologies, including feed supply, rearing, and processing, as well as a prudent use of antimicrobials during the rearing of insects destined for human consumption is strongly recommended.

4.4 Occurrence of transferable antibiotic resistances in commercialized ready-to-eat mealworms (*Tenebrio molitor* L.) (Paper I)

Worldwide, entomophagy is practised by around 2.5 billion people (van Huis et al., 2013). Despite that, hazards related to insects consumption are still scarcely studied. It is noteworthy that insects can be carriers of antibiotic resistant microorganisms which can horizontally transfer antibiotic resistance plasmids to human gut microbiota (Anacarso et al., 2016).

In this context, this study was aimed to assess the occurrence of transferable AR determinants in 40 samples of edible mealworms (*Tenebrio molitor* L.) marketed in the EU.

The prevalence of 12 selected determinants conferring resistance to tetracyclines [*tet(M)*, *tet (O)*, *tet(S)*, *tet(K)*], macrolide-lincosamide-streptogramin B (MLSB) [*erm (A)*, *erm(B)*, *erm(C)*], vancomycin (*vanA*, *vanB*), beta-lactams (*blaZ*, *mecA*) and aminoglycosides [*aac(6')-Ie aph(2'')-Ia*, abbreviated as *aac-aph*] was assessed through optimized PCR and nested PCR assays. These determinants correspond to those most frequently detected in both foodborne human pathogens (Rolain, 2013) and commensal bacteria (Aarts and Margolles, 2015; Devirgiliis et al., 2011).

4.4.1 Materials and methods

Purchase of edible insects

Forty samples of edible mealworms (boiled, dried and salted) were provided by four dealers located in the Netherlands (samples NL1 to NL10), Thailand (samples TH1 to TH10), Belgium

(samples BL1 to BL10) and France (samples FR1 to FR10). The samples were provided in sealed plastic boxes of varying weights and delivered via international transport. They were analyzed within their shelf life at ambient temperature, which ranged from nine to twelve months, depending on the producer.

Microbial enumeration

Ten grams of each sample were crushed with a mortar under sterile conditions followed by 10 min of homogenization at 260 rpm in 90 mL of peptone-saline solution with a Stomacher 400 Circulator apparatus (VWR International, Milan, Italy).

The resulting suspensions were diluted 10-fold and subjected to microbial enumeration of total mesophilic aerobes, Enterobacteriaceae, lactic acid bacteria, and spore forming bacteria in appropriate growth media by inclusion spreading as already described by Osimani et al. (2017a). All samples were analyzed in duplicate.

Bacterial DNA extraction

Microbial DNA, directly extracted from the mealworms, was iso- lated as described by Osimani et al. (2017b). Briefly, the PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) was used, followed by quantity and purity assessment via optical readings at 260, 280 and 234 nm using a UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) (Garofalo et al., 2015).

PCR and nested PCR amplification of AR determinants

The DNA extracts were subjected to a first set of PCR reactions, targeting the 12 selected AR determinants; negative samples were further subjected to a set of nested PCR reactions to improve amplification sensitivity. PCR and nested PCR reactions were carried out as previously described by Osimani et al. (2017b).

Briefly, 2 μL of DNA extract (~50 ng of bacterial DNA) or PCR product was amplified in a reaction mixture that was composed of 1× buffer, 50 pmol each primer, 0.2 mM dNTPs (2.5 mM for the amplification of erm genes in both assays) and 0.75 U Taq polymerase.

Thermal cycling conditions and primers used for PCR and nested PCR assays are reported in Tables 4.4.1 and 4.4.2.

In Table 4.4.3, reference cultures used as positive and negative controls are listed. PCR mixture supplemented with water instead of DNA was used as a blank. A PCR workstation with a built-in UV source (DNA/RNA UV-Cleaner UVC/T-M-AR, Biosan, Riga, Latvia) was used for the preparation of reaction mixtures.

All the amplifications were carried out in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, USA). PCR products were analyzed under UV light using the Complete Photo XT101 system (Explera, Jesi, Italy) as reported by Milanović et al. (2016). False-positive results and contamination in PCR assays were minimized by adopting basic pre- cautions suggested by Borst et al. (2004).

Table 4.4.1. *Primers used in the PCR assays targeting the 12 resistance genes of interest, and the corresponding annealing temperature (Ta), product size, reaction mixture, PCR program and references.*

AR gene	Primer sequence (5'-3')	Ta (°C)	Product size (bp)	Reaction mixture	PCR program	Reference
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-CAATACGAGAGCCGGGTTTC 2-ACAACGGGCTGGAATTTCAC	60	382	□	○	Milanović et al. (2017)
tet(K)	1-TCGATAGGAACAGCAGTA 2-CAGCAGATCCTACTCCTT	55	169	□	○○○	Ng et al. (2001)
erm(A)	1-CAGGAAAAGGACATTTTACCAA 2-CTTCGATAGTTTATTAATATTAGT	50	572	□□	○○	Garofalo et al. (2007)
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	□	○○○	Predari et al. (1991) Murakami et al. (1991)
blaZ	1-ACTTCAACACCTGCTGCTTTC 2-TAGGTTCAGATTGGCCCTTAG	58	240	□	○○○	Martineau et al. (2000) Garofalo et al. (2007)
aac-aph	1-GAGCAATAAGGGCATACCAAAAATC 2-CCGTGCATTTGTCTTAAAAAACTGG	58	505	□	○○○	Kao et al. (2000)

□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 0.2 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
□□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 3.0 mM MgCl2, 0.25 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 1 min., Ta 1 min, 72°C, 2 min. Final extension 72°C, 7 min.;
○○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 1 min., Ta 1 min., 72°C, 1 min. Final extension 72°C, 7 min.;
○○○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 30 sec., Ta 30 sec., 72°C, 30 sec. Final extension 72°C, 7 min.

Table 4.4.2. *Primers used in the nested-PCR assays targeting the 12 resistance genes of interest, and the corresponding annealing temperature (Ta), product size, reaction mixture, PCR program and references.*

Target gene	Primer sequence (5'-3')	Ta (°C)	Product size (bp)	Reaction mixture	PCR program	Reference
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 4-GGAAATCTGCTGGCGTACTG	64	106	□	○	Milanović et al. (2017)
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)
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. (2001)
aac-aph	1-GAGCAATAAGGGCATACCAAAAATC 4-GCCACACTATCATAACCACT	62	220	□	○	Garofalo et al. (2007)

□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 2.5 mM MgCl2, 0.2 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
□□ 1X buffer [60 mM Tris-HCl (pH 8.5 at 25°C); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100], 50 pmol of each primer, 3.0 mM MgCl2, 0.25 mM dNTPs and 0.75 U of Taq polymerase (Taq DNA polymerase, SybEnzyme Ltd., Novosibirsk, Russia);
○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 30 sec., Ta 30 sec., 72°C, 30 sec. Final extension 72°C, 7 min.;
○○ Initial denaturation 95°C, 5 min., 35 cycles at 94°C, 1 min., Ta 1 min., 72°C, 1 min. Final extension 72°C, 7 min;.
A blank control containing only molecular biology grade water added to the PCR mix (Sigma-Aldrich, St. Louis, MO, USA) was used in each PCR reaction to check for contaminants.

Table 4.4.3. *Bacterial reference strains used in this study.*

Bacterial reference strain	AR gene
Staphylococcus aureus M.P.	*erm*(A)
Enterococcus hirae Api 2.16	*erm*(B)
Staphylococcus spp. SE12	*erm*(C)
Enteroccus faecium PF3U	*vanA*
Enterococcus feacalis ATCC 51299	*vanB*
Lactobacillus casei/paracasei ILC2279	*tet*(M)
Streptococcus pyogenes 7008	*tet*(O)
Enterococcus italicus 1102	*tet*(S)
Staphylococcus aureus COL.	*tet*(K)
Staphylococcus aureus 27R	*mecA*
Staphylococcus aureus ATCC2921	*blaZ*
Enteroccus faecium M48	*aac-aph*
Enterococcus feacalis JH2-2	negative control

[a] Culture collection of Dipartimento di Scienze della Vita e dell'Ambiente (DiSVA), Università Politecnica delle Marche, Italy;
[b] Culture collection of Dipartimento di Scienze Agrarie, Alimentari ed Ambientali (D3A), Università Politecnica delle Marche, Italy;
[c] ATCC, American Type Culture Collection, Manassas, U.S.A.

Statistical analysis

The results of the viable counts were expressed as the mean of the log colony forming units (cfu) per gram of sample ± standard deviation. Given the high number of samples showing microbial counts below 1 log cfu g^{-1} for some microbiological parameters, two different statistical approaches were adopted. Briefly, viable counts of total mesophilic aerobes and spore forming bacteria (≥1 log cfu g^{-1} in >50% of the samples) were subjected to one way analysis of variance (ANOVA) with the following sources of variation: "Producer" (P), "Samples within Producer" (S[P]) and "error" (JMP 11.0.0, SAS Institute Inc., Cary, NC, USA). The variance among Producers was tested using "the among Samples within Producers variance" as denominator of the F test. The "among Samples within Producers" variance was tested using the error mean squares as denominator of the F test. Multiple comparisons among means were carried out through the Tukey's HSD test.

Viable counts of lactic acid bacteria were subjected to χ^2 test because of the low frequency of samples with counts ≥ 1 log cfu g^{-1} for this microbial group. An overall χ^2 test (3 degrees of freedom) was carried out by comparing the frequencies of samples below and above 1 log cfu g^{-1} across all the four producers, followed by χ^2 tests for specific comparisons.

Due to the very low frequency of samples showing viable counts of Enterobacteriaceae ≥ 1 log cfu g^{-1}, data for this microbiological parameter were not subjected to either ANOVA or χ^2 tests.

The analysis of AR determinants was started by creating a data matrix where PCR- and nested PCR-positive and -negative samples were scored as 1 or 0, respectively. A 40 × 40 matrix of Jaccard Similarity indexes was further obtained from the previous data matrix and a Principal Coordinate Analysis (PCoord) was conducted to study the grouping pattern of the whole set of 40 samples obtained from four different producers (NTSYS - Applied Biostatistics Inc., NY, USA).

Additionally, relative percentages of positivity for the 12 AR determinants were calculated for each producer as the number of PCR- or nested PCR-positive samples divided by the total number of samples assayed (=10). From this 4 × 12 data matrix the Principal Component Analysis (PCA) from the Pearson correlation matrix was performed. The eigenvectors' coefficients of the Principal Components allowed the quantification of the relative importance of each AR determinant in differentiating the four producers. The results were summarized gra- phically by plotting the four producers based on their respective PCA scores (NTSYS - Applied Biostatistics Inc., NY, USA). The overall percentages of positivity for the 12 determinants were also calculated as the number of PCR- or nested PCR-positive samples divided by whole set of 40 samples.

4.4.2 Results and discussion

In this study, 40 samples of edible mealworm larvae purchased from both EU and non-EU countries have been subjected to viable counting of various microbial groups and further screened for the occurrence of 12 transferable resistance genes. Among edible insects, mealworm larvae are easy to breed and represent a very promising source of protein and fats, providing 20% and 15% of these nutrients, respectively (Gasco et al., 2016; Zhao et al., 2016). In some EU countries, the farming of mealworms is carried out by family enterprises; in the Netherlands, edible insects belonging to this species can already be purchased for human consumption in specialized shops (van Huis et al., 2013).

Very recently, the potential use of mealworms for the industrial manufacture of minced meat-like products has successfully been ex- plored (Stoops et al., 2017).

The ANOVA results from the plate counting data collected from the 40 samples of mealworm larvae are reported in Table 4.4.4, and the mean values of viable counts and multiple comparisons among means are reported in Table 4.4.5.

Table 4.4.4. *ANOVA results for total mesophilic aerobes and spore-forming bacteria (log cfu g⁻¹) in 40 samples of edible mealworms according to producer.*

Source of variation	df	Total mesophilic aerobes		Spore-forming bacteria	
		Mean squares	F	Mean squares	*F*
Producer	3	59.528	68.61***	34.401	38.05***
Samples [producer]	36	0.868	13.05***	0.904	3.90***
Error	40	0.066		0.232	

Producer	Total mesophilic aerobes		Spore-forming bacteria	
	Mean ± SD	*	Mean ± SD	*
The Netherlands	3.14 ± 0.30	c	2.57 ± 0.34	b
Thailand	1.32 ± 0.96	d	0.79 ± 1.06	c
Belgium	4.05 ± 0.19	b	3.95 ± 0.27	a
France	5.43 ± 0.86	a	1.99 ± 0.94	b

*cfu, colony forming units; df, degrees of freedom; SD, standard deviation; within each column, means followed by different letters are significantly different (P < 0.05); *** Significant P < 0.0001.*

As a general trend, viable counts were in the range of those reported by other authors for the same microorganisms in heat processed and dried mealworms intended for human consumption (Garofalo et al., 2017a; Grabowski and Klein, 2017; Milanović et al., 2016; Osimani et al., 2017a). It is noteworthy that in this study, the samples produced in Thailand were determined to have the lowest mean viable counts for all the tested microorganisms; this evidence might be likely explained by the well-established tradition of Thailand in the production and marketing of edible insects (Maciel-Vergara and Ros, 2017). Among the tested microorganisms, spore-forming bacteria have been detected in both fresh and processed mealworms (Garofalo et al., 2017a; Stoops et al., 2016); it has previously been suggested that the guts of these insects naturally contain these microorganisms (Engel and Moran, 2013), though an environmental contamination during processing cannot be excluded. Moreover, the overall low occurrence of Enterobacteriaceae, defined as an indicator of processing hygiene (Brown et al., 2000; Petruzzelli et al., 2016), attests to the appropriateness of the heat treatment of the edible insects assayed.

Regarding total mesophilic aerobes, mean counts ranged between <1 and 6.73 log cfu g^{-1}. Multiple comparison among producers showed that, for this microbial group, the four dealers differed significantly (Table 4.4.4), with those located in France and Thailand showing the highest (5.43 log cfu g^{-1}) and the lowest (1.32 log cfu g^{-1}) counts, respectively. Furthermore, comparisons carried out within each producer (Table 4.4.5) highlighted the occurrence of a significant variation among samples produced in Thailand and France, with viable counts ranging from <1.00 to 2.40 log cfu g^{-1} and 3.78 to 6.73 log cfu g^{-1}, respectively. By contrast, samples from the Netherlands and Belgium were much more homogeneous (Table 4.4.5). For spore-forming bacteria, viable counts were between <1 and 4.24 log cfu g^{-1} (Table 4.4.5), with samples from Belgium and Thailand showing the maximum (4.24 log cfu g^{-1}) and minimum (0.65 log cfu g^{-1}) values, respectively (Table 4.4.4). Moreover, similarly to what observed for total mesophilic aerobes, even for spore-forming bacteria Thailand and France were characterized by a significant variation among samples, whereas no significant differences were seen among samples produced in the Netherlands and Belgium (Table 4.4.5).

Finally, for lactic acid bacteria, viable counts varied between <1 and 2.99 log cfu g^{-1} (Table 4.4.5). The χ^2 test showed that the producer located in France differed from the other three with a

significantly higher frequency of samples with counts of lactic acid bacteria above 1 log cfu g^{-1} ($\chi^2(1) = 8{,}54$, $P < 0.01$).

Table 4.4.5. *Mean counts (± standard deviation) of total mesophilic aerobes, spore-forming bacteria, lactic acid bacteria, and Enterobacteriaceae in samples of edible mealworms from the four producers located in the Netherlands (NL1-NL10), Thailand (TH1-TH10), Belgium (BL1-BL10) and France (FR1-FR10).*

Samples	Total mesophilic aerobes	*	Spore-forming bacteria	*	Lactic acid bacteria	Enterobacteriaceae
			log cfu g^{-1}			
NL1	2.63 ± 0.21	a	2.11 ± 0.20	a	1.93 ± 0.21	<1
NL2	3.10 ± 0.05	a	2.30 ± 0.14	a	2.99 ± 0.06	<1
NL3	3.03 ± 0.11	a	2.50 ± 0.10	a	<1	<1
NL4	3.54 ± 0.04	a	2.60 ± 0.02	a	<1	<1
NL5	3.20 ± 0.02	a	2.99 ± 0.06	a	<1	<1
NL6	3.04 ± 0.05	a	3.12 ± 0.11	a	<1	<1
NL7	3.45 ± 0.02	a	2.73 ± 0.05	a	<1	<1
NL8	3.40 ± 0.12	a	2.74 ± 0.06	a	<1	0.50 ± 0.41
NL9	3.30 ± 0.04	a	2.36 ± 0.15	a	<1	<1
NL10	2.73 ± 0.10	a	2.20 ± 0.04	a	<1	<1
TH1	0.65 ± 1.00	de	0.65 ± 0.92	ab	<1	<1
TH2	<1	e	<1	b	<1	<1
TH3	1.70 ± 0.10	abcd	0.80 ± 1.13	ab	<1	<1
TH4	2.24 ± 0.30	ab	<1	b	<1	<1
TH5	2.30 ± 0.00	ab	1.00 ± 1.40	ab	<1	<1
TH6	<1	e	<1	b	<1	<1
TH7	0.80 ± 1.13	cde	1.35 ± 1.91	ab	<1	<1
TH8	1.24 ± 0.30	bcd	1.45 ± 0.64	ab	<1	<1
TH9	2.40 ± 0.10	a	2.62 ± 0.05	a	1.54 ± 0.09	<1
TH10	1.90 ± 0.04	abc	<1	b	<1	0.50 ± 0.61
BL1	4.30 ± 0.03	a	4.01 ± 0.05	a	1.72 ± 0.17	<1
BL2	4.10 ± 0.20	a	3.53 ± 0.07	a	2.80 ± 0.23	<1
BL3	4.10 ± 0.23	a	4.20 ± 0.05	a	<1	<1
BL4	4.20 ± 0.12	a	4.24 ± 0.00	a	<1	<1
BL5	4.30 ± 0.10	a	4.10 ± 0.03	a	<1	<1
BL6	4.05 ± 0.40	a	3.97 ± 0.00	a	<1	0.52 ± 0.71
BL7	3.83 ± 0.05	a	3.70 ± 0.07	a	<1	<1
BL8	3.96 ± 0.08	a	4.20 ± 0.05	a	<1	<1
BL9	4.02 ± 0.00	a	3.50 ± 0.10	a	<1	<1
BL10	3.70 ± 0.02	a	3.99 ± 0.02	a	1.60 ± 0.00	<1
FR1	4.58 ± 0.08	ef	1.69 ± 0.12	abc	2.77 ± 0.02	<1
FR2	5.77 ± 0.04	abcd	2.34 ± 0.10	ab	2.50 ± 0.02	<1
FR3	5.56 ± 0.03	bcde	2.47 ± 0.07	ab	<1	<1
FR4	6.73 ± 0.06	a	2.26 ± 0.16	ab	1.35 ± 0.50	<1
FR5	3.78 ± 0.00	f	2.47 ± 0.10	ab	0.50 ± 0.70	<1
FR6	5.19 ± 0.13	cde	2.74 ± 0.12	a	2.45 ± 0.21	<1
FR7	4.89 ± 0.01	de	2.56 ± 0.12	ab	<1	<1
FR8	5.98 ± 0.00	abc	0.66 ± 0.92	bc	<1	0.65 ± 0.92
FR9	5.40 ± 0.02	bcde	2.73 ± 0.06	a	1.77 ± 0.10	<1
FR10	6.44 ± 0.13	ab	<1	c	1.30 ± 0.00	<1

Counts are expressed as log colony forming units (cfu) g^{-1};
** For total mesophilic aerobes and spore-forming bacteria, within each producer, multiple comparisons among means were carried out through the Tukey's HSD test; within each producer means followed by different letters are significantly different ($P < 0.05$).*

The results of PCR and nested PCR are reported in Table 4.4.6.

Table 4.4.6. *Results of PCR and nested-PCR amplification of AR determinants in of ready-to-eat edible mealworms samples from the four producers located in the Netherlands (NL1-NL10), Thailand (TH1-TH10), Belgium (BL1-BL10) and France (FR1-FR10).*

Samples	**Assays**	**Antibiotic resistance determinants**											
		erm(A)	*erm(B)*	*erm(C)*	*vanA*	*vanB*	*tet(M)*	*tet(O)*	*tet(S)*	*tet(K)*	*mecA*	*blaZ*	*aac-aph*
NL1	PCR	-	-	-	-	-	-	-	+	+	-	-	-
	n-PCR	-	-	-	-	-	+	-	n.d.	n.d.	+	-	-
NL2	PCR	-	-	-	-	-	+	-	+	-	-	-	-
	n-PCR	-	+	+	-	-	n.d.	-	n.d.	+	-	-	-
NL3	PCR	-	-	-	-	-	+	-	+	+	-	-	-
	n-PCR	-	-	+	-	-	n.d.	-	n.d.	n.d.	-	-	+
NL4	PCR	-	-	-	-	-	+	-	+	+	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	n.d.	n.d.	-	-	+
NL5	PCR	-	-	-	-	-	+	-	+	+	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	n.d.	n.d.	-	-	+
NL6	PCR	-	-	-	-	-	-	-	+	+	-	-	-
	n-PCR	-	-	-	-	-	+	-	n.d.	n.d.	-	-	-
NL7	PCR	-	-	-	-	-	+	-	+	-	-	-	-
	n-PCR	-	-	+	-	-	n.d.	-	n.d.	+	-	-	-
NL8	PCR	-	-	-	-	-	-	-	+	-	-	-	-
	n-PCR	-	+	-	-	-	+	-	n.d.	+	-	-	-
NL9	PCR	-	-	-	-	-	-	-	+	-	-	-	-
	n-PCR	-	-	+	-	-	+	-	n.d.	+	-	-	+
NL10	PCR	-	-	-	-	-	+	-	+	+	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	n.d.	n.d.	-	-	-
NL % of positivity for each determinant		0	20.0	40.0	0	0	100	0	100	100	10.0	0	40.0
TH1	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	+	-	-	-	-	-	-	-	-	-
TH2	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	-	-	-	+	-	-	-
TH3	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	-	-	+	-	-	+
TH4	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	-	-	+	-	-	-
TH5	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	+	-	-	-	-	-	+	-	-	-
TH6	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	+	-	+	+	-	-
TH7	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	-	-	+	-	-	-
TH8	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	-	-	+	-	-	+
TH9	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	-	-	-	+	-	-	-
TH10	PCR	-	-	-	-	-	-	-	+	-	-	-	-
	n-PCR	-	+	-	-	-	-	-	n.d.	+	-	-	-
TH % of positivity for each determinant		0	90.0	20.0	0	0	0	10.0	10.0	80.0	10.0	0	20.0

Samples	Assays	Antibiotic resistance determinants											
		erm(A)	*erm(B)*	*erm(C)*	*vanA*	*vanB*	*tet(M)*	*tet(O)*	*tet(S)*	*tet(K)*	*mecA*	*blaZ*	*aac-aph*
BL1	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	+	-	-	+	-	-	-
BL2	PCR	-	-	-	-	-	+	-	-	-	-	-	-
	n-PCR	-	+	-	-	-	n.d.	-	-	+	-	-	-
BL3	PCR	-	-	-	-	-	+	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	-	+	-	-	+
BL4	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	+	-	-	+	-	-	+
BL5	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	+	-	-	+	-	-	+
BL6	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	+	-	-	+	-	-	-
BL7	PCR	-	-	-	+	-	+	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	-	+	-	-	-
BL8	PCR	-	-	-	-	-	+	-	-	-	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	-	+	-	-	-
BL9	PCR	-	-	-	-	-	+	-	+	+	-	-	-
	n-PCR	-	-	-	-	-	n.d.	-	n.d.	n.d.	-	-	+
BL10	PCR	-	-	-	-	-	-	-	+	-	-	-	-
	n-PCR	-	-	-	-	-	+	-	n.d.	+	-	-	-
BL % of positivity for each determinant		0	20.0	0	10.0	0	100	0	20.0	100	0	0	40.0
FR1	PCR	-	-	-	-	-	-	-	+	-	-	+	-
	n-PCR	+	+	-	+	-	-	-	n.d.	+	-	n.d.	-
FR2	PCR	+	-	-	-	-	-	-	+	-	-	-	-
	n-PCR	n.d.	+	+	-	-	-	-	n.d.	+	-	-	-
FR3	PCR	-	-	-	-	+	-	-	+	-	-	+	-
	n-PCR	-	+	+	+	-	-	-	n.d.	+	-	n.d.	-
FR4	PCR	+	+	-	-	-	-	-	+	-	-	-	-
	n-PCR	n.d.	n.d.	-	+	-	-	-	n.d.	+	-	-	-
FR5	PCR	-	+	-	-	-	-	-	+	-	-	-	-
	n-PCR	-	n.d.	+	+	-	-	-	n.d.	+	-	-	-
FR6	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	+	+	-	-	-	-	+	-	-	-
FR7	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	-	+	-	-	-	-	+	-	-	-
FR8	PCR	-	-	-	-	-	-	-	-	-	-	-	-
	n-PCR	-	+	+	+	-	-	-	-	+	-	-	-
FR9	PCR	-	-	-	-	-	-	-	+	-	-	+	-
	n-PCR	-	+	+	+	-	-	+	n.d.	+	-	n.d.	-
FR10	PCR	-	-	-	-	-	-	-	+	-	-	-	-
	n-PCR	-	+	+	+	-	-	-	n.d.	+	-	-	-
FR % of positivity for each determinant		30.0	100	70.0	90.0	10.0	0	10.0	70.0	100	0	40.0	0
Overall % of positivity for each determinant		**7.5**	**57.5**	**32.5**	**25.0**	**2.5**	**50.0**	**5.0**	**50.0**	**95.0**	**5.0**	**10.**	**25.0**

PCR: positive after PCR; n-PCR: positive after PCR and nested PCR; n.d. not determined.

