MICROBIOLOGICAL QUALITY OF RAW EDIBLE INSECTS AND IMPACT OF PROCESSING AND PRESERVATION

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Preface - Voorwoord

Ik moet toegeven, toen ik begon als student in het hoger onderwijs had ik nooit gedacht dat ik zo'n tien jaar later het student-zijn zou beëindigen met een doctoraat. Zeker toen ik uiteindelijk in Geel terecht kwam (toen nog Katholieke Hogeschool Kempen (KHK), later Thomas More en uiteindelijk KU Leuven), had ik geen idee dat doctoreren ooit een optie zou zijn voor iemand met mijn profiel. Meer zelfs, ik wist eigenlijk totaal niet wat doctoreren inhield. De kaarten zijn echter anders geschud geweest en nu blijkt dat doctoraatsproefschrift er toch te liggen. En ik ben er nog trots op ook!

Het voorwoord wordt doorgaans als een van de laatste stukken geschreven, maar blijkt toch een van de moeilijkste onderdelen van dit boekje te zijn. Het is een stuk dat, denk ik, bijna iedereen zal lezen, benieuwd of zijn/haar bijdrage aan dit doctoraat, hoe klein dan ook, het dankwoord heeft gehaald (en misschien ook gewoon omdat dit het eerste stuk is dat je tegenkomt). Het is voor mij dus heel belangrijk om hier niets te vergeten, ook omdat ik vind dat alle bijdragen en alle steun die ik de voorbije jaren mocht ontvangen wel eens zwart op wit bedankt mogen worden. Verontschuldig mij alvast als er wel ergens iets door de mazen van het net geglipt mocht zijn ☺.

Door de sublieme manier waarop ik de afgelopen jaren begeleid ben geweest door mijn promotoren, kan ik niet anders dan hen de eerste plaats in dit voorwoord schenken. Het mag gezegd worden, ik kon me geen beter duo dan Leen en Bart wensen. Leen, jij hebt met voorsprong het meeste invloed op mij gehad in de evolutie van student naar (als ik dat mag zeggen) volwaardig onderzoeker. Al tijdens mijn masterproef liet je me kennismaken met de wondere wereld van het wetenschappelijk onderzoek en leerde je me dingen bekijken door de befaamde "microbiologische bril". Ik herinner me ook nog goed hoe je me voorstelde om te komen doctoreren bij Lab4Food. Na jouw uitvoerige uitleg over de inhoud van dat werkwoord, heb ik met plezier toegezegd. Een zeer intensieve training wapende me tegen het IWT-vragenvuur en zo kon ik beginnen aan een vierjarig doctoraatsproject (ik bedank ook graag het IWT/VLAIO voor de financiering). Als promotor heb je vervolgens je taak zeer ernstig genomen. De snelle, grondige en opbouwende manier waarop je mijn werk (onder)steunde en evalueerde heeft ertoe bijgedragen dat het niveau steeg tot een hoogte die ik alleen niet

had kunnen bereiken. Je hebt ervoor gezorgd dat ik niet enkel wetenschappelijk gezien mijn grenzen heb verlegd, maar ook geografisch gezien mocht ik de grens enkele malen over om zowel in Duitsland, Italië als Ethiopië de plaatselijke wetenschap te gaan ervaren. En alsof dat nog niet genoeg was, heb je er mee voor gezorgd dat ik op de koop toe nog een PDM heb kunnen binnenhalen! Ik mag dus nog even bij Lab4Food blijven, en daar ben ik zeer blij om. Een welgemeende dankjewel, Leen!

Bart, ook jij verdient een speciale vermelding in dit voorwoord. Toen ik begon met het schrijven van de IWT-aanvraag was ik, om het zacht uit te drukken, weinig onderlegd in de moleculaire biologie. Mede dankzij jouw bijdragen denk ik dat ik me ondertussen op z'n minst de meest courante technieken eigen heb kunnen maken. Jij was ook diegene die steeds volledig mee was met de nieuwste technologieën en inzichten, waardoor ik jouw suggesties bijna blindelings kon opvolgen bij het interpreteren en analyseren van moleculaire data (en het reviseren daarvan). Jouw deur stond altijd (ook letterlijk) open en ik was steeds welkom met mijn vele vragen of met mijn toch wel teleurgestelde stemming als er weer eens een PCR mislukt was. Gelukkig was jij er dan om weer de moed erin te brengen om het eens op een andere manier opnieuw te proberen. De aanhouder wint, dat heb ik wel geleerd. Bedankt Bart, en wellicht werken we in de toekomst ook nog wel eens samen!

Ik heb tijdens mijn doctoraat ook kunnen steunen op het toeziend oog van mijn begeleidings- en examencommissie. Chris en Stefaan/Marleen, jullie hebben als assessor reeds tijdens de vierjarige rit naar dit proefschrift de tijd genomen om me tussendoor van de nodige feedback te voorzien vanuit jullie eigen ervaring en expertise. Tijdens de laatste fase van de rit kwamen hier nog Liesbet, Annemie en Lucia bij. Ik apprecieer van harte dat jullie de taak in de commissie zo grondig hebben vervuld en me tot op het laatste moment waardevolle suggesties hebben aangereikt. Lucia, a special thanks to you for welcoming me at UnivPM in Ancona and to give me the opportunity to improve my qPCR skills. You and your team were also very dedicated to collaborate to jointly publish a paper and I would also really like to thank you for your constructive comments. It is therefore a pleasure to, on our turn, welcome you in Belgium for the public defence.

De afgelopen vier jaren hoefde ik gelukkig niet alleen te werken. Ik heb het geluk gehad vele toffe en enthousiaste collega's te mogen leren kennen. Sommigen onder hen hebben helaas

reeds andere oorden opgezocht, maar toch heeft iedereen zeker zijn of haar deel bijgedragen aan de aangename werksfeer die ik mocht ervaren. Het grootste deel van de tijd bracht ik door in Geel. Als lid van Lab4Food heb ik regelmatig van een ontspanningsmomentje (een etentje, een terrasje na het werk, een matchke volleybal, proevertjes in de keuken …) mogen genieten in het bijzijn van Jesse, Els, Tim, Maria, Karel, Riet, Sanne, (een van) beide Sofies, Eric, Mik, Johan, Kim, Enya, An, Ruben, Jeroen, Addisu, Ashenafi, Geoffrey, Forkwa en/of Habiba. Daarnaast stonden zij altijd paraat om eens een handje toe te steken bij een proef, om hun mening te geven over mijn werk of om me wat bij te leren. Ook de collega's van SusCroPP en "mede-eilandbewoner" Audrey speelden een prominente rol in de goede sfeer op de Geelse werkvloer en bij de collega's van de Mobilab-bureau was het eveneens aangenaam vertoeven. Collega's die zeker niet mogen ontbreken in dit voorwoord zijn Mandy, Isabelle en Patricia (de drie musketiers in E101), voor wie niets te moeilijk was om geregeld te krijgen, en Anneke, die onrechtstreeks ook heel wat ideeën heeft aangebracht en stiekem haar interesse in mijn doctoraat niet kon verbergen. Bedankt allemaal!

Hoewel Geel mijn hoofdverblijfplaats was, voelde ik me ook prima thuis bij de collega's van PME&BIM in Sint-Katelijne-Waver. Ik werd daar met open armen ontvangen en alles werd zo goed mogelijk geregeld om ervoor te zorgen dat SKW mijn tweede thuis werd. Bovendien stond het hele team steeds klaar voor mijn vragen en verzoeken. Bedankt Marijke, Lien, Caro, Christel, Ken en Tim! Sam, ik ben nog steeds niet helemaal mee met wat jij allemaal kan met die bio-informatica. Heel erg bedankt om me daar voor al mijn experimenten wegwijs in te maken (lees: het grotendeels voor mij te doen). Ook de collega's van Scientia Terrae verdienen een dankjewel om bij te dragen aan de leuke werksfeer. Zeker Liesbet om mijn talrijke bestellingen te managen en Anneleen om me te leren kloneren.

Although it was only a short trip of six weeks, my research stay in Ancona was a great experience. Thanks to the hospitality of my Italian colleagues of D3A, I could enjoy a nice combination of the Italian atmosphere and fascinating insect research. Vesna, thank you for all the effort and time you invested to teach me all your qPCR skills (and for trying and trying again). Taxi Andrea, thank you for driving me around so many times! Anse, bedankt voor het gezelschap en om me als gids op sleeptouw te nemen. Mocht er trouwens iemand nog twijfelen: internationale onderzoekservaringen zijn echt een verrijking!

Buiten de academische wereld werd mijn onderzoek ook door vele mensen gevolgd. “Hoe gaat het met de insecten?” was geen zeldzame vraag als ik mijn vrienden of familieleden tegenkwam. Ik vertelde natuurlijk met veel plezier over mijn onderzoek en vond misschien nog meer plezier in de interesse die ik uit verschillende hoeken (fluitisten uit de harde kern, KSA’ers, vrienden van LB, familie…) ontving. Ik herinner me nog de legendarische Zondag Josdag in het teken van insecten, waarbij de interesse in praktijk werd omgezet. Er wordt nog steeds over die wasmotrupsen en meelwormschnitzels gepraat! Het is dus duidelijk blijven hangen. Bedankt aan al mijn vrienden en de hele familie om me zo te steunen.

Zaterdag is voor mij maar zelden een doctoraatsdag geweest. Die dag werd steevast voorbehouden voor de KSA. Daar kon ik me uitleven en inzetten voor de Molse jeugd en dat bracht wekelijks de nodige afleiding. KSA is ook de plaats waar ik gedurende 22 jaar verschillende sterke vriendschappen heb gesmeed. Al deze KSA’ers (het zijn er écht te veel om op te noemen) verdienen daarom uitdrukkelijk een woordje van dank in dit voorwoord, want zij hielpen me ontspannen, maar vooral om me stevig te amuseren. Hoewel ik dit jaar afscheid nam bij KSA Mol, zal ik toch altijd KSA’er blijven en die vriendschappen, die duren gewoon voort (denk maar aan KSA Westende en de Partycrew ☺ of de oud-leiding).

Ten slotte nog een laatste keer “bedankt” (het worden er al veel!) aan enkele zeer belangrijke mensen. Mama en papa, er is geen woord sterk genoeg om mijn dankbaarheid naar jullie toe uit te drukken. Ik besef heel goed dat ik van jullie alle kansen heb gekregen en het is voor een groot deel dankzij jullie dat ik uiteindelijk hier geraakt ben. Bedankt voor de jarenlange verwennerij, op alle vlakken! Kasper, kameraad, vooral de laatste weken/maanden hebben we intensief onze vrije tijd gedeeld. Dat waren momenten waarop ik even niet aan dat doctoraat moest denken. En als ik dat wel deed, was jij er om je goede raad mee te geven. Merci daarvoor! En dan als laatste ook een grote, liefdevolle dankjewel aan jou, Maarten, voor de dagelijkse steun, het ontwerp van uitnodigingen en figuren, het delen van je mening als ik weer eens bevestiging zocht en vooral om altijd en overal klaar te staan!

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Samenvatting

Eetbare insecten vormen sinds enkele jaren een nieuwe voedingsmatrix in Westerse landen. Microbiologisch gezien zijn deze insecten en afgeleide producten echter nog niet uitgebreid gekarakteriseerd. Een uitvoerige microbiologische analyse van rauwe, onbehandelde eetbare insecten alsook van de impact van specifieke processtappen en bewaartechnieken kan bijdragen tot de voedselveiligheid en proceshygiëne binnen de insectensector. Door middel van zowel cultuurafhankelijke telmethoden als cultuuronafhankelijke technieken werd in dit proefschrift daarom beoogd om de microbiologische kwaliteit van enkele insectensoorten voor humane consumptie bloot te leggen. Door een stalencollectie die verschillende insectenkwekers en productiebatchen omvatte te onderzoeken, kon de microbiologische kwaliteit grondig beschreven worden.

Eetbare insecten worden voornamelijk verwerkt tot insectenproducten alvorens geconsumeerd te worden. Hiertoe kunnen insecten een of meerdere processtappen ondergaan, zoals een hittebehandeling, een droogstap of een rookstap. Deze processtappen worden enerzijds toegepast om het gewenste product te produceren, maar hebben anderzijds een effect op de microbiologische kwaliteit en houdbaarheid van het product. Om ook tijdens het productieproces en de bewaring van insectenproducten de microbiologische voedselveiligheid te kunnen garanderen, dient de impact van beide stappen in de voedselketen op de micro-organismen van insecten gekend te zijn. Ook het productieproces en de bewaring van insectenproducten werden daarom in deze doctoraatsstudie onderzocht, zowel op laboschaal als in industriële omgeving.

Ondanks de grote verschillen tussen insectensoorten, kwekers en kweekbatchen, kan algemeen gesteld worden dat rauwe, onbehandelde insecten steeds worden gekenmerkt door hoge aantallen micro-organismen (gemiddeld 7,5 à 8,5 log kve/g). Deze aantallen hoeven als dusdanig geen groot voedselveiligheidsrisico te vormen. De identificatie van bacteriën en bacteriesporen die bij de insecten aangetroffen werden, geeft echter aan dat bepaalde pathogene bacteriën zich in de insecten kunnen huisvesten en zo een voedselveiligheidsrisico kunnen vormen. Voornamelijk rond de sporevormende bacterie *Bacillus cereus* en aanverwante soorten bestaat een risico. Tevens wees dit

doctoraatsonderzoek uit dat rauwe insecten, mits enige variatie met betrekking tot de herkomst van het staal, ook hoge aantallen van een of zelfs meerdere tetracyclineresistentiegenen kunnen bevatten (tot $2{,}10 \times 10^8$ genkopijen/g), vergelijkbaar met andere voedingsmiddelen. De verspreiding van antibioticumresistentiegenen zoals deze vormt op zijn beurt ook een risico voor de humane gezondheid. Om deze risico's in te dijken is een gepaste actie zoals een hittebehandeling noodzakelijk voor consumptie.

Tijdens de productie en bewaring van insecten werd geconstateerd dat een milde hittebehandeling slechts voldoende is om vegetatieve micro-organismen af te doden (3,2 tot 6,4 log kve/g reductie). Minstens een deel van de bacteriële endosporenpopulatie is, voor alle onderzochte stalen, in staat om de toegepaste processtappen te overleven. Bovendien werd duidelijk dat het aantal micro-organismen dat door voorafgaande processtappen werd gereduceerd, tijdens de productie opnieuw kan toenemen. Redenen hiervoor zijn bijvoorbeeld nabesmetting en de mogelijkheid tot uitgroei van bacteriële endosporen. Ook tijdens gekoelde bewaring van hittebehandelde insecten kon dat laatste fenomeen opgemerkt worden, echter slechts na een microbiologisch stabiele bewaarperiode van 7 à 11 dagen. Diepgevroren bewaring en bewaring van gedroogde producten, daarentegen, konden een microbiologische houdbaarheid van minstens 6 maanden garanderen, uitgaande van een veilig beginproduct.

In dit doctoraatsonderzoek werd duidelijk dat, zowel in rauwe insecten als tijdens productie en bewaring van insectenproducten, sporevormende pathogenen zoals *B. cereus* mogelijks een risico vormen. Niet alleen bestaat de kans dat deze bacterie zich in het rauwe insect bevindt, de sporevorm van dit organisme is mogelijk in staat om processtappen te overleven en tijdens bewaring van insecten en afgeleide producten opnieuw uit te groeien. Het is daarom aangeraden om, in de verdere ontwikkeling van de insectensector en bijhorende wetgeving, rekening te houden met dit risico door passende microbiologische criteria op te stellen en te implementeren en geschikte behandelingen toe te passen waarbij sporevormende pathogenen zoals *B. cereus* kunnen worden beheerst.

Abstract

Since a few years, edible insects have formed a new food matrix in Western countries. These insects and their derived products are, however, not yet extensively microbiologically characterised. A detailed microbiological analysis of raw, unprocessed edible insects as well as the impact of specific processing steps and preservation techniques can contribute to food safety and process hygiene within the insect sector. By applying both culture-dependent counts and culture-independent techniques, this dissertation aimed to unravel the microbiological quality of a few selected edible insect species. By investigating a collection including samples obtained from several insect rearing companies and production batches, the microbiological quality could be explored thoroughly.

Edible insects are mainly processed into insect-based food products prior to being consumed. To this end, insects are subjected to one or more processing steps such as a heat treatment, a drying step or a smoking step. These processing steps are applied in the first place to produce the intended end product, but, on the other hand, also have an impact on the microbiological quality and shelf life of the product. To be able to guarantee food safety also during processing and preservation, the impact of both steps in the food chain on the microorganisms harboured by insects should be identified. Consequently, also processing and preservation of insect products were investigated in this PhD thesis, both on laboratory and industrial scale.

Generally, notwithstanding the large differences between insect species, rearers, and batches, raw, unprocessed insects are consistently characterised by high counts of microorganisms (on average 7.5 to 8.5 log cfu/g). As such, these counts do not necessarily pose a food safety risk. However, identification of bacteria and bacterial endospores that were encountered in insects indicate that certain pathogenic bacteria are able to reside in the insects and thus present a food safety risk. This risk especially exists for the spore-forming bacterium *Bacillus cereus* and related species. At the same time, this dissertation pointed out that raw insects, given some variation regarding the origin of the sample, can also contain high amounts of one or more tetracycline resistance genes (up to 2.10×10^8 gene copies/g), comparable to other food products. On its turn, the distribution of such antibiotic resistance

genes poses a human health risk as well. To address these hazards, appropriate measures such as a heat treatment are essential prior to consumption.

During processing and preservation of insects, it was observed that a mild heat treatment is merely sufficient to kill vegetative microorganisms (3.2 to 6.4 cfu/g reduction). For all samples investigated, at least a part of the bacterial endospore population is capable of surviving the processing steps applied. Moreover, it became clear that microbial numbers that were reduced during previous processing steps were able to increase again during production of end products. This can be caused, for example, by post-contamination and/or the outgrowth of bacterial spores. Likewise, during chilled preservation of heat-treated insects, the latter phenomenon could be observed, yet only after a microbiologically stable shelf life of 7 to 11 days. Frozen storage and preservation of dried products, on the other hand, could guarantee a microbiological shelf life of at least 6 months, starting from an initially safe product.

In this doctoral research, it was acknowledged that spore-forming bacteria such as *B. cereus* possibly pose a risk, both in raw insects and during production and preservation of insect-based food products. Additional to the possibility of raw insects to harbour this bacterium, the spore form of this organism may be capable of surviving processing and grow again during preservation of insects and insect products. In the further development of the insect sector and concomitant legislative framework, it is therefore advised to take into account this risk. This can be achieved by composing and implementing appropriate microbiological criteria and applying suitable treatments to control spore-forming pathogens such as *B. cereus*.

List of abbreviations

ANOVA	Analysis of variance
ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (France)
AR	Antibiotic resistance
a_w	Water activity
BIIF	Belgian Insect Industry Federation
BLAST	Basic Local Alignment Search Tool
BSF	Black soldier fly
cfu	Colony-forming units
DGGE	Denaturing gradient gel electrophoresis
DRBC	Dichloran rose bengal chloramphenicol agar
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EMA	European Medicines Agency
erm	Erythromycin resistance gene
FAO	Food and Agriculture Organisation of the United Nations
FASFC	Federal Agency for the Safety of the Food Chain (Belgium)
FCR	Feed conversion ratio
HACCP	Hazard Analysis and Critical Control Points
IPIFF	International Platform of Insects for Food and Feed
K_p	Nitrogen-to-protein conversion factor
kve	Kolonievormende eenheden (see cfu)
LAB	Lactic acid bacteria
LCA	Life cycle assessment
MRS	de Man, Rogosa and Sharpe agar
NA	Nutrient agar
NB	Nutrient broth
NFE	Nitrogen-free extract
NMDS	Non-metric multidimensional scaling

List of abbreviations

NVWA	Nederlandse Voedsel- en Warenautoriteit (Dutch Food and Consumer Product Safety Authority) (the Netherlands)
OGA	Oxytetracycline glucose agar
OTU	Operational taxonomic unit
PCA	Plate count agar
PCR	Polymerase chain reaction
PPS	Peptone physiological salt solution
qPCR	Real-time (quantitative) PCR
R^2	Coefficient of determination
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks (EU)
SCP	Single cell protein
SHC	Superior Health Council (Belgium)
SPI	Strategic platform insects (Flanders, Belgium)
s.l.	Sensu lato
s.s.	Sensu stricto
tet	Tetracycline resistance gene
UN	United Nations
UNU	United Nations University
VENIK	Verenigde Nederlandse Insectenkwekers (United Dutch Insect Rearers) (the Netherlands)
VRBG	Violet red bile glucose agar
WHO	World Health Organization of the United Nation

List of publications

Publications in peer-reviewed journals

Vandeweyer D., Milanović V., Garofalo C., Osimani A., Clementi F., Van Campenhout L., & Aquilanti L. (2019). Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and The Netherlands. *International Journal of Food Microbiology*, *290*, 288-295. http://doi.org/10.1016/j.ijfoodmicro.2018.10.027

Vandeweyer D., Wynants E., Crauwels S., Verreth C., Viaene N., Claes J., Lievens B., & Van Campenhout L. (2018). Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House Crickets (*Gryllodes sigillatus*) for Human Consumption. *Applied and Environmental Microbiology*, *84* (12), e00255-18. http://doi.org/10.1128/AEM.00255-18

Andeta A. F., Vandeweyer D., Woldesenbet F., Eshetu F., Hailemicael A., Woldeyes F., Crauwels S., Lievens B., Ceusters J., Vancampenhout K., & Van Campenhout L. (2018). Fermentation of enset (*Ensete ventricosum*) in the Gamo highlands of Ethiopia: Physicochemical and microbial community dynamics. *Food Microbiology*, *73*, 342-350. http://doi.org/10.1016/j.fm.2018.02.011

Vandeweyer D., Crauwels S., Lievens B., & Van Campenhout L. (2017). Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. *International Journal of Food Microbiology*, *261*, 11-18. http://doi.org/10.1016/j.ijfoodmicro.2017.08.018

Stoops J., Vandeweyer D., Crauwels S., Verreth C., Boeckx H., Van Der Borght M., Claes J., Lievens B., & Van Campenhout L. (2017). Minced meat-like products from mealworm larvae (*Tenebrio molitor* and *Alphitobius diaperinus*): microbial dynamics during production and storage. *Innovative Food Science & Emerging Technologies*, *41*, 1-9. http://doi.org/10.1016/j.ifset.2017.02.001

Vandeweyer D., Crauwels S., Lievens B., & Van Campenhout L. (2017). Microbial counts of mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing companies and different production batches. *International Journal of Food Microbiology*, *242*, 13-18. http://doi.org/10.1016/j.ijfoodmicro.2016.11.007

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

Presentations at international conferences

Vandeweyer D., Borremans A., Verreth C., Lievens B., & Van Campenhout L. (2018). Identification, heat-survival and outgrowth of endospore-forming bacteria harboured by edible insects. *International Symposium on Insects INSECTA 2018,* Gießen, Germany, September 05-07, 2018.

Poster presentations at international conferences

Wynants E., Vandeweyer D., Spaepen R., Crauwels S., Lievens B., & Van Campenhout L. (2017). Hygiene monitoring in a Belgian fries-producing company using next generation sequencing. *Conference on Food Microbiology*, Brussels, Belgium, September 21-22, 2017.

Andeta A. F., Vandeweyer D., Eshetu F., Vancampenhout K., Woldesenbet F., & Van Campenhout L. (2017). Traditional enset (*Ensete ventricosum*) fermentation in the Gamo highlands of Ethiopia: physiochemical and microbial dynamics. *Conference on Food Microbiology*, Brussels, Belgium, September 21-22, 2017.

Vandeweyer D., & Van Campenhout L. (2017). Tyndallisation as a treatment to reduce the bacterial endospore load in mealworms (*Tenebrio molitor* & *Alphitobius diaperinus*). *Conference on Food Microbiology*, Brussels, Belgium, September 21-22, 2017.

Vandeweyer D., & Van Campenhout L. (2016). Microbiological assessment of blanching and refrigerated storage for fresh edible mealworm larvae (*Tenebrio molitor*). *Conference on Food Microbiology*, Brussels, Belgium, September 15-16, 2016.

Vandeweyer D., Lievens B., & Van Campenhout L. (2015). Microbial quality of edible insects reared on industrial scale in Belgium and the Netherlands. *Conference on Food Microbiology*, Brussels, Belgium, October 08-09, 2015.

Vandeweyer D., Lievens B., Van Campenhout L. (2015). Microbial quality of edible insects reared on industrial scale in Belgium and the Netherlands. *Innovations in Food Packaging, Shelf Life and Food Safety*, Munich, Germany, September 15-17, 2015.

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Chapter 1 General introduction

"What we eat is, after all, more a matter of custom and fashion than anything else… It can be attributed only to prejudice, that civilized man of today shows such a decided aversion to including any six-legged creatures in his diet." - Joseph Charles Corneille Bequaert (1921)

As early as in 1921, the Belgian-American Joseph Charles Corneille Bequaert considered insects as a source of food. The tradition of entomophagy (the human consumption of insects, see also below), however, definitely has a much longer history, especially outside of Europe. In many African, Asian, Latin-American and Oceanian countries, entomophagy is of major importance to assure food security (Raheem et al., 2018; van Huis et al., 2013). Over 2141 insect species are being consumed worldwide (Payne & Van Itterbeeck, 2017) and most of them are consumed in tropical and subtropical regions (Figure 1.1), where availability is higher than in other regions.

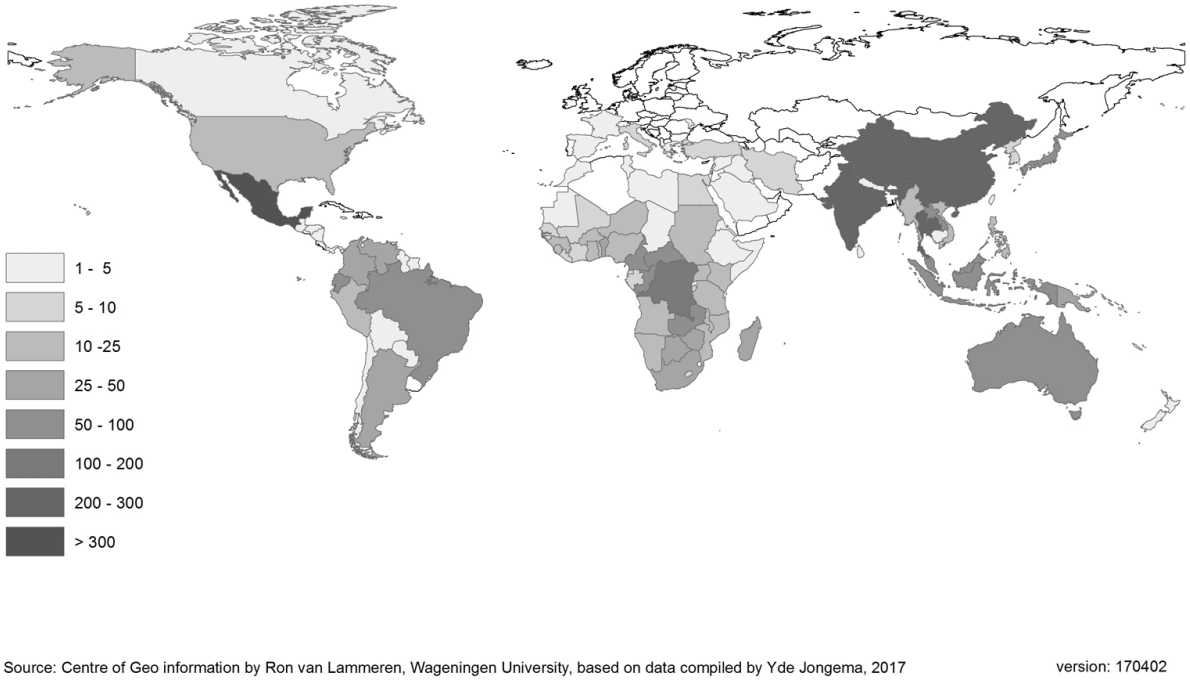


Figure 1.1 Recorded number of edible insect species, by country (Jongema, 2017).

Because insects are frequently considered as pests or disease-carrying animals, insect consumption in Western countries is still limited (Looy, Dunkel, & Wood, 2014). Yet, influencing factors such as population growth and the increasing concern of environmental

conservation have augmented interest in insect consumption as a sustainable alternative protein source in the last few years. The introduction of edible insects as a new food matrix requires profound and scientific insight into all aspects necessary to provide safe insect foods. This chapter presents the required introductory background including the current and developing use of edible insects in Belgium and Europe, the legislative framework, microbiological food safety risks regarding edible insects, common processing and preservation strategies, and finally the objectives of this thesis and an outline of the experimental chapters.

1.1 Edible insects as human food in Belgium and Europe

1.1.1 Definition of entomophagy

As reviewed by Evans et al. (2015), the term "entomophagy" is prone to discussion when it comes to the correct definition of the word. According to the Oxford Dictionary, entomophagy means "the practise of eating insects, especially by people". However, since the word "entomophagy" originates from the Greek words for "insect", ἔντομον (entomon) and "to eat", φᾰγεῖν (phagein) (Kouřimská & Adámková, 2016), strictly there is no specific reference to humans. To clarify this, the term "**anthropo-entomophagy**" was introduced (Ramos-Elorduy, 2009; Ramos-Elorduy, Landero-Torres, Murguía-González, & Pino M., 2008) by adding the Greek word for human, "anthropos" (ἄνθρωπος). Ever since, it has been sporadically used in literature (Grabowski & Klein, 2017; Premalatha, Abbasi, Abbasi, & Abbasi, 2011). Nevertheless, enthomophagy is often preferred and nowadays generally considered as a human practise, with the non-human counterpart being "insectivory" (Evans et al., 2015).

Another issue related to the term "entomophagy" is the proper definition of "insects". Also edible non-insect arthropods, such as spiders, scorpions and centipedes, are sometimes named "insects". While they are taxonomically not members of the class of Insecta, the consumption of those edible arthropods is often also covered by the term "entomophagy" (Evans et al., 2015). Additionally, insects and other edible arthropods are regularly categorised as "**minilivestock**", together with other small edible animals such as snails, rodents, frogs … (Hardouin, 1995).

Finally, the meaning of the adjective "edible" has to be clarified. The Oxford Dictionary defines the word edible as "fit to be eaten (often used to contrast with unpalatable or poisonous varieties)". Hence, edible insects should be non-toxic and appetising. While the latter requirement is an individual preference, human toxicity can be investigated using standard scientific methods. Indeed, some insect species or certain life stages contain toxic compounds (Dobermann, Swift, & Field, 2017), similar to for example mushrooms and plants. These insects are therefore unsuitable for consumption and defined as inedible. Toxins produced by microorganisms are not yet considered in this definition.

The existence of several linguistic terms – some of which may have different interpretations in different languages and cultures (Evans et al., 2015) – describing more or less the same act, can deliver ambiguous information to researchers. Evans et al. (2015) therefore recommend to use the term entomophagy as little as possible and, when used, clearly defined. Yet, in this dissertation, the term "entomophagy" will be used, indicating the **human consumption of edible insects, i.e. non-toxic stages of species from the taxonomic class of Insecta**.

1.1.2 History of entomophagy in Belgium and Europe

Entomophagy has a long history worldwide, and also in Europe, human consumption of insects was documented in the past. Dreon & Paoletti (2009), for example, describe the collection and consumption of local moths (*Zygaena* spp. and *Syntomis phegea*), bumblebees (*Bombus* spp.) and grasshoppers (*Decticus verrucivorus*) in Northern Italy up to 30 years ago. Other examples of prolonged insect consumption in Europe are the traditional cockchafer (*Melolontha* spp.) soup in France and Germany (van Huis et al., 2013) and the Italian Casu Marzu, a sheep milk cheese containing living larvae of the cheese fly *Piophila casei* (Mazzette et al., 2010).

Since the publication of the Food and Agriculture Organisation of the United Nations (FAO) report "Edible insects: Future prospects for food and feed security" (van Huis et al., 2013), interest in the use of insects for food and feed has substantially increased in Western countries (Halloran, Flore, Vantomme, & Roos, 2018; House, 2018). Although legislation, particularly EU legislation, was not yet adapted to include edible insects at that time, insect rearing and food production companies initiated the edible insect sector anyway. Both

Belgium and the Netherlands started off as leaders in insect rearing and processing. In 2014 and 2015, Belgium, the Netherlands, France and eventually the European Food Safety Authority (EFSA) published advisory documents (ANSES, 2015; EFSA Scientific Committee, 2015; NVWA, 2014; SHC & FASFC, 2014) in which insects were assessed as potential food source. Also in 2014, the Belgian Federal Agency for the Safety of the Food Chain (FASFC) tolerated the production and commercialisation of ten insect species through publishing a circular (FASFC, 2014), which was updated in 2016 to contain the new novel food legislation (FASFC, 2016). The publication of these documents had a leverage effect to the starting insect sector by creating the start of a legal framework.

In Belgium, the tolerance of ten edible insect species quickly caused insect-based products to be launched on the market (Van Thielen, Vermuyten, Storms, Rumpold, & Van Campenhout, 2018). Burgers, spreads, nuggets, schnitzels, etc. containing insects were introduced in Belgium, and some products were also exported to e.g. the Dutch market. Eco-shops, supermarkets and specialised web shops offered those insect products, hence providing easy access to possible consumers. Also the Belgian media gave special attention to the introduction of these new foodstuffs. Meanwhile, product development and research regarding edible insects were unrolled in Belgium and Europe (Caparros Megido et al., 2014; Grabowski & Klein, 2016; Osimani, Milanović, Cardinali, Roncolini, et al., 2018; Stoops et al., 2016, 2017). Rearers, food-producing companies and research facilities started to set up insect consortia and network organisations and joined in sector organisations such as the Belgian Insect Industry Federation (BIIF), the Flemish Strategic Platform Insects (SPI), the Verenigde Nederlandse Insectenkwekers (VENIK) and the International Platform of Insects for Food and Feed (IPIFF). These sector organisations aim to promote the insect sector, create general guideline documents for the sector and further commercialise insect food and feed products. Since the launch of insects and insect-based products in Belgium in 2014 and 2015, a number of them have already been withdrawn due to disappointing sales. Nevertheless, after the initial hype, on an international level insects and insect-based foods are still under attention of mostly small companies and researchers. Therefore, in the (near) future, products that have been withdrawn will probably be replaced by newly developed insect-based products (Cadesky, 2017; Stoops et al., 2017).

1.1.3 Insects used as or in food in Europe

1.1.3.1 Predominantly used species in Europe

While more than 2100 insect species are considered edible (see paragraph 1.1.1) and are effectively consumed (Jongema, 2017), not all of them are used in Europe. In most countries where entomophagy is common, insects are typically collected or harvested from the wild (Raheem et al., 2018). Depending on geographic location, only certain species are available. The history of insect consumption therefore strongly coincides with particular species being present in the environment. Consequently, in tropical regions, edible insects are much more considered as food source than in other regions. In Europe, insect consumers rely on (industrially) farmed insects, because the tradition of collecting insects is absent (Mlček, Rop, Borkovcova, & Bednarova, 2014). Only recently, edible insects gained the interest of European consumers and this induced the start of insect rearing for human consumption and insect food production. In contrast to other regions in the world, European entomophagy is based on controlled rearing rather than natural occurrence. Consequently, insects for human consumption in Europe should be able to be reared on large scale.

In an opinion composed by the EFSA Scientific Committee (2015), a list of insect species with the greatest potential to be used as food and feed in the EU was proposed (Table 1.1). While many reported species may have the potential to be used in both food and feed, others are preferred for feed production rather than for human consumption, i.e. the common housefly (*Musca domestica*) and the black soldier fly (BSF, *Hermetia illucens*). These species are considered edible (i.e. non-toxic under the condition they do not contain human pathogens or their toxins) and for example BSF is effectively consumed sporadically in Malaysia. Nevertheless, consumer acceptance for these insect species is very low due to unappetising odour and flavour and socio-cultural aversion (Wang & Shelomi, 2017).

Table 1.1 Insect species considered to have large potential to be used as food and feed (EFSA Scientific Committee, 2015).

Scientific name	Common name (English)
Musca domestica	Common housefly
Hermetia illucens	Black soldier fly
Tenebrio molitor	Mealworm
Zophobas atratus	Giant mealworm
Alphitobius diaperinus	Lesser mealworm
Galleria mellonella	Greater wax moth
Achroia grisella	Lesser wax moth
Bombyx mori	Silkworm
Acheta domesticus	House cricket
Gryllodes sigillatus	Tropical house cricket or banded cricket
Locusta migratoria migratorioides	African migratory locust
Schistocerca americana	American grasshopper

Among other, less frequently consumed insects, mealworms and crickets are the edible insects predominantly used in Europe. Important mealworm species include ***Tenebrio molitor*** (yellow mealworm) and ***Alphitobius diaperinus*** (lesser mealworm). Commonly reared cricket species are ***Acheta domesticus*** (house cricket) and ***Gryllodes sigillatus*** (tropical house cricket or banded cricket). Consequently, these four species were selected as subjects for this PhD dissertation. As further explained in paragraph 1.1.3.2, the former two present holometabolous species of which the larval stage is consumed, whereas the latter two are hemimetabolous species consumed in the (nearly) adult stage. Also noteworthy is the African migratory locust ***Locusta migratoria migratorioides***, which is also considered as a member of the so-called "**Big Four**", together with *T. molitor*, *A. diaperinus* and *A. domesticus*. The Big Four is a term that comprises the four most widely used and consumed edible insect species in Europe according to House (2018). These highlighted species (see Figure 1.2) were already occasionally reared as pet food and feed for zoo animals in Western countries before interest using them as human food arose. Hence, the available knowledge on rearing acted as a good basis for existing rearing companies to start cultivating these insects for human consumption as well. After introduction of specific prerequisites for human food production into their rearing facilities (FASFC, 2016) (see also paragraph 1.2), some insect rearers started producing human food grade insects. To date, some insect rearing companies are still combining the production of insects for food, feed and/or pet food purposes in their production facility.

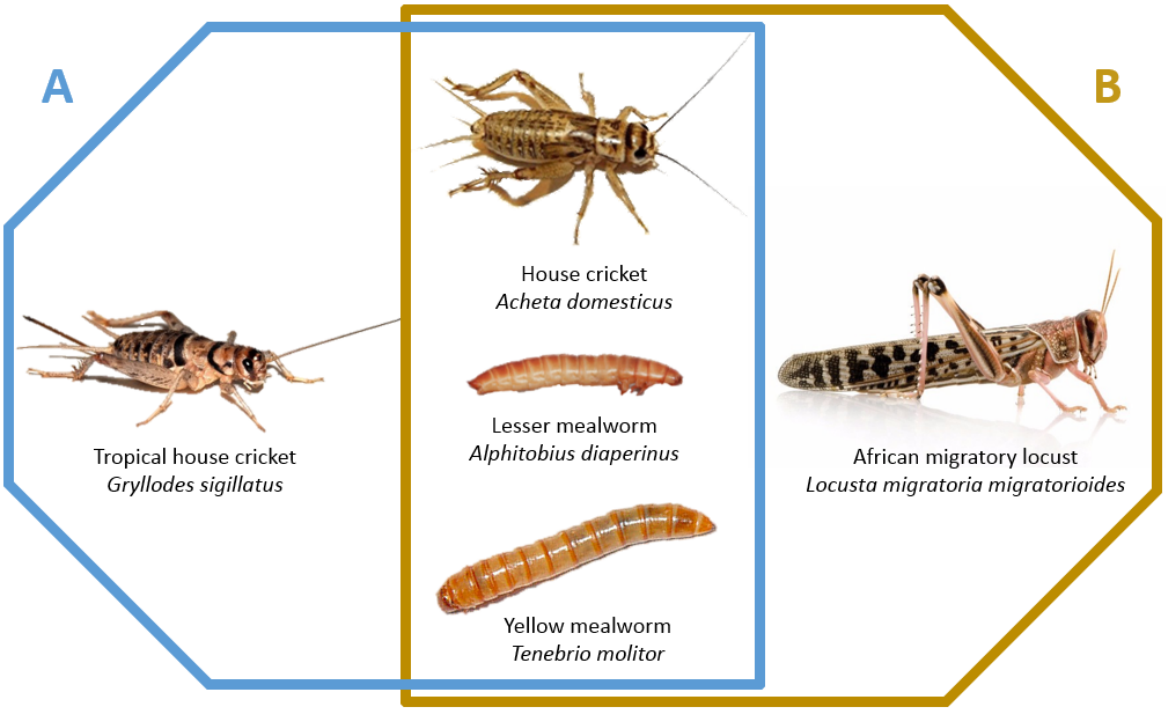


Figure 1.2 Edible insect species reported in this dissertation, depicted in the life stage suitable for human consumption. (A) The four species investigated in the practical part. (B) Members of the "big four" according to House (2018).
Figure constructed using input from https://e-insects.wageningenacademic.com, http://livecrickets.co.uk, http://www.lm-magazine.com, https://www.amazon.com, and https://www.pig333.com/.

1.1.3.2 Life cycles and industrial rearing

When insects are being reared, they are subjected to conditions that are selected and optimised by the rearer, in order to maximise yield and profit. For each species, other optimal conditions are required and also among rearing companies, slight differences can occur. Still, the life cycle and the general rearing practises for certain species are comparable between rearers. Even between different insect species that belong to the same taxonomic family, rearing practises are often very similar. This is for example the case for different mealworm species and different cricket species. Consequently, their life cycle and industrial rearing practises are considered per insect type (i.e. mealworms and crickets) in the following paragraphs.

Mealworms

Both yellow and lesser mealworms are holometabolous in their development, classifying them into the super order of Endopterygota. This means that their life cycle includes a pupal stage and a complete metamorphosis from larva into adult insect (Rumpold & Schlüter, 2013b). Mealworms, as part of the order of Coleoptera, transform into darkling beetles, i.e. the adult forms that are reproductive and (in case of female beetles) produce eggs. Yellow and lesser mealworms are close relatives in the Tenebrionidae family and follow a similar life cycle, which is illustrated in Figure 1.3A.

Briefly explained, the life cycle of mealworms consists of four stages: egg, larva, pupa and adult (Ghaly & Alkoaik, 2009; Simon, Baranyai, Braun, Fábián, & Tóthmérész, 2013). Eggs are laid by adult beetles and larvae hatch from the eggs after 1 to 2 weeks. During the larval stage, several instar phases and exuviations take place, resulting in the formation of a pupa after 22 to 100 days. The pupal stage lasts approximately 8 days until the beetle emerges from the pupa. Adults live for 2 to 3 months and are responsible for reproduction. As demonstrated by Oonincx, Van Broekhoven, Van Huis, & Van Loon (2015), the development time and growth efficiency of mealworms can highly depend on the feed administered. Also rearing conditions such as temperature and relative humidity have an important influence on the rearing process (EFSA Scientific Committee, 2015). Hence, a complete cycle can take from six months up to more than two years. In industrial rearing systems where optimal conditions are applied, mealworms for human consumption (final instar phase before pupating) are typically reared in 8 to 10 weeks, until they reach the desired size (NVWA, 2014).

In industrial rearing environments, mealworms are reared in plastic trays filled with a wheat-based substrate in which the larvae reside. In this way, larvae are continuously in contact with their feed source as well as with their exuviae and faeces. To provide the mealworms with moisture, for example carrots, apples, potatoes or brewer's spent grain are supplemented. Mealworms are reared at an optimal temperature of around 30 °C and a relative humidity level of 60% (NVWA, 2014). To harvest the fully grown larvae, they are separated from their substrate by (automated) sieving. In order to obtain adult forms that can reproduce and provide a continuous rearing process, a small fraction of the larvae is kept aside and allowed to enter the pupal stage.

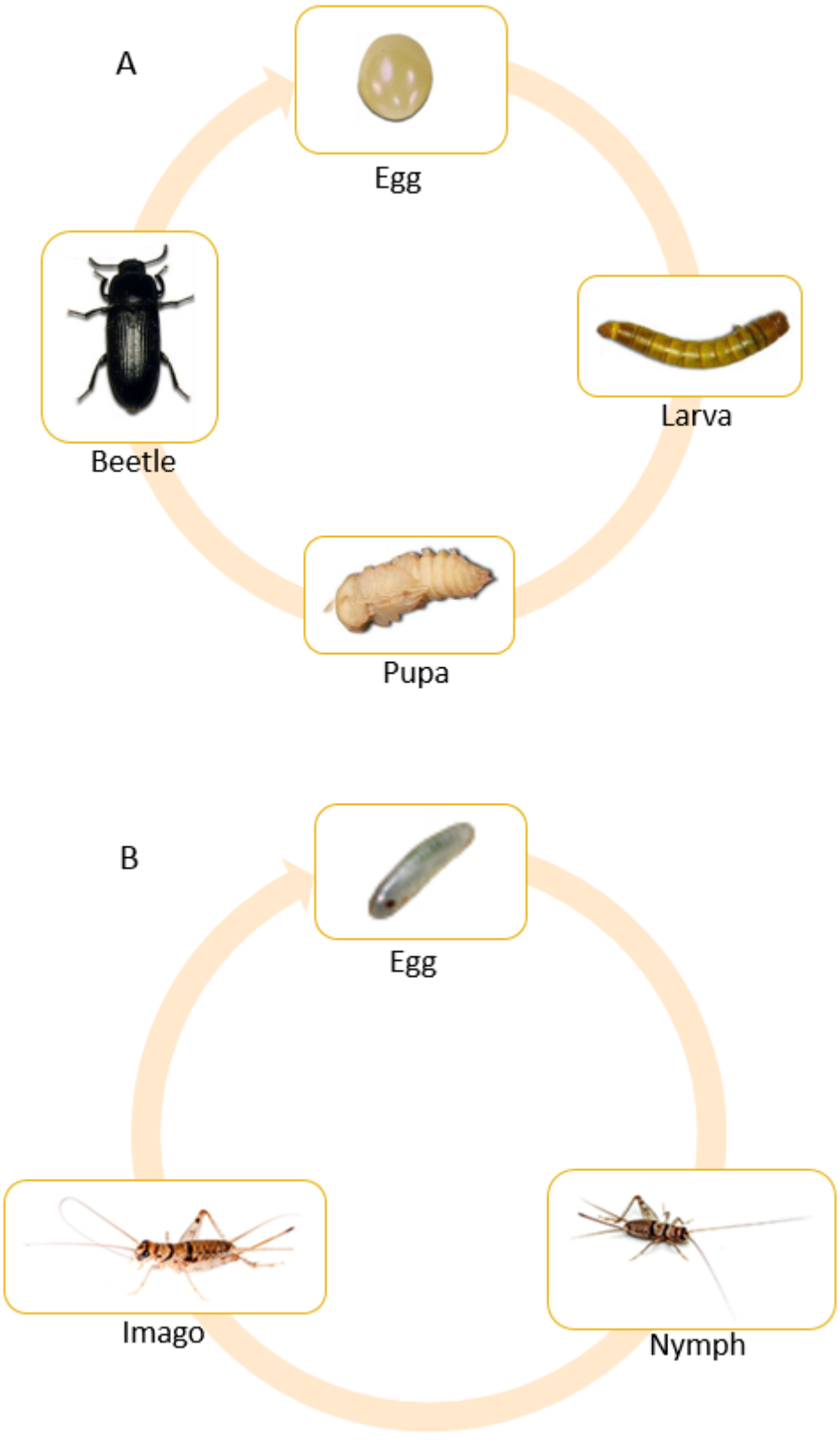


Figure 1.3 Life cycle of mealworms (A) and crickets (B).
Figure constructed using input from
https://i.pinimg.com/736x/32/a0/c8/32a0c86ae0dfa3e8c49e3e3dcf15f38f.jpg and
https://www.nomorebugs.com.

Crickets

Contrary to mealworms, crickets develop hemimetabolously and consequently lack a pupal stage in their development (Figure 1.3B). For this reason, they are classified as Exopterygota. Upon hatching, crickets appear as nymphs, which are small versions of the adult form (imago). Nymphs grow into imagos after moulting several times. During these moults, an incomplete metamorphosis takes place to provide the nymph with wings and reproductive organs. Their straight-shaped wings and elongated cylindrical body shape classifies them into the order of Orthoptera. Both *Acheta domesticus* and *Gryllodes sigillatus* are members of the family of Gryllidae or "true crickets" and share a similar life cycle. It consists of three subsequent stages: egg, nymph and imago. Crickets industrially reared for human consumption are typically produced in 2 to 4 months (EFSA Scientific Committee, 2015; Vandeweyer et al., 2018) and are harvested in their final nymphal stage or as an adult. Also for (house) crickets, this development time may be dependent of the feed provided (Oonincx et al., 2015) and the rearing conditions applied.

On industrial scale, crickets are currently reared in large plastic, wooden or Perspex cages containing egg cardboards to create dark crevices. Considering food hygiene, stainless steel cages may also constitute a valuable option (Rossoni & Gaylarde, 2000). Cricket feeds can be very diverse, for example based on chicken feed, brewer's spent grain, wheat, maize, soy, fungal mycelium, etc. Feed and water are provided in the cages, but are not constantly in contact with the crickets. After the rearing process at around 30 to 31 °C and 50 to 70% relative humidity (Clifford & Woodring, 1990; Vandeweyer et al., 2018), crickets are harvested by shaking them out of the cardboards. As is also the case for mealworms, a small fraction of the crickets are selected for reproduction and allowed to mature further, mate and lay their eggs in a matrix such as peat soil.

1.1.3.3 Belgian and European insect rearing companies

Belgium

In Belgium, 13 insect rearing facilities are registered at the Federal Agency for the Safety of the Food Chain (FASFC). They are authorised for the production of "insects other than

bees/bumblebees". Six of them produce insects for human consumption, while the others are specialised in insects for feed, pet food or aquaculture.

NuSect is an insect rearing facility producing approximately 15 different insect species. Most insects are reared for animal feed, but since several years, NuSect introduced production for human consumption, for example of yellow mealworms. NuSect is the largest Belgian insect rearing company and produces up to 50 tonnes per year (Schillewaert, 2018).

Little Food produces house crickets for human consumption. They are currently raising their production capacity to reach up to 30 tonnes per year (Schillewaert, 2018). In addition, Little Food processes the crickets into food products such as snacks, and sells them via local retailers.

Bugood Food, Tor Royal and B-bugs are three smaller rearing companies, all producing yellow mealworms for human consumption. Bugood Food and Tor Royal both provide cooking workshops and tastings using their own edible insects. A last small rearing company is Locousta, rearing tropical house crickets for human consumption.

Other European countries

In the same way as Belgium, the Netherlands counts several insect rearing facilities. As compiled by Schillewaert (2018), 14 companies in the Netherlands are active in insect rearing, of which 10 also produce insects for human consumption. The largest Dutch insect-producing company is Proti-farm, which focusses particularly on the large-scale production of lesser mealworms for human consumption. Apart from that, Proti-farm also produces yellow mealworms, crickets and grasshoppers for human consumption as well as several species for animal consumption. Three other Dutch edible insect producing companies Van de Ven (yellow mealworms), Nostimos (house crickets) and Meertens Insectenkwekerij (locusts) are grouped within the consortium Fair Insects, which has recently become part of Protix, a large company that processes insects for applications in food, pet food, animal feed, agriculture, aquaculture, etc.

Also in other European countries, insect rearing companies are established. Over 20 different rearing facilities for edible insects are located all over Europe, including France, the United

Kingdom, Denmark, Norway, Finland, Spain, Italy and Switzerland. Examples of large-scale insect rearers for human consumption are Micronutris (yellow mealworms and tropical house crickets, France), Crispy Crickets (house crickets, Finland) and Entomos (yellow mealworms, house crickets and locusts, Switzerland).

1.1.4 Strengths and opportunities of entomophagy

As indicated by several studies and reports (FAO, 2009; Foley et al., 2011; Tilman, Cassman, Matson, Naylor, & Polasky, 2002; van Huis et al., 2013), our world faces a growing population and a concurrent demand for food. Due to an increase in income in many developing countries, the **demand for meat as a high quality protein source** is rising as well (Godfray et al., 2018). Hence, both agricultural and natural resources are being put under pressure, while **sustainability and food security** present an increasing concern (Foley et al., 2011). Conventional protein sources such as meat, fish and imported soy are being used intensively, but have a high environmental impact (Dagevos & Voordouw, 2013; Godfray et al., 2018; Reijnders & Soret, 2003). To be able to provide the growing world population with food in a sustainable way, changes in diet should be adopted in the first place (Smith & Gregory, 2013). Although it has been repeatedly shown that plant-based diets can be more sustainable than omnivorous diets (Baroni, Cenci, Tettamanti, & Berati, 2007; Reijnders & Soret, 2003), diverse views regarding a change in meat consumption exist (Vinnari & Tapio, 2009) and the demand of animal-based food is still expected to rise globally (FAO, 2011; Godfray et al., 2018). Several promising **low-impact novel protein sources** were already proposed (van der Spiegel, Noordam, & van der Fels-Klerx, 2013) to substitute conventional protein sources and contribute to a more sustainable solution. Alternative protein sources include single cell proteins (SCPs; microbial biomass or protein extracts originating from fungi, bacteria or micro-algae) (Becker, 2007), seaweed (macro-algae) (Mohamed, Hashim, & Rahman, 2012), cultured meat (Post, 2012; Tuomisto & Teixeira De Mattos, 2011) and insects. At the same time, food waste has to be reduced and the efficiency in food production has to be improved by applying better agricultural technologies (e.g. precision agriculture) (Reijnders & Soret, 2003) to decrease environmental impact.

Edible insects show the opportunity to provide humans with high-quality animal proteins while having a small environmental impact compared to conventional animal protein sources.

With their interesting nutritional value and high sustainability, they have the potential to help reduce undernutrition in developing countries (Nadeau, Nadeau, Franklin, & Dunkel, 2015), provide food for the growing population and replace a substantial portion of protein sources with high environmental impact, e.g. meat (Schösler, Boer, & Boersema, 2012). Moreover, they can play an interesting role in the formulation of sustainable animal feeds as well (Makkar, Tran, Heuzé, & Ankers, 2014). Their nutritional value and the aspects that contribute to insects being a sustainable food source are described in more detail in the following paragraphs.

