MICROBIOLOGICAL DYNAMICS AND SAFETY RISKS DURING REARING OF INSECTS FOR FOOD AND FEED

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Preface - Voorwoord

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Samenvatting

Eetbare insecten krijgen steeds meer aandacht in westerse landen omwille van hun potentieel als duurzame eiwitbron voor voeding en diervoeders. Insecten worden beschouwd als “minivee” en kunnen potentieel duurzamer geproduceerd worden in vergelijking met traditioneel vee en veevoederingrediënten (bv. sojaschroot en vismeel), terwijl ze toch evenwaardige nutritionele eigenschappen bezitten. Er is echter weinig geweten over de endogene microbiota van industrieel gekweekte insecten en over de microbiologische dynamiek gedurende de kweekfase. Verder zijn de potentiële risico’s die de microbiologische veiligheid van gekweekte insecten kunnen beïnvloeden nog niet in detail onderzocht. In deze doctoraatsthesis werd de microbiota van een aantal gekweekte insecten gekarakteriseerd tijdens de kweekfase en tijdens naoogstbehandelingen op labo-, grote en/of industriële schaal. Daarnaast werden mogelijke microbiologische veiligheidsrisico’s geïdentificeerd door een selectie voedselpathogenen te bestuderen.

In eerste instantie werd de endogene microbiota van drie verschillende insectensoorten tijdens de kweekfase onderzocht. Larven van de zwarte wapenvlieg (*Hermetia illucens*), gekweekt voor hun potentieel als diervoederingrediënt, werden bestudeerd tijdens de kweek op vier verschillende locaties: één op laboschaal en drie op grote schaal. Kleine meelwormen (*Alphitobius diaperinus*) en bandkrekels (*Gryllodes sigillatus*) werden bestudeerd tijdens een kweekcyclus op industriële schaal voor humane consumptie. Voor de drie insectensoorten werden stalen genomen van de insecten, van de substraten voor toevoeging aan de kweek, en van de residuen (bestaande uit niet-geconsumeerd substraat, uitwerpselen en vervellingshuiden). De intrinsieke parameters, microbiële aantallen (via plaattellingen) en bacteriële gemeenschap (door sequenering van het 16S rRNA gen via het Illumina platform) van deze stalen werden geanalyseerd. Voor elk van de onderzochte insectensoorten werd een groot aantal van de bacteriële soorten, die gevonden werden in de insecten, ook teruggevonden in de substraten (hoewel vaak in erg verschillende relatieve abundanties). Het substraat bleek dus een belangrijke bron te zijn van bacteriën voor de microbiota van het insect. Toch verschilden zowel de microbiële aantallen als de bacteriële gemeenschappen sterk tussen substraten en insecten, voornamelijk voor kleine meelwormen en zwarte wapenvlieglarven. Zwarte wapenvlieglarven gekweekt op verschillende reststromen en op verschillende locaties verschilden ook sterk in

bacteriële gemeenschap. De bacteriële gemeenschap van insecten bleek dus niet zomaar een weerspiegeling van die van het substraat te zijn, maar was waarschijnlijk het resultaat van een selectief proces dat bepaalt welke bacteriën de darm kunnen koloniseren. Zo werd ook vastgesteld voor de kleine meelworm dat het enkele weken duurde vooraleer een stabiele bacteriële gemeenschap ontwikkeld werd. Echter, voor alle onderzochte insectensoorten werden bacteriële genera waargenomen die ook werden waargenomen in andere kweekcycli (voor de zwarte wapenvlieg) en/of in de literatuur. Hoewel meer onderzoek nodig is, zou de hypothese gesteld kunnen worden dat er tijdens de kweek een selectieproces plaatsvindt dat bepaalde bacteriële soorten of genera begunstigt. Deze zouden dan mogelijks zelfs een functionele rol kunnen vervullen in de insectendarm.

Stalen van de zwarte wapenvlieglarven, kleine meelwormen en bandkrekels gekweekt in externe kwekerijen op grote en/of industriële schaal werden ook onderzocht voor de aanwezigheid van een selectie van vier voedselpathogenen. *Listeria monocytogenes* en coagulase-positieve staphylococci werden nooit gedetecteerd in deze studie. Vermoedelijke *Bacillus cereus*-kolonies en *Salmonella* sp. werden tevens niet gedetecteerd in de kweek van de kleine meelworm of bandkrekel. Voor de kweek van de zwarte wapenvlieg daarentegen, werden deze laatste twee voedselpathogenen wel gedetecteerd. Meer specifiek werd *Salmonella* sp. gedetecteerd (aanwezig in 25 g) in het residu van één externe kwekerij, terwijl *B. cereus* gedetecteerd werd in één residustaal (200 kve/g) van een tweede kwekerij, en in alle residu- en larvenstalen van een derde kwekerij (aantallen tot 3.8 log kve/g). Dat toont aan dat bijzondere aandacht nodig is voor deze pathogenen wanneer zwarte wapenvlieglarven gebruikt worden in diervoeder. Microbiologische controle van de substraten die gevoederd worden aan insecten kan een belangrijke rol spelen in het bewaken van microbiologische veiligheid. Met betrekking tot die hypothese werd ook nagegaan of het mogelijk is dat voedselpathogenen die in het substraat aanwezig zijn, opgenomen worden door de insecten (horizontale transmissie). Dat werd onderzocht voor de casus waarbij *Salmonella* sp. aanwezig was in tarwezemelen en waarbij transmissie naar gele meelwormen (*Tenebrio molitor*) werd bestudeerd. Tarwezemelen, geplaatst in kweekcontainers op laboschaal, werden daarvoor artificieel gecontamineerd met *Salmonella* sp. in verschillende contaminatieniveaus. Na zeven dagen werd de aanwezigheid van *Salmonella* sp. in zowel de tarwezemelen als in de larven opnieuw nagegaan. Uit de resultaten bleek dat *Salmonella* sp. aanwezig bleef in de tarwezemelen gedurende zeven dagen wanneer larven afwezig waren,

maar dat het aantal gereduceerd werd in aanwezigheid van de larven. De larven zelf waren besmet met *Salmonella* sp. op dag 1, maar voor het laagste contaminatieniveau in de zemelen (2 log kve/g) werd geen *Salmonella* sp. meer gedetecteerd in de larven op dag 7. Dat suggereert dat het risico op aanwezigheid van *Salmonella* sp. in meelwormen afhangt van het contaminatieniveau in de zemelen. De studie toont echter wel aan dat meelwormen gecontamineerd kunnen worden wanneer *Salmonella* sp. aanwezig is in het substraat. Controle op de aanwezigheid van deze pathogeen in de zemelen bij levering in de kwekerij en in de meelwormen na oogst is dus aangewezen.

Nadat insecten geoogst worden, kunnen ze een variatie aan naoogstbehandelingen ondergaan zoals spoelen, uitvasten (om de darm te legen), en hittebehandelingen. Voor de gele meelworm werd de invloed van spoelen en uitvasten in detail onderzocht. Uit die studie bleek dat noch spoelen, noch uitvasten, noch een combinatie van beide behandelingen de microbiologische kwaliteit verbeterde door de microbiële aantallen in de larven te reduceren. Uitvasten had ook geen substantiële invloed op samenstelling van de bacteriële gemeenschap. Deze behandelingen blijken dus overbodig te zijn vanuit een microbiologisch oogpunt.

Als naoogstbehandeling na de industriële kweek van de kleine meelworm en de bandkrekel voor humane consumptie, werd in beide gevallen een hittebehandeling toegepast. Voor beide insecten bleek deze behandeling de meeste microbiële kiemgetallen te reduceren. Hoewel dit niet onderzocht werd voor de zwarte wapenvlieg, wordt ook hier aangeraden om een hittebehandeling of een andere decontaminatietechnologie toe te passen zodat potentieel aanwezige voedselpathogenen geëlimineerd worden. Bacteriële endosporen bleken echter weinig tot niet gereduceerd te worden door de hittebehandelingen uitgevoerd op de kleine meelworm en bandkrekel. Bacteriesporen zijn vaak in hoge aantallen aanwezig in insecten. In deze doctoraatsthesis werden zelfs aantallen tot 7,5 log kve/g geobserveerd. Aan kwekers wordt dus geadviseerd om de toegepaste hittebehandelingen te valideren zodat bacteriesporen gereduceerd worden, hoewel voor deze pathogeen in de wetgeving nog geen microbiologisch criterium bestaat. Gezien vermoedelijke *B. cereus* werd gedetecteerd in zwarte wapenvlieglarven in deze studie, alsook in andere insecten in de literatuur, is het van groot belang dat de aanwezigheid van (sporen van) deze bacteriesoort bepaald wordt.

Deze doctoraatsthesis verschaft basisinzicht in de microbiota van enkele industrieel gekweekte insectensoorten. De resultaten suggereren dat een goede beheersing van

microbiologische contaminatie in het substraat, alsook een goede beheersing van de risico's met betrekking tot sporenvormende pathogenen, belangrijke aandachtspunten zijn bij het ontwikkelen van goede hygiënepraktijken voor de sector. Voortbouwend op dit werk richt verder onderzoek zich best (1) op de microbiologische veiligheid van (nu nog niet toegelaten) substraten die gevoederd worden aan insecten, zodat in de toekomst mogelijks meer substraten kunnen toegelaten worden, (2) op de variatie in de microbiota bij herhaling van kweekcycli in identieke of variërende omstandigheden, (3) op het uitdiepen van de mycoflora, (4) op nog niet bestudeerde invloedsfactoren zoals de "huisflora" in een kwekerij, (5) op de transmissie van andere voedselpathogenen, zoals *B. cereus*, naar meelwormen maar ook andere insectensoorten om op die manier waardevolle risico-analyses te verkrijgen per pathogeen-insect-combinatie, (6) op het ontwikkelen van sporenreducerende behandelingen voor de insecten en het voorkomen van kieming van sporen en uitgroei van vegetatieve cellen in de eindproducten, en (7) op decontaminatietechnieken voor het residu die nieuwe toepassingen ervan zullen toelaten.

Summary

The potential of insects as novel protein source in food and feed is gaining increased attention in western countries. Insects are considered as “minilivestock” with potential of being produced more sustainably as compared to traditional livestock and livestock feed ingredients (e.g. soybean meal and fishmeal), while exhibiting equal nutritional qualities. However, little knowledge exists on the endogenous microbiota of industrially reared insects and its changes during the rearing process. Furthermore, little is known about potential hazards that may affect the microbiological safety of harvested insects. This dissertation aimed to characterise the microbiota of insects during rearing and during post-harvest procedures at laboratory, large and/or industrial scale. Potential microbiological safety risks were identified by studying a selection of food pathogens during rearing.

In a first series of studies, rearing cycles of three different insect species were characterised for their endogenous microbiota and its dynamics. For black soldier fly (BSF) larvae (*Hermetia illucens*), reared for their potential use in animal feed, rearing cycles were monitored at four different locations: one at laboratory scale and three at large scale. Lesser mealworms (*Alphitobius diaperinus*) and tropical house crickets (*Gryllodes sigillatus*) were investigated during rearing at industrial scale for human consumption. To this end, samples were taken for all three species of the insects themselves, as well as of the rearing substrates prior to administration, and of the residues in the rearing containers/cages (i.e. leftover substrate, faeces and exuviae). Intrinsic parameters, microbial quality (through plate counts) as well as bacterial community composition (through high-throughput 16S rRNA gene sequencing) were assessed. For all three insect species, a large portion of bacterial species observed in the insects was also present (although often in very different abundances) in the substrates. It appeared that the substrate was an important source of bacteria for the insect microbiota. Nevertheless, both microbial numbers and bacterial community compositions differed to a large extent between substrates and insects, especially for BSF larvae and lesser mealworms. In addition, even for BSF larvae reared with different substrates and at different facilities, large differences were observed in their microbiota. Thus, the insect bacterial community composition was not merely a reflection of the microbiota in the substrate, but was likely the result of a selective process determining the ability of specific bacterial species to colonize the insect gut.

As was shown for lesser mealworms, the establishment of a stable bacterial community may occur only after some weeks during the rearing process. Still, for all species studied, bacterial genera were recovered in our study that were also reported in other rearing cycles (for BSF) or in studies by other authors. Although more research is certainly necessary, the preference for certain bacterial genera or species, possibly even exhibiting functional roles in the insect gut, could be hypothesised.

Samples of BSF larvae, lesser mealworms and tropical house crickets at external facilities at large and/or industrial scale were assessed for the presence of a selection of four food pathogens. *Listeria monocytogenes* and coagulase-positive staphylococci were never detected. Presumptive *Bacillus cereus* and *Salmonella* sp. were not detected in lesser mealworm and tropical house cricket rearing, but were detected in larvae and residue samples of BSF rearing. More specifically, *Salmonella* sp. was detected (present in 25 g) in the residue of one rearing facility, while presumptive *B. cereus* was detected in one residue sample of a second (200 cfu/g) and both residues and larvae of a third rearing facility (up to 3.8 log cfu/g). Thus, specific attention should be paid to these pathogens when BSF larvae are to be used as feed ingredients. Monitoring microbial contamination in the substrates may play an important role in assuring the absence of food pathogens in reared insects. With regard to the latter hypothesis, the potential that food pathogens possibly present in the substrate are taken up by the insects (horizontal transmission), was studied. This was assessed in a case study on the transmission potential of *Salmonella* sp. present in wheat bran as a substrate for yellow mealworms (*Tenebrio molitor*). To this end, *Salmonella* sp. was artificially inoculated into wheat bran in laboratory scale rearing trays at different contamination levels and its presence in the bran and larvae was determined during seven days. Results showed *Salmonella* sp. to remain viable in the bran for seven days in the absence of larvae, but its number was reduced in the presence of larvae. Larvae did become contaminated with *Salmonella* sp., but also here, its number was reduced by day 7. For the lowest inoculation level (2 log cfu/g), no Salmonellae were detected after seven days in the larvae. Thus, it appears that the risk related to the presence of *Salmonella* sp. in mealworms may depend on the contamination level in the bran. Nevertheless, the study shows that mealworms can become contaminated with *Salmonella* sp. when it is present in the bran. Monitoring of the pathogen in the bran after arrival at the rearing facility and in harvested mealworms is thus advised.

After harvest, a variety of treatments may be applied to insects, such as rinsing, starvation (to empty the gut), and heat treatments. For the yellow mealworm, the

impact of starvation and rinsing was investigated in detail. It was shown that neither procedure, nor a combination of both procedures, enhanced the microbial quality by reducing microbial numbers. In addition, starvation did not substantially alter the bacterial community composition. Thus, these procedures appear redundant from a microbiological point of view.

After industrial rearing of both lesser mealworms and tropical house crickets for human consumption, a post-harvest heat treatment was applied. These treatments were shown to reduce most microbial numbers. Although the effect of heat treatments was not assessed for BSF larvae, also here, a heat treatment or other decontamination technology prior to or during further processing is advised in order to eliminate potentially present food pathogens. However, bacterial endospores were hardly affected by the treatments applied to lesser mealworms and tropical house crickets. Bacterial spores seem present in insects at high numbers. In this dissertation, numbers up to 7.5 log cfu/g were observed. Consequently, rearers are advised to validate heat treatments with respect to spore inactivation, even though no legislative criterion exists for endospores. In particular because presumptive *B. cereus* was encountered in insects in this study and in insects in other studies in literature, the risk for the presence of (spores from) this pathogen should be determined.

This PhD dissertation provides general insights into the microbiota of a selection of industrially reared insect species. The results suggest that monitoring of the microbial contamination of the substrate, as well as controlling possible risks related to spore-producing pathogens, are important points of attention for the development of good hygiene practices for the developing insect sector. Based on this dissertation, future research should focus (1) on exploring the microbiological safety of (currently not authorised) substrates, to allow more organic waste streams to be authorised in the future, (2) on exploring the variability of the microbiota during subsequent rearing cycles under identical and varying conditions, (3) on characterising the mycoflora and presence of mycotoxins in industrially reared insects, (4) on assessing the influence of factors so far not investigated, such as the “house flora” in an insect rearing facility, (5) on transmission of other food pathogens, such as *B. cereus*, to mealworms as well as to other insect species in order to provide valuable risk assessments for specific insect-pathogen combinations, (6) on developing and validating techniques to reduce (in particular) the number of endospores in insects as well as preventing germination of spores and growth of the vegetative cells in end products, and (7) on studying decontamination technologies for the residue towards allowing new applications.