In agreement with the results of previous studies (Garofalo et al., 2017a; Milanović et al., 2016; Osimani et al., 2017b), the nested PCR assays showed a higher sensitivity (up to 7 orders of

magnitude) with respect to the corresponding PCR assays, thus allowing the number of samples considered as positive to be increased from 9.4% to 30.4%.

Regarding tetracycline resistance genes, a high prevalence of *tet(K)* was noted in all the samples assayed, with percentages of positive PCR results that ranged from 80% (samples from Thailand) to 100% (samples from the Netherlands, Belgium and France). In addition, about half of the 40 samples analyzed were positive for both *tet(M)* and *tet(S)*. Specifically, *tet(M)* was detected in 100% of the samples produced in the Netherlands and Belgium whereas no positive samples for this gene were found in mealworms produced in Thailand and France. The occurrence of *tet(S)* was highest in mealworms from the Netherlands (100%) and France (70%). Finally, there was an overall low detection of *tet(O)*, with this gene occurring only in the 10% of samples produced in Thailand and France, respectively, and being not detected at all in Belgium and the Netherlands.

Tetracyclines represent one class of antibiotics most commonly used in human and veterinary clinical practice due to their broad spectrum of activity. Resistance to these antibiotics is usually conferred by the acquisition of a tetracycline resistance gene (*tet*) encoding for efflux pump systems [e.g., *tet(K)*] or ribosomal protection [e.g., *tet(M)*, *tet(O)*, *tet(S)*] (Hwang et al., 2017; Wilcks et al., 2005). The frequencies of the four *tet* genes observed in this study are consistent with those reported by Milanović et al. (2016) and Osimani et al. (2017b) in ready-to-eat edible insects, where *tet(K)*, *tet(M)* and *tet(S)* prevailed, and only a few samples were positive for *tet(O)*. Tetracycline resistance genes were also found by Larson et al. (2008) in feed mill insects and by Pai (2013) in Gram-positive and -negative bacteria isolated from cockroaches (Periplaneta americana and Blattella germanica). In addition, Levy and Marshall (2013) argued that the honeybee gut can harbor tetracycline resistance genes as the result of selective pressures exerted by the use of tetracyclines in the honeybee industry.

Regarding resistance to erythromycin, *erm(B)* prevailed (57.5%), being retrieved in 90% and 100% of the samples produced in Thailand and France, respectively. The genes *erm(A)* and *erm(C)* were retrieved with lower frequencies, at 7.5% and 32.5% of the total samples, respectively. However, 70% of mealworms produced in France were positive for *erm(C)*, which was also detected in samples from the Netherlands and Thailand. No samples positive for *erm(C)* were found in the samples produced in Belgium. Interestingly, all the samples positive for *erm(A)* were produced in France.

Macrolides inhibit the synthesis of bacterial proteins and represent one of the most important classes of antibiotics in the clinical treatment of community-acquired pneumonia, sexually transmitted diseases, salmonellosis, and shigellosis. Resistance to these antibiotics is encoded by erythromycin ribosomal methyltransferase genes (Fyfe et al., 2016).

In the present study, the detection frequencies of *erm* genes are comparable to those reported by Milanović et al. (2016) and Osimani et al. (2017b) in various edible insects; indeed, in both studies, *erm(A)* was undetected, whereas *erm(B)* and *erm(C)* were present with frequencies ranging from 16.7 to 45.4% and from 18.2 to 26.7%, respectively. To the authors' knowledge, no other published studies are available in the scientific literature to date on the occurrence of erythromycin resistance genes in either edible or non-edible insects. The overall low occurrence of *erm* genes in the 40 samples of mealworm larvae analyzed is in agreement with data reported by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) (EFSA and ECDC, 2016). Notwithstanding, EFSA and ECDC (2016) highlighted that, although resistance to erythromycin is considered as not being subject to transfer between different strains of bacteria, horizontal transfer of resistance genes to this class of antibiotics has been described in Asia, between bacteria ascribed to genus *Campylobacter*.

Concerning genes for resistance to vancomycin, *vanA* was detected in 90% and 10% of the samples produced in France and Belgium, respectively, whereas *vanB* was exclusively found in one sample produced in France. These findings are particularly interesting since an increasing emergence of vancomycin-resistant strains of *Enterococcus faecium* has recently been reported by the ECDC (2016).

To the best of the authors' knowledge, this report is the first to describe the occurrence of resistance genes to vancomycin in edible insects; indeed, in analogous studies carried out by Milanović et al. (2016) and Osimani et al. (2017b), neither *vanA* nor *vanB* could be detected using the same PCR-based approach. Moreover, in the study carried out by Pai et al. (2005) onto house cockroaches (*Periplaneta americana* and *Blattella germanica*), no vancomycin resistant gram-positive bacteria were isolated.

Regarding beta-lactamase genes, low occurrences for both *blaZ* and *mecA* were seen in the 40 samples assayed. More specifically, no samples produced in the Netherlands, Thailand and Belgium that were positive for *blaZ*, whereas 40% of the samples produced in France were positive for this gene. Similarly, just one sample produced in the Netherlands and one in Thailand was positive for *mecA*. Regarding this latter determinant, the overall low occurrence of beta-lactamase genes is in agreement with the results of Milanović et al. (2016) and Osimani et al. (2017b). Beta-lactams are one of the first described classes of antibiotics; their anti-bacterial activity is carried out by beta-lactamases, which are enzymes capable of hydrolysing the beta-lactam ring (Queener, 1986).

As reported by the ECDC, EFSA, and European Medicines Agency (EMA) (2009), methicillin-resistant *Staphylococcus aureus* and *Clostridium difficile* can enter the food chain; moreover, strains of *Streptococcus pneumoniae*, *S. aureus* and *Mycobacterium tuberculosis* are now showing an alarming increase in resistance to beta-lactams, and their efficacy is worryingly declining (Fisher and Mobashery, 2016).

Finally, regarding the gene *aac-aph* conferring resistance to aminoglycosides, 40% of the samples produced in the Netherlands and in Belgium and 20% of the samples produced in Thailand were positive for this determinant. By contrast, no samples positive for aac-aph were found in mealworms produced in France. Aminoglycosides are broad-spectrum antibiotics routinely used against aerobic Gram-negative bacteria, for their capacity of binding to bacterial ribosomes, and hence of inhibiting the synthesis of bacterial proteins (Mingeot-Leclercq et al., 1999). Resistance to this class of antibiotics can be exerted via N- acetylation, O-nucleotidy-lation, and/or O-phosphorylation inactivation and efflux pumps (Sheikhalizadeh et al., 2017). To the authors' knowledge, there is a shortage of scientific literature on resistance to aminoglycoside antibiotics in insects. The *aac-aph* detection frequency found in this study was similar to that reported by Osimani et al. (2017b) onto grasshoppers. The fact that edible insects can constitute a reservoir of resistance to aminoglycosides has first been suggested by Larson et al. (2008), in whose study strains of *Enterococcus gallinarum* isolated from *Sitophilus zeamais* Motschulsky (maize weevil) and *Trogoderma variabile* Ballion (warehouse beetle) were resistant to streptomycin and neomycin. As reported by the ECDC (2017), aminoglycoside resistance, in combination with third-generation cephalosporin and fluoroquinolone resistances, has significantly increased in the EU/EEA (European Economic Area) between 2012 and 2015 for both *Escherichia coli* and *Klebsiella pneumoniae*, thus representing a threat for patients suffering from illnesses caused by these two human pathogens.

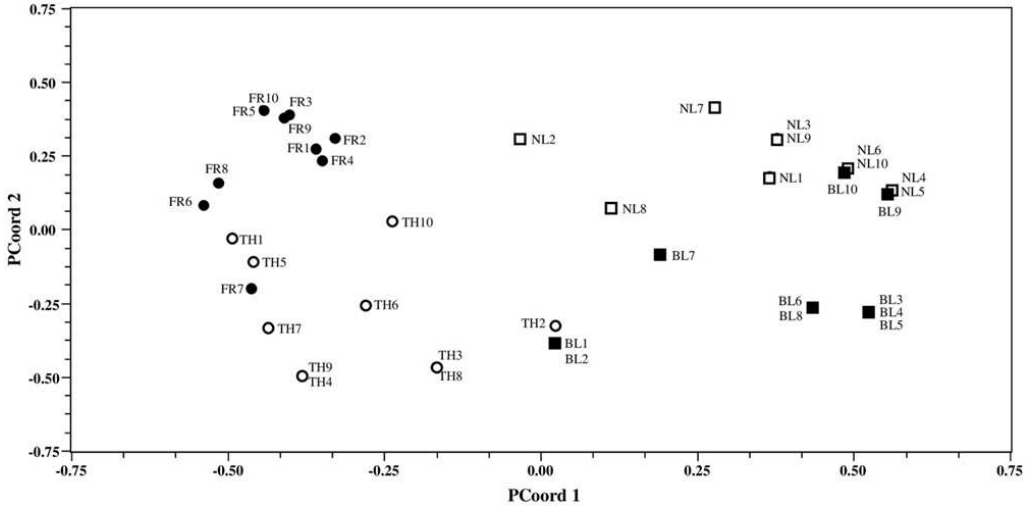
It is acknowledged that genes that confer resistance to a given antibiotic can be specifically associated, although not limited, to a specific bacterial genus or group, as already reported for the *erm* and *tet* genes (Chopra and Roberts, 2001; Leclercq, 2002; Milanović et al., 2017). To date, *tet* genes have been associated with numerous microbial genera, including *Bacillus*, *Clostridium*, *Streptococcus*,

Staphylococcus, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, and *Pseudomonas*, with *tet*+ microorganisms being increasingly isolated from various food matrices (Bulajić et al., 2015; Devirgiliis et al., 2013; Eid et al., 2015; Verraes et al., 2013). Regarding mealworms, recent studies aimed at identifying the microbiota of both fresh and dried larvae (Garofalo et al., 2017a; Osimani et al., 2017a; Stoops et al., 2016) have highlighted the presence of numerous species belonging to the above mentioned genera, thus potentially explaining the high occurrence of *tet* genes in the analyzed samples. Similarly, *erm* genes have been detected in a number of foodborne microorganisms, including *Bacillus*, *Enterobacter*, *Lactobacillus*, and *Pseudomonas* (Tang et al., 2015; Xing et al., 2014); representatives of these latter genera have previously been retrieved in mealworm larvae (Garofalo et al., 2017a; Osimani et al., 2017a; Stoops et al., 2016), thus supporting once again the hypothesis of a contribution of *erm*+ microorganisms belonging to these genera to the high occurrence of erm genes in the analyzed samples.

Further hypothesis can be formulated to correlate the detection of the remaining AR genes with microbial genera or species previously detected in mealworms larvae. At this regard, enterococci, which have very recently been detected by PCR-DGGE and pyrosequencing in dried mealworms larvae (Garofalo et al., 2017a), are known to contribute to the spread of vancomycin resistance genes in the food environment (Van den Braak et al., 1997). Regarding beta-lactamase genes, it has previously been proved that determinants responsible for resistance to beta-lactamases can effectively be carried by *Pseudomonas* spp. and *E. coli* (Wilke et al., 2005); members of both these taxa have already been detected in mealworm larvae by Stoops et al. (2016) and Osimani et al. (2017a). Finally, for the genes conferring resistance to aminoglycosides, a correlation with the occurrence of Enterobacteriaceae, such as *E. coli* and *Klebsiella* spp., recently found in the microbiota of mealworms larvae (Osimani et al., 2017a), can be hypothesized.

Results of PCoord Analysis (Figure 4.4.1) evidenced a good grouping of samples purchased from the same producer.

Figure 4.4.1. *Results of principal coordinates analysis of AR determinants showing the distribution of samples based on the first and second principal coordinates.*



Letters refer to the samples listed in Table 4.4.5.

The first principal coordinate separated samples produced in France and Thailand, which showed negative scores, from those produced in the Netherlands and Belgium, which showed positive scores. The second principal coordinate further distinguished within these two groups, separating the samples

produced in France and the Netherlands, with positive scores, from those produced in Thailand and Belgium, with negative scores. Overall, PCoord showed a separation of the four producers based on the distribution of the 12 AR determinants, though a partial overlapping of clusters of samples from the same producers was observed. PCA was further applied in order to identify the relative load of each AR determinant in the differentiation among the four producers. Eigenvalues and eigenvectors are reported in Table 4.4.7 and graphic of PCA scores in Figure 4.4.2.

Table 4.4.7*. Results of principal components analysis: eigenvalues, percentage and cumulative percentage of the variance explained by three principal component (panel a) and eigenvectors (panel b).*

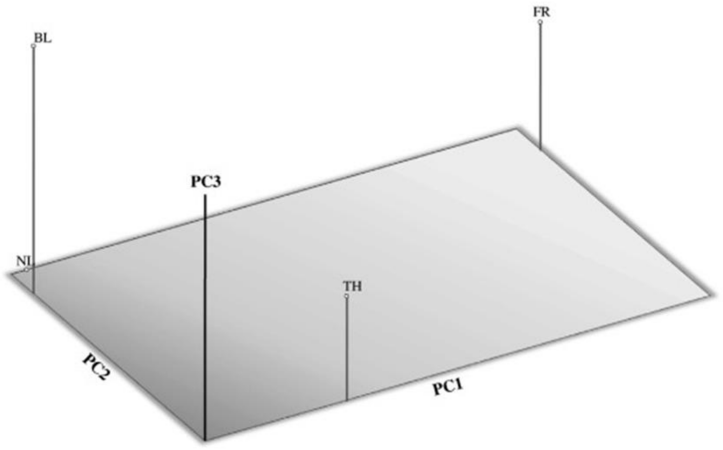
a

	PC1	**PC2**	**PC3**
Eigenvalues	7.391	3.231	1.378
Variance (%)	61.6%	26.9%	11.5%
Cumulative (%)	61.6%	88.5%	100%

b

	Eigenvectors		
	PC1	**PC2**	**PC3**
erm(A)	0.9627	0.2657	0.0515
erm(B)	0.8315	-0.5545	-0.0335
erm(C)	0.8201	0.2766	-0.5009
vanA	0.9400	0.3067	0.1491
vanB	0.9627	0.2657	0.0515
tet(M)	-0.7758	0.6296	0.0425
tet(O)	0.7758	-0.6296	-0.0425
tet(S)	0.1728	0.6924	-0.7005
tet(K)	0.0669	0.9927	0.1005
mecA	-0.4275	-0.4749	-0.7692
blaZ	0.9627	0.2657	0.0515
aac-aph	-0.9706	0.2409	-0.0013

Figure 4.4.2. *Results of Principal Component Analysis of AR determinants showing the variation characterizing the 4 producers based on AR determinant frequencies.*



Letters refer to the samples listed in Table 4.4.5.

The producer from France was clearly separated from the other three by PC1, based on a relatively higher frequency of *erm(A)*, *erm(B)*, *erm(C)*, *vanA*, *vanB*, *tet(O)*, and *blaZ*, which were characterized by highly positive eigenvector coefficients (close to +1), and a lower frequency of *tet(M)* and *aac-aph*, which were by contrast characterized by highly negative eigenvector coefficients (close to −1).

Concerning PC2, *erm(B)*, *tet(M)*, *tet(O)*, *tet(S)* and *tet(K)* discriminated the producer from Thailand from the other three producers on the basis of a lower detection frequency of *tet(M)* and *tet(S)* (characterized by highly positive eigenvector coefficients) and a higher detection frequency of *erm(B)* and *tet(O)* (characterized by highly negative eigenvector coefficients). Finally, PC3 separated the producer from the Netherlands from that from Belgium, the latter being characterized by a higher frequency of *tet(S)* and *mecA*, both characterized by highly negative eigenvector coefficients.

Overall, both PCoA and PCA could differentiate the four producers from each other; this finding suggests a potential role of geographical locations on such variability, as the result of the differences potentially occurring in clinical practices and antimicrobial usage in the respective countries of origin. This hypothesis also seems to be supported by the results previously obtained by the same authors on different edible insect species (Milanović et al., 2016; Osimani et al., 2017b).

4.4.3 Conclusions

The data overall collected in this study can effectively contribute to define a baseline for health risk assessment on edible insects as a food source. Indeed, in accordance with international food standards, the occurrence of foodborne ARs should be considered in the risk analysis of food products. Given the growing interest on the use of mealworms as a novel protein source, AR detection frequencies found in the present study suggest further insights on the use of antibiotics during rearing of this insect species and more extensive studies focused on the factors that can affect the spread of AR in the production chain. Until then, a prudent use of antibiotics during rearing of edible insects is recommended.

4.5 Investigation of the dominant microbiota in ready-to-eat grasshoppers and mealworms and quantification of carbapenem resistance genes by qPCR (Paper V)

Carbapenems are currently considered the last-line antibiotics for the treatment of severe human infections caused by multidrug-resistant Gram-negative bacteria which are able to produce carbapenemases, namely β-lactamases capable of hydrolyzing carbapenems and almost all β-lactams (Tzouvelekis et al., 2012; EFSA BIOHAZ Panel, 2013; Guerra et al., 2014; Woodford et al., 2014; Doi and Paterson, 2015; Fischer et al., 2017). Carbapenemases are encoded by genes that are easily transferable among bacteria by horizontal gene transfer events since they are located on mobile genetic elements, thus increasing their worldwide spread among bacteria in different reservoirs (Tzouvelekis et al., 2012; Woodford et al., 2014; Doi and Paterson, 2015; Fischer et al., 2017). Although carbapenemases were first discovered and mainly investigated at hospitals and healthcare facilities, carbapenemase-producers (CPE) and carbapenemase-encoding genes (CEG) were detected in animals, the environment and food (Guerra et al., 2014). In this context, since insects are increasingly considered as an alternative suitable protein source, they deserve great attention in terms of safety, including the assessment of the microbiota the incidence of AR genes, in particular CEG.

Therefore, in order to obtain an overview of the predominant bacterial species in samples of processed edible mealworms (*Tenebrio molitor* L.) and grasshoppers (*Locusta migratoria migratorioides*) samples from producers in Europe (Belgium and the Netherlands) and Asia (Thailand) were subjected to culture-independent PCR-DGGE and screening by quantitative PCR (qPCR) of five among the most common carbapenem resistance genes (*blaNDM−1*, *blaVIM*, *blaGES*, *blaOXA−48*, and *blaKPC*) (Monteiro et al., 2012).

4.5.1 Materials and methods

Sampling

Thirty samples of edible mealwors and thirty samples of grasshoppers (boiled, dried, and salted) were purchased via the internet from dealers located in Europe (Belgium and the Netherlands) and Asia (Thailand). Ten mealworm and ten grasshopper samples from each country were collected and marked as follows: TN1-TN10 (mealworms from the Netherlands, Producer 1), TB1-TB10 (mealworms from Belgium, Producer 2), TT1-TT10 (mealworms from Thailand, Producer 3), GN1-GN10 (grasshoppers from the Netherlands, Producer 1), GB1-GB10 (grasshoppers from Belgium, Producer 2), and GT1-GT10 (grasshoppers from Thailand, Producer 3). All the samples were provided in sealed plastic containers and delivered at ambient temperature via international shipping. No information was available on the rearing and hygiene conditions of processing, transport and storage applied to these edible insects before marketing.

Bacterial DNA Extraction

Total microbial DNA was extracted directly from the insect samples using PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) as described by Osimani et al. (2017a). The extracted DNA was quantified and checked for the purity using a NanoDrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and then standardized to 2 ng μL^{-1} for qPCR assays and to 25 ng μL^{-1} for PCR-DGGE analysis. The effective extraction of bacterial DNA was confirmed by conventional PCR amplification of 2 µL (50 ng) of extracted DNA suspensions in a My Cycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using universal prokaryotic primers 27F and 1495R as described by Osimani et al. (2015).

PCR-DGGE Analysis

The equal portions of DNAs extracted from insects were mixed together with the goal of obtaining six pooled samples (TB, TN, TT, GB, GN, and GT), each representing an insect type (mealworms and grasshoppers) and country of origin (Belgium, the Netherlands, and Thailand). The amplification products obtained from the 27F-1495R primer pair as described above were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Subsequently, 2 µL of the purified PCR products was reamplified using the universal prokaryotic primers U968GC (added with GC clamp) and L1401 (Muyzer et al., 1993; Randazzo et al., 2002) following the PCR conditions previously described by Aquilanti et al. (2013). Following amplification, 5 µL of the PCR reaction was loaded on a 1.5% agarose gel together with a 100 bp molecular weight marker (HyperLadderTM 100 bp) to check for the expected product size of 480 bp. Twenty microliters of these PCR amplicons were analyzed by DGGE (30–60% urea-formamide denaturing gradient; 4 h at 130 V) using DCode Universal Mutation Detection System (Bio-Rad Laboratories) as described by Garofalo et al. (2015). All DGGE bands visible under UV light were excised from the gel, and the DNA was eluted overnight at 4°C in 50 µL of molecular biology grade water (Garofalo et al., 2008) and reamplified via PCR as described above, but with the

forward primer U968 lacking the GC clamp. The PCR products were sent to Genewiz (Takeley, UK) for purification and sequencing, and the obtained sequences were identified at species level as described by Osimani et al. (2018c).

Reference Strains

DNA extracted from five reference strains (Table 4.5.1), each carrying one of the carbapenem resistance genes under study, was used as positive control in the qPCR reactions and for the construction of qPCR standard curves.

Table 4.5.1. *Bacterial reference strains carrying carbapenems resistance genes, used as positive controls in the qPCR reactions.*

Bacterial strain	Carbapenems resistance gene
BAA 2146_ Klebsiella pneumoniae	blaNDM-1
LEMC_VIM-1_ Pseudomonas aeruginosa	blaVIM-1
LEMC_GES-1_ Pseudomonas aeruginosa	blaGES-1
LEMC_OXA-48_ Klebsiella pneumoniae	blaOXA-48
ATCC 1705_ Klebsiella pneumoniae	blaKPC

qPCR

Absolute quantification of each carbapenemase gene (*blaNDM−1*, *blaVIM*, *blaGES*, *blaOXA−48*, and *blaKPC*) in the insect samples was performed by qPCR in a Mastercycler® ep realplex machine (Eppendorf, Hamburg, Germany) using the qPCR primers and cycling conditions described by Monteiro et al. (2012). To check for product specificity, all cycles were followed by a melt curve step analysis with temperature gradually increasing from 65 to 95°C by 0.2°C/s. Each qPCR reaction consisted of 4 µL (8 ng) of the extracted DNA; 5 µL of Type-it 2X HRM PCR Master Mix (Qiagen, Hilden, Germany) containing HotStarTaq Plus DNA Polymerase, EvaGreen Dye, an optimized concentration of Q-solution, dNTPs and MgCl2; 0.2 µM of forward and reverse primers for each gene; and nuclease-free molecular biology grade water to a final reaction volume of 10 µL. The exogenous standards for each gene were prepared by qPCR amplification of the DNA extracted from the reference strains (Table 4.5.1) as described above but in a final reaction volume of 25 µL. The correct melting temperatures (Tm) and sizes of the obtained PCR products were checked by melting curve analysis and electrophoresis on a 1.5% agarose gel, respectively. The Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) was used for the purification of the amplicons following the manufacturer's instructions. The quantity and the purity of the products were determined using the NanoDrop ND 1000 (Thermo Fisher Scientific). The calculation of each gene copy number was performed using an online calculator (www.idtdna.com) based on the mass and size of the purified qPCR products. The standard curves were created by qPCR amplification of 10-fold serial dilutions of exogenous standards. The amplification efficiencies were estimated from the slopes of the standard curves, and the correlation coefficients (R^2) (Stolovitzky and Cecchi, 1996) were calculated automatically by Mastercycler® ep realplex software. To determine the qPCR detection limit for each gene, the standard curves were generated in the range from ~1 to 10^7 gene copies per reaction.