1.1.4.1 Nutritional value

In contrast to their microbiological quality at the start of this PhD research, the nutritional value of edible insects has been investigated frequently. They are generally acknowledged to be a good source of **proteins, fats, vitamins, energy and minerals** (Rumpold & Schlüter, 2013a). Since insects are considered as an alternative protein source, typically their protein content and quality are assessed. According to a review by Belluco et al. (2013), insects contain a high protein content (see also Table 1.2), which is comparable to that of conventional protein sources. Their quality as determined by **digestibility and amino acid composition** is comparable as well and may even be superior compared to some (plant-based) protein sources. Indeed, digestibility of insect proteins varies, as reviewed by Kouřimská & Adámková (2016), between 76 and 96%. This is only slightly lower than for egg (95%) or beef (98%) proteins, but higher than for many plant proteins. Considering amino acid composition, Yi et al. (2013) reported amino acid patterns of lesser mealworms, yellow mealworms and house crickets, and concluded that the insect protein quality for both mealworm species is comparable to that of casein and higher than that of soybeans. The house cricket protein quality as based on amino acid composition was slightly lower compared to those conventional protein sources. These conclusions were based on the essential amino acid values, which were considered to be sufficient compared to the recommendations composed by several UN organisations (FAO, WHO, UNU).

Additionally, edible insects are typically appraised for their **vitamin B_{12}** content. For edible insects, this is an important micronutrient since they are promoted as alternative for meat products which are generally “a source of” or even “rich in” vitamin B_{12} (i.e. > 0.375 µg/100 g

and > 0.750 µg/100 g, respectively, as determined by Regulation (EU) N° 1169/2011 on the provision of food information to consumers) (Gille & Schmid, 2015). As an example, the vitamin B_{12} contents of mealworms and house crickets were reported as 0.47 µg ("source of") and 5.37 µg ("rich in") per 100 g fresh weight by Finke (2002).

Table 1.2 Ranges of proximate nutritional value of the edible insect species under study as documented in literature and compared to conventional protein sources[1].

	Moisture (%)[2]	Nutritional property (dry matter base)				
		Protein (%)[3]	Fat (%)[4]	Fibre (%)[5]	NFE (%)[6]	Energy (kcal/100 g)[7]
Mealworm	56.3 – 72.7	41.0 – 63.9	17.0 – 50.1	3.7 – 15.0	0.3 – 7.1	444.0 – 746.3
Lesser mealworm	66.7 – 70.0	60.0 – 65.0	13.4 – 29.0	N.D.[8]	N.D.	N.D.
House cricket	67.6 – 69.2	48.1 – 70.8	14.1 – 24.0	6.2 – 22.1	2.1 – 2.6	455.2 – 472.2
Tropical house cricket[9]	N.D.	70.0	18.2	3.7	0.1	452.4
Beef	65.3 – 74.1	53.6 – 87.2	5.8 – 49.8	0.0 – 1.2	N.D.	359.7 – 818.5
Pork	70.3	68.0	33.0	0.3	N.D.	572.4
Turkey	72.0	77.9	21.4	0.0	N.D.	503.6
Salmon	67.8	62.1	34.1	0.0	N.D.	555.9

[1]Values for raw edible insects were adapted from Adámková, Kourimská, Borkovcová, Kulma, & Mlček, 2016; Bednárová, Borkovcová, Mlcek, Rop, & Zeman, 2013; Finke, 2002; Janssen, Vincken, Van Den Broek, Fogliano, & Lakemond, 2017; Lenaerts et al., 2018; Nowak, Persijn, Rittenschober, & Charrondiere, 2016; Payne, Scarborough, Rayner, & Nonaka, 2016; Ravzanaadii, Kim, Choi, Hong, & Kim, 2012; Rumpold & Schlüter, 2013a; Siemianowska et al., 2013; van Broekhoven et al., 2015 and Zielińska, Baraniak, Karaś, Rybczyńska, & Jakubczyk, 2015. Values for conventional protein sources (raw) were obtained from the Dutch National Institute for Public Health and the Environment.

[2]Moisture contents were determined using lyophilisation or oven drying.

[3]Protein contents were determined using the Kjeldahl or Dumas method, using a Kp of 6.25. Consequently, protein contents should be interpreted as crude protein since also other nitrogen-containing compounds such as chitin are included.

[4]Fat contents were determined using the Soxhlet or Folch method.

[5]Fibre contents were reported as crude fibre, acid detergent fibre or neutral detergent fibre, depending on the method employed.

[6]Nitrogen-free extract, a calculated value representing carbohydrates other than fibre (Rumpold & Schlüter, 2013a).

[7]Energy content was calculated in the studies using standard calculations [g of protein × 4.0] + [g of fat × 9.0] + [g of NFE × 4.0] (Finke, 2002).

[8]N.D. = not determined in the selected studies.

[9]Values were obtained from one study.

Importantly, it should be remarked that significant **variation** in the nutritional values exists between different insect species, as well as between different populations and harvesting moments (Finke, 2002). This is clearly illustrated in Table 1.2, which shows the content ranges of macronutrients of the four insect species considered in this thesis as published by several studies. The large variation for nutritional values within the same insect species can be caused

by different feeds, rearing methods and/or methods of analysis applied (Lenaerts et al., 2018; van Broekhoven et al., 2015). Considering the **methods of analysis**, it should be noted that no specific standards are in place for insects. Nutritional values reported in literature may originate from different methods used and therefore are hard to compare. Also, the nitrogen-to-protein conversion factor (K_p) of 6.25, typically used for foods in e.g. the Kjeldahl analysis method, is prone to discussion for insects. The presence of chitin-derived nitrogen may cause an overestimation for the insect protein content. A specific K_p of 4.76 was suggested instead (Janssen, Vincken, Van Den Broek, Fogliano, & Lakemond, 2017).

1.1.4.2 Sustainability

The definition of sustainability has been discussed intensively. At the World Commission on Environment and Development in 1987, the concept of sustainable development was described for the first time (Brundtland, 1987). Later, at the World Summit on Social development in 2005, sustainability was conceptualised as an equilibrium in the conjunction of **social, economic and environmental considerations** (UN General Assembly, 2005). For a food system, this means that food security and nutrition can be delivered to everyone without harming social, economic and environmental sustainability for future generations (Halloran et al., 2018). While this three-pillared approach may be criticised (Adams, 2006; Magee et al., 2013), it offers a good basis to assess the sustainability of edible insects.

Since **wild insects** are part of the ecosystem, uncontrolled collection may cause overexploitation of the insect and thus harm the environment. On the other hand, a controlled collection of wild insects can positively contribute to the environment, e.g. when managing insect plagues and therefore reducing the use of pesticides. Additionally, these insects themselves are threatened by environmental problems, such as water pollution by pesticides. The collection of insects from the wild is consequently a matter of balance between conservation of the ecosystem and food, economic and social security (van Huis & Oonincx, 2017). It has been suggested that wild insect populations will not be sufficient when insects will be used in large quantities for food and feed (van Huis & Oonincx, 2017). Therefore, a shift towards production rather than collection will be necessary in tropical regions as well.

The **industrial production of insects** can provide a more sustainable protein source compared to livestock production. The sustainability of insect production was repeatedly investigated through comparative **life cycle assessment** (LCA), for example for mealworms (Oonincx & de Boer, 2012; Smetana, Mathys, Knoch, & Heinz, 2015), black soldier fly (Smetana, Palanisamy, Mathys, & Heinz, 2016) and house crickets (Halloran, Hanboonsong, Roos, & Bruun, 2017). The LCAs show that, depending on the substrate used during rearing and the processing technologies applied after rearing, the production of the investigated insects, compared to benchmarks such as meat (pork, chicken, beef) or milk production, can emit less **greenhouse gasses** such as CO_2, CH_4, NH_3 and N_2O. However, **energy use** to produce insects is reported to be equal to or even higher than for the benchmarks. A large amount of energy is used for example during processing insects into dried products. Even before processing, heat is applied to the insects during (part of) the rearing process to maintain body temperature, but at later stages of the rearing process, an excess amount of heat may be generated and reused. At the same time, the **poikilothermic** nature of insects ensures that the energy provided by their feed is not required for maintaining body temperature but can be employed completely for growth. As a result, the **feed conversion ratio** (FCR, mass of input divided by mass of output) of insects is low (i.e. efficient) compared to other farm animals and therefore reduces the environmental impact of insect cultivation (Halloran, Roos, Eilenberg, Cerutti, & Bruun, 2016). Moreover, insects can be reared on feeds currently not used for traditional livestock, such as food industry wastes or by-products. In addition, the fact that the edible fraction of insects is much higher than that of conventional livestock also contributes to the efficient FCR. Also the **use of land and water** is substantially lower for insect production than for conventional livestock production. Finally, other factors such as transport, storage, waste management, etc. have an impact on the outcome of the LCAs as well.

While the LCAs of the different insect species all conclude that insects can serve as a more sustainable source of protein than conventional sources, it is important to note that all these studies may have their shortcomings (Halloran et al., 2016). Several parameters (e.g. different units or system boundaries that were used) contribute to the outcome of the LCA and depending on how the LCA was conducted, results may be more or less straightforward or hard to compare. Also, since industrial insect rearing is constantly evolving due to upscaling

and automation, as also discussed in the next paragraph, LCAs need to be updated simultaneously. Additional and specific research is therefore still necessary.

1.1.5 Weaknesses and threats of entomophagy

To be able to produce insect food and feed products on a large scale with a reasonable price, large amounts of insects will be necessary. Consequently, insect rearers will, in time, have to be capable of producing several tonnes of edible insects per week. This requires a commercial rearing on industrial scale and therefore large investments in location, equipment and automation to accomplish **upscaling**. This involves **financial challenges** in order to create a profitable business. Given the fact that many existing insect rearing companies currently note an operation loss (Schillewaert, 2018), investment in upscaling constitutes a large hurdle for the development of the insect sector.

Large-scale production will also lead to challenges considering **insect diseases** (Eilenberg, Vlak, Nielsen-LeRoux, Cappellozza, & Jensen, 2015). Rearing companies should be aware of several species-specific insect pathogens that can affect the whole production without proper measures. Important entomopathogens include several viruses such as parvoviruses, baculoviruses and iridoviruses, bacteria such as *Bacillus thuringiensis*, *Brevibacillus laterosporus*, *Serratia* spp. and *Pseudomonas* spp., many fungi such as *Beauveria* spp., *Metarhizium* spp. and Entomophthorales spp. and parasites such as *Microsporidia* spp. (Bravo, Gill, & Soberón, 2007; EFSA Scientific Committee, 2015; Eilenberg et al., 2015; Halloran et al., 2018; Maciel-Vergara & Ros, 2017; Ruiu, 2013; van Huis et al., 2013). Infection of insects with these pathogens entails severe risks such as reduced fitness and fertility or mass mortality (Eilenberg et al., 2015), which of course affects production capacity and has financial consequences. As an example, the *A. domesticus* densovirus (AdDNV, Parvoviridae) (Szelei et al., 2011) frequently infects *A. domesticus* populations with a severe production loss as a result. To overcome this problem, *G. sigillatus* can be used as an AdDNV-resistant alternative edible cricket species (Eilenberg et al., 2015).

Only when the demand for insects can be met by a proper production quantity, insect food products with **prices** able to compete with meat products can be manufactured. While the prices of insect products currently available on the market are high (e.g. € 19.93/kg for frozen

mealworms, € 158.00/kg for dried mealworms and € 24.95/kg for pasta including mealworm flour at Bio-planet, Belgium, October 2018), a reduction of these prices may convince the consumer to choose for insects as an alternative to other protein sources. As a comparison, prices for pork and beef, for example, range between €4.48 and €15.95/kg for pork and € 9.96 and € 34.90/kg for beef (Colruyt, Belgium, October 2018), the latter being the price of one of the most expensive pieces of meat.

While price is an important factor in food choice (Hoek, Pearson, James, Lawrence, & Friel, 2017), it is not the only factor that contributes to the willingness of consumers to eat edible insects or insect-based products. **Consumer acceptance** for edible insects and insect-derived products was repeatedly assessed in Europe (House, 2016; Lensvelt & Steenbekkers, 2014) and in Belgium (Caparros Megido et al., 2014; Schouteten et al., 2016; Van Thielen et al., 2018; Verbeke, 2015). While different experimental set-ups were used, several findings were common. For example, young male consumers were found to be more eager to adopt edible insects in their diet and those people that experience a positive insect tasting show a high willingness to continue consuming insects after the first try. However, a large group of consumers are not interested in eating insects, contributing to a low general consumer acceptance. The main reasons for this attitude are aversion, dietary or cultural habits and the availability of other alternatives. In order to increase consumption and/or consumer acceptance, it may help to improve availability and visibility of insect products, to provide more information on edible insect consumption and preparation, to incorporate insects in familiar food products in an invisible way and to improve the taste of insect products (Van Thielen et al., 2018).

Other than consumer acceptance, also **food safety** is important to launch a new food matrix such as insects (SHC & FASFC, 2014). Like for other food products, chemical and microbiological food safety should be guaranteed for edible insects. Due to the lacking **legislation** applicable on edible insects, no specific criteria or regulations exist for edible insects. Yet, routine checks and quality control in the insect food sector are not optimised. The current state of insect-specific legislation will be discussed in paragraph 1.2.

Finally, also **animal welfare** should be acknowledged regarding the use of insects for human consumption. When insects are reared intensively, they can be considered as farm animals

(as they also are from a legal point of view). For all farm animals, the European Commission recognised the so-called "**Five Freedoms**" to promote animal welfare and protect animals of all species kept for farming purposes. The "Five Freedoms" include freedom (1) from hunger and thirst, (2) from discomfort, (3) from pain, injury and disease, (4) from fear and distress and (5) to express normal behaviour. These "Five Freedoms" are included in the European Convention for the Protection of Animals kept for Farming Purposes, which acted as a basis for Council Directive 98/58/EC on the protection of animals kept for farming purposes. While rearing insects for human consumption, these "Five Freedoms" should therefore be taken into account in parameters such as rearing density and feed selection as well as behaviour such as cannibalism (van Huis et al., 2013). Also the killing method preferably reduces suffering to the minimum by using for example freezing or instantaneous shredding. On the other hand, **uncertainty** exists regarding the extent to which **insects can experience pain, fear and discomfort** and have cognitive abilities (van Huis et al., 2013). Additional research regarding these aspects of animal welfare should provide more insights in the future, which may lead to practical implementations in insect rearing.

1.2 Legislation regarding edible insects as or in human food

Prior to the major introduction of insect food products into the market, the small-scale production and trade of edible insects was not considered important enough to be included in legislation. Since rearing has been scaled up and insect food products have become a segment in food retail, questions regarding proper regulation of this food chain emerged (Halloran et al., 2018). A clear legislative framework was therefore necessary.

Worldwide, the *Codex Alimentarius* provides a collection of guidelines, standards and codes of practise to contribute to food safety and quality in an internationally uniform way. While the *Codex* has a major influence on food legislation, it is not legally binding. In Europe, human food and feed are strictly regulated at European level, while leaving room for specific regulation on a national level. The European Food Safety Authority (EFSA) and the Federal Agency for Safety of the Food Chain (FASFC) guard the food safety risks in Europe and Belgium, respectively. Since the introduction of insects on the market in several European countries, EFSA and the national regulatory agencies have taken action to start constructing specific

regulations regarding insects for human and animal consumption. These regulations should focus on microbiological criteria applicable on edible insects and the status of insects as novel food. Additionally, general European (Regulations) and Belgian (Royal Decrees) legislation applicable for animal-based food and feedstuffs, covering for example aspects of traceability, hygiene, packaging, and Hazard Analysis and Critical Control Points (HACCP) is also valid for edible insects (FASFC, 2016):

- Regulation (EC) N^{o} 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety; **General Food Law;**
- Regulation (EC) N^{o} 852/2004 on the **hygiene of foodstuffs**;
- Regulation (EC) N^{o} 853/2004 laying down specific **hygiene rules for food of animal origin;**
- Regulation (EC) N^{o} 854/2004 laying down specific rules for the organisation of official **controls on products of animal origin** intended for human consumption;
- Regulation (EU) N° 1169/2011 on the provision of **food information** to consumers;
- Royal Decree of 13 September 1999: **labelling of pre-packed foods**;
- Royal Decree of 14 November 2003: **self-regulation, compulsory notification and traceability;**
- Royal Decree of 16 January 2006: **authorisations, approvals and pre-registrations** delivered by FASFC;
- Royal Decree of 13 July 2014: **hygiene of foodstuffs**, additions to Regulation (EC) N^{o} 852/2004;
- Royal Decree of 30 November 2015: **hygiene for food of animal origin.**

Additionally, also considering the feed administered for the rearing of insects for human consumption, following animal feed regulations are applicable (FASFC, 2016):

- Regulation (EC) N° 999/2001 laying down rules for the **prevention, control and eradication of certain transmissible spongiform encephalopathies;**
- Regulation (EC) N° 183/2005 laying down requirements for **feed hygiene;**
- Regulation (EC) N° 767/2009 on the **placing on the market and use of feed;**

- Regulation (EC) N° 1069/2009 laying down health **rules as regards animal by-products and derived products not intended for human consumption.**

1.2.1 Microbiological criteria and guidance documents

In Europe, microbiological criteria of food products are regulated by the Commission Regulation (EC) N° 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. This regulation defines specific criteria for certain (groups of) microorganisms in certain food categories, which determine the acceptability of a food product, batch or production process during production or after introduction on the market. Accordingly, this is regulated by two different types of criteria. A first type is the **food safety criterion**, defining the acceptability of a food product after being placed on the market. The second type is the **process hygiene criterion**, maintaining the hygiene during food production. All food operators are obliged to ensure that their products comply with the regulation by performing microbiological tests and introducing specific measures based on the test results.

For edible insects, no specific microbiological criteria are embedded in Regulation N° 2073/2005. However, microbiological food safety risks may exist (see paragraph 1.3), and specific criteria could guide insect rearers and insect food producers to maintain a high quality food production process, as well as control agencies to evaluate operators. The development of a microbiological criterion involves a laborious process, which requires sufficient scientific data (van Schothorst, Zwietering, Ross, Buchanan, & Cole, 2009). Given the relatively small insect food sector compared to other food categories and the developing scientific research regarding edible insects, it will probably require some time until specific criteria will be established. Yet, it has been stated that a legislative framework related to insects is under development in the EU (EFSA Scientific Committee, 2015).

Awaiting a European regulatory framework for the existing insect sector, several countries already published national **guidance documents**. These documents typically consist of a **risk assessment** and, based on the risks that were exposed, some **recommendations** for the edible insect sector. Such guidance documents were for example published in Belgium (SHC & FASFC, 2014), the Netherlands (NVWA, 2014) and France (ANSES, 2015). In 2015, preceded by the national documents already published, the European Food Safety Authority also

published a guidance document capturing risks and recommendations regarding insect consumption (EFSA Scientific Committee, 2015). So far, most risk assessments published envisaged edible insects in general, and to date, only one risk assessment on a specific species (house cricket) has been published (Fernandez-Cassi et al., 2018). This individual approach is desirable due to the clear differences between feeds, rearing protocols and post-harvest processing practices for individual insect species, and in the microbiota of different species as will be demonstrated and discussed in this manuscript.

Both the Belgian and the Dutch national risk assessment documents included recommendations for microbiological criteria to be applied for edible insects. They refer to process hygiene and food safety criteria currently available for comparable food products such as ready-to-eat foods, minced meat, meat preparations and crustaceans and molluscs. For these food categories, food safety criteria for *Salmonella* spp., *Listeria monocytogenes* and *E. coli* exist and are recommended to be adopted. Moreover, the Dutch guidance document advises to also include their national criteria for *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and *Campylobacter* spp.

As a result of the **Belgian** risk assessment, the FASFC has directed a list of **specific requirements regarding food safety** of insects for human consumption and insect-based food products (FASFC, 2016). These requirements include, among others, periodical tests on *Salmonella* (absence in 10 grams) and *Listeria monocytogenes* (absence in 25 grams or < 100 cfu/g, respectively prior to and after introduction on the market). Additionally, a heat treatment is required for all insect products before entering the market, but no specifications are provided as to the time-temperature combinations that have to be reached or microbial reductions that have to be obtained. Other microbiological recommendations published in the Belgian guidance document are currently not included in the circular as legal obligation.

Apart from the current legal requirements in Belgium, the FASFC has also updated its **action limits for microbial contaminants in foodstuffs** to include edible insects and insect-based products. These action limits are advisory microbiological limits used to evaluate microbiological analyses by the FASFC, to support food operators in their auto control system and as a check for compulsory notification. The latest list of action limits (January 2018) includes limits for *Salmonella* spp., *Bacillus cereus*, Enterobacteriaceae, *Escherichia coli*,

yeasts, moulds, coagulase positive staphylococci and total viable aerobic count, and are additional to the obligations posed in the circular. The action limits are based on recommendations by the several guidance documents on microbiological criteria included in Regulation (EC) N° 2073/2015 and should be interpreted accordingly. A full list of both legal criteria and recommended action limits is provided in Table S1.1 (Supporting information) and contains interesting reference values to compare obtained research data with.

1.2.2 The novel food regulation

Until 2015, Regulation (EC) N° 258/97 of the European parliament and of the council of 27 January 1997 concerning novel foods and novel food ingredients defined that foods and food ingredients which were not consumed to a significant degree by humans prior to 15 May 1997 were considered novel foods or novel food ingredients in the EU. Following this regulation, the placing of such novel foods or food ingredients on the European market should be requested by submitting an extensive technical dossier that assesses the novel food or novel food ingredient. Regulation N° 258/97 was not completely clear about the novel food status of edible insects. It was uncertain whether insects and insect-based food products could be categorised under "food ingredients isolated from animals" in the regulation or whether this category only covered isolated substances such as lipids and proteins. Additionally, the novel food regulation did not specifically mention insects. This evoked uncertainty whether or not whole edible insects should be considered as novel foods and were allowed on the European market without approval. This uncertainty was clarified by the publication of Regulation (EU) 2015/2283 of the European parliament and the council of 25 November 2015 on novel foods. This regulation stated that, as from 1 January 2018, whole insects and their parts would be considered as novel foods and require an approval by EFSA.

Prior to the publication of the new novel food regulation 2015/2283, the uncertainty on the novel food status of edible insects led to the publication of a circular by the Belgian FASFC that tolerated the rearing and marketing of insects and insect food products for human consumption (FASFC, 2014). This circular allowed ten insect species on the Belgian market (Table 1.3), under specific conditions described in the circular, including registration of activities at the FASFC, application of general principles regarding food legislation, and implementation of specific insect-related food safety aspects.

In 2016, the Belgian circular was updated including the recent publication of the new novel food regulation (FASFC, 2016). The circular of 2016 continued to tolerate the rearing and marketing of the ten insect species in Table 1.3 until 1 January 2018. After that date, only insect species for which a novel food dossier was submitted prior to 1 January 2018 in Belgium would be tolerated on the Belgian market. To date, this includes the yellow mealworm (*Tenebrio molitor*), the house cricket (*Acheta domesticus*) and the African migratory locust (*Locusta migratoria migratorioides*) as confirmed in a state of the play (Federal Public Service Health Food Safety and Environment, 2018). Consequently, only those three insect species and their applications, as described in the state of the play and provided in Table S1.2 (Supporting information) are currently tolerated. According to the EFSA website, novel food dossiers for the lesser mealworm (*Alphitobius diaperinus*) and the tropical house cricket (*Gryllodes sigillatus*) are currently submitted as well, be it in other member states. When a novel food dossier is approved, the use and marketing of that specific insect and its described food products will be allowed in the whole European Union.

Table 1.3 Insect species tolerated for rearing and marketing in Belgium as defined by the 2014 circular (FASFC, 2014)**.**

Scientific name	Common name (English)
Acheta domesticus	House cricket
Locusta migratoria migratorioides	African migratory locust
Zophobas atratus morio	Giant mealworm
Tenebrio molitor	(Yellow) mealworm
Alphitobius diaperinus	Lesser mealworm
Galleria mellonella	Greater wax moth
Schistocerca americana gregaria	American desert locust
Gryllodes sigillatus	Tropical house cricket or banded cricket
Achroia grisella	Lesser wax moth
Bombyx mori	Silkworm

1.2.3 Summarising overview of the current insect legislation in Belgium

For operators active in the Belgian insect sector, the current legislation regarding edible insects is not always clear and straightforward. As discussed in previous paragraphs, a combination of general (European and national) food legislation and specific requirements imposed by the FASFC apply for the insect industry. As such, a summary regarding the current legal obligations regarding insects in foodstuffs in Belgium is provided here. This overview is

limited to operators in rearing, processing and distribution of insects for food and does not include advisory recommendations.

1.2.3.1 Rearing operators

1. **General principles in food legislation** are applicable. These general principles include for example good hygienic practices, traceability, compulsory notification, labelling and the implementation of a self-checking system based on the HACCP principles;
2. Feed administered to edible insects should follow the **general feed legislation**, including feed hygiene and the prohibition of using e.g. manure, animal by-products, pharmacological agents such as antibiotics, and animal proteins in insect feed;
3. Operators rearing insects must be **registered** at the FASFC;
4. Currently, a transitional measure allows **only those insect species and their applications for which a novel food dossier was submitted** prior to 1 January 2018 to be commercialised on the Belgian market. Today, this comprises **three insects**: the yellow mealworm (*Tenebrio molitor*), the house cricket (*Acheta domesticus*) and the African migratory locust (*Locusta migratoria migratorioides*);
5. Some **specific advices** compiled by SHC & FASFC (2014) must be followed by rearers:
    a. Consequent cleaning and disinfection of rearing facilities and materials;
    b. Removal of faeces and dead insects;
    c. Regular provision and renewal of feed and water;
    d. Clear separation of insects reared for food from insects reared for other purposes (feed, pet food, …);
    e. The use of therapeutic pharmacological agents should comply with the Royal Decree of 14 December 2006 and Regulation (EU) N° 37/2010.

1.2.3.2 Processing and/or distribution operators

1. **General principles in food legislation** (see 1.2.3.1);
2. Operators in the processing and/or distribution sector must be **authorised** by the FASFC;
3. **Three insects** (for which a novel food dossier was submitted, see 1.2.3.1) and their applications are allowed for commercialisation;

4. Some **specific advices** compiled by SHC & FASFC (2014) must be followed by processing and/or distribution operators:
    a. Prior to commercialisation, insect products must be heat-treated to reduce the amount of microorganisms;
    b. Insect products put on the market should periodically be tested for *Salmonella* spp. and *Listeria monocytogenes*, following Regulation (EC) N° 2073/2005;
    c. For dried or freeze-dried insects, the number of pathogens that can grow in the product should be taken into account till the end of their shelf life;
    d. Labels should include clear instructions and preservation requirements;
    e. When applicable (e.g. for crickets, grasshoppers) labels should include that legs and/or wings should be removed;
    f. A warning should be labelled regarding allergies related to insects.

1.3 Microbiological quality and food safety risks of insects for foods

As required by the novel food regulation, edible insects should be assessed for their microbiological quality and safety before they can be introduced into the market. Prior to the research described in this PhD dissertation, microbiological data concerning edible insects for human consumption were scarce. The studies available at the start of the PhD are summarised in Table 1.4 for the species considered in this dissertation (studies published during the course of the PhD are discussed in the following chapters). The first description of microbial counts harboured by edible insects was made by Giaccone (2005). He reported a total viable aerobic count of 5 to 6 log cfu/g. Unfortunately, the reported count was an average of a combination of several insect species: the giant mealworm (*Zophobas morio*), the greater wax moth (*Galleria mellonella*), the butterworm (*Chilecomadia moorei*), the yellow mealworm (*Tenebrio molitor*), and the house cricket (*Acheta domesticus*). The same limitation was encountered in a preliminary study performed by Grabowski, Jansen, & Klein (2014) on edible insects sold as pet food. They reported total viable aerobic counts of 7.041 ± 0.434 log cfu/mg insect, being a random combination of nine different species. Also the amount of Enterobacteriaceae and fungi was reported, being 6.710 ± 0.639 and 4.554 ± 1.044 log cfu/mg, respectively. Noteworthy is the uncommon notation of the microbial count in log

cfu/mg, which would correspond with a log cfu/g value of 3 log units higher and hence results in values that are not in line with other studies.

A first study that reported microbial counts of individual insect species was performed by Klunder, Wolkers-Rooijackers, Korpela, & Nout (2012). While this study is still acknowledged as the start of microbiological research regarding edible insects, it had some limitations as well. The reported microbial counts were obtained from only one sample and the methods used for microbial enumeration were incomplete. However, it gives a first indication of both the microbial numbers that can be expected on edible insects and of the impact of a heat treatment on those numbers. The same study also included effects of preservation and fermentation of sorghum flour mixed with a small amount of insects, as well as the identification of a few bacteria isolated. Later, as a first publication of our research group on the microbiology of edible insects, Stoops et al. (2016) added new microbiological data based on experiments that included several raw insect samples from different rearing batches. Moreover, this study was the first to investigate the microbial composition of the insects using next generation sequencing. The organisms that reside in or on the insects could thus be identified in a culture-independent way.

Microorganisms that were reported on (one of) the four edible insect species considered prior to the research performed in this dissertation were assigned to genera including *Bacillus, Propionibacterium*, *Lactobacillus*, *Streptococcus*, *Haemophilus*, *Pseudomonas*, *Staphylococcus*, *Acidovorax*, *Varibaculum*, *Clostridium* and *Micrococcus* (Klunder et al., 2012; SHC & FASFC, 2014; Stoops et al., 2016), some of which include pathogenic species. Typical food pathogens such as *Salmonella* spp., *Listeria monocytogenes* and *Clostridium perfringens* were not yet encountered at that time (EFSA Scientific Committee, 2015; Grabowski et al., 2014; NVWA, 2014; SHC & FASFC, 2014). In some cases, however, the food pathogen *Bacillus cereus* was retrieved (NVWA, 2014).

Table 1.4 Summary of the microbial counts of the edible insects under study reported prior to the research described in this dissertation.

Insect species	Treatment	Study	Microbial counts (log cfu/g)				
			Total viable aerobic count	Entero-bacteriaceae	Lactic acid bacteria	Aerobic bacterial endospores	Fungi
Yellow mealworm	None (raw)	Klunder et al., (2012)	7.7	6.8	N.D.[1]	2.1	N.D.
		Stoops et al. (2016)	7.7 ± 0.3 – 8.3 ± 0.1	6.8 ± 0.1 – 7.6 ± 0.2	7.0 ± 0.1 – 7.6 ± 0.1	< 1.0 ± 0.0 – 3.5 ± 0.0	5.2 ± 0.6 – 5.7 ± 0.2
	Boiled (10 min)	Klunder et al. (2012)	< 1.7	< 1	N.D.	< 1	N.D.
	Boiled (10 min) and crushed	Klunder et al. (2012)	2.5	< 1	N.D.	2.5	N.D.
	Roasted (10 min)	Klunder et al., (2012)	< 1.7	2.2	N.D.	1.6	N.D.
	Roasted (10 min) and crushed	Klunder et al. (2012)	4.8	2.6	N.D.	< 1	N.D.
	Freeze-dried	NVWA (2014)[2]	< 5 – > 7	< 3 – > 5	N.D.	N.D.	N.D.
	Freeze-dried and processed into a snack	NVWA (2014)[2]	< 5	< 3	N.D.	N.D.	N.D.
	Frozen	SHC & FASFC (2014)[3]	7 - 9	N.D.	N.D.	4	N.D.
House cricket	None (raw)	Klunder et al. (2012)	7.2	4.2	N.D.	3.6	N.D.
	Boiled (1 min)	Klunder et al. (2012)	3.1	< 1	N.D.	2.0	N.D.
	Boiled (5 min)	Klunder et al. (2012)	1.7	< 1	N.D.	1.5	N.D.
	Stir-fried (5 min)	Klunder et al. (2012)	2.7	< 1	N.D.	1.5	N.D.
Lesser mealworm	Freeze-dried	NVWA (2014)[2]	< 5 – > 7	< 3 – > 5	N.D.	N.D.	N.D.

[1]N.D. = not determined

[2] Data were retrieved from a small-scale study performed by NVWA in 2010.

[3]Data were retrieved from a preliminary study performed at Lab4Food, KU Leuven in 2014.

While these first microbiological data provided the first insights into the microbiological quality and food safety of edible insects, many questions regarding the (food) microbiology of edible insects remained unanswered (EFSA Scientific Committee, 2015). The high microbial counts that were reported and the possible occurrence of pathogenic microorganisms in edible insects might involve food safety risks. Consequently, the urge for additional microbiological data from several insect species, rearing batches and rearing companies, as well as from insects that were subjected to processing and preservation remained high and inspired the research performed in this dissertation.

Concurrent with the research performed in this dissertation, other studies regarding the microbiological quality and food safety of edible insects were performed as well. Yet, these studies investigated other aspects than defined in this PhD dissertation (see paragraph 1.5) such as microbiological aspects during industrial or laboratory-scale rearing of insects (Osimani, Milanović, Cardinali, Garofalo, et al., 2018; Wynants et al., 2018), microbiological assessment of processed and online marketed edible insect products (Fasolato et al., 2018; Garofalo et al., 2017; Grabowski & Klein, 2016; Osimani, Garofalo, Milanović, et al., 2017; Osimani, Milanović, Garofalo, et al., 2018) and the microbiological effects of household cooking techniques (Caparros Megido et al., 2018). Undoubtedly, these studies have also contributed to the general microbiological knowledge considering edible insects.

1.4 State of the art in insect processing and preservation

1.4.1 Current post-harvest treatments and processing

The whole process including rearing and processing generally follows more or less the same flow for insects intended for feed as those intended for food purposes, as depicted in Figure 1.4. When the insects reach the desired size or stage, they are harvested. Prior to the harvesting step, some rearing companies apply a period of **starvation**, during which the insects are either separated from their feed or given only one specific type of feed (e.g. carrots). As a result, insects empty their guts, which may result, according to the rearers, in improved taste and/or cleaner (also including a lower microbial load) and more appetising insects. The **harvesting** step includes separation of the insects from their substrate. Depending on the size of the insect rearing facility, this can either be done manually or

automatically. While larvae such as mealworms and lesser mealworms can be sieved from their substrate, crickets and locusts typically live separated from their feed source as a natural behaviour. The latter species can be harvested by either picking them from their rearing cage or by shaking them out of the crevices they reside in. After harvest, edible insects are subjected to several post-harvest treatments prior to consumption.

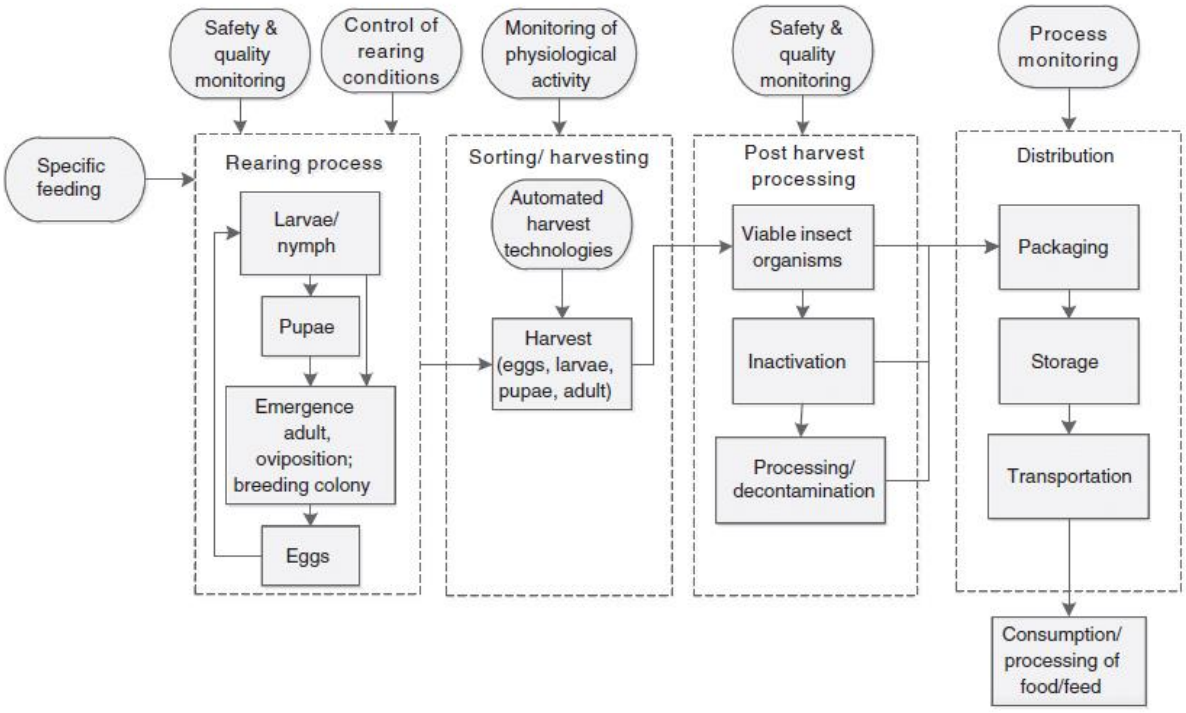


Figure 1.4 Schematic production process of food and feed derived from edible insects (Rumpold & Schlüter, 2013b).

A first step following the harvest is, when necessary, **killing** the insects. This may be achieved by either freezing them or submerging them in hot or boiling water. For some purposes, insects are not killed and sold as living species. For human consumption, however, insects are consistently killed and heat-treated (when killing was not accomplished by heat) before they enter the market. The aim of this **heat treatment** is to decontaminate the insects to reduce their health risks (EFSA Scientific Committee, 2015). The heat treatment can involve blanching, cooking or steaming, and should be designed properly as to process conditions in order to deliver low-risk edible insects.

After killing and decontamination of the insects, they may be **processed** into their desired commercial form. Insects can either be marketed as whole insects or fragmented and further processed. Specific fractions extracted from insects are also commercialised. Each product type requires a different way of processing (EFSA Scientific Committee, 2015). Whole insects are commercially available as dried, frozen or chilled products, which may be used directly by the consumer or the food producer. The fragmentation of insects into smaller parts results in a powder or a paste and allows insects to be incorporated in food products or directly in dishes prepared by consumers. Size reduction can be established by applying grinding, compression and/or impact, using equipment such as mills, shredders, cutters, etc. (Fellows, 2009). These operations can be applied on dried, frozen or raw insects. Finally, insects can be subjected to extraction to obtain different fractions such as fats, proteins and chitin. A combination of mechanical and chemical separation technologies will lead to the different fractions. The extraction method applied can also include drying or other processing steps.

Freezing and chilling do not need further processing. **Drying**, on the other hand, can be performed by oven drying or freeze-drying. Oven drying includes the application of heat and thus adds an additional decontamination step to the process, but it may also have an impact on the nutritional quality of the product (Fombong, Van Der Borght, & Vanden Broeck, 2017). Freeze-drying does not involve heat transport to the product, but requires a freezing step instead. Although this drying method may harm the product to a smaller extent, it includes a higher cost compared to alternative drying methods. In developing countries with a tradition in entomophagy, sun drying is often the preferred method, as it is the cheapest drying method available (Usub et al., 2010). However, this may include a risk of microbial growth and contamination and as a result a reduction of microbiological quality and food safety. Presently, other drying methods such as microwave drying are being investigated for edible insects, some of which show promising results (Lenaerts et al., 2018; Vandeweyer, Lenaerts, Callens, & Van Campenhout, 2017).

After processing insects into the desired product, they are ready to be packed, distributed, preserved and consumed. Prior to packaging, some insects such as members of the Orthoptera require the removal of physically harmful parts, including legs and wings. Some producers also add a seasoning to their products. Finally, insects in whatever form may serve

as an ingredient in industrially manufactured food products or may directly be used in the consumer's kitchen.

1.4.2 Preservation strategies

In order to extend the shelf life of edible insects and insect-based products, they are stabilised and/or preserved accordingly. Typically, raw edible insects or products containing raw insects are preserved in a chilled or frozen way. **Chilled** preservation generally slows down (part of) the microbiological growth and the chemical spoilage, but only allows a short preservation. In a **frozen** state, spoilage may be reduced to a larger extent, resulting in a shelf life of several months. In addition to low temperature storage, insect products may be subjected to other preservation techniques such as marination or fermentation (Borremans, Lenaerts, Crauwels, Lievens, & Van Campenhout, 2018) as well as modified atmosphere packaging or the addition of preservatives.

When an effective drying process was applied (reducing the moisture content to below 10% and water activity to below 0.60 (Jay, Loessner, & Golden, 2005)), **dried** insects and insect products are shelf-stable and do not require low temperature storage. A correct packaging design is important to maintain the dried conditions. It must be noted that, in the case of storage in the presence of oxygen, chemical spoilage can still occur as a result of oxidation.

1.4.3 Microbiological impact of processing and preservation

All treatments that are applied to edible insects in order to obtain edible insect ingredients or products may have an impact on the microbiological quality. Because raw edible insects contain a high microbial load, it should, as indicated by for example the EFSA risk profile (EFSA Scientific Committee, 2015), be reduced prior to consumption. A **thermal treatment** is a good practise to decrease the microbial load of food products (Fellows, 2009). Factors such as temperature and treatment time will influence the extent of the microbial reduction. A number of post-harvest steps, for example the killing or the drying step, also involve the supply of heat and can reduce the microbial load as a side effect next to the primary aim of killing or moisture reduction. As indicated by Klunder et al. (2012), the presence of bacterial

endospores can pose a risk for edible insects and may therefore require a specific spore-reducing treatment.

Apart from thermal treatments, several **non-thermal processing** steps may alter the microbiological properties of edible insects. Techniques such as non-thermal drying, salting, seasoning, fermenting, marinating, freezing, chilling, irradiation, modified atmosphere packaging or a combination strategy can reduce the microbial load and/or establish an environment in which microorganisms are inhibited in their growth. Combined with a proper decontamination technique, this will allow insects and insect-based products to be preserved in a microbiologically safe way. The processing technique(s) applied will determine the shelf life of the products.

Not all treatments applied by rearers and insect food producers, however, result in a reduction of microorganisms. For example, as mistakenly assumed by some insect rearers, starvation and/or rinsing insects with water prior to harvesting did not lower the microbial load or even alter the bacterial composition of mealworms, as demonstrated by Wynants et al. (2017). The effects of these manipulations on the fate of possible chemical contaminants, however, was not assessed. Also, it should be noted that some processing techniques can increase the amount of microorganisms in the insect product. This is, for example, the case for fermentation (intentional introduction of fermenting microorganisms) or seasoning (introduction of microorganisms harboured by herbs and spices (Sospedra, Soriano, & Mañes, 2010)), especially when applied on previously decontaminated insect products.

During the whole chain of rearing, processing and preservation, attention must be given to hygiene and process design, since cross-contamination, post-contamination and/or recontamination may occur during handling. A proper HACCP plan is therefore (legally) required to control the critical points.

1.5 Objectives and outline of the dissertation

Since the introduction of edible insects and insect-based products on the Belgian and European markets, specific questions arose from the insect sector. Uncertainties regarding legislation, food safety, processing and preservation, consumer acceptance, etc. existed.

Several of these lacunas were assembled and described in the different national guidance documents (ANSES, 2015; NVWA, 2014; SHC & FASFC, 2014), which on their turn contributed to the composition of a European insect risk profile by EFSA (EFSA Scientific Committee, 2015). In these documents, objectives were formulated regarding research required to further develop the insect sector and, eventually, a legislative framework. Based on the objectives stated in these guidance documents, as well as on interests of Belgian and Dutch collaborating insect rearing and processing facilities, the following research goals for this PhD dissertation were identified:

- **Objective 1:** To explore the microbiota of freshly reared, raw insects for food, with special attention to the occurrence of human bacterial pathogens;
- **Objective 2:** To reveal the occurrence of antibiotic resistance genes in freshly reared, raw insects for food;
- **Objective 3:** To elucidate the impact of specific processing steps on the microbiota of insects for food;
- **Objective 4:** To investigate the microbiological quality and shelf life during preservation of insects for food.

These research goals are relatively basic in the domain of food microbiology. However, due to the fact that insects present a new food matrix in Europe and the microbiological implications of typical Western food processing technologies on this matrix have not been studied before, a baseline study as aimed at in this PhD dissertation was necessary. As it is not possible to tackle all research questions for both the pre- and post-harvest stage of the insect value chain, this PhD focuses on post-harvest insect microbiology, since the pre-harvest stage is being covered by other projects within the research group Lab4Food. In addition, the four most important insect species for human consumption that are most available were selected: the yellow mealworm (*Tenebrio molitor*), the lesser mealworm (*Alphitobius diaperinus*), the house cricket (*Acheta domesticus*) and the tropical house cricket (*Gryllodes sigillatus*). Only insects that were harvested, i.e. ready for consumption, were considered.

Driven by the designated objectives, a research plan was composed as illustrated in Figure 1.5, which also presents the outline of the dissertation. Following this general introduction (**Chapter 1**), the first part of the research focusses on the microbiological characterisation of

raw insects shortly after rearing. In **Chapter 2**, several insect species, obtained from several rearers and production batches, are assessed for their intrinsic properties and microbial numbers. By using culture-dependent techniques implemented in the food industry and traditionally employed for microbiological quality assessment of foods, this chapter provides microbiological data that can easily be compared with data obtained for other food matrices. Included in Chapter 2 is also the detection of the foodborne pathogens *Salmonella* spp. and *Listeria monocytogenes*, hence contributing to Objective 1. The samples collected in Chapter 2 are also assessed in Chapter 3 and Chapter 4. Following the culture-dependent assessment in Chapter 2, **Chapter 3** investigates the bacterial composition of the collected samples in a culture-independent way, using next generation sequencing. This data set provides insight in the bacterial genera and eventually species harboured by edible insects, and whether or not these genera or species contain food pathogens (Objective 1). Next, **Chapter 4** investigates the occurrence of antibiotic resistance genes in the insect sample collection. The chapter describes a real-time PCR technique developed for edible insects as a matrix and the simultaneous detection and quantification of several antibiotic resistance genes. The results provide information to meet Objective 2.

The second part of the research concentrates on processing and preservation of edible insects. **Chapter 5** and **Chapter 6** both assess the microbiological impact of processing steps and subsequent preservation of mealworms and crickets, respectively. Both chapters contain processing steps currently applied in the insect sector. Specific attention is paid to the fate of bacterial endospores. Both Objective 3 and 4 are supported by data reported in Chapter 5 and 6. Finally, in **Chapter 7** all previous chapters are discussed, resulting in the formulation of conclusions and future prospects.

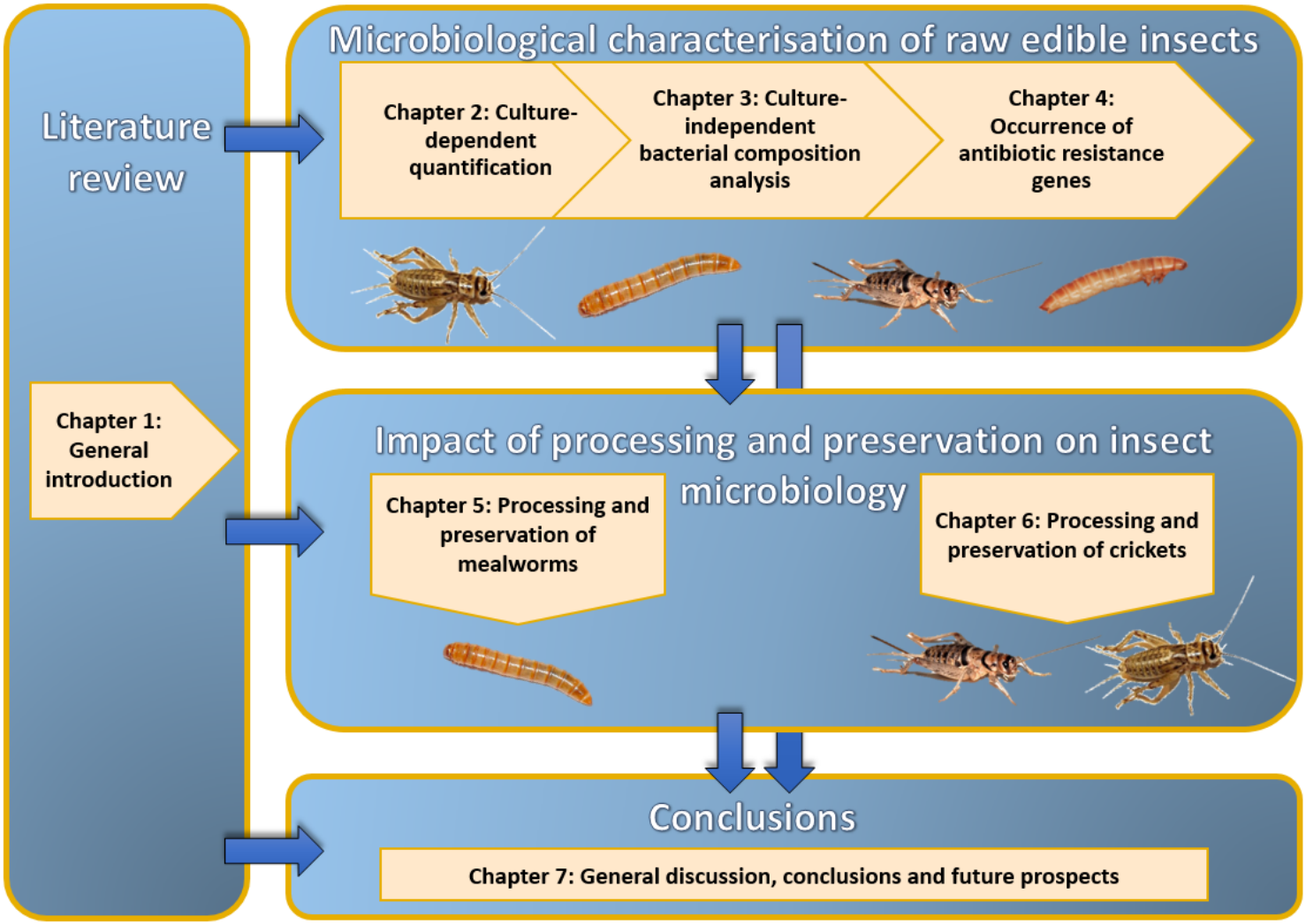


Figure 1.5 Schematic outline of the doctoral dissertation.

Chapter 2 Culture-dependent quantification of microorganisms in raw edible insects

This chapter was redrafted [1] after Vandeweyer D., Crauwels S., Lievens B., & Van Campenhout L. (2017). Microbial counts of mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing companies and different production batches. *International Journal of Food Microbiology*, *242*, 13-18. http://doi.org/10.1016/j.ijfoodmicro.2016.11.007

2.1 Introduction

Interest in human consumption of edible insects (entomophagy) in Western countries is increasing (Caparros Megido et al., 2014; Mlček et al., 2014) and more and more insect-based food products are being marketed. Compared with Asian, African, Oceanian and Latin American regions, Western society has no history of insect consumption (Siemianowska et al., 2013; van Huis, 2013; van Huis et al., 2013; Yen, 2015). However, insects are a promising and valuable alternative to conventional protein sources such as meat. They provide an opportunity to meet the increased protein demand of the growing world population (Mlček et al., 2014; Premalatha et al., 2011; van Huis et al., 2013). Moreover, rearing insects for food has a smaller ecological footprint compared to traditional animal husbandry (Oonincx et al., 2010; Oonincx & de Boer, 2012; van Huis et al., 2013).

Insects are being considered as novel food in Europe starting from January 2018, as stated in the new Regulation (EU) 2015/2283 on novel foods. Hence, more research data on the microbiological quality of edible insects reared in Europe are necessary to support risk assessments performed by the European Food Safety Authority (EFSA) (Belluco et al., 2013). Additionally, more quantitative data concerning the microbiological quality will be needed in order to establish microbiological criteria for edible insects in the future, similar to existing

[1] The complete content of this paper (Vandeweyer, Crauwels, Lievens, & Van Campenhout, 2017b) was included in Chapter 2, with only small alterations to keep the information provided up to date and to follow the logic course of this dissertation. As first author, D.V. contributed to all parts described in this work, from experimental design to the writing of the paper. The multivariate analysis was performed in collaboration with the Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), KU Leuven (Prof. B. Lievens).

criteria for other food products (Regulation (EC) N° 2073/2005 on microbiological criteria for foodstuffs).

Presently, the nutrient composition of several insect species has already been studied extensively (Finke, 2002; Nowak et al., 2016; Rumpold & Schlüter, 2013a; Sánchez-Muros, Barroso, & Manzano-Agugliaro, 2014; Siemianowska et al., 2013). Microbiological data, however, are only scarcely available, as highlighted in a recent EFSA opinion (EFSA Scientific Committee, 2015). Moreover, the few studies available containing microbiological data (Giaccone, 2005; Grabowski et al., 2014; Klunder et al., 2012; Rumpold et al., 2014) do not include analyses of different production batches or insects from different rearing companies. So far, there is only one study including different production batches (Stoops et al., 2016), but they originate from only one rearing company. None of the studies available contain data on intrinsic properties of edible insects, such as pH and water activity (a_w), although those factors have an important impact on the growth and survival of microorganisms (Madigan, Martinko, Dunlap, & Clark, 2009) and need to be taken into account when considering insects as a food matrix.

The objective of this study is to investigate the microbial load and intrinsic properties of freshly reared, raw mealworms (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) as a food product. In order to obtain a generalised view, different production batches and rearing companies were included in the study. pH, moisture content, and water activity (a_w) were determined, as well as a range of plate counts and presence-absence tests for pathogens typically determined for foods.

2.2 Materials and methods

2.2.1 Study materials

Three insect species commonly reared for human consumption were investigated: *Acheta domesticus* (house cricket), *Gryllodes sigillatus* (tropical house cricket) and *Tenebrio molitor* (yellow mealworm). Samples were obtained from seven rearing companies in Belgium and the Netherlands, including five companies specialised in rearing for human consumption and two companies for pet food. For each company, three production batches (i.e. rearing cycles)

were sampled between March and December 2015, resulting in 21 batches studied, consisting of 12 mealworm and 9 cricket batches (Table 2.1).