List of Abbreviations

ANOVA	Analysis of variance
ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (France)
a_w	Water activity
BLAST	Basic Local Alignment Search Tool
BSF	Black soldier fly
cfu	colony-forming units
DRBC	Dichloran rose Bengal chloramphenicol agar
DDGS	Dried distillers grains and solubles
DGGE	Denaturing gradient gel electrophoresis
DON	Deoxynivalenol
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organisation of the United Nations
FASFC	Federal Agency for the Safety of the Food Chain (Belgium)
GI	Gastro-intestinal
HACCP	Hazard Analysis and Critical Control Points
IPIFF	International Platform of Insects for Food and Feed
Kp	Nitrogen-to-protein conversion factor
LCA	Life cycle assessment
LOQ	Limit of quantification
MRS	de Man, Rogosa and Sharpe agar
MUFA	Mono-unsaturated fatty acid
NMDS	Non-metric multidimensional scaling
NVWA	Nederlandse Voedsel- en Warenautoriteit (Dutch Food and Consumer Product Safety Authority) (The Netherlands)
OGA	Oxatetracycline glucose agar
OTU	Operational taxonomic unit
PAP	Processed animal protein
PCA	Plate count agar
PCR	Polymerase chain reaction
PUFA	Poly-unsaturated fatty acid
SD	Standard deviation
SHC	Superior Health Council (Belgium)
TVC	Total viable count
TF	Total fat content
VRBG	Violet red bile glucose agar

List of Publications

Publications in peer-reviewed journals

Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., Bruyninckx, L. 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. https://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. https://doi.org/10.1016/j.fm.2017.09.012

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[1]D.V. and E.W. contributed equally to this work.

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CHAPTER 1: GENERAL INTRODUCTION

1.1 HISTORY AND IMPORTANCE OF INSECTS FOR HUMANS AND ANIMALS

Insects have been providing humans with a variety of valuable products for centuries, with insects as a food source being their most ancient utilisation. Human entomophagy, i.e. the consumption of insects by humans, has been practiced for thousands of years. In fact, humankind has evolved as an entomophagous species. Carbon isotope analysis of the bones and dental enamel of australopithecines already indicated an insect-rich diet. In addition, prehistoric tools were discovered, which are thought to have been used for scavenging of insect delicacies, for instance for digging in search of ants (Mlcek et al., 2014; Dobermann et al., 2017). To date, insects are still a widely consumed food throughout the world. It is estimated that over 2000 insect species are consumed worldwide in at least 113 countries, among which the majority belong to the orders of the Coleoptera (beetles, 31%), followed by the Lepidoptera (caterpillars, 18%), Hymenoptera (bees, wasps and ants, 14%), Orthoptera (grasshoppers, locusts and crickets, 13%) and Hemiptera (cicadas, leafhoppers, planthoppers, scale insects and true bugs, 10%) (Jongema, 2017). Entomophagy is mostly practiced in regions with a tropical climate. In those regions, insects tend to be larger and often congregate in significant numbers, facilitating wild capture. In addition, many species can be either found year-round, or optimal harvest times and places can be easily predicted (van Huis et al., 2013). In South-East Asia, export and import of insects even plays an important economic role, with the import market in Thailand alone estimated at 1.14 million USD/year (Dobermann et al., 2017). On the contrary, in developed regions such as Europe, insects are not traditionally consumed. When food production through agriculture spread from the Fertile Crescent throughout Europe, this led to the domestication of a variety of mammalian livestock, providing meat, milk, leather, warmth and working power (van Huis et al., 2013). In these temperate regions, insects did not offer the same benefits with regard to capture and availability as compared to tropical regions. Consequently, insects as agricultural product were of little interest, and were even experienced as a threat to farmed plants and animals (DeFoliart, 1999; van Huis et al., 2013). As a consequence, many western citizens have a negative attitude towards eating insects and associate them with illnesses and uncleanliness. Furthermore, many people experience “neophobia”, i.e. a

reluctance to try novel foods (Verbeke, 2015; Caparros Megido et al., 2016). In recent years, however, insects have received an increasing amount of attention as alternative source of proteins and fat for human food and animal feed. The potential of insects as a sustainable protein source for food and feed was highlighted in the publication "Edible insects: Future prospects for food and feed security" by the Food and Agriculture Organisation of the United Nations (FAO) in 2013 (van Huis et al., 2013), which gained a lot of attention. In 2015, the European Food Safety Authority (EFSA) published a risk profile related to the production and consumption of insects. Although no legislation specific for insects in food or feed existed at that time, this interest for insects as food source led to the worldwide emergence of an edible insect sector.

For feed, the use of insects is traditionally constricted mainly to fish bait, pet birds and reptiles (van Huis et al., 2013). In Africa and Asia, insects are widely used in smallholder farms for fish feed, although also often wild-captured (Dobermann et al., 2017). Recently, the potential of insects to be reared for animal feed is gaining increased attention. One of the most promising insect species appears to be the larva (or prepupa) of the black soldier fly (BSF, *Hermetia illucens*) (De Smet et al., 2018). Black soldier fly larvae may be reared on a variety of organic waste streams because of their nature as generalist detrivores (Makkar et al., 2014). Furthermore, as adult black soldier flies do not feed and are thus not attracted to human habitats or foods, they are not considered a nuisance or disease vector (Anankware et al., 2015). In addition, Spranghers (2017) stated that escaping flies are unlikely to establish populations in temperate regions due to their lack of cold hardiness. Currently, black soldier fly larvae are reared for aquaculture and/or livestock feed in western countries such as the USA, Canada and the Netherlands (De Smet et al., 2018).

During history, mankind discovered useful purposes for insects which do not involve direct consumption. Honeybees (*Apis mellifera*) present the first reared insect, even domesticated, for their honey, which is consumed but also serves other purposes, such as medicinal treatments (Mason, 1984). Another ancient example of domestication of insects are silkworms (*Bombyx mori*) for the production of silk, starting approximately 5000 years ago (Goldsmith et al., 2005). The pupae, which are produced as "by-products" of the silk and honey production cycles, are consumed by humans in some (mainly Asian) countries (van Huis et al., 2013). In Chinese medicine, insects have been used to cure diseases for more than 2000 years. With modern pharmaceutical analyses, many functions of extractions and/or secretions from insects have been identified, such as anti-bacterial, anti-inflammatory, and anti-oxidant

activities, immune regulation, and reducing blood sugar (Feng et al., 2009; Cherniack, 2010). Moreover, even living insects are used for medicinal purposes, such as cleaning wounds through maggot therapy (Cherniack, 2010). Nowadays, insects are being industrially reared for a variety of purposes, among which many are not related to consumption. A profound example of industrial application includes the production of carminic acid from the cochineal insects, used as a red dye in textile production, pharmaceuticals and as a food colorant. Other functions include the rearing of insects for use in agri- and horticulture for pollination and biocontrol purposes, for medicinal treatments, and so on. Recently, interest is arising for the potential use of isolated insect components, such as fats, protein and chitin, for industrial applications. Fats, for instance, may be used for the production of biodiesel (Li et al., 2011; Zheng et al. 2012a; Zheng et al., 2013a). Protein may be used in the food industry, either for their functional properties (stabilisation of foams and emulsions) or for their nutritional value. Chitin, as well as its derivatives chitosan and glucosamine have a high application potential in the food industry, medicine, agriculture, aquaculture, cosmetics, wastewater treatment, and so on (Park & Kim, 2010; Xia et al., 2011).

1.2 INDUSTRIAL REARING OF INSECTS FOR FEED AND FOOD

1.2.1 Rearing facilities worldwide and in Europe

The rearing of insects for food is a practice that only recently developed. In the past, industrial rearing of insects has mainly focused on commercially valuable products derived from insects, such as silk and honey. Insects used for food were traditionally captured in the wild. However, rearing facilities were built in tropical countries for the production of insects for food. The rearing of insects as replacement for wild capture has a number of advantages, such as quality control and no more stress on wild populations due to captivation (van Huis et al., 2013). For instance, in Thailand, in Laos, and in Vietnam, cricket farming is becoming increasingly popular to increase the livelihood of local people, and it is even becoming a million-dollar industry. Cricket farms are set up in backyards, rearing the insects with basic cages and little space, and feeding them mainly with household food wastes. Other recent examples of commercially farmed (or semi-cultivated) insects for human consumption include palm weevils and the giant water bug in Thailand and water beetles in China (Hanboonsong et al., 2013; van Huis et al., 2013; Halloran et al., 2017). As discussed in section 1.1, the rearing of insects in Europe traditionally included insects for other purposes than feed

or food. In recent years, with the increasing attention for insects as food and feed, rearing companies were established, and existing companies producing insects for pet food purposes introduced production lines of insects for human consumption. A variety of food products containing insects was launched on the market, such as burgers, spreads, nuggets, etcetera. Over 20 rearing companies are now located all over Europe rearing insects for human consumption, of which three in Belgium and nine in The Netherlands (Schillewaert, 2018). In 2018, species mostly reared are the following: the yellow mealworm (*Tenebrio molitor*), the lesser mealworm (*Alphitobius diaperinus*), the house cricket (*Acheta domesticus*), the tropical house cricket (*Gryllodes sigillatus*) and the migratory locust (*Locusta migratoria*).

Also for animal feed, large scale rearing companies were recently founded. For instance, house fly larvae (*Musca domestica*) are reared for livestock and aquaculture feed in South-Africa and China (EFSA Scientific Committee, 2015). BSF larvae and/or prepupae are grown on organic waste streams and used in feed for aquaculture and/or livestock feed, for example in the USA (Enviroflight), Canada (Enterra Feed), South-Africa (Agri-Protein), the Netherlands (Bestico, Protix), Belgium (Circular Organics, formerly named Millibeter) and Germany (Hermetia). Because legislation in Europe currently does not allow the use of insect proteins (with some exceptions, e.g. hydrolysed proteins) in livestock feed (see section 1.5.2), facilities in Europe mainly focus on pet food or aquaculture.

1.2.2 Rearing processes of insects for feed or food

The following paragraph aims to describe the general production process of insects, based on information found in literature and reports (Rumpold & Schlüter, 2013a; Nederlandse Voedsel- en Warenautoriteit (NVWA), 2014; Superior Health Council & Federal Agency for Safety of the Food Chain (FASFC), 2014; European Food Safety Authority (EFSA) Scientific Committee, 2015) and on personal communication with rearers. The rearing process for insects to be used in feed or food can be divided into a rearing phase, a harvesting phase and a post-harvest phase, as shown in Figure 1.1. During the rearing phase, insects are usually kept in containers or cages, depending on the species. A substrate is offered, which may be a commercial feed (e.g. chick feed, wheat bran) or a substrate especially developed by the rearing facility. Insect substrates must be approved by legislation (see section 1.5). During rearing, insects are either kept inside their substrate (e.g. mealworms, black soldier fly larvae), or substrate

is added in a separate container (e.g. crickets and grasshoppers). The substrate may be supplemented with water (either mixed through the substrate, or added in a separate recipient) or with a water-rich component, such as fruit and/or vegetable pieces. Environmental conditions are controlled, maintaining temperatures most often between 25 °C and 31 °C. Relative humidity is mostly controlled in the range of 50% to 70%. Insects are usually kept in the same rearing room until they are ready for harvest. The latter depends on the species, with some species being harvested in the final juvenile stage (e.g. mealworms and black soldier fly larvae) and other species in the adult stage (e.g. crickets and grasshoppers). Harvest, i.e. the separation of the insects from the containers/cages and from the residues, may be performed manually or by use of an automated sieving system. After harvest, insects may undergo treatments such as starving, rinsing, and/or killing (e.g. by submerging them in hot water or by freezing). Sometimes, the killing step also serves as a heat treatment. Living insects may be submerged in (close to) boiling water, killing them and simultaneously reducing their microbial load (see section 1.8.3). After heat treatment, insects are further processed into food products using a variety of techniques (e.g. drying, freeze-drying, smoking, or a combination of processing steps). A variety of hygiene measures may be applied during the production process, such as wearing hair caps and/or gloves, washing of hands prior to entering the rearing facility, disinfection of infrastructure and materials, etcetera.

In the following sections, the life cycles and rearing processes of the insect species under study in this PhD dissertation are discussed, i.e. mealworms, crickets, and the black soldier fly. It should however be noted that even within one species, variation in rearing methods exist. The following paragraphs aim to provide an overview of the general rearing practices.

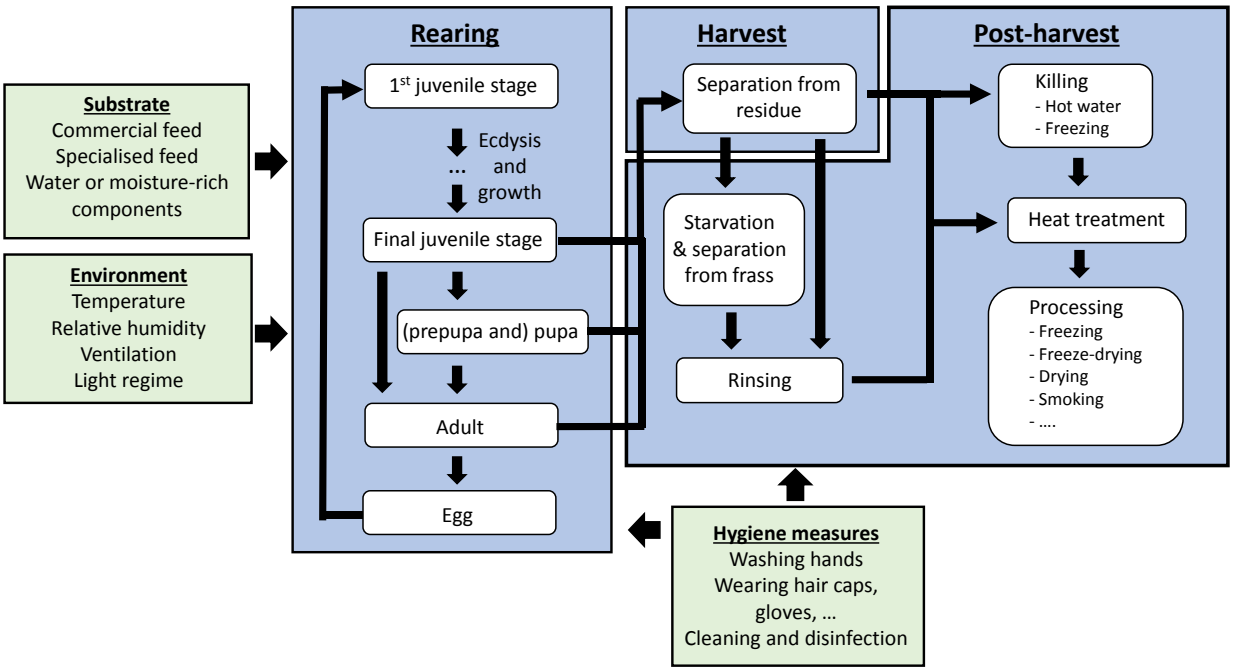


Figure 1.1 General overview of the rearing process of insects for food and feed.

Rearing of mealworms. The yellow mealworm (*T. molitor*) and lesser mealworm (*A. diaperinus*), both belonging to the family of the Tenebrionidae, are comparable in life cycle and rearing methods. These species are reared for human consumption in several European countries, but they may also be used in aquafeed and show potential for use in other animal feeds. The life cycles of both species include a complete metamorphosis and four life stages, i.e. egg, larva, pupa and adult (Figure 1.2a). In rearing facilities, eggs are laid in a substrate which is usually placed in plastic crates. The substrate usually consists of either wheat bran or it is especially composed (lesser) mealworm feed. Hatchlings are kept in the crates, without removing the residue, until they develop into harvest-ready late-instar larvae. The temperature is usually kept at 28 to 30 °C and relative humidity at 60%. Moisture-rich components are added such as carrots, chicory, cucumbers, or brewer's spent grains to provide water to the larvae. Larvae are harvested after eight to ten weeks for yellow mealworms (NVWA, 2014; SHC & FASFC, 2014), and after six weeks for lesser mealworms (Wynants et al., 2018a). Harvesting takes places by sieving larvae (manually or automated) from residue (existing of left substrate, faeces and exuviae). Larvae are often rinsed and/or starved for up to two days in order to remove their gut content (NVWA, 2014; SHC & FASFC, 2014). Prior to further processing, larvae may be heat-treated by placing them in hot water of more than 80 °C, which also serves as a killing step (Wynants et al., 2018a). A

fraction of the larvae are excluded from harvest, and allowed to pupate and reproduce in order to establish the next mealworm generation.