For the absolute quantification of CEG, the DNAs extracted from mealworms and grasshoppers were run along with the 10-fold serial dilutions of the standards prepared as described above. The gene copy number of each gene detected in the analyzed insect samples was determined using the slope of the corresponding standard curves. The baseline and threshold calculations were performed automatically by the Mastercycler® ep realplex software. In addition to melting curve analysis, the correct sizes of the amplification products were checked by electrophoresis on 1.5% agarose gels using 100 bp DNA Ladder (HyperLadderTM 100 bp, Bioline, UK) as a molecular weight marker.

Moreover, the accuracy of the amplification reactions was validated by the sequencing (Genewiz) of randomly selected positive samples (TB6, GT7, and GN1 for the *blaOXA−48* gene; GT2 and TT8 for the *blaNDM−1* gene; GT7 and GT8 for the blaVIM gene). The resulting sequences were compared with those from the GenBank database (http://www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). All qPCR reactions were performed in triplicate and the results were expressed as the mean gene copy number per ng of DNA ± standard deviation for each gene.

Statistical Analysis

Descriptive statistics, calculated on 20 samples for each producer and on 30 samples for each insect species were carried out for the *blaOXA−48*, *blaNDM−1*, and *blaVIM* gene copies by computing the means ± standard deviation.

After first checking for conformance to a normal distribution and identification of outliers, analysis of variance (ANOVA) was carried out using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA) to test the following main effects: producers (Belgium, Thailand, the Netherlands), insect species (mealworm, grasshoppers) and producer x species.

Principal component analysis (PCA) was applied to discriminate among mealworms and grasshoppers coming from different producers (Belgium, the Netherlands, and Thailand), and the presence of genes related to resistance to carbapenems (*blaNDM−1*, *blaVIM*, *blaGES*, *blaOXA−48*, and *blaKPC*). PCA was carried out using the Unscrambler 7.5 software (CAMO ASA, Oslo, Norway). The mean data were normalized to neutralize any influence of hidden factors. PCA provides a graphical representation of the overall differences in terms of distribution of genes between the insects coming from different producers.

4.5.2 Results and discussion

In the present study, the microbiota of commercialized read-to-eat grasshoppers and mealworms from different countries was investigated via PCR-DGGE, as well as the quantification and distribution of five common CEG within the same matrices, in order to have a more complete picture of some safety aspects related to edible insects.

To have an overview of the predominant bacterial species found in the edible insects considered in this study, the total microbial DNA was extracted from the samples, the DNAs were mixed in order to obtain six pooled samples, each representing an insect type (mealworms, grasshoppers) and country of origin (Belgium, the Netherlands, Thailand) and then analyzed by a culture-independent PCR-DGGE method. The results obtained are reported in Table 4.5.2.

The dominant species found in mealworms from Belgium and grasshoppers from the Netherlands belonged to the genus *Staphylococcus*, and species in the genus *Bacillus* were found in mealworms and grasshoppers from Thailand. Grasshoppers from Belgium were positive for *Weissella cibaria/confusa/*spp., while bacterial species with a percentage identity below 97% were found in mealworms from the Netherlands. It is interesting to note that the predominance of LAB, and in particular the *Weissella* spp., has been reported for processed and fresh grasshoppers from Belgium and the Netherlands (Stoops et al., 2016; Garofalo et al., 2017a; Osimani et al., 2017a), thus suggesting that the specific rearing conditions may have selected for this microbial group or that this bacterial species is intrinsically associated with this edible insect.

Table 4.5.2*. Sequencing results of the bands excised from the DGGE gel obtained from the amplified fragments of pooled bacterial DNA extracted directly from mealworms and grasshoppers.*

Sample	Closest relative	% Identity[a]	Acc.no[c]
TB	*Staphylococcus warneri*	98%	MH211317
	Staphylococcus pasteuri	98%	MH158278
	Staphylococcus sp.	98%	MH191108
	Staphylococcus kloosii	99%	CP027846
	Staphylococcus cohnii	99%	KY012323
	Staphylococcus sp.	99%	KY865752
TN	*Exiguobacterium sp.*	82%	MG859628
	Eikenella corrodens	90%	KU663108
	Eikenella sp.	90%	KU738863
	Neisseria shayeganii	90%	KM462144
TT	*Bacillus sp.*	99%	MG757948
	Bacillus sp.	99%	LT899995
GB	*Weissella cibaria*	99%	CP027427
	Weissella confusa	99%	MF327674
	Weissella sp.	99%	MG814036
GN	*Staphylococcus haemolyticus*	99%	MH179468
	Staphylococcus argenteus	99%	LC378381
	Staphylococcus sp.	99%	MH021651
	Staphylococcus hominis	99%	MF327701
	Staphylococcus aureus	99%	MG976640
GT	*Bacillus sonorensis*	99%	KY243955
	Bacillus subtilis	99%	KU172428
	Bacillus amyloliquefaciens	99%	KJ126909
	Bacillus axarquiensis	99%	KJ126897
	Bacillus sp.	100%	LT899995

TB- pool of 10 (TB1-TB10) mealworm samples from Belgium; TN- pool of 10 (TN1-TN10) mealworm samples from the Netherlands; TT- pool of 10 (TT1-TT10) mealworm samples from Thailand; GB- pool of 10 (GB1 GB10) grasshopper samples from Belgium; GN- pool of 10 (GN1-GN10) grasshopper samples from the Netherlands; GT- pool of 10 (GT1-GT10) grasshopper samples from Thailand;

[a]Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database;

[b]Accession number of the sequence of the closest relative found by a BLAST search.

The genera *Staphylococcus* and *Bacillus* identified among the other pooled samples of mealworms and grasshoppers may contain pathogenic species such as *Staphylococcus aureus* and *Bacillus cereus*, and these data are in agreement with other studies on the microbiota in fresh and processed mealworms and grasshoppers (Stoops et al., 2016; Garofalo et al., 2017a). The presence of *Staphylococcus* members may be due to an environmental contamination occurring during human handling or processing. Indeed, these insects were boiled and salted, and since *Staphylococcus* spp. is a halophile bacterium usually predominating in environments with low microbial competition, it could have found conditions suitable for growth. The lack of detection of bacterial DNA belonging to the Enterobacteriaceae family has been already reported by Osimani et al. (2018c), although it is generally reported that Enterobacteriaceae represents a predominant bacterial group in the edible insect gut microbiota (Stoops et al., 2016; Garofalo et al., 2017; Osimani et al., 2017c). These data suggest that good manufacturing practices were applied during rearing and processing of the insects and/or that a degutting step may have been applied. A further explanation is that members of the Enterobacteriaceae family could be present within the processed insect samples but with a lower abundance in respect with other microbial groups. This latter hypotesis is supported by the fact that these microorganisms had previously found below the detection limit of microbial counts (<1 log cfu g^{-1}) in the same samples (Osimani et al., 2017b,c). Additionally, the lack of detection of bacterial DNA belonging to Pseudomonadaceae is unusual, but it is possible, as suggested for Enterobacteriaceae,

that PCR-DGGE was not able to detect members of this microbial group if they were in the minority relative to the others.

This study represents the first report on the screening of five carbapenemase encoding genes (*blaNDM−1*, *blaVIM*, *blaGES*, *blaOXA−48*, and *blaKPC*) in processed edible mealworms (*T. molitor* L.) and edible grasshoppers (*L. migratoria migratorioides*) from producers located in Europe (Belgium and the Netherlands) and Asia (Thailand).

The identification of the genes coding for these carbapenemases in the samples of edible insects under investigation was conducted by using qPCR. As previously underlined by Monteiro et al. (2012), molecular assays are considered the best solutions for the rapid detection of carbapenem resistance genes and for the identification of the resistance mechanism.

The detection limit, defined as the lowest concentration at which 95% of the positive samples are detected was <10 gene copies per reaction for all the genes. The efficiencies of the qPCR reactions were 1.00 for the genes *blaOXA−48*, *blaVIM*, and *blaGES*; 1.01 for the gene *blaKPC* and 0.96 for the gene *blaNDM−1*. The R^2 was 1.000 for the genes *blaOXA−48* and *blaNDM−1*; 0.999 for the gene *blaGES*, 0.996 for the gene *blaKPC* and 0.995 for the gene *blaVIM*. Moreover, the specificity of the primers used for the amplification of carbapenem resistance genes was confirmed by the results of the sequencing of randomly selected positive samples, which showed >97% similarity with the corresponding gene sequences deposited in the GenBank database. In more detail, the results of the BLAST analysis for the *blaOXA−48* gene showed 98% of the similarity with the sequences deposited in GenBank such as *K. pneumoniae* (CP031374), *Citrobacter freundii* (MG430338), *Enterobacter ludwigii* (MG436907), *Pantoea agglomerans* (MG436898), *Escherichia coli* (NG_055499), *Shewanella* sp. (NG_055475), and *Proteus mirabilis* (KT175900); for the *blaNDM−1* gene 99% similarity with the *Enterobacter hormaechei* (AP018835*), K. pneumoniae* (AP018834), *Raoultella planticola* (MH257689), *E. coli* (CP021206), *Pseudomonas aeruginosa* (KT364224), and *Acinetobacter baumannii* (KU180703); and for the *blaVIM* gene 98% similarity with the *K. pneumoniae* (MH071811), *C. freundii* (NG_061412), *Paenibacillus* sp. (KR822172), *E. coli* (MF169879), *E. hormaechei* (LT991955), *Kluyvera cryocrescens* (MG228427), *Alcaligenes faecalis* (KY623659), *Klebsiella oxytoca* (NG_050362), and *Enterobacter cloacae* (CP030081).

The results of the qPCR quantification of carbapenem resistance genes in samples of ready-to-eat edible mealworms and grasshoppers produced in the Netherlands, Belgium and Thailand are reported in Tables 4.5.3 and 4.5.4.

Regarding mealworms, none of the samples were positive for the genes blaGES, blaKPC, and blaVIM, while only sample TB6 was found positive for *blaOXA−48* (3% positivity) and samples TN2, TN8, and TT8 were positive for *blaNDM−1* (10% positivity) (Table 4.5.3).

Concerning grasshoppers, the genes *blaGES* and *blaKPC* were not detected in any of the analyzed samples while only two samples from Thailand (GT7 and GT8) were positive for the blaVIM gene (7% positivity). Interestingly, a high prevalence of *blaOXA−48* was noted (57% positivity), followed by blaNDM−1 (27% positivity) (Table 4). Specifically, *blaOXA−48*was prevalent in 80% of the samples from Belgium, in 50% of the samples from the Netherlands and in 40% of the samples from Thailand. The highest frequency of *blaNDM−1* was found among samples from Thailand (40%), followed by samples from Belgium (30%) and the Netherlands (10%) (Table 4.5.4).

Table 4.5.3. *Results of qPCR quantification of carbapenemase AR genes in samples of ready-to-eat edible mealworms produced in The Netherlands (TN1-TN10), Belgium (TB1-TB10) and Thailand (TT1-TT10).*

Producer	**Samples**	**Carbapenemase resistant genes (gene copies ng^{-1}± standard deviation)**				
		GES-1	*KPC*	*OXA 48*	*NDM-1*	*VIM-1*
1	TN1	n.d.	n.d.	n.d.	n.d.	n.d.
	TN2	n.d.	n.d.	n.d.	2,29±0.31	n.d.
	TN3	n.d.	n.d.	n.d.	n.d.	n.d.
	TN4	n.d.	n.d.	n.d.	n.d.	n.d.
	TN5	n.d.	n.d.	n.d.	n.d.	n.d.
	TN6	n.d.	n.d.	n.d.	n.d.	n.d.
	TN7	n.d.	n.d.	n.d.	n.d.	n.d.
	TN8	n.d.	n.d.	n.d.	0,94±0.13	n.d.
	TN9	n.d.	n.d.	n.d.	n.d.	n.d.
	TN10	n.d.	n.d.	n.d.	n.d.	n.d.
	TN % of positivity for each determinant	n.dr.	n.dr.	n.dr.	20	n.dr.
2	TB1	n.d.	n.d.	n.d.	n.d.	n.d.
	TB2	n.d.	n.d.	n.d.	n.d.	n.d.
	TB3	n.d.	n.d.	n.d.	n.d.	n.d.
	TB4	n.d.	n.d.	n.d.	n.d.	n.d.
	TB5	n.d.	n.d.	n.d.	n.d.	n.d.
	TB6	n.d.	n.d.	1,81±0.01	n.d.	n.d.
	TB7	n.d.	n.d.	n.d.	n.d.	n.d.
	TB8	n.d.	n.d.	n.d.	n.d.	n.d.
	TB9	n.d.	n.d.	n.d.	n.d.	n.d.
	TB10	n.d.	n.d.	n.d.	n.d.	n.d.
	TB % of positivity for each determinant	n.dr.	n.dr.	10	n.dr.	n.dr.
3	TT1	n.d.	n.d.	n.d.	n.d.	n.d.
	TT2	n.d.	n.d.	n.d.	n.d.	n.d.
	TT3	n.d.	n.d.	n.d.	n.d.	n.d.
	TT4	n.d.	n.d.	n.d.	n.d.	n.d.
	TT5	n.d.	n.d.	n.d.	n.d.	n.d.
	TT6	n.d.	n.d.	n.d.	n.d.	n.d.
	TT7	n.d.	n.d.	n.d.	n.d.	n.d.
	TT8	n.d.	n.d.	n.d.	3,38±0.18	n.d.
	TT9	n.d.	n.d.	n.d.	n.d.	n.d.
	TT10	n.d.	n.d.	n.d.	n.d.	n.d.
	TT % of positivity for each determinant	n.dr.	n.dr.	n.dr.	10	n.dr.
	Overall % of positivity for each determinant	n.dr.	n.dr.	3	10	n.dr.

n.d., not detected; n.dr., not determined.

Table 4.5.4. *Results of qPCR quantification of carbapenemase AR genes in samples of ready-to-eat edible grasshoppers produced in The Netherlands (GN1-GN10), Belgium (GB1-GB10) and Thailand (GT1-GT10).*

Producer	**Samples**	**Carbapenemase resistant genes (gene copies ng^{-1}± standard deviation)**				
		GES-1	*KPC*	*OXA 48*	*NDM-1*	*VIM-1*
1	GN1	n.d.	n.d.	1,17±0.19	n.d.	n.d.
	GN2	n.d.	n.d.	0,24±0.02	n.d.	n.d.
	GN3	n.d.	n.d.	n.d.	n.d.	n.d.
	GN4	n.d.	n.d.	0,86±0.09	n.d.	n.d.
	GN5	n.d.	n.d.	n.d.	n.d.	n.d.
	GN6	n.d.	n.d.	n.d.	n.d.	n.d.
	GN7	n.d.	n.d.	0,89±0.01	n.d.	n.d.
	GN8	n.d.	n.d.	0,30±0.04	n.d.	n.d.
	GN9	n.d.	n.d.	n.d.	2,64±0.19	n.d.
	GN10	n.d.	n.d.	n.d.	n.d.	n.d.
	GN % of positivity for each determinant	n.dr.	n.dr.	50	10	n.dr.
2	GB1	n.d.	n.d.	0,57±0.02	n.d.	n.d.
	GB2	n.d.	n.d.	0,73±0.01	1,85±0.05	n.d.
	GB3	n.d.	n.d.	0,30±0.08	2,25±0.12	n.d.
	GB4	n.d.	n.d.	0,83±0.01	n.d.	n.d.
	GB5	n.d.	n.d.	0,35±0.11	n.d.	n.d.
	GB6	n.d.	n.d.	0,24±0.01	n.d.	n.d.
	GB7	n.d.	n.d.	n.d.	n.d.	n.d.
	GB8	n.d.	n.d.	n.d.	n.d.	n.d.
	GB9	n.d.	n.d.	0,58±0.03	n.d.	n.d.
	GB10	n.d.	n.d.	0,25±0.05	6,37±0.22	n.d.
	GB % of positivity for each determinant	n.dr.	n.dr.	80	30	n.dr.
3	GT1	n.d.	n.d.	0,30±0.1	10,31±0.52	n.d.
	GT2	n.d.	n.d.	8,65±1.29	70,38±3.01	n.d.
	GT3	n.d.	n.d.	n.d.	n.d.	n.d.
	GT4	n.d.	n.d.	n.d.	24,88±0.71	n.d.
	GT5	n.d.	n.d.	n.d.	n.d.	n.d.
	GT6	n.d.	n.d.	n.d.	n.d.	n.d.
	GT7	n.d.	n.d.	16,56±0.48	51,13±1.59	392,25±1.77
	GT8	n.d.	n.d.	n.d.	n.d.	42,83±6.82
	GT9	n.d.	n.d.	0,59±0.01	n.d.	n.d.
	GT10	n.d.	n.d.	n.d.	n.d.	n.d.
	GT % of positivity for each determinant	n.dr.	n.dr.	40	40	20
	Overall % of positivity for each determinant	n.dr.	n.dr.	57	27	7

n.d., not detected; n.dr., not determined.

All the insect samples analyzed in this study were previously screened for the presence of 12 selected genes coding for resistance to tetracyclines [*tet(M)*, *tet(O)*, *tet(S)*, and *tet(K)*], macrolide-lincosamide-streptogramin B (MLSB) [*erm(A)*, *erm(B)*, *erm(C)*], vancomycin (vanA and vanB), beta-lactams (*blaZ* and *mecA*) and aminoglycosides [*aac(6')-Ie aph(2'')-Ia* referred as *aac-aph*] through PCR and nested PCR assays (Osimani et al., 2017b,c). It is interesting to note that the mealworms samples TN2 and TN8 from the Netherlands were also found to be positive for the presence of genes coding for resistance to tetracyclines [*tet(M)*, *tet(K)*, *tet(S)*] and erythromycin [*erm(B)*, *erm(C)*], while sample TB6 was positive for genes coding for resistance to tetracyclines [*tet(M)*, *tet(K)*], and sample TT8 from Thailand was positive for genes coding for resistance to tetracyclines [*tet(K)*], erythromycin [*erm(B)*], and aminoglycosides (*aac-aph*) (Osimani et al., 2017c). Moreover, among grasshoppers, almost all of the samples that were found to be positive *blaOXA−48*, *blaNDM−1*, and *blaVIM* previously showed positivity for AR genes coding for resistance to tetracyclines [*tet(M)*, *tet(S)*, *tet(K)*], erythromycin [*erm(B)*, *erm(C)*], aminoglycosides (*aac-aph*) and beta-lactams (*blaZ*) (Osimani et al., 2017b). The average levels of gene copies ng^{-1} in the 60 samples of edible insects were as follows: 0.59 ± 2.39 with a minimum value of 0 and a maximum value of 16.56 for *blaOXA−48*, 2.94 ± 11.53 with a minimum value of 0 and a maximum value of 70.37 for *blaNDM−1*, 7.25 ± 50.85 with a minimum value of 0 and a maximum value of 392.25 for *blaVIM*.

Descriptive statistics on 20 samples from each producer are shown in Table 4.5.5. In addition, descriptive statistics on 30 samples from each insect species are reported in Table 4.5.6.

Table 4.5.5. *Descriptive statistics on 20 samples for each producer.*

	Belgium			**Thailand**			**The Netherland**		
	OXA-48	*NDM*	*VIM*	*OXA-48*	*NDM*	*VIM*	*OXA-48*	*NDM*	*VIM*
Mean	0.28	0.52	0.00	1.31	8.00	21.75	0.17	0.29	0.00
SD	0.45	1.51	0.00	4.07	19.22	87.73	0.36	0.77	0.00
Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Maximum	1.81	6.37	0.00	16.56	70.37	392.25	1.17	2.64	0.00

SD standard deviation values are expressed as gene copies ng^{-1}.

Table 4.5.6. *Descriptive statistics on 30 samples for each insect species.*

	Mealworm			**Grasshoppers**		
	OXA-48	*NDM*	*VIM*	*OXA-48*	*NDM*	*VIM*
Mean	0.06	0.22	0	1.11	5.66	14.5
SD	0.33	0.74	0	3.31	15.96	71.77
Minimum	0	0.00	0	0	0	0
Maximum	1.81	3.37	0	16.56	70.37	392.25

SD standard deviation; values are expressed as gene copies ng^{-1}.

The analysis of variance (Table 4.5.7) showed that all the variables (producers, species, and producers × species) had significant effects ($P < 0.05$) on the frequency of *blaNDM−1*, whereas for *blaOXA−48* and *blaVIM*, no significant effects were detected for the same source of variation.

Table 4.5.7. *ANOVA results for blaOXA-48, blaNDM-1, blaVIM-1.*

Source of variation	df	blaOXA-48			blaNDM-1			blaVIM		
		SS	MS	P	SS	MS	P	SS	MS	P
Producer	2	15.60	7.80	0.24	769.50	384.75	0.04	6309.68	3154.84	0.30
Species	1	16.64	16.64	0.08	443.85	443.85	0.05	3154.84	3154.84	0.27
Producer × species	2	18.26	9.31	0.19	736.79	368.39	0.04	6309.68	3154.84	0.30

*n.s. not significant; * significant at $P < 0.05$.*

Regarding the distribution of *blaOXA−48* and *blaVIM*, multiple comparisons between ACC Least Square Means (LSM) carried out using the Tukey test showed no significant differences among samples from different producers or insect species. Regarding *blaNDM−1*, multiple comparisons (Tukey HSD) showed that insect species, but not the origin of the sample, had a significant correlation ($P < 0.05$) with the frequency of the gene.

PCA did not discriminate between the presence of genes encoding resistance to carbapenems among mealworms and grasshoppers coming from different producers. In contrast, in the previous studies on the occurrence of transferable ARs in ready-to-eat edible insects, PCA showed a differentiation among producers, thus suggesting that different rearing and clinical practices associated with different countries may have played a role in the variability observed (Milanovic´ et al., 2016; Osimani et al., 2017b,c).

As reported by Schlüter et al. (2017), it is presumed that the rearing and processing conditions applied to edible insects will comply with the same food safety regulations as for livestock farming. The use of carbapenems is prohibited in food-producing animals in all countries (OIE, 2015; Webb et al., 2016). Notwithstanding, scientific studies reporting CPE and CEG in livestock and their environment are progressively more frequent (Guerra et al., 2014; Webb et al., 2016; Zurfluh et al., 2016; Fischer et al., 2017). Furthermore, in the last EFSA report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015, the presumptive extended-spectrum beta-lactamase (ESBL)- /AmpC-/carbapenemase-production in *Salmonella* and *E. coli* was monitored in humans, meat (pork and beef), fattening pigs and calves for the first time (EFSA and ECDC, 2017). Varying occurrence/prevalence rates of ESBL-/AmpC-producers were observed between countries, and carbapenemase-producing *E. coli* were detected in single samples of pig meat and from fattening pigs from two Member States (EFSA and ECDC, 2017). These data indicate that other antimicrobial classes could indirectly select CPE outside the hospital setting and that the rapid dissemination of CPE is also promoted by CEG located on plasmids transmissible by horizontal gene transfer events (Tzouvelekis et al., 2012; Woodford et al., 2014). As reviewed by Caniça et al. (2015), AR comprises a dynamic network that involves several environmental niches (e.g., water, soil, and plants) and different reservoirs (e.g., husbandry, hospitals, wild animal, human settings, human hand, food and global trade in foodstuffs) in which the path of dissemination and dynamics of AR genes has to be taken into consideration in order to understand and prevent the AR transmission and spread. Therefore, it is possible to hypothesize that, irrespective of the use of carbapenems in the edible insect rearing, the CEG may derive from the substrates used for feed or from surfaces and hands of operators or from treatments applied for processing, in addition to transport and storage. It is also interesting to note that grasshoppers and mealworms have different dietary habits since grasshoppers are grass-feeders whereas mealworms are usually reared on cereal-based matrices; therefore, the differences in terms of presence and distribution of CEG among these insect species may derive from different rearing practices and substrates.

4.5.3 Conclusions

Edible insects such as grasshoppers and mealworms represent a novel food that deserves attention in terms of safety, including the assessment of the incidence of AR genes. The investigation of the microbiota of the mealworm and grasshopper samples in this study revealed the presence of potential pathogenic and non-pathogenic species. Scientific studies reporting carbapenemase-producing microorganisms and CEG in animals, the environment and food are increasingly frequent.

The data presented in this study is the first attempt aimed at determining the incidence of CEG among samples of commercialized ready-to-eat grasshoppers and mealworms from Belgium, the Netherlands and Thailand. Although further studies are necessary to understand the correlation of CEG with the insect microbiota and to assess the possible role of edible insects as reservoirs of resistance to carbapenems, an intensified surveillance plan examining the occurrence of CEG in the food chain and in different environmental compartments, along with a prudent use of carbapenems and antimicrobials in general, are primary measures that should be applied.

5. Development of new insect-based food products

The present chapter reports three studies about the development of insect-based bread and other baked goods aimed to improve their nutritional quality and to assess consumers acceptance about food products containing edible insects. In more detail, a focus on microbiological and technological analyses was performed in the course of the bread-making assays using cricket (*A. domesticus*) (cfr. paragraph 5.1), mealworm (*T. molitor*) (cfr. paragraph 5.2) or lesser mealworm (*A. diaperinus*) powder (cfr. paragraph 5.3). The results presented in the following chapter regard the activities carried out by Food Microbiology research area of D3A of UNIVPM within already published or under review collaboration studies. Further investigations (proximate, aminoacids, fats and minerals composition) about studies included in paragraphs 5.1, 5.2 and 5.3 can be found in Paper III, Paper VI and Paper X.