Table 2.1 Sample information.

Sample ID	Rearing company	Batch	Sampling month (2015)	Insect type	Species	Purpose (human/pet food)
MW 1.1	1	1	March	Mealworm	*T. molitor*[1]	Human food
MW 1.2	1	2	May	Mealworm	*T. molitor*	Human food
MW 1.3	1	3	September	Mealworm	*T. molitor*	Human food
MW 2.1	2	1	March	Mealworm	*T. molitor*	Human food
MW 2.2	2	2	June	Mealworm	*T. molitor*	Human food
MW 2.3	2	3	October	Mealworm	*T. molitor*	Human food
MW 3.1	3	1	May	Mealworm	*T. molitor*	Pet food
MW 3.2	3	2	July	Mealworm	*T. molitor*	Pet food
MW 3.3	3	3	November	Mealworm	*T. molitor*	Pet food
MW 4.1	4	1	July	Mealworm	*T. molitor*	Pet food
MW 4.2	4	2	August	Mealworm	*T. molitor*	Pet food
MW 4.3	4	3	September	Mealworm	*T. molitor*	Pet food
CR 1.1	5	1	March	Cricket	*A. domesticus*[2]	Human food
CR 1.2	5	2	June	Cricket	*A. domesticus*	Human food
CR 1.3	5	3	September	Cricket	*A. domesticus*	Human food
CR 2.1	6	1	April	Cricket	*A. domesticus*	Human food
CR 2.2	6	2	July	Cricket	*A. domesticus*	Human food
CR 2.3	6	3	October	Cricket	*A. domesticus*	Human food
CR 3.1	7	1	August	Cricket	*G. sigillatus*[3]	Human food
CR 3.2	7	2	October	Cricket	*G. sigillatus*	Human food
CR 3.3	7	3	December	Cricket	*G. sigillatus*	Human food

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

2.2.2 Sampling and sample preparation

Samples (± 500 g as provided by the rearer) of fully grown and living insects ready for consumption were transported to the laboratory at ambient temperature and immediately processed upon arrival. Prior to analysis, dead insects were removed and insects were sedated by cooling (± 4 °C, 1 h). Subsequently, three subsamples of 30 g were taken aseptically from each batch and pulverised (Bosch CNHR 25, max speed) as described previously (Stoops et al., 2016).

2.2.3 Intrinsic properties

All pulverised subsamples were directly (i.e. undiluted) subjected to measurements of pH, water activity (a_w) and moisture content. pH was measured in threefold using a digital pH meter (Portamess 911, Knick, Berlin, Germany with SI analytics electrode, Mainz, Germany). A single a_w measurement was performed on a 7 g aliquot of each subsample using a water activity meter (LabMaster a_w, Novasina, Lachen, Switzerland), until water activity and temperature (20 °C) were stable for 15 and 5 min, respectively. Moisture content was calculated from the weight loss of 2 to 3 g from each subsample after oven drying overnight at 105 °C.

2.2.4 Microbial plate counts

Since it was not clear whether pulverisation of the insects before homogenisation would affect microbial counts, a preliminary experiment was executed. Several counts (mesophilic aerobic count, aerobic endospores and Enterobacteriaceae, see below) were determined using a procedure with and without pulverisation (as described in paragraph 2.2.2). Both approaches were performed on five subsamples of a mealworm sample obtained from company 4 (Table 2.1). Because pulverisation was found to be necessary for optimal extraction of microorganisms from their matrix (see paragraph 2.3.1), the step was included in all further analyses.

To obtain a primary dilution, 5 g of each pulverised subsample and 45 g of peptone physiological salt solution (PPS, 0.85% NaCl, 0.1% peptone, Biokar Diagnostics, Beauvais, France) were mixed together in a stomacher bag. After homogenisation for 60 s in a Bagmixer® (Interscience, Saint Nom, France), a tenfold dilution series was prepared and plated using the pour-plate technique, according to the ISO standards assembled by Dijk et al. (2015). Bacterial endospores and yeasts and moulds were determined according to Dijk et al. (2007). Total viable mesophilic and psychrotrophic aerobic counts were assessed after aerobic incubation on Plate Count Agar (PCA, Biokar diagnostics) for respectively 72 h at 30 °C and 10 days at 6.5 °C. Lactic acid bacteria (LAB) were incubated on de Man, Rogosa & Sharpe agar (MRS, Biokar diagnostics) for 72 h at 30 °C, Enterobacteriaceae on Violet Red Bile Glucose agar (VRBG, Biokar diagnostics) for 24 h at 37 °C, and yeasts and moulds on Oxytetracycline

Glucose Agar (OGA, Biokar diagnostics) supplemented with oxytetracycline (50 mg/550 ml OGA, Biokar diagnostics) for 5 days at 25 °C. Aerobic bacterial endospores were determined on PCA for 24 h at 37 °C after a pasteurisation treatment of the 10^{-1} dilution at 80 °C for 10 min.

2.2.5 Pathogen detection

Pulverised samples were also used for detection of *Salmonella* spp. and *Listeria monocytogenes*. Detection of *Salmonella* spp. was performed according to ISO 6579 (absence in 25 g) and detection of *Listeria monocytogenes* according to AFNOR BRD 07/4-09/08 (absence in 25 g). Two samples (one mealworm sample, one cricket sample), were analysed in fivefold (i.e. as five pulverised subsamples) as described in Regulation (EC) N° 2073/2005 on microbiological criteria for foodstuffs. A single analysis for both pathogens was performed on all other samples.

2.2.6 Statistics and multivariate analysis

To determine statistical differences between production batches of individual rearing companies, between different rearing companies, and between the different insect types studied, data were analysed with SPSS Statistics 20 (IBM, New York, USA). To determine significant differences in intrinsic factors and microbial counts between rearing companies and between batches, one-way Kruskal-Wallis analysis was used, followed by a multiple comparison using the Dunn-Bonferoni post hoc test. For the Kruskal-Wallis tests, a distinction between weakly significant ($0.05 < p < 0.10$) and significant ($p < 0.05$) was adopted. Mann-Whitney U tests were performed to analyse differences between mealworms and crickets, with a level of significance of 0.05. In the preliminary experiment comparing pulverised and non-pulverised subsamples, an independent samples t-test with significance level 0.05 was used. Additionally, all data (means) from intrinsic and microbiological parameter analyses were used to perform multivariate non-metric multidimensional scaling (NMDS) to visualise differences between batches, rearing companies and insects. Batches lacking one or more parameters were excluded from this analysis. The NMDS plot was created using the vegan package in R (v12.2.1) (Oksanen et al., 2012; R Development Core Team, 2013).

2.3 Results and discussion

2.3.1 Intrinsic properties and microbial counts

With total mean pH values of 6.7 and 6.4 for mealworms (Table 2.2) and crickets (Table 2.3) respectively, a near-neutral pH was observed for both insect types. Together with a high mean water activity of 0.96 for both insect types, the new food matrices can be considered as well-suitable for the growth of a broad range of microorganisms. It should be noted that these values only apply for the insect as a crushed matrix and can differ from the intrinsic properties of individual parts. Therefore, conclusions on the distribution and growth rates of microorganisms within the different parts (e.g. surface versus intestinal tract) of the insect cannot be made based on the data presented here.

The preliminary experiment investigating the importance of pulverisation demonstrated that microbial counts obtained from non-pulverised mealworms are an underestimation of the total amount of countable microorganisms. With mean values of 7.0 ± 0.2 (standard error of the mean) log cfu/g for total aerobic count, 2.5 ± 0.2 log cfu/g for aerobic endospores and 5.9 ± 0.2 log cfu/g for Enterobacteriaceae, counts from non-pulverised mealworms were strongly significantly lower ($p = 0.000$ for all counts) than those obtained from pulverised larvae (8.6 ± 0.1, 4.7 ± 0.1 and 7.7 ± 0.1 log cfu/g respectively). Pulverising insects as a first step in microbiological analysis thus resulted in counts that are 1.6 to 2.2 log cycles higher.

Table 2.2 Intrinsic properties and microbial counts of raw mealworms (*T. molitor*) from different rearing companies and batches[1].

Rearing company	Sample ID	Intrinsic properties			Microbial counts (log cfu/g)					
		pH (-)	a_w (-)	Moisture content (%)	Total viable aerobic count	Lactic acid bacteria	Entero-bacteriaceae	Aerobic bacterial endospores	Psychro-trophic aerobic count	Yeasts and moulds
1	MW 1.1	6.73 ± 0.02^{a}	0.97 ± 0.00^{a}	61.4 ± 0.4^{a,b}	8.3 ± 0.0^{a}	7.4 ± 0.0^{a}	6.8 ± 0.1^{a}	4.2 ± 0.1^{a,b}	5.8 ± 0.0^{a,b*}	4.8 ± 0.1^{a}
	MW 1.2	6.75 ± 0.01^{a}	0.97 ± 0.00^{a}	60.8 ± 0.1^{a}	8.4 ± 0.0^{a}	7.4 ± 0.0^{a}	7.2 ± 0.1^{a,b}	5.0 ± 0.6^{a}	4.8 ± 0.6^{a}	4.5 ± 0.1^{a,b*}
	MW 1.3	6.76 ± 0.01^{a}	0.95 ± 0.00^{b}	62.7 ± 0.1^{b}	8.3 ± 0.0^{a}	7.7 ± 0.0^{b*}	7.6 ± 0.1^{b}	2.6 ± 0.1^{b}	7.0 ± 0.0^{b*}	4.2 ± 0.1^{b*}
	Mean	**6.75 ± 0.01^{A}**	**0.96 ± 0.00^{A}**	**61.6 ± 0.5^{A}**	**8.3 ± 0.0^{A}**	**7.5 ± 0.1^{A}**	**7.2 ± 0.2^{A}**	**3.9 ± 0.7^{A}**	**5.9 ± 0.6^{A}**	**4.5 ± 0.2^{A}**
2	MW 2.1	6.73 ± 0.02^{a}	0.97 ± 0.00^{a}	64.8 ± 0.0^{a}	8.5 ± 0.2[2]^{,a}	8.2 ± 0.0[2]^{,a}	6.9 ± 0.2[2]^{,a}	2.3 ± 0.1^{a,b*}	6.5 ± 0.0[2]^{,a}	5.3 ± 0.0[2]^{,a}
	MW 2.2	6.68 ± 0.01^{b}	0.97 ± 0.00^{a,b}	65.3 ± 0.1^{b}	8.2 ± 0.0^{b}	7.6 ± 0.0^{b*}	7.4 ± 0.1^{a}	2.0 ± 0.1^{a}	6.6 ± 0.0^{a}	5.3 ± 0.1^{a}
	MW 2.3	6.75 ± 0.01^{a}	0.95 ± 0.00^{b}	70.7 ± 0.3^{b}	8.2 ± 0.0^{a,b}	8.1 ± 0.0^{a,b*}	7.5 ± 0.1^{a}	2.5 ± 0.2^{b*}	6.7 ± 0.1^{a}	5.3 ± 0.1^{a}
	Mean	**6.72 ± 0.02^{A}**	**0.96 ± 0.01^{A}**	**66.9 ± 1.9^{B*}**	**8.3 ± 0.1^{A}**	**8.0 ± 0.2^{A}**	**7.3 ± 0.2^{A}**	**2.3 ± 0.1^{B*}**	**6.6 ± 0.1^{A}**	**5.3 ± 0.0^{A}**
3	MW 3.1	6.68 ± 0.01^{a}	0.96 ± 0.00^{a}	64.3 ± 0.1^{a,b}	8.0 ± 0.0^{a}	7.4 ± 0.0^{a,b*}	7.1 ± 0.0^{a}	4.2 ± 0.1^{a}	5.3 ± 0.4^{a}	5.6 ± 0.1^{a,b}
	MW 3.2	6.61 ± 0.00^{b}	0.97 ± 0.00^{a}	64.8 ± 0.1^{a}	8.2 ± 0.1^{a,b}	7.3 ± 0.0^{a}	7.5 ± 0.1^{a,b}	3.5 ± 0.7^{a}	7.0 ± 0.1^{a,b}	4.6 ± 0.1^{a}
	MW 3.3	6.71 ± 0.01^{a}	0.96 ± 0.00^{a}	63.4 ± 0.1^{b}	9.3 ± 0.1^{b}	8.1 ± 0.1^{b*}	8.3 ± 0.0^{b}	4.1 ± 0.1^{a}	9.1 ± 0.0^{b}	7.5 ± 0.0^{b}
	Mean	**6.67 ± 0.03^{A}**	**0.96 ± 0.00^{A}**	**64.2 ± 0.4^{A,B*}**	**8.5 ± 0.4^{A}**	**7.6 ± 0.3^{A}**	**7.6 ± 0.4^{A}**	**3.9 ± 0.2^{A}**	**7.1 ± 1.1^{A}**	**5.9 ± 1.5^{A}**
4	MW 4.1	N.D.[3]	N.D.	N.D.	8.3 ± 0.1^{a}	N.D.	7.8 ± 0.1^{a}	2.2 ± 0.2^{a,b}	N.D.	N.D.
	MW 4.2	N.D.	N.D.	N.D.	8.3 ± 0.0^{a}	N.D.	7.8 ± 0.1^{a}	1.7 ± 0.1^{a}	N.D.	N.D.
	MW 4.3	6.76 ± 0.02	0.97 ± 0.00	65.9 ± 0.1	8.3 ± 0.0^{a}	8.2 ± 0.0	6.9 ± 0.1^{b*}	3.4 ± 0.1^{b}	7.6 ± 0.0	6.0 ± 0.1
	Mean	**6.76^{A}**	**0.97^{A}**	**65.9^{B}**	**8.3 ± 0.0^{A}**	**8.2^{A}**	**7.5 ± 0.3^{A}**	**2.4 ± 0.5^{B*}**	**7.6^{A}**	**6.0^{A}**

[1]Data are the mean values of three replicates ± standard error of the mean; ^{a,b}Means per sample with the same superscript (small letter) within the same columns from the same rearing company do not differ significantly ($p > 0.10$); ^{A,B}Means per rearing company with the same superscript (capital) within the same columns do not differ significantly ($p > 0.05$). *Superscripts with an asterisk indicate a weak significance ($0.05 < p < 0.10$).

[2]Mean value of two replicates ± standard error of the mean.

[3]N.D. = not determined.

Table 2.3 Intrinsic properties and microbial counts of raw crickets (*A. domesticus* and *G. sigillatus*) from different rearing companies and batches[1].

Rearing company	Sample ID	Intrinsic properties			Microbial counts (log cfu/g)					
		pH (-)	a_w (-)	Moisture content (%)	Total viable aerobic count	Lactic acid bacteria	Entero-bacteriaceae	Aerobic bacterial endospores	Psychro-trophic aerobic count	Yeasts and moulds
5	CR 1.1	6.58[2,a]	0.96[2,a]	N.D.[3]	8.3 ± 0.1[4,a,b*]	7.6 ± 0.3[4,a,b*]	8.0 ± 0.0[4,a,b*]	2.9 ± 0.0[4,a]	5.3 ± 0.3[4,a]	6.1 ± 0.0[4,a,b]
	CR 1.2	6.03 ± 0.01[b]	0.97 ± 0.00[a]	65.3 ± 0.1[a]	8.1 ± 0.1[a]	7.4 ± 0.2[a]	7.5 ± 0.2[a]	2.8 ± 0.0[a,b*]	6.4 ± 0.7[a]	5.9 ± 0.0[a]
	CR 1.3	6.61 ± 0.01[a]	0.98 ± 0.00[b]	73.3 ± 0.1[b]	8.8 ± 0.1[b*]	8.1 ± 0.0[b*]	8.3 ± 0.2[b*]	2.6 ± 0.0[b*]	4.9 ± 0.2[a]	7.2 ± 0.4[b]
	Mean	**6.41 ± 0.19[A]**	**0.97 ± 0.00[A]**	**69.3 ± 4.0[A]**	**8.4 ± 0.2[A]**	**7.7 ± 0.2[A]**	**7.9 ± 0.2[A]**	**2.8 ± 0.1[A]**	**5.5 ± 0.5[A]**	**6.3 ± 0.4[A]**
6	CR 2.1	6.44 ± 0.01[a]	0.97 ± 0.00[a]	73.0 ± 0.1[a]	8.3 ± 0.1[a]	7.8 ± 0.1[a,b*]	7.7 ± 0.1[a]	4.2 ± 0.9[a]	5.2 ± 0.5[a]	6.1 ± 0.4[a]
	CR 2.2	6.34 ± 0.01[b]	0.99 ± 0.00[b]	69.9 ± 0.1[b]	8.2 ± 0.1[a]	8.0 ± 0.1[a]	7.4 ± 0.1[a]	3.9 ± 0.1[a]	< 3.0 ± 0.0[b]	7.1 ± 0.2[a]
	CR 2.3	6.69 ± 0.01[c]	0.97 ± 0.00[a,b]	70.8 ± 0.2[b]	8.2 ± 0.0[4,a]	7.7 ± 0.0[4,b*]	7.6 ± 0.0[4,a]	4.3 ± 0.0[a]	3.7 ± 0.2[4,a,b]	6.4 ± 0.0[4,a]
	Mean	**6.49 ± 0.10[A]**	**0.98 ± 0.00[A]**	**71.3 ± 0.9[A]**	**8.2 ± 0.0[A]**	**7.8 ± 0.1[A]**	**7.6 ± 0.1[A]**	**4.1 ± 0.1[B*]**	**< 4.0 ± 0.6[A]**	**6.5 ± 0.3[A]**
7	CR 3.1	6.37 ± 0.01[a]	0.97 ± 0.00[a]	69.0 ± 0.6[a,b]	8.7 ± 0.2[a]	8.3 ± 0.0[a]	7.9 ± 0.2[a]	3.8 ± 0.1[a,b*]	4.5 ± 0.3[4,a]	6.2 ± 0.2[a,b]
	CR 3.2	6.23 ± 0.01[b]	0.96 ± 0.00[a,b]	67.5 ± 0.1[a]	8.4 ± 0.1[a]	8.2 ± 0.1[a]	7.2 ± 0.1[b*]	3.4 ± 0.3[a]	< 3.2 ± 0.1[b*]	5.6 ± 0.1[a]
	CR 3.3	6.37 ± 0.02[a]	0.92 ± 0.00[b]	70.0 ± 0.1[b]	8.6 ± 0.1[a]	7.9 ± 0.1[b*]	7.6 ± 0.1[a,b*]	4.9 ± 0.5[b*]	4.1 ± 0.5[a]	7.2 ± 0.1[b]
	Mean	**6.32 ± 0.05[A]**	**0.95 ± 0.02[A]**	**68.9 ± 0.7[A]**	**8.6 ± 0.1[A]**	**8.1 ± 0.1[A]**	**7.6 ± 0.2[A]**	**4.0 ± 0.4[B*]**	**< 3.9 ± 0.4[A]**	**6.3 ± 0.5[A]**

[1]Data are the mean values of three replicates ± standard error of the mean; [a,b,c]Means per sample with the same superscript (small letter) within the same columns from the same rearing company do not differ significantly ($p > 0.05$); [A,,B]Means per rearing company with the same superscript (capital) within the same columns do not differ significantly ($p > 0.05$). *Superscripts with an asterisk indicate a weak significance ($0.05 < p < 0.10$).

[2]Value of a single replicate.

[3]N.D. = not determined.

[4]Mean value of two replicates ± standard error of the mean.

Average counts for mealworms and crickets were generally high (Table 2.4), with total counts of at least 8 log cfu/g for all samples. For mealworms, Klunder et al. (2012) found 7.7 log cfu/g for total viable count, 6.8 log cfu/g for Enterobacteriaceae and 2.1 cfu/g for bacterial spores in a single sample analysis. Those values are lower than the averages obtained in this work (see Table 2.4: 8.4, 7.4 and 3.1 log cfu/g). That can be explained by the fact that insects were not pulverised in the study of Klunder et al. (2012), likely resulting in an incomplete extraction of countable microorganisms from the matrix. A complete recovery during analysis is of utmost importance as insects are consumed or processed into foods as a whole, i.e. without prior removal of the intestines. The same remark can be made for the data obtained from the cricket sample by Klunder et al. (2012). Sample preparation in Stoops et al. (2016), however, was done the same way as in our study and their values for total viable count, LAB, Enterobacteriaceae, and yeasts and moulds of mealworms are highly comparable to the data obtained in this work.

Table 2.4 Total mean values for intrinsic properties and microbial counts of raw crickets and mealworms[1].

		Mealworms	Crickets
Intrinsic properties	pH (-)	6.70 ± 0.02^{A}	6.40 ± 0.07^{B}
	a_W (-)	0.96 ± 0.00^{A}	0.96 ± 0.01^{A}
	Moisture content (%)	64.4 ± 0.9^{A}	69.9 ± 0.9^{B}
Microbial counts (log cfu/g)	Total viable aerobic count	8.4 ± 0.1^{A}	8.4 ± 0.1^{A}
	Lactic acid bacteria	7.7 ± 0.1^{A}	7.9 ± 0.1^{A}
	Enterobacteriaceae	7.4 ± 0.1^{A}	7.7 ± 0.1^{A}
	Aerobic bacterial endospores	3.1 ± 0.3^{A}	3.6 ± 0.3^{A}
	Psychrotrophic aerobic count	6.6 ± 0.4^{A}	4.5 ± 0.4^{B}
	Yeasts and moulds	5.3 ± 0.3^{A}	6.4 ± 0.2^{B}

[1]Data are the total mean values of all investigated batches per insect ± standard error of the mean. ^{A,,B}Means per row with the same superscript do not differ significantly ($p > 0.05$).

Psychrotrophic counts were never investigated before on edible insects, although it is an important parameter regarding chilled preservation of edible insects. Crickets contained on average 4.5 log cfu/g psychrotrophic organisms, but the average for mealworms was 6.6 log cfu/g (Table 2.4), with some batches even up to 9.1 log cfu/g (Table 2.2). That might involve a risk of spoilage in a chilled environment as well as the outgrowth of psychrotrophic pathogens such as *L. monocytogenes* (which was not detected in this study though, see

section 2.3.5) or *Bacillus cereus* (Hwang & Tamplin, 2005; Martínez, Borrajo, Franco, & Carballo, 2007).

2.3.2 Variation between batches of individual rearing companies

Because the intra-batch variation cannot completely be exposed based on the few subsamples analysed per batch, the inter-batch variation is also hard to uncover. However, it is still very interesting to compare the results from different batches in order to reveal the variation within a single rearing company. Although insects are generally reared using established protocols, variation between batches from the same company was commonplace (Figure 2.1), especially for bacterial endospores, psychrotrophic counts, and yeasts and moulds (Table 2.2 and Table 2.3). By contrast, variation in intrinsic properties was only marginal (Table 2.2 and Table 2.3), suggesting that the differences in microbiological parameters are caused by other variables such as feed supply and/or rearing practices which are not yet fully standardised. For example, mealworm batches from company 1 showed a significantly lower endospore count ($p = 0.039$) and a higher number of psychrotrophs (weakly significant, $p = 0.055$) for one particular batch (MW 1.3) compared to the other two batches.

Furthermore, company 3 produced a mealworm batch (MW 3.3) which showed significantly higher counts than the other two for almost all microbiological parameters ($p = 0.027$ for total aerobic count, Enterobacteriaceae, psychrotrophs, yeasts and moulds). That was in line with deviating visual (moister look) and olfactory (staler odour) observations for this sample. Additionally, batches MW 3.1 and MW 3.2 differed from each other, numerically but not significantly, in counts for psychrotrophs and yeasts and moulds (Table 2.2). The findings on the overall variation are in agreement with Stoops et al. (2016), who particularly found variation in the number of bacterial endospores, ranging from not detected (< 1.0 log cfu/g) to 3.5 log cfu/g. In our study, spore counts even ranged from 1.7 to 5.0 log cfu/g over all samples investigated.

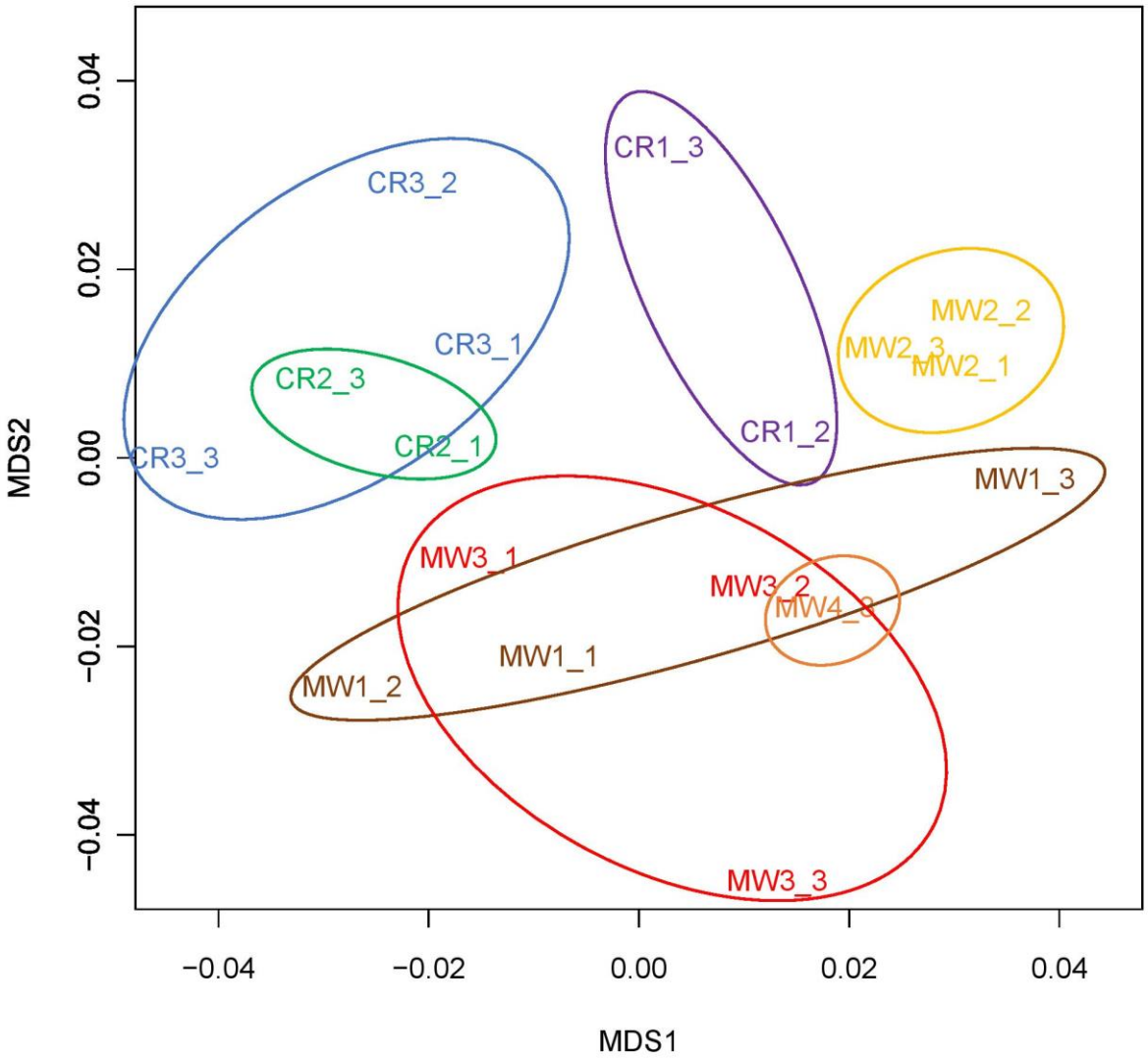


Figure 2.1 Non-metric multidimensional scaling (NMDS) of the samples analysed in this study. Batches MW 4.1, MW 4.2, CR 1.1 and CR 2.2 were excluded from this analysis because of lacking parameters or data lower than the detection limit. All intrinsic and microbiological parameters (mean values) were included in the analysis for the other batches. Batches from the same rearing company are represented by the same colour. The distance between samples on the plot reflects their similarity level: the more similar two samples are, the smaller their distance is. Sample IDs correspond with those in Table 2.1.

Similar observations were made for crickets (Table 2.3; Figure 2.1). For example, for company 5, batch CR 1.3 diverged from the other batches, mainly for the yeasts and moulds count ($p = 0.044$). The insects from batch CR 1.3 were younger (subadult) than those from the other two batches, which may have influenced their microbiota (Yun et al., 2014). Further, batch CR 2.2 from company 6 was different from the other two batches, particularly for its

low amount of psychrotrophs (around or below detection limit) ($p = 0.044$) and the high number of yeasts and moulds (not significant). Company 7 provided samples which were particularly different in the amount of psychrotrophs (weakly significant, $p = 0.077$) and yeasts and moulds ($p = 0.027$). The highest amount of yeasts and moulds was observed in batch CR 3.3. This may be related to the (slightly) lower water activity measured, because fungi better resist lower a_w values than bacteria (Madigan et al., 2009).

2.3.3 Variation between rearing companies

Large differences between different insect batches from each individual company resulted in large standard errors of the mean and hence few statistically significant differences between rearing companies. For mealworms (Table 2.2; Figure 2.1), only (weakly) significant differences between companies were found for the moisture content (ranging between 61.6 and 66.9%, $p = 0.051$) and the number of bacterial endospores (mean values between 2.3 and 3.9 log cfu/g, $p = 0.059$). Nevertheless, water activity has more impact on microbial growth in foods than moisture content and those values were highly comparable between companies ($p = 0.861$). The moisture content and the water activity of insects are a result of the relative air humidity in the rearing environment, the presence or absence of water supply and the moisture content of the feed. Mealworms are typically provided with water by offering a moist food product, such as carrots or apples. The varying bacterial endospore load may be reflected by differing endospore numbers in the feed, but this remains to be investigated. Notably, microbiological parameters for the two companies rearing mealworms for pet food (company 3 and 4; Table 2.2) did not differ significantly from those for the companies rearing for human consumption (company 1 and 2). However, psychrotrophs and yeasts and moulds show slightly higher counts for the former. As such, microbial counts could not clearly identify samples as reared for human or pet food, but different practises during rearing should still be implemented for insects for human consumption, which may have (minor) impact on the microbiology.

For crickets (Table 2.3; Figure 2.1), similar trends were observed. More specifically, our data indicate a weakly significant difference ($p = 0.061$) between different companies in bacterial endospore counts (ranging from 2.8 to 4.1 log cfu/g). Soil, used as a substrate for crickets to lay their eggs, can be a major source of contamination for bacterial endospores (Klunder et

al., 2012; Madigan et al., 2009). In contrast to the moisture content of mealworms, the moisture content of crickets was similar among the different companies. Crickets are supplied with pure water instead of a moist food product and that may result in lower variations in moisture content. The cricket moisture content obtained in our study is comparable with findings in literature (Finke, 2002). Altogether, no significant differences in mean intrinsic properties or microbial counts were observed between the two cricket species *Acheta domesticus* (company 5 and 6) and *Gryllodes sigillatus* (company 7).

Although the exact rearing history of the insect samples studied is unknown, some explanations for the observed differences can be formulated. Influencing factors are likely to be situated in the feed, the rearing environment and an (optional) starvation procedure (Jones, Sanchez, & Fierer, 2013; Yun et al., 2014). It is also plausible that rearing practices and hygiene measures may result in a company-specific "house flora", which can also affect the insect microbiota (Li, Xie, Dong, Wang, & Liu, 2016).

2.3.4 Variation between different insect types

When comparing data from mealworms and crickets (Table 2.4; Figure 2.1), pH, moisture contents, psychrotrophic counts and the amounts of yeasts and moulds were significantly different. The difference in pH was statistically significant ($p = 0.000$) but numerically rather small. In line with Finke (2002), crickets contained significantly ($p = 0.001$) more moisture (total mean value of 69.9%) than mealworms (64.4%). The significant difference ($p = 0.001$) found for psychrotrophic counts might be due to a difference in starvation temperature. Furthermore, crickets show significantly higher results for the overall fungal counts than mealworms ($p = 0.004$). Interestingly, mealworms were dominated by moulds, whereas for crickets yeasts dominated the fungal biota. Correspondingly, mealworms are generally fed with cereal products, while crickets are reared on very diverse substrates such as chicken or pig feed, organic vegetables and brewer's spent grain.

Altogether, our results show that crickets and mealworms should be considered as different food matrices in terms of intrinsic properties and microbiological quality. Therefore, when reporting on the microbiological quality of edible insects, it is important to distinguish between distinct insect types and not to report average values over several types or to pool

samples of different types, as was done in the past (e.g. Giaccone, 2005; Grabowski et al., 2014).

2.3.5 Compliance with microbiological criteria for foodstuffs

Many authors mention the possibility for insects to contain pathogenic microorganisms such as *Bacillus cereus*, *Campylobacter* spp., *Clostridium* spp., *Salmonella* spp. and *Staphylococcus aureus* (Crippen et al., 2012; EFSA Scientific Committee, 2015; Giaccone, 2005; Mpuchane et al., 2006; Rumpold & Schlüter, 2013b; Stoops et al., 2016; Templeton, De Jong, Blackall, & Miflin, 2006; Zheng et al., 2012). Furthermore, edible insects harbour a large amount of Enterobacteriaceae, a bacterial family containing foodborne pathogens. The Belgian Superior Health Council and the Federal Agency for the Safety of the Food Chain (SHC & FASFC, 2014) and the Dutch Food and Consumer Product Safety Authority (NVWA, 2014) both composed an opinion on food safety aspects of edible insects. The microbiological criteria referred to in those opinions are based on culture-dependent microbial counting, as is also the case for other food products described in Regulation (EC) N° 2073/2005 on microbiological criteria for foodstuffs. Both opinions refer to food safety criteria for *Salmonella*, *Listeria monocytogenes* and *Escherichia coli* for ready-to-eat meals, minced meat and live bivalve molluscs. The opinions suggest to use those criteria for edible insects, since no specific criteria for insects exist yet on European level. That led to the screening of each batch on *Salmonella* spp. and *L. monocytogenes* in this work. Both pathogens were not detected (in 25 g) in any of the samples, which corresponds to the findings from Giaccone (2005) and Grabowski et al. (2014). Nevertheless, the recently updated circular from the Belgian FASFC states that products available on the marked should still be periodically tested on the presence of pathogens *Salmonella* and *Listeria monocytogenes* (FASFC, 2016). Also the FASFC action limits recommend the assessment of *Salmonella* spp. in raw edible insects (Table S1.1, Supporting information). The samples investigated comply with those requirements.

In addition to food safety criteria, the opinions proposed process hygiene criteria based on comparable food matrices, e.g. minced meat. All total viable counts obtained in this study (8.2 – 9.3 log cfu/g) strongly exceed the value for the low criterion (m = 5.7 log cfu/g) for minced meat and even that for the high criterion (M = 6.7 log cfu/g). In the Belgian opinion, it is noted that this criterion is expected to be difficult to attain for raw insects, as demonstrated here,

but that it should be possible to be reached after a heat treatment in a controlled and hygienic process. The relevance of criteria for other foodstuffs for edible insects can be questioned, however, and the development of criteria specific for insects is desirable. A first attempt was made in the FASFC action limits, where specific process hygiene recommendations are presented for insects and insect-based food products. Four recommendations are applicable for the microbiological parameters determined in this experiment. The recommendations for total viable aerobic count (m = 6 log cfu/g, M = 7 log cfu/g) and Enterobacteriaceae (m = 3.7 log cfu/g, M = 5 log cfu/g) were exceeded for all samples, while the recommendations for yeasts (m = 3.7 log cfu/g, M = 5 log cfu/g) and moulds (m = 3.7 log cfu/g, M = 5 log cfu/g) could not be assessed, since their counts were not individually determined. However, given the fact that some samples were already below the lower (m) limit for the combined yeasts and moulds count, it is clear that the recommendations could be met for at least a few samples. Altogether, most samples did not comply with the process hygiene recommendations for edible insects, which urges for a proper decontamination prior to consumption, which is required in the form of a heat treatment by the FASFC circular (FASFC, 2016).

2.4 Conclusions

Our study provides a general view on the intrinsic properties and microbial counts of three edible insect species. Large variations in microbial counts between batches originating from a single rearer resulted in the variations between companies being smaller. Especially for aerobic bacterial endospores, aerobic psychrotrophic organisms, and yeasts and moulds, counts varied remarkably. Intrinsic properties of the insects, on the other hand, varied to a smaller extent and cannot explain the differences in microbiological parameters completely. While culture-dependent analyses are very useful to quantify microbial loads and to compare counts with other food matrices or with microbiological criteria, further research using culture-independent methods is needed to reveal the composition of the edible insect microbiota and variations in the composition between batches and companies. In addition, further research is needed to explore the relation between rearing conditions, including hygiene, and the insect microbiota.

Chapter 3 Culture-independent bacterial composition analysis of raw edible insects

This chapter was redrafted[1] after Vandeweyer D., Crauwels S., Lievens B., & Van Campenhout L. (2017). Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. *International Journal of Food Microbiology*, *261*, 11-18. http://doi.org/10.1016/j.ijfoodmicro.2017.08.018

3.1 Introduction

Although consumer acceptance of edible insects and insect-derived foods is still limited (Caparros Megido et al., 2014; House, 2016; Lensvelt & Steenbekkers, 2014; Verbeke, 2015; Yen, 2009), insect-based products are increasingly being investigated (Tan, Verbaan, & Stieger, 2017) as well as developed (Cadesky, 2017; Stoops et al., 2017) and insects are getting progressively more attention as food source in Western countries (Mlček et al., 2014). While insect products are entering the market – despite the novel food status of insects and their derived products as from 2018 (Regulation (EC) 2015/2283) – the microbiological quality of the insects is still not fully revealed. Some studies, including Chapter 2, have already assessed the microbiological quality of raw edible insects (Klunder et al., 2012; Stoops et al., 2016) and/or insect-derived products (Caparros Megido et al., 2017; Garofalo et al., 2017; Grabowski & Klein, 2016; Stoops et al., 2017). However, except for Chapter 2, these studies did not compare different production batches and rearing companies. Furthermore, most studies only used culture-dependent methods for microbiological analysis, leading to an observed microbial diversity which may be incomplete and/or biased (Justé, Thomma, & Lievens, 2008). Garofalo et al. (2017) and Stoops et al. (2016) recently investigated the bacterial composition of respectively processed and raw edible insects using culture-independent 454 pyrosequencing of partial 16S ribosomal RNA (rRNA) genes. These studies

[1] The complete content of this paper (Vandeweyer, Crauwels, Lievens, & Van Campenhout, 2017a) was included in Chapter 3, with only small alterations to keep the information provided up to date and to follow the logic course of this dissertation. As first author, D.V. contributed to all parts described in this work, from experimental design to the writing of the paper. The metagenetic analysis was performed in collaboration with the Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), KU Leuven (Prof. B. Lievens).

revealed that some potential food pathogen and spoilage genera can be present, which could not be proved on this taxonomic level by general culture-dependent counts alone. Both the edible insect sector and the legislative authorities (ANSES, 2015; EFSA Scientific Committee, 2015; SHC & FASFC, 2014) are highly interested in additional microbiological (and other) data from different sources. The data are also useful for insect rearing and processing companies to gain further insight into insects as a food matrix and to complete the novel food dossiers they are currently preparing.

The purpose of this study was to assess and compare the bacterial compositions of freshly reared, raw mealworms (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different production batches, produced at industrial rearing companies in Belgium and The Netherlands. In both countries, crickets and mealworms are produced intensively for human consumption, but raw crickets have never been investigated with next-generation sequencing techniques before and raw mealworms only once on a small scale in a preliminary study (Stoops et al., 2016). In addition to the intrinsic properties and the traditional culture-dependent microbial counts previously determined and described in Chapter 2, this study reports on the metagenetic data obtained for the samples collected in the aforementioned study.

3.2 Materials and methods

3.2.1 Sample preparation and DNA extraction

Concurrent with the culture-dependent analyses performed in Chapter 2, DNA extractions were executed on all samples collected in that study, except on samples MW 4.1, MW 4.2, MW 4.3, and CR 1.1. (Table 2.1). A 30 g subsample of living insects from each production batch was pulverised as described earlier (Stoops et al., 2016) and used to execute two extractions using 2 g starting material (manufacturer's protocol, Power Soil DNA Elution Accessory Kit, Mo Bio laboratories, Carlsbad, CA, USA). DNA samples were stored at -80 °C until further use.

3.2.2 Metagenetic analysis

To perform the metagenetic analysis, a tenfold dilution of each DNA extract was amplified in twofold by PCR targeting the V4 region of the 16S rRNA gene using sample-specific barcode-labelled versions of primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') (Caporaso et al., 2011; dual-index sequencing strategy, Kozich, Westcott, Baxter, Highlander, & Schloss, 2013; Table S3.1, Supporting information). Each 20 µl PCR reaction contained 1x Titanium Taq PCR buffer, 150 µM of each dNTP, 0.5 µM of each primer, 1x Titanium Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France) and 1 µl 10-times diluted DNA. The reaction was initiated by denaturation at 95 °C for 120 s, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 45 s. Replicate amplification products were combined and loaded on an agarose gel. Next, visible bands of the expected size were excised and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). After quantification of all purified DNA amplicons (Qubit High Sensitivity Fluorometer kit, Invitrogen, Carlsbad, CA, USA), DNA samples were equimolarily combined into a library and purified once again (Agencourt AMPure XP kit, Beckman Coulter Life Sciences, Indianapolis, IN, USA). The library was diluted to 2 nM and sequenced at the Centre of Medical Genetics Antwerp (University of Antwerp, Antwerp, Belgium), using an Illumina MiSeq sequencer with v2 500 cycle reagent kit (Illumina, San Diego, CA, USA).

Resulting sequences were received as a de-multiplexed FASTQ file (data deposited in a Sequence Read Archive; BioProject accession PRJNA390238; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA390238). Paired-end reads were merged using USEARCH (v.8.1) to form consensus sequences (Edgar, 2013) with no more than 10 mismatches allowed in the overlap region. Subsequently, sequences were truncated at the 250th base. Shorter reads or reads with a total expected error threshold above 1.0 for all the bases were discarded. Due to uneven sequencing depth, the number of sequences was rarefied to 54,000 sequences per sample. Remaining sequences with a minimum abundance of two were grouped into operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the UPARSE greedy algorithm in USEARCH, during which chimeric sequences were also removed (Edgar, 2013). Global singletons (i.e. OTUs represented by only

a single sequence in the entire dataset) were not taken into account to minimise the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud, Busschaert, Ruyters, Jacquemyn, & Lievens, 2014). Subsequently, OTUs were assigned taxonomic identities using the "classify.seqs" command in Mothur (v. 1.36.1) (Schloss et al., 2009) against the Silva taxonomy database v1.23 (Quast et al., 2013). With Mothur's "remove.lineage" command, OTUs originating from chloroplasts or mitochondria were deleted.

The taxonomic origin (genus level) of each OTU was determined with the SINTAX algorithm implemented in USEARCH, (Edgar, 2016) based on the Silva Living Tree Project v123 (LTP v123) database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.80. Additionally, OTU representative sequences (selected by UPARSE) were subjected to a BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) search against GenBank (Benson et al., 2013), excluding uncultured/environmental entries. Principally, assignments were based on SINTAX results, but BLAST results were used when SINTAX assignment was inconclusive or produced assignment scores below 0.80. Finally, nonmetric multidimensional scaling (NMDS) and Chao1 (Chao, 1984) and Shannon-Wiener (Shannon, 1948) diversity indices calculations were performed on the microbial composition of the samples using R-packages (R Development Core Team, 2013) Vegan (v.2.41) and Phyloseq (v. 1.19.0).

3.2.3 Statistical analyses

Statistical analyses were conducted using SPSS Statistics (version 23, IBM, New York, NY, USA). Chao1 and Shannon-Wiener diversity indices, as well as observed richness and coverage were compared between production batches per company, between rearing companies per insect species and between insect species. To this end, independent samples t-tests were used when comparing two conditions, while one-way ANOVA was used in all other cases. When necessary, pairwise comparison was performed using Tukey's post hoc test. All tests considered a 0.05 significance level.

3.3 Results and discussion

3.3.1 Comparison of the mealworm bacterial composition between rearing companies and production batches

Differences in bacterial composition between samples from different production batches as well as between rearing companies are visualised through NMDS in Figure 3.1. Both DNA extracts per sample are displayed with the same icon. A near position of these icons illustrates that the bacterial communities in both extracts were highly similar. Furthermore, it is clear from these results that the bacterial composition of mealworms is mainly driven by the rearing company rather than by the production batch (Figure 3.1A).

The mean bacterial composition with OTUs in a relative abundance of at least 2% (for an overview of all bacterial OTUs, see Table S3.2 and Table S3.3, Supporting information) for the different mealworm companies is exposed in Figure 3.2. These results suggest that mealworms from different rearing locations have at least a part of their bacterial composition in common. However, important differences can be designated as well. In all mealworm samples, a bacterium related to a *Spiroplasma* species (OTU 1; Table S3.3, Supporting information) and a bacterium assigned as an *Erwinia* species (OTU 2; Table S3.2, Supporting information) were abundantly present. For rearing company 1, together they comprised over 50% of all sequences recovered, while for companies 2 and 3, both OTUs only accounted for approximately 35% of all sequences recovered, with especially OTU 1 being less abundant. Furthermore, samples from rearing company 1 also harboured substantial quantities of a *Citrobacter* species (OTU 904; Table S3.2, Supporting information) and a *Brevibacillus* species (OTU 10; Table S3.3, Supporting information), while these OTUs were scarcely present in the other rearing companies. The high abundance of the *Brevibacillus* OTU, a spore-forming bacterium mainly present in sample MW 1.2, can most probably explain the high aerobic endospore count (5.0 log cfu/g) for this sample reported in Chapter 2. Company 2, on the other hand, clearly produced larvae with a high abundance of both an Enterobacteriaceae (OTU 14; Table S3.2, Supporting information) and a *Lactococcus* (OTU 5; Table S3.3, Supporting information) species. Production batches from company 3 contained higher amounts of two other Enterobacteriaceae OTUs (OTU 979 and 46; Table S3.2, Supporting

information) and a *Pseudomonas* species (OTU 4; Table S3.2, Supporting information). The diversity indices (Table 3.1) based on these bacterial compositions also show highly significant differences between the mealworm rearing companies. Because of the high abundance of two predominant OTUs, the mean observed richness of company 1 was rather low, but covered the estimated richness (Chao1 index) for 75%. Also the Shannon-Wiener index, which denotes the diversity based on both richness and abundance, was lower for company 1 than for the other mealworm rearing companies. It was only significantly different ($p = 0.003$) from rearing company 2, which shows the highest amount of OTUs (richness) and the highest general diversity.

On Figure 3.2, the error bars representing the standard deviation are an indication of the differences between production batches from the same company. In accordance with the culture-dependent approach in Chapter 2, standard deviations for the most abundant OTUs from company 2 were the smallest (all below 11%), indicating that the different production batches for this company deliver insects with a comparable bacterial composition. Unlike company 2, standard deviations from company 1 (up to 15%) and especially from company 3 (up to 20%) were large. Consequently, striking differences could be found between different production batches (see also Figure S3.1, Supporting information). For company 1, batch 2 was the most divergent, because it was the only batch that contained a *Brevibacillus* OTU (OTU 10), even in an abundance of approximately 28%. For company 3, all batches can be considered as very different from each other. The bacterial composition of batch 1 consisted for over 80% of the *Spiroplasma* (OTU 1) and *Erwinia* OTUs (OTU 2), while batch 2 and 3 contained only around 30% and 5% of these OTUs, respectively. Sample MW 3.2 (batch 2) contained an Enterobacteriaceae OTU (OTU 979; 28%) instead, and sample MW 3.3 (batch 3) harboured large quantities of two *Pseudomonas species* (OTU 4; 39% and OTU 23; 11%; Table S3.2, Supporting information) and an Enterobacteriaceae species (OTU 46; 21%). When comparing these findings with the diversity indices in Table 3.1, the only sample showing a significant difference was MW 3.2, which had a larger diversity (Shannon-Wiener index, $p = 0{,}010$) compared to the other production batches from company 3. Obviously, this implies that samples with a similar diversity level can still differ in terms of microorganisms present.

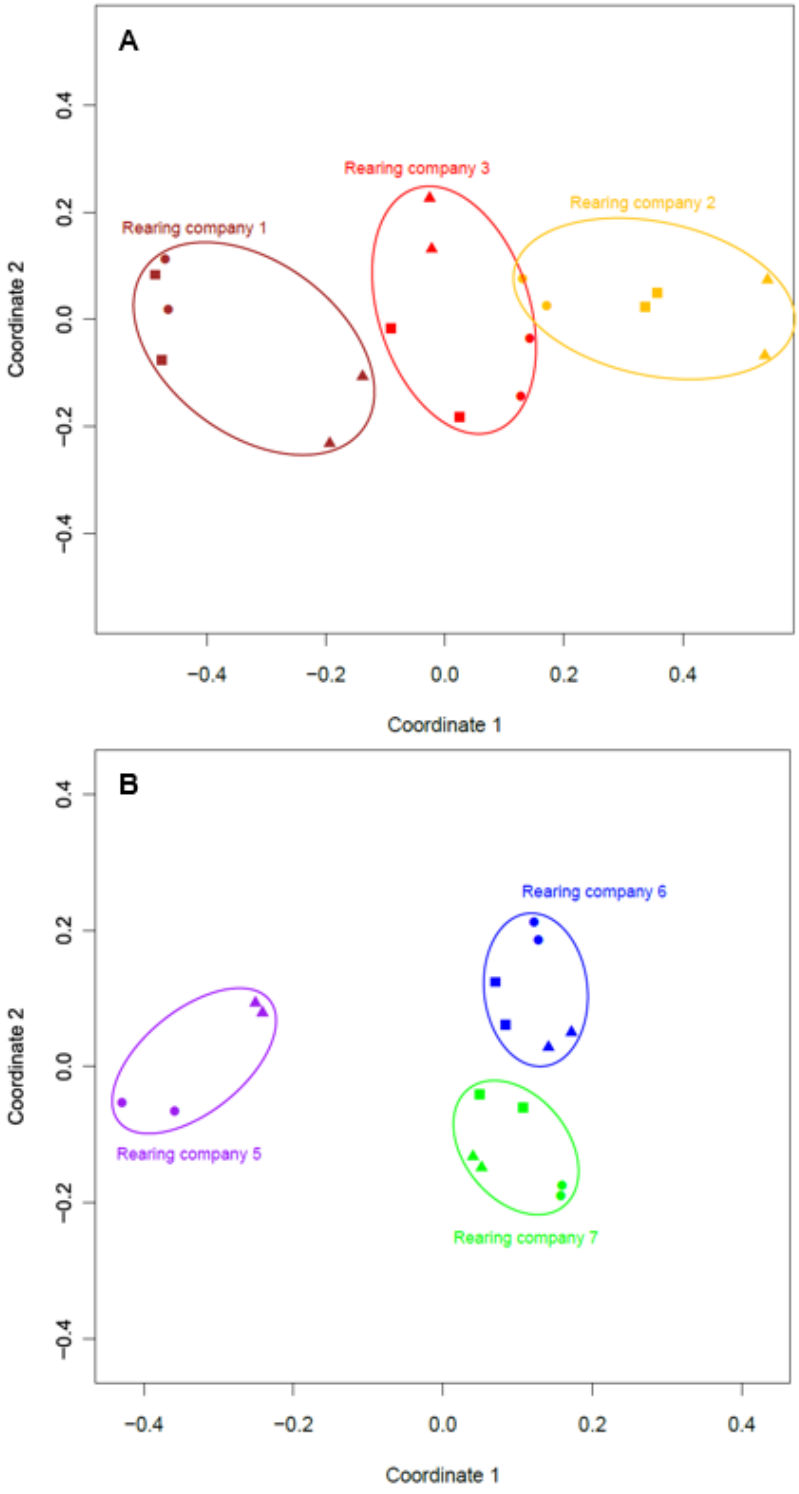


Figure 3.1 Non-metric multidimensional scaling (NMDS) ordinations composed of the bacterial composition data for both mealworms (Fig. 3.1A; stress value of 0.017) and crickets (Fig. 3.1B; stress value of 0.041). Samples from the same rearing company are represented by the same colour, while extracts from the same production batch (see Table 2.1) are represented by ■ (batch 1), ● (batch 2) or ▲ (batch 3). The distance between different points on the plot reflects their similarity level: the more similar the bacterial communities, the smaller the distance between the points. Plots were constructed based on the bacterial data for the most abundant OTUs, altogether representing 50% of the sequence data obtained.

Table 3.1 Diversity indices for the mealworm and cricket samples investigated in this study[1].