Rearing of crickets. Crickets typically consumed by humans in Europe include *Acheta domesticus* and *Gryllodes sigillatus*, both belonging to the Gryllidae family or "true crickets". The life cycles and rearing practices for the two species are very similar (Figure 1.2b) In contrast to mealworms, crickets do not undergo metamorphosis. Hatchlings, which are called "nymphs" look similar to the adults (imagos), except for the absence of wings and fully developed reproductive organs. Eggs hatch after approximately two weeks, and a measured quantity of hatchlings are placed in rearing cages. The nymphs develop into adults through eight to ten instar stages over a period of three to four months. Crickets are industrially reared in cages made of wood, Perspex, and/or plastic, that are equipped with piled-up egg cardboard boxes, creating dark crevices for the crickets to reside in. Feed is administered next to or on top of the cardboard pile (Vandeweyer et al., 2018). Crickets are natural omnivores, but when reared to be consumed by humans, however, the feed should comply to legislation (see section 1.5) and is often specially developed. In contrast to mealworms, crickets do normally not excrete their faeces and exuviae into the feed. The feed is also frequently replenished. Crickets are reared for three to four months until adulthood, with a temperature usually of 30 to 31 °C and relative humidity of 50 to 70% (Clifford & Woodring, 1990; Vandeweyer et al., 2018). The final days before harvest, feed may be removed in order to empty the gut content of the crickets (EFSA scientific committee, 2015), or carrots may be provided to obtain a better taste (Vandeweyer et al., 2018). Harvested crickets are killed by freezing (EFSA scientific committee, 2015) or by submerging them in hot (e.g. 65 °C) water (Vandeweyer et al., 2018). Next a heat treatment is most often applied prior to further processing. Furthermore, when crickets reach the adult stage, trays containing an egg deposition medium, e.g. soil, or a mixture of peat soil and coconut peel, are placed inside the cage. Eggs are then allowed to hatch in smaller, plastic ventilated boxes. A controlled number of hatchlings are then placed again in the larger crates to be reared until they reach the adult stage (Vandeweyer et al., 2018).

Rearing of the black soldier fly. The black soldier fly (*Hermetia illucens*) is a true fly of the family Stratiomyidae, also undergoing complete metamorphosis (Figure 1.2c). Black soldier fly larvae and prepupae are reared in industrial facilities for pet food,

aquafeed and sometimes other animal feed, but not for human food, due to consumer acceptance issues (Wang & Shelomi, 2017). Adult flies are often kept in tents from meshed textile, provided with a (plastic) plant as habitat for mating. An attractant for egg deposition is placed in the tent, which mimicks decaying material and often contains residue from a previous rearing cycle (Dortmans et al., 2017). Pieces of corrugated cardboard or biplex plates are placed close to the attractant for egg deposition. Eggs are collected and placed onto a nutrient rich substrate such as chick/hen feed (Dortmans et al., 2017). Those substrate types provide optimal growth for young hatchlings which emerge after approximately four days. An organic waste stream may be added simultaneously with the chick/hen feed, or after a few days when larvae are larger and more resilient (Wynants et al., 2018b). Under optimal rearing conditions, at a temperature of 29 to 31 °C and a relative humidity of 50 to 70% (Makkar et al., 2014), larvae are fully grown and approach the prepupation stage in thirteen to eighteen days (De Smet et al., 2018). When larvae are to be harvested, they are separated from the residue by sieving (Wynants et al., 2018b). When prepupae are to be harvested, they are allowed to "self-harvest" by crawling out of the substrate in search of a dryer place to pupate (Wang & Shelomi, 2017). Some larvae are left to go into adulthood to procreate, after emerging from the pupal stage, which may last from ten days up to months (de Smet et al., 2018). Adult flies lack functional mouth parts and do not feed, they solely focus on reproduction, and die after five to eight days after eclosion (Wang & Shelomi, 2017).

1.2.3 Challenges for the insect rearing industry

As a relatively new industry, the insect rearing sector faces challenges. The rearing of insects is currently labour-intensive and thus expensive, limiting their economic potential as alternative protein source for food and/or feed. Furthermore, as rearing protocols are constantly being optimised, frequently involving a "trial and error" approach, the production of insects with constant quality poses a challenge (van Huis, 2013). In addition, the occurrence of insect diseases (see section 1.7) may impact quality and availability of mass-reared insects (Eilenberg et al., 2015). It is therefore of importance that automated rearing techniques are being developed. Advantages to automation, aside from cost reduction, possibly include an increased product performance and consistency, a reduction in microbiological contamination by personnel, and increased space utilisation (van Huis, 2013).

As discussed in section 1.1, insects are not traditionally consumed in western countries, posing another challenge with regard to consumer acceptance for insects as food products. A study conducted by Van Thielen et al. (2019) in Belgium revealed that 57% of nearly 400 respondents was not willing to try foods with processed insects. The main reasons were aversion, cultural habits and the lack of necessity for eating insects when alternatives are available. The authors suggest the best strategies to encourage people to eat insects include improving the supply, visibility and taste of insect-containing products. Altough not investigated in that study, a reduction of prices for insect products will likely also improve the public's willingness to buy them.

Opportunities and challenges of insects for food and feed related to nutritional value, sustainability, legislation, and animal welfare are discussed in detail in the following sections (section 1.3 to section 1.6).

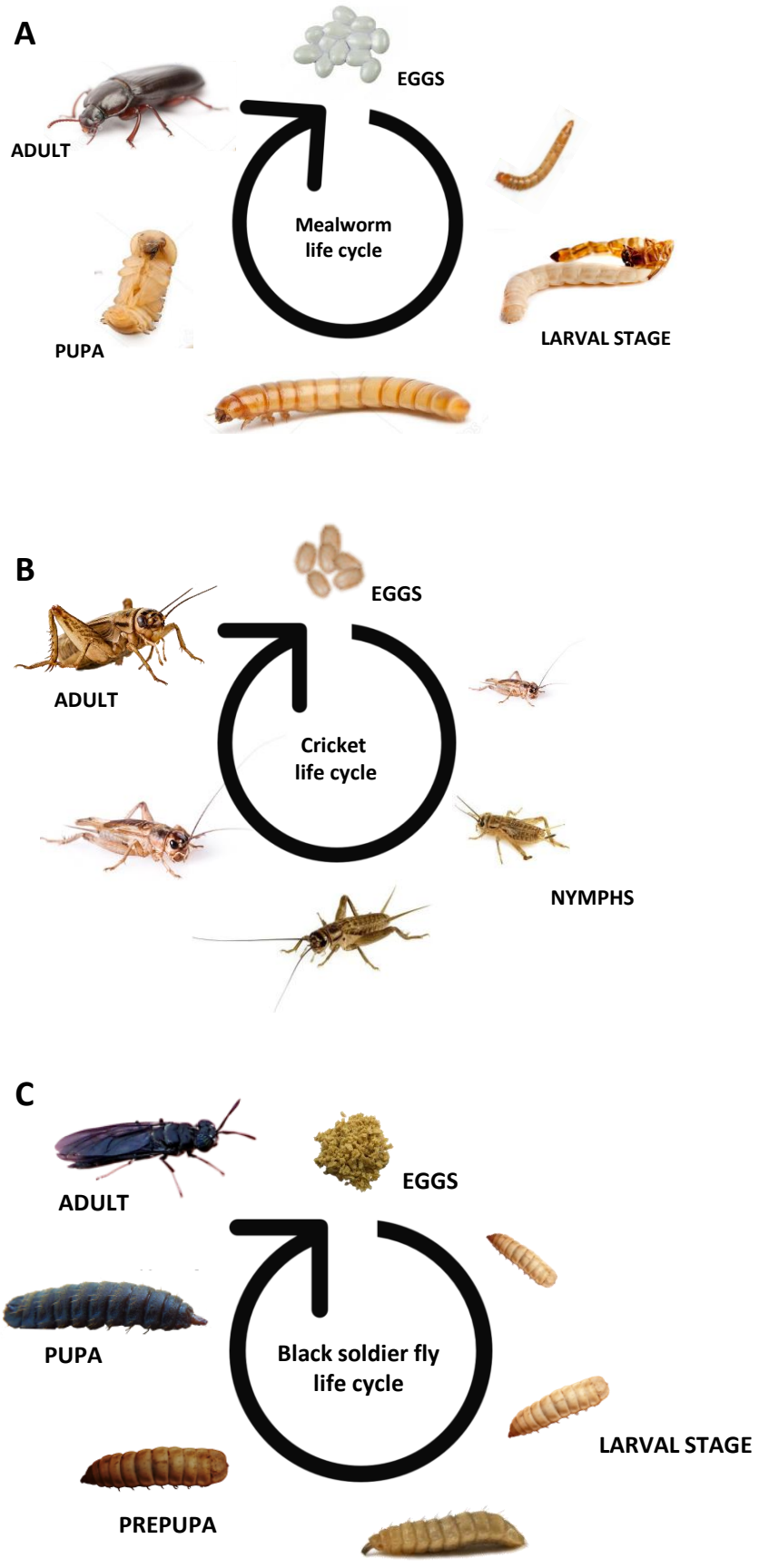


Figure 1.2 Overview of life cycles of **A)** mealworms (*T. molitor* and *A. diaperinus*), **B)** crickets (*A. domesticus* and *G. sigillatus*) and **C)** the black soldier fly (*H. illucens*).

1.3 NUTRITIONAL VALUE

Insects provide a good source of proteins, fats, many vitamins and minerals, and are energetically dense (Ramos-Elorduy et al., 1997; Rumpold & Schlüter, 2013b). Nevertheless, insects differ largely in nutritional composition depending on the species, the developmental stage and even the substrate and origin (Rumpold & Schlüter, 2013b; Kouřimská & Adámková, 2016). Table 1.1 shows the nutritional values of insect species currently sold for human consumption in Europe, i.e. *Tenebrio molitor*, *Alphitobius diaperinu*s, *Acheta domesticus*, *Gryllodes sigillatus, and Locusta migratoria,* as well as *Hermetia illucens* which is reared for aquaculture and/or animal feed. It should be noted that a large variability exists between the specific insects used for each study (substrate, origin, age, …) as well as between the analysing methods used.

Many species contain over 60% of protein on a dry matter basis (Belluco et al., 2013; Rumpold & Schlüter, 2013b), and generally provide a satisfactory amino acid profile to meet the requirements of human adults (Rumpold & Schlüter, 2013b). Edible insect protein contents and amino acid profiles are also comparable or even superior to those of fish meal and soybean meal (Veldkamp et al., 2012; Yi et al., 2013; Makkar et al., 2014). However, the presence of chitin, although valued for its fibre content, may reduce the protein digestibility. Hence, suitability of insect protein for human and animal nutrition should be further assessed in experimental trials (Dobermann et al., 2017).

Most insect species are high in fats, while the fat content varies to a large extent between species and between developmental stages, with larval and pupal stages generally being higher in fat content compared to adult stages. For most insects, among which those sold for human consumption in Europe (such as lesser/yellow mealworms, crickets and locusts), unsaturated fatty acids predominate. Their fatty acid composition is comparable to poultry and fish in their degree of unsaturation (Rumpold & Schlüter, 2013b; Mlcek et al., 2014). Nevertheless, the fatty acid composition may be influenced by the substrate, and thus may vary greatly (Vrabec et al., 2015). Compared to soybean meal and fish meal, insect are higher in lipids. Of particular interest for animal feed is the use of BSF larvae, as they provide the possibility to valorise organic waste streams into a high quality source of protein and fat (see section 1.1). A challenge may be their amount of saturated fats, which is higher than in other insect species (Table 1.1). This was shown to result in negatively altered fatty acid profiles of fish and poultry meat when the animals were fed with BSF larvae (Li et al.,

2016a; Schiavone et al., 2017). On the other hand, BSF larvae are high in lauric acid (Barragan-Fonseca et al., 2017), which is valued for its anti-microbial properties (Zeiger et al., 2017). Research suggests that the fatty acid composition of BSF larvae can be influenced by optimising the substrate composition (Makkar et al., 2014; Wang & Shelomi, 2017), as studies show BSF fat composition to depend on the substrate choice (Barroso et al., 2017; Sphrangers et al., 2017).

Insects reared for human consumption in Europe (i.e. lesser and yellow mealworms, (tropical) house crickets, and grasshoppers) are a good source of minerals such as copper, iron, magnesium, manganese, and zinc (Rumpold & Schlüter, 2013b; Siemianowska et al., 2013), and vitamins such as riboflavin, pantothenic acid and biotin (Rumpold & Schlüter, 2013b). BSF larvae are, in addition to the afore mentioned micronutrients, also rich in calcium (Barragan-Fonseca et al., 2017). It is suggested that the quantity of minerals and vitamins in insects may also be controlled via the substrate used (Oonincx & Van Der Poel, 2011; Rumpold & Schlüter, 2013b). The content of cobalamine, also known as vitamin B12, is important when insects are used as a meat alternative. The vitamin is well represented in yellow mealworms and house crickets. Many other species, however, are low in vitamin B12, and more research is needed to identify edible insects that provide a good source of B vitamins (Finke, 2002; Lenaerts et al., 2018).

As large inter-species variability exists regarding macro-and micronutrients, species will have to be selected for the provision to humans and/or animals of the desired nutritional components (Rumpold & Schlüter, 2013b). For BSF, promising results were obtained in animal trials mainly with poultry, but also with pigs and fish (Makkar et al., 2014; Maurer et al., 2016). Depending on the traditional farm animal species, however, the extent to which soy bean meal or fish meal may be replaced by BSF meal will vary. Moreover, some dietary components which are insufficiently present in BSF meal may have to be artificially included in the diet (Makkar et al., 2014).

Table 1.1 Overview of the crude protein content, total fat content and saturated and unsaturated fatty acids, and caloric value of various fresh insects in comparison to traditional food and feed protein sources.

	Nutritional components (dry matter base)					
	Crude protein (%)[1]	Total fat content (TF,%)	Saturated fatty acids (% of TF)	Unsaturated fatty acids (% of TF)		Energy (kcal/100 g)
				MUFA[2]	PUFA[3]	
Tenebrio molitor (larva)	44 - 64	17 - 43	22 - 35	33 - 52	12 - 42	539 - 577
Alphitobius diaperinus (larva)	58 - 65	22 - 29	41	38	22	N.D.
Acheta domestica (adult)	58 - 74	14 - 23	30 - 36	25 - 26	36 - 39	455
Gryllodes sigillatus (adult)	70	18	34	34	32	452
Locusta migratoria (adult)	56 - 65	13 - 30	35 - 39	28 - 29	32 - 37	568
Hermetia illucens (larva)	32 -52	12 - 42	75 - 87	9 - 10	4 - 13	528
Beef (ground, raw)	59	35	47	42	5	602
Pork (ground, raw)	43	49	41	49	10	675
Chicken (broiler meat, raw)	87	10	32	37	15	485
Atlantic salmon (wild, raw)	63	18	17	37	45	451
Fish meal	48 - 75	9 - 11	N.D.	N.D.	N.D.	454 - 523
Soybean meal	44 - 54	2 - 3	N.D.	N.D.	N.D.	471

Based on the following publications: Finke, 2002; Diener et al., 2009; Oonincx & van der Poel 2011; Ravzanaadii et al., 2012; Bednarova et al., 2013; Rumpold & Schlüter, 2013b; Siemianowska et al., 2013; Makkar et al., 2014; Oonincx et al., 2015; Tschirner & Simon, 2015; Vrabec et al., 2015; Adámková et al., 2016; Ramos-Bueno et al., 2016; Schiavone et al., 2017; Lenaerts et al., 2018; De Smet et al., 2019; and the United States Department of Agriculture National Nutrient Database for Standard Reference Legacy Release (consulted December 2018).

[1]Protein contents were determined using the Kjeldahl or Dumas method, using a Kp of 6.25. As a consequence, they should be interpreted as crude protein contents since other nitrogen-containing components such as chitin are included as well.