5.1 Cricket (*Acheta domesticus*) powder-based bread: a real opportunity for dietary high quality nutrients intake? (Paper III)

Recently, edible insects are increasingly attracting the attention of researchers and food industry for their high nutritional value (Rumpold and Schlüter, 2013). Even though, entomophagy is a customary practice in Asia, Africa and America, in Western European Countries is still far from being achieved for psychological rather than rational reasons (Poma, et al., 2017). Indeed, only in 2015 EU has dealt edible insects including them in the "*novel food*" category (Regulation EU 2015/2283). Moreover in the same year, EFSA Scientific Committee edited an opinion in which listed the insect species to be consumed as food and feed in the EU and declared the necessity to get in depth information about potential risks related to them (EFSA, 2015).

In this context, a possible approach to overcome the consumers reluctance towards insects as food could be to develop food products where insects are masked (Stoops et al., 2017). Furthermore, a microbiological analysis of these products is a useful approach to get useful information for the evaluation of the risks related to their consumption.

In particular, the aim of this study was the evaluation of cricket (*Acheta domesticus*) powder in bread-making to obtain bread with an enhanced nutritional value. In more detail, technological and microbiological traits of the obtained doughs and bread loaves were carried out to investigate about their behaviour and microbial dynamics during leavening and after baking, respectively. Finally, obtained bread loaves were subjected to sensory analysis to test the consumers acceptance.

5.1.1 Materials and methods

Raw materials

Wheat flour (WF) was purchased from a local mill (Molino Mariani, Senigallia, Italy), whereas cricket (*A. domesticus*) powder (CP) from a producer located in Thailand. Samples of cricket powder were shipped in 1 kg plastic bag packages by international express transport and stored at ambient temperature until use. As reported by the producer, crickets used to produce powder were fed a diet of mixed grains and vegetables; no other information was available on the rearing of crickets as well as hygiene conditions during powder processing, transport and storage before purchasing.

Lactic acid bacteria strains

For the production of sourdough, the following five lactic acid bacteria (LAB) strains were used: *Lactobacillus sanfranciscensis* PB276, *Lactobacillus sanfranciscensis* PB223, *Lactobacillus plantarum* PB11, *Lactobacillus plantarum* PB24, *Lactobacillus fermentum* PB162. All LAB belonged to the Dipartimento di Scienze Agrarie, Alimentari ed Ambientali (D3A) culture collection. Selected strains were those isolated from type I sourdoughs collected in the Marche region (central Italy) and already characterized by Osimani et al. (2011) for their acidifying activity, amylase activity, starch hydrolysis and production of lactate, acetate and CO2. Before use, the frozen stored cultures were grown on appropriate media. All strains but *Lactobacillus sanfranciscensis* PB276 and PB223 were grown on modified De Man, Rogosa and Sharp (mMRS) agar, supplemented with 1% maltose (w/v) plus 5% fresh yeast extract (v/v) incubated under anaerobiosis using the AnaeroGen 2.5 System (Oxoid, Basingstoke, UK) at 30°C for 48 h (Taccari et al., 2016). *Lactobacillus sanfranciscensis* PB276 and PB223 were grown on Sourdough Bacteria (SDB) medium modified according to Vogel et al. (1994) incubated under anaerobic conditions using the AnaeroGen 2.5 System (Oxoid) at 30°C for 48 h.

Production of sourdough

Selected LAB were used as pool to start wheat flour as previously described by Coda et al. (2010), with some modifications. Briefly, the five LAB cultures were separately grown in mMRS broth at 30°C for 12 h, each broth culture was harvested by centrifugation at 4000 rpm for 5 min, washed and resuspended in sterile water. The sourdough (SD) was obtained by mixing the pool of LAB, wheat flour and sterilized water to reach dough yield 167 (60 g/100 g wheat flour plus 40 g/100 g water). The obtained dough, at a LAB concentration of 8 log cfu g^{-1}, was left to ferment at 30°C for 16 h.

Doughs composition and bread-making

Two different concentrations of CP (10% and 30%) were used to produce experimental bread loaves. Moreover, bread loaves produced with the sole WF were used as control. The flour:sourdough ratio was kept constant and equal to 3:1; water was added in order to reach a dough yield 160.

All the tested formulations (Table 5.1.1) included bakers' yeast at 2% concentration. Before bread making, the leavening of the doughs (WD, WDS, CD10, CDS10, CD30 and CDS30) was assessed as previously described by Zannini et al. (2009). Briefly, each dough, prepared as described in Table 5.1.1, was manually mixed for 10 min and then placed in a graduated glass cylinder (2 L); for a more precise estimation of volume increase, 40 mL paraffin were added at the top of the doughs. The volume of the doughs (in mL) was recorded immediately (t0) and after a 2-h incubation (t2) at 30 °C.

The leavening was calculated using the following formula:

$$[(V2-V0) / V0] \times 100$$

where V0 was the volume at t0 and V2 was the volume after 2-h fermentation.

The results were expressed as means ± standard deviations of triplicates.

The bread loaves (B) (Table 5.1.1) made with WD (namely WB), WDS (namely WBS), CD10 (namely CB10), CDS10 (namely CBS10), CD30 (namely CB30) and CDS30 (namely CBS30), were produced through a onestep fermentation process (30 °C for 2 h) with baking at 200°C for 1 h. Bread-making was carried out in duplicate.

Table 5.1.1. *Formulations of doughs (D) obtained with the use of wheat flour (W) or different blends of wheat flour and 10% or 30% cricket powder (C) and admixed with sourdough (S) and baker's yeast as leavening agents.*

Ingredients	**Experimental doughs**					
	WD	**WDS**	**CD10**	**CDS10**	**CD30**	**CDS30**
Wheat flour (g/100 g dough)	61.25	52.06	55.13	46.86	42.88	36.45
Cricket powder (g/100 g dough)	n.a.	n.a.	6.13	5.21	18.38	15.62
Water (g/100 g dough)	36.75	30.63	36.75	30.63	36.75	30.63
Sourdough (g/100 g dough)	n.a.	15.31	n.a.	15.31	n.a.	15.31
Baker's yeast (g/100 g dough)	2.00	2.00	2.00	2.00	2.00	2.00
Obtained bread (B) loaves	**WB**	**WBS**	**CB10**	**CBS10**	**CB30**	**CBS30**

n.a. not added.

Analysis of bread firmness

The crumb texture was measured according to the AACC method 74-09.01 (AACC, 2000) using a CT3-4500 texture analyzer (Brookfield Engineering Laboratories Inc., Middleboro MA, USA) equipped with a 36mm diameter bread probe (mod. TA-AACC36). Briefly, bread loaves were centre sliced (25mm thick) with an electric bread knife. Bread slices were positioned between the load cell and the fixture base table of the instrument. A 4500 g load cell was used. The probe compressed the crumb to a 40% compression limit (10mm compression depth) at a speed of 100 mm min^{-1}. The analysis was performed in triplicate at three positions (left, centre, and right) of each assayed slice.

Microbiological analyses

Wheat flour, cricket powder, sourdough and doughs were subjected to viable counting of LAB, yeasts and spore-forming bacteria. This latter bacterial group was also counted in the bread crumbs soon after baking to investigate about its surviving potential to the baking step.

Briefly, 10 g of each sample was homogenized into 90 mL peptone water with a stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm. For spore-forming bacteria, the homogenized samples were subjected to thermal treatment at 80°C for 15 min followed cooling in iced water, to inactivate the vegetative cells (Osimani et al., 2017c). Aliquots of ten-fold dilutions were then inoculated into appropriate growth media as follows: LAB were enumerated in both MRS and SDB, prepared as described above, supplemented with cycloheximide (0.2 g L^{-1}) to inhibit the growth of yeasts; yeast counts were carried out on Rose Bengal Agar supplemented with chloramphenicol (0.1 g L^{-1}) (Oxoid) as already described by Cardinali et al. (2016). Spore-forming bacteria were grown in Standard Plate Count Agar (Oxoid) incubated at 30 °C for 48 h.

The results of the viable counts were expressed as mean of log colony forming units (cfu) per gram of sample ± standard deviation. After counting, plates of spore-forming bacteria that showed a number of colonies comprised between 30 and 300 were used for bulk formation in accordance with the method already described by Garofalo et al. (2015). When colonies grown on plates were below 30, bulk cells were collected from the first dilution that showed any growth.

PCR-DGGE analysis

The PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA) was used to extract the microbial DNA directly from spore-forming bacteria bulk cells (Garofalo et al., 2015). The DNA template quantity and purity was assessed by optical readings at 260, 280 and 234 nm, respectively, with a UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). One hundred ng of each DNA extract was amplified through PCR in 50 μL reaction volume with the following primer pair 338f (5'-ACT CCT ACG GGA GGC AGC AGC AG-3') and 518r (5'-ATT ACC GCG GCT GCT GG-3') for the amplification of the V3 region of the 16S rRNA gene. A GC clamp was attached to the 338f primer as proposed by Ampe, Ben Omar, Moizan, Wacher, and Guyot (1999). The reaction mixtures and amplification conditions were those already described by Osimani et al. (2015). A vertical DCode electrophoresis system (Bio-Rad Laboratories) was used for the DGGE analysis which was carried out as already described by Garofalo et al. (2015).

Sequencing of the DGGE bands and sequence analysis were carried out as previously described by Osimani et al. (2015). The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) search tool (Altschul, Gish, Miller, Myers, & Lipman, 1990).

Determination of pH and total titratable acidity (TTA)

Assessment of pH values were performed with a solid electrode (HI2031, Hanna Instruments, Padova, Italy) of a pH meter (HI2031, Hanna Instruments, Padova, Italy) directly inserted into the doughs or sourdoughs. Total titratable acidity (TTA) was determined as already described by Minervini, Lattanzi, De Angelis, Di Cagno, and Gobbetti (2012). Briefly, 10 g of each dough or sourdough was blended with 90 mL distilled water, the obtained suspensions were then titrated with 0.1 N NaOH to a final pH of 8.5. The results, reported as mean ± standard deviation, were expressed as the amount of NaOH (mL) used. All the assays were carried out in triplicate.

Sensory analysis

The sensory analysis of the experimental bread loaves was carried out soon after baking as already described by Mariotti et al. (2014). Briefly, loaves were left to cool at room temperature and then small-scale acceptance test was performed (Svensson, 2012). To this end 9 nontrained panelists (4 males and 5 females, non-smokers, age: 23–60) familiar with the taste/consumption of bread were chosen.

The sensory evaluation was carried out as suggested by Resurreccion (1998). The degree of global liking was ranked using a 9-point hedonic scale, ranging from 1 (dislike extremely) to 9 (like extremely) (Peryam & Pilgrim, 1957). Results represent the means of three independent experiments.

Statistical analysis

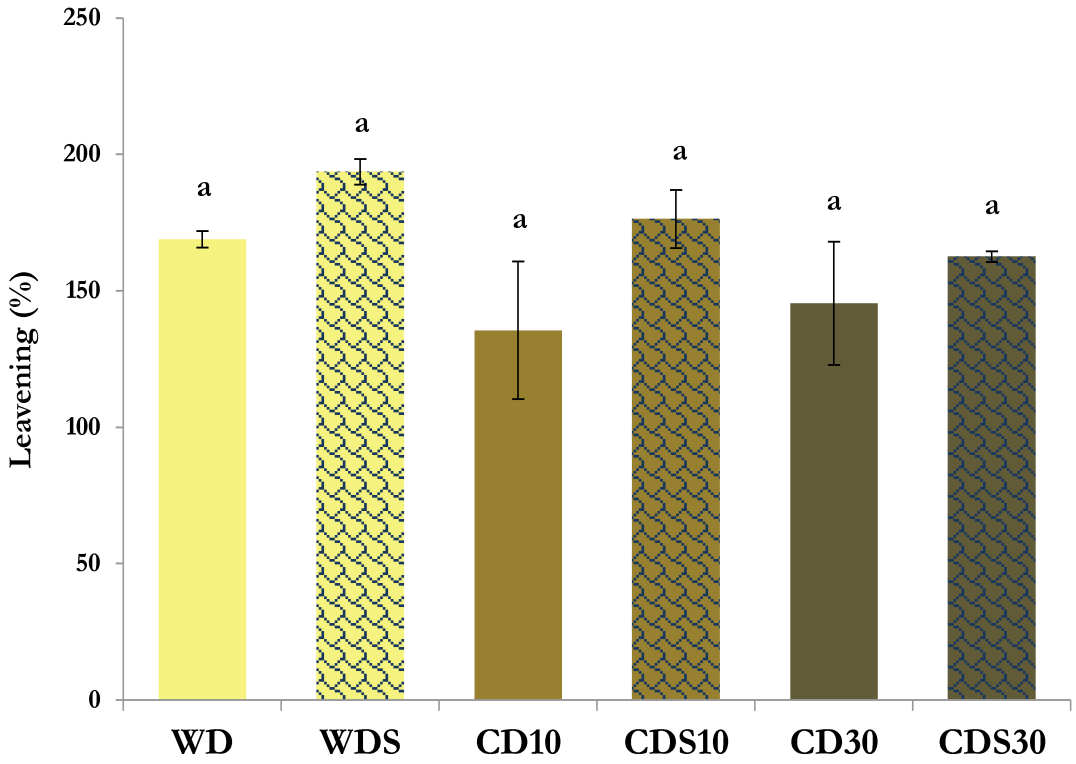
Statistical analyses were conducted using the software JMP® Version 11.0.0 (SAS Institute Inc., Cary, NC). Correlations between variables were determined on autoscaled data matrix by the Pearson product-moment correlation coefficient (r). Variable reduction was achieved by PCA (Principal Component Analysis) on correlation matrix. A one-way analysis of variance (ANOVA) was carried out to evaluate differences among flour blends, doughs, bread loaves. Multiple means comparison was carried out through the Tukey-Kramer's Honest Significant Difference (HSD) test and level of significance was set to 0.05.

5.1.2 Results and discussion

Leavening

Leavening performances of the doughs prepared with the different flour blends (WF, CP10 and CP30) and fermenting agents (bakers' yeast and sourdough) after 2 h fermentation are reported in Figure 5.1.1. Based on the results of ANOVA no significant differences were measured among doughs, although average leavening values ranging between 140 ± 20% and 190 ± 20% for WDS and CDS10, respectively, were observed.

Figure 5.1.1. *Leavening of the doughs (D) prepared with the different blends of wheat flour (WF) and cricket powder (CP) and admixed with sourdough (S) and baker's yeast as leavening agents.*



Samples are codified as reported in Table 5.1.1; means ± standard deviations of triplicate independent experiments are shown; means with different superscripts are significantly different (P > 0.05).

Viable counts, pH and TTA and PCR-DGGE

The results of microbiological enumeration, pH and Total Titratable Acidity (TTA) of WF, CP, sourdough (after 16 h fermentation), doughs (after 2 h fermentation) and experimental bread loaves are shown in Table 5.1.2.

Microbial load data have a fundamental role in the assessment of microbial risk related to insects as food requested by EFSA.

Table 5.1.2 *Results of microbiological characterization of wheat flour (W), cricket powder (C), sourdough (S) (after 16 h fermentation), doughs (D) (after 2 h fermentation) admixed with sourdough (S) and baker's yeast as leavening agents, and experimental bread loaves (B).*

Samples		LAB on MRS	LAB on MSDB	Yeasts	Spore-forming bacteria	pH	TTA
		log cfu g^{-1}					
Flour	WF	<1	<1	2.70±0.26	<1	n.d.	n.d.
	CP	3.86±0.01	3.86±0.01	<1	5.52±0.02	n.d.	n.d.
Sourdough (16 h)	S	9.27 ± 0.01	9.24 ± 0.03	2.15 ± 0.21	<1	3.69 ± 0.01	7.70 ± 0.14
Dough (2 h)	WD	<1	<1	8.00 ± 0.02	<1	5.62 ± 0.01	3.30 ± 0.14
	WDS	8.94 ± 0.02	8.89 ± 0.01	7.88 ± 0.10	<1	4.73 ± 0.02	3.05 ± 0.64
	CD10	5.88 ± 0.04	5.88 ± 0.04	8.36 ± 0.05	4.34±0.03	5.71 ± 0.01	8.30 ± 0.42
	CDS10	9.80±0.02	9.84±0.03	8.32±0.10	4.13±0.07	5.46 ± 0.01	6.10 ± 0.70
	CD30	6.53 ± 0.06	6.54 ± 0.04	8.25±0.00	4.30 ± 0.10	5.84 ± 0.01	4.25 ± 0.07
	CDS30	8.60 ± 0.05	8.60 ± 0.02	4.30±0.10	4.30±0.10	5.30 ± 0.01	6.00 ± 0.14
Bread	WB	n.d.	n.d.	n.d.	<1	6.09 ± 0.01	1.05 ± 0.21
	WBS	n.d.	n.d.	n.d.	<1	5.87 ± 0.01	2.05 ± 0.07
	CB10	n.d.	n.d.	n.d.	3.10 ± 0.21	5.86 ± 0.02	4.40 ± 0.14
	CBS10	n.d.	n.d.	n.d.	<1	5.90 ± 0.02	4.60 ± 0.42
	CB30	n.d.	n.d.	n.d.	3.70 ± 0.05	5.65 ± 0.01	3.10 ± 0.00
	CBS30	n.d.	n.d.	n.d.	3.55 ± 0.09	5.20 ± 0.01	4.20 ± 0.14

Samples are codified as reported in Table 5.1.1;
TTA was expressed as ml of 0.1 N NaOH;
n.d. not determined.

In the present study, the enumeration of pro-technological LAB and yeasts was carried out along the bread-making process from raw materials to sourdough and doughs, while spore-forming bacteria load were assessed also in bread loaves because of their ability to survive to hugh temperature during baking.

In detail, wheat flour was characterized by counts < 1 log cfu g^{-1} for LAB (on both MRS and SBD media) and spore-forming bacteria and around 3 log cfu g^{-1} for yeasts. These results were similar to those reported by Alfonzo et al. (2017) and Valerio et al. (2012) for various semolinas, respectively. Contrarily, cricket powder was characterized by high counts of both LAB (grown on both MRS and SBD media) and spore-forming bacteria which reached up to 3.8 log cfu g^{-1} and 5 log cfu g^{-1}, respectively; whereas yeast counts were < 1 log cfu g^{-1}. The results of viable counts carried out on cricket powder were comparable with those reported by Osimani, et al. (2017) for the same substrate. High levels of LAB were ascertained produced with selected inoculated strains on both MRS and SBD media, thus suggesting the good adaptation of the artificially inoculated LAB strains to the wheat flour subjected to fermentation was certified by high counts in the 16 h-sourdough (up to 9 log cfu g^{-1}). On the contrary, yeast counts attested at about 2 log cfu g^{-1} while spore-forming bacteria resulted under the detection limit. As a consequence, in the doughs produced with sourdough and bakers' yeast (WDS, CDS10, and CDS30) LAB ranged between 8.6 and 9.8 log cfu g^{-1} in both MRS and SBD media. Instead the doughs produced with the sole use of bakers' yeast were characterized by lower LAB counts in both media. Indeed, their counts were below 1 log cfu g^{-1} in WD, whereas CD10, and CD30 showed counts of 5.8 and 6.5 log cfu g^{-1}, respectively. As expected, doughs did not differ in the yeast counts, which ranged between 7.8 and 8.3 cfu g^{-1}.

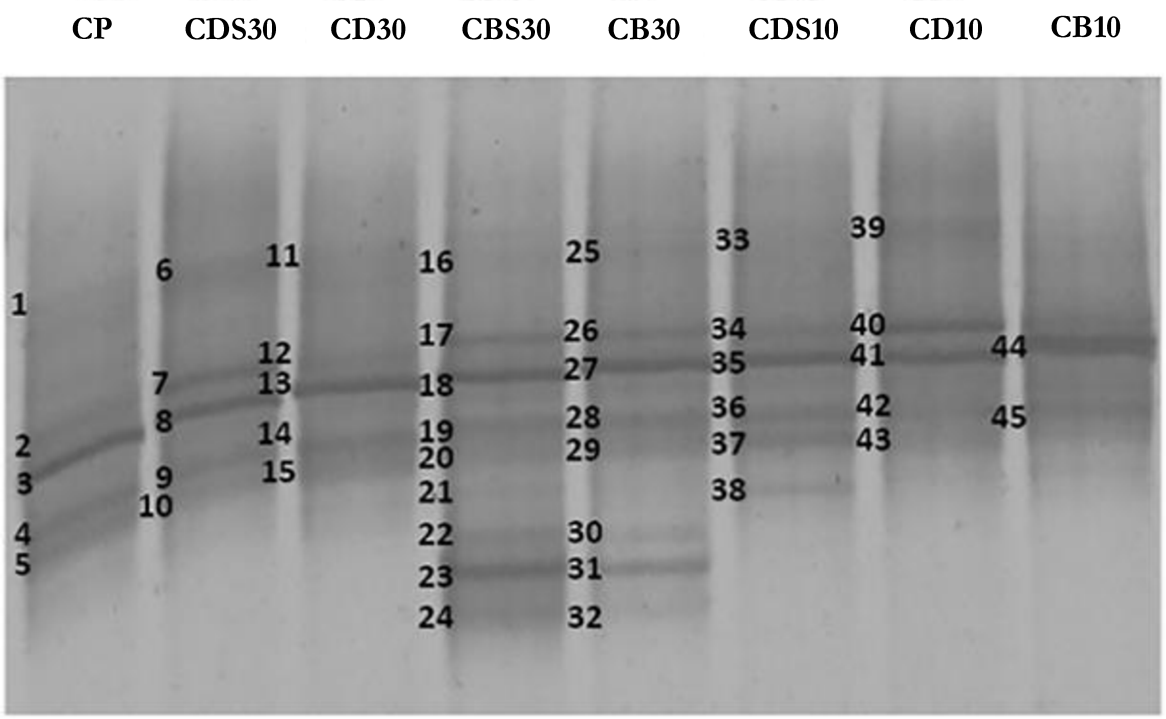
Concerning spore-forming-bacteria, differences were found in doughs and bread loaves. In more detail, CD10, CDS10, CD30, and CDS30 were characterized by spore-forming bacteria counts comprised between 4.1 and 4.3 log cfu g^{-1}. On the contrary, both WD and WDS showed spore-forming bacteria counts <1 log cfu g^{-1}. Regarding bread loaves, for CB10, CBS10, CB30 and CBS30

spore-forming bacteria, counted soon after baking, ranged between 3.1 and 3.7 log cfu g^{-1}, with the only exception of CBS10; whereas on WB and WBS they were below 1 log cfu g^{-1}.

A good metabolic activity of the artificially inoculated LAB was underlined by pH and TTA measurements in sourdough due to their ability in producing organic acids (Mariotti et al., 2014). Indeed, a pH of 3.69 ± 0.01 and a TTA of 7.70 ± 0.14 were measured after 16 h fermentation. Consequently all the doughs containing sourdough showed a lower pH and a higher TTA in respect to the ones with only baker's yeast as leavening agent. In general, a higher TTA was observed in doughs and bread loaves containing cricket powder in respect with control bread loaves produced with the sole wheat flour. This finding can be likely correlated with the higher ash content of cricket powder which can have an influence on dough buffering capacity (Mariotti et al., 2014; Taccari et al., 2016).

The DGGE profiles obtained by analyzing the DNA extracted from the bulk cells harvested from plates with counts >1 log cfu g^{-1} used for spore-forming bacteria viable counts are shown in Figure 5.1.2.

Figure 5.1.2*. DGGE profiles of the DNA extracted from the spore- forming bacteria bulk cells washed off the PCA medium and amplified with primers 338fGC and 518r. The bands indicated by the numbers were excised, ream- plified and subjected to sequencing.*



The identification of the bands is reported in Table 5.1.3. Samples are codified as reported in Table 5.1.1.

The closest relatives, the percent identities, and the accession numbers of sequences obtained from the selected PCR-DGGE bands are reported in Table 5.1.3.

For the majority of these bands, a % identity between 95 and 97% to known reference strains was seen, whereas for a few other bands, a lower % identity to known sequences was observed, thus allowing the occurrence of unknown microbial species to be hypothesized.