	Rearing company	Sample ID[2]	Observed richness	Chao1[3]	Coverage (%)[4]	Shannon – Wiener[5]
Mealworms	1	MW 1.1	56 ± 14[a]	75.85 ± 16.29[a]	73.52 ± 2.85[a]	1.82 ± 0.03[a]
		MW 1.2	68 ± 4[a]	86.67 ± 10.97[a]	78.25 ± 5.83[a]	1.91 ± 0.02[a]
		MW 1.3	74 ± 1[a]	97.80 ± 3.96[a]	75.76 ± 4.51[a]	1.84 ± 0.01[a]
		Mean	**66 ± 9[A]**	**86.77 ± 10.97[A]**	**75.84 ± 2.37[A]**	**1.86 ± 0.04[A]**
	2	MW 2.1	110 ± 19[a]	121.81 ± 22.36[a]	89.97 ± 0.84[a]	2.16 ± 0.09[a]
		MW 2.2	151 ± 3[a]	158.13 ± 5.76[a]	95.52 ± 1.69[a]	2.33 ± 0.07[a]
		MW 2.3	117 ± 4[a]	127.72 ± 8.09[a]	91.31 ± 3.01[a]	2.56 ± 0.16[a]
		Mean	**126 ± 22[B]**	**135.89 ± 19.49[B]**	**92.27 ± 2.90[B]**	**2.35 ± 0.20[B]**
	3	MW 3.1	88 ± 4[a]	97.67 ± 3.30[a]	90.23 ± 7.39[a]	1.76 ± 0.00[a]
		MW 3.2	110 ± 31[a]	120.95 ± 35.29[a]	91.07 ± 0.85[a]	2.38 ± 0.15[b]
		MW 3.3	83 ± 1[a]	95.13 ± 0.01[a]	87.25 ± 1.47[a]	1.87 ± 0.00[a]
		Mean	**94 ± 14[C]**	**104.59 ± 14.23[A]**	**89.51 ± 2.01[B]**	**2.00 ± 0.33[A]**
	Mean MW[6]		**95 ± 30**	**109.08 ± 24.86**	**85.87 ± 8.80**	**2.07 ± 0.26**
Crickets	5	CR 1.2	248 ± 4[a]	265.77 ± 0.48[a]	93.31 ± 1.76[a]	4.06 ± 0.02[a]
		CR 1.3	288 ± 18[a]	299.47 ± 12.92[a]	96.13 ± 1.99[a]	4.07 ± 0.01[a]
		Mean	**268 ± 28[A]**	**282.62 ± 23.82[A]**	**94.72 ± 1.99[A]**	**4.06 ± 0.01[A]**
	6	CR 2.1	352 ± 25[a]	362.75 ± 24.40[a]	97.02 ± 0.49[a]	4.48 ± 0.01[a]
		CR 2.2	320 ± 11[a]	328.32 ± 18.76[a]	97.53 ± 2.13[a]	4.43 ± 0.04[a]
		CR 2.3	363 ± 25[a]	378.75 ± 27.05[a]	95.72 ± 0.30[a]	4.43 ± 0.07[a]
		Mean	**345 ± 22[B]**	**356.61 ± 25.77[B]**	**96.76 ± 0.93[A]**	**4.45 ± 0.03[B]**
	7	CR 3.1	334 ± 16[a]	341.60 ± 20.94[a]	97.82 ± 1.44[a]	4.22 ± 0.01[a]
		CR 3.2	314 ± 8[a]	326.37 ± 10.97[a]	96.22 ± 0.63[a]	4.07 ± 0.05[a]
		CR 3.3	330 ± 1[a]	349.21 ± 15.67[a]	94.58 ± 3.84[a]	4.13 ± 0.10[a]
		Mean	**326 ± 11[B]**	**339.06 ± 11.63[B]**	**96.21 ± 1.62[A]**	**4.14 ± 0.07[A]**
	Mean CR[6]		**313 ± 40**	**326.10 ± 38.66**	**95.89 ± 1.05**	**4.22 ± 0.20**

[1]Data are the mean values of two analysed DNA-extracts from the same sample ± standard deviations; [a,b]Means per production batch and per rearing company with the same superscript (small letter) within the same columns do not differ significantly ($p > 0.05$); [A,B]Means per rearing company per insect (bold) with the same superscript (capital) within the same column do not differ significantly ($p > 0.05$).

[2]Sample IDs correspond with the sample details shown in Table 2.1. MW = mealworm, CR = cricket.

[3]Chao1 richness estimator: the total number of OTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1984).

[4]Coverage = (Observed richness / Chao1 estimate) * 100.

[5]Shannon-Wiener diversity index: index to characterise species diversity based on species richness as well as their relative abundance. A higher value represents more diversity (Shannon, 1948).

[6]All mean indices for mealworms are strongly significantly different from those calculated for crickets ($p = 0.000$).

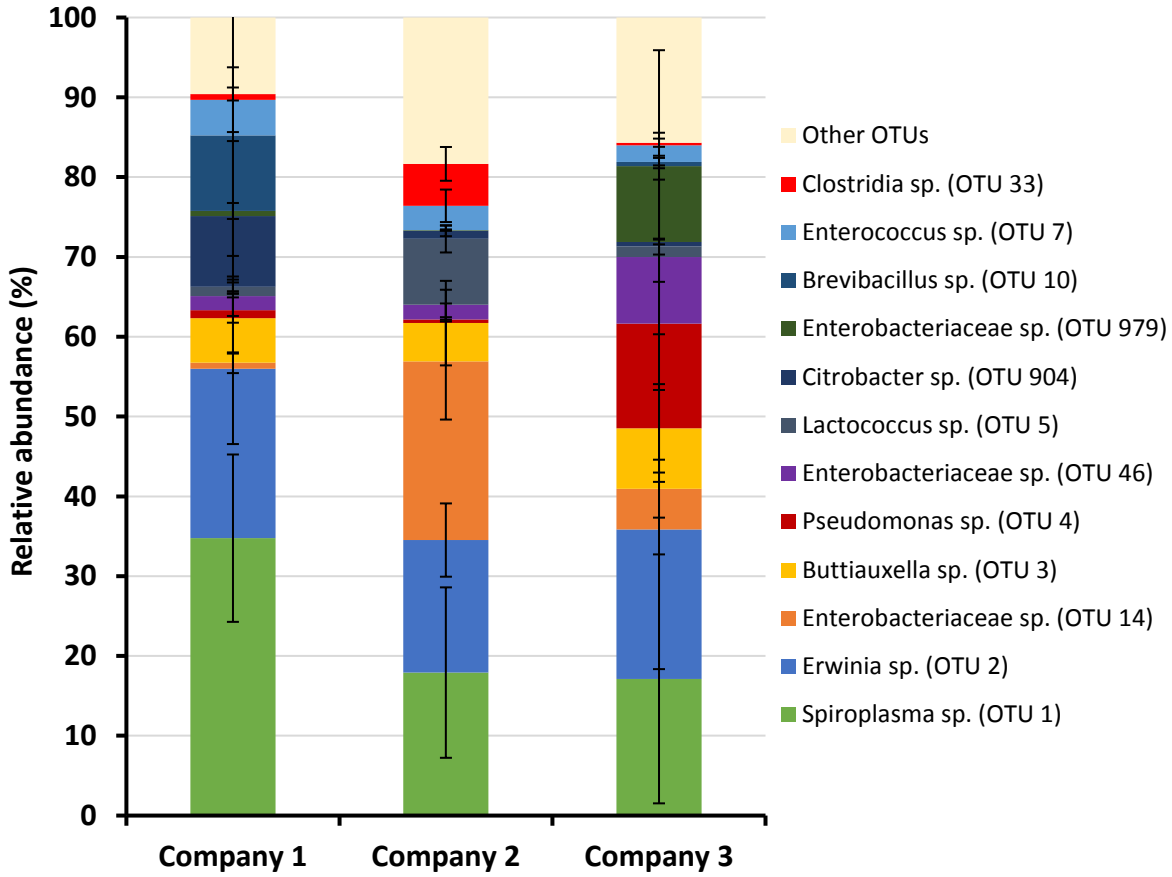


Figure 3.2 Bacterial composition at OTU level for mealworms from three rearing companies. Only the most abundant OTUs (i.e. with > 2% sequence abundance) are indicated. All other OTUs were grouped together in "Other OTUs". Data represent mean values of two extracts per sample from three samples per rearing company. Error bars represent standard deviations per OTU.

3.3.2 Comparison of the cricket bacterial composition between rearing companies and production batches

Concerning crickets, Figure 3.1B visually shows the distinction between the studied samples of each rearing company and Figure 3.3 shows the mean bacterial composition per cricket rearing company. Many dominant (abundance > 2%) OTUs were found in all three rearing companies and the bacterial communities seem to be less different from each other than for mealworm companies. Correspondingly, the diversity indices (Table 3.1) demonstrate the smaller variety between cricket rearing companies in comparison with the mealworm rearing companies. Based on the diversity indices, samples from cricket company 5 harboured a significantly (p = 0,000) lower amount of OTUs and show to be the least diverse. The most

abundant OTUs for company 5 corresponded to a *Buttiauxella* species (OTU 3; 8%; Table S3.2, Supporting information), a bacterium assigned to the genus *Parabacteroides* (OTU 8; 8%; Table S3.3, Supporting information) and a species from the family of Pseudomonadaceae (OTU 6; 8%; Table S3.2, Supporting information). Company 6 and 7 were dominated by another *Parabacteroides* species (OTU 9; 9% and 14%, respectively; Table S3.3, Supporting information) and species assigned to the genera *Photorhabdus* (OTU 12; 5%; Table S3.3, Supporting information) and *Bacteroides* (OTU 11; 5%; Table S3.3, Supporting information), respectively. In this respect, the cricket bacterial communities of company 6 and 7 were the most similar (Figure 3.1B), and this confirms the culture-dependent findings in Chapter 2.

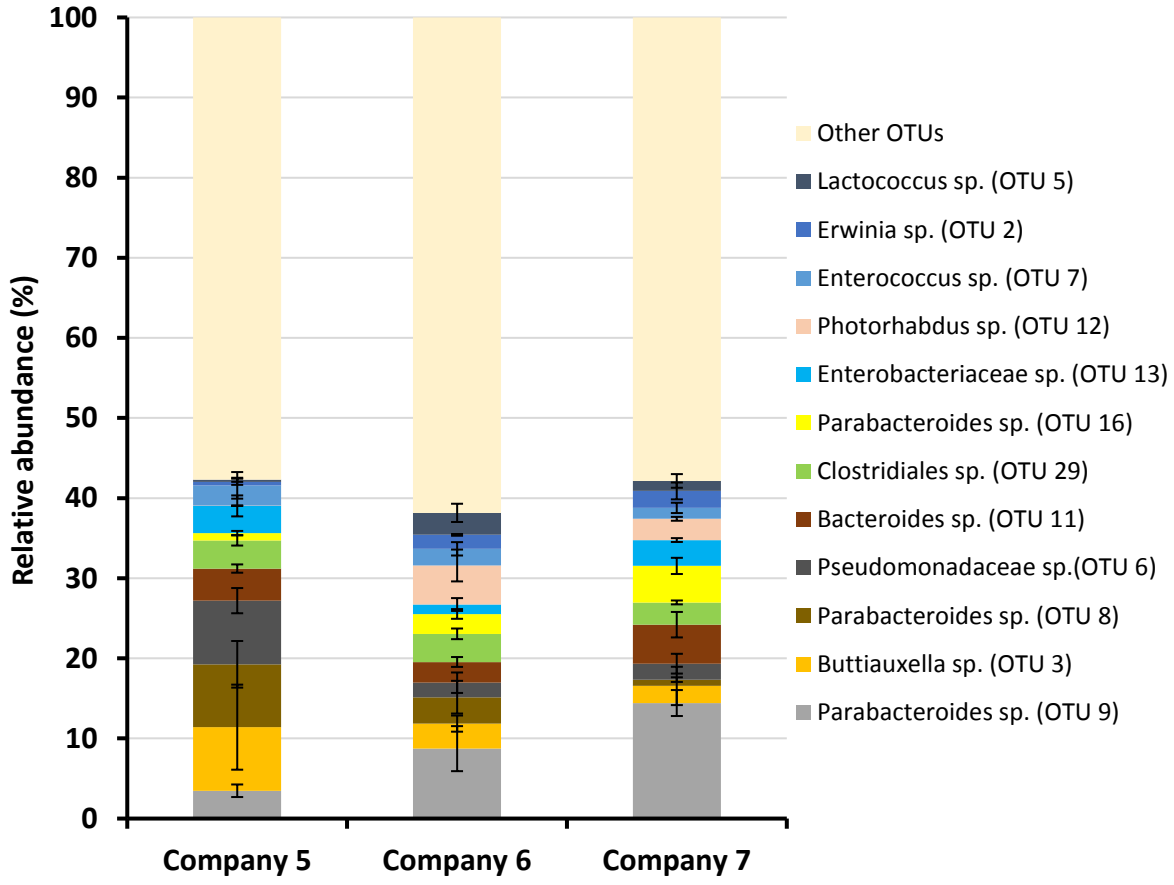


Figure 3.3 Bacterial composition at OTU level for crickets from three rearing companies. Only the most abundant OTUs (i.e. with > 2% sequence abundance) are indicated. All other OTUs were grouped together in "Other OTUs". Data represent mean values of two extracts per sample from two (company 5) or three samples (companies 6 and 7) per rearing company. Error bars represent standard deviations per OTU.

Again, the error bars shown in Figure 3.3 represent the variety between different production batches within the same company. While company 5 shows the highest standard deviations on the mean OTU abundance (up to 6%, while below 3% for company 6 and 7) and therefore produced the most variable cricket samples (see also Figure S3.2, Supporting information), the variability between samples from all cricket companies was small and not statistically significant. This small variation between samples can also be seen on Figure 3.1B. In contrast to the mealworms, reared crickets appear to contain a more uniform bacterial composition over different batches and can be delivered with a more constant bacterial quality.

3.3.3 Comparison of the bacterial composition between mealworm and cricket species

Although they belong to different genera, the house cricket (*A. domesticus,* rearing companies 5 and 6) and the tropical house cricket (*G. sigillatus,* rearing company 7) possess a highly similar mean bacterial composition, as shown in Figure 3.3 and Figure 3.4. Likewise, the calculated diversity indices (Table 3.1) deliver no significant differences between both species. These results are in agreement with the culture-dependent microbial counts presented in Chapter 2, and can most probably be explained by similar intrinsic parameters such as pH and water activity (a_w). It is reasonable to believe that a comparable diet and rearing process for both cricket species contributes to the development of a similar microbial composition (Yun et al., 2014).

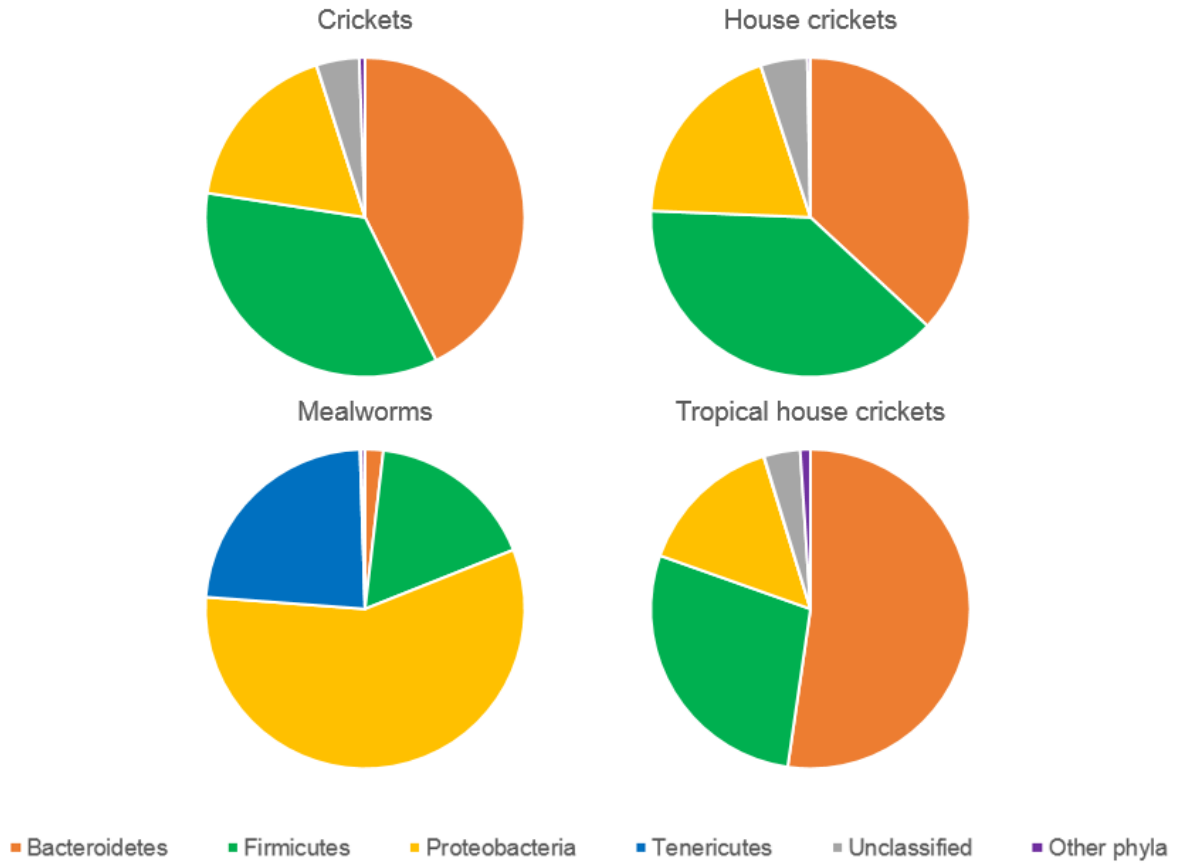


Figure 3.4 Distribution of the relative abundance (%) of the major bacterial phyla in the investigated insect species. Data represent mean values of two extracts per sample from three (tropical house crickets), five (house crickets), eight (crickets, i.e. tropical house crickets and house crickets combined) or nine samples (mealworms) investigated. Only the most abundant phyla (i.e. with > 2% sequence abundance) are indicated. Sequences assigned to less abundantly present phyla were grouped together in "Other phyla". Sequences without reliable assignment on phylum level were grouped in "Unclassified".

To allow further comparison between the bacterial compositions of mealworms and crickets as two different insect types, the bacterial compositions of both house crickets and tropical house crickets were combined (Figure 3.4 and Figure 3.5). Regarding phylum level taxonomy (Figure 3.4), mealworms showed a larger relative abundance of Proteobacteria (57%) and Tenericutes (23%), while the cricket bacterial composition was dominated by Bacteroidetes (43%) and Firmicutes (35%). When comparing these results to those obtained by Stoops et al. (2016) and Garofalo et al. (2017), mealworms were dominated by Proteobacteria in both studies, but the second most abundant phylum Tenericutes was not reported in Stoops et al. (2016). Since this latter study was executed using the same sequencing approach as Garofalo

et al. (2017), the reason for the absence of Tenericutes remains unclear. For crickets, Garofalo et al. (2017) reported a similar distribution of phylum abundance.

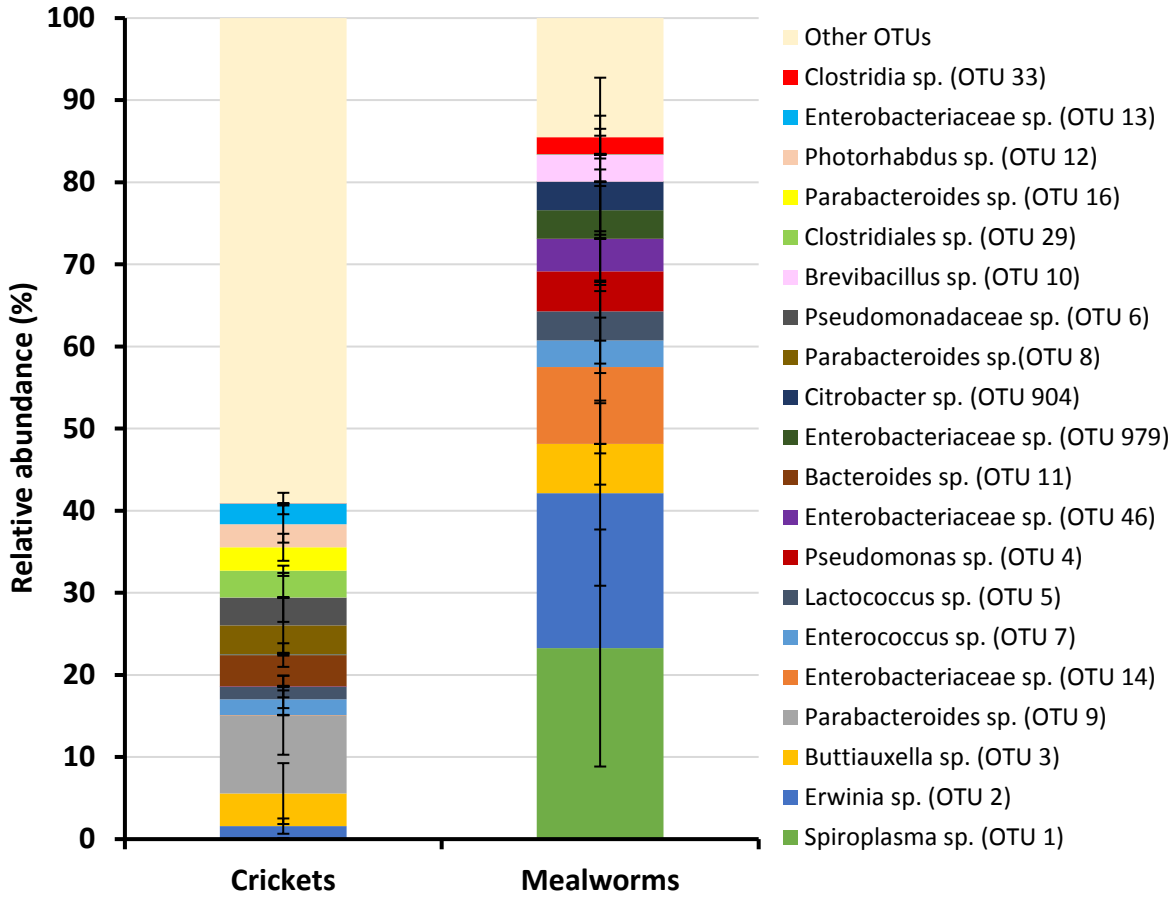


Figure 3.5 Bacterial composition at OTU level for mealworms and crickets. Only the most abundant OTUs (i.e. with > 2% sequence abundance) are indicated. All other OTUs were grouped together in "Other OTUs". Data represent mean values of two extracts per sample from eight (crickets) or nine (mealworms) samples per insect type. Error bars represent standard deviations per OTU.

Focussing on a lower taxonomic level, it can be seen that the mean cricket bacterial composition was more diverse than the mean mealworm bacterial composition (Figure 3.5). In particular, for crickets all OTUs had a relative abundance below 10%, and on average 60% of the mean cricket bacterial composition consisted of OTUs with a relative abundance below 2% (Figure 3.3 and Figure 3.5). In contrast, for mealworms around 85% of the recovered sequences could be attributed to the 12 most dominant OTUs, with even 2 to 4 OTUs comprising half of the bacterial composition (Figure 3.2 and Figure 3.5). From all reported OTUs from both insects, many belong to the family of Enterobacteriaceae or the order of

lactic acid bacteria (Lactobacillales). Microbial counts performed on the same samples (Chapter 2) already predicted a high abundance of these bacterial groups for both mealworms and crickets.

However, our sequencing approach revealed that only a few OTUs were found in both insects (when considering an abundance threshold of 0.1%), i.e. OTUs corresponding to species from the genera *Erwinia* (OTU 2), *Buttiauxella* (OTU 3), *Lactococcus* (OTU 5) and *Enterococcus* (OUT 7; Table S3.3, Supporting information). Altogether, these results indicate strong differences in the bacterial composition between mealworms and crickets, which is in line with the differences in microbial counts, pH, moisture content and water activity (a_w) observed in Chapter 2. The differences in bacterial composition can also be derived from the calculated diversity indices (Table 3.1). Both the observed (i.e. amount of OTUs) and predicted (Chao1 index) OTU richness were remarkably and statistically ($p = 0.000$) higher for crickets. Likewise, the Shannon-Wiener index was statistically ($p = 0.000$) higher for crickets compared to mealworms.

In the quickly evolving field of microbial ecology, sequencing techniques are constantly being assessed and reviewed. It has been reported that the relative abundances obtained through this sequencing approach may not entirely mirror the actual species abundance (Aird et al., 2011). However, the sequencing results obtained here still provide valuable information for the insect sector and can be improved in future research. In summary, our results clearly show a large difference between the bacterial compositions of both insect types, with the cricket bacterial compositions being much more diverse. Not only their taxonomic distinction, with *Tenebrio* belonging to the Coleoptera order and both cricket species being Orthoptera, but also their life cycle and feed source are likely to be important determinants of the insect microbiome (Colman, Toolson, & Takacs-Vesbach, 2012; Jones et al., 2013; Yun et al., 2014).

3.3.4 Biological relevance of the bacteria encountered

The most dominant OTUs found on mealworms were related to a *Spiroplasma* species (Tenericutes, OTU 1) and an *Erwinia* species (Proteobacteria, OTU 2). *Spiroplasma* was already found in raw mealworms previously (Garofalo et al., 2017; Jung et al., 2014; Y. Wang & Zhang, 2015) and in a mealworm-based minced meat-like product (Stoops et al., 2017). While the

bacterial communities may differ largely between different rearing companies and production batches, some bacteria, such as *Spiroplasma* spp., may be typically associated with mealworms and mealworm-derived products. *Spiroplasma* spp. are typically found as endosymbiont in the insect gut, but are hard to culture *in vitro* (Madigan et al., 2009). While some *Spiroplasma* species may protect the host insect against entomopathogens (Shokal et al., 2016), other species of the genus may be pathogenic for insects (Ammar, Fulton, Bai, Meulia, & Hogenhout, 2004) or even humans (Aquilino et al., 2015). *Spiroplasma* is generally not considered as a foodborne pathogen, but further research is needed to unravel the role of *Spiroplasma* spp. or related bacteria in edible insects.

The second most abundant OTU for mealworms was assigned to the genus *Erwinia* (OTU 2). Although this OTU was assigned *E. oleae* with an assignment score of 0.86 (Table S3.2, Supporting information), the short 250 bp read length of the investigated 16S rRNA gene amplicon does not ensure a 100% correct species determination. Further research, e.g. using other genetic markers is needed to confirm its identification. *Erwinia* species are often associated with plants as phytopathogens (Madigan et al., 2009) and may infect diverse fruits and vegetables (Aremu & Babalola, 2015; Farrar, Nunez, & Davis, 2000; Moretti et al., 2011). Therefore, it is reasonable to assume that introduction of plant pathogens such as *Erwinia* species can occur via infected carrots or apples that are provided in the diet of mealworms as a source of moisture. In a previous study on the microbial composition in mealworm-derived products, *Erwinia* was also detected (Stoops et al., 2017; relative abundance of 11%). Besides plant pathogens, *Erwinia* spp. have also been reported as spoilage organisms, but so far, no reports are available about potential human health risks (Madigan et al., 2009).

When screening the list of OTUs for possible human (food) pathogens, mealworm rearing company 2 was found to harbour an abundant OTU corresponding to an Enterobacteriaceae species (OTU 14; 22%). While phylogenetic assignment via SINTAX (Table S3.2, Supporting information) was not conclusive on genus level (assignment score of 0.28), BLAST analysis against GenBank (Table S3.3, Supporting information) related this OTU to the genus *Cronobacter* with 96-98% sequence identity (248/253 to 250/253 bp). Since most *Cronobacter* species are human pathogens (Grim et al., 2013), the consumption of mealworms contaminated with *Cronobacter* might pose a health risk without appropriate processing.

Other Enterobacteriaceae OTUs, as well as OTU 33, related to the spore-forming class of Clostridia (Table S3.3, Supporting information), might correspond with pathogenic bacteria as well. OTU 33 was also present as one of the most abundant OTUs for mealworms. Spore-forming bacteria are especially important in processing edible insects and for the food industry in general as they are easily maintained after processing (Chapter 5).

The other abundant OTUs found in mealworms do not pose severe health risks as food pathogen, but some OTUs such as those associated with *Pseudomonas* might have a role in spoilage of the mealworms as foodstuffs. Many *Pseudomonas* spp. are involved in the process of food spoilage (Dijk et al., 2015; Madigan et al., 2009). The fact that some *Pseudomonas* species (OTU 4 and OTU 23) were abundantly found on mealworms and that many *Pseudomonas* species can grow at low temperatures (Carrión, Miñana-Galbis, Montes, & Mercadé, 2011; Reddy, Matsumoto, Schumann, Stackerbrandt, & Shivaji, 2004), holds the risk of spoilage during preservation at refrigerator temperatures. Sample MW 3.3 contained up to 50% of those (psychrotrophic?) *Pseudomonas* OTUs, which may correspond with the high psychrotrophic count (9.1 log cfu/g) reported in Chapter 2. The results in both Chapter 2 and Chapter 3 prove that psychrotrophic *Pseudomonas* spp. can easily colonise mealworms. Good practices (e.g. a proper heating step early in the food production process) are therefore necessary to use insects as a food ingredient.

While dominant OTUs were less apparent for crickets, OTUs assigned to the genera *Bacteroides* or *Parabacteroides* (Bacteroidetes) were common and repeatedly present among the most abundant OTUs encountered on crickets. Both genera are commensal intestinal inhabitants of humans and other animals. They were also found in processed crickets by Garofalo et al. (2017). Despite being very dominantly present in e.g. the human large intestine, they can also act as pathogens when occurring outside the gut, in other parts of the human body (Awadel-Kariem, Patel, Kapoor, Brazier, & Goldstein, 2010; Madigan et al., 2009; Wexler, 2007). Consequently, a good processing practice is necessary for raw crickets as well before using them as a food ingredient.

3.4 Conclusions

In this study, culture-independent microbiological data were obtained to compare bacterial communities of edible insects from different rearing companies and several production batches. The results demonstrate that different rearing companies can provide the same insect species with a different bacterial composition, as was demonstrated especially for mealworms. Even between several production batches from the same company, a different bacterial composition could be seen, which was also reflected by culture-dependent microbial counts. For crickets, the bacterial composition compared between production batches and rearing companies differed to a much lesser extent than for mealworms. At the same time, mealworms and crickets harbour largely different bacterial communities, but different cricket species are much more alike. Concerning food safety, only a few possible risks were identified as some of the OTUs found could be related to families (e.g. Enterobacteriaceae) or larger taxonomic groups (e.g. Clostridia class) that may contain pathogenic species. Since fungi and viruses, which may have an important role in the overall microbiological quality of edible insects as well, are not considered in this study, future research on this topic is desirable.

Chapter 4 Occurrence of antibiotic resistance genes in raw edible insects

This chapter was redrafted[1] after Vandeweyer D., Milanović V., Garofalo C., Osimani A., Clementi F., Van Campenhout L., & Aquilanti L. (2018). Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and the Netherlands. *International Journal of Food Microbiology 290*, 288-295. http://doi.org/10.1016/j.ijfoodmicro.2018.10.027

4.1 Introduction

Since the recent introduction of edible insects in several European countries, the microbiological quality and safety of insects used for human consumption was repeatedly investigated, not only in Chapters 2 and 3, but also by other researchers (Garofalo et al., 2017; Klunder et al., 2012; Osimani, Garofalo, Milanović, et al., 2017; Rumpold & Schlüter, 2013a; Stoops et al., 2016, 2017; van der Spiegel et al., 2013; Wynants et al., 2018). This was already recommended by different scientific opinions and advices (ANSES, 2015; EFSA Scientific Committee, 2015; NVWA, 2014; SHC & FASFC, 2014), but the new European novel food regulation (EU 2015/2283), which took effect in January 2018, has evoked an increase in edible insect research as well.

As recently reviewed by Dobermann, Swift, & Field (2017), the main challenges of mass rearing of edible insects include the bacterial contamination of the end products, e.g. high counts of spore-forming bacteria, total mesophilic aerobes, and Enterobacteriaceae, and the potential occurrence of human pathogens as well as the risks of antibiotic usage in such mass rearing. Concerning this latter aspect, the use and misuse of antibiotics are known to have a

[1] The complete content of this paper (Vandeweyer et al., 2019) was included in Chapter 4, with only small alterations to keep the information provided up to date and to follow the logic course of this dissertation. As first author, D.V. contributed to all parts described in this work, from experimental design to the writing of the paper. The second author, V.M., contributed intensely during design and writing, and continued the analyses after D.V. left back from Italy to Belgium after a research stay. The whole experiment, including the writing of the paper, was performed in close collaboration with the other co-authors of the Department of agricultural, environmental and food science (Dipartimento di Scienze Agrarie, Alimentari ed Ambientali), Marche Polytechnic University, Italy (Prof. L. Aquilanti).

major effect on the prevalence of antibiotic resistant microorganisms, for example in primary production, food, feed, and the environment (Clementi & Aquilanti, 2011; Verraes et al., 2013). Hence, for 2016-2020 the European Medicine Agency (EMA) expressed a joint opinion with the European Food Safety Authority (EFSA) on measures to reduce the use of antimicrobial agents in animal husbandry (also known as the 'RONAFA' opinion) (EMA & EFSA, 2017). Even earlier reports published jointly by EMA and European bodies including the European Centre for Disease Prevention and Control (ECDC), EFSA, and the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) have emphasised the need for the prudent use of antibiotics in animals (ECDC, EFSA, EMA, & SCENIHR, 2009). Concerning edible insects, no data are currently available about the use of antibiotics in mass rearing and the occurrence of antibiotic resistances (ARs) in edible insects is limitedly assessed (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017), despite the recommendations posed in the EFSA opinion (EFSA Scientific Committee, 2015) and two joint reports of the ECDC, EFSA, and EMA (ECDC, EFSA, & EMA, 2015, 2017).

Antibiotic resistances may pose a risk in animal and human health, since they are easily transferred through horizontal gene transfer between microorganisms, including pathogens (Gogarten, Gogarten, & Olendzenski, 2009; Verraes et al., 2013). For edible insects, which typically contain high microbial counts (Chapter 2), the transfer of such ARs can establish important food safety risks. Food, especially that of animal origin, is an important vehicle in the transfer of antibiotic resistance genes into the human digestive tract and its associated microbiome (Verraes et al., 2013). Because edible insects are generally used as a whole in food products and because starvation has shown not to alter the microbiome, at least in mealworms (Wynants et al., 2017), the complete microbial composition (i.e. also intestinal) is included in the foodstuffs.

Edible insects are typically processed prior to consumption (Fombong et al., 2017; van Huis et al., 2013) (see also Chapter 5). To lower microbial counts, many processing steps involve a heat treatment causing a number of lesions in microbial cells. These include membrane damage, loss of nutrients and ions, ribosome aggregation, and even DNA strand breaks (Mañas & Pagán, 2005). However, less vulnerable microorganisms (e.g. bacterial spores) and

their AR genes may survive the minimal heating treatments frequently applied for insects (Chapter 5) or even be triggered in AR transfer (Verraes et al., 2013). Processing may also cross-contaminate insects with ARs initially not carried by their associated microbiota (Verraes et al., 2013). Recently, a few studies have been performed to investigate the occurrence and distribution of AR genes in processed, ready-to-eat insects available on the European market (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017), but so far, raw insects, i.e. living insects at the end of their rearing cycle collected from industrial rearing facilities, have not been subjected to AR assessment, yet. In all former studies, a number of genes inducing resistance against classes of antibiotics commonly used in both human and animal therapy (e.g. tetracyclines and macrolides) were detected by qualitative nested PCR. According to Penders, Stobberingh, Savelkoul, & Wolffs (2013), three different metagenomic approaches are currently applied to study the AR pool: PCR-based metagenomics, functional metagenomics, and sequence-based metagenomics. Although nested PCR assays are characterised by an extremely high sensitivity for detection of target AR genes (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017), this technique does not allow an effective quantification of the amount of gene copies occurring in a given sample. To date, real-time PCR (qPCR) techniques have been applied in a number of food matrices to detect and quantify both tetracycline and erythromycin resistance genes (Flórez et al., 2014). However, to the authors' knowledge, no qPCR assays have been used or optimised for the analysis of ARs in edible insects, yet.

Based on these premises, the present study was aimed at detecting and quantifying a set of tetracycline and erythromycin resistance genes in freshly reared, raw edible insects to be used as an ingredient for insect food production. To this end, raw mealworms and crickets collected from different rearing facilities in Belgium and the Netherlands and from different rearing batches per facility were analysed. After DNA extraction, all samples were screened by qPCR for tetracycline *tet*(K), *tet*(O), *tet*(M), *tet*(S) and erythromycin *erm(*B) resistance genes previously found in edible insects (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017).

4.2 Materials and methods

4.2.1 Raw insect sampling

A total of 30 raw insect samples were obtained from 9 rearing companies located in Belgium and the Netherlands (Table 4.1). Samples (± 500 g as provided by the rearer) were obtained from rearing stages used for consumption (except for one cricket sample taken at nymph stage: BCR 1.4a). Insect species investigated included yellow mealworms (*T. molitor*, 17 samples), lesser mealworms (*Alphitobius diaperinus*; 3 samples), house crickets (*Acheta domesticus*; 5 samples), and tropical house crickets (*Gryllodes sigillatus*; 5 samples). Most rearing companies were sampled several times, thus investigating different production batches from the same facility. All insects were reared according to company-specific optimised protocols, which were only partly revealed. Important rearing details and post-harvest handlings are detailed in Table 4.1 together with references to previously performed microbiological analyses on the same samples. Samples from the same batch which were slightly different (e.g. post-harvest treatment) were given a different letter in the sample code. After transportation from the rearing facility to the laboratory and removal of dead insects, samples were frozen (-21 °C) until DNA extraction, to preserve the DNA.

4.2.2 Reference strains

DNA extracted from five reference strains (Table 4.2), each carrying one of the AR genes under study, was used for the construction of qPCR standards and as positive controls in the qPCR runs. The strain *Enterococcus faecalis* JH2-2 (Jacob & Hobbs, 1974) was used as a negative control. Prior to DNA extraction, the five reference strains were grown on de Man, Rogosa and Sharpe (MRS) agar supplemented with tetracycline or erythromycin, incubated at 37 °C for 48 h. The concentrations of added antibiotics were chosen according to the microbiological breakpoint levels reported by Clinical and Laboratory Standards Institute (2017).

4.2.3 Sample preparation and DNA extraction

Five grams of each thawed (ambient temperature, 1 hour) insect sample were aseptically crushed and homogenised in 45 ml of sterile peptone water (peptone, 1 g/l) for 2 minutes at 260 rpm using a Stomacher 400 Circulator (PBI, Milan, Italy). Subsequently, 1.5 ml of each homogenate was centrifuged at 16,000 g for 5 minutes to produce a pellet containing the bacterial cells. Total bacterial DNA was extracted from 0.2 grams of each pellet using a PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. The quantity and the purity of the extracted DNA were determined using a Nanodrop ND 1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The concentration of the extracted DNA was standardised to 2 ng/µl for all the samples. To check the effectiveness of bacterial DNA extraction, the DNA suspensions were amplified by end-point PCR using the universal prokaryotic primers 338F-518R, as previously described (Osimani, Garofalo, Aquilanti, et al., 2017). DNA from the reference strains was extracted following the procedure previously detailed by Osimani et al. (2015).

Table 4.1 Sample information.

Sample ID	Rearing company	Batch	Sampling period	Country	Insect species	Post-harvest treatment	Remarks	Previous microbiological analyses
MW 1.1	1	1	March 2015	Belgium	*T. molitor*[1]	24 h starving	During starving, carrots were provided	Yes, sample MW 1.1[A]
MW 1.2	1	2	May 2015	Belgium	*T. molitor*	48 h starving		Yes, sample MW 1.2[A]
MW 1.3	1	3	September 2015	Belgium	*T. molitor*	48 h starving		Yes, sample MW 1.3[A]
MW 2.1	2	1	March 2015	The Netherlands	*T. molitor*	None		Yes, sample MW 2.1[A]
MW 2.2	2	2	June 2015	The Netherlands	*T. molitor*	None		Yes, sample MW 2.2[A]
MW 2.3	2	3	October 2015	The Netherlands	*T. molitor*	12 h starving		Yes, sample MW 2.3[A]
MW 3.1	3	1	May 2015	The Netherlands	*T. molitor*	96 h starving		Yes, sample MW 3.1[A]
MW 3.2	3	2	July 2015	The Netherlands	*T. molitor*	96 h starving		Yes, sample MW 3.2[A]
MW 3.3	3	3	November 2015	The Netherlands	*T. molitor*	96 h starving	Refrigerated starving	Yes, sample MW 3.3[A]
MW 4.1a	4	1	February 2016	Belgium	*T. molitor*	48 h starving		Yes, sample 2.3[B]
MW 4.1b	4	1	February 2016	Belgium	*T. molitor*	None		Yes, sample 2.3 control[B]
MW 4.2a	4	2	February 2016	Belgium	*T. molitor*	None		No
MW 4.2b	4	2	February 2016	Belgium	*T. molitor*	48h starving	Refrigerated starving	No
MW 4.3a	4	3	March 2016	Belgium	*T. molitor*	None		Yes, sample 3.3 control[B]
MW 4.3b	4	3	March 2016	Belgium	*T. molitor*	48 h starving	Refrigerated starving	Yes, sample 3.3[B]
MW 4.4	4	4	August 2017	Belgium	*T. molitor*	None		No
MW 5.1	5	1	February 2017	Belgium	*T. molitor*	None		No
LMW 1.1a	6	1	July 2016	The Netherlands	*A. diaperinus*[2]	None		Yes, sample L35 first crate[C]
LMW 1.1b	6	1	July 2016	The Netherlands	*A. diaperinus*	24 h starving		Yes, sample L36 first crate[C]
LMW 1.2	6	2	July 2016	The Netherlands	*A. diaperinus*	None		Yes, sample L35 second crate[C]
HCR 1.2	7	2	June 2015	The Netherlands	*A. domesticus*[3]	12 h starving	Refrigerated starving	Yes, sample CR 1.2[A]
HCR 1.3	7	3	September 2015	The Netherlands	*A. domesticus*	12 h starving	Refrigerated starving	Yes, sample CR 1.3[A]

HCR 2.1	8	1	April 2015	The Netherlands	*A. domesticus*	None		Yes, sample CR 2.1[A]
HCR 2.2	8	2	July 2015	The Netherlands	*A. domesticus*	None		Yes, sample CR 2.2[A]
HCR 2.3	8	3	October 2015	The Netherlands	*A. domesticus*	12 h starving		Yes, sample CR 2.3[A]
BCR 1.1	9	1	August 2015	Belgium	*G. sigillatus*[4]	12 h starving		Yes, sample CR 3.1[A]
BCR 1.2	9	2	October 2015	Belgium	*G. sigillatus*	12 h starving	Refrigerated starving	Yes, sample CR 3.2[A]
BCR 1.3	9	3	December 2015	Belgium	*G. sigillatus*	None		Yes, sample CR 3.3[A]
BCR 1.4a	9	4	November 2016	Belgium	*G. sigillatus*	None	Nymph stage (26 days old)	Yes, sample Crickets day 26[D]
BCR 1.4b	9	4	November 2016	Belgium	*G. sigillatus*	None	Adult stage	Yes, sample Crickets day 40[E]

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

[A] Table 2.1, Chapter 2.

[B] Wynants et al. (2017).

[C] Wynants et al. (2018).

[D] Vandeweyer et al. (2018).

[E] Chapter 6.

Table 4.2 Bacterial reference strains used as positive controls in qPCR runs.

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

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

4.2.4 Construction of qPCR standards

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

4.2.5 Real-time qPCR quantification

Bacterial DNA extracted from the insect samples was screened by qPCR for the absolute quantification of the gene *erm*(B), coding for resistance to erythromycin, and the genes *tet*(O), *tet*(M), *tet*(S), and *tet*(K), coding for resistance to tetracyclines. The qPCR reactions were performed using the Mastercycler® ep realplex (Eppendorf) with the qPCR primers described by Flórez et al. (2014). Four µl (8 ng) of the extracted DNA was amplified in a total volume of

10 µl including 5 µl of QPCR Green Master Mix LRox 2X (Biotechrabbit GmbH, Hennigsdorf, Germany) and 900 nM of the forward and reverse primer. In each assay, the opportune positive and negative controls were run together with a blank (molecular grade water instead of DNA).

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

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

4.2.6 Statistical analyses

To investigate statistical differences among insect species, samples and rearing facilities, as well as influences of graphical distribution, sampling period and post-harvest treatments, one-way ANOVA with Tukey's post hoc test was performed for all AR genes. In case of unequal variances, Welch's ANOVA with Games-Howell post hoc test was used instead. All tests were performed with SPSS Statistics 23 (IBM, New York, NY, USA) and considered a 0.05 significance

level. Finally, nonmetric multidimensional scaling (NMDS) was performed on the total AR gene composition of all samples (i.e. including results of all five genes assessed) using the R-package (R Development Core Team, 2013) "Vegan" (v.2.43) in RStudio (v1.1.442).

4.3 Results and discussion

4.3.1 Accuracy of qPCR assessments

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

4.3.2 Quantitative detection of antibiotic resistance genes in insect samples

All qPCR assessments, each detecting and quantifying one target AR gene, were applied for all 30 samples investigated. The results, expressed as gene copy number per gram of insect sample, are shown in Table 4.3. *Tet* genes were present in several samples with mean quantities ranging between 2.78×10^4 and 2.10×10^8 gene copies per gram of insect. Raw edible insects have been reported to harbour up to 8 or 9 log cfu/g microorganisms (Chapter 2), thus suggesting that a large fraction of the microorganisms occurring in some samples carried at least one AR gene under study. More specific, *tet*(O), *tet*(K), *tet*(M) and *tet*(S) genes were encountered in 37%, 40%, 100% and 70% of the analysed samples, respectively. The *tet* genes investigated in this study are typically (but not uniquely) associated with gram positive bacteria (Chopra & Roberts, 2001). Therefore, the recovered *tet* genes may have been particularly carried by gram positive members of the edible insect microbiota, such as lactic acid bacteria (LAB), which have previously been found in large quantities (up to 8 log cfu/g) (Chapter 2). Indeed, it was suggested that LAB play an important role in the preservation and transfer of AR genes in foodstuffs and the animal gastrointestinal tract (Clementi & Aquilanti, 2011). Yet, especially the *tet*(M) gene is also occasionally encountered in gram negative bacteria, e.g. in members of the genus *Bacteroides* (Barbeyrac, Dutilh, Quentin, Renaudin, & Bébéar, 1991; Chopra & Roberts, 2001), which are known to be abundantly present in raw

edible crickets (Chapters 2 and 6). Interestingly, only one sample (MW 4.2b) contained a detectable number of *erm*(B) gene copies, coding for erythromycin (macrolide) resistance. The *erm*(B) gene is mostly associated with streptococci and enterococci (Leclercq, 2002) and may often be detected in combination with *tet*(M) because of their possible co-occurrence on the same transposon (Chopra & Roberts, 2001). Nevertheless, the absence of the *erm*(B) gene in most samples investigated here, suggests there was no co-occurrence with *tet*(M) in the insect microbiota associated with the samples.

Except for one mealworm sample (MW 4.2b), *tet*(O) was exclusively found in cricket samples at levels up to 4.24×10^7 gene copies. This finding agrees well with previous research, where *tet*(O) was detected in samples of processed edible crickets (*A. domesticus*), but rarely in other insects (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017). Since the microbiome is known to be specific for different edible insects (Garofalo et al., 2017; Osimani, Garofalo, Milanović, et al., 2017; Stoops et al., 2016) (see also Chapter 3), AR genes may concomitantly be present or absent in different insects, depending on the microbiota composition of these insects. Previously, *tet*(O) has been detected in streptococci and campylobacteria (Chopra & Roberts, 2001), two microbial groups that were already recovered from cricket samples in Chapter 3, though at very low levels (Table S3.2 and Table S3.3, Supporting information). The high copy numbers of *tet*(O) detected in the cricket samples analysed in this study suggest that also microorganisms other than streptococci and campylobacteria might carry this determinant. In this regard, a number of previous studies have clearly indicated that the microbial compositions of crickets and mealworms are influenced largely by their diet (Colman et al., 2012; Wynants et al., 2018; Yun et al., 2014), thus explaining potential differences occurring in the distribution of specific AR genes as well.

Table 4.3 Mean antibiotic resistance gene copy number per g insect sample[$].

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

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

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

[+]N.D. = not detected.

In contrast to *tet*(O), the detection of *tet*(K) was more widespread among samples, although significantly ($p = 0.043$) higher average copy numbers were observed for mealworms compared to crickets. Significant differences were also seen between rearing companies, with rearer 3 producing mealworms with significantly the highest numbers of *tet*(K) copies ($p = 0.000$). Also between different batches from a single rearer, significant differences were observed (Table 4.3). In previous investigations on processed edible insects, *tet*(K) has frequently been detected as well (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017), thus suggesting a wide distribution of *tet*(K) in edible insects. This might be explained by its location on small transferable plasmids that can easily integrate in the chromosome of different gram-positive bacteria (Chopra & Roberts, 2001). *Tet*(K) has been detected in numerous genera, including *Bacillus*, *Clostridium, Enterococcus, Streptococcus* and *Staphylococcus* (Chopra & Roberts, 2001), all of which have already been found in various edible insect species in Chapter 3 and 6 and by Wynants et al. (2018).

Concerning their geographical origin, an unequal distribution of *tet*(K) was observed among samples. Indeed, only 13% of the Belgian samples (2 out of 16) harboured *tet*(K), while 71% of the samples collected in the Netherlands (10 out of 14) were positive for this gene. Statistical analysis confirmed this evidence, and also revealed a significant difference ($p = 0.023$) in the number of *tet*(K) gene copies between Dutch and Belgian samples. Overall, these findings suggest that the occurrence of *tet*(K) and even its relative abundance might be geographically determined for freshly reared, raw insects. A previous study investigating the occurrence of antibiotic resistances in processed mealworms by nested PCR did not report any significant difference between Belgian and Dutch samples, all being positive for *tet*(K) (Osimani, Cardinali, et al., 2017). However, most of those Belgian samples were positive only after the second set of PCR runs, whereas in 60% of the Dutch samples, *tet*(K) had already been amplified after the first set of PCR runs, thus suggesting a different abundance of the target sequence.

Regarding *tet*(M), a ubiquitous occurrence of this determinant was revealed by qPCR analysis. Although it was detected in all samples, mealworms contained, on average, a statistically ($p = 0.042$) higher copy number of *tet*(M) than crickets. When comparing different batches

produced by the same company, the highest variety in *tet*(M) copies between samples was found for company 9 (*G. sigillatus*). Also company 4 (*T. molitor*) shows a high variation between different samples for *tet*(M) copy number, but the high standard deviations obtained did not allow for a statistical confirmation of this observation. Besides the type of insect species and production batches, other potentially influencing factors considered, such as the geographical origin or the type of post-harvest treatment, were not found to exert a significant influence on the distribution of *tet*(M) among the samples analysed. Our results agree well with other studies (Milanović et al., 2016; Osimani, Garofalo, Aquilanti, et al., 2017), where *tet*(M) was frequently detected in various specimens of marketed edible insects, including processed mealworms and crickets. Overall, these findings suggest a wide distribution of *tet*(M) in the microbiome of edible insects. Its frequent detection as well as its occasionally high copy numbers (up to 4.52×10^7) might be attributed to the fact that *tet*(M) is typically located on conjugative transposons (e.g. Tn916 - Tn1545 family) and can therefore easily be transferred from one bacterial species to another (Doherty, Trzcinski, Pickerill, Zawadzki, & Dowson, 2000). Accordingly, *tet*(M) has been detected in numerous food matrices, including dairy (Flórez et al., 2014) and meat products (Hölzel, Huther, Schwaiger, Kämpf, & Bauer, 2011). Interestingly, in the present study, two samples (MW 1.1 and MW 1.2) did not carry any other AR gene than *tet*(M).

Concerning *tet*(S), a lower occurrence was observed in comparison with *tet*(M), with 70% (21 out of 30) of the samples found to be positive. Again, the presence and copy numbers of *tet*(S) were significantly higher ($p = 0.000$) in mealworms than in crickets. *Tet*(S) was first discovered in *Listeria monocytogenes* (Charpentier, Gerbaud, & Courvalin, 1994), but, to the authors' knowledge, *L. monocytogenes* has never been detected in either industrially (Giaccone, 2005; Grabowski & Klein, 2016; Milanović et al., 2016; Osimani, Garofalo, Milanović, et al., 2017; Osimani, Milanović, Garofalo, et al., 2018; Vandeweyer et al., 2018; Wynants et al., 2018) (see also Chapter 2) or laboratory reared edible insects (Osimani, Milanović, Cardinali, Garofalo, et al., 2018). However, Charpentier et al. (1994) reported the transfer of *tet*(S) from *Listeria* to *Enterococcus*, a genus whose representatives have been detected in most of the samples analysed here (Chapter 3) (Wynants et al., 2018). If *tet*(S) was effectively carried by enterococci, its higher detection frequency in mealworms might be explained by the higher relative abundance of *Enterococcus* spp. in mealworms compared to crickets, as revealed by

Next Generation Sequencing in Chapter 3. In addition to *Listeria* and *Enterococcus* spp., *tet*(S) has mainly been detected in *Lactococcus* (Ishihara et al., 2013; S.-R. Kim, Nonaka, & Suzuki, 2004) and *Streptococcus* (Gevers et al., 2003). Since these genera were previously encountered in numerous mealworm and cricket samples (Chapter 3) (Wynants et al., 2018), they might as well have introduced the *tet*(S) gene in the samples investigated if they indeed carried the *tet*(S) determinant. Significantly different gene copy numbers were also found among different insect batches collected from the same rearing company, with rearer 2 showing the highest *tet*(S) copy numbers, up to 2.10×10^8. Our findings agree well with previous research (Milanović et al., 2016; Osimani, Cardinali, et al., 2017; Osimani, Garofalo, Aquilanti, et al., 2017), revealing a widespread occurrence of *tet*(S), with more than 50% of the samples found to be positive by nested PCR.

Figure 4.1 summarises the differences emerged among the samples analysed in terms of detected AR genes. Distances between different points are a measure of the dissimilarity between different samples. A clear distinction between mealworm (green) and cricket (blue) samples is shown, as a consequence of the differences in the occurrence and relative abundance of *tet*(O), *tet*(M), *tet*(K) and *tet*(S) in mealworms and crickets. Generally, mealworms contained higher copy numbers of *tet*(K), *tet*(M) and *tet*(S) than crickets, and contrary to the cricket samples, only occasionally harbour a detectable level of *tet*(O). In Figure 4.1, the grouping of different rearing batches is also shown. Overall, batches from the same company are (at least moderately) clustered together, with the exception of MW 1.3 which differed from both other batches produced by rearing company 1 (MW 1.1 and MW 1.2) for the presence of *tet*(S). Also for company 4, one sample (MW 4.2b) differs greatly from the other six for the occurrence of four out of five AR genes investigated. According to the statistical analyses, sampling period (autumn/winter vs. spring/summer) and post-harvest treatment had no influence on the occurrence and abundance of the AR genes analysed. Furthermore, differences were seen between mealworms and crickets, but not among different insect species (lesser mealworm vs. yellow mealworm and house cricket vs. tropical house cricket) within the same insect order.