[2]Mono-unsaturated fatty acids

[3]Poly-unsaturated fatty acids

N.D. not determined in the selected studies.

1.4 SUSTAINABILITY

The rearing of insects for food and feed is often praised for its low ecological footprint as compared to traditional animal protein. Indeed, insects were shown to score better than traditional livestock (e.g. cattle, pigs, poultry, …) and feed ingredients on multiple sustainability aspects. A first aspect is their feed conversion efficiency, i.e. their capacity to convert feed mass into increased body mass. Typically, 1 kg of live animal weight requires 2.5 kg of feed in the case of chicken, 5 kg in the case of pork and 10 kg in the case of beef (Smil, 2002). Insects, in contrast, require far less amounts of feed due to their poikilothermic nature. For crickets, for example, only 1.7 kg of feed is required to produce 1 kg of crickets (van Huis et al, 2013). Moreover, the edible and digestable percentage of insects (e.g. up to 80% of live weight for crickets) is far greater when compared to the traditionally consumed percentage for meat (e.g. chicken (55%), pigs (55%) and cattle (40%) (van Huis, 2013). Crickets and grasshoppers are only stripped from their legs and wings, while larval stages such as mealworms are even consumed or processed completely. A second aspect is the lower greenhouse gas (GHG) and ammonia emissions of insects compared to traditional farm animals. Edible insects such as mealworms, crickets and locusts compared favourably with pigs and beef cattle for their GHG emissions (CH_4, N_2O and CO_2), as was shown by Oonincx et al. (2010). In that same study, those insects were also proven to produce less ammonia emissions as compared to pigs when expressed per kg biomass gain. It should, however, be noted that these experiments were conducted at small scale in the laboratory, so caution is advised while extrapolating them to large industrial rearing facilities. A third aspect is the lower requirement of insects for land and water (Oonincx & De Boer, 2012; Miglietta et al., 2015). On the other hand, energy use is not always lower for insect rearing. Especially when insects are reared in temperate regions, a large amount of energy is needed to maintain the rearing temperature, which is most often higher than 25 °C. Energy use for the rearing of mealworms is lower than for pork and beef, but higher than for milk and chicken (Oonincx & De Boer, 2012).

When assessing the sustainability of a food product, it is important to take into account the complete production chain, starting from the sustainability of the raw materials, to production and processing steps, and finally including waste management. The sustainability of insects was assessed by different authors through life cycle assessments (LCAs), for example for mealworms (Oonincx & de Boer, 2012; Smetana et al., 2015), BSF larvae (Salomone et al., 2017; Smetana et al., 2016; Smetana

et al., 2019) and crickets in Thailand (Halloran et al., 2017). In general, these studies showed insects to be a potentially more sustainable protein source than conventional meat products (Oonincx & de Boer, 2012; Halloran et al., 2017), than feed ingredients (Salomone et al., 2017), and than other meat alternatives such as mycoprotein, dairy-based and gluten-based meat alternatives (Smetana et al., 2015). Smetana et al. (2016) compared the environmental impact of BSF production using a variety of substrates. Their most important finding was that the most critical aspect to BSF sustainability is the substrate used. The authors thus suggest that the feeding of a low value agri-food product with a good nutritional profile, such as dried distillers grains and solubles (DDGS), would be advisable. A low-quality waste stream may be used if the increased amount of substrate needed does not outweigh the benefits of waste utilisation (Smetana et al., 2016). However, when considering the total feed sector, BSF larvae should preferably be reared on side streams that are not used in feed for traditional livestock and that otherwise would be directed to composting or anaerobic digestion. In addition to substrate choice, upscaling and the use of renewable energy are among the most important factors increasing sustainability of BSF rearing and processing (Smetana et al., 2019).

Nevertheless, as described by Halloran et al. (2016), each of these LCA studies have their shortcomings. Indeed, different LCAs defined different system boundaries or functional units, impeding comparisons between them. Furthermore, the rearing practices at industrial facilities are constantly evolving due to upscaling and automation, and therefore LCAs need to be updated simultaneously (Vandeweyer, 2018). Hence, more research is necessary in order to assess whether edible insects meet the sustainability claims that promote their use as feed and food.

1.5 LEGISLATION IN THE EU AND IN BELGIUM

1.5.1 Legislation with respect to substrates allowed in insect diets

The Transmissible Spongiform Encephalopathies (TSE) crisis at the end of the twentieth century, caused by prions when proteins from infected animals such as cattle and sheep were fed to cattle, gave rise to the TSE regulation EC 999/2001 or “feed ban”. Since then, the regulation prohibits the feeding to livestock of all processed animal proteins (PAPs), with some exceptions (EC 999/2001, 767/2009, 1069/2009). As insects themselves are viewed as “minilivestock”, feeding animal products to insects is also prohibited, with the exception of a limited number of animal materials

(e.g. hydrolysed protein, fish meal, blood products from non-ruminants, egg products, milk products, etcetera) (EC 1069/2009). Consequently, substrates allowed in insect diets currently include those authorised as feed for food producing animals (EC 68/2013), vegetable substrates, dairy and eggs. Food waste may be divided into pre-consumer food waste, i.e. food products handled according to good hygiene practices (GHP) to be sold for consumption, but which did not reach the consumer (i.e. off-specification products, supermarket waste, …) and post-consumer food waste of which hygienic handling (e.g. with regard to treatment, storage, …) cannot be monitored (e.g. household waste, catering/restaurant waste). Pre-consumer food waste is allowed only if it does not include animal products other than dairy and eggs. The use of post-consumer food waste, slaughterhouse by-products, other types of plant-based organic waste (e.g. gardening waste), and intestinal content (of both human and animal origin) is not authorised (Figure 1.3). When more research is performed with regard to the possible health risks related to different substrates, the International Platform of Insects for Food and Feed (IPIFF) pleads for a relaxation of EU legislation in the future, so that more substrates will be authorised (IPIFF, 2017). Possibly, substrates allowed will differ depending on the intended use of insects, i.e. either for animal consumption, human consumption or applications that do not relate to the food chain, e.g. biochemicals.

1.5.2 Legislation with respect to the use of insects in animal feed

When insects are used in feed in the EU, several legislative documents need to be obeyed. Today, the "feed ban" (EC 999/2001) only allows a limited number of animal products in animal feed (see section 1.5.1.). Consequently, insect proteins and whole insects are not allowed as animal feed in the EU except for aquafeed. Living insects, insect-derived oils and hydrolysed insect proteins, on the other hand, are allowed as animal feed (with the exception of living insects for ruminants). However, as insects are considered livestock, legislation until recently required insects to be slaughtered in a registered slaughterhouse. Consequently, the use of insects in aquaculture was not practiced. In 2017, Regulation EC 2017/893 authorised the use of insect proteins from seven species (*H. illucens, M. domestica, T. molitor, A. diaperinus, A. domesticus, G. sigillatus* and *Gryllus assimilis*) for fish feed and allowed insects to be "slaughtered" in the rearing facility. With regard to other species, IPIFF states on their website (consulted January 2019) that the European Commission services are currently

exploring the possibilities for revising the feed ban in order to authorise insect proteins in poultry feed.

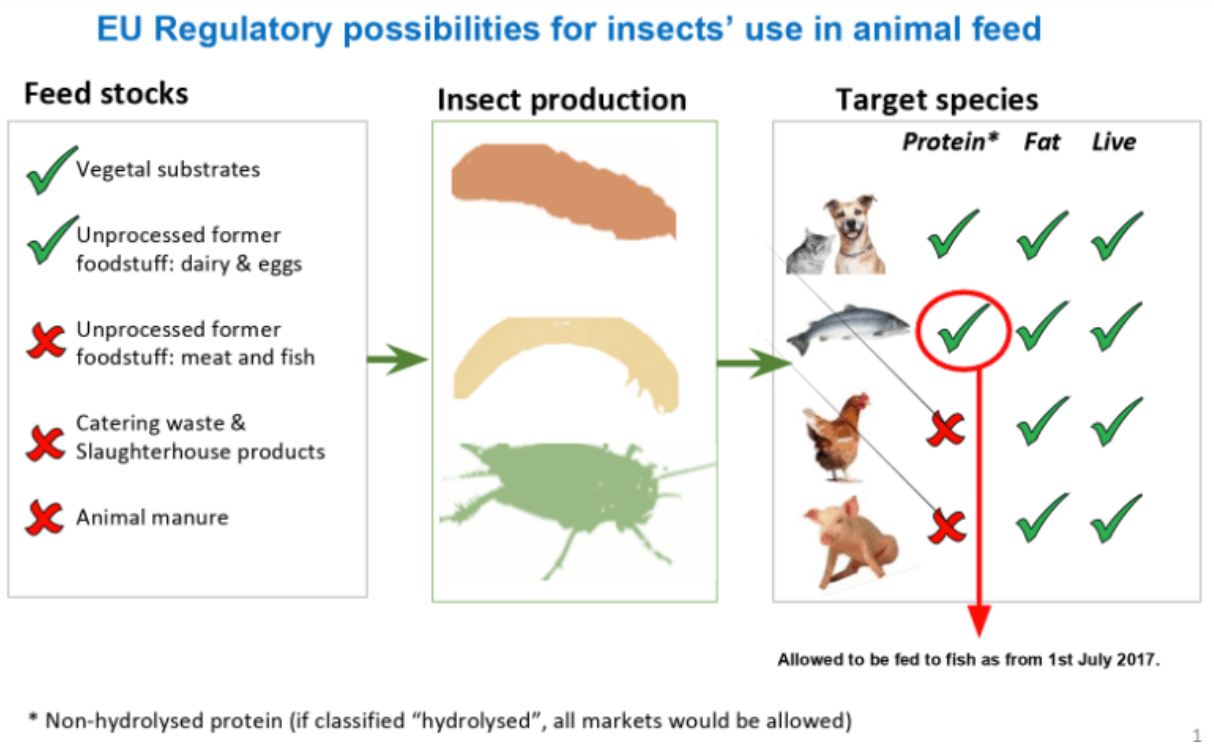


Figure 1.3 Summary of the current EU legislation regarding substrates allowed as insect diets, and regarding insects allowed as feed for vertebrate species (source: www.ipiff.org)

1.5.3 Legislation with respect to insects in food

Food products not consumed to a significant degree by humans in Europe prior to 1997 are covered by the Novel Food regulation (EC 258/97), stating that prior to marketing these food products, an extensive dossier assessing the safety of the novel food should be submitted to the EFSA. With regard to the previous version of this regulation, uncertainty existed until the end of 2017 as to whether whole processed insects should be considered as novel foods, as the legislation only considered "food ingredients isolated from animals". Hence, in the lack of a clear legislative framework, several European countries, such as Belgium, the Netherlands, and France, published national guidance documents for insect producers (NVWA, 2014; SHC & FASFC, 2014; Agence Nationale de Sécurité Sanitaire de l'alimentation, de l'environnement et du travail (ANSES), 2015), followed by a risk assessment by the EFSA (2015). In Belgium, specifically, a circular was published in 2014 (FASFC, 2014), tolerating the commercialisation of a selection of 10 insect species. In 2015, the novel food regulation was updated (EC 2015/2283), considering edible insects in whatever form as novel

foods as from the 1st of January 2018. Since then, insects require approval by the EFSA, based on an extensive dossier assessing the safety of the novel food, before they can be marketed in the EU. In Belgium, the FASFC further tolerates the commercialisation of insects only for those species for which a novel food dossier was submitted prior to January 1st 2018. Three insect species and foods including these species are currently tolerated on the Belgian market: yellow mealworm (*Tenebrio molitor*), house cricket (*Acheta domesticus*) and migratory locust (*Locusta migratoria;* Federal Public Service Health, Food Chain Safety and Environment, 2018). In other EU states, novel food dossiers were also submitted for the lesser mealworm (*Alphitobius diaperinus*) and the tropical house cricket (*Gryllodes sigillatus*; EFSA website, consulted January 2019).

1.5.4 Microbiological criteria and guidance documents

As for any food product, it is important to monitor microbial food quality and safety during production and processing of insects. On EU level, microbiological criteria for food products are given in regulation EC 2073/2005, comprising both food safety criteria as well as process hygiene criteria. For insects used as food, however, no specific criteria exist in this regulation. In the national guidance documents, microbiological criteria applied to minced meat and meat products, crustaceans, and/or ready-to-eat food products (as suitable growth matrix for *Listeria monocytogenes*) are proposed (NVWA, 2014; SHC & FASFC, 2014). These matrices are highly different from freshly harvested insects, which usually contain a total viable aerobic count of approximately 8 log cfu/g (Vandeweyer, 2018), which is higher than the proposed limits, for instance for minced meat in EC 2073/2005 (m = 5.7 log cfu/g and M = 6.7 log cfu/g). Indeed, in contrast to traditional meat, insects are consumed as a whole, including their intestinal content, which explains their high microbial load. In 2019, IPIFF published a EU sector guide for the insect rearing industry which focusses on good hygiene practices (IPIFF, 2019). The objective of this guide is to help insect producers effectively apply EU food and feed safety legislation, and to provide an aid for developing a food and feed safety management system. In this document, a number of microbiological parameters are suggested to be monitored for insects and insect-based products intended for human consumption. These parameters include the aerobic count (30 °C) and number of *E. coli* as hygiene indicators, as well as a number of food pathogens such as coagulase-positive staphylococci, *Listeria monocytogenes*, *Salmonella* sp., *Cronobacter* spp., *Clostridium perfringens*, *Bacillus*

cereus, and *Campylobacter* sp. In Belgium, specifically, some mandatory measures are in place. A circular published in 2014 and updated in 2016 and 2018 (FASFC, 2014; FASFC, 2016; FASFC, 2018a) requires periodical testing of marketed insect products for *Salmonella* sp. and *Listeria monocytogenes* (Table 1.2). Furthermore, based on the recommendations by the several guidance documents mentioned above, the FASFC provides on its website advisory food safety action limits for the presence of *Salmonella* sp., *L. monocytogenes*, *B. cereus* and coagulase-positive staphylococci. In addition, these action limits contain process hygiene criteria for total aerobic counts, Enterobacteriaceae and fungi. When insect products exceed these limits, the FAVV requires specific actions, depending on the limit and product, to be taken. An overview of Belgian legal microbiological criteria for *Salmonella* sp. and *L. monocytogenes* and action limits is provided in Table 1.2.

Producers of PAPs such as insects for feed purposes must comply to regulation 1069/2009 (animal by-products). According to that regulation, insects must be processed according to regulated processing methods (EC 142/2011) prior to being fed to food-producing animals. According to EC 142/2011, category 3 materials such as insects (i.e. non-pathogenic) must be processed using methods described in the regulation (with specified process parameters such as fractionation sizes and temperature-time combinations) or using methods that are proven sufficient for resulting products to comply to microbiological criteria which need to be daily monitored for a 30-day production period. More specifically, *C. perfringens* (absent in 1 g) should be analysed in samples taken immediately after processing. *Salmonella* sp. (absent in five samples of 25 g), and Enterobacteriaceae (five samples, of which at least three are lower than 10 cfu/g and maximally two are between 10 and 300 cfu/g) should be monitored during storage or prior to leaving the facility.

Table 1.2 Summary of Belgian microbiological criteria (FASFC, 2018a) and action limits (as listed on the FASFC website) for the application of insects in food as published in a circular.

Microbiological parameter (type)	Sampling plan[1]		Limits[2]		Unit	Type of criterion	Type of product
	n	c	m	M			
Salmonella sp. (detection)	5	0	Absence		/25 g	Criterion[3]	Insects and insect-based products ready for consumption
	5	0	Absence		/10 g	Criterion[3]	Insects, provided a heat treatment will be applied prior to consumption, freeze-dried insects
Listeria monocytogenes (count)[4]	5	0	100	100	cfu/g	Criterion[3]	Insects and insect-based products, if proven counts will remain < 100 cfu/g during the shelf life
Listeria monocytogenes (detection)[4]	5	0	Absence		/25 g	Criterion[3]	Insects and insect-based product, if it cannot be proven that counts will remain < 100 cfu/g during the shelf life
Bacillus cereus (count)[5]	5	2	5000	100000	cfu/g	Action limit	Insects and insect-based products
Coagulase-positive staphylococci (count)[5]	5	2	5000	100000	cfu/g	Action limit	Insects and insect-based products
Escherichia coli (count)	5	2	500	5000	cfu/g	Action limit	Insects and insect-based products
Total viable aerobic count (30°C)	5	2	1000000	10000000	cfu/g	Action limit	Insects and insect-based products
Enterobacteriaceae (count)	5	2	5000	100000	cfu/g	Action limit	Insects and insect-based products
Yeasts (count)	5	2	5000	100000	cfu/g	Action limit	Insect-based products
Moulds (count)	5	2	5000	100000	cfu/g	Action limit	Insects and insect-based products

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

[2]A value below M is acceptable under the following requirements: (1) the mean value observed is ≤ m, (2) a maximum of c/n values observed are between m and M and (3) no values observed exceed the limit of M.