Table 5.1.3. *Sequencing results from the bands cut from the denaturing gradient gel elec- trophoresis (DGGE) gels obtained from the amplified fragments of the DNA extracted directly from the spore-forming bacteria bulk cells washed off the PCA medium.*

Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
CP	1	*Bacillus licheniformis*	97%	GQ392053
	2	*Bacillus subtilis*	97%	KP050498
		Bacillus licheniformis	97%	GQ392052
	3	*Bacillus subtilis*	95%	KJ603239
	4	*Bacillus subtilis*	97%	HQ290081
		Bacillus sp.	97%	GQ392045
	5	*Bacillus subtilis*	97%	KJ603239
CDS30	6	*Bacillus licheniformis*	97%	GQ392053
	7	*Bacillus subtilis*	95%	KP050498
		Bacillus licheniformis	95%	GQ392052
	8	*Bacillus subtilis*	95%	KJ603239
	9	*Bacillus subtilis*	97%	HQ290081
		Bacillus sp.	97%	GQ392045
	10	*Bacillus subtilis*	97%	KJ603239
CD30	11	*Bacillus licheniformis*	93%	GQ392053
	12	*Bacillus subtilis*	95%	KP050498
		Bacillus licheniformis	95%	GQ392052
	13	*Bacillus subtilis*	97%	KJ603239
		Bacillus sp.	97%	GQ392045
	14	*Bacillus subtilis*	95%	KJ603239
		Bacillus sp.	95%	GQ392045
	15	*Bacillus subtilis*	97%	KJ603239
CBS30	16	*Bacillus licheniformis*	93%	GQ392053
	17	*Bacillus subtilis*	97%	KJ603239
	18	*Bacillus subtilis*	97%	KJ603239
	19	*Bacillus subtilis*	97%	KJ603239
	20	*Bacillus subtilis*	95%	KJ603239
		Bacillus sp.	95%	GQ392045
	21	*Brevibacillus borstelensis*	95%	GQ392051
	22	*Brevibacillus borstelensis*	95%	GQ392051
	23	*Bacillus subtilis*	97%	KJ603239
		Bacillus sp.	97%	FJ649344
	24	*Brevibacillus borstelensis*	90%	GQ392051
CB30	25	*Bacillus licheniformis*	93%	GQ392053
	26	*Bacillus subtilis*	92%	KJ603239
		Bacillus licheniformis	92%	GQ392053
	27	*Bacillus subtilis*	97%	HQ290081
		Bacillus sp.	97%	GQ392045
	28	*Bacillus subtilis*	93%	KJ603239
	29	*Bacillus subtilis*	97%	KJ603239
		Bacillus sp.	97%	GQ392045
	30	*Brevibacillus borstelensis*	95%	GQ392051
	31	*Bacillus subtilis*	92%	KJ603239
		Bacillus sp.	92%	FJ649344
	32	*Brevibacillus borstelensis*	90%	GQ392051
CDS10	33	*Bacillus subtilis*	97%	KJ603239
		Bacillus licheniformis	97%	GQ392053
	34	*Bacillus subtilis*	97%	KJ603239
		Bacillus licheniformis	97%	GQ392053
	35	*Bacillus subtilis*	97%	HQ290081
		Bacillus sp.	97%	GQ392045
	36	*Bacillus subtilis*	97%	HQ268531
	37	*Bacillus subtilis*	97%	HQ268531
		Bacillus licheniformis	97%	GQ392052
	38	*Bacillus subtilis*	97%	EU532192
		Bacillus licheniformis	97%	GQ392052

Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
CD10	39	*Bacillus subtilis*	97%	KJ603239
		Bacillus licheniformis	97%	GQ392053
	40	*Bacillus subtilis*	97%	KJ603239
		Bacillus licheniformis	97%	GQ392053
	41	*Bacillus subtilis*	97%	HQ290081
		Bacillus sp.	97%	GQ392045
	42	*Bacillus subtilis*	97%	HQ268531
	43	*Bacillus subtilis*	97%	HQ268531
		Bacillus licheniformis	97%	GQ392052
CB10	44	*Bacillus sp.*	97%	GQ392045
	45	*Bacillus subtilis*	97%	HQ268531

PCA, Plate Count Agar. CP, cricket powder. Samples of doughs (D) admixed with baker's yeast and sourdough (S) as leavening agents and experimental bread loaves (B) are codified as reported in Table 5.1.1;

[a] Selected bands excised from the agarose gels and subjected to sequencing are numbered;

[b] Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database;

[c] Accession number of the sequence of the closest relative found by a BLAST search.

In all the analyzed samples, species of *Bacillus* were detected. In more detail, closest relatives to *Bacillus subtilis* were found in almost all doughs and bread loaves; moreover closest relatives to *Bacillus licheniformis* and *Brevibacillus borstelensis* were sporadically found. As already reported by Valerio et al. (2015), a load of 2 log cfu g^{-1} spores of *Bacillus* in flour can reach up to 7 log cfu g^{-1} in bread crumb within 2 days, causing spoilage. In particular *Bacillus subtilis* and *B. licheniformis* are known to be the causative agents of ropy spoilage in bread and, if present in high loads (≥8 log cfu g^{-1}), of illness to the consumer (Osimani et al., 2018a; Rosenkvist & Hansen, 1998;).

It is known that ropy spoilage occurs through the combined effect of proteolytic and amylolytic enzymes produced by bacteria which leads to a bread characterized by pineapple-like odor and discoloration of the crumb wchich become soft and sticky to the touch (Valerio et al., 2008). The amylase activity and spoilage ability of strains of *B. amyloliquefaciens*, *B. subtilis* and *Bacillus pumilus* after heat treatment (100°C, 10 min) has recently been reported by Valerio et al. (2012). In the present study, the core temperature of the experimental bread loaves measured soon after baking was comprised between 97.1°C and 98.5°C, hence it is likely that, most of the spore-forming bacteria in CB10, CB30 and CBS30 survived the baking process. It is noteworthy that, after baking, CBS10 showed counts <1 log cfu g^{-1}. This finding could be likely explained by the lowest quantity of cricket powder (and spore-forming bacteria) in the flour blend used.

Specific volume and texture

Regarding bread production, Figure 5.1.3 shows the bread slices obtained from the different loaves (panel a and b respectively).

Specific volume and firmness of bread loaves are showed in Figure 5.1.4 (panel a and b respectively).

The highest specific volume was recorded for WB (3.49 ± 0.33 g cm^{-3}) whereas the lowest value was recorded for CBS30 (3.37 ± 0.03 g cm^{-3}). WBS, CB10, CBS10, and CB30 showed intermediate specific volume values which were not significantly different among each other.

Concerning the results of bread firmness CB30 and CBS30 showed the highest mean values (1402.25 ± 30.05 and 1488.00 ± 4.24 g, respectively), whereas WBS reported the lowest (439.5 ± 7.78 g).

Figure 5.1.3. *Slices obtained from the loaves of experimental bread loaves. Samples are codified as reported in Table 5.1.1.*

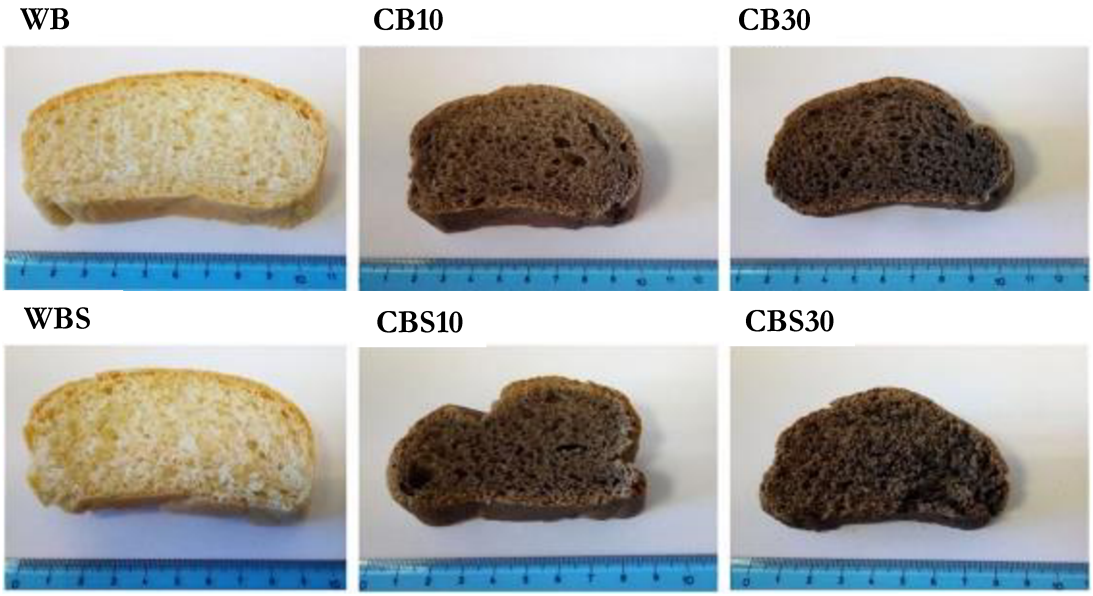
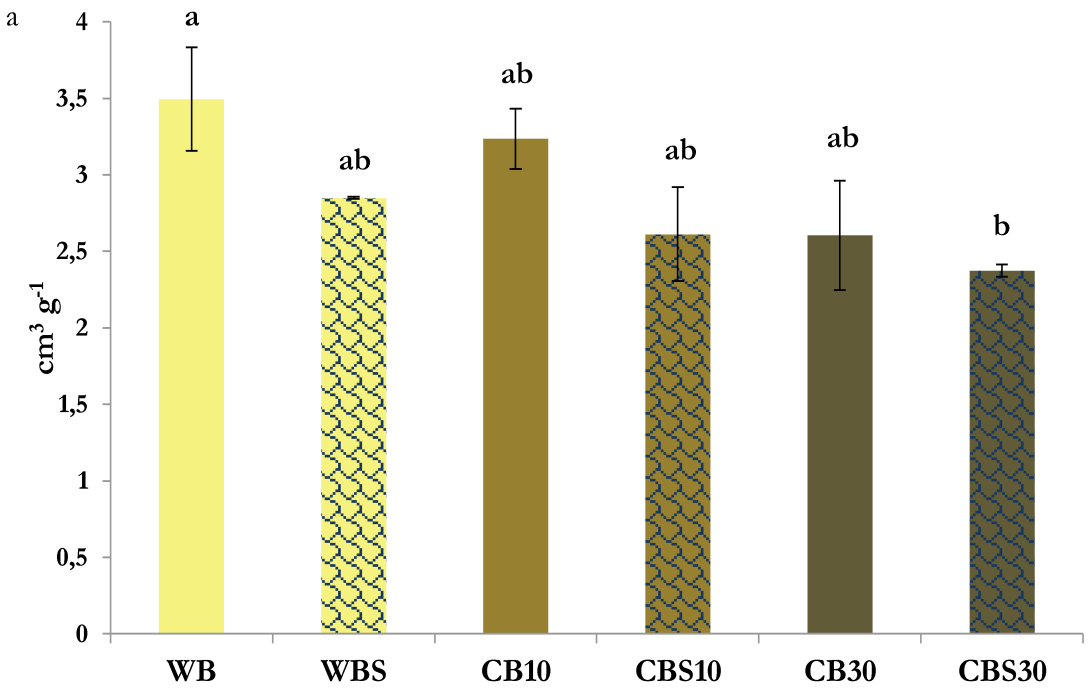
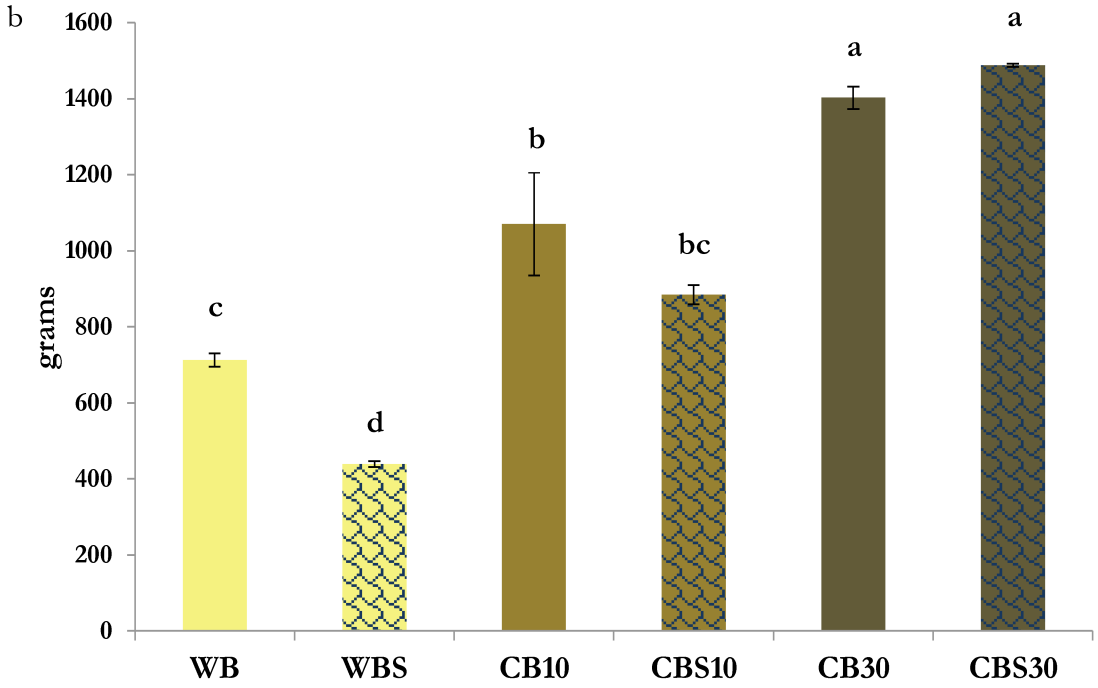


Figure 5.1.4. *Specific volume (panel a) and firmness (panel b) of bread (B) loaves prepared with the different blends of wheat flour (W) and cricket powder (C) and admixed with sourdough (S) and baker's yeast as leavening agents.*





Samples are codified as reported in Table 5.1.1; means ± standard deviations of triplicate independent experiments are shown; means with different letters are significantly different (P > 0.05).

The obtained data are in agreement with the recent study of de Oliveira et al. (2017) regarding specific volumes and firmness of bread enriched with flour from cinereous cockroach (*Nauphoeta cinerea*), in which a linear correlation between volume and hardness of the experimental bread loaves and the amount of insect powder added to the dough was found (de Oliveira et al., 2017).

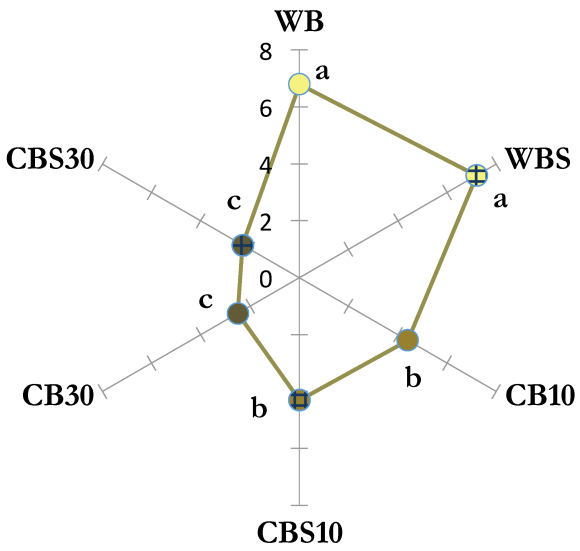
Sensory analysis

The results of acceptance tests carried out on the experimental bread loaves are reported in Figure 5.1.5.

As a general trend, global liking data showed an opposite trend with the amount of cricket powedr added.

In detail, the highest average values were obtained by control bread (WD and WDS), whereas bread produced with 30% cricket powder schowed the lowest average values. Finally, bread containing 10% cricket powder obtained intermediate average values of global liking. Based on these results, it might be hypothesized that the amount of added cricket powder markedly affects the acceptance of the products due to the notable flavor and taste of the insect-based ingredient. Moreover, it is likely that the unusual nature of this novel bread influenced the non-trained panelists who were not used to eat insect-based foods.

Figure 5.1.5. *Global liking of experimental bread loaves (B) produced with wheat flour and dif- ferent blends of wheat flour (WF) and cricket powder (CP) admixed with sourdough (S) and baker's yeast as leavening agents.*



The degree of global liking was ranked in accordance with a 9-point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely); samples are codified as reported in Table 5.1.1; different letters are significantly different (P < 0.05).

5.1.3 Conclusions

As a general trend, a negative linear correlation between the amount of added cricket powder and the dough technological parameters was seen. However, the addition of 10% cricket powder produced doughs apparently suitable for bread-making.

Of note, the high load of spore-forming bacteria in the bread loaves containing cricket powder, as revealed by the microbiological analyses, which highlighted potential safety issues for consumers. Accordingly, preventive treatments (e.g., blenching, microwave drying, high-pressure, etc.) able to reduce the load of such a bacterial group are recommended for processing of insect powder to be used as a food ingredient. Finally, bread enriched with 10% cricket powder showed a discrete appreciation by untrained panelists, which might potentially be increased by the addition of natural bread improvers (e.g., enzymes, sugars, etc.). The results of sensory analysis suggested that foods where insects are not directly visible could be appreciated by European consumers.

Further studies are needed to better understand the interactions between cereal-based matrices and insect powders and to adjust insects powder composition to obtain a better balanced products regarding fat content (González, Garzón, & Rosell, 2018).

5.2 Protein fortification with mealworm (Tenebrio molitor L.) powder: Effect on textural, microbiological, nutritional and sensory features of bread (Paper VI)

Edible insects potential as novel ingredient for protein fortification of bread was already demonstrated (de Oliveira et al., 2017; Gonzàlez et al., 2018; Osimani et al., 2018e). In particular, Osimani et al. (2018e) highlighted the development of a bread characterized by higher nutritional qualities but at the same time a lower appreciation rate with the increasing of the amount of insect powder. As a matter of fact, insect tastes and flavour depend on a number of factors, such as: (i) pheromones occurring at the insect surface; (ii) type of feed; (iii) presence of an exoskeleton (Kouřimskà and Adàmkovà, 2016). In particular, cricket (*A. domesticus*) powder is different than mealworm (*T. molitor* L.) powder in terms of color, flavor and taste due to the above cited factors. Indeed, cricket powder is produced from adult insects, generally fed with grass, whose external anatomy is made up of exoskeleton, head, eyes, mandibles, antennae, legs and wings; the result is a powder characterized by a strong crustacean-like, cooked legumes-like and earthy aroma and a medium to dark brown color with some coarse particulates visible, deriving from insect exoskeleton parts. By contrast, mealworm powder is produced from larvae typically fed on cereal bran or flour and is characterized by a sweet, almost nutty flavor, a nutty/cocoa smell, and a light to medium brown color.

Given these premises, the present study was intended to explore the use of mealworm powder for fortification of soft wheat (*Triticum aestivum* L.) bread with high protein content and more appealing sensory properties. In more detail, the present study was carried out performing, technological, microbiological and sensory analysis during the bread-making assay to investigate about microbial dynamics and consumers acceptance.

5.2.1 Material and methods

Flour and insect powder

Soft wheat (*T. aestivum* L.) flour (WF), classified as type 00 and used for both technological evaluation of blends and bread making, was provided by a local mill (Molino Stacchiotti, Ancona, Italy), whereas mealworm (*T. molitor* L.) powder (MP), containing the whole lipid fraction, was purchased from Kreca Ento-Food BV (Ermelo, The Netherlands). Samples of MP were packaged in 100 g plastic bags each and stored at ambient temperature until use. No information was available on the rearing conditions of mealworms or hygiene practices applied during powder production, storage and transport before buying.

Sourdough production with selected lactic acid bacteria strains

The sourdough used as a leavening agent was produced through the inoculation of five LAB strains, namely: *Lactobacillus fermentum* PB162, *Lactobacillus plantarum* PB11, *Lactobacillus plantarum* PB24, *Lactobacillus sanfranciscensis* PB276, and *Lactobacillus sanfranciscensis* PB223.

The selected strains, belonging to the culture collection of the Dipartimento di Scienze Agrarie, Alimentari ed Ambientali (D3A) (Universita Politecnica delle Marche), were previously isolated from wheat sourdoughs produced in the Marche region (central Italy) and characterized for their technological traits (acidification, amylase activity, starch hydrolysis and lactate, acetate and CO_2 production) by Osimani et al. (2009). The frozen stored cultures were grown as reported by Osimani

et al. (2018). Briefly, *L. fermentum* PB162, *L. plantarum* PB11 and *L. plantarum* PB24 were cultivated on modified De Man, Rogosa and Sharp (MRS) agar (Oxoid, Basingtoke, UK) added with 1% maltose (w/v) and 5% fresh yeast extract (v/v) prepared according to Gobbetti et al. (1996), whereas *L. sanfranciscensis* PB276 and *L. sanfranciscensis* PB223 were cultivated on Sourdough Bacteria (SDB) medium modified in accordance with Vogel et al. (1994). Sourdough was produced as previously reported by Osimani et al. (2018e). Briefly, selected LAB pure cultures were separately cultivated in modified MRS broth at 30°C for 12 h. The obtained broth cultures were centrifuged at 4,000 rpm for 5 min, washed, and resuspended in sterile tap water (Coda et al., 2010). The pool of selected LAB strains, wheat flour and sterilized water were admixed to reach dough yield 167 (60 g/100 g wheat flour plus 40 g/100 g water). The obtained dough, at an initial LAB concentration of 8 log cfu g^{-1}, was left to ferment at 30°C for 16 h in order to obtain the mature sourdough (SD) used for bread making trials.

Dough composition and bread-making

Two different amounts of MP were used in addition to WF in order to produce experimental bread loaves, whereas the sole WF was used to produce control bread loaves. In more detail, MP was used at a substitution level of WF equal to 5 and 10% (w/w) regardless of the sourdough WF content. In bread added with sourdough, the flour:sourdough ratio was set at 3:1. In all the dough formulations, water was added in order to reach a dough yield 160 whereas baker's yeast was added at a concentration of 2% (Table 5.2.1). The leavening performance of the doughs (WD, WDS, MD_5, MDS_5, MD_{10} and MDS_{10}), carried out at 30°C, was evaluated using graduated glass cylinders (2 L) as previously described (Zannini et al., 2009). Briefly, each dough was prepared as described in Table 5.2.1 and manually kneaded for about 10 minutes up until a proper gluten development was reached. In order to estimate the volume increase of doughs, they were placed in graduated glass cylinders and poured with 40 mL paraffin at their top to prevent moisture loss and drying. The volume of each dough (in mL) was recorded soon after placement in the cylinders (t_0) and after a 2 hour fermentation (t_2). The leavening was calculated using the following formula:

$$[(V_2 — V_0)/V_0] \times 100$$

where V_0 was the volume at t_0 and V_2 was the volume after fermentation.

The results were expressed as means ± standard deviations of duplicate experiments.

The bread loaves (B) (Table 5.2.1) made with WD, WDS, MD_5, MDS_5, MD_{10} and MDS_{10} were obtained through a one-step fermentation process (30°C for 1 h) followed by 1 h oven baking at 200°C as previously described by Osimani et al. (2018). Core temperature of baked bread loaves was measured soon after baking using a portable thermometer (Checktemp 1—HI 98509, Hannah Instruments, Padova, Italy). Bread-making trials were carried out in duplicate.

Table 5.2.1. *Formulations of doughs (D) obtained with the use of wheat flour (W) or different blends of wheat flour and 5% or 10% mealworm powder (M) admixed with baker's yeast as leavening agents and sourdough (S).*

Ingredients	**Experimental doughs**					
	WD	**WDS**	**MD_5**	**MDS_5**	**MD_{10}**	**MDS_{10}**
Wheat flour (g/100 g dough)	61.25	52.06	58.19	49.46	55.13	46.86
Mealworm powder (g/100 g dough)	n.a.	n.a.	3.06	2.60	6.13	5.21
Water (g/100 g dough)	36.75	30.63	36.75	30.63	36.75	30.63
Sourdough (g/100 g dough)	n.a.	15.31	n.a.	15.31	n.a.	15.31
Baker's yeast (g/100 g dough)	2.00	2.00	2.00	2.00	2.00	2.00
Obtained bread (B) loaves	**WB**	**WBS**	**MB5**	**MBS5**	**MB10**	**MBS10**

n.a. not added

Determination of pH and total titratable acidity (TTA)

Direct potentiometric pH assessment of sourdoughs and doughs was carried out with a pH meter (HI2031, Hanna Instruments, Padova, Italy) equipped with a solid electrode (HI2031, Hanna Instruments, Padova, Italy). Total titratable acidity (TTA) was assessed in accordance with the method already described by Minervini et al. (2012). Briefly, 10 g of each sourdough or dough was homogenized in 90 mL distilled water and the suspensions were then titrated with 0.1 N NaOH to a final pH of 8.5. The TTA results were expressed as the amount of NaOH (mL) used. Both pH and TTA analyses were carried out in duplicate and the results reported as mean ± standard deviation.