The data collected in this study suggest that edible insects can effectively harbour AR genes that might be mobilised at any stage of the food chain, from rearing up until processing and

even consumption. In this regard, the quantification of AR genes in edible insects can contribute to a better evaluation of the health risks associated with the consumption of this novel food, since a higher AR gene copy number is intrinsically associated with a higher risk. Compared to other food matrices, the resistance gene quantity carried by edible insects varies within the same range. For example, for cheeses, the *tet*(S) gene was observed ranging from 4.5 up to 8 log copies/g (Manuzon et al., 2007). Likewise, the *tet*(M) gene was reported to be present almost up to 7 and 8 log copies/cm² chicken and pork meat, respectively (Hölzel et al., 2011). In the latter study, the unit in which the AR gene occurrence was presented does not correspond with the unit employed for the investigated insect samples. A recalculation to log copies/g would not be appropriate because of the different nature of both matrices and a strict comparison is therefore not possible. Still, a comparison can be made with the microbiologically most contaminated part of the meat products, i.e. the surface. On the other hand, when considering a homogenous meat sample, the log copy number/g would probably be lower and be dependent of the dimensions (surface area vs. volume) of the meat pieces. In this regard, edible insects may pose a risk which is similar to or even higher than for other food matrices in terms of antibiotic resistance genes carried, depending on the product compared with.

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

genes in insects during further processing into food is necessary. While AR genes in edible insects can pose a health risk, it was also noted by Cai et al. (2018) that insects and their intestinal microbiota may play a role in the degradation of e.g. tetracyclines. This includes an interesting path for future research as well.

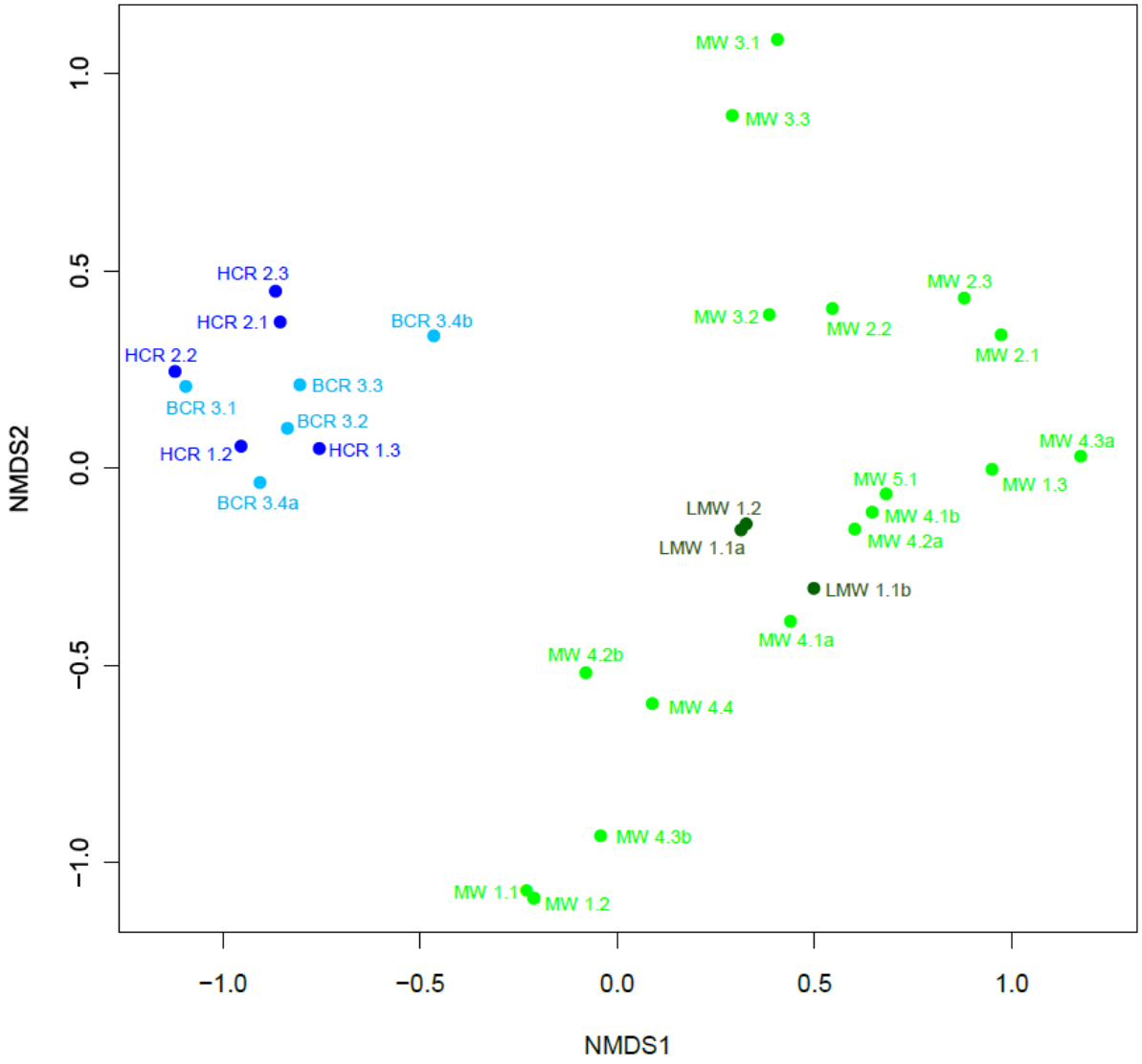


Figure 4.1 Non-metric multidimensional scaling (NMDS) ordination composed of all antibiotic resistance gene data for all 30 samples investigated (stress value of 0.146). Mealworm samples are represented by a green colour (light green: yellow mealworms, dark green: lesser mealworms) and cricket samples by a blue colour (dark blue: house crickets, light blue: tropical house crickets). The distance between different points on the plot reflects their similarity level: the more similar the AR gene composition, the smaller the distance between the points. The plot was constructed based on the AR gene copy number per gram of insect for all five genes assessed. Sample IDs correspond with those in Table 4.1.

4.4 Conclusions

The occurrence of antibiotic resistance genes in food products may pose a risk for human health. Insects, considered as an emerging source of proteins in Western countries, are currently intensively being investigated for their food safety. This study provides quantitative data on the presence of a selected pool of AR genes in 30 samples of freshly reared, raw mealworms and crickets from different industrial rearers. As a whole, genes conferring resistance to tetracyclines were detected with a high frequency, ranging from 37% up to 100% of the samples, for *tet*(O) and *tet*(M), respectively. A significantly different distribution of these genes was seen in raw mealworms compared to crickets, with mealworms harbouring a higher copy number of *tet*(K*), tet*(M), and *tet*(S), while *tet*(O) occurred exclusively in crickets. Based on the results collected in this study as well as in a previous one on the same samples (Chapter 3), these differences might be ascribed to differences in the microbial composition and the feed source of the insects analysed. Also, clear correlations between sample microbiota previously reported and the occurrence of certain genes known to be carried by specific genera were observed. Moreover, a geographical distribution seems to exist for *tet*(K), with a significantly higher occurrence in samples from the Netherlands than from Belgium. A remarkably lower occurrence of *erm*(B) was observed, with only one mealworm sample found to be positive by qPCR. In conclusion, fresh edible insects can contain antibiotic resistance genes up to levels comparable with other food matrices and may pose a similar health risk. Further research is needed to elucidate the sources of these AR genes during the rearing of the insects as well as their distribution during and after processing into foodstuffs.

Chapter 5 Microbiological assessment of processing and preservation of mealworms

A part of this chapter[1] was published as Vandeweyer D., Lenaerts S., Callens A., & Van Campenhout L. (2017). Effect of blanching followed by refrigerated storage or industrial microwave drying on the microbial load of yellow mealworm larvae (*Tenebrio molitor*). *Food Control*, *71*, 311-314. http://doi.org/10.1016/j.foodcont.2016.07.011

5.1 Introduction

Several ways to process insects into food products and to store insects and derived products are currently being explored. After rearing, mealworms are typically frozen to gently kill them and additionally they may be freeze-dried. Those practices enable long term storage and transport, thus facilitating the supply chain management of the insects. However, they are both expensive technologies, freezing especially with respect to the concomitant frozen transport and freeze-drying as to investment as well as operational costs (energy consumption). Both unit operations add to the cost of edible insects as a raw material for food companies. Alternative ways for processing and preservation need to be explored, which are preferably also more sustainable. Alternatives are only valuable when they are feasible on an industrial scale, when they are payable and, last but not least, when they result in intermediate or end products of good nutritional and microbiological quality.

The first aim of this work was to consider chilling of mealworms as alternative for freezing. The focus was on the effect of the technology on the microbial load of mealworms. The impact of chilling on the microbiota of mealworms has not been studied before. The microbial load of raw mealworms after rearing is high, with total viable counts generally being about 7 – 8.5 log cfu/g (Klunder et al., 2012; Rumpold et al., 2014; Stoops et al., 2016) (Chapter 2). In

[1] The paper on which a part of Chapter 5 was based (Vandeweyer, Lenaerts, et al., 2017), describes two main experiments. Only the first experiment regarding blanching and chilled preservation of mealworms was included in Chapter 5. The second experiment in this paper, describing microwave drying of mealworms, was not included. D.V. and S.L. are joint co-authors: D.V. was mainly involved in the first experiment and S.L. in the second experiment. Additionally, Chapter 5 was supplemented with data generated by D.V. and not published so far regarding the effect of blanching and chilled preservation on spore-forming bacteria harboured by mealworms.

Belgium, the Federal Agency for the Safety of the Food Chain postulates that a heating step is necessary to reduce microbial numbers on insects before they are placed on the market (FASFC, 2016). In this study, chilling was therefore preceded by blanching.

The concept of blanching is frequently applied on fruits and vegetables as a method to destroy enzymes prior to further processing such as canning, freezing or drying (Fellows, 2009). Another result of blanching is the reduction of microbial numbers, which benefits preservation since a lower initial level of microorganisms is attained. The two most commonly performed blanching methods are the use of hot (70 – 100 °C) water or saturated steam but microwave blanching can be applied as well. During the blanching process, food is heated rapidly to a certain temperature and subsequently held for a pre-set time after which it is typically cooled rapidly to near-ambient temperature (Fellows, 2009). Blanching time depends on the size and type of the food as well as the blanching method and temperature. For insects, a short heat treatment such as blanching is typically used as a pre-treatment for freezing or freeze-drying. However, no information is available on log reductions that can be obtained for specific time and temperature combinations.

In a first experiment in this study, freshly reared, raw mealworms were first blanched using several blanching times and then stored under chilled conditions. The larvae were assessed for their microbial counts prior to blanching, after blanching and after chilled preservation. The second aim of this study was to assess the specific impact of a suitable blanching treatment and consecutive chilled preservation on the bacterial endospores harboured by edible insects. Previous research including bacterial endospores in the investigated microbial counts pointed out that insects harbour a varying and sometimes high amount of endospores (Klunder et al., 2012; Stoops et al., 2016) (Chapter 2) and that those spores can survive a heat treatment (Klunder et al., 2012). Given the possible food safety risks associated with spore-forming bacteria such as *Clostridium perfringens*, *C. botulinum* and *Bacillus cereus*, the fate of bacterial endospores in insects should be investigated. The second experiment in this study involved the counting and identification of endospores present in raw edible mealworm samples and the monitoring of endospore counts after blanching and during chilled preservation of the mealworms.

5.2 Materials and methods

5.2.1 Insect samples

Living yellow mealworms (*Tenebrio molitor*) were purchased and transported to the laboratory and stored at ambient temperature until analysis. For the first experiment (assessment of blanching times), three batches of larvae (1.5 kg) were obtained from an eco-shop in Antwerp (Belgium). For the second experiment (effect of blanching and chilled preservation on endospores), one batch of mealworms (1.5 kg) was obtained from each of two rearing companies, both located in Belgium and one of them rearing for pet food and one for human food. Dead insects were removed prior to analysis.

5.2.2 Blanching and chilled preservation

For the investigation of the microbiological impact of blanching times, duplicate samples of 400 g larvae per batch (i.e. three batches x two samples) were blanched by transferring them into 4 l of boiling water and keeping them in the boiling water for respectively 10, 20 or 40 s. After blanching, they were transferred immediately into 9 l sterile cooling water using an autoclaved sieve. The cooling water was chilled to approximately 0 °C in advance in an ice water bath. The larvae were kept there for 60 s and drained by placing them in a sterile sieve for 30 s. In the second experiment, two samples per rearing company were investigated (i.e. two batches x two samples). All samples were subjected to a 40 s blanching step without subsequent cooling. During the blanching, the same insect/boiling water ratio was applied as in the first experiment. Only one of the two batches was used to study the effect of chilled preservation.

In both experiments, mealworms were packed under air in plastic bags (VAC090 PA/PE 20/70, thickness 80 µm, width 20 cm, Euralpack, Schoten, Belgium) each containing 100 g larvae (resulting in a gas/product (G/P) value of 1.24) prior to the chilled preservation. The bags were sealed (including air) using a packaging machine (C 200, Multivac, Mechelen, Belgium). For the first experiment, two bags per blanching time were stored for 6 days in a home-type refrigerator (Miele, Belgium) with set point 3 °C. For the second experiment, six bags from one batch were stored under the same conditions. During a preservation period of 24 days,

one bag was used for (destructive) analyses on day 7, 11, 14, 18, 21 and 24. The temperature during chilled preservation was monitored by a logger (Escort iLog internal sensor, VWR International, Leuven, Belgium).

5.2.3 Microbial plate counts

Microbial counts were performed on raw larvae, blanched larvae and larvae during chilled storage. In the first experiment, the untreated insects were anesthetised before analysis by incubating them in 100% nitrogen gas (Praxair, Schoten, Belgium) for at least 1.5 min. In the second experiment, larvae were anesthetised by refrigeration for at least 1 hour at 3 °C. All samples were analysed in threefold. Prior to microbiological analyses, subsamples of 30 g were taken aseptically from each sample and pulverised using an ethanol-sterilised handheld mixer (Bosch CNHR 25, speed 12, 2 min) as described previously (Stoops et al., 2016). Hence, microorganisms on the surface as well as in the intestine were counted, as larvae are consumed or processed entirely. Of each pulverised subsample, 5 g was transferred into a stomacher bag together with 45 g of peptone physiological salt solution (PPS, 0.85% NaCl, 0.1% peptone, Biokar Diagnostics, Beauvais, France). After homogenisation for 60 s in a Bagmixer® (Interscience, Saint Nom, France), a tenfold dilution series was prepared and plated on different agar media (Biokar diagnostics) using the pour plate technique, according to the ISO standards assembled by Dijk et al. (2015). Total viable aerobic and anaerobic mesophilic counts were determined on Plate Count Agar (PCA) after incubation for 72 h at 30 °C, lactic acid bacteria (LAB) on de Man, Rogosa & Sharpe agar for 72 h at 30 °C, Enterobacteriaceae on Violet Red Bile Glucose agar for 24 h at 37 °C, yeasts and moulds on spread plates of Dichloran Rose-Bengal Chloramphenicol Agar (DRBC) after incubation for 5 days at 25 °C. Psychrotrophs were counted on PCA for 10 days at 6.5 °C. Aerobic and anaerobic bacterial endospores were determined after a pasteurisation treatment of the 10^{-1} dilution at 80 °C for 10 min, followed by dilution and incubation on PCA for 48 h at 37 °C. Anaerobic conditions were generated in anaerobic containers (Anaerocult 2.5L, VWR International) using 'AnaeroGen 2.5L atmosphere generation systems' (Thermo Fisher Scientific, Asse, Belgium). Anaerobic conditions were checked using resazurin indicators (BR0055B, Thermo Fisher Scientific).

5.2.4 Isolation and identification of endospore-forming bacteria

During incubation, bacterial spores that survived the pasteurisation treatment incorporated in the ISO-based endospore counting technique (Dijk et al., 2015) are able to germinate. The colonies that develop on the PCA plate therefore originate from bacterial spores present in the insect matrix. In the second experiment, isolates were prepared from spore-forming bacteria from both raw mealworm batches. After picking several colonies with various morphologies from both the aerobically and the anaerobically incubated endospore count plates, they were inoculated and incubated respectively aerobically or anaerobically on Nutrient Agar (NA, Biokar Diagnostics) for 24 h at 37 °C to form individual colonies and hence axenic cultures. For long-term storage, they were subsequently incubated overnight (17 h) at 37 °C in Nutrient Broth (NB, Biokar Diagnostics) and stored at -80 °C after addition of glycerol to reach a final concentration of 50% (v/v). In this way, 67 spore-forming bacteria were isolated from the mealworms.

All isolates were subjected to phenol-chlorophorm DNA extraction according to the protocol described by Lievens et al. (2003). Next, the 16 S ribosomal RNA (rRNA) gene was amplified by PCR (T100™ Thermal Cycler, Biorad, Belgium), using the primers 27F (3'-AGAGTTTGATCCTGGCTCAG-5') and 1492R (3'-TACGGYTACCTTGTTACGACTT-5'). Reactions were performed in a 20 µl volume, containing 1.25 units of TaKaRa ExTaq polymerase, 1× Ex Taq Buffer (Clontech Laboratories, Palo Alto, CA, USA), 312.5 µM of each dNTP, 1.0 µM of each primer, and 5 ng genomic DNA (measured by a Nanodrop spectrophotometer). PCR conditions included an initial denaturation for 2 min at 95 °C followed by 34 cycli of denaturation for 45 s at 95 °C, annealing at 59 °C for 45 s and elongation for 45 s at 72 °C, and a final elongation of 72 °C for 10 min. After PCR, the amplicons were sequenced by Macrogen Europe (Amsterdam, the Netherlands) using the 1492R primer. To allow a reliable identification, high quality sequences with a read length of more than 750 bp were grouped into operational taxonomic units (OTUs) based on 98% sequence similarity. In this way, 40 out of 67 isolates were assigned to an OTU. Representative sequences of the OTUs were identified by subjecting them to a BLAST (Altschul et al., 1990) search against GenBank (Benson et al., 2013), excluding uncultured/environmental entries. Other isolates that were not assigned to an OTU but that were sequenced properly were identified individually by BLAST against

GenBank. OTUs and individual isolates that did not correspond with spore-forming bacteria (based on spore-forming characteristics published in literature) were discarded, leaving 50 spore-forming isolates identified.

5.2.5 Statistical analysis

SPSS Statistics 23 (IBM, New York, NY, USA) was used. Differences within the same batch or sample between initial microbial values, values obtained after blanching and, where applicable, during chilled storage were investigated with one-way ANOVA, followed by the Duncan post hoc test. Initial values from different batches were compared as well with an identical one-way ANOVA test. All tests were performed with a significance level of 0.05.

5.3 Results and discussion

5.3.1 Effect of different blanching times and chilled preservation on microbial numbers in the first experiment

For vegetables and fruits, blanching is mainly applied as a pre-treatment to inactivate enzymes and to reduce the microbial load prior to further treatment (Fellows, 2009; Xu et al., 2012). In our study, the main purpose of blanching mealworms was to reduce their microbial load prior to further preservation or processing. Mealworms were blanched using hot water, since this technique is easily accessible for insect producers. Due to the small size of the mealworms, short blanching times (10, 20 and 40 s) were assessed, which is an important difference compared to e.g. boiling, which aims to cook a food product and therefore requires longer heating time.

As presented in Table 5.1, the microbial counts before blanching were very similar for the three batches investigated. No statistical differences for the individual counts were observed between batches, except for the LAB (although the range was only 0.5 log cfu/g) and the psychrotrophs (ranging between 6.0 and 7.2 log cfu/g). The total count, the number of Enterobacteriaceae and the number of LAB reported by Stoops et al. (2016), who used similar procedures for sample treatment and counts, were in line with our counts. However, Stoops et al. (2016) found a highly variable number of aerobic spores (3.5, <1.0 and <1.0 in three

batches), whereas our data ranged between 2.6 and 3.1 log cfu/g. High numbers of psychrotrophs (ranging between 6.0 and 7.2 log cfu/g) were observed in our study, demonstrating the potential for microbial spoilage when storing raw mealworms in chilled conditions.

Blanching resulted in a very pronounced and also statistically significant reduction of all types of counts and for all blanching times, except for the spores. For total counts, reductions of 4.4, 6.4 and 5.6 log cycles were observed for blanching times of 10, 20 and 40 s respectively. The data do not show a relation between blanching time and magnitude of reduction. Even blanching during 10 s decreased microbial counts to below the detection limit, except for the total count and the spores. The effect of blanching on the spore count was not consistent: blanching during 10 s yielded similar spore counts after the treatment as before, whereas blanching during 40 s resulted in a numerical reduction with 0.8 log cfu/g, and blanching at 20 s caused a substantial and unexplainable rise in the spore count. Similarly, Klunder et al., (2012) reported 2.1 and less than 1 log cfu/g spores on raw mealworms and larvae which were boiled (rather than blanched) for 10 min, respectively. In our study the microbiological effect of shorter blanching times was studied. If effective in reducing the microbiota, shorter times are more desirable for industrial processing of mealworms, also avoiding overprocessing and excessive loss of nutrients such as vitamins (in particular vitamin B_{12} is important when consuming insects as meat replacer). Hence, although in our study the number of spores before blanching was the lowest when all microbial subgroups are compared, after blanching the spore count was the highest. That suggests that blanching causes the microbiota of mealworms to be reduced to mainly bacterial spores. The small disparity between the numbers of bacterial spores and the total counts (except for batch II) may originate from inherent variation caused by the counting technique or by the survival of a few organisms other than spores, for example thermophiles.

Table 5.1 Microbial counts of mealworms before and after blanching for different treatment times and after subsequent chilled preservation of six days at 3.7 ± 1.7 °C. Data are the mean values of three to six replicates ± standard deviation.

Batch	Treatment	Microbial counts (log cfu/g)					
		Total viable aerobic count	Aerobic bacterial endospores	Enterobacteriaceae	Lactic acid bacteria	Yeasts and moulds	Psychrotrophic aerobic count
I	None (initial count)	$7.9 \pm 0.3^{a, A}$	$2.6 \pm 0.3^{a, A}$	$7.3 \pm 0.5^{a, A}$	$7.4 \pm 0.2^{a, A}$	$3.8 \pm 0.5^{a, A}$	$7.2 \pm 0.4^{a,A}$
	10 s blanching	3.5 ± 0.8^{b}	2.8 ± 1.0^{a}	$< 1.0 \pm 0.0^{b}$	$< 1.0 \pm 0.0^{b}$	$< 2.0 \pm 0.0^{b}$	$< 1.0 \pm 0.0^{b}$
	Chilled preservation	2.7 ± 0.2^{c}	2.3 ± 0.1^{a}	$< 1.0 \pm 0.0^{b}$	1.3 ± 0.4^{b}	$< 2.0 \pm 0.0^{b}$	1.1 ± 0.1^{b}
II	None (initial count)	$8.2 \pm 0.7^{a, A}$	$2.8 \pm 0.4^{a, A}$	$7.5 \pm 0.6^{a, A}$	$7.0 \pm 0.1^{a, B}$	$3.5 \pm 0.2^{a, A}$	$6.0 \pm 0.2^{a, B}$
	20 s blanching	1.8 ± 0.4^{b}	4.7 ± 0.7^{b}	$< 1.0 \pm 0.0^{b}$	$< 1.0 \pm 0.0^{b}$	$< 2.0 \pm 0.0^{b}$	$< 1.0 \pm 0.0^{b}$
	Chilled preservation	2.9 ± 0.2^{b}	2.4 ± 0.1^{a}	1.2 ± 0.3^{b}	$< 1.0 \pm 0.0^{b}$	$< 2.0 \pm 0.0^{b}$	1.3 ± 0.1^{b}
III	None (initial count)	$7.6 \pm 0.4^{a, A}$	$3.1 \pm 0.1^{a, A}$	$7.1 \pm 0.9^{a, A}$	$6.9 \pm 0.2^{a, B}$	$3.5 \pm 0.4^{a, A}$	$6.5 \pm 0.6^{a, A, B}$
	40 s blanching	2.0 ± 0.4^{b}	2.3 ± 0.1^{a}	$< 1.0 \pm 0.0^{b}$	1.5 ± 0.8^{b}	$< 2.0 \pm 0.0^{b}$	$< 1.0 \pm 0.0^{b}$
	Chilled preservation	3.5 ± 0.3^{c}	1.4 ± 1.1^{b}	1.1 ± 0.1^{b}	1.3 ± 0.3^{b}	$< 2.0 \pm 0.0^{b}$	2.8 ± 0.8^{c}

^{a,b,c}Mean values per treatment with the same superscript within the same batch and column are not statistically different ($p > 0.05$).

^{A,B}Mean initial counts from different batches with the same superscript within the same column are not statistically different ($p > 0.05$).

For insects, no specific microbiological criteria exist for total bacterial endospore counts. Even in the action limits published by FASFC (Table S1.1, Supporting information), no recommendations were included. However, for insects and insect-based products, process hygiene action limits are described for e.g. total viable aerobic count at 30 °C and for the amount of Enterobacteriaceae, yeasts, moulds and *Bacillus cereus.* For all these action limits, the limits for primary, distribution and processing sector are the same. Since all counts determined in this experiment, except total viable aerobic counts and endospore counts, were easily reduced below their detection limits, the action recommendations for Enterobacteriaceae, yeasts, and moulds were easily achieved. Also the total viable aerobic counts were reduced below the low process hygiene limit (m) of 6 log cfu/g for all blanching times.

The action limit for *B. cereus* is of interest regarding the endospore counts, since it is the only endospore-forming bacterium included in the action limits. The limit is defined as n = 5, c = 2, m = 3.7 log cfu/g and M = 5 log cfu/g and referring to the official method of analysis to be used. In the Dutch guidance document (NVWA, 2014), it is advised to investigate insects periodically on the presence of certain pathogens, including *Bacillus cereus* where numbers should be below 5 log cfu/g or ml. It is noted by NVWA that this criterion does not apply for unprocessed, raw foods nor for processed foods that were not subjected to a microbial killing step but that will only be consumed after heating by the consumer. Consequently, the criterion can be considered for blanched but not for raw mealworms. In this study, no specific counts for *Bacillus cereus* (including both vegetative cells and spores) were performed nor were five samples per batch analysed. However, all total aerobic spore counts of blanched mealworms were below 5 log cfu/g (one of which above 3.7 log cfu/g), and therefore it can be reasoned -although the comparison is very rough- that also the *B. cereus* counts will likely be situated below the *B. cereus* criterion.

After blanching, the larvae were packaged in air and stored for 6 days in chilled conditions. The temperature during storage was 3.7 ± 1.7 °C as recorded by the temperature logger. The data in Table 5.1 show that there was no microbial growth during chilled preservation, except for a slight growth of mesophilic and psychrotrophic organisms in batch III (i.e. 40 s blanching). This is surprising, as one would expect that the longest blanching time reduces the microbiota

the most and results in the lowest growth during chilled preservation. For all three batches, the spore count was reduced during chilled preservation and the reductions were statistically significant for batches II and III. Possibly, part of the spores germinated during chilled preservation and contributed to other counts. From this first experiment, it can be carefully concluded that after blanching, mealworms can be kept for 6 days without spoilage under chilled conditions, but research using different temperatures and atmospheres is necessary to confirm this. Even though some microbial numbers can increase slightly during chilled preservation, they stay far below the spoilage level of 7 log cfu/g (Sperber & Doyle, 2009). While it is still being debated as from which microbial numbers in foods microbial spoilage can occur (and even if microbial spoilage can be related to certain microbial numbers, not taking into account spoilage metabolites), Sperber & Doyle (2009) proposed a general spoilage level for foods of 7 log cfu/g for the total viable count. At this threshold level, food spoilage can be observed by odour, taste or sight.

In a follow-up study based on the results presented here (Borremans et al., 2018), blanched (40 s) mealworms were subjected to a similar chilled storage up to 17 days. Similar results for microbial counts were obtained after blanching and after 7 days of storage, but as from day 7, a rapid increase of both total viable aerobic count and the number of Enterobacteriaceae was reported. The spoilage level of 7 log cfu/g was reached between day 7 and day 14 of storage. As a result, the second experiment in this chapter focussed on the effect of blanching and longer chilled preservation, especially on endospore-forming bacteria.

5.3.2 Effect of blanching and chilled preservation on the endospore-forming bacteria in the second experiment

5.3.2.1 Total viable and bacterial endospore counts

In the second experiment, a blanching treatment (40 s) on mealworms similar to that of experiment 1 was assessed. Since the experiment focussed on endospore-forming bacteria, also anaerobic bacterial spore counts and total anaerobic counts were included. As shown in Table 5.2, raw mealworms contained, as expected, a typically high total viable aerobic count, but also the total viable anaerobic count, which was not yet assessed for mealworms before, reached values of ± 7 to 8 log cfu/g. Although the insect samples used in the second

experiment originated from two other rearing companies than in the first one, the total viable counts show to be more or less comparable between different rearers. It was already reported that small differences between the companies may exist due to differences in insect feed, rearing environment and hygiene (Chapter 2). Bacterial spore counts, on the other hand, may vary to a larger extent between rearing companies as well as between and within batches (Stoops et al., 2016) (Chapter 2). Also in this experiment, aerobic bacterial endospores varied between 2.3 and 3.4 log cfu/g for raw mealworms. Anaerobic bacterial spores even showed a larger variation between 1.6 and 3.7 log cfu/g. It must be noted that aerobic and anaerobic microbial counts have an important overlap, since facultative anaerobic organisms are embedded in both counts.

Table 5.2 Microbial counts of mealworms before and after heat treatment. Data are the mean values of three replicates ± standard deviation.

Rearing company	Sample n°	Treatment	Microbial counts (log cfu/g)			
			Total viable aerobic count	Total viable anaerobic count	Aerobic bacterial endospores	Anaerobic bacterial endospores
1	1	None[1]	7.5 ± 0.1^{a,A}	7.4 ± 0.2^{a,A}	2.6 ± 0.5^{a,A}	N.D.[2]
		40 s blanching	1.8 ± 0.2^{b}	1.2 ± 0.3^{b}	< 1.0 ± 0.0^{b}	N.D.
	2	None	8.0 ± 0.0^{a,A}	7.7 ± 0.6^{a,A}	2.3 ± 0.0^{a,A}	1.6 ± 0.4^{a,A}
		40 s blanching	1.9 ± 0.2^{b}	1.4 ± 0.2^{b}	< 1.2 ± 0.4^{b}	< 1.4 ± 0.7^{a}
2	1	None	8.1 ± 0.1^{a,A}	8.1 ± 0.0^{a,A}	2.6 ± 0.9^{a,A}	2.6 ± 0.9^{a,A,B}
		40 s blanching	2.5 ± 1.0^{b}	< 2.0 ± 1.0^{b}	< 1.0 ± 0.0^{b}	< 1.0 ± 0.0^{b}
	2	None	7.7 ± 0.5^{a,A}	7.8 ± 0.5^{a,A}	3.4 ± 0.5^{a,B}	3.7 ± 0.6^{a,B}
		40 s blanching	4.4 ± 0.1^{b}	4.2 ± 0.2^{b}	3.3 ± 0.2^{a}	3.4 ± 0.1^{a}

[1]Initial count.
[2]N.D. = not determined.
^{a,b}Mean values per treatment with the same superscript within the same sample and column are not statistically different ($p > 0.05$). ^{A,B}Mean initial counts from different samples with the same superscript within the same column are not statistically different ($p > 0.05$).

When results before and after blanching are compared, a significant reduction in amount of total viable cells was observed, both for aerobic and anaerobic counts. This is comparable with the results of the first experiment for all different blanching temperatures. Surprisingly, the amount of endospores (aerobic and anaerobic) was significantly reduced as well for three out of four samples investigated. The fourth sample showed higher initial spore counts than the other three and these counts remained high after the heat treatment (but yet equal to the lower limit m for *B. cereus* in the FAVV action limits (Table S1.1, Supporting information)).

The low amount of colonies on the PCA plates in the former samples may have impeded a reliable calculation of spore counts, while the higher counts are more easily determined. Overall, in both the first and the second experiment, a subgroup of the bacterial composition which consists, for an important part, of endospores, survives the heating process applied in this study.

After the blanching treatment of 40 s, the second sample of rearing company 2 was subjected to a 24-day storage at 4.03 ± 0.74 °C, according to the temperature monitoring. Figure 5.1 shows the course of the microbial counts investigated during the chilled preservation. During the first 7 days of storage, total viable counts remained stable, similar to the chilled preservation of 6 days in the first experiment. As from day 11, however, the total counts started to rise and at day 14, they reached a value slightly below the 7 log cfu/g spoilage threshold (Sperber & Doyle, 2009). Compared to the findings observed by Borremans et al. (2018), the rapid increase of total viable count can be confirmed, although the microbial numbers at storage day 14 are slightly lower in this experiment. The amount of spores decreased slightly during the first 11 days of storage. They may have started to germinate into vegetative cells which, in turn, multiply. As from day 14 of storage, both total and spore counts remain constant until day 18, where the total counts start to rise slowly up to the initial counts of the raw insects at day 24. The amount of spores fluctuates as from day 18, where some spores may have germinated while also vegetative cells may have sporulated. Noteworthy is that, also as from day 18, the aerobic and anaerobic counts start to differ from each other. The aerobic environment during preservation was probably more suitable for aerobic organisms to multiply, after which these organisms could have sporulated as well.

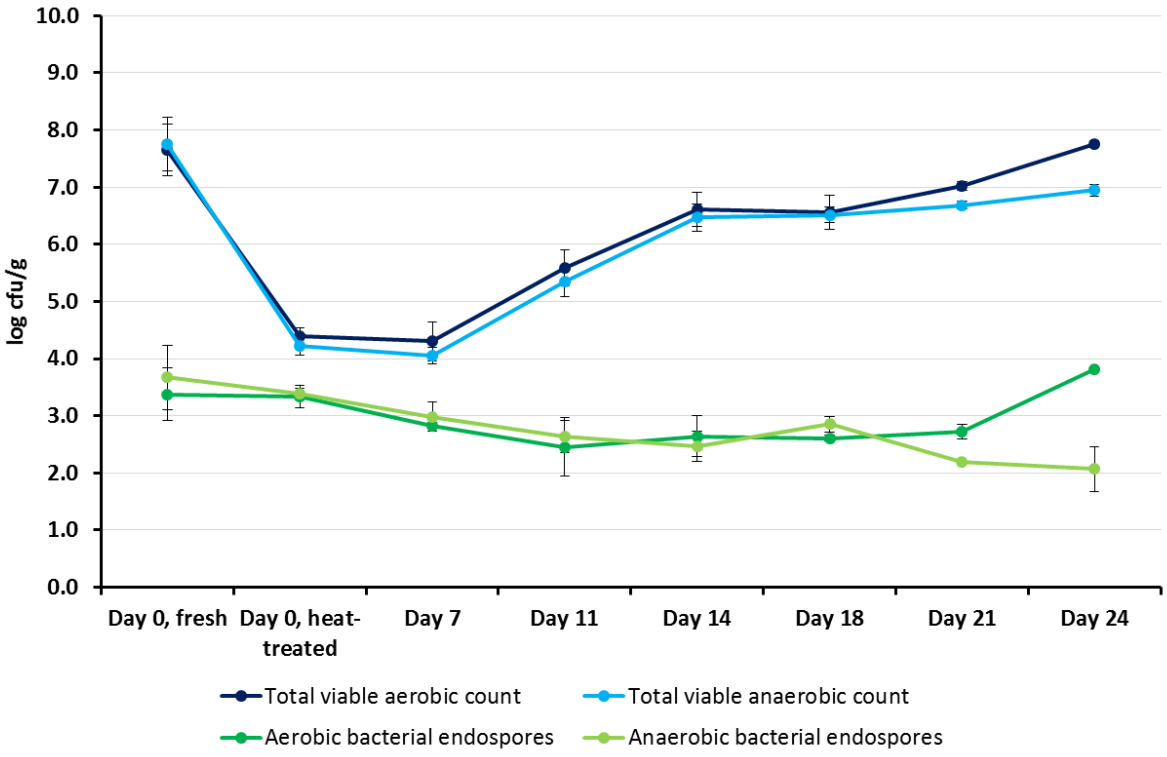


Figure 5.1 Microbial counts during chilled preservation of heat-treated mealworms. Error bars represent standard deviations.

Currently, edible insects are subjected to a heat treatment prior to further processing, with boiling or blanching being commonly used techniques. Since spores may be capable of surviving these heat treatments, they are able to cause the insect products to spoil, even in chilled conditions. After approximately 14 days, blanched and chilled insects nearly reach the level of spoilage. The survival of bacterial endospores, some of which may be pathogenic, thus poses a food safety risk.

To tackle the problem of surviving spores after a heat treatment, a small proof-of-concept tyndallisation experiment was executed (data not shown). To this end, mealworms were blanched during 40 s as described above and subsequently incubated at 37 °C for 2.5 hours to allow spores to germinate. Afterwards, a second identical heat treatment was applied, with the intention to kill the germinated vegetative cells. Tyndallisation has shown to effectively kill bacterial spores in the past (H. Kim et al., 2012), however, in our experiment, no or only small reductions of endospores were obtained (up to 0.4 log cfu/g reduction) after the second heating step. Optimisation to e.g. a longer tyndallisation treatment (3-step tyndallisation

and/or longer incubation times) may improve the reduction of spores, but may not be interesting from an economical and/or industrial point of view. Additionally, the two successive heating steps may be harmful for the nutritional, physicochemical and sensory quality of the mealworms (Fellows, 2009).

Alternative heat treatments such as steaming at a temperature above 100 °C or high pressure inactivation (Herdegen & Vogel, 1998) as well as an additional heat treatment prior to consumption (i.e. not a tyndallisation which comprises an incubation step between heat treatments) may reduce the food safety risks. Better heat treatment allows the chilled preservation to start with lower amounts of spores that then can germinate and the second heat treatment prior to consumption may kill the high amounts of vegetative cells that may have grown in the food.

5.3.2.2 Identification of bacterial endospores

As demonstrated by the microbial counts of both experiments, even a short heat treatment (e.g. blanching for 40 s or less) can result in a large reduction of microbial counts. One large exception is the group of bacterial endospores, which is capable of surviving a short term heat treatment as applied in this study. Whether or not this poses microbiological health risks for the consumer depends on the pathogenicity of the bacterial species present and their possibility to grow to numbers capable of causing foodborne illnesses.

The identification of the 50 bacterial isolates collected from the two batches of raw mealworms resulted in the detection of 8 OTUs (Table 5.3). Of the 50 isolates, 20 were identified as a species belonging to the *Bacillus cereus* group or *B. cereus sensu lato*. The *B. cereus* group consists, among other recently described new species, of the closely related bacteria *B. cereus sensu stricto*, *B. thuringiensis*, *B. weihenstephanensis, B. wiedmannii, B. anthracis*, *B. mycoides*, *B. pseudomycoides, B. cytotoxicus,* and *B. toyonensis* (EFSA Panel on Biological Hazards, 2016; Rasigade, Hollandt, & Wirth, 2018). Due to the close genetic relationships within the *B. cereus* group, it is hard to distinguish between the different species compiled in this group. Indeed, it was demonstrated that the 16 S rRNA gene is extremely similar (97.34 to 100% inter-species similarity) between all *B. cereus* group members (Ceuppens, Boon, & Uyttendaele, 2013; Liu et al., 2015). Moreover, also other typical

classification methods, e.g. based on virulence genes, are prone to discussion (Liu et al., 2015). The method applied for identification of bacterial endospores in this study was therefore insufficient to further discriminate the *B. cereus s.l.* isolates. Nevertheless, the results in Table 5.3 indicate a high presence of the *B. cereus* group and it is clear that most isolates of this group were obtained from rearing company 2 (i.e. rearer for pet food). However, given the random selection of spores from the plate count dishes, comparing both companies with respect to frequency of occurrence of the *B. cereus* group would not result in correct conclusions. Most *B. cereus s.l.* isolates (12 out of 20) were sorted in OTU 1. Next, one isolate was assigned each to OTUs 9 and 11. Finally six additional isolates that were not assigned to an OTU corresponded with a *B. cereus* group member according to BLAST analysis.

The *B. cereus* group contains many relevant species with respect to human health, agriculture or food safety. In the case of edible insects, presence of the well-known pathogen *B. cereus s.s.* may pose a severe food safety risk for consumers, but also some *B. weihenstephanensis* and *B. cytotoxicus* strains were reported as potential human (foodborne) pathogens (Guinebretière et al., 2013; Stenfors, Mayr, Scherer, & Granum, 2002). For insect rearing, *B. thuringiensis* may be harmful because of its insecticidal properties (Bravo et al., 2007). Whichever *B. cereus* group species were present in the mealworms, the *B. cereus* group poses an important threat for the edible insect sector and should be monitored. Also, given the fact that the *B. cereus* group contains psychrotrophic strains (Liu et al., 2015; Martínez et al., 2007), chilled preservation of heat-treated insects can still allow the growth of food pathogens such as *B. cereus s.s.*

Table 5.3 Identification of isolated spore-forming bacteria harboured by mealworms. Two batches were considered, each originating from another rearing company (termed 1 and 2).

Isolate number	Rearing company	Incubation	OTU assignment	BLAST identification	Bit score S'	Expected value	Sequence identity (%)
1	1	Anaerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
2	1	Anaerobic	None[1]	*Bacillus sp.* (non-cereus group)	1088.8	0.0	589/589 (100.0)
3	1	Anaerobic	None	*Bacillus sp.*	643.754	2.78×10^{-180}	537/630 (85.2)
4	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
5	1	Anaerobic	OTU 9	*Bacillus cereus* group	817.339	0.0	657/761 (86.3)
6	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
7	1	Aerobic	None	*Bacillus sp.* (non-cereus group)	1820.07	0.0	993/997 (99.6)
8	1	Aerobic	OTU 4	*Brevibacillus laterosporus*	1404.57	0.0	760/760 (100.0)
9	1	Aerobic	None	*Bacillus sp.* (non-cereus group)	1160.82	0.0	632/634 (99.7)
10	1	Anaerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
11	1	Aerobic	None	*Brevibacillus laterosporus/halotolerans*	1123.88	0.0	610/611 (99.8)
12	1	Aerobic	None	*Lysinibacillus sp.*	1153.43	0.0	624/624 (100.0)
13	1	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
14	1	Aerobic	None	*Bacillus sp.* (non-cereus group)	579.122	7.62×10^{-161}	330/338 (97.6)
15	1	Aerobic	None	*Brevibacillus laterosporus*	789.64	0.0	427/427 (100.0)
16	1	Aerobic	None	*Bacillus cereus* group	424.003	3.78×10^{-114}	263/279 (94.3)
17	1	Aerobic	OTU 4	*Brevibacillus laterosporus*	1404.57	0.0	760/760 (100.0)
18	1	Aerobic	None	*Bacillus sp.* (non-cereus group)	1072.18	0.0	580/580 (100.0)
19	1	Aerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
20	1	Aerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
21	1	Aerobic	OTU 4	*Brevibacillus laterosporus*	1404.57	0.0	760/760 (100.0)
22	1	Aerobic	OTU 6	*Bacillus subtilis*	1216.22	0.0	729/758 (96.2)
23	1	Aerobic	OTU 10	*Bacillus pumilus*	1062.94	0.0	705/762 (92.5)
24	1	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
25	1	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)

26	2	Aerobic	None	*Bacillus cereus* group	1040.78	0.0	563/563 (100.0)
27	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
28	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
29	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
30	2	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
31	2	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
32	2	Aerobic	None	*Lysinibacillus sp.*	1002.0	0.0	542/542 (100.0)
33	2	Anaerobic	None	*Bacillus cereus* group	1035.24	0.0	563/564 (99.8)
34	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
35	2	Anaerobic	None	*Bacillus cereus* group	1171.9	0.0	634/634 (100.0)
36	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
37	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
38	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
39	2	Aerobic	None	*Bacillus cereus* group	1040.78	0.0	563/563 (100.0)
40	2	Aerobic	None	*Bacillus cereus* group	961.378	0.0	522/523 (99.8)
41	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
42	2	Aerobic	OTU 4	*Brevibacillus laterosporus*	1404.57	0.0	760/760 (100.0)
43	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
44	2	Aerobic	None	*Lysinibacillus sp.*	1044.48	0.0	565/565 (100.0)
45	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
46	2	Aerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
47	2	Aerobic	OTU 11	*Bacillus cereus* group	983.538	0.0	692/759 (91.2)
48	2	Anaerobic	None	*Lysinibacillus fusiformis/sphaericus*	1112.8	0.0	664/690 (96.2)
49	2	Anaerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
50	2	Anaerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)

[1]Not assigned to an OTU.

The pathogenicity of *B. cereus s.s.* in chilled heat-treated insects depends on several factors. *B. cereus s.s.* knows two pathogenic pathways, both involving toxin production (Ehling-Schulz, Frenzel, & Gohar, 2015). A first toxin, cereulide, is produced in the food matrix and is heat-resistant, while the second pathway involves consumption of *B. cereus s.s.* cells and production of several heat-sensitive enterotoxins in the gastrointestinal tract. For both pathways, a minimal amount of approximately 5 log cfu/g *B. cereus s.s.* cells (i.e. the upper (M) FASFC action limit, Table S1.1, Supporting information) is necessary to produce the toxins (Berkeley, Heyndrickx, Logan, & De Vos, 2008; Finlay, Logan, & Sutherland, 2000). Also, for cereulide production, temperature may be important, as well as the food matrix in which *B. cereus s.s.* resides (Agata, Ohta, & Yokoyama, 2002).

A few previous studies that report on the microbiota harboured by edible insects also encountered members of the *B. cereus* group. For example, *B. cereus* and *B. weihenstephanensis* were detected by PCR-DGGE (Denaturing Gradient Gel Electrophoresis) in edible processed (boiled and dried) mealworms and crickets and in cricket powder bought in Belgium and the Netherlands (Osimani, Garofalo, Milanović, et al., 2017). Another study by Fasolato et al. (2018) focussed on the prevalence of *B. cereus* group members in processed (freeze-dried, dried, roasted and/or cooked) edible insects (crickets, mealworms, mole crickets and silk worms). *B. cereus* counts were determined on mannitol egg yolk polymyxine (MYP) agar and colonies were identified through 16 S rRNA gene sequencing combined with analysis of selected housekeeping and virulence genes. All investigated samples produced *B. cereus* colonies on MYP agar, some up to 6.6 log cfu/g. Sequencing and biomolecular identification of MYP (and other) isolates could identify *B. cytotoxicus*, *B. cereus s.s.* and *B. thuringiensis*.

The second most abundant OTU (OTU 2, 7/50 isolates, 14%) was assigned to *Lysinibacillus fusiformis* or its relative *L. sphaericus*. A *Lysinibacillus* sp. was also detected previously in edible insects by Fasolato et al. (2018). This spore former, renamed from *Bacillus fusiformis/sphaericus* (Ahmed, Yokota, Yamazoe, & Fujiwara, 2007), rarely acts as a human pathogen, but especially *L. sphaericus* has been reported as a potent insect pathogen. *L. sphaericus* preferably targets mosquitos, but activity against other insects species was observed as well (Berry, 2012).

OTU 3 (5/50 isolates, 10%) corresponded with a *Bacillus* sp. other than the *B. cereus* group. According to the BLAST results, OTU 3 may correspond to several species including *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*. The latter three species are closely related and together, comparable to the *B. cereus* group, sometimes called the *B. subtilis* group (L. T. Wang, Lee, Tai, & Kasai, 2007). Other bacteria related to *B. subtilis* encountered in the BLAST results for OTU 3 were e.g. *B. pumilus*, *B. tequilensis*, and *B. velezensis*. As was the case with the *B. cereus* group, the 16 S rRNA gene does not allow for species-level identification for *B. subtilis* and its related species (L. T. Wang et al., 2007). *B. subtilis* and its relatives are generally not considered as human pathogens, but may act as spoilage organisms instead (De Vos et al., 2009). They are also frequently described as beneficial for plants and/or animals e.g. as biocontrol organism or probiotic (Serrano, Manker, Brandi, & Cali, 2013; Tactacan, Schmidt, Miille, & Jimenez, 2013), which may pose opportunities for industrial valorisation. *Bacillus* species were regularly found in edible insects (Fasolato et al., 2018; Klunder et al., 2012; Osimani, Garofalo, Milanović, et al., 2017; Osimani, Milanović, Cardinali, Garofalo, et al., 2018; Y. Wang & Zhang, 2015), however, due to the difficulty and uncertainty of identification, rarely described on species level.

Finally, four isolates (8%) were ascribed to *Brevibacillus laterosporus* (OTU 4). *B. laterosporus* has also been reported as insect pathogen (Ruiu, 2013). In Chapter 3, a *Brevibacillus* sp. was already detected by Illumina sequencing in mealworms, in abundances up to 28% in sample MW 1.2 (Figure S3.1, Supporting information). Together with *B. thuringiensis*, to which OTUs 1, 9 and/or 11 might be assigned, and *Lysinibacillus sphaericus* that might correspond with OTU 2, a realistic risk for edible insect rearing exists when these entomopathogenic OTUs are present. On the other hand, *B. laterosporus* has also been described as a broad-spectrum antimicrobial species against phytopathogenic bacteria and fungi (Ruiu, 2013), which may pose agricultural benefits.

While both aerobic and anaerobic bacterial endospore counts were performed and isolates were picked randomly from all plates, it surprises that only aerobic and/or facultative anaerobic organisms were identified. Anaerobic spore formers such as specific *Clostridium* spp. were already encountered in edible insects, including mealworms (Garofalo et al., 2017; Osimani, Garofalo, Milanović, et al., 2017; Osimani, Milanović, Garofalo, et al., 2018; Stoops

et al., 2016). The genus *Clostridium* is of concern regarding food safety, since it contains the food pathogens *C. perfringens* and *C. botulinum* (Madigan et al., 2009). Also in Chapter 3, OTUs that correspond with bacterial classes (Clostridia) or orders (Clostridiales) which may contain spore-forming anaerobes were reported, yet obtained from different samples. All these previous observations of (possible) anaerobic spore-forming bacteria were, however, based on culture-independent methods. The method used to isolate axenic bacterial cultures may not have been sufficient to retain the strict anaerobic species that might have been present in the samples investigated. Accordingly, the isolation and identification of anaerobic bacterial spore formers would form an interesting addition to this research.

5.4 Conclusions

The objective of this study was to investigate blanching and chilled preservation of mealworms from a microbiological perspective, with focus on bacterial endospores. Regardless of treatment times, blanching resembles a pasteurisation treatment, i.e. killing vegetative cells but not or hardly spores. Blanched mealworms can be stored in chilled conditions for approximately 7 days without substantial microbial growth. Afterwards, microbial growth may occur, up to the initial count of untreated larvae of approximately 8 log cfu/g after 24 days. The general spoilage level of 7 log cfu/g is almost reached after 14 days of storage. During the whole chilled preservation, bacterial spores are capable of germinating and growing out.

Identification of bacterial endospores present in raw mealworms revealed that many isolates may belong to the *B. cereus* group. Further research based on PCR specifically designed to detect or quantify *B. cereus* can be lead to identification of the isolates to species level. Since this group consists of several pathogens for either humans or insects themselves, the presence of spores in insects poses risks for consumption as well as rearing. Based on the obtained results, a few recommendations can be formulated. To reduce the amount of endospores in larvae, a better heat treatment may be necessary. It is not clear whether measures can be taken earlier in the chain, during the rearing process. The relation between the microbiota in the feed and the microbiota in the insects has not been studied extensively so far and was also beyond the scope of this study. Next, during chilled preservation, levels of

microorganisms should not exceed the threshold level for pathogens to cause food infection and/or intoxication. For *B. cereus s.s.* this threshold is 5 log cfu/g, which is also the upper limit (M) of the action limits defined by the FASFC. During chilled preservation in this study, the 5 log cfu/g threshold was reached between 7 and 11 days of storage. Finally, a heating step immediately before to consumption is useful to kill (part of) the microorganisms that were able to grow during chilled preservation, thus lowering the risk of food infection. While the heat-resistant cereulide toxin of *B. cereus* will not be destroyed or inactivated by these measures, the bacterial cells and/or spores may be affected.

Chapter 6 Microbiological assessment of processing and preservation of crickets

A part of this chapter[1] was published as Vandeweyer D., Wynants E., Crauwels S., Verreth C., Viaene N., Claes J., Lievens B., & Van Campenhout L. (2018). Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House Crickets (*Gryllodes sigillatus*) for Human Consumption. *Applied and Environmental Microbiology*, *84* (12), e00255-18. http://doi.org/10.1128/AEM.00255-18

6.1 Introduction

As already shown in Chapter 5 as well as in previous research (Klunder et al., 2012; Rumpold et al., 2014; Stoops et al., 2017), postharvest processing of insects has a major impact on the microbial load. Processing techniques such as blanching, cooking, freezing, oven drying, and freeze-drying are presently applied for edible insects (Fombong et al., 2017; Hanboonsong, Jamjanya, & Durst, 2013; van Huis et al., 2013; Wynants et al., 2018) (Chapter 5). Usually, companies rearing edible insects produce different end products from the same insect species, including frozen, dried, and seasoned insects. Many of these products are already introduced into the market. Nevertheless, the impact of the processing steps on the microbiological quality of the products has not yet been thoroughly assessed. Additionally, given their different production processes, which likely result in different intrinsic properties per product, each product may differ in shelf life. Therefore, research is needed on the microbiological stability of these different insect products. Whereas the previous chapter concentrated on mealworms, this chapter envisages crickets, with different post-harvest processing as to mealworms.

The first goal of this study was, comparable to experiment 2 in chapter 5, to specifically investigate the identities and fate of bacterial endospores present in freshly reared, raw

[1] The paper on which a part of Chapter 6 was based (Vandeweyer et al., 2018), consists of two parts. The first part of the paper, describing microbial dynamics during cricket rearing, is not included in this chapter. The second part regarding processing and preservation of fully grown crickets is included. D.V and E.W. are joint co-authors: E.W. mainly responsible for the first part and D.V. for the second. Additionally, Chapter 6 was supplemented with data not published so far regarding the effect of blanching and chilled preservation on spore-forming bacteria harboured by crickets.