[3]For *Salmonella* sp. and *Listeria monocytogenes*, the criteria as described here are also included as action limits by the FASFC.

[4]Only food matrices allowing the growth of *L. monocytogenes* are considered.

[5]Depending on the product and sector (primary, processing, distribution), the presence of toxins specific to these pathogens must be tested in 25 g of sample by the Scientific Institute of Public Health in case numbers of the pathogen exceed 5 log cfu/g.

1.6 ETHICAL CONSIDERATIONS

The UK Farm Animal Welfare Council identified in 1979 the elements that determine an animals perception of its welfare state and the provisions necessary to promote that state. These elements were encapsulated as the “Five Freedoms” and address both physical fitness and mental suffering (Webster, 2001). These five freedoms include (1) freedom from thirst, hunger and malnutrition, (2) freedom from discomfort, (3) freedom from pain, injury and disease, (4) freedom to express normal behaviour, and (5) freedom from fear and distress. These criteria are included by the Council of the European Union into a directive (Council Directive 98/58/EC) laying down minimum standards for the protection of animals bred or kept for farming purposes. This directive does, however, not apply to invertebrates. Nevertheless, for insects, most criteria could be met by ensuring enough feed and moisture as well as enough individual space (taking into account, however, the natural clustering behaviour of the species) and mimicking the natural habitat (e.g. using cardboard to mimic dark crevices, providing a suitable light regime, etcetera). Nevertheless, little is known about the extent to which insects experience pain and discomfort (Erens et al., 2012; Adamo, 2016). It is generally assumed that insects do not feel pain in the same way as vertebrates, and that their nervous system is smaller (Adamo, 2016). Still, it is suggested to kill insects using methods that are either gentle (e.g. freezing) or instantaneous (e.g. submerging them in hot water, shredding, …) (van Huis et al., 2013). In the future, more research should be conducted on the wellbeing of insects, eventually leading to practical guidelines for the insect rearing industry.

1.7 MANAGING QUALITY AND SAFETY DURING REARING OF INSECTS

The EFSA stated in a risk assessment on edible insects in 2015 that the rearing practices will likely be an important factor for the chemical and microbiological quality and safety of insects (EFSA Scientific Committee, 2015). The nutritional composition of insects can be affected based on substrate choice, as was already shown for mealworms (Ramos-Elorduy et al., 2002; van Broekhoven et al., 2015) and BSF larvae (St. Hilaire et al., 2007). Even more important than managing nutritional quality is ensuring food safety. Multiple chemical and microbiological contaminations may occur during rearing, resulting in food safety risks when harvested insects are (processed and) consumed. Chemical hazards may comprise both inorganic (e.g. heavy metals) as well as organic (e.g. pesticides, mycotoxins, antibiotics, prions …) materials. The intake

and possible accumulation of these contaminants in the insects can be largely controlled by monitoring the contaminant level in the substrate and insects (EFSA Scientific Committee, 2015). Microbiological hazards may include bacteria, fungi, viruses, and parasites. For microbiological contaminants, the substrate is likely an important source, but a variety of other contamination routes may exist in addition. For instance, microorganisms may be introduced to the rearing facility through the environment (e.g. air, water), personnel (through manual work) and vermin (e.g. rodents, birds, feral insects). Furthermore, cross-contamination may occur from one batch to another (i.e. horizontal transmission), or from one generation to the next (i.e. vertical transmission). Microbiological hazards during insect rearing can be divided in two categories: (1) those harmful to the insect itself (i.e. entomopathogenic) and (2) those harmful to the human/animal consuming the insect. The most relevant examples of the first category include viruses (e.g. parvoviruses, baculoviruses and iridoviruses), bacteria (e.g. *Bacillus thuringiensis*, *Serratia* spp*., Pseudomonas* spp.), fungi (e.g. *Beauveria* spp., *Metarhizium* spp.) and parasites (e.g. *Microsporidia* spp.) (Eilenberg et al., 2015). Due to the large difference in physiology between insects and vertebrates, entomopathogens likely do not cause problems in vertebrate species (EFSA Scientific Committee, 2015). In this PhD research, the rearing of insects is studied with focus those microorganisms relevant for food and feed safety and quality, and not on those relevant for insect health. Microbiological hazards related to food and feed safety during the rearing phase are further discussed in section 1.8.

For insects intended for human consumpstion, national and international guidance documents (NVWA, 2014; SHC & FASFC, 2014; ANSES, 2014), the IPIFF sector guide (IPIFF, 2019), as well as Belgian legislation (FASFC, 2018) include advisory and mandatory measures for insect producers in order to warrant and/or improve the food safety of harvested insects. In this paragraph, a brief summary of these measures is provided.

The following measures are mandatory for insects for food in Belgium, as described in the circular by the FASFC (2018):

- Operators rearing insects must be registered at the FASFC. Operators also participating in the processing and/or distribution sector must be authorised by the FASFC.
- General principles in food and feed legislation are applicable, i.e. the following of Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP), similar

to conventional food and feed products. Good practices apply to the whole production process, including obtaining of substrates, rearing, transport and slaughter, and also include traceability, compulsory notification and adequate labelling. The implementation of a HACCP plan in insect rearing facilities is mandatory.

- Feed administered to edible insects should also follow the general feed legislation (see section 1.5).
- The administration of therapeutic pharmacological agents should comply with EU legislation. Prescription and administration of the agents has to be conducted by a registered veterinarian.
- The use of fungicides or pharmacological substances such as antibiotics in insect substrates is prohibited, as little knowledge exists on the residual content of these substances in the harvested insects.
- If possible, dry substrates should be used, faeces and dead insects should be removed, and the substrate and water source should be frequently replenished during rearing.
- Consequent cleaning (and disinfection, e.g. in case of increased mortality) of all used rearing materials and infrastructure (e.g. rearing rooms, rearing cages/containers, etcetera) must be applied in between rearing cycles.
- Production cycles of insects destined for human consumption must be kept strictly separate from those destined for use as animal and/or pet feed.
- Products should be periodically tested for the presence of *Salmonella* sp. and *Listeria monocytogenes* (see section 1.5.4 for criterion details).
- Insect products must be heat-treated prior to commercialisation in order to reduce the microbial load.
- The possibility of pathogen outgrowth during the shelf life, which is often long for dried and freeze-dried products, should be taken into account when assessing food safety.
- Products should be adequately labelled with regard to preparation, storage, physical hazards (e.g. removal of wings and legs) and allergy risks.

Although only mandatory for human consumption in Belgium, most of these measures are also advised for insects reared for both food and feed in other countries according to the IPIFF sector guide (IPIFF, 2019).

The following paragraph summarises the most specific advisory measures - in addition to the mandatory measures in the previous paragraph - for insect rearing for food and feed, including non-binding recommendations proposed by various guidance and legislative documents (NVWA, 2014; SHC & FASFC, 2014; ANSES, 2014; FASFC, 2018) and the IPIFF sector guide (IPIFF, 2019).

- Incoming substrates must be stored in dry and hygienic conditions and free from vermin.
- Both the chemical as well as the microbiological safety of the rearing substrate should be monitored by regular testing for organic contaminants (e.g. pesticides, veterinary substances, …) and the presence of food pathogens such as *Salmonella* sp. and *Campylobacter* sp. Analyses for process hygiene criteria such as the total aerobic mesophilic count are also recommended. Furthermore, substrates should be pre-treated when necessary.
- Only certified food contact equipment should be used to provide the substrates to the insects.
- Mesh size of harvesting sieves should enable effective one or two-step separation of insects from their residue. In the case of volatile faeces, separation must take place in a confined area. The frass must be dried (if necessary), controlled, and stored in a dedicated area in case of reuse (e.g. as soil conditioner), or be disposed of.
- Harvesting equipment should be cleaned thoroughly on a regular basis to limit microbiological contamination and the proliferation of larvae from unhatched eggs.
- After harvest and prior to killing, insects are advised to be kept chilled (0 – 10 °C depending on the species).
- Insects killed through freezing should be frozen at temperatures below -5 °C.
- Insects and insect-based products should be tested, in addition to *Salmonella* sp. and *L. monocytogenes*, for the presence of other food pathogens such as *B. cereus*, coagulase-positive staphylococci, *Campylobacter* sp., *C. perfringens* etcetera (see section 1.5.4) as well as determining the total aerobic mesophilic count (30 °C) and the number of *E. coli* as hygiene indicators.
- Heat treatments using water should be conducted while taking into account (1) the levels of temperatures used (duration not specified), (2) bacterial spores and their survival on the insects, and (3) any other insects reared in the rearing environment.

- Heat treatments not based on the use of water include microwaving and infra-red tunnels. Process parameters such as time of exposure or layer thickness should be adapted to the specific insect species.

The above mentioned recommendations and regulations are often undetailed with regard to specific measures to improve food or feed safety (e.g. specific process parameters for treatments of substrates and insects, sampling plans for microbiological and chemical testing, specific rearing practices, and so on). Indeed, all national guidance documents, as well as the EFSA scientific committee (2015) have urged for further research on the safety of reared insects and the impact of specific rearing and processing procedures.

1.8 MICROBIOLOGICAL QUALITY AND SAFETY DURING REARING

1.8.1 Microbiota of insects

As insects are living organisms, they harbour a complex microbiota. Microorganisms can be found on their body surface and mouthparts, but it is assumed that the highest number of microorganisms is situated within the gastro-intestinal (GI) tract (Cazemier et al., 1997; Rumpold et al., 2014). Insects acquire microorganisms vertically, through the ovary, the egg capsule, or during egg laying, and via horizontal transmission from their environment and their diet (Schlüter et al., 2017). In most insects, gut communities are dominated by widely distributed bacteria that appear to colonize hosts opportunistically. Physicochemical conditions, such as pH, redox potential, in (different compartments of) the insect gut, as well as interactions with the present microbiota, seem selective for particular species. Microorganisms in insect guts show a variety of functions, such as nutritional symbioses (e.g. aid in digestion and nutrient provisioning), affecting insect development, detoxification of toxic components, and protection against pathogenic microorganisms by means of colonisation resistance (i.e. nutrient competition, niche occupation, and/or immune priming) (Rajagopal, 2009; Engel & Moran, 2013). For the insects under study in this PhD thesis, multiple studies have characterised their bacterial composition through DNA-based techniques, such as DGGE and high-throughput sequencing. These studies report a large number of bacterial species, although some genera appear to reoccur as abundant community members across studies and across rearing batches. For fresh yellow and lesser mealworms, for instance, the genera *Spiroplasma*, *Lactococcus*,

Entercoccus, and *Erwinia*, are reported in multiple studies (Stoops et al., 2016; Stoops et al., 2017; Vandeweyer et al., 2017a, Osimani et al., 2018a,b). For black soldier fly larvae, some genera such as for instance *Providencia*, *Morganella*, *Pseudomonas* and *Klebsiella* were also identified in more than one study (Jeon et al., 2011; Zheng et al, 2013b,c; Bruno et al., 2019). This suggests that, even though insects in those different studies were reared on different substrates and with differing rearing methods, some bacterial species or genera might generally occur in the insect, possibly because they play (a) beneficial role(s) (De Smet et al., 2018; Osimani et al., 2018a).

When assessing the microbial load of fresh insects from a food perspective, many studies have assessed the total viable count of insects marketed for consumption in Europe. Indeed, various studies show total aerobic viable counts for fresh insects such as yellow/lesser mealworms, crickets, and grasshoppers to approximate 8 log cfu/g (ranging from 7.5 to 9.3 log cfu/g) (Stoops et al., 2016; Stoops et al., 2017; Vandeweyer et al., 2017b,c; Osimani et al., 2018a), clearly exceeding the action limits (m = 5.7 log cfu/g and M = 6.7 log cfu/g) as described in section 1.5.4. Those studies also report other culture-dependent microbial counts in order to characterise the microbiological quality of fresh harvested insects as food/feed matrices. In general, high counts are reported for lactic acid bacteria (6.8 to 8.3 log cfu/g), Enterobacteriaceae (6.1 to 8.3 log cfu/g), and more varying counts for fungi (3.5 to 7.2 log cfu/g) and bacterial endospores (0.5 to 5.0 log cfu/g). These numbers indicate that freshly harvested insects are a suitable matrix for a variety of microorganisms, including possible spoilage organisms or food pathogens. Thus, attention should be paid to the high numbers of these microbial groups when edible insects are processed and stored (Klunder et al., 2012).

1.8.2 Microbial dynamics during rearing

For traditional farm animals, knowledge exists on the fact that the microbiota of the gastro-intestinal (GI) tract contributes to the performance and health of the animal, and that its composition is highly variable depending on the feed and farm management (Chaucheyras-Durand & Durand, 2010). In the previous section, the high microbial load of fresh insects was discussed. As highlighted by Engel & Moran (2013), insects acquire most of their gut microbiota from the environment and from their diet. It is suggested that the rearing infrastructure and practices, as well as the substrate choice affect the insect microbiota (EFSA scientific committee, 2015; Boccazzi et al.,

2017; Osimani et al., 2018a). To date, only a couple of studies investigated the microbiota of reared insects in relation to rearing practices and/or diet choice. For mealworms and crickets (house crickets and tropical house crickets), Vandeweyer et al. (2017b) showed that these insects differed largely in some microbial counts, such as fungi and aerobic endospores, as well as in their bacterial community composition. These differences were observed between different production cycles (batches) within one rearing facility, and between batches from different facilities. Although no information was given on the specific rearing methods and substrates, this shows that the composition of the gut microbiota is not universal for a certain species and likely determined by a variety of rearing factors. For mealworms, Li et al. (2016b) compared the microbial community between larvae reared on the same diet either in a closed artificial environment or in an open environment, and found substantial differences in the microbial communities. Bacterial species richness, however, was very similar between larvae from the two groups. Osimani et al. (2018), who studied the microbiota of substrate, harvested larvae and residues, suggested two sources of contamination during mealworm rearing, i.e. the substrate (wheatmeal in that specific study) and vertical transmission from mother to offspring. For the black soldier fly, Zheng et al. (2013c) found substantial differences in bacterial communities between successive live stages. Furthermore, it was shown that larvae reared on different diets, differed in both their bacterial (Jeon et al., 2011; Bruno et al., 2019) and fungal community (Boccazzi et al., 2017). De Smet et al. (2018) suggested that the microbiota in the rearing substrate and in the insect GI tract (the two of which are likely interchangeable) play a crucial role during the larval development. On the other hand, multiple studies suggest that BSF larvae, in turn, affect the microbiota of their substrate, e.g. by reducing the number of specific bacteria, including pathogens such as *Salmonella* sp. and *Escherichia coli* (Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2015). In contrast, Bruno et al. (2019) did not observe large alterations of the substrate bacterial community after contact with BSF larvae. Indeed, the interplay between the microbiota of the rearing environment, substrate, and the insects themselves is still largely unexplored. Besides diet choice, other rearing factors are also hypothesised to affect the insect microbial community, such as environmental conditions (e.g. pH and moisture content of the rearing substrate, temperature, relative humidity, …), manipulations during rearing, and even the specific insect genotype reared. However,

very little research has been performed to unravel the mechanisms that determine the gut microbial community composition in reared insects.