Analysis of bread firmness

Bread firmness was assessed as previously described by Osimani et al. (2018e). Briefly, experimental bread loaves were centre sliced (25 mm thick) and bread slices positioned on the sample table under the load cell of a CT3-4500 texture analyzer (Brookfiled Engineering Laboratories Inc., Middleboro MA, USA) equipped with a 36 mm diameter bread probe (mod. TA-AACC36). The probe compressed the crumb to a 40% compression limit at a speed of 100 mm min^{-1}. A 4500 g load cell was used. All measurements were carried out in duplicate and the results reported as mean ± standard deviation.

Microbiological analyses

MP, WF, sourdough, doughs, and bread loaves underwent plate counting of LAB, yeasts and aerobic bacterial spores, depending on the type of sample. LAB occurring in MP, WF, sourdoughs (t0 and t16h) and doughs (t1h) were counted on both MRS (Oxoid) and SDB agar supplemented with cycloheximide (0.2 g L-1) (Merck KGaA, Darmstadt, Germany) to inhibit the growth of yeasts as previously described by Osimani et al. (2009). Yeasts occurring in MP, WF, sourdoughs (t0 and t16h), and doughs (t1h) were enumerated on Rose Bengal Agar (Oxoid) supplemented with chloramphenicol (0.1 g L-1) (Oxoid) as already described by Cardinali et al. (2016). Aerobic bacterial spores occurring in all samples except for sourdoughs (t0 and t16h) and doughs (t0) were counted on Standard Plate Count Agar (Oxoid) as previously described (Osimani et al., 2017c). Finally, counts of *Bacillus cereus* and *Clostridium perfringens* in all bread samples were carried out in accordance with UNI EN ISO 7932:2005 and ISO 7937:2004 standard methods, respectively. The results of viable counting were expressed as mean of log colony forming units (cfu) per gram of sample ± standard deviation.

PCR-DGGE analysis

Plates of aerobic bacterial spores showing a number of colonies comprised between 30 and 300 were used for bulk formation prior to Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis, as previously described (Osimani et al., 2018e). When colonies grown on plates were below 30, bulk cells were collected from the first dilution plates that showed any growth. The DNA was extracted from bulk colonies as described by Osimani et al. (2015). The quantity and the purity of the extracted DNA was assessed by Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and standardised to a concentration of 25 ng µL-1 for further analysis. Two µL (about 50 ng) of each DNA extract was amplified by PCR in a My Cycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using the universal prokaryotic primers 338F and 518r (Alessandria et al., 2010) and PCR conditions described by Osimani et al. (2015). As proposed by Ampe et al. (1999), the forward primer 338F was added with a GC clamp, as required for the subsequent DGGE analysis. The DGGE analysis as well as the sequencing and identification of the excised DGGE bands were performed as detailed by Osimani et al. (2018c). Only the sequences showing more than 97% of similarity with the sequences deposited in the GenBank database (http://www.ncbi.nlm.nih.gov) were clearly assigned to a species or a genus.

Sensory analysis

Overall liking for fortified and control bread loaves was assessed as previously described (Osimani et al., 2018e). Briefly, 9 untrained panellists, including 5 females and 4 males with age comprised between 23–60 years, regularly consuming wheat bread were chosen. Liking of experimental bread loaves was ranked with a 9-point hedonic scale, where 1 and 9 correspond to extreme disliking and liking, respectively. Data were expressed as mean ± standard deviation of three independent experiments. All assessors involved in the sensory analysis were informed about the aim of the study and provided their informed written consent to the D3A. Moreover, the need for approval of the sensory analysis was prospectively waived by the Ethical Committee of the Università Politecnica delle Marche.

Statistical analysis

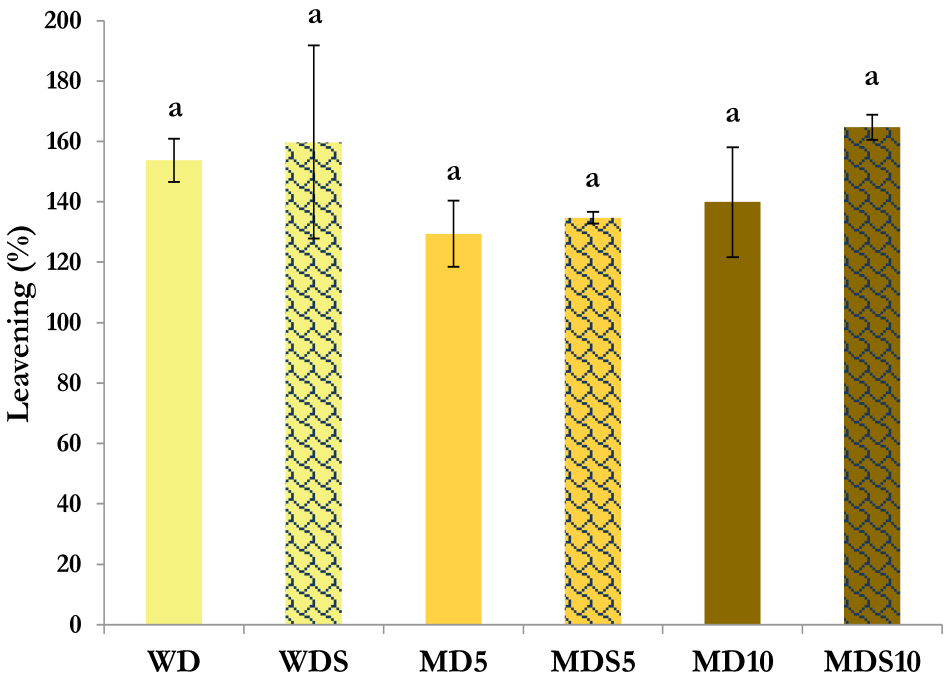
The Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used to evaluate differences inside groups (blends of WF and MP, bread loaves), by one-way analysis of variance (ANOVA). Experimental data were explored by Principal Component Analysis (PCA); normalization was used as data pre-treatment procedure. The software JMP Version 11.0.0 (SAS Institute Inc., Cary, NC) was used to carry out all tests.

5.2.2 Results and discussion

Leavening

As shown in Figure 5.2.1, all doughs exhibited the same leavening ability. Indeed no significant differences were observed among them irrespective of the use of baker's yeast alone or baker's yeast in combination with sourdough as leavening agents or the amount of added MP.

Figure 5.2.1. *Leavening of the doughs (D) prepared with the different blends of wheat flour (W) and mealworm powder (M) and admixed with baker's yeast as leavening agent and sourdough (S).*



Samples are codified as reported in Table 5.2.1; means ± standard deviations of triplicate independent experiments are shown; means with different superscripts are significantly different (P > 0.05).

These results are in agreement with the trends reported by Osimani et al. (2018e) for the leavening ability of wheat doughs enriched with cricket powder.

Viable counts, pH and TTA and PCR-DGGE

It is well known that edible insects and insect-based ingredients harbor complex microbial communities that originate from their intestinal tract or from the rearing and processing environments. Among the reported species, saprophytic, spoilage or potentially pathogenic microorganisms can likely be present (Milanović et al., 2016; Garofalo 2017a; Osimani et al., 2017a, 2017b, 2017c; Vandeweyer et al., 2017a, 2017b; Winants et al., 2017; Osimani et al., 2018a, 2018c; Osimani et al., 2018e; Roncolini 2018).

In the present study, microbiological enumeration was carried out to evaluate the quantity of pro-technological (LAB and yeasts) and spore-forming bacteria in the raw materials, sourdough, and doughs. Moreover, the presence of endospores in the final bread loaves was assessed, together with the occurrence of the following human pathogens: *B. cereus* and *C. perfringens*. The results of viable counting are reported in Table 5.2.2, as well as pH and TTA analysis data.

Table 5.2.2. *Results of microbiological characterization of wheat flour (W), mealworm powder (M), sourdough (S0) (before fermentation), doughs (D) (before fermentation and after 1h fermentation) and mixed with baker's yeast as leavening agents and sourdough (S), and experimental bread loaves (B).*

Samples		LAB on MRS	LAB on SBD	Yeasts	Spore-forming bacteria	pH	TTA
		(log cfu g^{-1})					
Flour	MP	3.46±0.65	3.81±0.81	0.22±0.53	2.81±0.56	n.d.	n.d.
	WF	1.45±1.21	1.44±0.93	0.84±0.99	1.04±1.26	n.d.	n.d.
Sourdough (0 h)	S0	7.34±0.15	7.33±0.16	<1	n.d.	5.85±0.02	1.05±0.22
Sourdough (16 h)	S16	9.16±0.08	9.16±0.12	<1	n.d.	3.69±0.10	9.33±1.04
Dough (0 h)	WD	n.d.	n.d.	n.d.	n.d.	5.60±0.04	1.71±0.19
	WDS	n.d.	n.d.	n.d.	n.d.	5.01±0.06	2.82±0.82
	MD5	n.d.	n.d.	n.d.	n.d.	5.70±0.12	2.30±0.28
	MDS5	n.d.	n.d.	n.d.	n.d.	5.15±0.06	3.92±0.14
	MD10	n.d.	n.d.	n.d.	n.d.	5.88±0.10	3.65±0.50
	MDS10	n.d.	n.d.	n.d.	n.d.	5.39±0.06	4.41±0.72
Dough (1 h)	WD	3.95±0.91	3.89±0.83	7.81±0.16	1.04±1.65	5.47±0.01	1.93±0.32
	WDS	8.37±0.12	8.39±0.17	6.92±0.47	0.25±0.46	4.83±0.05	2.84±0.92
	MD5	3.58±0.50	3.58±0.69	7.67±0.40	<1	5.57±0.05	2.02±0.17
	MDS5	8.39±0.04	8.22±0.33	7.89±0.20	<1	5.05±0.04	3.88±0.59
	MD10	4.59±0.32	4.56±0.35	7.91±0.54	1.35±0.99	5.82±0.01	3.57±0.80
	MDS10	8.34±0.09	8.39±0.14	8.00±0.04	<1	5.24±0.04	4.34±0.65
Bread	WB	n.d.	n.d.	n.d.	0.38±0.52	6.15±0.09	1.64±0.45
	WBS	n.d.	n.d.	n.d.	0.58±1.07	5.52±0.18	2.63±0.46
	MB5	n.d.	n.d.	n.d.	<1	6.01±0.03	2.80±0.31
	MBS5	n.d.	n.d.	n.d.	<1	5.57±0.05	2.68±0.42
	MB10	n.d.	n.d.	n.d.	<1	6.24±0.11	2.28±0.16
	MBS10	n.d.	n.d.	n.d.	<1	5.61±0.02	3.72±0.50

Samples are codified as reported in Table 5.2.1;
TTA was expressed as mL of 0.1 N NaOH;
n.d. not determined.

Regarding MP, the counts of LAB on both MRS and SDB media were in the range of 3 to 4 log cfu g^{-1}, whereas the aerobic bacterial spores were present at 2.81 ± 0.56 log cfu g^{-1}. Low counts of yeasts were recorded, with a mean value of 0.22 ± 0.53 log cfu g^{-1}. The counts of LAB were slightly greater than those reported by Osimani et al. (2017b, 2017c) in whole dried edible mealworms, whereas the numbers of yeast counts were consistent with the values reported in the studies cited

above. Finally, the counts of spore-forming bacteria were in the range of those reported by Klunder et al. (2012) in crushed and boiled mealworms.

The microbiological characterization of WF revealed LAB loads in the range of 1 to 2 log cfu g^{-1}. LAB are naturally present at low levels in cereal-based matrices and results of viable counts performed on WF were consistent with those reported by Alfonzo et al.(2017) for samples of semolina collected in Italy. In the present study, counts of 0.84 ± 0.99 log cfu g^{-1} for yeasts were detected in WF, whereas, in the same matrix the aerobic bacterial spore counts exhibited a mean value of 1.04 ± 1.26 log cfu g^{-1}. Regarding bacterial that, different authors have already reported the occurrence of *Bacillus* strains in wheat semolina and grains, thus suggesting a potential spoilage or pathogenic effect in the final bread (Valerio et al., 2012; Pepe et al., 2013; De Bellis et al., 2015).

Microbial viable counts performed on sourdough at both t_0 and after 16 h of fermentation evidenced high loads of LAB in both MRS and SDB, with mean counts that reached 9.16 ± 0.08 log cfu g^{-1}, thus attesting the appropriateness of the inoculum and the growth of the inoculated selected strains in the dough during incubation. The LAB activity was also reflected by the pH reduction from 5.85 ± 0.02 (t0) to 3.69 ± 0.01 (t16) and the TTA increase from 1.05 ± 0.22 (t0) to 9.33 ± 1.04 (t16) mL of 0.1 N NaOH. The recorded TTA values are caused by the production of organic acids by sourdough LAB (Mariotti et al., 2014). Yeast counts performed in sourdough at both t0 and after 16 h of fermentation revealed counts below 1 log cfu g^{-1}.

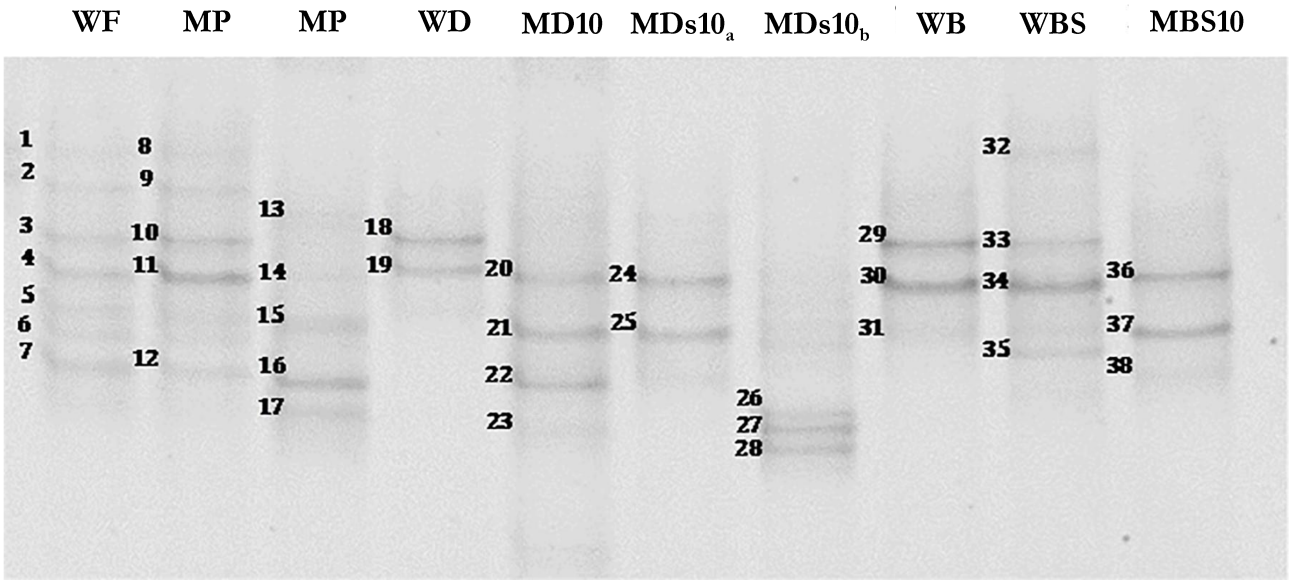
The bread doughs produced with the sole use of baker's yeast as leavening agent (namely, WD, MD5, and MD10) analyzed after 1 h of fermentation exhibited LAB counts between 3.58 ± 0.05 log cfu g^{-1} (MD5 on MRS) and 4.59 ± 0.32 log cfu g^{-1} (MD10 on MRS). The recorded values were slightly lower than those reported by Osimani et al. (2018e) for doughs con- taining WF or a blend of WF and cricket powder. As expected, doughs produced with the use of both baker's yeast and sourdough as leavening agents (namely, WDS, MDS5, and MDS10) exhibited LAB counts as high as 8.39 ± 0.17 log cfu g^{-1}, thus showing the growth of sourdough LAB in the final doughs. The LAB counts in doughs containing sourdough were in accordance with those reported by Osimani et al. (2018e) for doughs inoculated with the same amount of 16-h- fermented sourdough. Regarding yeast counts, the doughs exhibited mean values between 6.92 ± 0.47 log cfu g^{-1} (WDS) and 8.00 ± 0.04 log cfu g^{-1} (MDS10), thus illustrating the effects of the baker's yeast.

Finally, the spore counts just before baking exhibited relatively low mean values that ranged between less than 1 and 1.35 ± 0.99 log cfu g^{-1}. Moreover, the lowest values were found in the bread crumb analyzed soon after baking, with viable counts between less than 1 and 0.58 ± 1.07 log cfu g^{-1}. Overall, the occurrence of aerobic bacterial spores in bread crumb can be explained by their baking heat resistance. In this study, the core temperature of bread loaves ranged between 96.65 ± 0.86 and 97.83 ± 0.59°C, thus likely leading to an incomplete inactivation of the spores.

As reported by different authors, edible insects are natural carriers of bacterial spores that can be transferred to the food matrix when edible insects are used as food ingredients (Grabowsky et al., 2017Osimani 2017a; Stoops et al., 2017; Vandeweyer 2017c; Osimani et al., 2018). Hence, to obtain a more detailed picture of the viable spores occurring in the analyzed samples, bulk cells collected from PCA agar plates with spore counts greater than 1 log cfu g^{-1} were subjected to PCR-DGGE analysis. Since the late 1990s, PCR-DGGE has been widely applied to the study of food and environmental microbial communities, and it still rep- resents one of the most powerful molecular tools to investigate the microbiota of food matrices (Cocolin et al., 2013). Figure 5.2.2 shows DGGE profiles obtained from the microbial DNA extracted from the bulk cells, whereas the closest relatives, the percent

identities, and the accession number of sequences obtained from selected DGGE bands are reported in Table 5.2.3.

Figure 5.2.2. *DGGE profiles of the DNA extracted form the spore-forming bacteria bulk cells washed off the PCA medium and amplified with primers 338fGC and 518r.*



The bands indicated by the numbers were excised, reamplified and subjected to sequencing; the identification of the bands is reported in Table 5.2.3; MDS10$_a$ and MDS10$_b$ correspond to the spore forming bacteria collected from the first and the second bread making trials, respectively; PCA, Plate Count Agar; MP, mealworm powder; samples are codified as reported in Table 5.2.1.

Table 5.2.3. *Sequencing results from the bands cut from the denaturing gradient gel electrophoresis (DGGE) gels obtained from the amplified fragments of the DNA extracted directly from the colonies washed off the PCA plates for the spore counts.*

Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
WF	1	*Bacillus amyloliquefaciens*	99%	KJ603230
	2	*Bacillus amyloliquefaciens*	100%	KJ603230
	3	*Bacillus subtilis*	100%	KJ603239
	4	*Bacillus subtilis*	100%	KJ603239
	5	*Bacillus* sp.	100%	GQ169106
		Bacillus amyloliquefaciens	99%	KJ603230
	6	*Bacillus* sp.	100%	GQ169106
		Bacillus subtilis	100%	KJ603239
		Bacillus amyloliquefaciens	99%	KJ603230
	7	*Bacillus amyloliquefaciens*	99%	KJ603230
MP	8	*Bacillus subtilis*	100%	KJ603239
		Bacillus vietnamensis	100%	LC325200
		Bacillus velezensis	100%	CP023320
	9	*Bacillus amyloliquefaciens*	100%	KJ603230
	10	*Bacillus subtilis*	100%	KJ603239
	11	*Bacillus subtilis*	100%	KJ603239
	12	*Bacillus amyloliquefaciens*	99%	KJ603230
	13	*Bacillus* sp.	100%	GQ169106
	14	*Paenibacillus* sp.	99%	MF139331
	15	*Bacillus* sp.	100%	GQ169106
	16	*Bacillus amyloliquefaciens*	99%	KJ603230
	17	*Bacillus* sp.	100%	GQ169106
		Paenibacillus sp	99%	MF139331

Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
WD	18	*Bacillus subtilis*	100%	KJ603239
	19	*Bacillus* sp.	100%	GQ169106
MD10	20	*Paenibacillus* sp.	99%	MF139331
	21	*Bacillus* sp.	100%	GQ169106
	22	FAILED		
	23	*Bacillus* sp.	100%	GQ169106
MDS10	24	Paenibacillus sp.	99%	MF139331
	25	Bacillus sp.	100%	GQ169106
	26	*Brevibacillus agri*	99%	KX783536
	27	*Brevibacillus agri*	99%	KX783536
	28	*Brevibacillus agri*	99%	KX783536
WB	29	*Bacillus subtilis*	100%	KJ603239
	30	*Bacillus subtilis*	100%	KJ603239
	31	*Bacillus* sp.	100%	GQ169106
WBS	32	*Bacillus amyloliquefaciens*	99%	KJ603230
	33	*Bacillus subtilis*	100%	KJ603239
	34	*Bacillus subtilis*	100%	KJ603239
	35	*Bacillus amyloliquefaciens*	99%	KJ603230
MBS10	36	*Bacillus* sp.	100%	GQ169106
	37	*Bacillus* sp.	100%	GQ169106
	38	*Bacillus* sp.	100%	GQ169106

[a] *Selected bands excised from the agarose gels and subjected to sequencing are numbered;*

[b] *Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database;*

[c] *Accession number of the sequence of the closest relative found by a BLAST search;*
PCA, Plate Count Agar. MP, mealworm powder. Samples of doughs (D) admixed with baker's yeast as leavening agents and sourdough (S) and experimental bread loaves (B) are codified as reported in Table 5.2.1.

High percent identities, between 99 and 100%, were obtained for all of the sequenced bands. The most prevalent genus detected in all the samples belonged to the closest relatives to *Bacillus*.

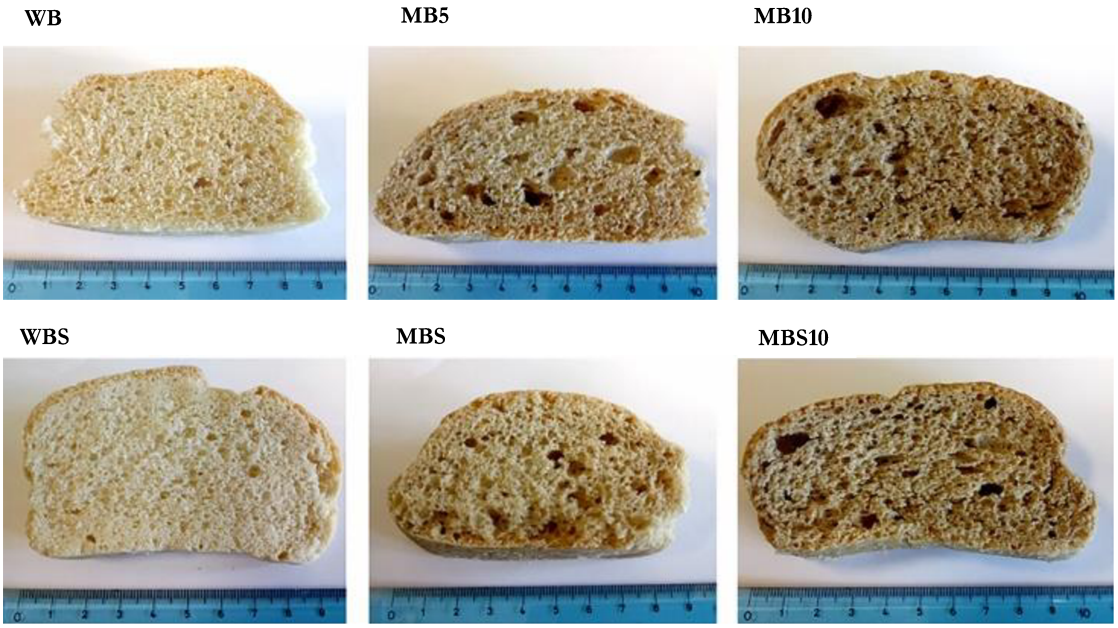
In more detail, the closest relatives to the spoilage species *Bacillus amyloliquefaciens* and *Bacillus subtilis* were broadly detected. These two *Bacillus* species, known to be the causative agent of the ropy spoilage of bread, have been frequently detected in raw materials used in bread making. For example, Valerio et al. (2012) reported the presence of spoilage activity of strains of *B. amyloliquefaciens, B. subtilis* and *B. pumilus* in bread after heat treatment at 100°C for 10 min.

In such a context, the use of sourdough can represent a possible mitigation strategy. Indeed, as reported by Ziane et al. (2014), low pH values prevent spores from germinating and can also exert an inhibitory activity against the recovery of injured spores. Based on the above mentioned available scientific literature, the characterization of bacterial spores that survive a heat treatment is useful for a proper risk assessment, which should also comprise the determination of spores from anaerobic spore-formers, such as *Clostridium* species. Though no microbiological criteria are set by Regulation 2073/2005 as amended by Regulation 1441/2007 for *Clostridium perfringens* and *Bacillus cereus* in ready-to-eat foods, including backed goods, aliquots of experimental bread loaves fortified with mealworm powder were subjected to enumeration of both these spore forming human pathogens, which showed viable counts below 1 log cfu g^{-1}.