Acheta domesticus crickets (with common name and further referred to as 'house cricket') and during (laboratory scale) blanching and subsequent chilled preservation. While a few insect rearing and food safety risks related to bacterial endospores were revealed for mealworms (Chapter 5), the different microbiological quality of cricket species (Chapters 2 and 3) does not allow for an unfounded generalisation of those conclusions. Hence, a similar assessment specifically targeting crickets was performed in a first experiment.

A second experiment investigated the microbial dynamics, including microbial numbers and bacterial composition, from industrial processing and preservation of *Gryllodes sigillatus* crickets (with common name and further referred to as 'tropical house cricket') reared for human consumption. For this purpose, fully grown (industrially reared), raw crickets and crickets during processing were analysed, as well as three end products after packaging as they are commercialised, including crickets that were frozen, oven-dried, and smoked and subsequently oven-dried (smoked/dried). Next, these products were analysed during their 6-month shelf life, as proposed by the manufacturer. To this end, samples were investigated for their intrinsic parameters, microbial numbers and bacterial composition.

6.2 Materials and methods

6.2.1 Sampling, blanching and chilled preservation of the house cricket

For the first experiment, performed at laboratory scale, one batch of raw house crickets (1.5 kg) was obtained each from two different rearing companies in Belgium. Dead insects were removed prior to analysis. For each batch, 2 samples (i.e. two batches x two samples) were subjected to a 90 s blanching step without subsequent cooling, using an insect/boiling water ratio of 1:4. Given the different dimensions of crickets compared to mealworms, a longer blanching time of 90 s was determined in a preliminary experiment (data not shown). All samples were used for microbiological analysis before and after blanching as well as for bacterial endospore isolation.

After blanching, one batch was further used for the chilled storage experiment in a home-type refrigerator (Miele, Belgium) with set point 3 °C. Six bags (packed in air as described in paragraph 5.2.2) with 100 g of crickets were stored for a period of 24 days. One bag was used

for (destructive) analyses on day 7, 11, 14, 18, 21 and 24. During the entire preservation (chilled and frozen), temperatures were monitored using data loggers (Escort iLog internal sensor, VWR International, Leuven, Belgium).

6.2.2 Microbiological analysis of the house cricket and endospore identification

All cricket samples were pulverised prior to the analyses as described by Stoops et al. (2016). Next, samples taken before and after blanching as well as during chilled preservation were analysed for total viable aerobic and anaerobic counts and for aerobic and anaerobic endospore counts. The microbial counts were performed in threefold as described in Chapter 5.

From the aerobic and anaerobic spore count plates, 95 isolates were randomly obtained using the exact same method as described in paragraph 5.2.4. After extracting their DNA using the phenol-chlorophorm method described by Lievens et al. (2003), the 16 S rRNA gene was amplified by PCR (27F - 1492R primer combination, see paragraph 5.2.4) and sequenced by Macrogen Europe (Amsterdam, the Netherlands) using the 1492R primer. Also here, obtained results were grouped into OTUs with 98% sequence identity. Of the 95 cricket isolates, 74 were assigned to an OTU. Next, both the OTUs and the individual unassigned isolates were identified using the BLAST tool against GenBank (excluding uncultured/environmental entries). Isolates and OTUs not corresponding with spore-forming bacteria were discarded, which resulted in 92 identified spore-forming isolates.

6.2.3 Industrial production, sampling and preservation of tropical house crickets

Prior to the analyses described in this PhD dissertation (second experiment), a complete rearing cycle in a Belgian company rearing crickets for human consumption was monitored and microbiologically investigated (data not shown). An overview of the whole industrial cricket production process, including post-harvest treatments, is given in Figure 6.1A and was described in detail in Vandeweyer et al. (2018). After harvesting the crickets, they were killed by submersion in hot water (60 °C) and rinsed (5 min) with regular tap water. Next, they were

given a heat treatment by placing them in a kettle with boiling water and keeping them submerged until the water boiled again (after 5 to 10 minutes). Finally, heat-treated crickets were further processed into three end products. Crickets were either frozen to -20 °C (frozen crickets), oven-dried overnight at 80 °C (dried crickets) or smoked. The smoking process involved a combination of salting (submerging in brine of 62.5 g NaCl/litre for 40 min), freezing to -20 °C (crickets were stored in the freezer until they were smoked), thawing, smoking (traditional beech wood smoker for 40 min at 80 °C) and finally oven drying overnight at 80 °C (smoked/dried crickets).

For the second experiment, crickets were sampled and analysed immediately after harvest and after the heat treatment applied by the rearing company (Figure 6.1B). At every sampling point, three replicates (50 g) were obtained per sample (i.e. 3-fold sampling). After processing, nine packed samples of all three end products (frozen (50 g), dried (8 g) and smoked/dried crickets (8 g)) were obtained as well. Three packages of the finished products were analysed immediately after sampling, while the remaining samples were kept aside for long-term storage and evaluated in threefold after three and six months of storage (Figure 6.1B). Frozen cricket samples were stored in sealed plastic bags (VAC090 PA/PE 20/70, thickness 80 µm, width 20 cm, Euralpack, Schoten, Belgium; sealed with a C 200 Multivac packing machine, Mechelen, Belgium) at -25 °C. Dried and smoked/dried cricket samples were stored at ambient temperature in individual glass tubes with cork stop (which is the packaging used by the company to commercialise the crickets).

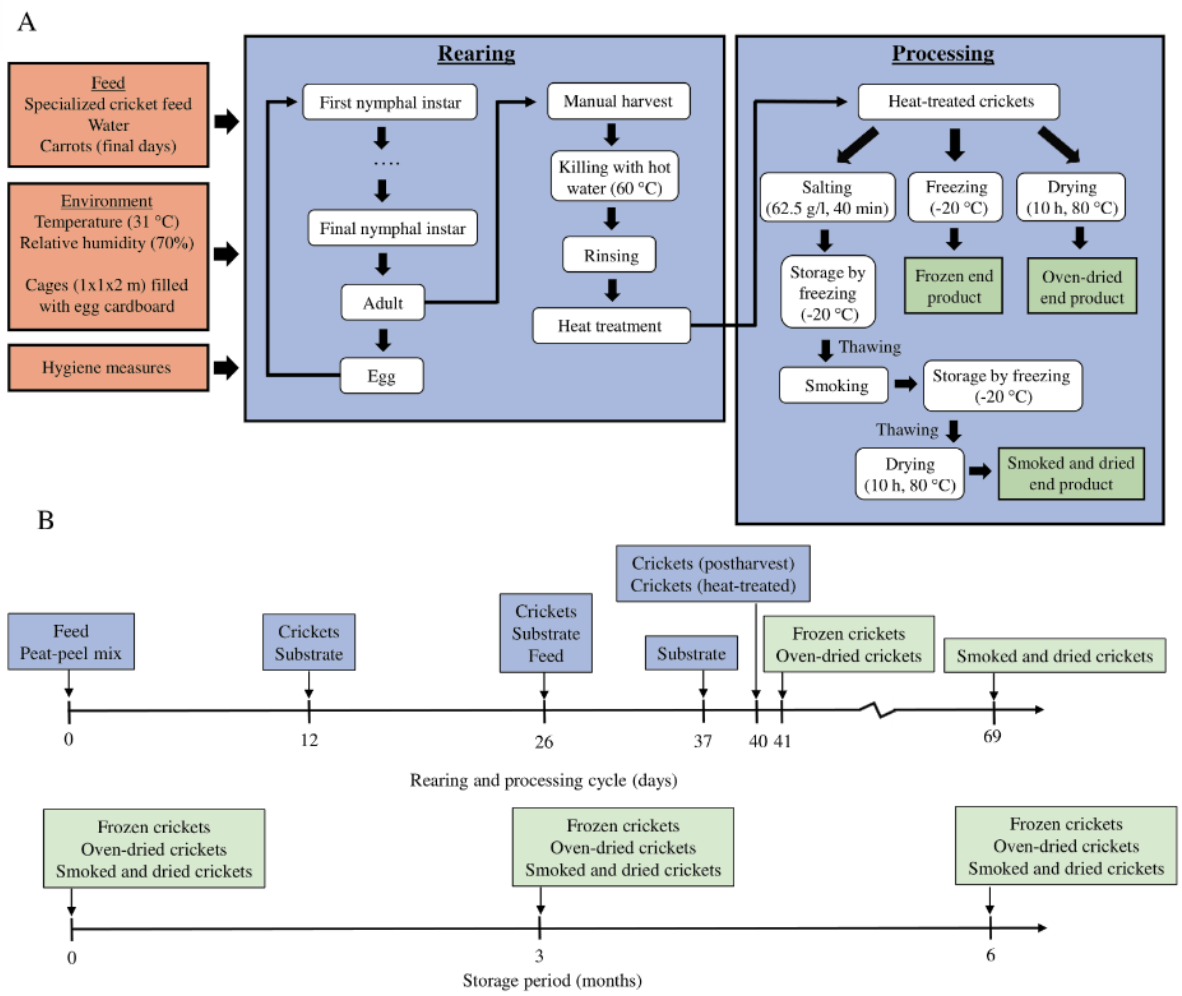


Figure 6.1 (A) Schematic representation of the rearing and processing cycle of tropical house crickets. The three final end products are depicted in green. The rearing period, from first instar to harvested adult cricket, took 40 days. In the manual harvesting step, crickets were harvested by shaking them out of the cardboard trays into a circular plastic container. The crickets were then killed by submerging them in hot water inside the container. The crickets were then rinsed thoroughly in a colander using running tap water for 5 min per batch. For the heat treatment step, the crickets were submerged in boiling water and until the water boiled again, which took 5 to 10 min. The crickets were salted after heat treatment by submerging them per batch in 4 litres of salted water (62.5 g/litre) for 40 min. Crickets were smoked using a traditional beech wood smoker for 40 min at 80 °C. In the drying step, crickets were spread out over a baking tray and dried overnight (10 h at 80 °C). (B) Sampling plan throughout rearing, processing, and preservation. Analyses of feed, peat-peel mix, substrate and crickets during rearing were not included in this PhD dissertation.

6.2.4 Microbiological analysis of the tropical house cricket

Similar to experiment 1, all analyses on tropical house crickets were executed on pulverised samples, after removal of dead insects. Frozen end products were thawed for 4 hours at 3 °C before pulverisation. Intrinsic parameters pH, water activity and moisture content were

analysed as described in Chapter 2. Additionally, for raw and heat-treated crickets, total viable aerobic count and the number of Enterobacteriaceae, lactic acid bacteria, aerobic endospores and fungi were determined. During preservation of the end products, the total viable count and the number of Enterobacteriaceae, endospores and fungi was monitored. All microbiological analyses were performed according to the ISO standard compiled by Dijk et al. (2015) as described in either Chapter 2 or Chapter 5. For the determination of lactic acid bacteria, sorbic acid (0.14%) was added in this experiment to prevent fungal growth.

The bacterial composition of the raw and heat-treated cricket samples as well as the three end products was determined using Illumina MiSeq sequencing of partial 16S rRNA gene amplicons (V4 region, 250 bp). To this end, two replicates of each sample were pulverised as described for the intrinsic properties and plate count analyses. Subsequently, DNA extraction, PCR amplification (primer design shown in Table S6.1, Supporting information), library preparation, sequencing, sequence processing and diversity analyses were performed as described by Wynants et al. (2018). For each pulverised replicate, genomic DNA was extracted in duplicate, resulting in a total of 4 DNA extracts per sample. Downstream diversity analyses used data rarefied to 1700 sequences per DNA extract. For the harvested (raw) and the smoked/dried crickets, only two DNA extractions (of one replicate) delivered useful sequences; the others were not retained for data analysis. Sequences were clustered into Operational Taxonomic Units (OTUs) based on a 97% similarity cut-off as proxies for species. The taxonomic origin of each OTU was determined up to genus level with the SINTAX algorithm implemented in USEARCH (Edgar, 2016) based on the Silva Living Tree Project v123 (LTP v123) database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.80 (Table S6.2, Supporting information). In case genus level could not be determined reliably (bootstrap value < 0.80) based on the Silva database, OTU representative sequences were compared to the nucleotide database in GenBank (excluding uncultured/environmental entries; Table S6.3, Supporting information). Chao1 and Shannon-Wiener diversity indices were calculated using the R package Phyloseq (v. 1.19.0) (R Development Core Team, 2013).

6.2.5 Statistical analyses

Differences in microbial counts before and after blanching in the first experiment were analysed using independent samples t-tests, while initial counts between samples were investigated using one-way ANOVA. For the second experiment, differences in the intrinsic parameters, microbial counts and diversity parameters (OTU richness, Chao1, coverage and Shannon-Wiener indices) for raw crickets as well as during processing and preservation of the crickets were analysed by one-way ANOVA followed by Tukey's post hoc test. In case of unequal variances, Welch's ANOVA with Games-Howell post hoc test was used. All tests were performed with SPSS Statistics 23 (IBM, New York, NY, USA) and considered significant at a p-value below 0.05.

6.2.6 Accession numbers

Sequences obtained from the Illumina Miseq platform were deposited in a Sequence Read Archive (SAMN08032682 - SAMN08032721) under BioProject accession PRJNA418072 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA418072).

6.3 Results and discussion

6.3.1 Effect of laboratory blanching and chilled preservation on the endospore-forming bacteria harboured by house crickets in the first experiment

6.3.1.1 Total viable and bacterial endospore counts

The house crickets used in the first experiment harboured total viable aerobic and anaerobic counts ranging between 7.8 and 8.7 log cfu/g, both aerobic and anaerobic counts being very similar within the same sample (Table 6.1). Likewise, aerobic and anaerobic bacterial endospores varied only slightly within the same sample and ranged between 2.2 and 4.2 log cfu/g over all samples. As frequently discussed already, both in previous chapters and in literature, high total viable counts and varying bacterial endospore counts are common for raw edible insects. Also for the house crickets considered in experiment 1, similar

observations could be made. Noteworthy was the significant difference in endospore counts (both aerobic and anaerobic) between both rearers. This difference between insect producers was also described in Chapter 2.

Table 6.1 Microbial counts of house crickets before and after heat treatment. Data are the mean values of three replicates ± standard deviation.

Rearing company	Sample n°	Treatment	Microbial counts (log cfu/g)			
			Total viable aerobic count	Total viable anaerobic count	Aerobic bacterial endospores	Anaerobic bacterial endospores
1	1	None[1]	8.7 ± 0.3^{a,A}	8.5 ± 0.2^{a,A}	3.1 ± 0.1^{a,A}	3.3 ± 0.5^{a,A,B}
		90 s blanching	2.3 ± 0.3^{b}	1.5 ± 0.0^{b}	1.8 ± 0.4^{b}	1.6 ± 0.5^{b}
	2	None	8.3 ± 0.0^{a,A}	8.3 ± 0.1^{a,A}	3.4 ± 0.5^{a,A}	2.2 ± 0.7^{a,A}
		90 s blanching	2.4 ± 0.1^{b}	2.3 ± 0.5^{b}	1.7 ± 0.2^{b}	1.0 ± 0.0^{b}
2	1	None	8.7 ± 0.3^{a,A}	8.7 ± 0.3^{a,A}	4.2 ± 0.5^{a,B}	3.9 ± 0.9^{a,C}
		90 s blanching	5.5 ± 0.6^{b}	5.4 ± 0.7^{b}	3.9 ± 0.5^{a}	3.9 ± 0.7^{a}
	2	None	7.8 ± 0.0^{a,A}	7.8 ± 0.0^{a,A}	3.9 ± 0.4^{a,B}	4.0 ± 0.9^{a,B,C}
		90 s blanching	2.8 ± 0.1^{b}	2.7 ± 0.1^{b}	3.1 ± 0.7^{a}	2.4 ± 0.6^{a}

[1]Initial count.

^{a,b}Mean values per treatment with the same superscript within the same sample and column are not statistically different ($p > 0.05$). ^{A,B}Mean initial counts from different samples with the same superscript within the same column are not statistically different ($p > 0.05$).

After blanching, as expected, the total viable counts were drastically (up to 6.4 log cfu/g reduction) and significantly reduced for all samples. For sample 1 from company 2, the decrease in total viable count was the smallest, leaving up to 5.5 log cfu/g microorganisms, probably in particular as a result from the high amount of bacterial spores (which were able to survive the heat treatment) in this sample. This count, however, is still below the lower (m) action limit for total viable aerobic count recommended by the FASFC (Table S1.1, Supporting information). The number endospores, on the other hand, was clearly reduced (up to 1.7 log cfu/g reduction) after blanching for samples from company 1, while endospore counts in samples from company 2 experienced no or only a small, insignificant reduction (Table 6.1). This may suggest that not all endospores present have a similar heat-resistance, which was already documented for different spore-forming species (Kort et al., 2005; Luu-Thi, Khadka, & Michiels, 2014). From the obtained results, it can be concluded that the blanching treatment applied clearly reduced the total viable counts, but only marginally the amount of bacterial endospores, similar to what was observed for mealworms in Chapter 5.

Changes in total viable and bacterial endospore counts during chilled preservation of sample 1 obtained from company 2 are displayed in Figure 6.2. After a first reduction as a result from the blanching treatment, total viable counts declined further to approximately 4 log cfu/g at the 11th day of storage. Also the amount of bacterial spores followed the same pattern. Consequently, when blanched crickets were stored in chilled conditions in this experiment, a more or less microbiologically stable product could be retained up to 11 days. After day 11, both total counts started to rise slightly, but never rose above the spoilage threshold of 7 log cfu/g (Sperber & Doyle, 2009). Based on the total counts, crickets could be stored for a longer period of time until spoilage occurs in comparison with the chilled preservation of mealworms (paragraph 5.3.2). However, the presence of pathogenic species in the crickets was not assessed during the storage experiment. While at least a fraction of the total endospore count may survive a short heat treatment (Table 6.1) and given the possible presence of pathogenic endospore-forming bacteria in that subpopulation, a food safety risk may exist, even at total viable counts below 7 log cfu/g.

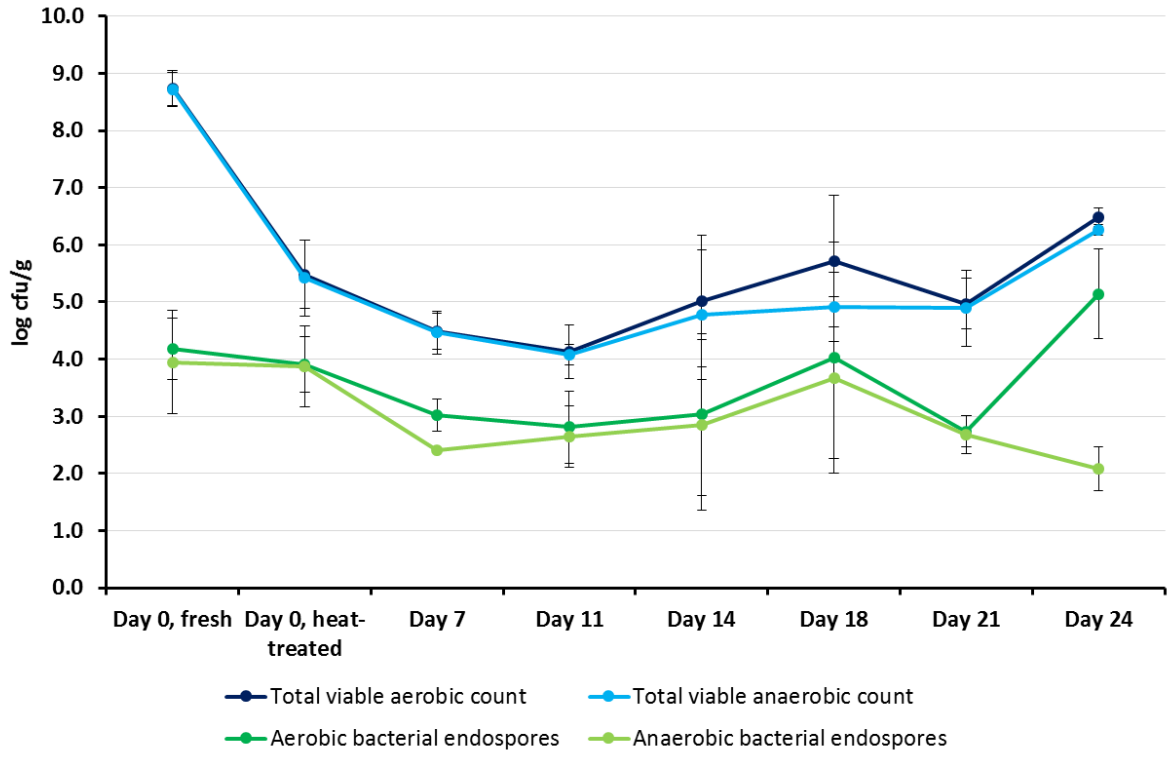


Figure 6.2 Microbial counts during chilled preservation of heat-treated house crickets. Error bars represent standard deviations.

In this experiment, the amount of bacterial spores remained stable till day 14 of storage. From then on, the counts became more variable, showing both sporulation and germination. At day 24 of storage, a distinct difference between aerobic and anaerobic endospore counts was observed. The aerobic storage conditions may have favoured the aerobic spore-forming bacteria in growth and sporulation.

6.3.1.2 Identification of bacterial endospores

The 92 endospore-forming isolates that were identified were assigned to 5 OTUs (Table 6.2, Figure 6.3). Three of these OTUs (OTU 1, 7 and 14) corresponded, according to GenBank, with a member of the *Bacillus cereus* group. In total, 63 isolates were assigned to a *B. cereus* group organism and an additional 14 isolates that were not grouped into an OTU corresponded with a *B. cereus* group member. In total, 84% of all isolates were identified as *B. cereus* group species. The second most abundantly present OTU was OTU 2 (7%), corresponding with *Lysinibacillus fusiformis/sphaericus* and the other isolates were grouped into OTU 3 (*Bacillus sp.,* non-cereus; 3%).

The results of the identification of spore-forming bacteria not only suggest that the recovered species were able to reside and perhaps multiply (prior to spore formation) in the crickets, but also indicate an important risk for cricket rearing and consumption. The *B. cereus* group includes the food pathogen *B. cereus* and the insect pathogen *B. thuringiensis*, together with other species that were previously described as possibly harmful for human or insect (see also paragraph 5.3.2.2). Moreover, the ability of spore formation and their possible survival of heat treatments applied, increases the food safety risks. Accordingly, high abundances of these species are not desired. Also the possible presence of the insect pathogen *L. sphaericus* poses a risk for the cricket sector.

Similar to the isolates obtained from mealworm samples (paragraph 5.3.2.2), the identification results from the house crickets suggest a high abundance of *B. cereus* group members within the group of endospores. However, as is clear from Figure 6.3, the relative abundance in crickets is substantially higher compared to that in mealworms. This may indicate that crickets are more susceptible to colonisation with *B. cereus* group members. On the other hand, the random selection of endospores from the plates may have resulted in a

coincidental disproportionate composition of the viable endospores. Anyhow, it is sure that at least *B. cereus* group members, *Lysinibacillus fusiformis/sphaericus* and a *Bacillus species* (non-cereus) may be present as spores in house crickets reared for human consumption. *Brevibacillus laterosporus* and *Bacillus pumilus*, two OTUs retrieved from mealworm samples, were not recovered from crickets. Additionally, as was also the case in Chapter 5 for mealworms, no endospores of anaerobic bacteria (such as specific *Clostridium* spp.) were retrieved from crickets using the technique applied in this experiment (Table 6.2), while the order of Clostridiales was found in the research described in Chapter 3.

Table 6.2 Identification of isolated spore-forming bacteria harboured by house crickets.

Isolate number	Rearing company	Incubation	OTU assignment	BLAST identification	Bit score S'	Expected value	Sequence identity (%)
1	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
2	1	Anaerobic	None[1]	*Bacillus cereus* group	1171.9	0.0	634/634 (100.0)
3	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
4	1	Anaerobic	None	*Bacillus cereus* group	985.385	0.0	533/533 (100.0)
5	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
6	1	Aerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
7	1	Aerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)
8	1	Aerobic	OTU 7	*Bacillus cereus* group	771.173	0.0	493/527 (93.5)
9	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
10	1	Aerobic	None	*Bacillus cereus* group	1171.9	0.0	634/634 (100.0)
11	1	Aerobic	None	*Bacillus cereus* group	1040.78	0.0	563/563 (100.0)
12	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
13	1	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
14	1	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
15	1	Aerobic	None	*Lysinibacillus fusiformis/sphaericus*	1044.48	0.0	565/565 (100.0)
16	1	Anaerobic	None	*Bacillus cereus* group	966.918	0.0	523/523 (100.0)
17	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
18	1	Anaerobic	None	*Bacillus cereus* group	1168.2	0.0	632/632 (100.0)
19	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
20	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
21	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
22	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
23	1	Aerobic	None	*Bacillus cereus* group	989.078	0.0	535/535 (100.0)
24	1	Aerobic	None	*Bacillus cereus* group	1040.78	0.0	563/563 (100.0)
25	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
26	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
27	1	Anaerobic	None	*Bacillus cereus* group	1142.35	0.0	618/618 (100.0)

28	1	Aerobic	None	*Lysinibacillus sp.*	983.538	0.0	532/532 (100.0)
29	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
30	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
31	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
32	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
33	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
34	1	Anaerobic	None	*Bacillus cereus* group	808.106	0.0	437/437 (100.0)
35	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
36	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
37	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
38	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
39	1	Aerobic	None	*Bacillus cereus* group	1027.86	0.0	556/556 (100.0)
40	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
41	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
42	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
43	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
44	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
45	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
46	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
47	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
48	1	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
49	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
50	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
51	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
52	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
53	1	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
54	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
55	1	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
56	1	Anaerobic	OTU 3	*Bacillus sp.* (non-cereus group)	1408.27	0.0	762/762 (100.0)

57	2	Aerobic	None	*Bacillus cereus* group	1249.46	0.0	676/676 (100.0)
58	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
59	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
60	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
61	2	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
62	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
63	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
64	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
65	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
66	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
67	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
68	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
69	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
70	2	Aerobic	None	*Bacillus cereus* group	878.279	0.0	475/475 (100.0)
71	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
72	2	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
73	2	Aerobic	None	*Bacillus cereus* group	1040.78	0.0	563/563 (100.0)
74	2	Aerobic	OTU 14	*Bacillus cereus* group	904.132	0.0	549/576 (95.3)
75	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
76	2	Aerobic	OTU 2	*Lysinibacillus fusiformis/sphaericus*	1410.11	0.0	763/763 (100.0)
77	2	Aerobic	None	*Bacillus cereus* group	1286.39	0.0	714/723 (98.8)
78	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
79	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
80	2	Aerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
81	2	Aerobic	None	*Lysinibacillus sp.*	785.946	0.0	425/425 (100.0)
82	2	Aerobic	None	*Lysinibacillus fusiformis/sphaericus*	1107.26	0.0	629/642 (98.0)
83	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
84	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
85	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)

86	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
87	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
88	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
89	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
90	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)
91	2	Anaerobic	None	*Bacillus cereus* group	1441.51	0.0	782/783 (99.9)
92	2	Anaerobic	OTU 1	*Bacillus cereus* group	1406.42	0.0	761/761 (100.0)

[1]Not assigned to an OTU.

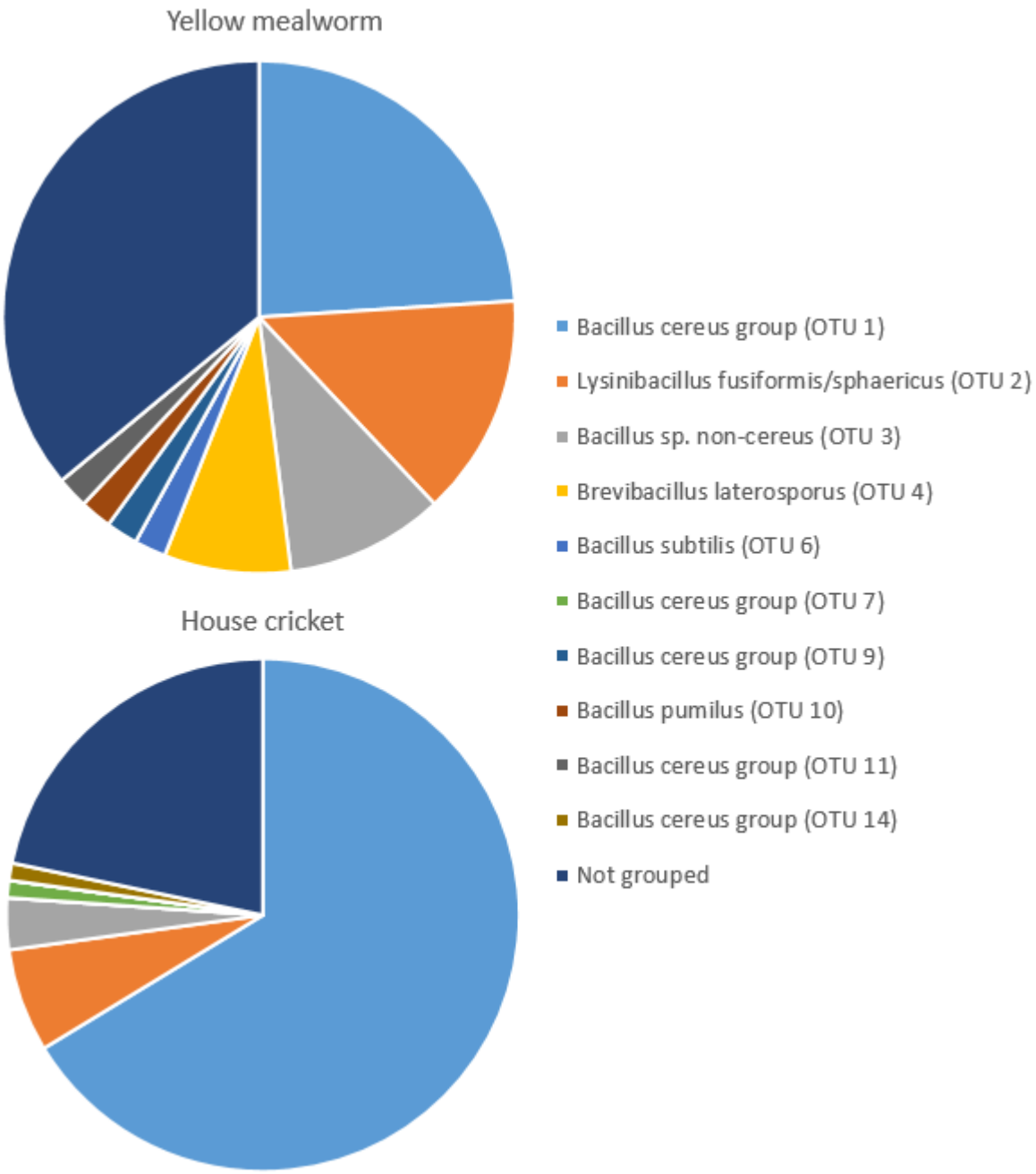


Figure 6.3 Relative abundance (%) of bacterial endospore isolates assigned to the different OTUs for yellow mealworm (upper graph) and house cricket (lower graph). Isolates not assigned to an OTU were brought together in "Not grouped".

6.3.2 Industrial processing and preservation of tropical house crickets in the second experiment

6.3.2.1 Intrinsic parameters

Water activity (a_w), moisture content, and pH were determined for homogenised raw, heat-treated, and frozen crickets in experiment 2. Due to the small sample size, only water activity and moisture content were determined for the dried and the smoked/dried crickets. After harvest, crickets were high in water activity and moisture content (0.97 and 71.5% on average, respectively) and showed a near-neutral pH of 6.64 on average (Table 6.3).

Table 6.3 Intrinsic properties during processing and preservation of tropical house crickets[1].

Product	Storage time (months)	Intrinsic property		
		pH (-)	a_w (-)	Moisture content (%)
Crickets	0	6.64 ± 0.10^{A}	0.97 ± 0.01^{A}	71.5 ± 0.7^{A}
Heat-treated[2] crickets	0	6.84 ± 0.05^{B}	0.98 ± 0.00^{A}	73.8 ± 0.4^{B}
Frozen crickets	0	6.85 ± 0.06^{a,B}	0.98 ± 0.00^{a,A}	73.6 ± 0.6^{a,B}
	3	6.95 ± 0.04^{a}	0.97 ± 0.01^{a}	74.3 ± 1.0^{a}
	6	6.89 ± 0.01^{a}	0.98 ± 0.00^{a}	75.6 ± 2.0^{a}
Oven-dried crickets	0	N.D.[3]	0.35 ± 0.08^{a,B}	5.1 ± 1.0^{a,C}
	3	N.D.	0.36 ± 0.01^{a}	4.4 ± 0.4^{a}
	6	N.D.	0.36 ± 0.01^{a}	6.0 ± 0.3^{a}
Smoked and dried crickets	0	N.D.	0.24 ± 0.03^{a,B}	2.2 ± 0.1^{a,b,C}
	3	N.D.	0.30 ± 0.03^{a,b}	1.9 ± 0.2^{a}
	6	N.D.	0.34 ± 0.02^{b}	5.0 ± 0.9^{b}

[1]Data are the mean ± standard deviation values of three replicates. ^{a,b,c}Means per product with the same lowercase letter within the same column do not differ significantly ($p > 0.05$); ^{A,B,C}Means from different products with the same uppercase letter within the same column do not differ significantly ($p > 0.05$).
[2]The heat treatment consisted of bringing the crickets to a boil in a kettle with water.
[3]N.D. = not determined.

Following heat treatment (bringing to a boil) of the crickets, the mean pH and moisture content significantly increased to 6.84 ($p = 0.031$) and 73.8% ($p = 0.006$), respectively. No difference was seen for water activity (Table 6.3). The frozen crickets did not show any

difference in intrinsic parameters compared to heat-treated crickets (Table 6.3). Obviously, the oven-dried and smoked/dried crickets were significantly lower in a_w ($p = 0.015$ and 0.001, respectively) and moisture content ($p = 0.000$). During the six-month storage of the products, a_w ($p = 0.024$) and moisture content ($p = 0.018$) of the smoked/dried crickets increased slightly, but significantly (Table 6.3). This indicates that the packaging technique used (glass tube with cork stop) allows moisture to enter the product. The a_w value, however, never rose above 0.60 to allow microbial growth (Jay et al., 2005).

6.3.2.2 Plate counts

In the second experiment, immediately after rearing as well as after the heat treatment, crickets were analysed for their total viable aerobic count and the number of Enterobacteriaceae, LAB, aerobic bacterial endospores and fungi (Table 6.4). After production and during preservation, the finalised cricket products were analysed for total viable aerobic count and the number of Enterobacteriaceae, aerobic bacterial endospores and fungi. Raw crickets harboured an average total viable aerobic count of 8.5 log cfu/g. Mean counts of Enterobacteriaceae, LAB, aerobic bacterial endospores and fungi were 7.2, 7.8, 3.7 and 5.6 log cfu/g, respectively. These numbers were comparable to those obtained for tropical house crickets and house crickets (*Acheta domesticus)* in Chapter 2, as well as to the counts obtained in experiment 1.

After heat treatment, all microbial counts were reduced, with those for LAB and fungi even below the detection limit (1 and 2 log cfu/g, respectively). The reduction was significant for all counts ($p = 0.000$, 0.018, 0.000, 0.002 and 0.004 for total viable aerobic count, Enterobacteriaceae, LAB, endospores and fungi, respectively). A substantial number of endospores remained, however, as was expected based on previous research on (lesser) mealworms and crickets (Klunder et al., 2012; Wynants et al., 2018) and based on the results of Chapter 5 and experiment 1 in this Chapter. Next, after production of the end products, the microbial counts of the frozen crickets remained unchanged. For the dried and the smoked/dried crickets, the amount of Enterobacteriaceae and fungi remained below the detection limit and the amount of aerobic endospores increased slightly, but not significantly. However, the total viable aerobic counts of both dried ($p = 0.002$) and smoked/dried crickets ($p = 0.000$) were significantly higher compared to that of crickets that were only heat-treated.

Additionally, the total viable aerobic count of smoked/dried crickets was higher compared to that of the oven-dried crickets ($p = 0.013$).

The unexpected increase of total counts of dried and smoked/dried crickets can likely be explained by several factors, including cross-contamination through the equipment and installations used, post-contamination through human interaction while removing legs and during packaging, and/or, for smoked/dried crickets, the possibility for microbial growth during subsequent processing steps, including freezing and thawing cycles. Repeatedly freezing and thawing can have a substantial influence on the microbiological quality of the product. The freeze-thaw cycles can cause, for example, germination of bacterial spores and subsequent multiplication, sporulation of vegetative cells, nutrient leakage out of damaged insect tissues facilitating microbial growth … (Fellows, 2009). To reduce risks for such contamination, especially the freeze-thaw cycles should be avoided or at least limited to the minimum. Also, it is advised to incorporate good hygiene and manufacturing practices such as the wearing of gloves and proper cleaning and disinfection of equipment.

Table 6.4 Microbial counts during preservation of processed tropical house crickets[1].

Product	Storage time (months)	Microbial counts (log cfu/g)			
		Total viable aerobic count	Enterobacteriaceae	Lactic acid bacteria	Aerobic bacterial endospores	Fungi
Crickets	0	8.5 ± 0.5^{A}	7.2 ± 0.1^{A}	7.8 ± 0.4^{A}	3.7 ± 0.3^{A,C}	5.6 ± 0.3^{A}
Heat-treated crickets[2]	0	2.6 ± 0.5^{B}	< 1.5 ± 0.9^{B}	< 1.0 ± 0.0^{B}	2.4 ± 0.4^{B,C}	< 2.0 ± 0.0^{a,B}
Frozen crickets	0	2.4 ± 0.4^{a,B}	< 1.0 ± 0.0^{a,B}	N.D.[3]	2.0 ± 0.4^{a,B}	< 2.0 ± 0.0^{a,B}
	3	2.5 ± 0.3^{a}	< 1.1 ± 0.1^{a}	N.D.	2.4 ± 0.4^{a}	< 2.1 ± 0.1^{a}
	6	2.2 ± 0.1^{a}	< 1.0 ± 0.0^{a}	N.D.	2.2 ± 0.0^{a}	< 2.0 ± 0.0^{a}
Oven-dried crickets	0	4.3 ± 0.0^{a,C}	< 1.0 ± 0.0^{a,B}	N.D.	2.4 ± 0.4^{a,B,C}	< 2.0 ± 0.0^{a,B}
	3	3.9 ± 0.8^{a}	< 1.0 ± 0.0^{a}	N.D.	2.3 ± 0.5^{a}	< 2.0 ± 0.0^{a}
	6	3.9 ± 0.8^{a}	< 1.1 ± 0.1^{a}	N.D.	2.5 ± 0.5^{a}	< 2.1 ± 0.1^{a}
Smoked and dried crickets	0	7.9 ± 0.1^{a,D}	< 1.0 ± 0.0^{a,A}	N.D.	3.4 ± 0.6^{a,C}	< 2.0 ± 0.0^{a,B}
	3	7.4 ± 0.3^{a,b}	< 1.0 ± 0.0^{a}	N.D.	3.9 ± 0.6^{a}	< 2.0 ± 0.0^{a}
	6	7.0 ± 0.2^{b}	< 1.0 ± 0.0^{a}	N.D.	3.2 ± 0.0^{a}	< 2.0 ± 0.0^{a}

[1]Data are the mean value of three replicates ± standard deviation. ^{a,b}Means per product with the same superscript (small letter) within the same column do not differ significantly ($p > 0.05$); ^{A,B,C,D}Means from unstored (0 months) products with the same superscript (capital letter) within the same column do not differ significantly ($p > 0.05$).
[2]The heat treatment consisted of bringing the crickets to a boil in a kettle with water.
[3]N.D. = not determined.

Following processing into the different end products, frozen crickets were stored for six months at -25 ± 1 °C, dried crickets at 21 ± 1 °C and smoked/dried crickets at 22 ± 2 °C. During the six-month storage period, all counts remained stable, except the total viable aerobic count of the smoked/dried crickets, which slightly (but significantly, $p = 0.009$) decreased over time (Table 6.4). The frozen preservation and the low a_w thus resulted in a stable microbiological quality during the proposed shelf life, for frozen and dried or smoked/dried end products, respectively.

6.3.2.3 Culture-independent analyses

Samples from the crickets after harvest, after heat treatment, and after production of the end products (Figure 6.1), were subjected to high-throughput, amplicon-based bacterial 16 S ribosomal RNA gene sequencing. Relative abundances and diversity indices were averaged over all DNA extracts of replicate samples. Average coverages, based on the Chao1 estimator, ranged between 96.5% and 98.2%, indicating that the majority of bacterial OTUs was recovered (Table 6.5). Indices for species richness (observed richness and Chao1 (Chao, 1984)) showed that the raw crickets contained most bacterial species, while the least diversity was observed in crickets after smoking. Likewise, the mean Shannon index (Shannon, 1948) was significantly lower for smoked/dried crickets (Table 6.5).

Table 6.5 Diversity indices for samples subjected to metagenetic analysis[1].

Cricket sample	Diversity indices			
	Observed richness	Chao1[2]	Coverage (%)[3]	Shannon[4]
End of rearing	115 ± 11^{a,b,c}	117.05 ± 8.27^{a,b,c,d}	97.75 ± 2.15^{a}	3.53 ± 0.01^{a}
After heat treatment	101 ± 4^{a,c}	104.43 ± 4.77^{c,d}	96.52 ± 1.47^{a}	3.18 ± 0.02^{b}
After freezing	104 ± 2^{a}	106.05 ± 1.41^{a,c,d}	97.83 ± 1.13^{a}	3.16 ± 0.03^{b}
After oven drying	114 ± 5^{a,b}	116.35 ± 4.11^{a,b}	98.19 ± 0.71^{a}	3.20 ± 0.18^{b}
After smoking	94 ± 1^{c}	96.10 ± 0.14^{d}	97.82 ± 1.62^{a}	2.66 ± 0.05^{c}

[1]Data are the mean values of two analysed DNA extracts from two replicates per sampling moment ± standard deviations; ^{a,b,c,d}Means per product with the same superscript within the same column do not differ significantly ($p > 0.05$);

[2]Chao1 richness estimator: the total number of OTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1984).

[3]Coverage = (Observed richness / Chao1 estimate) * 100.

[4]Shannon-Wiener diversity index: index to characterise species diversity based on species richness as well as their relative abundance. A higher value represents more diversity (Shannon, 1948).

Identification of the operational taxonomic units (OTUs) (Table S6.2 and Table S6.3, Supporting information) revealed that the most abundant phylum in the crickets was Bacteroidetes (ranging from 35.8 ± 0.2% in smoked/dried crickets to 71.2 ± 1.7% in frozen crickets), followed by Firmicutes (ranging from 5.4 ± 0.3% in frozen crickets to 56.6 ± 1.9% in smoked/dried crickets) and Proteobacteria (ranging from 4.9 ± 1.4% in smoked/ dried crickets to 16.0 ± 2.5% in heat-treated crickets). Other phyla were present in abundances below 10% in any sample of the dataset.

A total of 337 OTUs was identified throughout the whole dataset, including metagenetic analysis of the peat-peel mix, the feed and the substrate (data not shown). For the raw crickets, more than 40% of sequences recovered belonged to OTUs represented by < 3% of sequences in all samples (Table 6.4). Consequently, the cricket bacterial composition was highly diverse, as also indicated by the diversity indices. This observation was also reported in Chapter 3, where both diversity indices and the number of OTUs represented by less than 3% were higher for crickets compared to the values observed for mealworms. Crickets thus harbour a remarkably more complex bacterial composition than mealworms with also more dominating organisms.

Both processed and unprocessed cricket samples were abundant (> 5% mean abundance in any sample) in OTUs corresponding to members of the family Porphyromonadaceae (OTUs 2 and 4), which also contains the genus *Parabacteroides* (OTU 6). The cricket samples were also abundant in a *Bacteroides* sp. (OTU 3), a *Fusobacterium* sp. (OTU 5), and an *Erwinia* sp. (OTU 10). In addition, raw crickets showed a high abundance of two *Acinetobacter* sp. (OTUs 34 and 52) and smoked/dried crickets showed a large abundance (> 40%) of a *Bacillus* species (OTU 9). Also here, a large percentage of OTUs showed a relative abundance below 3% in any sample. Considering the bacterial composition, similarities were observed between the crickets in the present study and those analysed in Chapter 3. Indeed, the presence of OTUs corresponding to the families Enterobacteriaceae and Pseudomonadaceae, and the genera *Bacteroides, Parabacteroides*, *Erwinia,* and *Fusobacterium* was also reported in raw crickets there. Most members of the genus *Erwinia* are plant-associated and plant-pathogenic bacteria (Brenner et al., 2005), and the presence of OTU 10 may be caused by the plant-based diet of the crickets. Indeed, the feed administered in this study contained a substantial

fraction (> 20% relative abundance) of OTU 10 as well (Vandeweyer et al., 2018). The other observed OTUs belonged to genera/families existing of bacteria typically isolated from different parts of the human body such as the oral cavity, the gastrointestinal tract the and urogenital tract (Krieg et al., 2010).

Altogether, it is reasonable to assume that *Parabacteroides* spp., among other Porphyromonadaceae spp., and *Bacteroides* spp. are typical members of the endogenous intestinal bacterial composition of crickets. Also *Fusobacterium* was already observed as member of the tropical house cricket microbiome in Chapter 3. Noteworthy is the presence of *Acinetobacter* sp. in our dataset, e.g. OTUs 34 and 52 in the sample of harvested crickets. *Acinetobacter* species are widely distributed in nature, and commonly occur in soil and water, but also in insect guts and plant-related environments. Furthermore, it contains multiple nosocomial opportunistic pathogens among which *A. baumanii* is the most well-known (Van Assche et al., 2017). Interestingly, *Acinetobacter* species were previously found in carrots (Dahiru & Enabulele, 2015) and their wash water (Hausdorf, Fröhling, Schlüter, & Klocke, 2011), which may explain their appearance in the harvested crickets, since exclusively carrots were administered the final days before harvest.

Heat-treated crickets had a similar bacterial composition compared to the crickets immediately after harvesting. It should however be noted that the heat treatment, although reducing microbial numbers significantly, possibly did not break down all bacterial DNA, which can explain the comparable abundances of the recovered OTUs. Many genera observed in this study were also encountered in previous research on processed crickets performed by Garofalo et al. (2017). Highly remarkable, however, is the appearance of a strongly abundant *Bacillus* species (OTU 9, 40.9%), a typical spore-forming bacterium, in the smoked/dried crickets. The succession of processing steps to obtain the smoked/dried crickets may have triggered subsequent cycles of spore formation and germination, while the background microbiota was reduced, and hence caused the high abundance of this OTU. The high abundance of this OTU also explains the significantly lower bacterial diversity in the smoked/dried crickets.

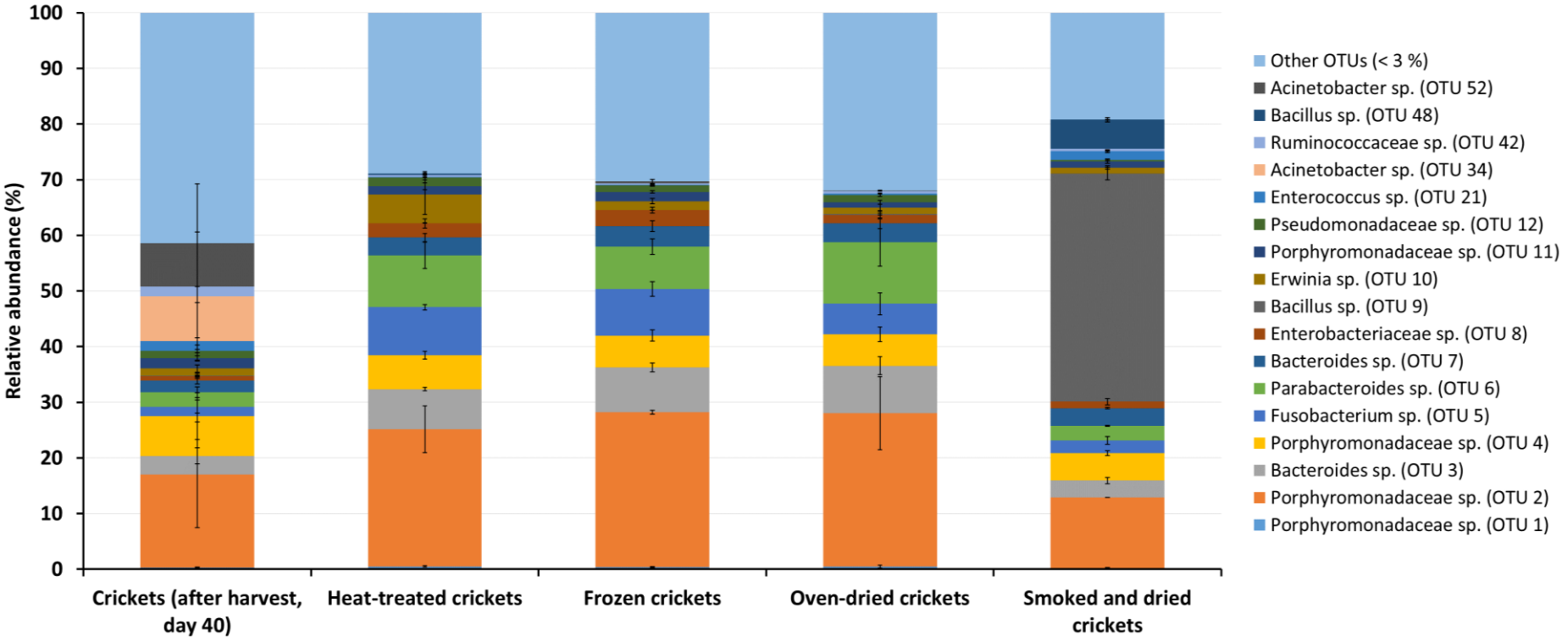


Figure 6.4 Relative abundance (%) of OTUs in the crickets during processing. Data are mean values of two extracts per sample from one (crickets at day 40 and smoked/dried crickets) or two cricket samples. Error bars represent the standard deviation. Only OTUs represented by an average relative abundance of more than 3% of sequences in any sample are shown. OTUs with a mean relative abundance of less than 3% are grouped in “Other OTUs (< 3%)”.

While the technique applied for identification did not allow for reliable identification on species level, the 250 bp read of OTU 9 corresponded, according to GenBank, the closest to a *B. cereus* group member. Also previous research on edible crickets was able to identify *Bacillus* species as well. For example, Osimani, Garofalo, Milanović, et al. (2017) were able to identify both *B. cereus* group members and *B. subtilis* in edible crickets and cricket powders and Fasolato et al. (2018) retrieved several species of the *B. cereus* group from cricket samples as well. Additionally, experiment 1 identified several spores present on house crickets as *B. cereus s.l.* Given the small differences previously described in bacterial composition between the two cricket species *G. sigillatus* and *A. domesticus* (Chapter 3), the results from the first experiment may be of interest for both cricket species.

Since this *Bacillus cereus* group contains the food pathogen *B. cereus*, special attention should be paid to the heat treatment, even though *B. cereus* was not detected using the classical enumeration method (ISO 7932) performed on the crickets immediately after harvest (Vandeweyer et al., 2018). It is advised to apply a heat treatment which is sufficient to eliminate endospores and to decrease the amount of freeze-thaw cycles in the production process of such insect products to a minimum. Although no legal criteria for endospore count or *B. cereus* count exist, it is advisable to at least reduce the number of bacterial endospores and/or the *B. cereus* count below the FASFC action limits (Table S1.1, Supporting information).

6.4 Conclusions

The first experiment in this study investigated the fate of bacterial endospores harboured by house crickets when subjected to a heat treatment and subsequently stored in chilled conditions for 24 days. Identification of several isolates from raw crickets revealed that many isolates could be assigned to members of the *Bacillus cereus* group, which may contain both human and insect pathogens. Also *Lysinibacillus fusiformis/sphaericus* and non-cereus *Bacillus* species were encountered. After blanching, the amount of microorganisms was more or less reduced to the amount of endospores, which were in their turn reduced as well in some cases. Still, a fraction was able to survive the heating step in all cases. During chilled preservation of the blanched crickets, microbial growth only occurred as from day 11, without reaching the spoilage threshold considered by Sperber & Doyle (2009) at the end of the

storage period. Since possible pathogens were harboured by the freshly harvested, raw house crickets and at least a part of the endospore population survived the heat treatment, a food safety risk still exists during the chilled preservation.

In a second experiment, the microbial dynamics during processing and preservation of tropical house crickets were investigated. OTUs assigned to the family of Porphyromonadaceae and the genera *Bacteroides, Parabacteroides, Erwinia* and *Fusobacterium* were found to be important members of the cricket microbiome. Microbial numbers of raw crickets were high, yet comparable to other studies, and were significantly reduced by a heat treatment. After processing into dried and smoked/dried end products, in contrast to the frozen end products, an increase in total microbial count could be detected as a result of post-contamination, but the microbial composition remained comparable to those of the raw crickets. However, a high abundance of a *Bacillus sp.* was observed in the smoked/dried crickets. During their six-month shelf life, the microbiological quality of the cricket products, as indicated by their microbial numbers, remained stable. A shelf life of six months as used by the manufacturer is acceptable from a microbiological point of view, if pathogens remain absent or below a recommended level.

In both experiments, *Bacillus* species that were assigned to the *B. cereus* group were encountered. In terms of food safety, this observation urges for a sufficiently effective elimination treatment for bacterial endospores. A proper legal criterion for *Bacillus cereus* would be valuable in order to optimise the spore-eliminating treatment. Additionally, during processing of crickets, it is advised to also minimise the number of freeze-thawing cycles.