1.8.3 Microbial dynamics during post-harvest treatments

Aside from factors that are relevant during the rearing phase as discussed in the previous section, a variety of harvest and post-harvest procedures may be applied at the rearing facility, each of which may affect the insect microbiota. As stated by the NVWA (2014), insects are starved after harvest in order to empty their gut content. It is assumed that this may enhance the microbial quality of the insects by reducing their microbial load (personal communication with rearers). It is suggested that the stagnation of the feed passage during starvation will likely cause shifts in the microbial community (Dillon & Dillon, 2004). A study by Dillon et al. (2010) indeed showed the microbiota of starved grasshoppers to differ from that of well-fed grasshoppers. However, the impact of starvation was not yet investigated in the light of microbiological food quality and research on this topic was advised by the advisory rapport by the SHC & FASFC (2014). The starvation treatment may be followed by a rinsing step in order to clean the larval surfaces, another step of which the effect on microbial quality is poorly investigated thus far.

After harvest, and possibly after starvation and/or rinsing, larvae are most often killed. The killing may entail a freezing step, of which the effect on the insect microbiota has, to our knowledge, not yet been investigated. A final step in most rearing facilities, prior to further processing, most often consists of a heat treatment applied to frozen or living insects (personal communication with rearers). Next, insects are stored in a frozen state or directly further processed. Klunder et al. (2012) and Vandeweyer et al. (2017c) investigated the effect of boiling or different blanching treatments, respectively, on the microbial quality of mealworms, and found that most microbial counts substantially decreased to below the proposed lower limit of 6 log cfu/g as proposed by the FASFC (see section 1.5.4 for detailed criterion). Noteworthy, bacterial endospores were affected hardly or not and were still present in numbers up to 4.7 log cfu/g. Indeed, national guidance documents as well as Belgian legislation emphasize the importance of the application of a heat treatment prior to consumption (ANSES, 2014; NVWA, 2014; SHC & FASFC, 2014; FASFC, 2016). However, no guidelines exist on the time-temperature conditions to be applied. Companies currently optimise their own heat treatments, which may vary in their effectiveness in reducing total viable

counts and in the number of surviving endospores. Indeed, Fasolato et al. (2018) reported endospore counts ranging from 1.6 to 8.1 log cfu/g for marketed, processed insect products, showing that processes applied across countries, e.g. freeze-drying and drying, are often ineffective against bacterial spores.

1.8.4 Microbiological safety aspects

The fact that fresh edible insects contain a high microbial load implies that they can harbour food pathogens, such as bacterial food pathogens or mycotoxinogenic fungi. As the rearing environment and diet are proposed sources for bacteria and fungi in insects, it is reasonable to assume that pathogens might contaminate insects during rearing. For instance, for lesser mealworms, the uptake of *Salmonella* sp. and *Campylobacter* from highly concentrated solutions was extensively investigated in the light of the insect's potential of being a vector for *Salmonella* sp. in the poultry industry (McAllister et al., 1994; Strother et al., 2005; Templeton et al., 2006; Hazeleger et al., 2008; Roche et al., 2009; Crippen et al., 2009; Leffer et al., 2010; Zheng et al., 2012b). Transmission routes and circumstances in poultry houses are very different from those in an insect rearing facility, and those studies used very different inoculation methods that do not represent realistic contamination of substrates in insect rearing facilities. Nevertheless, those studies do show that the lesser mealworm may be contaminated with food pathogens from its environment. Questions emerge whether food pathogens, possibly present in the insect diet or environment, may contaminate insects. A following question then would be whether this results in health risks when the insects are subsequently consumed by humans or animals. Bacterial food pathogens *Salmonella* sp. and *Listeria monocytogenes* were not encountered through classical analyses in fresh insects sold for consumption in Europe thus far (Giaccone, 2005; Grabowski et al., 2014; Grabowski and Klein, 2016; Vandeweyer et al., 2017b; Garofalo et al., 2017). *Salmonella* sp., however, was encountered in grasshoppers consumed in Cameroon (Ali et al., 2010). In addition, food pathogens *Staphyloccoccus aureus* and presumptive *Bacillus cereus*, in contrast, have been detected in marketed insect products in Europe and/or in Africa based on lesser mealworms, crickets, locusts and/or rhinoceros beetles (Banjo et al., 2006; NVWA, 2014; Grabowski and Klein, 2016; Osimani et al., 2017; Fasolato et al., 2018). It should, however, be noted that analysis for *B. cereus* according to classical ISO-standards does not discriminate between *B. cereus sensu stricto* and other members of the *Bacillus cereus* group. The latter

group consists of a number of species of the *Bacillus* genus, which are genetically almost identical and some of which are pathogenic to mammals and/or insects, including *Bacillus cereus sensu stricto*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus* and *B. toyonensis* (Ceuppens et al., 2013; EFSA Panel on Biological Hazards, 2016). Nevertheless, the group deserves particular attention, as heat treatments applied post-rearing might not suffice in reducing number of *Bacillus* spores. Also through DNA-based techniques, *Bacillus* species were identified in commercialised edible insects in previous studies (Garofalo et al., 2017; Osimani et al., 2017; Fasolato et al., 2018). Vandeweyer (2018) performed a characterisation of the spore community of mealworms and crickets through the identification of isolates after pasteurisation of the samples, and found that many isolates corresponded to the *Bacillus cereus* group. Thus, the presence of *B. cereus* might be a risk in edible insects. Consequently, Vandeweyer (2018) suggested the inclusion of a food safety criterion for *Bacillus cereus* in EU legislation, even though ISO-methods are unable to distinguish between different members of the group. For instance, *B. thuringiensis* is a well-known insect pathogen and is even commercialised as biopesticide in agriculture. Some *B. thuringiensis* are also capable of producing enterotoxins, and the Belgian FASFC (2018b) advises to apply the same action limits for *B. thuringiensis* as for *B. cereus*.

As fresh insects harbour fungal species (Stoops et al., 2016; Stoops et al., 2017; Vandeweyer et al., 2017b,c; Boccazzi et al., 2017), it can be possible that the fungal community includes mycotoxinogenic fungi. In addition to the fact that insects are often fed with grain-based products, the presence of mycotoxins in harvested insects is possible. A previous study by Charlton et al. (2015) showed the presence of mycotoxins (Beauvericin, Enniatin A and Enniatin A1) in housefly larvae, but concluded that these mycotoxins in the observed quantities would not pose a safety risk when used in feed. For mealworms, Van Broekhoven (2014) showed that mealworms fed with DON-contaminated substrate excreted contaminated frass, but the residual load in the insects themselves was below the limit of quantification (LOQ) (Van Broekhoven, 2014 in EFSA Scientific Committee, 2015). Other microbiological food safety aspects include vertebrate viruses and parasites that may be carried by insects within their GI tract, if - for instance - present in the substrate. These microbiological hazards are considered of less concern when evaluating the microbiological safety of edible insects. The risk involved with these pathogens may be easily managed by applying proper processing techniques such as freezing and cooking (EFSA Scientific Committee, 2015).

1.9 OUTLINE AND SCOPE OF THE PHD DISSERTATION

For traditional livestock animals, research has been performed for decennia in order to understand the relation between farming practices and the (microbiological) quality of the products derived from the animals. Sector guides are developed, and farmers and food producers are well informed on how to ensure microbiological quality and mitigate food safety risks. For insects, however, it becomes clear from the previous sections that little knowledge exists on the microbiota and the microbiological quality and safety of insects in relation to the rearing substrates, environmental conditions and rearing protocols. Moreover, the microbial dynamics throughout the rearing phase until harvest, and during application of post-harvesting procedures are still largely unexplored. Furthermore, it remains unclear to date to what extent rearing practices and the substrate affect the microbiological safety of harvested insects. This PhD was conducted in the framework of a research project titled "EDINCO – Microbial and chemical food safety risks during rearing of insects" funded by the Federal Public Service Health, Food Chain Safety and Environment. It was requested to investigate - as it was formulated originally - "*the food safety risks in the industrial context of insect rearing*". The request focussed in general on insects for both food and feed, implying that several insect species needed to be included in the study. As a consequence, the microbiological aspects investigated in this PhD (1) focussed on several insect species, (2) targeted many "levels" (rearing and post-rearing, substrates and insects, endogenous and potential microbiota, …) and (3) were mainly studied in an industrial environment. More specifically, the following research goals were defined:

- **Objective 1:** Characterisation of the microbial dynamics during industrial rearing of insects, in particular of *H. illucens*, *A. diaperinus* and *G. sigillatus*
- **Objective 2:** Studying selected microbiological safety risks during industrial rearing of *H. illucens*, *A. diaperinus* and *G. sigillatus* for feed and food
- **Objective 3:** Assessing the horizontal transmission of a food pathogen to the insect during rearing, with focus on the case study of transmission of *Salmonella* sp. from wheat bran to mealworms (*T. molitor*)
- **Objective 4:** Assessing the effect of post-harvest practices on the microbial load and the bacterial community, with focus on the case study of starvation and rinsing of mealworms (*T. molitor*)

The outline of the PhD is visualised in Figure 1.4. In *Objective 1*, the aim is to provide a microbiological characterisation of the rearing phase during the production of insects at industrial and/or large scale rearing facilities. To this end, samples are taken from insects, as well as from substrates and residues (i.e. the mixture of left-over substrate, faeces and exuviae after separation from the larvae) during rearing. In addition, the microbiota of insects is characterised at harvest and, if applicable, after post-harvest procedures performed in the rearing facility. Microbiological characterisation of the samples includes analyses through classical plate counts as well as through high-throughput 16S rRNA gene sequencing in order to determine the bacterial community composition. In **Chapter 2**, the microbiota during the rearing of black soldier fly larvae (*H. illucens*) as insect for feed is characterised. In **Chapter 3** and **Chapter 4**, a rearing cycle is studied at industrial scale for the lesser mealworm (*A. diaperinus*) and the tropical house cricket (*G. sigillatus*), respectively, both used for human consumption. In those chapters, a selection of fungal isolates from substrates, insects and residues, is identified through sequencing in order to gain a general insight into the most abundant members of the fungal community. Furthermore, for those rearing cycles, samples are taken after post-harvest heat treatments in order to study the effect on the microbiological quality. For all three species studied in Chapters 2 to 4, food safety aspects is assessed in the light of *Objective 2.* The presence of *Salmonella* sp., *L. monocytogenes*, presumptive *B. cereus*, and coagulase-positive staphylococci is monitored in larvae and residues of the external rearing facilities. In addition, the data obtained via high-throughput sequencing are also screened for pathogenic genera and fungal isolate identifications are screened for possibly mycotoxinogenic fungi.

In *Objective 3*, the risk of contamination of mealworms with *Salmonella* sp*.,* if present in their rearing substrate, is assessed. The transmission of *Salmonella* sp. from contaminated wheat bran to yellow mealworms (*T. molitor*) is studied under laboratory conditions. In this way, a first baseline assessment can be conducted (**Chapter 5**) on the potential hazard when *Salmonella* sp. is present in wheat bran fed to yellow mealworms.

While Chapters 2 to 5 mainly focus on the rearing phase, **Chapter 6** focusses on the effect of two often applied post-harvest treatments, being starvation and rinsing, on yellow mealworms (*T. molitor*). This work is conducted in the light of research *Objective 4*. Research on this topic was advised in the advisory report by the Belgian SHC and FASFC (2014). The effect of both procedures is assessed at laboratory scale in terms of microbiological quality (through microbial counts) and, in the case of starvation, in

terms of the bacterial community composition (through high-throughput 16S rRNA gene sequencing).

Finally, in **Chapter 7**, the results obtained in Chapters 2 to 6 are synthesised into conclusions. Suggestions are given for further research and for valorisation opportunities of the suggested research projects.

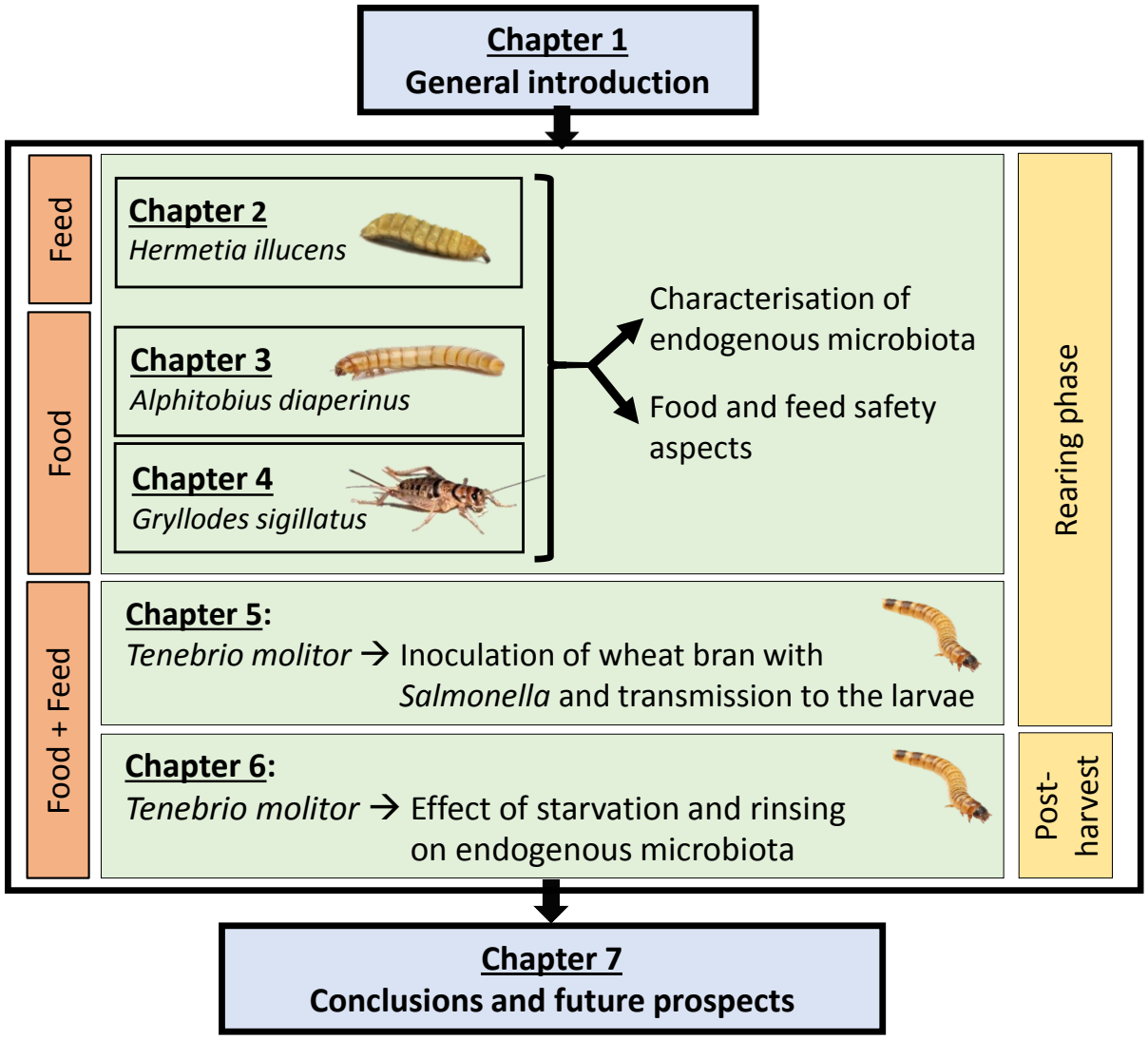


Figure 1.4 Schematic overview of the PhD dissertation.

CHAPTER 2

Assessing the microbiota of black soldier fly larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale

Modified from:

Wynants, E.*, Frooninckx, L., Crauwels, S., Verreth, C., De Smet, J., Sandrock, C., Wohlfahrt, J., Van Schelt, J., Depraetere, S., Lievens, B., Van Miert, S., Claes, J., Van Campenhout, L. (2018). Assessing the microbiota of black soldier fly larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. *Microbial Ecology* (in press).

*The complete content of this paper 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, E.W. contributed to all parts described in this work, from experimental design, sampling of rearing cycles and subsequent analyses, to the writing of the paper. High-throughput 16S rRNA gene sequencing and bio-informatic analysis of sequencing data were performed in collaboration with the Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), KU Leuven (Prof. B. Lievens).