Specific volume and texture

Figure 5.2.3 shows the slices obtained from the experimental bread loaves, whereas the results of the specific volume and firmness measurements are shown in Figure 5.2.4. Concerning the specific volume, significantly higher values than for the other conditions were recorded for MB5 and MBS5 with mean values of 3.76 ± 0.38 and 4.02 ± 0.36 cm^3 g^{-1}, respectively. The lowest mean value was recorded for WB (2.55 ± 0.17 cm^3 g^{-1}).

Figure 5.2.3. *Slices obtained from the loaves of experimental bread loaves.*

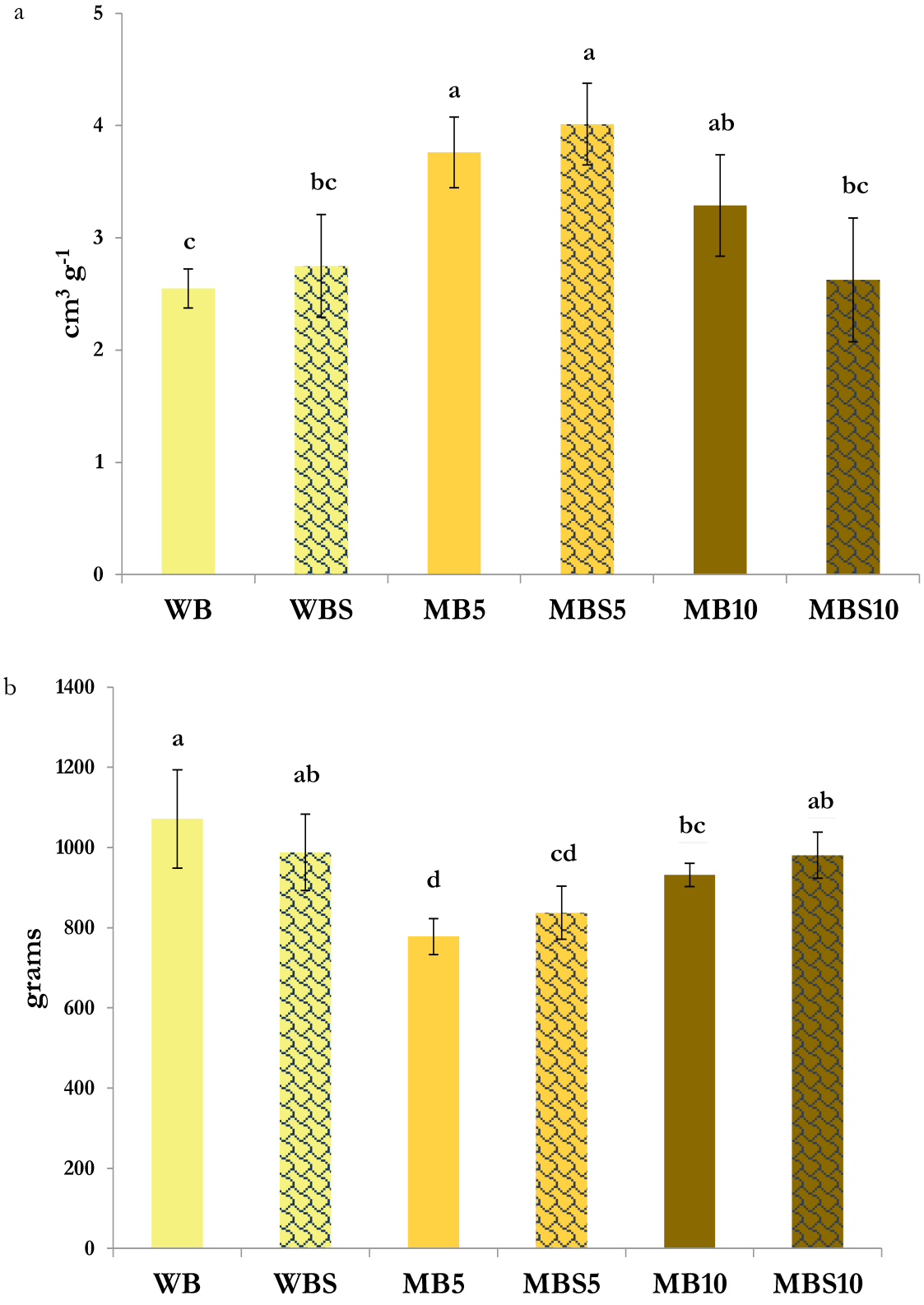


Samples are codified as reported in Table 5.2.1.

Regarding the bread firmness, the highest mean value was measured in WB (1071.06 ± 122.77 g), whereas MB5 exhibited the lowest (777.87 ± 45.00 g). It is noteworthy that de Oliveira et al. (2017) and Osimani et al. (2018e) reported a linear correlation between specific volume and bread firmness in their study regarding bread prepared with insect powder, which can also explain that the bread loaves with the lowest specific volume exhibiting the highest hardness in this study.

In the present study, the addition of MP yielded an enhancement of the specific volume and a softer bread compared with the control bread loaves. These findings can be likely ascribed to the fat fraction of the added MP; it is known that in bread making fat is often incorporated as an antistaling agent and volume improver (Mouliney et al., 2011). As reported by Pareyt et al.(2011), added fats plasticize and lubricate the dough, with an increase in air incorporation during mixing. In such a system, lipid crystals complex with the gluten network, thus leading to dough stabilization and strengthening. Moreover, fats, being adsorbed at the gas cell-dough interface, increase gas retention during leavening. During baking, the melting of fats stabilizes the expanding gas cells)Pareyt et al., 2011).

Figure 5.2.4. *Specific volume (panel a) and firmness (panel b) of bread (B) loaves prepared with the different blends of wheat flour (W) and mealworm powder (M) and admixed with baker's yeast as leavening agent and sourdough (S). Samples are codified as reported in Table 5.2.1. Means ± standard deviations of triplicate independent experiments are shown. Means with different superscripts are significantly different (P > 0.05).*



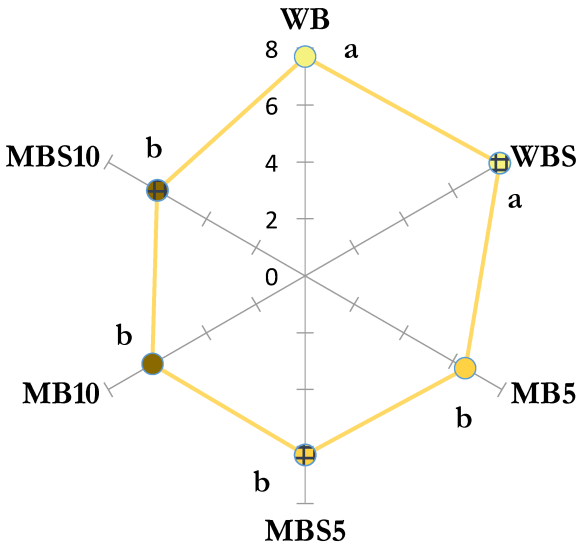
Means ± standard deviations of triplicate independent experiments are shown; means with different superscripts are significantly different (P > 0.05).

Sensory analysis

Consumer acceptability is undoubtedly a further key parameter for launching a new food product into the market. The results of sensory evaluation of the fortified bread loaves containing different levels of MP substitution compared to the controls are shown in Figure 5.2.5.

Overall a significantly highest liking for control bread loaves in respect with fortified bread loaves was seen, irrespective of the type of leavening agent used. Moreover, for the latter bread loaves, no differences were seen in the overall liking, irrespective of the amount of MP added. If these data are compared with those collected by Osimani et al. (2018e) in a similar study, where the effect of cricket powder on sensory properties of fortified bread loaves was assessed, a higher acceptability was scored by mealworm bread loaves in comparison to cricket bread loaves. This latter finding might be explained by the occurrence of differences in the sensory features of the two insect-based powders used in bread-making, namely color, flavor and taste. It is known that different insect species are characterized by quite different tastes and flavor (Kouřimskà and Adámková, 2016; Ramos-Elorduy et al., 1998). For instance, mealworms have a more attractive as food from different sensory point of views.

Figure 5.2.5. *Overall liking of experimental bread loaves (B) produced with wheat flour and different blends of wheat flour (W) and mealworm powder (M) admixed with sourdough (S) and baker's yeast as leavening agents.*



The degree of global liking was ranked in accordance with a 9-point hedonic scale ranging from 1 (dislike extremely) to 9 (like extremely); samples are codified as reported in Table 5.2.1; means followed by different letters are significantly different (P < 0.05).

Given these premises, either the texture or the strong aroma and smell of cricket powder might have negatively contributed to the consumers appreciation of the corresponding fortified bread than those assayed in the present study.

5.2.3 Conclusions

Among edible insects, mealworm larvae are easy to rear and can provide protein of high nutritional value. In the present study, proof of concept was provided for the inclusion of MP into bread doughs leavened with sourdough and/or baker's yeast. Moreover, MP contributed to the improvements in both bread volume and softness, likely due to its lipid fraction. At this regard, MP

appears as an adequate ingredient for bread fortification. Whereas technically, there are no major limitations to incorporate MP into bread dough at the assayed levels (5 and 10%), some considerations regarding microbial and sensory quality traits of bread enriched with mealworm powder can now be made. First, acceptability of fortified breads was negatively affected by comparison with control breads produced with the sole soft wheat flour. However, if overall liking scores collected in the present study were compared with those of a previous research from the same laboratory on breads fortified with cricket powder (at 10 and 30% substitution level), mealworm breads were characterized by a higher acceptability, feasibly due to the acknowledged differences in flavor and aroma of mealworm and cricket powders. Concerning the microbial quality, the occurrence of spore-forming bacteria in both doughs and breads fortified with mealworm powder highlighted potential spoilage and even human safety issues that still need to be resolved. At this regards, in future studies, it might be valuable to investigate the effect of strategies to reduce the amount of endospores in bread and, more in general, in baked leavened goods, such as the use of preservatives, e.g. propionate or mitigation technologies, e.g. dehydration or toasting.

5.3 Lesser mealworm (*Alphitobius diaperinus*) powder as a novel baking ingredient for manufacturing high-protein, mineral-dense snacks (Paper X)

Recently, food fortification in terms of protein, vitamin, aminoacid and mineral contents drew consumers attention. The interest in consuming foods with higher nutritional properties is actually leading to the growth of the fortified snacks market (Ahuja and Deb, 2018). As a consequence, the food industry is selecting innovative and sustainable raw materials to produce fortified foods (Ahuja and Deb 2018).

In this context, the use of edible insects as food ingredients can represent an emerging strategy for the fortification of conventional foods due to their positive nutritional features in terms of proteins, unsaturated fatty acids, minerals and vitamins.. Edible insects consumption is expected to increase in next years. For instance, it is expected that the market for snacks produced from edible insects could exceed 240 million dollars in 2024 (Rumpold and Schlüter 2013; Ahuja and Deb 2018).

Even the research sector has recently exploited edible insect powder in bread-making (Aguilar-Miranda et al. 2002; de Oliveira et al. 2017; González et al. 2018; Osimani et al. 2018b; Roncolini et al. 2019a). In all the latter studies, edible insect powders were successfully incorporated into leavened baked goods in terms of nutritional traits enhancement. Since the inclusion of insect powder in bread-making can alter the technological, nutritional, microbiological and sensory features of the end product, depending on both the amount of insect powder added (Osimani et al. 2018e; Roncolini et al. 2019a) and the insect species (Rumpold and Schlüter 2013; Roncolini et al. 2019), further research is needed to evaluate their effects on baked goods properties, such as technological, nutritional, microbiological and sensory qualities.

The present study was aimed at (i) making formulations containing lesser mealworm (*Alphitobius diaperinus*) powder for protein and mineral fortification of soft wheat (*Triticum aestivum* L.) crunchy

snacks and (ii) evaluating the effects of increasing amounts of lesser mealworm powder on the technological, microbiological, and sensory characteristics of the fortified crunchy snacks.

To achieve these goals, physico-chemical, microbiological and sensory analyses were carried out on experimental samples belonging to the different baking steps from raw materials to rusks produced using different blends of wheat flour and lesser mealworm powder and different leavening agents.

5.3.1 Materials and methods

Flour and insect powder

Lesser mealworm powder (LP) containing the whole lipid fraction was purchased from Kreca Ento-Food BV (Ermelo, The Netherlands); LP samples were shipped in 100 g plastic bag packages and stored at ambient temperature until use. Soft wheat flour (WF), classified as type 00, was purchased from a local mill (Molino Stacchiotti, Ancona, Italy).

Sourdough production with selected lactic acid bacteria strains

Five lactic acid bacteria strains belonging to the Culture Collection at the Department of Agricultural, Food and Environmental Sciences (D3A) (Università Politecnica delle Marche) were used as sourdough starter cultures, namely Lactobacillus fermentum PB162, Lactobacillus plantarum PB11, Lactobacillus plantarum PB24, Lactobacillus sanfranciscensis PB276, and Lactobacillus sanfranciscensis PB223. These lactic acid bacteria strains were cultured and added to a mixture of water and WF in accordance with the conditions already described by Roncolini et al. (2019) and Osimani et al. (2018b).

The pool of selected lactic acid bacteria strains, WF and sterilized tap water were mixed to reach a dough yield of (60 g of WF plus 40 mL of water per 100 g of dough). The dough, which had an initial lactic acid bacteria concentration of 8 log cfu g^{-1}, was left to ferment at 30°C for 16 h. The mature sourdough (S) was then used for bread making trials.

Dough composition and rusk production

The dough (D) formulations are reported in the flowchart shown in Figure 5.3.1. In brief, two different substitution levels of WF with LP (10% and 30%) were used to produce the experimental rusks. Furthermore, the rusks produced with WF alone were used as a control. The flour:sourdough ratio was kept constant and equal to 3:1; sterilized tap water was added to reach a dough yield of 160. All the formulations included 2% (w/w) bakers' yeast.

The leavening performance of the doughs (LD10, LDS10, LD30, LDS30, WD, and WDS) was evaluated as previously reported by Osimani et al. (2018e). Each dough was prepared as described in Figure 1 and then placed in a graduated glass cylinder (2 L) and left to ferment. The volumes of the doughs (in mL) were recorded immediately (t0) and after a 2-h fermentation (t2) at 30°C. The leavening was calculated using the following formula:

$$[(V2-V0) / V0] \times 100$$

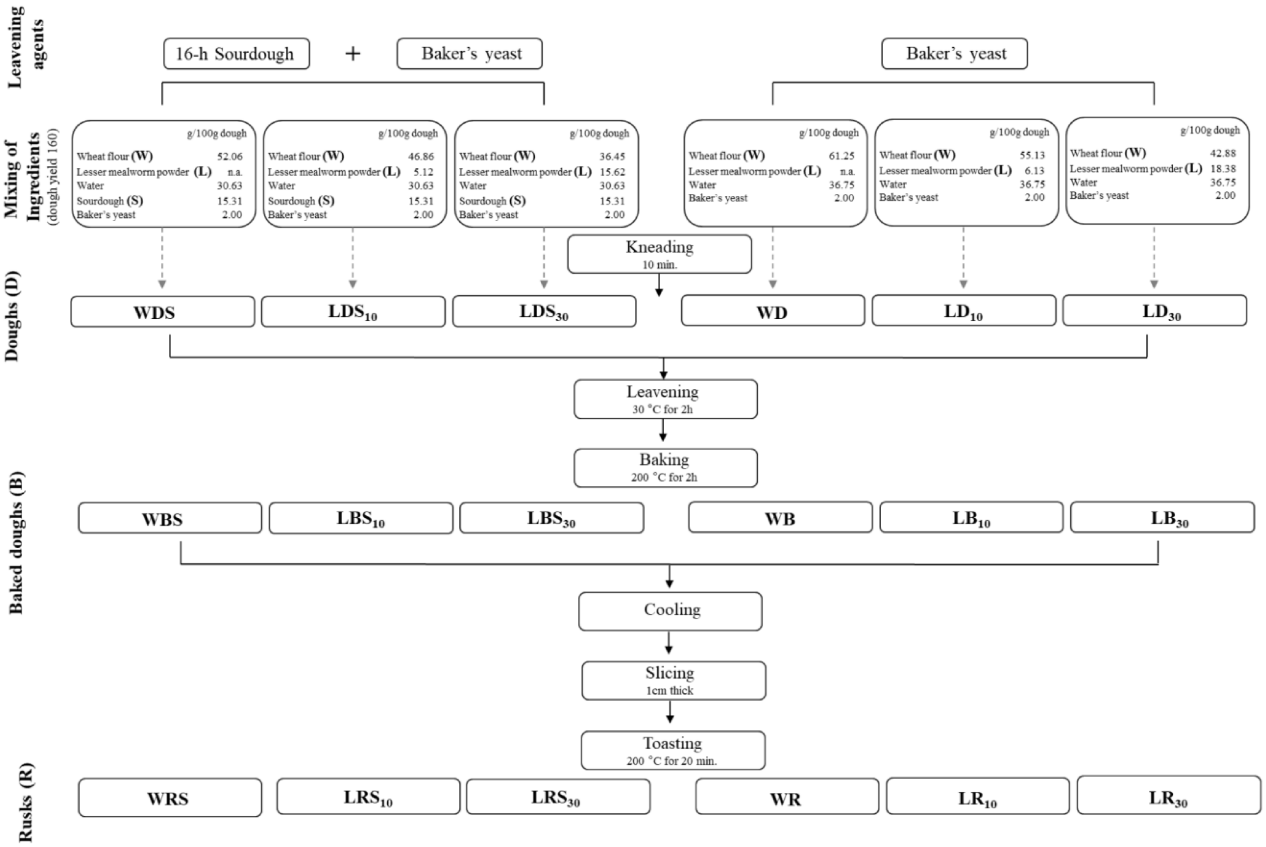
where V0 was the volume at t0 and V2 was the volume after a 2-h fermentation.

The results were expressed as the means ± standard deviations of triplicates.

The baked doughs (B) made with LD10 (LB10), LDS10 (LBS10), LD30 (LB30), LDS30 (LBS30), WD (WB), and WDS (WBS) used for rusk production (Figure 5.3.1) were produced through a one-step fermentation process (30 °C for 2 h) with baking at 200 °C for 1 h (Osimani et al. 2018).

The production of rusks was performed as proposed by Rey-Salgueiro et al. (2008). In brief, slices (approximately 8 x 5 x 1.5 cm) of cooled, baked doughs were subjected to 20 min of roasting at 200 °C. The process parameters are reported in the flowchart shown in Figure 5.3.1. The rusk production was performed in duplicate.

Figure 5.3.1. *Flow chart for the production of doughs (D), baked doughs (B) and rusks (R) obtained with the use of wheat flour (W) or different blends of wheat flour and 10% or 30% lesser mealworm powder (L) and admixed with the sole baker's yeast or with baker's yeast plus sourdough (S) as leavening agents.*



Physico-chemical measurements of baked products

The water activity (a_w) was measured in accordance with the ISO 21807:2004 standard method using an Aqualab 4TE apparatus (Meter Group, Pullman, USA). The pH was determined using a solid electrode (HI2031, Hanna Instruments, Padova, Italy) pH meter (HI2031, Hanna Instruments, Padova, Italy) directly inserted into the food matrix. The total titratable acidity (TTA) was determined as previously described by Minervini et al. (2012). Ten g of each sourdough, dough and baked dough was blended with 90 mL of distilled water and then titrated with 0.1 N NaOH to a final pH of 8.5. The results, as reported as the means ± standard deviation, were expressed as the amount of NaOH (mL) used. All the assays were performed in triplicate.

Microbial counts

For the viable microbial counts, 10 g of each sample was homogenized into 90 mL of peptone water (bacteriological peptone 1 g L^{-1}, Oxoid, Basingstoke, UK) with a stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 260 rpm. To determine the aerobic bacterial

spores, the homogenized samples were further subjected to thermal treatment at 80°C for 15 min followed by cooling in iced water (Osimani et al. 2017c). Aliquots of ten-fold dilutions were then inoculated into the appropriate growth media as follows: presumptive lactic acid bacteria were enumerated on both MRS and SDB (Vogel et al. 1994) media and supplemented with cycloheximide (0.2 g L^{-1}) to inhibit the growth of yeasts. The yeasts were enumerated on Rose Bengal Agar supplemented with chloramphenicol (0.1 g L^{-1}) (Oxoid) as described by Cardinali et al. (2016). The aerobic bacterial spores were enumerated on Standard Plate Count Agar (Oxoid) incubated at 30°C for 48 h. The viable 176 counts were expressed as the mean log colony forming units (cfu) per gram of sample ± standard deviation.

PCR-DGGE analyses

Plates of spore-forming bacteria showing colony counts between 30 and 300 were used for bulk formation according to Garofalo et al. (2015). When there were fewer than 30 colonies growing on the plates, the bulk cells were collected from the first dilution that showed any growth. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analyses were performed in accordance with the methods described by Roncolini et al. (2019). In brief, the microbial DNA was extracted directly from the bulks using a PowerSoil Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, USA).

After a DNA quantity and purity assessment with a UV-Vis spectrophotometer (Shimadzu UV-1800, Shimadzu Corporation, Kyoto, Japan), 100 ng of each template was subjected to PCR in a 50 µL reaction volume with the primer pair 338f and 518r to amplify the V3 region of the 16S rRNA gene. As proposed by Ampe et al. (1999), a GC clamp was attached to the 338f primer. The DGGE analysis was performed using the vertical DCode electrophoresis system (Bio-Rad Laboratories) as already described by Garofalo et al. (2015). The DGGE bands were sequenced as previously described by Osimani et al. (2015).

The sequences obtained in FASTA format were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990).

Sensory analysis

The sensory evaluation of the rusks was performed soon after they cooled. In brief, the rusks were left to cool at room temperature and then a small-scale acceptance test was performed (Svensson 2012). To this end, 9 untrained panelists (5 males and 4 females, non-smokers, aged 22–58) familiar with the taste/consumption of leavened baked goods were recruited.

The sensory analysis was performed as suggested by Resurreccion (1998). Overall participant liking was ranked using a 9-point hedonic scale, ranging from 1 (dislike extremely) to 9 (like extremely) as previously suggested by Peryam and Pilgrim (1957). The results represent the means of three independent experiments. All the assessors involved in the sensory analyses were informed about the aim of the study, and they provided their informed written consent.

Statistical analysis

The Tukey-Kramer's Honest Significant Difference (HSD) test (level of significance 0.05) was used to evaluate the differences within groups (blends of WF and LP, and baked goods) by one-way analysis of variance (ANOVA). The experimental data were explored by Principal Component Analysis (PCA); normalization was used as a data pre treatment procedure. JMP Version 11.0.0 software (SAS Institute Inc., Cary, NC) was used to perform all the tests.

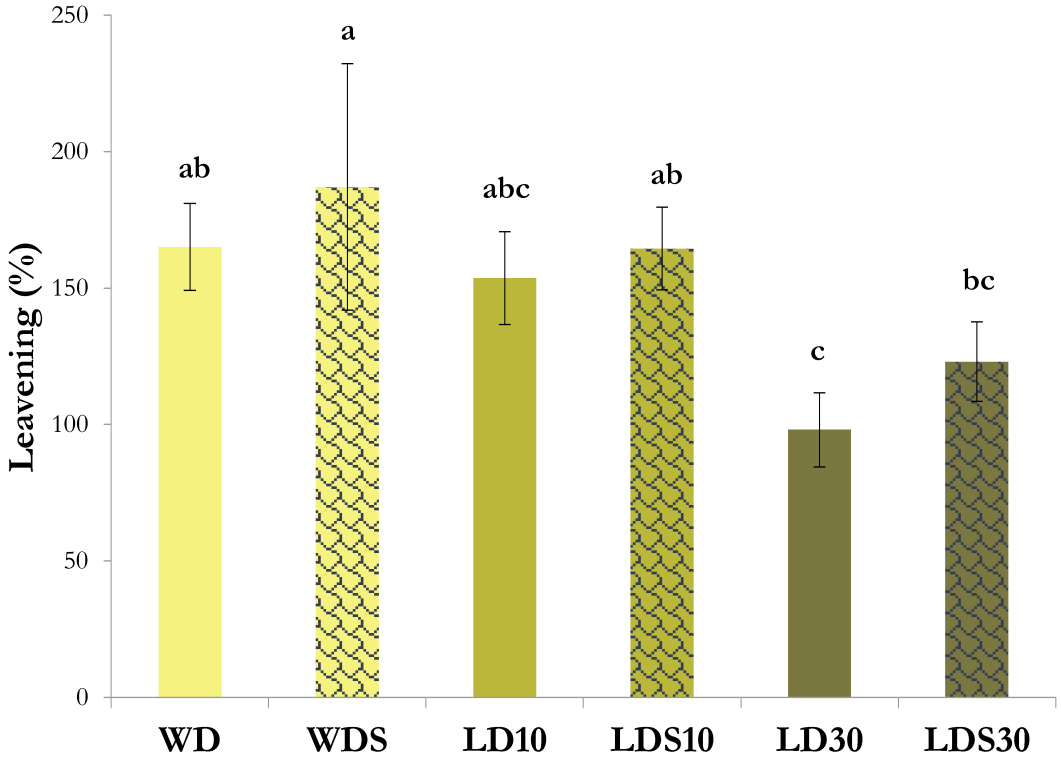
5.3.2 Results and discussion

Leavening

The results of the leavening ability measurements are reported in Figure 5.3.2.