Chapter 7 General discussion, conclusions and future prospects

7.1 Outcome of research objectives

7.1.1 Objective 1: To explore the microbiota of freshly reared, raw insects for food, with special attention to the occurrence of human bacterial pathogens

This dissertation aimed to address four research objectives, inspired by risk assessments from several countries and by inputs from the insect sector. The first objective aimed to explore the microbiota of raw edible insects immediately after harvest. Particularly Chapter 2 and 3 contribute to this research goal. Samples were collected from 7 insect producers located in Belgium and the Netherlands and 3 batches per producer and they originated from 3 different insect species (*Tenebrio molitor*, *Acheta domesticus* and *Gryllodes sigillatus*). By analysing these 21 samples for 6 different microbiological culture-dependent parameters (total viable aerobic count, lactic acid bacteria count, Enterobacteriaceae count, aerobic bacterial endospore count, psychrotrophic aerobic count, and yeasts and moulds count), an extensive microbiological data set could be obtained. These data are supported by intrinsic parameters pH, water activity, and moisture content, that characterise the insect matrix and which contribute to the interpretation of the microbiological data. The culture-dependent approach allows to evaluate insects in the same way as the microbiological quality of other food products is monitored in the food industry. In this work, this approach is supported by culture-independent (i.e. DNA-based) characterisation of 17 samples of the same sample collection. Hence, counts and identification of microbiota in freshly reared, raw edible insects can be assessed simultaneously.

Additional to Chapters 2 and 3, also Chapters 5 and 6 contribute to Objective 1 with microbial counts, including anaerobic counts, obtained from raw insect samples. In Chapter 6, the bacterial composition of one raw cricket sample is reported as well. Moreover, the focus on characterisation of bacterial endospores in Chapters 5 and 6 results in the identification of 92 endospore-forming isolates harboured by 4 insect samples. Altogether, a total of 171

microbial counts obtained from 33 raw insect samples from 9 rearing companies are provided in this dissertation. Furthermore, the bacterial composition of 18 samples and the identification of 92 bacterial spore-forming isolates present in 4 samples can be supplemented to the microbial counts.

Contained in Objective 1 was the special attention for human pathogens. This focus is addressed through the specific search for the foodborne pathogens *Salmonella* spp. and *Listeria monocytogenes* following established ISO-methods, as well as by monitoring the results of the bacterial composition analyses and spore identifications. While no specific human foodborne pathogens are unambiguously detected throughout this dissertation, the experiments performed identify microbial genera that may contain pathogenic species. As a result, evidence exists that pathogenic species, among which especially members of the *Bacillus cereus* group, may pose a realistic risk for human health.

The **outcome** of the microbiological data generated in the several chapters is **threefold**:

- Firstly, it should be concluded that mealworms and crickets are completely different food matrices in terms of microbiological quality. Taking also into account other research results obtained in our research group and reported in literature, it is clear that the **microbiological profile of an insect species is relatively unique** and it is not desirable or meaningful to discuss the microbiota of edible insects in general;
- Secondly, **the microbiological quality can vary substantially between rearing companies and between production batches**, which demonstrates that, at this moment, it is not possible for the insect sector to produce insects with a constant microbial composition;
- Finally, it is concluded that **the three edible insect species investigated <u>can</u> harbour foodborne pathogens such as *Bacillus cereus***. *Bacillus cereus* is, however, mainly of concern when they can reach numbers able to cause a foodborne illness, i.e. 4 to 5 log cfu/g food.

7.1.2 Objective 2: To reveal the occurrence of antibiotic resistance genes in freshly reared, raw insects for food

Chapter 4 provides research data that address the second objective, which aimed to identify antibiotic resistance genes carried by raw edible insects. A real-time PCR protocol, especially established to provide data on the presence of 5 antibiotic resistance genes, detected and quantified merely tetracycline resistance genes in four different insect species. Additionally, information is provided considering the distribution of these genes among different species, as well as related to the geographical origin of the samples. It can be concluded that:

- **Certain antibiotic resistances occur** in specific samples;
- The occurrence of antibiotic resistance genes in insects for foods is **comparable to other foods**;
- **The microbial composition of that sample affects the presence of the antibiotic resistances**.

7.1.3 Objective 3: To elucidate the impact of specific processing steps on the microbiota of insects for food

As required by the third objective, the impacts of processing on microbiological quality of both mealworms and crickets are assessed in Chapter 5 and 6 in this dissertation. The application of heat to whole insects after harvesting allows most microbial counts to be reduced to below the detection limit. Two exceptions are the total viable count and the bacterial endospore count. It is consistently noted that **at least a part of the bacterial endospores is able to survive the heat treatments applied in this work**. Also the **application of subsequent heat treatments**, e.g. boiling combined with drying, **does not always fully eliminate the endospores**. Hence, it is concluded that, depending on time-temperature combinations used, heat treatments may reduce the microbiota, but mainly leave bacterial endospores unharmed and potentially even trigger their germination in the heat-treated product with a reduced background microbiota. Evidently, after the heat treatments, specific attention to post-contamination or microbial outgrowth during further processing of the products is required.

7.1.4 Objective 4: To investigate the microbiological quality and shelf life during preservation of insects for food

The processing of insects into stable products such as dried or frozen insects can deliver products with a long shelf life. The initial microbial load of the stabilised products thus determines the microbiological quality during shelf life. Additionally, the stability of the product should be guaranteed by e.g. applying correct packaging and preservation conditions. Following the outcome of Objective 3, the risk of remaining (possibly pathogenic, see Objective 1) **bacterial spores should be taken into account during preservation**. Especially when edible insects are stored in chilled conditions, these remaining spores may be able to germinate and spoil the insects, resulting in a limited shelf life. In turn, sporulation can occur as well during chilled preservation. **For mealworms and crickets, different shelf lives in chilled conditions are proposed**, given the different behaviour of their microbiota during preservation. These findings from Chapters 5 and 6 contribute to the fourth objective, which required the investigation of the microbiological quality during preservation of edible insects.

7.2 Future prospects for academia, governments and industry

7.2.1 Suggestions for further scientific research on the microbiological quality of insects for food

In the 4-year period this PhD research was established, extensive research data on the subject of edible insects have become available, originating from either research performed in the context of this PhD dissertation or from other authors. Yet, not all aspects regarding the microbiological quality and safety of edible insects have been elucidated and several follow-up studies can be formulated.

Firstly, the research in this dissertation was delineated to only four edible insect species, of which only three are thoroughly assessed. As listed by Jongema (2017), over 2000 edible insect species exist worldwide and also the report by the EFSA Scientific Committee (2015) indicates the potential of several other insect species as human food source. It is demonstrated in Chapters 2, 3, and 4 that different insect species are very distinct in their

microbiological quality. Future research specifically focussed on the **microbiological quality of other insect species** should therefore be performed prior to adopting them in the human diet as well. Two considerable suggestions for microbiological quality assessment are the African migratory locust (*Locusta migratoria migratorioides*) and the lesser mealworm (*Alphitobius diaperinus*). These insect species are both included in the EFSA list of promising insects for food and are also described as members of the "big four", being the most widely used species in Europe (House, 2018). In Belgium and the Netherlands, rearing companies for both insect species are already established and for both species, a novel food dossier was already submitted. To date, however, only limited microbiological data exist publically regarding the African migratory locust (Osimani, Garofalo, Aquilanti, et al., 2017; Stoops et al., 2016) and the lesser mealworm (Stoops et al., 2017; Wynants et al., 2018) (Chapter 4) for human consumption. To be able to assess these edible species thoroughly, extensive data should be made available in future research projects. Also in more tropical regions, where improved insect collection, rearing and preservation are currently being explored (e.g. in the VLIR-UOS TEAM project "Using the edible insect *Ruspolia differens* to enhance food security in East Africa"), specific insect species should be subjected to microbiological assessment. Since different legislation may apply there or even be less established than in Europe, similar microbiological techniques should be employed to allow for a universal comparison. While the microbiological focus in this dissertation was on bacteria, **other microorganisms such as fungi and viruses** are encouraged to be included in future microbiological assessments.

Secondly, research has shown that insects may contain possible human and also insect pathogens, some of which are spore-forming bacteria resistant to certain forms of processing while in their spore stage. To decrease the food safety risks associated with these organisms, the **evaluation of strategies to reduce the amount of endospores** would be a useful subject for further research. Alternative strategies worth investigation are for example steaming (Cenkowski, Pronyk, Zmidzinska, & Muir, 2007) and high pressure decontamination (Herdegen & Vogel, 1998) or even a combined approach (Reineke, Mathys, & Knorr, 2011). A preliminary study already performed by KU Leuven, Lab4Food (De Smet et al., unpublished data) indicated variable results for the reduction of bacterial endospores by steaming (5 min.). A more thorough study is therefore necessary. Also irradiation (De Lara, Fernández, Periago, & Palop, 2002) and microwave radiation (Celandroni et al., 2004) have proven their efficacy

in killing bacterial spores (De Lara et al., 2002). A last interesting treatment to reduce the amount of bacterial endospores may be (deep) frying. A future research project titled for example "*Advanced treatments to eliminate bacterial endospores in edible insects*" would bring added value to the field. Additionally, it may be of strategical importance to identify the contamination source of the particular microorganisms in the whole insect food production chain, for example in the rearing or processing environment. Also for the presence of antibiotic resistance genes, mitigation strategies such as monitoring of antibiotic usage, effective disinfection, and attempts for detection of AR genes in the environment should be undertaken.

Thirdly, it is suggested to **investigate alternative preservation techniques for edible insects**. In this dissertation, edible insects were stored after heat treatment and/or after drying, in chilled or frozen conditions or at ambient temperature. A research project considering preservation techniques including modified atmosphere or vacuum packaging, the use of preservatives, fermentation, salting, canning … can result in preservation concepts with prolonged shelf life and eventually the development of new products.

While the methods used in the microbiological assessment of edible insects in this dissertation definitely produced valuable results for the sector, they come with some considerations. In the field of microbial ecology, quantification of dominant taxa or OTUs becomes more important, as advanced insight in microbial systems is desired (Zhang et al., 2017). Relative abundances, however, obtained from high-throughput sequencing as used in this dissertation, not always correctly reflect the actual abundance of the species they represent (Aird et al., 2011). These biases are caused for example by competition of target DNA fragments for primer binding or by the presence of amplification targets in different copy numbers in microbial species. Even though the impact of these factors on relative abundances obtained in high-throughput sequencing is still being debated, and even though these considerations were carefully adopted in the interpretation of results in this thesis, new insights with regard to microbiome sequencing should also be implemented in edible insect microbiological research. A technique such as qPCR can be employed in the future to determine the abundance of the dominant insect microbiota more precisely. As a result, a final proposal for future research suggests the **investigation of the relation between (1)**

microbial abundances determined using established methods for microbial counting, (2) absolute quantification through qPCR-based methods and (3) relative abundances obtained through microbial composition analysis. This can, for example, be performed in a project titled "*Relating microbial counts and quantitative qPCR results to relative next-generation sequencing abundances for dominant insect microbiota*".

7.2.2 Suggestions to improve the legislative framework

The current lack of a well-elaborated legislative framework is a burden for the edible insect sector. As described in the introductory chapter (Chapter 1), in Belgium, a few specific microbiological requirements for edible insects are included in the FASFC circular of 2016 and some additional food safety and process hygiene recommendations are mentioned in a list of action limits composed by the FASFC. However, for other food sectors, a European legislative framework exists in the form of specific microbiological criteria embedded in Regulation (EG) N° 2073/2005. Likewise, it is suggested to **compose specific criteria for edible insects** as well.

The microbiological data obtained in this dissertation, provided along the whole post-harvesting chain, can contribute to microbiological risk assessments that precede the development of microbiological criteria (van Schothorst et al., 2009). To date, three publications containing microbiological data generated in this dissertation have proven to contribute to a risk assessment of the house cricket (Fernandez-Cassi et al., 2018). This risk assessment, which was first published on 28 August 2018 in the EFSA journal, is the first to specifically investigate a single insect species, being the house cricket, and assemble all currently available data regarding microbiological and chemical food safety of that particular insect species. While this risk assessment in the first place aims to provide data to evaluate the novel food status of this insect species, it can also provide a scientific basis to construct microbiological criteria. Likewise, **future specific risk assessments** of other insect species can rely on data provided in this and other research.

The results obtained in this dissertation were repeatedly compared with the currently existing requirements and action limits in Belgium. Consequently, the usefulness of these requirements and action limits could be evaluated. With prudence, it can be concluded that *Bacillus cereus* has a higher probability to be present in edible insects than *Salmonella* spp.

and *Listeria monocytogenes,* the two organisms that are currently embedded in the legal requirements. As a result, a suggestion to **include a food safety criterion for *Bacillus cereus*** additional to *Salmonella* spp. and *L. monocytogenes* is made. It should be noted, however, that if an insect product that contains small numbers of *B. cereus* (i.e. below approximately 4 log cfu/g) is properly monitored and if growth of the bacterium can be controlled, the health risk can be kept low. Additional research, for example including tests to study the transmission of *B. cereus* from elements presents during rearing (feed, egg lay matrices, water …) and the detection of the *B. cereus* toxins when the organism is present in high numbers, can add information to determine the exact microbial values in the criterion. Action limits such as those formulated for total viable count or a few other pathogenic species are definitely valuable as well. Given the high numbers of microorganisms carried by insects and the obliged heat treatment to reduce those counts, specific targets to achieve with such reduction are welcome and can be used to optimise heating (and other decontamination) processes and specify treatment parameters.

7.2.3 Suggestions for industrial producers and processors of insects as food

Given the variations that were observed during this PhD research, which was exclusively dedicated to the post-harvest stage of the insect value chain, future research focussing on establishing rearing protocols that deliver edible insects with constant (microbiological) quality and high safety can benefit insect producers and processors in the insect sector. **Upscaling and automation** of insect rearing and insect-based food production is desired on the short term. It can reduce manual interventions and facilitate cleaning and disinfection. While to date a large variability exists in insect microbiological quality, the introduction of automation can equalise quality and perhaps allows for safer products. This could be investigated as a post-project trajectory through intense collaboration between academia and industry.

Several times in this dissertation, the **role of the rearing substrate or insect feed** was discussed. To date, it is not completely elucidated how this probably important factor contributes to the microbiological quality of edible insects. Yet, the high temperature and relative humidity during rearing could ease the survival and growth of microorganisms in the

substrate. Not only it was observed that certain microorganisms harboured by the insects could have originated from the feed, also the differences in microbiological quality observed between several rearing companies could have been (partially) caused by different feeding concepts. Additionally, also antibiotic resistance genes may be transferred via (bacteria in) the insect feed or substrate. In a few studies (Vandeweyer et al., 2018; Wynants et al., 2018), the microbiological quality of insect feed was already investigated alongside that of the insects reared, but additional research could still greatly improve knowledge on this topic.

Regarding the relationship between microbiological quality of insects and **rearing and processing hygiene**, a few hypotheses were formulated in this dissertation. In Chapter 3, for example, it was hypothesised that a company-specific "house flora" might exist. In this view, thorough cleaning and disinfection or even sterile rearing may cause a loss of this useful microflora. On the other hand, it was already stated by some rearing companies that their insects show the best growth in a recently cleaned rearing environment. Therefore, it may be valuable to further investigate the role of hygiene in an industrial insect rearing environment.

Also for insect sector organisations, suggestions can be formulated based on this PhD research. As is the case in other food sectors, the **composition of a sector guidebook for the insect sector** would allow both starting and established insect rearers and processors to set up their business including a self-regulation system. Important sections to be embedded in such sector guidebook are for instance food safety risks and strategies to control those risks, good hygiene practises, HACCP, methods of analysis … At the moment of finalising this PhD, in Belgium, in the Netherlands and also by the European sector organisation IPIFF (International Platform of Insects for Food and Feed), the compilation of such (a) sector guidebook(s) is being discussed. Microbiological data such as those generated in this dissertation can contribute to the composition of those guidebooks.

References

Adámková, A., Kouřimská, L., Borkovcová, M., Kulma, M., & Mlček, J. (2016). Nutritional values of edible Coleoptera (*Tenebrio molitor*, *Zophobas morio* and *Alphitobius diaperinus*) reared in the Czech Republic. *Potravinarstvo Scientific Journal for Food Industry*, *10*(1), 663–671. http://doi.org/10.5219/609

Adams, W. M. (2006). *The Future of Sustainability: Re-thinking Environment and Development in the Twenty-First Century*. *Report of the IUCN Renowned Thinkers Meeting*. Retrieved from http://www.vda.de/en/service/jahresbericht/aut

Agata, N., Ohta, M., & Yokoyama, K. (2002). Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. *International Journal of Food Microbiology*, *73*(1), 23–27. http://doi.org/10.1016/S0168-1605(01)00692-4

Ahmed, I., Yokota, A., Yamazoe, A., & Fujiwara, T. (2007). Proposal of Lysinibacillus boronitolerans gen. nov. sp. nov., and transfer of Bacillus fusiformis to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *57*(5), 1117–1125. http://doi.org/10.1099/ijs.0.63867-0

Aird, D., Ross, M. G., Chen, W. S., Danielsson, M., Fennell, T., Russ, C., … Gnirke, A. (2011). Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. *Genome Biology*, *12*(2), R18. http://doi.org/10.1186/gb-2011-12-2-r18

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. http://doi.org/10.1016/S0022-2836(05)80360-2

Ammar, E.-D., Fulton, D., Bai, X., Meulia, T., & Hogenhout, S. A. (2004). An attachment tip and pili-like structures in insect- and plant-pathogenic spiroplasmas of the class Mollicutes. *Archives of Microbiology*, *181*(2), 97–105. http://doi.org/10.1007/s00203-003-0630-8

ANSES. (2015). *Opinion of the French Agency for Food, Environmental and Occupational Health & 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."* Maisons-Alfort, France. Retrieved from https://www.anses.fr/en/system/files/BIORISK2014sa0153EN.pdf

Aquilino, A., Masiá, M., López, P., Galiana, A. J., Tovar, J., Andrés, M., & Gutiérrez, F. (2015). First human systemic infection caused by *Spiroplasma*. *Journal of Clinical Microbiology*, *53*(2), 719–721. http://doi.org/10.1128/JCM.02841-14

Aremu, B. R., & Babalola, O. O. (2015). Classification and taxonomy of vegetable macergens. *Frontiers in Microbiology*, *6*, 1361. http://doi.org/10.3389/fmicb.2015.01361

Awadel-Kariem, F. M., Patel, P., Kapoor, J., Brazier, J. S., & Goldstein, E. J. C. (2010). First report of *Parabacteroides goldsteinii* bacteraemia in a patient with complicated intra-abdominal infection. *Anaerobe*, *16*(3), 223–225. http://doi.org/10.1016/j.anaerobe.2010.01.001

Barbeyrac, B. de, Dutilh, B., Quentin, C., Renaudin, H., & Bébéar, C. (1991). Susceptibility of *Bacteroides ureolyticus* to antimicrobial agents and identification of a tetracycline resistance determinant related to *tet*M. *Journal of Antimicrobial Chemotherapy*, *27*(6), 721–731. http://doi.org/10.1093/jac/27.6.721

Baroni, L., Cenci, L., Tettamanti, M., & Berati, M. (2007). Evaluating the environmental impact of various dietary patterns combined with different food production systems. *European Journal of*

Clinical Nutrition, *61*(2), 279–286. http://doi.org/10.1038/sj.ejcn.1602522

Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnology Advances*, *25*(2), 207–210. http://doi.org/10.1016/J.BIOTECHADV.2006.11.002

Bednárová, M., Borkovcová, M., Mlček, J., Rop, O., & Zeman, L. (2013). Edible insects - Species suitable for entomophagy under condition of Czech Republic. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, *61*(3), 587–593. http://doi.org/10.11118/actaun201361030587

Belluco, S., Losasso, C., Maggioletti, M., Alonzi, C. C., Paoletti, M. G., & Ricci, A. (2013). Edible Insects in a Food Safety and Nutritional Perspective: A Critical Review. *Comprehensive Reviews in Food Science and Food Safety*, *12*(3), 296–313. http://doi.org/10.1111/1541-4337.12014

Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2013). GenBank. *Nucleic Acids Research*, *41*(D1), D36–D42. http://doi.org/10.1093/nar/gks1195

Berkeley, R., Heyndrickx, M., Logan, N., & De Vos, P. (2008). *Applications and systematics of Bacillus and relatives*. Blackwell Science. http://doi.org/10.1002/9780470696743

Berry, C. (2012). The bacterium, Lysinibacillus sphaericus, as an insect pathogen. *Journal of Invertebrate Pathology*, *109*(1), 1–10. http://doi.org/10.1016/j.jip.2011.11.008

Borremans, A., Lenaerts, S., Crauwels, S., Lievens, B., & Van Campenhout, L. (2018). Marination and fermentation of yellow mealworm larvae (*Tenebrio molitor*). *Food Control*, *92*, 47–52. http://doi.org/10.1016/j.foodcont.2018.04.036

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon*, *49*(4), 423–435. http://doi.org/10.1016/j.toxicon.2006.11.022

Brenner, D. J., Krieg, N. R., Staley, J. T., Garrity, G. M., Boone, D. R., De Vos, P., … Schleifer, K.-H. (2005). *Bergey's Manual of Systematic Bacteriology Volume 2, parts A, B and C* (2nd ed.). New York, NY, USA: Springer Science+Business Media. http://doi.org/10.1007/0-387-28022-7

Brown, S. P., Veach, A. M., Rigdon-Huss, A. R., Grond, K., Lickteig, S. K., Lothamer, K., … Jumpponen, A. (2015). Scraping the bottom of the barrel: Are rare high throughput sequences artifacts? *Fungal Ecology*, *13*, 221–225. http://doi.org/10.1016/j.funeco.2014.08.006

Brundtland, G. H. (1987). *Our Common Future: Report of the World Commission on Environment and Development*. *United Nations Commission* (Vol. 4). Retrieved from http://www.un-documents.net/our-common-future.pdf

Cadesky, L. (2017). Edible insect derived products and processes for the manufacture and use thereof. Canada. Retrieved from https://worldwide.espacenet.com/publicationDetails/biblio?II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20170427&CC=WO&NR=2017066880A1&KC=A1

Cai, M., Hu, R., Ma, S., Tomberlin, J. K., Yu, C., Zhang, K., … Zhang, J. (2018). The degradation of tetracycline by black soldier fly (Diptera: Stratiomyidae) larvae with intestinal microorganisms. In *Insects to feed the world* (p. 22). Wuhan, Hubei, China: Journal of Insects as Food and Feed 4, Supplement 1.

Caparros Megido, R., Desmedt, S., Blecker, C., Béra, F., Haubruge, É., Alabi, T., & Francis, F. (2017). Microbiological Load of Edible Insects Found in Belgium. *Insects*, *8*(1), 12. http://doi.org/10.3390/insects8010012

Caparros Megido, R., Poelaert, C., Ernens, M., Liotta, M., Blecker, C., Danthine, S., … Francis, F. (2018).

Effect of household cooking techniques on the microbiological load and the nutritional quality of mealworms (*Tenebrio molitor* L. 1758). *Food Research International*, *106*, 503–508. http://doi.org/10.1016/j.foodres.2018.01.002

Caparros Megido, R., Sablon, L., Geuens, M., Brostaux, Y., Alabi, T., Blecker, C., … Francis, F. (2014). Edible Insects Acceptance by Belgian Consumers: Promising Attitude for Entomophagy Development. *Journal of Sensory Studies*, *29*(1), 14–20. http://doi.org/10.1111/joss.12077

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(1), 4516–4522. http://doi.org/10.1073/pnas.1000080107

Carrión, O., Miñana-Galbis, D., Montes, M. J., & Mercadé, E. (2011). *Pseudomonas deceptionensis* sp. nov., a psychrotolerant bacterium from the antarctic. *International Journal of Systematic and Evolutionary Microbiology*, *61*(10), 2401–2405. http://doi.org/10.1099/ijs.0.024919-0

Celandroni, F., Longo, I., Tosoratti, N., Giannessi, F., Ghelardi, E., Salvetti, S., … Senesi, S. (2004). Effect of microwave radiation on *Bacillus subtilis* spores. *Journal of Applied Microbiology*, *97*(6), 1220–1227. http://doi.org/10.1111/j.1365-2672.2004.02406.x

Cenkowski, S., Pronyk, C., Zmidzinska, D., & Muir, W. E. (2007). Decontamination of food products with superheated steam. *Journal of Food Engineering*, *83*(1), 68–75. http://doi.org/10.1016/j.jfoodeng.2006.12.002

Ceuppens, S., Boon, N., & Uyttendaele, M. (2013). Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. *FEMS Microbiology Ecology*. Oxford University Press. http://doi.org/10.1111/1574-6941.12110

Chao, A. (1984). Non-parametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, *11*(4), 265–270. http://doi.org/10.2307/4615964

Charpentier, E., Gerbaud, G., & Courvalin, P. (1994). Presence of the *Listeria* Tetracycline Resistance Gene *tet*(S) in *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy*, *38*(10), 2330–2335. http://doi.org/10.1128/AAC.38.10.2330

Chopra, I., & Roberts, M. (2001). Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews*, *65*(2), 232–260. http://doi.org/10.1128/MMBR.65.2.232-260.2001

Clementi, F., & Aquilanti, L. (2011). Recent investigations and updated criteria for the assessment of antibiotic resistance in food lactic acid bacteria. *Anaerobe*, *17*(6), 394–398. http://doi.org/https://doi.org/10.1016/j.anaerobe.2011.03.021

Clifford, C. W., & Woodring, J. P. (1990). Methods for rearing the house cricket, *Acheta domesticus* (L.), along with baseline values for feeding rates, growth rates, development times, and blood composition. *Journal of Applied Entomology*, *109*(1–5), 1–14. http://doi.org/10.1111/j.1439-0418.1990.tb00012.x

Clinical and Laboratory Standards Institute. (2017). *Performance Standards for Antimicrobial Susceptibility Testing* (M100 27th). Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Colman, D. R., Toolson, E. C., & Takacs-Vesbach, C. D. (2012). Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology*, *21*(20), 5124–5137. http://doi.org/10.1111/j.1365-294X.2012.05752.x

Crippen, T. L., Zheng, L., Sheffield, C. L., Tomberlin, J. K., Beier, R. C., & Yu, Z. (2012). Transient gut

retention and persistence of *Salmonella* through metamorphosis in the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). *Journal of Applied Microbiology*, *112*(5), 920–926. http://doi.org/10.1111/j.1365-2672.2012.05265.x

Dagevos, H., & Voordouw, J. (2013). Sustainability and meat consumption: Is reduction realistic? *Sustainability: Science, Practice, and Policy*, *9*(2), 60–69. http://doi.org/10.1080/15487733.2013.11908115

Dahiru, M., & Enabulele, O. I. (2015). Incidence of *Acinetobacter* in Fresh Carrot (*Daucus carota* subsp. *sativus*). *International Journal of Nutrition and Food Engineering*, *9*(12), 1192–1195. Retrieved from http://waset.org/publications/10003296/incidence-of-acinetobacter-in-fresh-carrot-daucus-carota-subsp.-sativus-

De Lara, J., Fernández, P. S., Periago, P. M., & Palop, A. (2002). Irradiation of spores of *Bacillus cereus* and *Bacillus subtilis* with electron beams. *Innovative Food Science and Emerging Technologies*, *3*(4), 379–384. http://doi.org/10.1016/S1466-8564(02)00053-X

De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., … Whitman, W. B. (2009). *Bergey's Manual of Systematic Bacteriology Volume 3* (2nd ed.). New York, NY, USA: Springer Science+Business Media. http://doi.org/10.1007/978-0-387-68489-5

Dijk, R., Beumer, R., de Boer, E., Brinkman, E., van Dijk, J., Dijkstra, A., … van Woerden, M. (2007). *Microbiologie van voedingsmiddelen: Methoden, principes en criteria* (4th ed.). Houten, The Netherlands: Noordervliet Media.

Dijk, R., van den Berg, D., Beumer, R., de Boer, E., Dijkstra, A., Mout, L., … in 't Veld, S. (2015). *Microbiologie van voedingsmiddelen: Methoden, principes en criteria* (5th ed.). Capelle aan den Ijssel, The Netherlands: MYbusinessmedia.

Dobermann, D., Swift, J. A., & Field, L. M. (2017). Opportunities and hurdles of edible insects for food and feed. *Nutrition Bulletin*, *42*(4), 293–308. http://doi.org/10.1111/nbu.12291

Doherty, N., Trzcinski, K., Pickerill, P., Zawadzki, P., & Dowson, C. G. (2000). Genetic diversity of the *tet*(M) gene in tetracycline-resistant clonal lineages of *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy*, *44*(11), 2979–2984. http://doi.org/10.1128/AAC.44.11.2979-2984.2000

Dreon, A. L., & Paoletti, M. G. (2009). The wild food (plants and insects) in Western Friuli local knowledge (Friuli-Venezia Giulia, North Eastern Italy). *Contributions to Natural History*, *12*(12), 461–488. Retrieved from http://www.bio.unipd.it/agroecology/download/pdf/papers/2009/Ethnobiology Dreon Paoletti Thaler Memoral book Reduced dimension.pdf

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

ECDC, EFSA, & EMA. (2017). ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. *EFSA Journal*, *15*(7). http://doi.org/10.2903/j.efsa.2017.4872

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

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature*

Methods, *10*(10), 996–998. http://doi.org/10.1038/nmeth.2604

Edgar, R. C. (2016). SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *BioRxiv*, 074161. http://doi.org/10.1101/074161

EFSA Panel on Biological Hazards. (2016). Risks for public health related to the presence of *Bacillus cereus* and other *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs. *EFSA Journal*, *14*(7), 93. http://doi.org/10.2903/j.efsa.2016.4524

EFSA Scientific Committee. (2015). Risk profile related to production and consumption of insects as food and feed. *EFSA Journal 2015*, *13*(10), 60 pp. http://doi.org/10.2903/j.efsa.2015.4257

Ehling-Schulz, M., Frenzel, E., & Gohar, M. (2015). Food-bacteria interplay: Pathometabolism of emetic *Bacillus cereus*. *Frontiers in Microbiology*, *6*, 704. http://doi.org/10.3389/fmicb.2015.00704

Eilenberg, J., Vlak, J. M., Nielsen-LeRoux, C., Cappellozza, S., & Jensen, A. B. (2015). Diseases in insects produced for food and feed. *Journal of Insects as Food and Feed*, *1*(2), 87–102. http://doi.org/10.3920/JIFF2014.0022

EMA, & EFSA. (2017). EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). *EFSA Journal*, *15*(1), 245. http://doi.org/10.2903/j.efsa.2017.4666

Evans, J., Alemu, M. H., Flore, R., Frøst, M. B., Halloran, A., Jensen, A. B., … Eilenberg, J. (2015). 'Entomophagy': an evolving terminology in need of review. *Journal of Insects as Food and Feed*, *1*(4), 293–305. http://doi.org/10.3920/JIFF2015.0074

FAO. (2009). *How to Feed the World in 2050*. Retrieved from http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf

FAO. (2011). *World Livestock 2011 - Livestock in Food Security*. (A. McLeod, Ed.). Rome: FAO. http://doi.org/10.1080/00036841003742587

Farrar, J. J., Nunez, J. J., & Davis, R. M. (2000). Influence of soil saturation and temperature on *Erwinia chrysanthemi* soft rot of carrot. *Plant Disease*, *84*(6), 665–668. http://doi.org/10.1094/PDIS.2000.84.6.665

FASFC. (2014). *Circular concerning the breeding and marketing of insects and insect-based food for human consumption*. Brussels, Belgium. Retrieved from http://www.afsca.be/foodstuffs/insects/_documents/2014-05-21_Circular_insects_version11_EN.pdf

FASFC. (2016). *Circular concerning the breeding and marketing of insects and insect-based food for human consumption*. Brussels, Belgium. Retrieved from http://www.afsca.be/levensmiddelen/omzendbrieven/_documents/2016-04-26_circ-ob_NL_insecten_V2_clean.pdf

Fasolato, L., Cardazzo, B., Carraro, L., Fontana, F., Novelli, E., & Balzan, S. (2018). Edible processed insects from e-commerce: Food safety with a focus on the *Bacillus cereus* group. *Food Microbiology*, *76*, 296–303. http://doi.org/10.1016/j.fm.2018.06.008

Federal Public Service Health Food Safety and Environment. (2018). *State of the play concerning the commercialisation of insects or products on the basis of insects for human consumption after 1 January 2018 on the Belgian market*. Brussels, Belgium. Retrieved from https://www.health.belgium.be/sites/default/files/uploads/fields/fpshealth_theme_file/2018_01_revision_insects_stateoftheplay_en.pdf

References

Fellows, P. (Peter). (2009). *Food processing technology: Principles and practice* (3rd ed.). Cambridge, UK: Woodhead Publishing Limited and CRC Press LLC.

Fernandez-Cassi, X., Supenu, A., Jansson, A., Boqvist, S., Vagsholm, I., Boqvist, S., & Vagsholm, I. (2018). Novel foods: a risk profile for the house cricket (*Acheta domesticus*). *EFSA Journal*, *16*. http://doi.org/10.2903/j.efsa.2018.e16082

Finke, M. D. (2002). Complete nutrient composition of commercially raised invertebrates used as food for insectivores. *Zoo Biology*, *21*(3), 269–285. http://doi.org/10.1002/zoo.10031

Finlay, W. J. J., Logan, N. A., & Sutherland, A. D. (2000). *Bacillus cereus* produces most emetic toxin at lower temperatures. *Letters in Applied Microbiology*, *31*(5), 385–389. http://doi.org/10.1046/j.1472-765X.2000.00835.x

Flórez, A. B., Alegría, Á., Rossi, F., Delgado, S., Felis, G. E., Torriani, S., & Mayo, B. (2014). Molecular Identification and Quantification of Tetracycline and Erythromycin Resistance Genes in Spanish and Italian Retail Cheeses. *BioMed Research International*, *2014*, 746859. http://doi.org/10.1155/2014/746859

Foley, J. A., Ramankutty, N., Brauman, K. A., Cassidy, E. S., Gerber, J. S., Johnston, M., … Zaks, D. P. M. (2011). Solutions for a cultivated planet. *Nature*, *478*(7369), 337–342. http://doi.org/10.1038/nature10452

Fombong, F. T., Van Der Borght, M., & Vanden Broeck, J. (2017). Influence of Freeze-Drying and Oven-Drying Post Blanching on the Nutrient Composition of the Edible Insect *Ruspolia differens*. *Insects*, *8*(3), 102–116. http://doi.org/10.3390/insects8030102

Garofalo, C., Osimani, A., Milanović, V., Taccari, M., Cardinali, F., Aquilanti, L., … Clementi, F. (2017). The microbiota of marketed processed edible insects as revealed by high-throughput sequencing. *Food Microbiology*, *62*, 15–22. http://doi.org/10.1016/j.fm.2016.09.012

Gevers, D., Masco, L., Baert, L., Huys, G., Debevere, J., & Swings, J. (2003). Prevalence and Diversity of Tetracycline Resistant Lactic Acid Bacteria and Their *tet* Genes Along The Process Line of Fermented Dry Sausages. *Systematic and Applied Microbiology*, *26*(2), 277–283. http://doi.org/10.1078/072320203322346137

Ghaly, A. E., & Alkoaik, F. N. (2009). The Yellow Mealworm as a Novel Source of Protein. *American Journal of Agricultural and Biological Sciences*, *4*(4), 319–331.

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

Gille, D., & Schmid, A. (2015). Vitamin B12 in meat and dairy products. *Nutrition Reviews*, *73*(2), 106–115. http://doi.org/10.1093/nutrit/nuu011

Godfray, H. C. J., Aveyard, P., Garnett, T., Hall, J. W., Key, T. J., Lorimer, J., … Jebb, S. A. (2018). Meat consumption, health, and the environment. *Science*, *361*(6399). http://doi.org/https://doi.org/10.1126/science.aam5324

Gogarten, M. B., Gogarten, J. P., & Olendzenski, L. C. (Eds.). (2009). *Horizontal Gene Transfer* (Vol. 532). Totowa, NJ: Humana Press. http://doi.org/10.1007/978-1-60327-853-9

Grabowski, N. T., Jansen, W., & Klein, G. (2014). Microbiological status of edible insects sold as pet feed in Germany. In *Conference "Insects to feed the world."* Wageningen University and Research Centre, Wageningen, The Netherlands.

Grabowski, N. T., & Klein, G. (2016). Microbiology of processed edible insect products – Results of a

preliminary survey. *International Journal of Food Microbiology*, *243*, 103–107. http://doi.org/10.1016/j.ijfoodmicro.2016.11.005

Grabowski, N. T., & Klein, G. (2017). Bacteria encountered in raw insect, spider, scorpion, and centipede taxa including edible species, and their significance from the food hygiene point of view. *Trends in Food Science and Technology*. Elsevier. http://doi.org/10.1016/j.tifs.2017.01.007

Grim, C. J., Kotewicz, M. L., Power, K. A., Gopinath, G., Franco, A. A., Jarvis, K. G., … Tall, B. D. (2013). Pan-genome analysis of the emerging foodborne pathogen *Cronobacter* spp. suggests a species-level bidirectional divergence driven by niche adaptation. *BMC Genomics*, *14*(1), 366. http://doi.org/10.1186/1471-2164-14-366

Guinebretière, M. H., Auger, S., Galleron, N., Contzen, M., de Sarrau, B., de Buyser, M. L., … Sorokin, A. (2013). *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the *Bacillus cereus* group occasionally associated with food poisoning. *International Journal of Systematic and Evolutionary Microbiology*, *63*(1), 31–40. http://doi.org/10.1099/ijs.0.030627-0

Halloran, A., Flore, R., Vantomme, P., & Roos, N. (2018). *Edible Insects in Sustainable Food Systems*. Cham, Switzerland: Springer International Publishing. http://doi.org/10.1007/978-3-319-74011-9

Halloran, A., Hanboonsong, Y., Roos, N., & Bruun, S. (2017). Life cycle assessment of cricket farming in north-eastern Thailand. *Journal of Cleaner Production*, *156*, 83–94. http://doi.org/10.1016/j.jclepro.2017.04.017

Halloran, A., Roos, N., Eilenberg, J., Cerutti, A., & Bruun, S. (2016). Life cycle assessment of edible insects for food protein: A review. *Agronomy for Sustainable Development*, *36*(4), 57. http://doi.org/10.1007/s13593-016-0392-8

Hanboonsong, Y., Jamjanya, T., & Durst, P. B. (2013). *Six-legged livestock: edible insect farming, collecting and marketing in Thailand*. Retrieved from http://www.fao.org/3/a-i3246e.pdf

Hardouin, J. (1995). Minilivestock: from gathering to controlled production. *Biodiversity and Conservation*, *4*(3), 220–232. http://doi.org/10.1007/BF00055969

Hausdorf, L., Fröhling, A., Schlüter, O. K., & Klocke, M. (2011). Analysis of the bacterial community within carrot wash water. *Canadian Journal of Microbiology*, *57*(5), 447–452. http://doi.org/10.1139/w11-013

Herdegen, V., & Vogel, R. F. (1998). Strategies for High Pressure Inactivation of Endospore-forming Bacteria. *High Pressure Food Science, Bioscience and Chemistry*, 394–402. http://doi.org/10.1533/9781845698379.5.394

Hoek, A. C., Pearson, D., James, S. W., Lawrence, M. A., & Friel, S. (2017). Healthy and environmentally sustainable food choices: Consumer responses to point-of-purchase actions. *Food Quality and Preference*, *58*, 94–106. http://doi.org/10.1016/j.foodqual.2016.12.008

Hölzel, C. S., Huther, S. K., Schwaiger, K., Kämpf, P., & Bauer, J. (2011). Quantity of the Tetracycline Resistance Gene *tet*(M) Differs Substantially between Meat at Slaughterhouses and at Retail. *Journal of Food Science*, *76*(6), M318–M323. http://doi.org/10.1111/j.1750-3841.2011.02239.x

House, J. (2016). Consumer acceptance of insect-based foods in the Netherlands: Academic and commercial implications. *Appetite*, *107*, 47–58. http://doi.org/10.1016/j.appet.2016.07.023

House, J. (2018). Insects as food in the Netherlands: Production networks and the geographies of edibility. *Geoforum*, *94*, 82–93. http://doi.org/10.1016/j.geoforum.2018.05.011

Hwang, C.-A., & Tamplin, M. L. (2005). The influence of mayonnaise pH and storage temperature on

the growth of *Listeria monocytogenes* in seafood salad. *International Journal of Food Microbiology*, *102*(3), 277–85. http://doi.org/10.1016/j.ijfoodmicro.2004.11.019

Ishihara, K., Nakajima, K., Kishimoto, S., Atarashi, F., Muramatsu, Y., Hotta, A., … Tamura, Y. (2013). Distribution of antimicrobial-resistant lactic acid bacteria in natural cheese in Japan. *Microbiology and Immunology*, *57*(10), 684–691. http://doi.org/10.1111/1348-0421.12090

Jacob, A. E., & Hobbs, S. J. (1974). Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. *Journal of Bacteriology*, *117*(2), 360–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4204433

Janssen, R. H., Vincken, J. P., Van Den Broek, L. A. M., Fogliano, V., & Lakemond, C. M. M. (2017). Nitrogen-to-Protein Conversion Factors for Three Edible Insects: *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*. *Journal of Agricultural and Food Chemistry*, *65*(11), 2275–2278. http://doi.org/10.1021/acs.jafc.7b00471

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). *Modern Food Microbiology* (7th ed.). New York, NY, USA: Springer Science+Business Media. http://doi.org/10.1007/b100840

Jones, R. T., Sanchez, L. G., & Fierer, N. (2013). A cross-taxon analysis of insect-associated bacterial diversity. *PloS One*, *8*(4), e61218. http://doi.org/10.1371/journal.pone.0061218

Jongema, Y. (2017). List of edible insects of the world (April 1, 2017). Retrieved June 5, 2018, from https://www.wur.nl/en/Research-Results/Chair-groups/Plant-Sciences/Laboratory-of-Entomology/Edible-insects/Worldwide-species-list.htm

Jung, J., Heo, A., Woo Park, Y., Ji Kim, Y., Koh, H., & Park, W. (2014). Gut microbiota of *Tenebrio molitor* and their response to environmental change. *Journal of Microbiology and Biotechnology*, *24*(7), 888–897. http://doi.org/10.4014/jmb.1405.05016

Justé, A., Thomma, B. P. H. J., & Lievens, B. (2008). Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes. *Food Microbiology*, *25*(6), 745–761. http://doi.org/10.1016/j.fm.2008.04.009

Kim, H., Kim, H., Bang, J., Kim, Y., Beuchat, L. R., & Ryu, J. H. (2012). Reduction of *Bacillus cereus* spores in sikhye, a traditional Korean rice beverage, by modified tyndallization processes with and without carbon dioxide injection. *Letters in Applied Microbiology*, *55*(3), 218–223. http://doi.org/10.1111/j.1472-765X.2012.03278.x

Kim, S.-R., Nonaka, L., & Suzuki, S. (2004). Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiology Letters*, *237*(1), 147–156. http://doi.org/10.1016/j.femsle.2004.06.026

Klunder, H. C., Wolkers-Rooijackers, J., Korpela, J. M., & Nout, M. J. R. (2012). Microbiological aspects of processing and storage of edible insects. *Food Control*, *26*(2), 628–631. http://doi.org/10.1016/j.foodcont.2012.02.013

Kort, R., O'Brien, A. C., van Stokkum, I. H. M., Oomes, S. J. C. M., Crielaard, W., Hellingwerf, K. J., & Brul, S. (2005). Assessment of Heat Resistance of Bacterial Spores from Food Product Isolates by Fluorescence Monitoring of Dipicolinic Acid Release. *Applied and Environmental Microbiology*, *71*(7), 3556 LP-3564. Retrieved from http://aem.asm.org/content/71/7/3556.abstract

Kouřimská, L., & Adámková, A. (2016). Nutritional and sensory quality of edible insects. *NFS Journal*, *4*, 22–26. http://doi.org/10.1016/j.nfs.2016.07.001

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and Environmental Microbiology*, *79*(17), 5112–

5120. http://doi.org/10.1128/AEM.01043-13

Krieg, N. R., Staley, J. T., Brown, D. R., Hedlund, B. P., Paster, B. J., Ward, N. L., … Whitman, W. B. (2010). *Bergey's Manual of Systematic Bacteriology Volume 4* (2nd ed.). New York, NY, USA: Springer Science+Business Media. http://doi.org/10.1007/978-0-387-68572-4

Leclercq, R. (2002). Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. *Clinical Infectious Diseases*, *34*(4), 482–492. http://doi.org/10.1086/324626

Lenaerts, S., Van Der Borght, M., Callens, A., & Van Campenhout, L. (2018). Suitability of microwave drying for mealworms (*Tenebrio molitor*) as alternative to freeze drying: Impact on nutritional quality and colour. *Food Chemistry*, *254*, 129–136. http://doi.org/10.1016/j.foodchem.2018.02.006

Lensvelt, E. J. S., & Steenbekkers, L. P. A. (2014). Exploring Consumer Acceptance of Entomophagy: A Survey and Experiment in Australia and the Netherlands. *Ecology of Food and Nutrition*, *53*(5), 543–561. http://doi.org/10.1080/03670244.2013.879865

Li, L., Xie, B., Dong, C., Wang, M., & Liu, H. (2016). Can closed artificial ecosystem have an impact on insect microbial community? A case study of yellow mealworm (*Tenebrio molitor* L.). *Ecological Engineering*, *86*, 183–189. http://doi.org/10.1016/j.ecoleng.2015.09.015

Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Lévesque, C. A., Cammue, B. P. A., & Thomma, B. P. H. J. (2003). Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. *FEMS Microbiology Letters*, *223*(1), 113–122. http://doi.org/10.1016/S0378-1097(03)00352-5

Liu, Y., Lai, Q., Göker, M., Meier-Kolthoff, J. P., Wang, M., Sun, Y., … Shao, Z. (2015). Genomic insights into the taxonomic status of the *Bacillus cereus* group. *Scientific Reports*, *5*(1), 14082. http://doi.org/10.1038/srep14082

Looy, H., Dunkel, F. V., & Wood, J. R. (2014). How then shall we eat? Insect-eating attitudes and sustainable foodways. *Agriculture and Human Values*, *31*(1), 131–141. http://doi.org/10.1007/s10460-013-9450-x

Luu-Thi, H., Khadka, D. B., & Michiels, C. W. (2014). Thermal inactivation parameters of spores from different phylogenetic groups of *Bacillus cereus*. *International Journal of Food Microbiology*, *189*, 183–188. http://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2014.07.027

Maciel-Vergara, G., & Ros, V. I. D. (2017). Viruses of insects reared for food and feed. *Journal of Invertebrate Pathology*, *147*, 60–75. http://doi.org/10.1016/j.jip.2017.01.013

Madigan, M. T., Martinko, J. M., Dunlap, P. V, & Clark, D. P. (2009). *Brock Biology of microorganisms* (12th ed.). San Francisco, CA, USA: Pearson/Benjamin Cummings.

Magee, L., Scerri, A., James, P., Thom, J. A., Padgham, L., Hickmott, S., … Cahill, F. (2013). Reframing social sustainability reporting: Towards an engaged approach. *Environment, Development and Sustainability*, *15*(1), 225–243. http://doi.org/10.1007/s10668-012-9384-2

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. *Animal Feed Science and Technology*, *197*, 1–33. http://doi.org/10.1016/j.anifeedsci.2014.07.008

Mañas, P., & Pagán, R. (2005). Microbial inactivation by new technologies of food preservation. *Journal of Applied Microbiology*, *98*(6), 1387–1399. http://doi.org/10.1111/j.1365-2672.2005.02561.x

Manuzon, M. Y., Hanna, S. E., Luo, H., Yu, Z., Harper, W. J., & Wang, H. H. (2007). Quantitative Assessment of the Tetracycline Resistance Gene Pool in Cheese Samples by Real-Time TaqMan PCR. *Applied and Environmental Microbiology*, *73*(5), 1676–1677. http://doi.org/10.1128/AEM.01994-06

Martínez, S., Borrajo, R., Franco, I., & Carballo, J. (2007). Effect of environmental parameters on growth kinetics of *Bacillus cereus* (ATCC 7004) after mild heat treatment. *International Journal of Food Microbiology*, *117*(2), 223–7. http://doi.org/10.1016/j.ijfoodmicro.2006.08.002

Mazzette, R., Colleo, M. M., Riu, G., Piras, G., Piras, F., Addis, M., … Lentini, A. (2010). Production under controlled conditions of "Casu Marzu" cheese: Effect of the *Piophila casei* colonization on microbial and chemical composition of the cheeses. *Italian Journal of Food Safety*, *A.I.V.I. m*(7), 45–54. http://doi.org/10.4081/ijfs.2010.1102

Milanović, V., Osimani, A., Aquilanti, L., Tavoletti, S., Garofalo, C., Polverigiani, S., … Clementi, F. (2017). Occurrence of antibiotic resistance genes in the fecal DNA of healthy omnivores, ovo-lacto vegetarians and vegans. *Molecular Nutrition and Food Research*, *61*(9), 1601098. http://doi.org/10.1002/mnfr.201601098

Milanović, V., Osimani, A., Pasquini, M., Aquilanti, L., Garofalo, C., Taccari, M., … Clementi, F. (2016). Getting insight into the prevalence of antibiotic resistance genes in specimens of marketed edible insects. *International Journal of Food Microbiology*, *227*, 22–28. http://doi.org/10.1016/j.ijfoodmicro.2016.03.018

Mlček, J., Rop, O., Borkovcova, M., & Bednarova, M. (2014). A Comprehensive Look at the Possibilities of Edible Insects as Food in Europe – a Review. *Polish Journal of Food and Nutrition Sciences*, *64*(3), 147–157. http://doi.org/10.2478/v10222-012-0099-8

Mohamed, S., Hashim, S. N., & Rahman, H. A. (2012). Seaweeds: A sustainable functional food for complementary and alternative therapy. *Trends in Food Science and Technology*, *23*(2), 83–96. http://doi.org/10.1016/j.tifs.2011.09.001

Moretti, C., Hosni, T., Vandemeulebroecke, K., Brady, C., de Vos, P., Buonaurio, R., & Cleenwerck, I. (2011). *Erwinia oleae* sp. nov., isolated from olive knots caused by *Pseudomonas savastanoi* pv. *savastanoi*. *International Journal of Systematic and Evolutionary Microbiology*, *61*(11), 2745–2752. http://doi.org/10.1099/ijs.0.026336-0

Mpuchane, S., Allotey, J., Matsheka, I., Simpanya, M., Coetzee, S., Jordaan, A., … Gashe, B. A. (2006). Carriage of micro-organisms by domestic cockroaches and implications on food safety. *International Journal of Tropical Insect Science*, *26*(03), 166–175. http://doi.org/10.1079/IJT20063141

Nadeau, L., Nadeau, I., Franklin, F., & Dunkel, F. (2015). The Potential for Entomophagy to Address Undernutrition. *Ecology of Food and Nutrition*, *54*(3), 200–208. http://doi.org/10.1080/03670244.2014.930032

Nowak, V., Persijn, D., Rittenschober, D., & Charrondiere, U. R. (2016). Review of food composition data for edible insects. *Food Chemistry*, *193*, 39–46. http://doi.org/10.1016/j.foodchem.2014.10.114

NVWA. (2014). *Advisory report on the risks associated with the consumption of mass-reared insects*. Utrecht, The Netherlands. Retrieved from https://www.nvwa.nl/binaries/nvwa/documenten/consument/eten-drinken-roken/overige-voedselveiligheid/risicobeoordelingen/advies-consumptie-gekweekte-insecten/Advies+over+de+risico%27s+van+consumptie+van+gekweekte+insecten.pdf

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., … Wagner, H. (2012).

Vegan: Community Ecology. In *R package ver. 2.0–5*. Vienna, Austria: R Foundation for Statistical Computing.