2.1 INTRODUCTION

Every year, an increasing volume of solid waste is generated worldwide. A large fraction of this waste exists of organic material, such as pre- and postconsumer food waste and animal manure (Lalander et al., 2015). In Europe alone, approximately 100 million tonnes of food products remain unused annually (European Commission, 2015). Furthermore, if left untreated, livestock waste products such as manure cause pollution to water bodies through eutrophication, to air through ammonia and greenhouse gas emissions (and thus contributing to global warming), and to soil through nutrient accumulation (Martinez et al., 2009). One method for valorisation of organic waste products consists of their use as a substrate for mass-rearing of edible insects (Diener et al., 2009; van Huis, 2013; Salomone et al., 2017). Some insect species, such as the black soldier fly (*Hermetia illucens* (Diptera: Stratiomyidae), further referred to as "BSF"), can be reared on a variety of organic side streams such as food waste (Jeon et al., 2011; Nguyen et al., 2015a; Salomone et al., 2017) livestock manure (Sheppard et al., 1994; Myers et al., 2008; Li et al., 2011), and faecal sludge (Lalander et al., 2013; Banks et al., 2014). The larvae as well as specific compounds isolated from the larvae (e.g. protein, fat and chitin) show a large potential to be used in aquaculture (Henry et al., 2015; Stadtlander et al., 2017); livestock feed (Maurer et al., 2015; Nguyen et al., 2015a), human food (Wang & Shelomi, 2017) or other applications such as biofuel (Li et al., 2011; Zheng et al., 2012a) and bioactive coatings (Elieh-Ali-Komi & Hamblin, 2016). The residue after harvest, existing of unconsumed substrate, faeces and exuviae, exhibits similar characteristics to immature compost (Xiao et al., 2018).

So far, little attention has been paid to microbial dynamics associated with the rearing of BSF larvae on waste streams. Research has suggested that the gut microbial community in insects may be greatly influenced by the substrate (Dillon & Dillon, 2004; Engel & Moran, 2013; EFSA Scientific Committee, 2015). For BSF, such an effect was already demonstrated for the bacterial microbiota of larvae reared on either food waste, cooked rice or calf forage (Jeon et al., 2011), for the bacterial microbiota of larvae reared on a standard Diptera diet, fruit and vegetables, and fish feed (Bruno et al., 2019) and for the mycobiota of BSF larvae reared on chicken feed and/or vegetable waste (Boccazzi et al., 2017). As the microbiological safety of the larvae is of great importance for their use as feed ingredient (Wang & Shelomi, 2017), the selection of the substrate can be an important factor in assuring food/feed safety (EFSA Scientific Committee, 2015). Indeed, food pathogens that may be present in the substrate may

be transferred to the larval intestinal tract and subsequently cause illness in the traditional farm animals given a BSF-based feed or in people consuming the derived animal products (Erickson et al., 2004; Čičková et al., 2015; Wang & Shelomi, 2017). On the other hand, multiple studies show that BSF larvae possess antimicrobial capacities and are able to reduce pathogenic bacteria such as *Salmonella* sp. and *E. coli* in their substrate (Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2013; Čičková et al., 2015; Lalander et al., 2015; Nguyen et al., 2015a). In addition to the importance of the BSF microbiota for food and/or feed safety, microorganisms present in the rearing environment may have a potential towards optimising growth performance of the insect and insect-derived antimicrobial strategies (De Smet et al., 2018; Xiao et al., 2018). However, the variability in the microbiota of BSF larvae reared in different facilities, each with their own rearing methods and on different organic waste streams, is still unexplored. Therefore, this study aimed to gain insight into the variability in microbiological quality, safety, and bacterial community composition of BSF larvae reared at different facilities and in relation to the rearing substrate used. To this end, samples were taken during the rearing process of BSF larvae at laboratory scale as well as in three external facilities in Belgium, the Netherlands and Switzerland. As a consequence of considering different locations, larvae were cultivated on different organic waste streams, using slightly different practices and in slightly different environmental conditions. Samples of the larvae, but also of the substrates (i.e. the waste streams) and the residues (i.e. the mixture of non-consumed substrate, faeces or frass and exuviae) were analysed for their intrinsic parameters, microbial numbers, and bacterial community composition (using high-throughput Illumina sequencing of 16S rRNA genes). In addition, larvae and residues from the three external facilities were also assessed for their microbiological safety (through detection of a selection of food pathogens).

2.2 MATERIALS AND METHODS

2.2.1 Laboratory rearing cycles

BSF larvae were reared at laboratory scale on four different waste streams (Table 2.1 and Table S2.1, Supporting Information). For each waste stream, one rearing cycle was conducted that consisted of three batches. Fruit/vegetable waste (LAB 1), consisting of a mixture of strawberries, apples, lettuce, cucumbers, red bell peppers, broccoli, carrots, and chicory, was obtained from the local supermarket (Colruyt group,

Geel, Belgium) and homogenised using a home-type blender (Espressions, Eindhoven, The Netherlands) and stored at -21 °C. Supermarket and restaurant waste (consisting of vegetable and animal products; LAB 2) was obtained from a local waste management company (Renewi, Mol, Belgium), where it had been collected, unpacked and mixed into a slurry. Upon arrival at the laboratory, the slurry was stored at -21 °C. Poultry blood (LAB 3) was obtained from a local poultry slaughtering facility (Pluvera-Klaasen & Co, Ravels, Belgium) and stored at -21 °C. Finally, poultry manure (including shavings; LAB 4) was obtained from a local broiler farm (Proefbedrijf pluimveehouderij, Geel, Belgium) and stored at 3 °C. Substrates were obtained maximally one week prior to the start of the rearing cycle, except for the fruit/vegetable waste which was obtained 6 months before and kept frozen. No further treatments except from cooled/frozen storage were applied to the substrates prior to administering them to the larvae. Frozen substrates were thawed for one to three days at 3 °C, and all substrates were placed at room temperature for four hours before administering to the larvae. At the start of each rearing cycle (day 0), 0.2 g of BSF eggs were placed in an open plastic cup (200 ml) with 15 g of apple slices and 15 g of commercial chicken starter feed (startmeel voor kuikens 259, AVEVE, Leuven, Belgium) and incubated at 30 °C (= nursery phase). On day 3, the same quantity of apple slices and chick feed was added. On day 4, the larvae, including the residue, were transferred into a larger plastic beaker without lid (1 l) containing 150 g of chick feed moisturised with 150 ml of tap water and placed in a large insect-rearing room (i.e. phase I, from day 4 to 6). On day 7, the larvae were placed in larger plastic container (20 l, 36 x 26 x 28 cm), and the specific side stream was added (phase II). Chick feed and/or water were also added depending on the cycle (Table S2.2, Supporting Information), in order to maintain a suitable moisture content of approximately 70%. That moisture content was chosen to allow proper larval growth and at the same time efficient drying of the residue towards the end of the cycle (Cheng et al., 2017). Larvae were harvested, by manually picking them out of the residue using sterile forceps, at day 14.

2.2.2 External rearing facilities

Three external, large scale rearing facilities specialized in the cultivation of BSF larvae for commercial or research purposes contributed on this study (Table 2.1 and Table S2.1, Supporting Information). These facilities were located in Belgium (Millibeter, now named Circular Organics, EXT-BE), the Netherlands (Bestico B.V.,

Koppert Biological Systems, EXT-NL) and Switzerland (FiBL Research Institute of Organic Agriculture, EXT-CH). Each rearing facility was studied with respect to its own specific rearing infrastructure and methods. Here, all rearing cycles could also be divided into two phases during each of which a different substrate was administered. Similar to the laboratory cycles, one cycle was conducted by each facility, during which samples were taken from three batches of larvae.

Briefly, at EXT-BE, larvae were supplemented during phase I with chick feed that was mixed with the phase II substrate (see further; ratio not determined by the rearer) and with supplementation of methylparaben (0.1%). Methylparaben was included in order to prevent moulding of the substrate, which can have detrimental effects on larval development as experienced by that rearer. The other rearers did not make use of methylparaben. The whole cycle was completed in crates (50 l), and no separation of the larvae from the residue took place after phase I. After a three days phase I period, phase II substrate was added to the crates (solely phase II substrate, without chick feed and methylparaben), which consisted of a mixture of dried distillers grains with solubles (DDGS, 20%), an apple waste stream from apple juice production (60%) and water (20%). Those ingredients were stored at room temperature for two months prior to homogenising them into a mixture, after which they were administered without further treatment to the larvae. The larvae were kept in a temperature-controlled, ventilated room during the complete cycle, and were harvested at day 14 (batches 1 and 2) or at day 21 (batch 3), depending on the larval development.

At EXT-NL, larvae were grown for the first 7 days (= phase I) on a substrate of fine wheat bran (30%) and water (70%) in 50 l crates, after which they were separated from the bran by automated sieving and transferred into new crates of the same volume. In phase II, larvae were grown on a mixture of fermented potato peels (40%), yeast concentrate (40%) and wheat flour (20%). These ingredients were homogenised into a mixture which was stored for maximally 6 weeks before administering to the larvae without further treatment. Both phases took place in a temperature-controlled room (Table S2.1, Supporting Information). Larvae were considered harvest-ready at day 14.

At EXT-CH, larvae were reared on feed for laying hens (34%) supplemented with water (66%) in phase I. During this phase (days 0-7), larvae were kept in 10 l plastic crates, which were placed in a climate chamber (days 0 to 4) or rearing room (days 4 to 7; Table 2.1 and Table S2.1, Supporting Information). After 7 days, larvae were separated from phase I substrate by manual sieving and transferred into larger containers (550 l) containing phase II substrate, and then housed in another room. The

phase II substrate consisted of a homogenised mixture of fruit and vegetable wastes (40%), brewer's spent grains (30%), and off-specification, pre-cooked ravioli/tortellini pasta (30%), which were kept for a maximum of two weeks in a barn at temperatures below 10 °C (approaching outside temperatures during winter in Switzerland). After homogenisation and before administering, the mixture was brought to rearing temperature without further treatment. In this cycle, larvae were harvested at day 19. More details on the rearing practices and feeding regimes of the cycles are shown in tables S2.1 and S2.2.

2.2.3 Sampling and sample pre-treatment

For each rearing cycle, samples were taken from each of the three batches per cycle. From these batches, samples were taken of both larvae and residues (one sample per batch, randomly collected from different places in each rearing crate) at harvest day. In addition, phase I and phase II substrates were sampled in triplicate for each rearing cycle. These samples were taken immediately before administering, after homogenising them with a sterile spoon and as they were added to the rearing crates (i.e. brought to room temperature after storage for LAB cycles and EXT-CH). For the cycles at the large scale facilities (EXT-BE, EXT-NL and EXT-CH), samples were also taken from larvae and residues from each batch at subsequent sampling moments during the rearing phase. After sampling, larvae were washed with running tap water on a sieve (1 mm mesh size) for 1 min in order to remove remaining residue from the larval surface. This procedure was shown in a preliminary experiment to be sufficient to report reliable counts for the interior microbiota of the larvae, excluding microorganisms from their outer surface. In that experiment, three samples of BSF larvae were subjected to the rinsing procedure as described above ("rinsed larvae"), while three other samples were subjected to the rinsing procedure and an additional disinfection protocol ("rinsed + disinfected larvae"). The disinfection protocol existed of five grams of larvae being subjected to three washing steps in 100 ml of 70% ethanol followed by three washing steps in 100 ml sterile distilled water. Each step was performed during 1 min at 200 rpm on a laboratory shaker (Unimax 1010, Heidolph, Germany). This experiment was repeated for two batches. The results showed that average microbial counts between rinsed and rinsed + disinfected larvae per batch maximally differed 0.6 log unit for any count, and thus that counts obtained for larvae that were only rinsed are representative for the interior larval microbiota. Because the

larval gut was not dissected, it cannot be concluded that only the gut microbiota was included in our analysis, as other organs may also harbour microorganisms.

For all cycles under study, larval weights at harvest were determined prior to further analysis and the mean was calculated from three times ten larvae from each batch. All samples were stored at 3 °C for a maximum of 24 h until analyses. After storage, larvae were homogenised prior to analysis according to Stoops et al. (2016). Substrate and residue samples were analysed without rinsing or homogenisation.

2.2.4 Analyses

Intrinsic parameters. Water activity was measured using a water activity meter (LabMaster a_w, Novasina, Lachen Switzerland), until water activity and temperature (25 °C) were stable for 15 and 5 min, respectively. The moisture content was determined by calculating the difference in weight of 5 g of the initial sample before and after oven drying for 17 hours at 105 °C. The pH was measured using a digital pH meter (Portamess 911, Knick, Berlin, Germany with SI analytics electrode, Mainz, Germany). For phase I substrate and residue samples, demineralised water was added prior to pH measurement in a 1:1 or 1:2 ratio (sample:water).

Microbial counts. All samples were subjected to microbiological analyses via plate count methods as described by Dijk et al. (2015). Total viable aerobic counts (TVC, also referred to in the text of this dissertation as “total viable counts” or “total aerobic mesophilic counts”) were determined on Plate Count Agar (PCA, Biokar Diagnostics, Beauvais, France) and incubated at 30 °C for 72 h. Enterobacteriaceae were determined on Violet Red Bile Glucose agar (VRBG, Biokar Diagnostics) after incubation at 37 °C for 24 h. Lactic acid bacteria were determined on De Man, Rogosa and Sharpe agar (MRS, Biokar Diagnostics) and incubated at 30 °C for 72 h. Aerobic bacterial endospores were determined by giving the 10^{-1} dilution a heat shock (10 min at 80 °C), followed by a tenfold serial dilution, plating onto PCA and incubation at 37 °C for 48 h. Fungi were determined on Dichloran Rose Bengal Chloramphenicol agar (DRBC, Biokar Diagnostics) and incubated at 25 °C for six days.

Pathogen detection. Larvae and residue samples taken at harvest from the large scale rearing cycles (EXT-CH, EXT-BE and EXT-NL) were investigated for a selection of food pathogens. For samples of EXT-BE and EXT-NL, the presence of *Salmonella* sp. and

Listeria monocytogenes was investigated using ISO methods ISO 6579 B" and AFNOR BRD 07/4-09/98 B". For samples of EXT-CH, enrichment was performed according to ISO 6579 (*Salmonella* sp.) and ISO 11290-1 (*L. monocytogenes*), respectively, and detection was for both pathogens performed using real-time PCR. Coagulase-positive staphylococci were determined according to AFNOR 3M-01/9-04/03 B (EXT-BE/NL) or ISO 6888-2 (EXT-CH). Presumptive *Bacillus cereus* colonies were enumerated according to ISO 7932 (EXT-BE/NL/CH).

16S rRNA gene amplicon sequencing. For each rearing cycle, the bacterial community composition of the phase I and II substrates (two biological replicates before administration), as well as of the residues and larvae at harvest (three replicates, one of each of the three batches) was determined using Illumina MiSeq sequencing of partial 16S rRNA gene amplicons (V4 region, 250 bp; Figure 2.1). To this end, each sample was prepared as described above. Subsequently, DNA extraction, using the DNeasy Soil Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany), was performed in duplicate on each biological replicate, thus resulting in a total of four DNA extracts for the substrates (n = 2 x 2), and six for the larvae and residues (n = 3 x 2). PCR amplification (primer design shown in Table S2.3, Supporting Information) was performed on the V4 region of the 16S rRNA gene using different primer barcodes (Kozich et al., 2013; dual indexing strategy) of the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'; Caporaso et al., 2011). Briefly, PCR reactions were performed in duplicate in a 20 µl reaction volume, containing 150 µM of each dNTP, 0.5 µM of each primer, one unit of Titanium Taq DNA polymerase (Clontech, Saint-Germain-en-Laye, France), 1x Titanium Taq PCR buffer and 1 µl 1/10 diluted DNA. The PCR amplification protocol consisted of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 59°C for 45 s and primer extension at 72 °C for 45 s, and a final extension of 10 min at 72 °C. Next, amplicons were purified by Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified using the Qubit fluorometer (HS reaction kit, Invitrogen, Carlsbad, CA, USA) and combined into a library in equimolar concentrations. Finally, the library was diluted to 2 nM concentration and sequenced at the Center of Medical Genetics Antwerp (University of Antwerp, Belgium) using an Illumina MiSeq sequencer (V2 500 cycle kit, Illumina, San Diego, CA, USA).