As expected, an inverse trend was observed, with a higher dough volume at the lower WF substitution level for LP.

Figure 5.3.2. *Leavening of the doughs (D) prepared with the different blends of wheat flour (W) and lesser mealworm powder (L) and admixed with baker's yeast as leavening agent and sourdough (S).*



Samples are codified as reported in Figure 5.3.1; means ± standard deviations of triplicate independent experiments are shown; means with different letters are significantly different (P > 0.05).

Viable counts, pH and TTA and PCR-DGGE

The results of the microbiological analyses on the sourdough, doughs and rusks are shown in Table 5.3.1. The viable counts of lactic acid bacteria (on both MRS and SBD) and yeasts almost completely overlapped those previously reported by Osimani et al. (2018e), thus attesting to the good standardization of the 16 h sourdough inoculation process and the adopted bread-making parameters. Moreover, on both culture media, LAB enumeration was higher in doughs containing sourdough than in those with the sole baker's yeast as a leavening agent. The viable counts of spore-forming bacteria were notably lower than those reported by Osimani et al. (2018e) for leavened doughs containing cricket powder but slightly higher than those found by Roncolini et al. (2019a) in leavened doughs containing mealworm powder; the cause of that coulb be related to a few variables such as the differences in the rearing conditions, the feeding substrates, and the processing and storage conditions as well as the composition of the intrinsic microflora specifically associated with each insect species. The microbiological analyses performed on rusks soon after toasting (Table 5.3.1) revealed the presence of aerobic bacterial spores in all the samples, with values ranging between 0.25 ± 0.50 (samples WR) and 1.45 ± 0.17 (samples LRS30) log cfu g^{-1} .

Finally, TTA and pH values (Table 5.3.1) reflected the metabolic activities of the inoculated protechnological microorganisms. Indeed, doughs and the corresponding bread with sourdough showed the lowest pH and the highest TTA in comparison with respective samples without sourdough.

Table 5.3.1. *Results of microbiological characterization of sourdough before (S0) and after 16-h fermentation (S16), doughs (before and after 1-h fermentation), bread loaves and rusks.*

Samples		**LAB on MRS**	**LAB on SBD**	**Yeasts**	**Spore-forming bacteria**	**pH**	**TTA**	**aw**
		log cfu g^{-1}						
Flour	WF	1.65±0.26	1.19±0.24	2.10±1.11	0.62±0.74	n.d.	n.d.	0.54±0.01
	LP	4.03±0.04	4.06±0.06	0.58±1.15	3.41±0.28	n.d.	n.d.	0.08±0.02
Sourdough (0 h)	S0	7.54±0.22	7.61±0.08	1.56±0.11	n.d.	5.84±0.00	1.25±0.10	n.d.
Sourdough (16 h)	S16	9.25±0.14	9.25±0.16	0.33±0.65	n.d.	3.59±0.04	9.17±0.05	n.d.
Dough (0 h)	WD	n.d.	n.d.	n.d.	n.d.	5.50±0.08	2.22±0.17	n.d.
	WDS	n.d.	n.d.	n.d.	n.d.	4.86±0.14	3.50±0.09	n.d.
	LD10	n.d.	n.d.	n.d.	n.d.	5.88±0.08	3.02±0.12	n.d.
	LDS10	n.d.	n.d.	n.d.	n.d.	5.27±0.00	3.88±0.19	n.d.
	LD30	n.d.	n.d.	n.d.	n.d.	6.22±0.01	4.80±0.00	n.d.
	LDS30	n.d.	n.d.	n.d.	n.d.	5.80±0.00	4.90±0.09	n.d.
Dough (1 h)	WD	3.90±0.54	4.27±0.92	8.16±0.13	0.90±0.62	5.38±0.06	3.30±0.66	n.d.
	WDS	8.62±0.16	8.69±0.11	7.29±0.36	<1	4.48±0.01	4.25±0.15	n.d..
	LD10	4.97±0.22	5.01±0.04	8.25±0.16	2.06±0.13	5.64±0.05	3.98±0.17	n.d.
	LDS10	8.60±0.09	8.69±0.06	8.15±0.07	2.06±0.21	5.07±0.01	4.47±0.15	n.d.
	LD30	5.45±0.66	5.41±0.65	8.23±0.13	2.22±0.21	6.08±0.07	5.75±0.30	n.d.
	LDS30	8.76±0.05	8.83±0.05	8.26±0.11	2.32±0.19	5.58±0.01	6.83±0.15	n.d.
Bread	WB	n.d.	n.d.	n.d.	<1	6.92±0.14	1.58±0.08	0.97±0.00
	WBS	n.d.	n.d.	n.d.	<1	5.02±0.03	2.96±0.26	0.97±0.00
	LB10	n.d.	n.d.	n.d.	0.83±0.57	5.96±0.07	2.12±0.10	0.95±0.03
	LBS10	n.d.	n.d.	n.d.	0.94±0.66	5.32±0.05	3.68±0.13	0.98±0.01
	LB30	n.d.	n.d.	n.d.	0.58±0.68	6.31±0.14	3.58±0.10	0.96±0.03
	LBS30	n.d.	n.d.	n.d.	1.23±0.15	5.81±0.06	4.82±0.12	0.96±0.01
Rusk	WR	n.d.	n.d.	n.d.	0.25±0.50	n.d.	n.d.	0.61±0.02
	WRS	n.d.	n.d.	n.d.	1.23±0.29	n.d.	n.d.	0.59±0.01
	LR10	n.d.	n.d.	n.d.	0.83±0.57	n.d.	n.d.	0.61±0.06
	LRS10	n.d.	n.d.	n.d.	0.50±0.58	n.d.	n.d.	0.65±0.10
	LR30	n.d.	n.d.	n.d.	1.39±0.10	n.d.	n.d.	0.72±0.19
	LRS30	n.d.	n.d.	n.d.	1.45±0.17	n.d.	n.d.	0.70±0.17

TTA was expressed as ml of 0.1;
Samples are codified as reported in Figure 5.3.1;
n.d. not determined.

The PCR-DGGE analyses on the experimental rusks revealed the presence of the closest relatives of the *Bacillus* and *Paenibacillus* genera (Table 5.3.2). Despite the occurrence of the closest relatives to the spore forming bacteria in these rusks, the a_w values ranged from 0.59 ± 0.01 to 0.72 ± 0.19, suggesting that they did not represent a favorable growth substrate for these microorganisms. Hence, although the bacterial endospores survived the baking and toasting conditions, the latter treatment considerably reduces the risk of microbial multiplication, thus partly mitigating concerns over the safety issues regarding insect-based bread loaves (Osimani et al. 2018e; Roncolini et al. 2019a). Even regarding spoilage concerns, the strong a_w reduction due to oven toasting is expected to prevent the germination and further multiplication of endospores from the species, primarily ascribed

to Bacillus sp., that were responsible for ropiness. Indeed, low moisture conditions and pH values < 5.3 are known to limit the growth of these spoilage microorganisms (Rumeus and Turtoi 2013).

Table 5.3.2. *Sequencing results from the bands cut from the denaturing gradient gel electrophoresis (DGGE) gels obtained from the amplified fragments of the DNA extracted directly from the colonies washed off the Plate Count Agar (PCA) plates for the spore counts.*

Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
WF	1	*Bacillus* sp.	98%	MK300870
LP	2	*Bacillus* sp.	100%	KF751675
	3	*Bacillus subtilis*	98%	MK294226
	4	*Paenibacillus lautus*	98%	MK256307
	5	*Bacillus sp.*	98%	MH290495
	6	*Bacillus subtilis*	99%	MG208038
WD	7	*Bacillus* sp.	100%	MK300870
	8	*Bacillus* sp.	99%	MH569422
LD10	9	*Bacillus subtilis*	100%	MG208038
	10	*Paenibacillus oryzae*	98%	NR_153707
	11	*Paenibacillus* sp.	99%	MG755306
	12	*Bacillus subtilis*	98%	MG208038
LDS10	13	*Paenibacillus oryzae*	98%	NR_153707
	14	*Paenibacillus yonginensis*	98%	NR_148743
	15	*Paenibacillus* sp.	99%	MG755306
	16	*Paenibacillus* sp.	99%	MG755306
	17	*Bacillus subtilis*	99%	MG208038
LD30	18	*Paenibacillus oryzae*	98%	NR_153707
	19	*Paenibacillus yonginensis*	98%	NR_148743
	20	*Paenibacillus* sp.	100%	MK249747
	21	*Paenibacillus* sp.	99%	MG755306
	22	*Bacillus subtilis*	99%	MG208038
LDS30	23	*Bacillus subtilis*	100%	MK288147
	24	*Paenibacillus yonginensis*	98%	NR_148743
	25	*Paenibacillus* sp.	100%	MK249747
	26	*Paenibacillus segetis*	100%	NR_151983
	27	*Bacillus subtilis*	99%	MG208038
LB10	28	*Bacillus subtilis*	100%	HM475276
	29	*Paenibacillus segetis*	100%	NR_151983
LBS10	30	FAILED		
LB30	31	*Paenibacillus* sp.	99%	KY635885
	32	*Paenibacillus yonginensis*	98%	NR_148743
	33	*Paenibacillus* sp.	100%	LC336777
LBS30	34	*Paenibacillus yonginensis*	98%	NR_148743
	35	*Paenibacillus yonginensis*	98%	NR_148743
	36	*Paenibacillus* sp.	98%	KX636168
WR	37	*Bacillus subtilis*	100%	NR_118383
	38	*Bacillus subtilis*	100%	MG208038
WRS	39	*Bacillus* sp.	98%	KY914197
	40	*Bacillus* sp.	100%	MK294218
	41	*Bacillus subtilis*	100%	MK301273
	42	*Bacillus* sp.	100%	MH032861
	43	FAILED		
LR10	44	*Bacillus* sp.	98%	MK322937
	45	*Bacillus subtilis*	100%	MK101047
	46	*Bacillus subtilis*	100%	NR_118383
	47	*Bacillus* sp.	100%	MK300870
	48	*Bacillus subtilis*	98%	KM008605

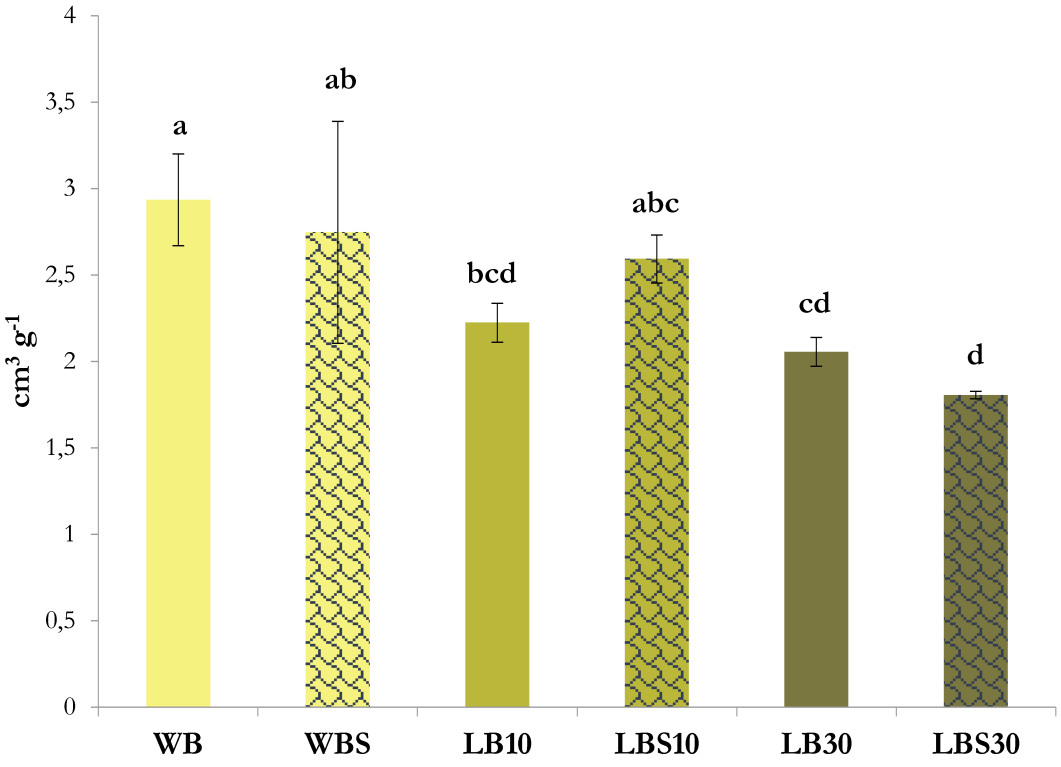
Samples	Bands[a]	Closest relative	% Ident.[b]	Acc. No.[c]
LRS10	49	*Bacillus amyloliquefaciens*	100%	MK301464
	50	*Bacillus subtilis*	98%	KM008605
	51	*Bacillus* sp.	100%	MK300870
	52	*Bacillus subtilis*	99%	MK288147
LR30	53	*Paenibacillus* sp.	100%	LC336777
	54	*Bacillus* sp.	100%	MH032861
	55	*Bacillus* sp.	100%	MG461855
LRS30	56	*Paenibacillus yonginensis*	100%	NR_148743
	57	FAILED		
	58	*Bacillus* sp.	99%	MG461855

[a]Selected bands excised from the agarose gels and subjected to sequencing are numbered;
[b]Percentage of identical nucleotides in the sequence obtained from the DGGE bands and the sequence of the closest relative found in the GenBank database;
[c]Accession number of the sequence of the closest relative found by a BLAST search. Samples are codified as reported in Figure 5.3.1.

Specific volume

Specific volumes of baked doughs are shown prior to slicing in Figure 5.3.3.

Figure 5.3.3. *Specific volume of baked doughs (B) prepared with the different blends of wheat flour (W) and lesser mealworm powder (L) and admixed with baker's yeast as leavening agent and sourdough (S).*

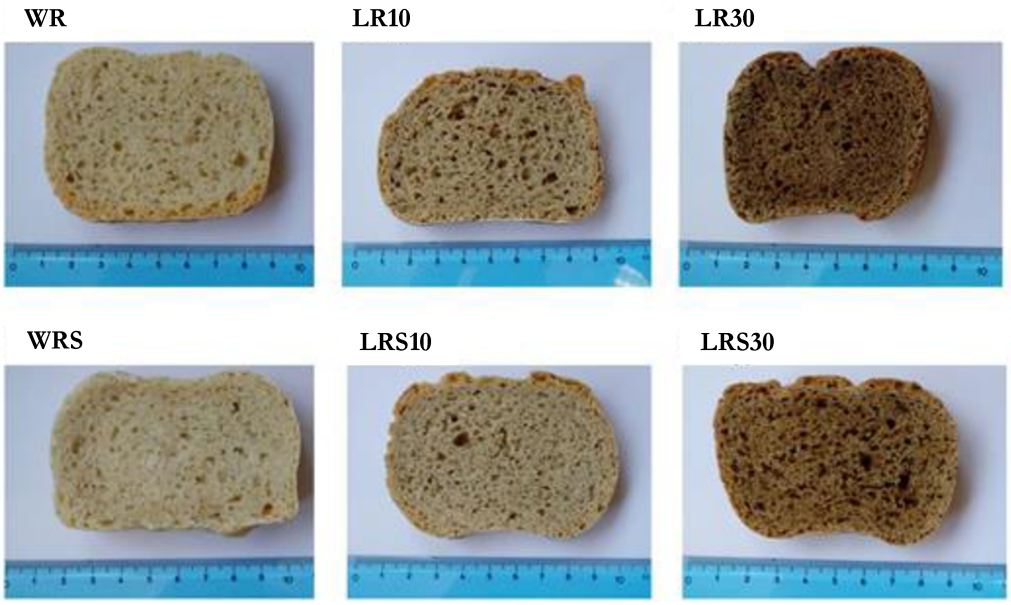


Samples are codified as reported in Figure 5.3.1; means ± standard deviations of triplicate independent experiments are shown; means with different superscripts are significantly different (P > 0.05).

All the data were fully consistent with those previously found by Osimani et al. (2018e) and Roncolini et al. (2019a) in bread loaves produced with cricket and mealworm powders, respectively, again with an inverse trend between the specific volume and the LP content.

The rusks produced from sliced baked doughs and oven toasting are shown in Figure 5.3.4.

Figure 5.3.4. *Rusks obtained from oven toasted slices of experimental baked goods.*

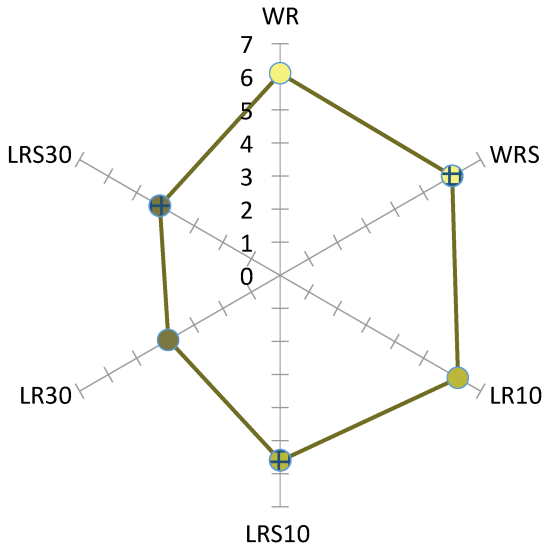


Samples are codified as reported in Figure 5.3.1.

Sensory analysis

Figure 5.3.5 reports the results of the sensory evaluation performed by the 9 untrained panelists on the experimental rusks.

Figure 5.3.5 *Global liking of experimental rusks (R) produced with wheat flour (W) and different blends of wheat flour and lesser mealworm powder (L) and admixed with baker's yeast as leavening agent and sourdough (S).*



Means of triplicate independent experiments are shown. For each rusk typology, means with different letters are significantly different (P > 0.05).

LR10 and LR30 received the highest and the lowest score, respectively, whereas no significant differences were seen among WR, WRS, LRS10, and LRS30. The preference of the panelists for rusks enriched with 10% LP undoubtedly represents a sound starting point for the formulation of

industrially produced insect-based snacks. In previous studies by Osimani et al. (2018e) and Roncolini et al. (2019a), the same panel expressed a much more modest appreciation of bread loaves produced with cricket and mealworm powders, respectively. On the contrary, the rusks with the highest amount of LP (30%) were less appreciated than those produced with 10% of this ingredient; this result is in accordance with Osimani et al. (2018e) for bread containing 30% cricket powder, thus suggesting 10% insect powder as a threshold. In this study, either the choice of the insect species or the peculiar processing of fortified cereal-based snacks might have exerted a positive impact on consumer acceptance. As reported by Ramos-Elorduy et al. (1998) and Kouřimská and Adámková (2016), insects are strongly characterized by peculiar sensory traits that can be influenced by pheromones, feed, and even the presence of an exoskeleton. In this regard, edible Coleoptera, including *A. diaperinus*, are usually reared on cereal-based feed that confers a sweet and nutty flavor to the larvae, along with a cocoa smell and a brownish color (Ramos-Elorduy et al. 1998; Kouřimská and Adámková 2016), which in turn are expected to positively affect the sensory attributes of foods produced with these insects, as verified in the present study.

5.3.3 Conclusions

In the present study, microbiological, physico-chemical and sensory analysis where performed along an insect-based rusks production process in order to have information about food safety and consumers acceptance regarding these specific baked goods.The results collected about microbial safety confirmed the suitability of LP for manufacturing leavened crunchy snacks (rusks). Indeed, even if *Bacillus* and *Paenibacillus* spores were found in both bread loaves and rsusks, a_w detected was low, thus avoiding germination and hence the multiplication of such bacteria. Moreover results regarding sensory analysis were encouraging for further investigations. Indeed, rusks containing 10% powder were appreciated by consumers.

6. Conclusions and perspectives

Edible insects are considered a suitable alternative to the traditional protein sources for both environmental and nutritional reasons.

Entomophagy is actually practised in many countries worldwide but in Europe insects have been taken into account as food and feed only recently. In 2015, due to the lack of knowledge about edible insects safety as food and feed, EFSA requested further research to collect systematical information about microbiological and chemical hazards associated to their consumption.

It is noteworthy that edible insects microbiota is associated both with their intrinsic flora, influenced by insect species, gender, life stage, and external factors, such as rearing or environmental conditions, handling and processing procedures.

In this context, the present PhD. thesis was aimed to collect information about the microbiota related to edible insects. To this objective, a polyphasic approach including culture-dependent and culture-independent methods was adopted. In particular, edible insects samples were subjected to microbial viable counts and molecular biology analysis, such as, PCR-DGGE, pyrosequencing and real-time qPCR in order to get information about microbial load and microbial population identification.

Overall collected data showed interesting aspects.

First of all, edible insects microbiota is characterized by the presence of symbiont, commensal, spoilage and potentially pathogenic agents. For instance, the presence of genera with demonstrated probiotics activity for insects, such as *Lactobacillus*, was highlighted in black ants and rhino beetles by both viable counts and culture-independent analysis; furthermore, black ants microbiota was found to include *Stenotrophomonas* spp., with demonstrated ability in degrading organophosphorus insecticides, thus inducing host resistance to insecticides.

Interestingly, microbiota of the edible insects under study was largely dominated by spore-forming bacteria, mainly belonging to genera *Bacillus* and *Clostridium*, which include both spoilage and pathogenic agents; this finding might be actually due to the treatment under which edible insects samples were subjected before the purchase, i.e. boiling and drying. *Pseuodomonas* and *Staphylococcus* species, which can include among others, pathogen agents, were also found in the analyzed samples. Regarding that, a positive result was the absence of *P. aeruginosa* via real-time qPCR in giant water bugs, black ants, winged termite alates, rhino beetles, mole crickets and silkworm pupae.

In general, results showed that edible insects microbiota is influenced by several factors: i) different insect species showed microbial variability which can be related to their intrinsic flora; ii) growth substrate might have a microbial modulatory effect, as seen by feeding *H. illucens* with coffee silverskin or vegetable substrate; iii) microbiota composition differences were found among insects belonging to different batches, thus highlighting the influence of rearing procedures; iv) finally a country of origin effect was observed in mealworms, grasshoppers and small crickets. These findings confirmed those already reported in literature.

Part of the present PhD. thesis was aimed at investigating about the occurrence of transferable antibiotic resitance genes. Insects can act as a reservoir of antibiotic resistance genes which can be horizontally transferred among bacteria. Insects microbiota can include bacteria, even pathogens, which are potential antibiotic resistance genes carriers; thus including insects in the health issue related to antibiotic resistance. In detail, both nested-PCR and real-time qPCR were performed to get in depth information about the occurrence of transferable resistance genes to commonly used and last generation (carbapenems) antibiotics in edible insects microbiota.

A high frequency of genes conferring resistance to the most commonly used antibiotic, mainly tetracycline and erythromycin, was observed. Carbapenems resistance genes were found in grasshoppers and mealworms even in much lower abundance than the previously cited antibiotic resistance genes. As well as for edible insects microbiota composition, data showed the influence of country of origin and rearing conditions on the occurrence of antibiotic resistance genes.

Collected data about microbiota related to edible insects as well as transferable resistance genes occurrence highlighted that a full standardization of production technologies and procedures is recommended to ensure a safe product when insects are intended for commercialization.

Finally, in the European Union, edible insects introduction in human diet is obstructed by consumers perception of insects as food. Insects are still associated with primitive behaviours and considered with scepticism and disgust. The strategy used in this PhD. to stimulate insects consumption was represented by the inclusion of edible insects powder in food products (bread and rusks) to mask their presence. Microbiological, technological and sensory analysis were performed on the above cited products to investigate about insect powder suitability in baked goods production. In particular, microbial viable counts and PCR-DGGE were carried out to get information about their safety mainly focusing on spore-forming bacteria, which can survive to the high temperatures of the baking process.

Obtained results showed that spore-forming bacteria were present in the final products confirming their capability in surviving to heat treatments. An encouraging data is represented by the low a_w measured in rusks containing *A. diaperinus* powder. Indeed a low a_w avoids bacteria germination in food.

Mealworm powder contributed to improve both bread volume and softness whereas cricket powder and lesser mealworm powder had not the same effect.

Finally, consumers acceptance of cricket powder-based bread was very low showing a negative linear correlation depending on its amount in bread. On the contrary, encouraging data were reported by the sensory analysis of bread and rusks made with mealworm powder and lesser mealworm powder.

These encouraging data may suggest the need of further research on formulations (use of other insect species powder or additives) or baking procedures and treatments in order to improve technolgical and safety properties of edible insect-based food products.

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