Oonincx, D. G. A. B., & de Boer, I. J. M. (2012). Environmental Impact of the Production of Mealworms as a Protein Source for Humans – A Life Cycle Assessment. *PLoS ONE*, *7*(12), e51145. http://doi.org/10.1371/journal.pone.0051145

Oonincx, D. G. A. B., Van Broekhoven, S., Van Huis, A., & Van Loon, J. J. A. (2015). Feed Conversion, Survival and Development, and Composition of Four Insect Species on Diets Composed of Food By-Products. *PLoS ONE*, *10*(12), e0144601. http://doi.org/10.1371/journal.pone.0144601

Oonincx, D. G. A. B., van Itterbeeck, J., Heetkamp, M. J. W., van den Brand, H., Van Loon, J. J. A., & van Huis, A. (2010). An exploration on greenhouse gas and ammonia production by insect species suitable for animal or human consumption. *PLoS One*, *5*(12), e14445. http://doi.org/10.1371/journal.pone.0014445

Osimani, A., Cardinali, F., Aquilanti, L., Garofalo, C., Roncolini, A., Milanović, V., … 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. http://doi.org/10.1016/j.ijfoodmicro.2017.10.009

Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., Cardinali, F., Taccari, M., … Clementi, F. (2017). Transferable Antibiotic Resistances in Marketed Edible Grasshoppers (*Locusta migratoria migratorioides*). *Journal of Food Science*, *82*(5), 1184–1192. http://doi.org/10.1111/1750-3841.13700

Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., & Clementi, F. (2015). Unpasteurised commercial boza as a source of microbial diversity. *International Journal of Food Microbiology*, *194*, 62–70. http://doi.org/10.1016/j.ijfoodmicro.2014.11.011

Osimani, A., Garofalo, C., Milanović, V., Taccari, M., Cardinali, F., Aquilanti, L., … Clementi, F. (2017). Insight into the proximate composition and microbial diversity of edible insects marketed in the European Union. *European Food Research and Technology*, *243*(7), 1157–1171. http://doi.org/10.1007/s00217-016-2828-4

Osimani, A., Milanović, V., Cardinali, F., Garofalo, C., Clementi, F., Pasquini, M., … Aquilanti, L. (2018). The bacterial biota of laboratory-reared edible mealworms (*Tenebrio molitor* L.): From feed to frass. *International Journal of Food Microbiology*, *272*, 49–60. http://doi.org/10.1016/j.ijfoodmicro.2018.03.001

Osimani, A., Milanović, V., Cardinali, F., Roncolini, A., Garofalo, C., Clementi, F., … Aquilanti, L. (2018). Bread enriched with cricket powder (*Acheta domesticus*): A technological, microbiological and nutritional evaluation. *Innovative Food Science & Emerging Technologies*, *48*, 150–163. http://doi.org/10.1016/J.IFSET.2018.06.007

Osimani, A., Milanović, V., Garofalo, C., Cardinali, F., Roncolini, A., Sabbatini, R., … 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. http://doi.org/10.1016/j.ijfoodmicro.2018.04.013

Payne, C. L. R., Scarborough, P., Rayner, M., & Nonaka, K. (2016). A systematic review of nutrient composition data available for twelve commercially available edible insects, and comparison with reference values. *Trends in Food Science and Technology*, *47*, 69–77. http://doi.org/10.1016/j.tifs.2015.10.012

Payne, C. L. R., & Van Itterbeeck, J. (2017). Ecosystem Services from Edible Insects in Agricultural Systems: A Review. *Insects*, *8*(1), 24. http://doi.org/10.3390/insects8010024

Penders, J., Stobberingh, E. E., Savelkoul, P. H. M., & Wolffs, P. F. G. (2013). The human microbiome as a reservoir of antimicrobial resistance. *Frontiers in Microbiology*, *4*, 87. http://doi.org/10.3389/fmicb.2013.00087

Post, M. J. (2012). Cultured meat from stem cells: Challenges and prospects. *Meat Science*, *92*(3), 297–301. http://doi.org/10.1016/j.meatsci.2012.04.008

Premalatha, M., Abbasi, T., Abbasi, T., & Abbasi, S. A. (2011). Energy-efficient food production to reduce global warming and ecodegradation: The use of edible insects. *Renewable and Sustainable Energy Reviews*, *15*(9), 4357–4360. http://doi.org/10.1016/j.rser.2011.07.115

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., … Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. http://doi.org/10.1093/nar/gks1219

R Development Core Team. (2013). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://scholar.google.com/scholar?cluster=8103611549594844363&hl=en&oi=scholarr#0

Raheem, D., Carrascosa, C., Oluwole, O. B., Nieuwland, M., Saraiva, A., Millán, R., & Raposo, A. (2018). Traditional consumption of and rearing edible insects in Africa, Asia and Europe. *Critical Reviews in Food Science and Nutrition*, 1–75. http://doi.org/10.1080/10408398.2018.1440191

Ramos-Elorduy, J. (2009). Anthropo-entomophagy: Cultures, evolution and sustainability. *Entomological Research*, *39*(5), 271–288. http://doi.org/10.1111/j.1748-5967.2009.00238.x

Ramos-Elorduy, J., Landero-Torres, I., Murguía-González, J., & Pino M., J. M. (2008). Biodiversidad antropoentomofágica de la región de Zongolica, Veracruz, México. *Revista de Biología Tropical*, *56*(1). http://doi.org/10.15517/rbt.v56i1.5526

Rasigade, J. P., Hollandt, F., & Wirth, T. (2018). Genes under positive selection in the core genome of pathogenic *Bacillus cereus* group members. *Infection, Genetics and Evolution*, *65*, 55–64. http://doi.org/10.1016/j.meegid.2018.07.009

Ravzanaadii, N., Kim, S.-H., Choi, W. H., Hong, S.-J., & Kim, N. J. (2012). Nutritional Value of Mealworm, *Tenebrio molitor* as Food Source. *International Journal of Industrial Entomology*, *25*(1), 93–98. http://doi.org/10.7852/ijie.2012.25.1.093

Reddy, G. S. N., Matsumoto, G. I., Schumann, P., Stackerbrandt, E., & Shivaji, S. (2004). Psychrophilic pseudomonads from Antarctica: *Pseudomonas antarctica* sp. nov., *Pseudomonas meridiana* sp. nov. and *Pseudomonas proteolytica* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, *54*(3), 713–719. http://doi.org/10.1099/ijs.0.02827-0

Reijnders, L., & Soret, S. (2003). Quantification of the environmental impact of different dietary protein choices. *The American Journal of Clinical Nutrition*, *78*(3 Suppl), 664S–668S. http://doi.org/10.1093/ajcn/78.3.664S

Reineke, K., Mathys, A., & Knorr, D. (2011). The impact of high pressure and temperature on bacterial spores: Inactivation mechanisms of *Bacillus subtilis* above 500 MPa. *Journal of Food Science*, *76*(3), M189–M197. http://doi.org/10.1111/j.1750-3841.2011.02066.x

Rossoni, E. M. M., & Gaylarde, C. C. (2000). Comparison of sodium hypochlorite and peracetic acid as sanitising agents for stainless steel food processing surfaces using epifluorescence microscopy. *International Journal of Food Microbiology*, *61*(1), 81–85. http://doi.org/https://doi.org/10.1016/S0168-1605(00)00369-X

Ruiu, L. (2013). Brevibacillus laterosporus, a Pathogen of Invertebrates and a Broad-Spectrum Antimicrobial Species. *Insects*. Multidisciplinary Digital Publishing Institute (MDPI).

http://doi.org/10.3390/insects4030476

Rumpold, B. A., Fröhling, A., Reineke, K., Knorr, D., Boguslawski, S., Ehlbeck, J., & Schlüter, O. K. (2014). Comparison of volumetric and surface decontamination techniques for innovative processing of mealworm larvae (*Tenebrio molitor*). *Innovative Food Science & Emerging Technologies*, *26*, 232–241. http://doi.org/10.1016/j.ifset.2014.09.002

Rumpold, B. A., & Schlüter, O. K. (2013a). Nutritional composition and safety aspects of edible insects. *Mol Nutr Food Res*, *57*(5), 802–823. http://doi.org/10.1002/mnfr.201200735

Rumpold, B. A., & Schlüter, O. K. (2013b). Potential and challenges of insects as an innovative source for food and feed production. *Innovative Food Science & Emerging Technologies*, *17*, 1–11. http://doi.org/10.1016/j.ifset.2012.11.005

Sánchez-Muros, M.-J., Barroso, F. G., & Manzano-Agugliaro, F. (2014). Insect meal as renewable source of food for animal feeding: a review. *Journal of Cleaner Production*, *65*, 16–27. http://doi.org/10.1016/j.jclepro.2013.11.068

Schillewaert, S. (2018). *Overzicht van bedrijven betrokken in de eetbare insecten sector regio België - Nederland: Marktverkenning in het kader van Entomospeed*. Retrieved from http://www.insectinfo.be/DNN_DropZone/Nieuws/4475/Overzicht bedrijven insecten sector.pdf

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., … Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, *75*(23), 7537–7541. http://doi.org/10.1128/AEM.01541-09

Schösler, H., Boer, J. de, & Boersema, J. J. (2012). Can we cut out the meat of the dish? Constructing consumer-oriented pathways towards meat substitution. *Appetite*, *58*(1), 39–47. http://doi.org/10.1016/j.appet.2011.09.009

Schouteten, J. J., De Steur, H., De Pelsmaeker, S., Lagast, S., Juvinal, J. G., De Bourdeaudhuij, I., … Gellynck, X. (2016). Emotional and sensory profiling of insect-, plant- and meat-based burgers under blind, expected and informed conditions. *Food Quality and Preference*, *52*, 27–31. http://doi.org/10.1016/j.foodqual.2016.03.011

Serrano, L., Manker, D., Brandi, F., & Cali, T. (2013). The Use of *Bacillus subtilis* QST 713 and *Bacillus pumilus* QST 2808 as Protectant Fungicides in Conventional Application Programs for Black Leaf Streak Control. *Acta Horticulturae*, *986*, 149–156. http://doi.org/10.17660/ActaHortic.2013.986.15

Shannon, C. E. (1948). A Mathematical Theory of Communication. *Bell System Technical Journal*, *27*(3), 379–423. http://doi.org/10.1002/j.1538-7305.1948.tb01338.x

SHC, & FASFC. (2014). *Food safety aspects of insects intended for human consumption (Sci Com dossier 2014/04; SHC dossier n° 9160)*. Brussels, Belgium. Retrieved from http://www.favv.be/scientificcommittee/advices/_documents/ADVICE14-2014_ENG_DOSSIER2014-04.pdf

Shokal, U., Yadav, S., Atri, J., Accetta, J., Kenney, E., Banks, K., … Eleftherianos, I. (2016). Effects of co-occurring *Wolbachia* and *Spiroplasma* endosymbionts on the *Drosophila* immune response against insect pathogenic and non-pathogenic bacteria. *BMC Microbiology*, *16*(1), 16. http://doi.org/10.1186/s12866-016-0634-6

Siemianowska, E., Kosewska, A., Aljewicz, M., Skibniewska, K. A., Polak-Juszczak, L., Jarocki, A., & Jędras, M. (2013). Larvae of mealworm (*Tenebrio molitor* L.) as European novel food. *Agricultural*

Sciences, *04*(06), 287–291. http://doi.org/10.4236/as.2013.46041

Simon, E., Baranyai, E., Braun, M., Fábián, I., & Tóthmérész, B. (2013). Elemental Concentration in Mealworm Beetle (*Tenebrio molitor* L.) During Metamorphosis. *Biological Trace Element Research*, *154*(1), 81–87. http://doi.org/http://dx.doi.org/10.1007/s12011-013-9700-1

Smetana, S., Mathys, A., Knoch, A., & Heinz, V. (2015). Meat alternatives: life cycle assessment of most known meat substitutes. *International Journal of Life Cycle Assessment*, *20*(9), 1254–1267. http://doi.org/10.1007/s11367-015-0931-6

Smetana, S., Palanisamy, M., Mathys, A., & Heinz, V. (2016). Sustainability of insect use for feed and food: Life Cycle Assessment perspective. *Journal of Cleaner Production*, *137*, 741–751. http://doi.org/10.1016/j.jclepro.2016.07.148

Smith, P., & Gregory, P. J. (2013). Climate change and sustainable food production. In *Proceedings of the Nutrition Society* (Vol. 72, pp. 21–28). Cambridge University Press. http://doi.org/10.1017/S0029665112002832

Sospedra, I., Soriano, J. M., & Mañes, J. (2010). Assessment of the Microbiological Safety of Dried Spices and Herbs Commercialized in Spain. *Plant Foods for Human Nutrition*, *65*(4), 364–368. http://doi.org/10.1007/s11130-010-0186-0

Sperber, W. H., & Doyle, M. P. (2009). *Compendium of the Microbiological Spoilage of Foods and Beverages*. New York, NY, USA: Springer. http://doi.org/10.1007/978-1-4419-0826-1

Stenfors, L. P., Mayr, R., Scherer, S., & Granum, E. (2002). Pathogenic potential of fifty *Bacillus weihenstephanensis* strains. *FEMS Microbiology Letters*, *215*(1), 47–51. http://doi.org/10.1111/j.1574-6968.2002.tb11368.x

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

Stoops, J., Vandeweyer, D., Crauwels, S., Verreth, C., Boeckx, H., Van Der Borght, M., … Van Campenhout, L. (2017). Minced meat-like products from mealworm larvae (*Tenebrio molitor* and *Alphitobius diaperinus*): Microbial dynamics during production and storage. *Innovative Food Science & Emerging Technologies*, *41*, 1–9. http://doi.org/10.1016/j.ifset.2017.02.001

Szelei, J., Woodring, J., Goettel, M. S., Duke, G., Jousset, F.-X., Liu, K. Y., … Tijssen, P. (2011). Susceptibility of North-American and European crickets to *Acheta domesticus* densovirus (AdDNV) and associated epizootics. *Journal of Invertebrate Pathology*, *106*(3), 394–399. http://doi.org/10.1016/J.JIP.2010.12.009

Tactacan, G. B., Schmidt, J. K., Miille, M. J., & Jimenez, D. R. (2013). A *Bacillus subtilis* (QST 713) spore-based probiotic for necrotic enteritis control in broiler chickens. *Journal of Applied Poultry Research*, *22*(4), 825–831. http://doi.org/10.3382/japr.2013-00730

Tan, H. S. G., Verbaan, Y. T., & Stieger, M. (2017). How will better products improve the sensory-liking and willingness to buy insect-based foods? *Food Research International*, *92*, 95–105. http://doi.org/10.1016/j.foodres.2016.12.021

Templeton, J. M., De Jong, A. J., Blackall, P. J., & Miflin, J. K. (2006). Survival of *Campylobacter* spp. in darkling beetles (*Alphitobius diaperinus*) and their larvae in Australia. *Applied and Environmental Microbiology*, *72*(12), 7909–7911. http://doi.org/10.1128/AEM.01471-06

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., & Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature*, *418*(6898), 671–677.

http://doi.org/10.1038/nature01014

Tuomisto, H. L., & Teixeira De Mattos, M. J. (2011). Environmental Impacts of Cultured Meat Production. *Environmental Science and Technology*, *45*(14), 6117–6123. http://doi.org/10.1021/es200130u

UN General Assembly. (2005). 2005 World Summit Outcome. *A/Res/60/1*, (October), 1–38. http://doi.org/10.1016/0262-1762(95)90889-7

Usub, T., Lertsatitthankorn, C., Poomsa-ad, N., Wiset, L., Siriamornpun, S., & Soponronnarit, S. (2010). Thin layer solar drying characteristics of silkworm pupae. *Food and Bioproducts Processing*, *88*(2), 149–160. http://doi.org/https://doi.org/10.1016/j.fbp.2009.04.002

Van Assche, A., Álvarez-Pérez, S., de Breij, A., De Brabanter, J., Willems, K. A., Dijkshoorn, L., & Lievens, B. (2017). Phylogenetic signal in phenotypic traits related to carbon source assimilation and chemical sensitivity in *Acinetobacter* species. *Applied Microbiology and Biotechnology*, *101*(1), 367–379. http://doi.org/10.1007/s00253-016-7866-0

van Broekhoven, S., Oonincx, D. G. A. B., van Huis, A., & Van Loon, J. J. A. (2015). Growth performance and feed conversion efficiency of three edible mealworm species (Coleoptera: Tenebrionidae) on diets composed of organic by-products. *Journal of Insect Physiology*, *73*, 1–10. http://doi.org/https://doi.org/10.1016/j.jinsphys.2014.12.005

van der Spiegel, M., Noordam, M. Y., & van der Fels-Klerx, H. J. (2013). Safety of Novel Protein Sources (Insects, Microalgae, Seaweed, Duckweed, and Rapeseed) and Legislative Aspects for Their Application in Food and Feed Production. *Comprehensive Reviews in Food Science and Food Safety*, *12*(6), 662–678. http://doi.org/http://dx.doi.org/10.1111/1541-4337.12032

van Huis, A. (2013). Potential of insects as food and feed in assuring food security. *Annu Rev Entomol*, *58*, 563–583. http://doi.org/10.1146/annurev-ento-120811-153704

van Huis, A., & Oonincx, D. G. A. B. (2017). The environmental sustainability of insects as food and feed. A review. *Agronomy for Sustainable Development*. Springer-Verlag France. http://doi.org/10.1007/s13593-017-0452-8

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

van Schothorst, M., Zwietering, M. H., Ross, T., Buchanan, R. L., & Cole, M. B. (2009). Relating microbiological criteria to food safety objectives and performance objectives. *Food Control*, *20*(11), 967–979. http://doi.org/10.1016/j.foodcont.2008.11.005

Van Thielen, L., Vermuyten, S., Storms, B., Rumpold, B. A., & Van Campenhout, L. (2018). Consumer acceptance of foods containing edible insects in Belgium two years after their introduction to the market. *Journal of Insects as Food and Feed*, 1–10. http://doi.org/10.3920/JIFF2017.0075

Vandeweyer, D., Crauwels, S., Lievens, B., & Van Campenhout, L. (2017a). Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. *International Journal of Food Microbiology*, *261*, 11–18. http://doi.org/10.1016/j.ijfoodmicro.2017.08.018

Vandeweyer, D., Crauwels, S., Lievens, B., & Van Campenhout, L. (2017b). Microbial counts of mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus* and *Gryllodes sigillatus*) from different rearing companies and different production batches. *International Journal of Food Microbiology*, *242*, 13–18. http://doi.org/10.1016/j.ijfoodmicro.2016.11.007

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

Vandeweyer, D., Milanović, V., Garofalo, C., Osimani, A., Clementi, F., Van Campenhout, L., & Aquilanti, L. (2019). Real-time PCR detection and quantification of selected transferable antibiotic resistance genes in fresh edible insects from Belgium and the Netherlands. *International Journal of Food Microbiology*, *290*, 288–295. http://doi.org/10.1016/j.ijfoodmicro.2018.10.027

Vandeweyer, D., Wynants, E., Crauwels, S., Verreth, C., Viaene, N., Claes, J., … Van Campenhout, L. (2018). Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House Crickets (*Gryllodes sigillatus*) for Human Consumption. *Applied and Environmental Microbiology*, *84*(12), AEM.00255-18. http://doi.org/10.1128/AEM.00255-18

Verbeke, W. (2015). Profiling consumers who are ready to adopt insects as a meat substitute in a Western society. *Food Quality and Preference*, *39*, 147–155. http://doi.org/10.1016/j.foodqual.2014.07.008

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., … Herman, L. (2013). Antimicrobial Resistance in the Food Chain: A review. *International Journal of Environmental Research and Public Health*, *10*(7), 2643–2669. http://doi.org/10.3390/ijerph10072643

Vinnari, M., & Tapio, P. (2009). Future images of meat consumption in 2030. *Futures*, *41*(5), 269–278. http://doi.org/10.1016/j.futures.2008.11.014

Wang, L. T., Lee, F. L., Tai, C. J., & Kasai, H. (2007). Comparison of *gyr*B gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. *International Journal of Systematic and Evolutionary Microbiology*, *57*(8), 1846–1850. http://doi.org/10.1099/ijs.0.64685-0

Wang, Y.-S., & Shelomi, M. (2017). Review of Black Soldier Fly (*Hermetia illucens*) as Animal Feed and Human Food. *Foods*, *6*(10), 91. http://doi.org/10.3390/foods6100091

Wang, Y., & Zhang, Y. (2015). Investigation of gut-associated bacteria in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae using culture-dependent and DGGE methods. *Annals of the Entomological Society of America*, *108*(5), 941–949. http://doi.org/10.1093/aesa/sav079

Waud, M., Busschaert, P., Ruyters, S., Jacquemyn, H., & Lievens, B. (2014). Impact of primer choice on characterization of orchid mycorrhizal communities using 454 pyrosequencing. *Molecular Ecology Resources*, *14*(4), 679–699. http://doi.org/10.1111/1755-0998.12229

Wexler, H. M. (2007). Bacteroides: The good, the bad, and the nitty-gritty. *Clinical Microbiology Reviews*, *20*(4), 593–621. http://doi.org/10.1128/CMR.00008-07

Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., … Van Campenhout, L. (2017). Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworm larvae (*Tenebrio molitor*). *Innovative Food Science and Emerging Technologies*, *42*, 8–15. http://doi.org/10.1016/j.ifset.2017.06.004

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

Xu, Y., Sismour, E., Pao, S., Rutto, L., Grizzard, C., & Ren, S. (2012). Textural and Microbiological Qualities of Vegetable Soybean (Edamame) Affected by Blanching and Storage Conditions. *Journal of Food Processing & Technology*, *3*(6). http://doi.org/10.4172/2157-7110.1000165

Yen, A. L. (2009). Edible insects: Traditional knowledge or western phobia? *Entomological Research*, *39*(5), 289–298. http://doi.org/10.1111/j.1748-5967.2009.00239.x

Yen, A. L. (2015). Insects as food and feed in the Asia Pacific region: current perspectives and future directions. *Journal of Insects as Food and Feed*, *1*(1), 33–55. http://doi.org/10.3920/JIFF2014.0017

Yi, L., Lakemond, C. M. M., Sagis, L. M. C., Eisner-Schadler, V., Huis, A. Van, & Boekel, M. A. J. S. V. (2013). Extraction and characterisation of protein fractions from five insect species. *Food Chemistry*, *141*(4), 3341–3348. http://doi.org/10.1016/j.foodchem.2013.05.115

Yun, J.-H., Roh, S. W., Whon, T. W., Jung, M.-J., Kim, M.-S., Park, D.-S., … Bae, J.-W. (2014). Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and Environmental Microbiology*, *80*(17), 5254–64. http://doi.org/10.1128/AEM.01226-14

Zhang, Z., Qu, Y., Li, S., Feng, K., Wang, S., Cai, W., … Deng, Y. (2017). Soil bacterial quantification approaches coupling with relative abundances reflecting the changes of taxa. *Scientific Reports*, *7*(1), 4837. http://doi.org/10.1038/s41598-017-05260-w

Zheng, L., Crippen, T. L., Sheffield, C. L., Poole, T. L., Yu, Z., & Tomberlin, J. K. (2012). Evaluation of *Salmonella* Movement Through the Gut of the Lesser Mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). *Vector-Borne and Zoonotic Diseases*, *12*(4), 287–292. http://doi.org/10.1089/vbz.2011.0613

Zielińska, E., Baraniak, B., Karaś, M., Rybczyńska, K., & Jakubczyk, A. (2015). Selected species of edible insects as a source of nutrient composition. *Food Research International*, *77*, 460–466. http://doi.org/10.1016/j.foodres.2015.09.008

Supporting information

Supporting information for Chapter 1

Table S1.1 List of regulatory criteria and action limits for microbial contaminants in insects and insect-based products as published by the Belgian Federal Agency for the safety of the food chain (January 2018)[1].

Food product	Treatment	Microbiological parameter (type)	Food sector activity	Sampling plan[2]		Limits[3]		Unit	Type of action limit
				n	c	m	M		
Insects	Fried or boiled	*Listeria monocytogenes* (count)[4]	Distribution	5	0	100	100	cfu/g	Food safety criterion (legal requirement)[5]
		Salmonella spp. (detection)	Distribution	5	0	Absence		/25g	Food safety recommendation
	Grilled	*Listeria monocytogenes* (count)[4]	Distribution	5	0	100	100	cfu/g	Food safety criterion (legal requirement)
		Salmonella spp. (detection)	Distribution	5	0	Absence		/25g	Food safety recommendation
	Freeze-dried	*Listeria monocytogenes* (count)[4]	Distribution	5	0	100	100	cfu/g	Food safety criterion (legal requirement)
		Listeria monocytogenes (count)	Processing	5	0	100	100	cfu/g	Food safety criterion (legal requirement)
		Salmonella spp. (detection)	Distribution	5	0	Absence		/10g	Food safety recommendation
		Salmonella spp. (detection)	Processing	5	0	Absence		/10g	Food safety recommendation
	Raw	*Salmonella* spp. (detection)	Primary production	5	0	Absence		/10g	Food safety recommendation
Insect-based products	N.S.[7]	*Listeria monocytogenes* (detection)[6]	Processing	5	0	Absence		/25g	Food safety criterion (legal requirement)
		Salmonella spp. (detection)	Distribution	5	0	Absence		/25g	Food safety recommendation
		Salmonella spp. (detection)	Processing	5	0	Absence		/25g	Food safety recommendation
Insects and insect-based products	N.S.	*Bacillus cereus* (count)	Distribution	5	2	5000	100000	cfu/g	Distribution recommendation
		Bacillus cereus (count)	Primary production	5	2	5000	100000	cfu/g	Process hygiene recommendation
		Bacillus cereus (count)	Processing	5	2	5000	100000	cfu/g	Process hygiene recommendation
	N.S.	Enterobacteriaceae (count)	Distribution	5	2	5000	100000	cfu/g	Distribution recommendation
		Enterobacteriaceae (count)	Primary production	5	2	5000	100000	cfu/g	Process hygiene recommendation

Insects and insect-based products	Enterobacteriaceae (count)	Processing	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Escherichia coli (count)	Distribution	5	2	500	5000	cfu/g	Distribution recommendation
	Escherichia coli (count)	Primary production	5	2	500	5000	cfu/g	Process hygiene recommendation
	Escherichia coli (count)	Processing	5	2	500	5000	cfu/g	Process hygiene recommendation
	Yeasts (count)	Distribution	5	2	5000	100000	cfu/g	Distribution recommendation
	Yeasts (count)	Primary production	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Yeasts (count)	Processing	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Moulds (count)	Distribution	5	2	5000	100000	cfu/g	Distribution recommendation
	Moulds (count)	Primary production	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Moulds (count)	Processing	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Coagulase-positive staphylococci (count)	Distribution	5	2	5000	100000	cfu/g	Distribution recommendation
	Coagulase-positive staphylococci (count)	Primary production	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Coagulase-positive staphylococci (count)	Processing	5	2	5000	100000	cfu/g	Process hygiene recommendation
	Total viable aerobic count at 30 °C	Distribution	5	2	1000000	10000000	cfu/g	Distribution recommendation
	Total viable aerobic count at 30 °C	Primary production	5	2	1000000	10000000	cfu/g	Process hygiene recommendation
	Total viable aerobic count at 30 °C	Processing	5	2	1000000	10000000	cfu/g	Process hygiene recommendation

[1]Full list accessible via http://www.favv.be/thematischepublicaties/_documents/2018-01-16_Narval_microbio_VCT_JWS_ADK__CKS_5_jan_2018NL.xlsx.

[2]n = number of units comprising the sample; c = number of sample units giving values over m or between m and M.

[3]Satisfactory, if the following requirements are fulfilled: (1) the mean value observed is ≤ m, (2) a maximum of c/n values observed are between m and M, (3) no values observed exceed the limit of M. Unsatisfactory, if the mean value observed exceeds m or more than c/n values are between m and M or one or more of the values observed are >M.

[4]For products placed on the market during their shelf life.

[5]Legal requirements are published in the FASFC circular (FASFC, 2016).

[6]Before the food has left the immediate control of the food business operator, who has produced it.

[7]N.S. = not specified.

Table S1.2 List of insect species and their applications for which the extended Belgian tolerance applies (Federal Public Service Health Food Safety and Environment, 2018).

Acheta domesticus
100% packaged whole heat-treated *A. domesticus* adults
100% dried whole *A. domesticus*
100% whole *A. domesticus* flour
100% fresh whole *A. domesticus* dough
Pasta[1]
Protein products excluding dairy analogues[1]
Meat products[1]
Confectionery[1]
Salads and savoury based sandwich spreads[1]
Bakery wares[1]
Nut spreads[1]
Ready-to-eat savouries and snacks[1]
Tenebrio molitor
100% packaged whole heat-treated *T. molitor* larvae
100% dried/drained whole *T. molitor*/ 100% *T. molitor* flour
100% sterilised whole *T. molitor*
100% fresh whole *T. molitor* dough
100% roasted whole *T. molitor*
Flours and other milled products and starches[2]
Pasta[2]
Protein products excluding dairy analogues[2]
Confectionery[2]
Salads and savoury based sandwich spreads[2]
Bakery wares[2]
Nut spreads[2]
Soups and broths[2]
Sauces[2]
Ready-to-eat savouries and snacks[2]
Locusta migratoria
100% packaged frozen whole heat-treated *L. migratoria* last nymphal instar
100% packaged frozen whole heat-treated *L. migratoria* adults
100% packaged dried whole heat-treated *L. migratoria* adults
Protein products excluding dairy analogues[3]
Confectionery[3]
Salads and savoury based sandwich spreads[3]
Bakery wares[3]
Ready-to-eat savouries and snacks[3]
Nut spreads[3]
Soups and broths[3]
Sauces[3]

[1]with whole heat-treated *A. domesticus* adults [2]with whole heat-treated *T. molitor* larvae [3]with whole heat-treated *L. migratoria* last nymphal instars or adults.

Supporting information for Chapter 3

Table S3.1 Primer design and sample-specific barcodes.

Illumina Primer Design

Forward	<Forward Adapter> <i5> <pad> <link> <Forward Target Specific Primer>
Reverse	<Reverse Adapter> <i7> <pad> <link> <Reverse Target Specific Primer>
Adapter (Forward)	AATGATACGGCGACCACCGAGATCTACAC
Adapter (Reverse)	CAAGCAGAAGACGGCATACGAGAT
i5 (Forward)	see sample barcodes
i7 (Reverse)	see sample barcodes
pad (Forward)	TATGGTAATT
pad (Reverse)	AGTCAGTCAG
link (Forward)	GT
link (Reverse)	CC
Forward primer (515F)	5' - GTG CCA GCM GCC GCG GTA A - 3'
Reverse primer (806R)	5' - GGA CTA CHV GGG TWT CTA AT - 3'

Sample Barcodes

Insect	Rearing company	Rearing batch	Sample ID	Extract	Index (i5)	Sequence	Index (i7)	Sequence
Mealworm (*Tenebrio molitor*)	1	1	MW 1.1	1	SA705	TGAGTACG	SA501	ATCGTACG
				2	SA706	CTGCGTAG	SA502	ACTATCTG
		2	MW 1.2	1	SA707	TAGTCTCC	SA503	TAGCGAGT
				2	SA708	CGAGCGAC	SA504	CTGCGTGT
		3	MW 1.3	1	SA709	ACTACGAC	SA505	TCATCGAG
				2	SA710	GTCTGCTA	SA506	CGTGAGTG
	2	1	MW 2.1	1	SA711	GTCTATGA	SA507	GGATATCT
				2	SA712	TATAGCGA	SA508	GACACCGT
		2	MW 2.2	1	SA701	CGAGAGTT	SA502	ACTATCTG
				2	SA702	GACATAGT	SA503	TAGCGAGT
		3	MW 2.3	1	SA703	ACGCTACT	SA504	CTGCGTGT
				2	SA704	ACTCACTG	SA505	TCATCGAG
	3	1	MW 3.1	1	SA705	TGAGTACG	SA506	CGTGAGTG
				2	SA706	CTGCGTAG	SA507	GGATATCT
		2	MW 3.2	1	SA707	TAGTCTCC	SA508	GACACCGT
				2	SA708	CGAGCGAC	SA501	ATCGTACG
		3	MW 3.3	1	SA709	ACTACGAC	SA502	ACTATCTG
				2	SA710	GTCTGCTA	SA503	TAGCGAGT

House cricket (*Acheta domesticus*)	5	2	KR 1.2	1	SA701	CGAGAGTT	SA501	ATCGTACG
				2	SA702	GACATAGT	SA502	ACTATCTG
		3	KR 1.3	1	SA703	ACGCTACT	SA503	TAGCGAGT
				2	SA704	ACTCACTG	SA504	CTGCGTGT
	6	1	KR 2.1	1	SA705	TGAGTACG	SA505	TCATCGAG
				2	SA706	CTGCGTAG	SA506	CGTGAGTG
		2	KR 2.2	1	SA707	TAGTCTCC	SA507	GGATATCT
				2	SA708	CGAGCGAC	SA508	GACACCGT
		3	KR 2.3	1	SA709	ACTACGAC	SA501	ATCGTACG
				2	SA710	GTCTGCTA	SA502	ACTATCTG
Tropical house cricket (*Gryllodes sigillatus*)	7	1	KR 3.1	1	SA711	GTCTATGA	SA503	TAGCGAGT
				2	SA712	TATAGCGA	SA504	CTGCGTGT
		2	KR 3.2	1	SA701	CGAGAGTT	SA505	TCATCGAG
				2	SA702	GACATAGT	SA506	CGTGAGTG
		3	KR 3.3	1	SA703	ACGCTACT	SA507	GGATATCT
				2	SA704	ACTCACTG	SA508	GACACCGT

Table S3.2 Identification of operational taxonomic units (OTUs) according to the SILVA reference database. Taxonomic assignment scores are provided between brackets. Identification can be considered reliable when a score value >0.80 is found (indicated in bold).

Table S3.2 was published as Table S2 in Vandeweyer D., Crauwels S., Lievens B., & Van Campenhout L. (2017). Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. *International Journal of Food Microbiology*, *261*, 11-18. http://doi.org/10.1016/j.ijfoodmicro.2017.08.018

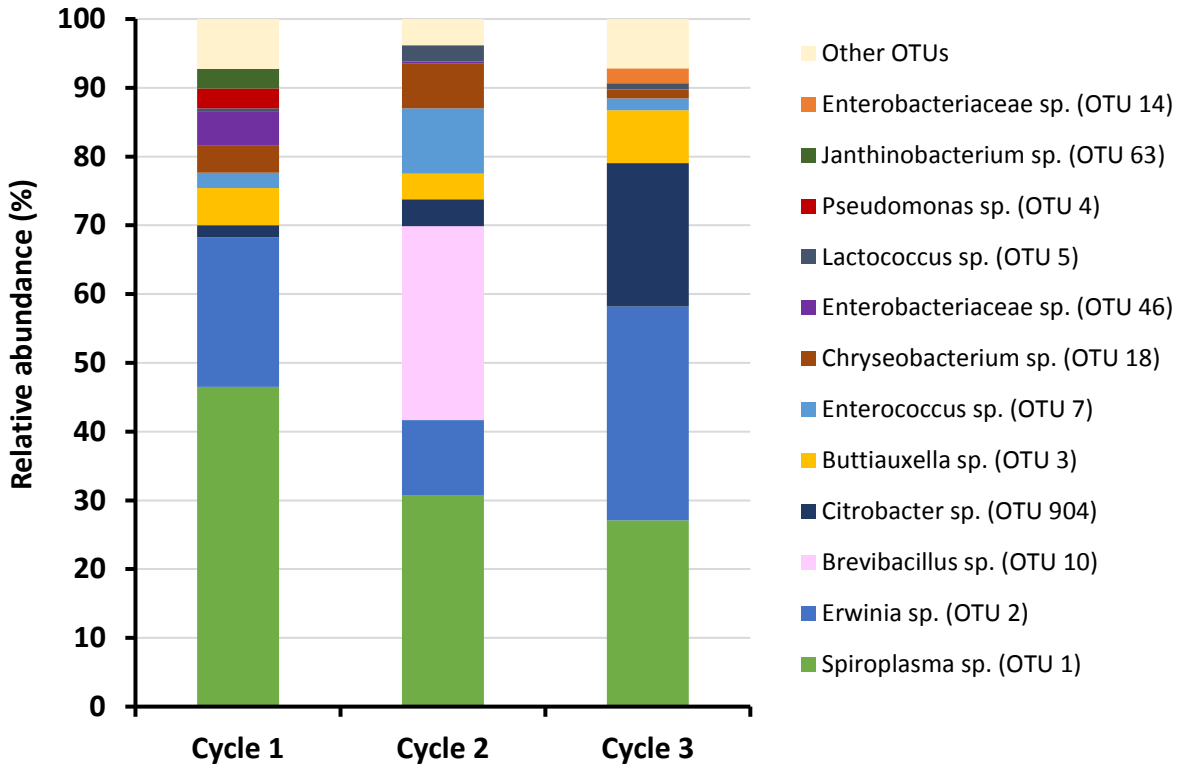
Table S3.2 can be consulted directly via QR code or
https://ars.els-cdn.com/content/image/1-s2.0-S0168160517303628-mmc1.zip.

Table S3.3 Identification of operational taxonomic units (OTUs) by BLAST search against the GenBank nucleotide (nt) reference database. Uncultured/environmental sample sequences were excluded. Only top 5 hits are displayed.

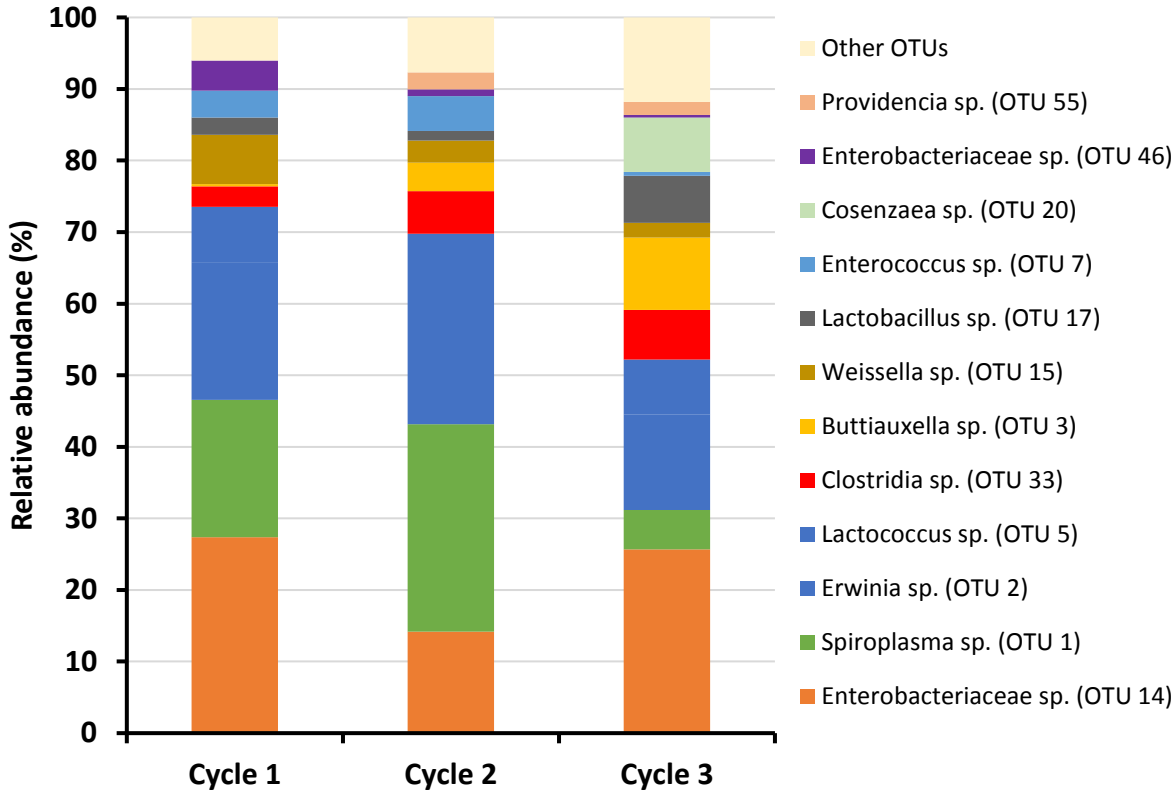
Table S3.3 was published as Table S3 in Vandeweyer D., Crauwels S., Lievens B., & Van Campenhout L. (2017). Metagenetic analysis of the bacterial communities of edible insects from diverse production cycles at industrial rearing companies. *International Journal of Food Microbiology*, *261*, 11-18. http://doi.org/10.1016/j.ijfoodmicro.2017.08.018

Table S3.3 can be consulted directly via QR code or
https://ars.els-cdn.com/content/image/1-s2.0-S0168160517303628-mmc1.zip.

Company 1



Company 2



Company 3

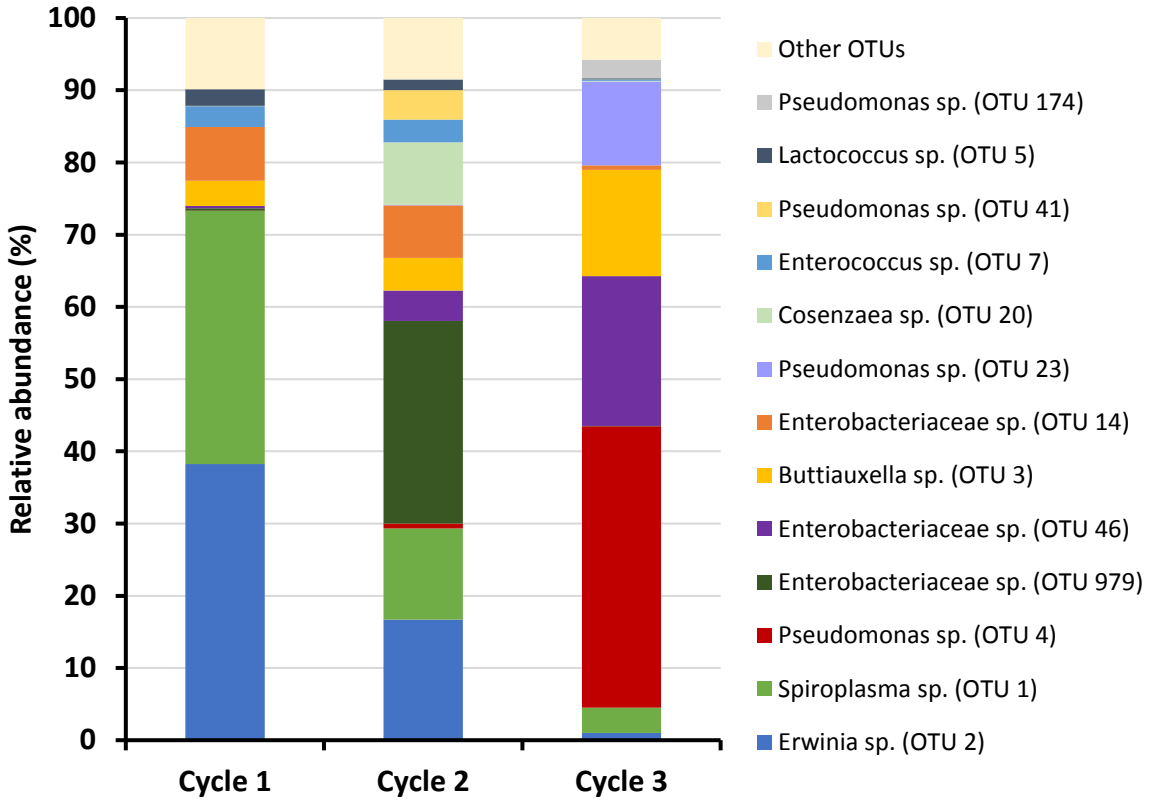
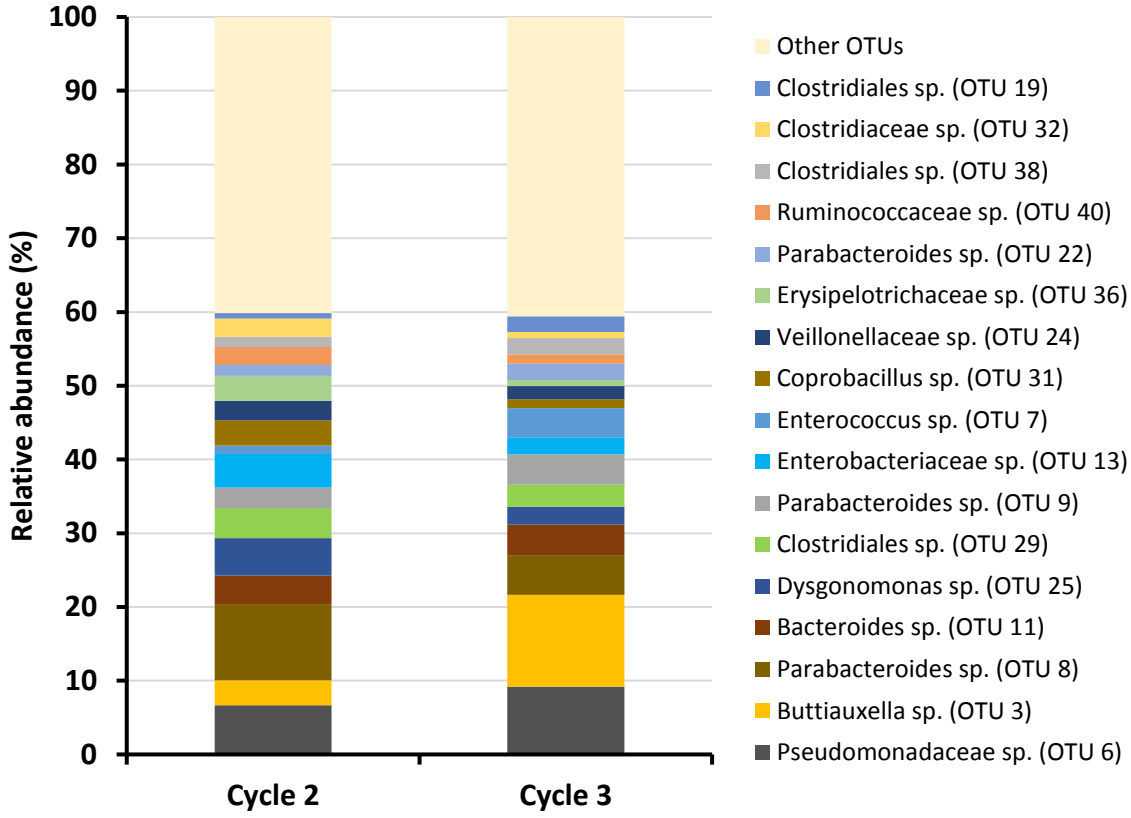
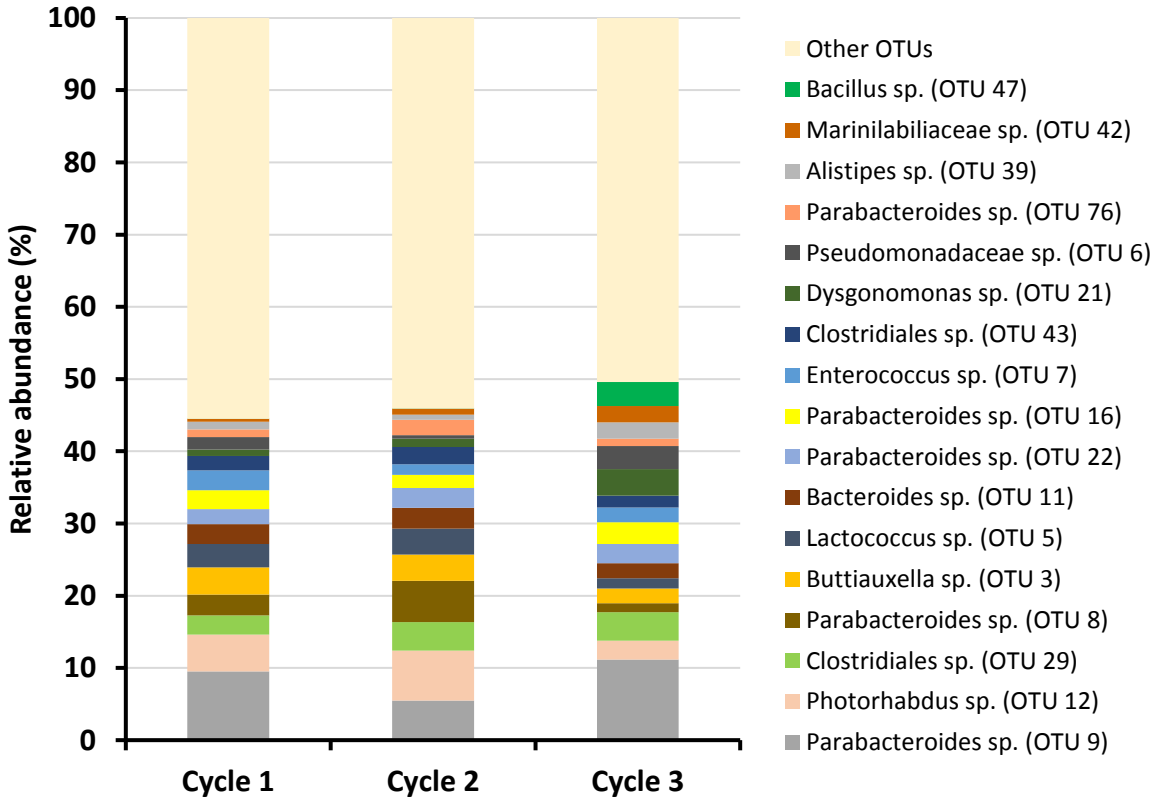


Figure S3.1 Bacterial composition at OTU level for mealworms from three production batches per company. Only the most abundant OTUs (i.e. with > 2% sequence abundance) are indicated. All other OTUs were grouped together in "Other OTUs". Data represent mean values of two extracts per sample from three samples per rearing company.

Company 5



Company 6



Company 7

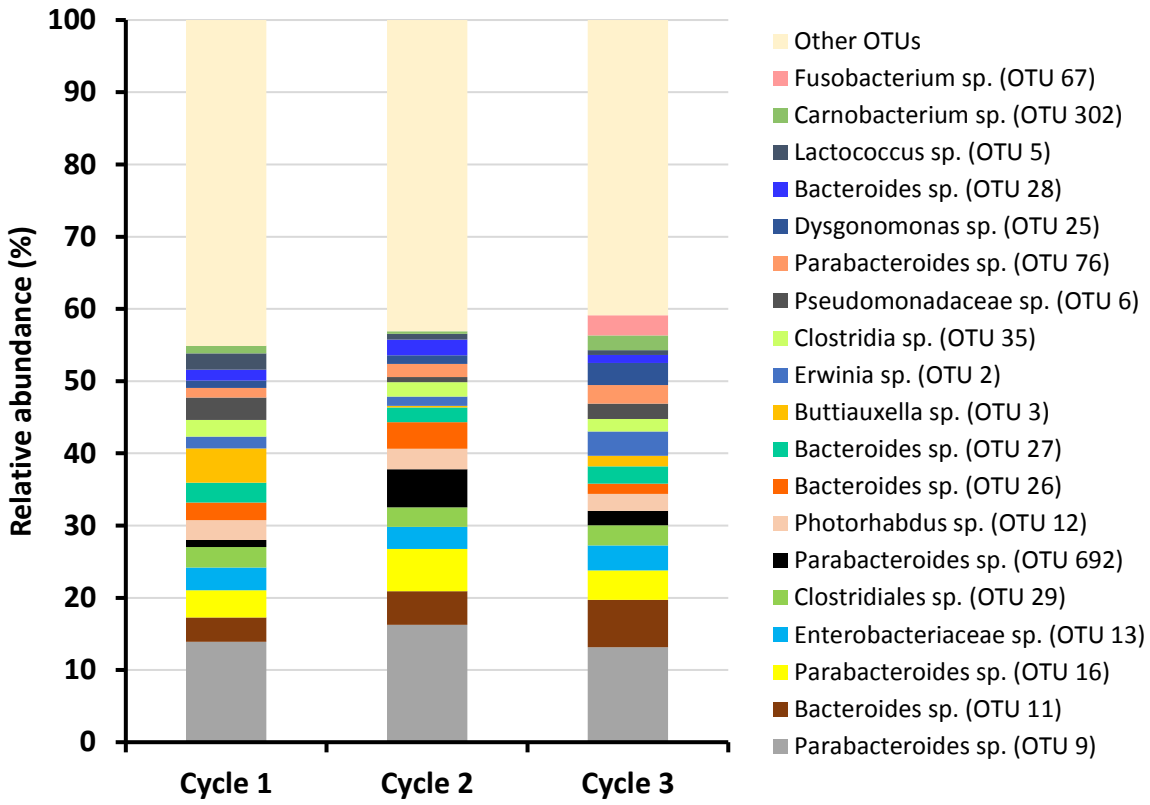


Figure S3.2 Bacterial composition at OTU level for crickets from two or three production batches per company. Only the most abundant OTUs (i.e. with > 2% sequence abundance) are indicated. All other OTUs were grouped together in "Other OTUs". Data represent mean values of two extracts per sample from three samples per rearing company.

Supporting information for Chapter 6

Table S6.1 Primer design and sample-specific barcodes.

Illumina Primer Design

Forward	<Forward Adapter> <i5> <pad> <link> <Forward Target Specific Primer>
Reverse	<Reverse Adapter> <i7> <pad> <link> <Reverse Target Specific Primer>
Adapter (Forward)	AATGATACGGCGACCACCGAGATCTACAC
Adapter (Reverse)	CAAGCAGAAGACGGCATACGAGAT
i5 (Forward)	see sample barcodes
i7 (Reverse)	see sample barcodes
pad (Forward)	TATGGTAATT
pad (Reverse)	AGTCAGTCAG
link (Forward)	GT
link (Reverse)	CC
Forward primer (515F)	5' - GTG CCA GCM GCC GCG GTA A - 3'
Reverse primer (806R)	5' - GGA CTA CHV GGG TWT CTA AT - 3'

Sample Barcodes

Sample	Sample moment	Replicate	Extract	Index (i5)	Sequence	Index (i7)	Sequence
Crickets (*Gryllodes sigillatus*)	rearing end, day 40	1	1	SA508	GACACCGT	SA702	GACATAGT
			2	SA508	GACACCGT	SA705	TGAGTACG
	after heat treatment	1	1	SA502	ACTATCTG	SA703	ACGCTACT
			2	SA502	ACTATCTG	SA706	CTGCGTAG
		2	1	SA503	TAGCGAGT	SA703	ACGCTACT
			2	SA503	TAGCGAGT	SA706	CTGCGTAG
	after freezing	1	1	SA504	CTGCGTGT	SA703	ACGCTACT
			2	SA504	CTGCGTGT	SA706	CTGCGTAG
		2	1	SA505	TCATCGAG	SA703	ACGCTACT
			2	SA505	TCATCGAG	SA706	CTGCGTAG
	after oven drying	1	1	SA506	CGTGAGTG	SA703	ACGCTACT
			2	SA506	CGTGAGTG	SA706	CTGCGTAG
		2	1	SA507	GGATATCT	SA703	ACGCTACT
			2	SA507	GGATATCT	SA706	CTGCGTAG
	after smoking	1	1	SA508	GACACCGT	SA703	ACGCTACT
			2	SA508	GACACCGT	SA706	CTGCGTAG

Table S6.2 Abundance and identification of operational taxonomic units (OTUs) according to the SILVA reference database. The numbers between brackets indicate the replicate sample (1 or 2), the letters between brackets indicate the technical replicate (a or b). Taxonomic assignments with highest bootstrap confidence value are shown and can be considered reliable when a confidence value > 0.80 was found (indicated in bold).

Table S6.2 was published as Data set S3 in Vandeweyer D., Wynants E., Crauwels S., Verreth C., Viaene N., Claes J., Lievens B., & Van Campenhout L. (2018). Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House Crickets (*Gryllodes sigillatus*) for Human Consumption. *Applied and Environmental Microbiology*, *84* (12), e00255-18. http://doi.org/10.1128/AEM.00255-18

Table S6.2 can be consulted directly via QR code or https://aem.asm.org/highwire/filestream/68495/field_highwire_adjunct_files/1/zam012188543sd2.xlsx.

Table S6.3 Identification of operational taxonomic units (OTUs) by BLAST search against the GenBank nucleotide (nt) reference database. Uncultured/environmental sample sequences were excluded. Only top 5 hits are displayed.

Table S6.3 was published as Table S4 in Vandeweyer D., Wynants E., Crauwels S., Verreth C., Viaene N., Claes J., Lievens B., & Van Campenhout L. (2018). Microbial Dynamics during Industrial Rearing, Processing, and Storage of Tropical House Crickets (*Gryllodes sigillatus*) for Human Consumption. *Applied and Environmental Microbiology*, *84* (12), e00255-18. http://doi.org/10.1128/AEM.00255-18

Table S6.3 can be consulted directly via QR code or https://aem.asm.org/highwire/filestream/68495/field_highwire_adjunct_files/0/zam012188543s1.pdf.