Resulting sequences were received in the format of a de-multiplexed FASTQ file. Paired-end reads were merged using USEARCH (v.9.2.64) (Edgar, 2013) to form consensus sequences originating from each sample. A maximum number of 5 mismatches was allowed in the overlap region. The number of mismatches represents the number of differences in basecall between forward and reverse reads. For each mismatch, the most probable base is calculated, and in case no sufficient probability can be attained, the read will eventually still be discarded. Next, sequences were truncated at the 250th base and shorter reads or reads with a total expected error threshold above 0.10 for all bases after truncation were discarded. Subsequently, reads were assigned taxonomic identities using the "classify.seqs" command in Mothur (v.1.39.3; Schloss et al., 2009) against the Silva taxonomy database (release v.1.2.3; Gurevich et al., 2013). DNA-sequences originating from chloroplasts or mitochondria were eliminated with Mothur's "remove lineage" command. Due to uneven sequencing depth, the number of sequences was rarefied to 7000 sequences per DNA extract. Error correction (denoising) was performed by means of the UNOISE algorithm (Edgar, 2016a) command implemented in USEARCH as follows: (i) reads with sequencing errors were identified and corrected, (ii) chimeras were removed, and (iii) PhiX were removed. Remaining sequences with a minimum abundance of two sequences were grouped into species-level operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off using the UPARSE greedy algorithm implemented in USEARCH (Edgar, 2013). This step resulted in the removal of global singletons (i.e. OTUs representing only a single sequence in the entire dataset), minimising the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud et al., 2014). The taxonomic origin of each OTU was determined with the SINTAX algorithm implemented in USEARCH (Edgar, 2016b) based on the Silva Living Tree Project v123 (LTP v123) database. Taxonomic assignments were considered reliable when bootstrap confidence values exceeded 0.80 (Table S2.4, Supporting Information). Sequences were deposited in the Sequence Read Archive (SAMN09425406 to SAMN09425515) under BioProject accession PRNJA476046. Unfortunately, all replicates from phase I substrate yielded too few sequence reads (most likely due to the lower microbial load) and were discarded from the analysis. For the same reason, two DNA extracts from larval batches of EXT-NL were discarded (each belonging to a different biological replicate), resulting in 4 instead of 6 extracts. However, a separate data analysis, rarified to 250 sequence reads and including only phase I substrates, was

performed in order to gain insight into the most abundant community members of the phase I substrates (Figure S2.1, Supporting Information).

Statistical analyses. Means for intrinsic parameters and plate counts between different rearing cycles for each sample type (Tables 2.2 and 2.3), as well as means of larvae and of residues between sampling moments within one cycle for the external facilities (Tables 2.4 and 2.5) were compared using One-Way ANOVA in SPSS (v.23) followed by Tukey's (in case of equal variances) or Games Howell's (in case of unequal variances) post hoc test. The same statistical approach was used for the diversity indices of samples subjected to metagenetic analyses (Table 2.6), which were calculated using Phyloseq (1.19-0): Chao1 index (representing the estimated species richness in the samples), equitability index (indicating the evenness in species abundances), and Shannon-Wiener index (a combined measure for species richness and relative abundances) (Shannon; 1948; Pielou, 1966; Chao, 1984). Furthermore, pearson correlation analyses were performed to detect pair-wise correlations of average intrinsic parameters and average counts between phase I and II substrates, residues and larvae of all cycles (Figures S2.2 and S2.3). In all statistical analyses, a significance level of $\alpha = 0.05$ was considered. In addition, the R-package vegan (v.2.5-2) (R Development Core Team, 2013) was used to create a non-metric multidimensional scaling (NMDS) for larvae, phase II substrates and residues using the 200 most abundant OTUs found in the entire dataset (Figure 2.2). The R-package was also used to conduct a cluster analysis (using the single linkage agglomeration method) on larvae from different rearing cycles based on all OTUs present in the larval samples. Resulting clusters were projected on top of the heat map in Figure 2.3, which was constructed based on percentage abundances of each OTU in the larval samples (limited to OTUs present in at least 1% abundance in any larval sample). Both analyses were based on the Bray-Curtis similarity matrix.

Table 2.1 Overview of the rearing cycles under study. For each rearing cycle, three replicate batches were studied. A more detailed description of the rearing procedures is shown in Table S2.1.

Rearing cycle		Substrate composition (w/w%)	Average temperature (°C)	Average humidity (%)	Age at harvest (days)	Larval weight at harvest (g)
Laboratory	LAB 1	Day 0 – 4 (nursery): chick feed (50%) + apple (50%) Day 4 – 7 (phase I): chick feed (50%) + water (50%) Day 7 – 14 (phase II): fruit/vegetable waste*	26.5	20.7	14	0.233 ± 0.009
	LAB 2	Day 0 – 4 (nursery): chick feed (50%) + apple (50%) Day 4 – 7 (phase I): chick feed (50%) + water (50%) Day 7 – 14 (phase II): supermarket/restaurant waste*	26.5	20.7	14	0.231 ± 0.008
	LAB 3	Day 0 – 4 (nursery): chick feed (50%) + apple (50%) Day 4 – 7 (phase I): chick feed (50%) + water (50%) Day 7 – 14 (phase II): poultry blood*	25.2	24.2	14	0.136 ± 0.037
	LAB 4	Day 0 – 4 (nursery): chick feed (50%) + apple (50%) Day 4 – 7 (phase I): chick feed (50%) + water (50%) Day 7 – 14 (phase II): poultry manure and shavings†	24.9	38.8	14	0.158 ± 0.019
Large scale facilities	Belgium (EXT-BE)	Day 0 – 3 (phase I): phase II substrate + chick feed (ratio N.D.) + methylparaben (0.1%) Day 3 – 21 (phase II): DDGS (20%) + apple waste stream (60%) + water (20%)	29.1	71.0	14 (batches 1&2) 21 (batch 3)	0.096 ± 0.026
	The Netherlands (EXT-NL)	Day 0 – 7 (phase I): fine wheat bran (30%) + water (70%) Day 7– 14 (phase II): fermented potato peel (40%) + wheat flour (20%) + yeast concentrate (40%)	29 (N.D.)° 27.7°	60 (N.D.)° 29.2°	14	0.076 ± 0.005
	Switzerland (EXT-CH)	Day 0 – 7 (phase I): laying hen feed (34%) + water (66%) Day 7 – 19 (phase II): fruit/vegetable waste (40%) + brewer's spent grains (30%) + off-specification pre-cooked pasta (30%)	28.8 (days 0-4)° 27.5 (days 4-6)° 27.3°	55.1 (days 0-4)° 32.2 (days 4-6)° 42.6°	19	0.219 ± 0.030

*additional chick feed was added during phase II (see Table S2.2).
†additional water was added during phase II (see Table S2.2).
°average temperature and humidity differ during the rearing cycle due to transfer of larvae from one rearing chamber to another.
N.D. not determined by measurement (but indicated by the rearer in case of temperature and humidity).

2.3 RESULTS

2.3.1 Intrinsic parameters

In the first place, the intrinsic parameters will be compared between all cycles and locations. These are all values obtained from the substrates before administering, and larvae and residues obtained at harvest (Table 2.2). For the cycles performed at large scale, also values during rearing were obtained from larvae and residues (Table 2.4) and these will be described in the second place. Results of larvae and residues at harvest are displayed in both tables, in order to be included in different comparisons (between cycles in Table 2.2 and between times within a cycle in Table 2.4). For all cycles, except for EXT-BE, the final sampling moment in Table 2.4 represents the larvae and residues at harvest as shown in Table 2.2. For EXT-BE - for which two batches were harvested at day 14 and one at day 21 - results were represented at harvest in Table 2.2 and dependent on the sampling day in Table 2.4.

Table 2.2 demonstrates that both pH and moisture content of the phase I substrates, which were all grain-based, showed significant differences between cycles (and thus locations). The moisture content was the lowest for the laboratory cycles (ranging from 53.7% to 55.3%) and the highest for EXT-BE (94.5%). The pH value of phase I substrates was the lowest in the latter cycle (4.65), while the pH of other cycles ranged from 5.69 to 6.58. Water activity, on the other hand, did not differ significantly between cycles and was always higher than 0.95. For the phase II substrates, which were all organic waste streams, significant differences between cycles (and locations) were found for each intrinsic parameter. The pH ranged from 3.51 for the fruit/vegetable waste in LAB 1 to 7.29 for the poultry blood in LAB 3. Although the moisture content differed largely, ranging from 65.8% to 92.1%, the water activity remained higher than 0.95 for all phase II substrates. Also in the residue samples taken at harvest, the water activity was higher than 0.95 for all cycles/locations except for EXT-NL (a_w = 0.83; Table 2.2), indicating a larger drying efficiency of the residue towards the end of that rearing cycle. The moisture content was also the lowest for that residue (23.2%), while the residues from other cycles showed a large variation and ranged from 46.3% to 73.8% on average. The average pH values of the residues at harvest, ranging between 7.57 and 9.09, were higher than those of the administered substrates. An exception to this was cycle LAB 3, where the residue at harvest showed a lower pH of 5.80 than the near-neutral pH (7.29) of the substrate, being poultry blood. However, the pH of this residue showed a high standard deviation, indicating large differences

between batches. For larval samples, the water activity and the moisture content at harvest respectively ranged from 0.96 to 0.97 and from 67.8% to 76.8% between cycles. Remarkably, the pH of larvae at harvest from cycle EXT-CH (6.32) was significantly lower ($p < 0.001$) compared to all other cycles (averages ranging from 7.13 to 7.26). No correlations were detected between the intrinsic parameters of either phase I and II substrate and the residue or the larvae at harvest (Figure S2.2, Supporting Information). One exception was the pH of the phase II substrate, which was negatively correlated to the pH of the residue ($p = 0.026$), although this result should be interpreted with caution given the high standard deviation for the residue pH of LAB 3.

Many intrinsic parameters showed prominent changes depending on the sampling moment, as is shown for the external rearing facilities (Table 2.4). This was expected for cycles which included a separation step after phase I (EXT-CH and EXT-NL), but it was also observed (although non-significant) for that cycle during which the residue contained – aside from faeces and exuviae - both the leftover of phase I substrate and also phase II substrate (EXT-BE). Also for the laboratory cycles, the pH of the residue at harvest (day 14) was on average 1.17 to 3.58 higher (depending on the cycle) when compared to the pH of the residue measured at day 9 (data not shown). Noteworthy, the pH of the larvae at EXT-CH, which showed a lower pH at harvest when compared to the other cycles with a value of 6.32, was 7.18 at the first sampling day (day 7, after feeding with the phase I substrate), thus showing a decrease in larval pH during rearing.

Table 2.2 Intrinsic parameters of the phase I and phase II substrates, residues at harvest and larvae at harvest. Results are presented as the average of three replicates ± standard deviation.

Sample	Rearing cycle	Intrinsic parameters		
		pH	Water activity	Moisture content (%)
Phase I substrate	LAB 1	5.59 ± 0.01^{a}	0.97 ± 0.00^{a}	53.7 ± 0.3^{ab}
	LAB 2	5.62 ± 0.02^{a}	0.97 ± 0.00^{a}	53.7 ± 0.6^{abc}
	LAB 3	5.81 ± 0.04^{bc}	0.97 ± 0.00^{a}	55.3 ± 0.3^{cd}
	LAB 4	5.86 ± 0.04^{b}	0.96 ± 0.01^{a}	54.3 ± 2.4^{bd}
	EXT-BE	4.65 ± 0.09^{d}	0.98 ± 0.00^{a}	94.5 ± 3.1^{acef}
	EXT-NL	6.58 ± 0.01^{e}	0.97 ± 0.00^{a}	73.2 ± 0.3^{f}
	EXT-CH	5.64 ± 0.02^{ac}	0.97 ± 0.00^{a}	68.7 ± 0.3^{e}
Phase II substrate	LAB 1	3.51 ± 0.01^{e}	0.97 ± 0.00^{bc}	92.1 ± 0.6^{a}
	LAB 2	4.40 ± 0.00^{b}	0.97 ± 0.00^{cd}	78.0 ± 0.2^{b}
	LAB 3	7.29 ± 0.01^{a}	0.98 ± 0.00^{b}	90.1 ± 0.2^{a}
	LAB 4	4.52 ± 0.04^{b}	0.95 ± 0.00^{e}	65.8 ± 0.8^{c}
	EXT-BE°	4.12 ± 0.12^{c}	0.99 ± 0.00^{a}	78.7 ± 0.4^{b}
	EXT-NL	3.74 ± 0.03^{d}	0.96 ± 0.00^{d}	69.3 ± 0.8^{c}
	EXT-CH°	4.42 ± 0.12^{b}	0.97 ± 0.00^{bc}	77.3 ± 1.4^{b}
Residue at harvest	LAB 1	8.45 ± 0.35^{ab}	0.98 ± 0.00^{ab}	65.8 ± 4.5^{ac}
	LAB 2	7.57 ± 0.47^{ab}	0.96 ± 0.01^{b}	53.2 ± 3.8^{a}
	LAB 3	5.80 ± 1.63^{ab}	0.96 ± 0.01^{ab}	63.3 ± 12.0^{abcd}
	LAB 4	9.09 ± 0.07^{a}	0.98 ± 0.00^{a}	67.9 ± 0.9^{abc}
	EXT-BE†	7.94 ± 0.43^{ab}	0.97 ± 0.01^{ab}	46.3 ± 5.9^{abd}
	EXT-NL	7.80 ± 0.13^{b}	0.83 ± 0.01^{c}	23.2 ± 0.6^{d}
	EXT-CH	8.06 ± 0.11^{b}	0.97 ± 0.00^{ab}	73.8 ± 3.3^{c}
Larvae at harvest	LAB 1	7.26 ± 0.08^{a}	0.97 ± 0.00^{ab}	69.1 ± 0.8^{ab}
	LAB 2	7.26 ± 0.06^{a}	0.97 ± 0.00 ^{ab}	67.8 ± 0.7^{a}
	LAB 3	7.21 ± 0.16^{a}	0.98 ± 0.00 ^{ab}	77.0 ± 2.1^{d}
	LAB 4	7.24 ± 0.11^{a}	0.97 ± 0.01 ^{ab}	72.4 ± 2.3^{bc}
	EXT-BE†	7.13 ± 0.12^{a}	0.98 ± 0.01^{b}	76.8 ± 1.5^{d}
	EXT-NL	7.22 ± 0.12^{a}	0.97 ± 0.01^{ab}	73.2 ± 0.6^{cd}
	EXT-CH	6.32 ± 0.12^{b}	0.96 ± 0.00^{a}	72.5 ± 0.4^{bc}

°Results are the average from two substrate batches, each represented by three replicate samples (n = 2 x 3)

†Results are the average of batch 1 and 2 at harvest (day 14) and batch 3 at harvest (day 21).

^{a,b,c,d,e,f}Means of one parameter per sample sharing any letter in superscript do not differ significantly ($p \geq 0.05$) between rearing cycles.

Table 2.3 Microbial counts of the phase I and phase II substrates, residues at harvest and larvae at harvest. Results are presented as the average of three replicates ± standard deviation.

Sample	Rearing cycle	Microbial counts (log cfu/g)				
		Total viable count	Enterobacteriaceae	Lactic acid bacteria	Aerobic endospores	Fungi
Phase I substrate	LAB 1	4.9 ± 0.0^{ab}	3.9 ± 0.3^{ab}	2.2 ± 0.0^{a}	3.4 ± 0.1^{a}	3.2 ± 0.2^{ae}
	LAB 2	4.4 ± 0.3^{b}	3.6 ± 0.2^{a}	2.3 ± 0.2^{a}	3.4 ± 0.1^{a}	3.4 ± 0.2^{a}
	LAB 3	5.4 ± 0.3^{ac}	4.4 ± 0.7^{abc}	2.7 ± 0.4^{a}	3.3 ± 0.9^{abc}	3.5 ± 0.2^{a}
	LAB 4	5.3 ± 0.1^{a}	4.6 ± 0.2^{b}	3.8 ± 0.2^{b}	2.9 ± 0.2^{ab}	4.1 ± 0.1^{b}
	EXT-BE	8.9 ± 0.1^{e}	4.3 ± 0.4^{ab}	9.0 ± 0.1^{d}	6.3 ± 0.1^{c}	5.9 ± 0.1^{d}
	EXT-NL	5.9 ± 0.2^{c}	4.4 ± 0.4^{ab}	<1.1 ± 0.2^{c}	1.4 ± 0.5^{ab}	2.8 ± 0.2^{e}
	EXT-CH	2.6 ± 0.1^{d}	<1.0 ± 0.1^{c}	<1.0 ± 0.1^{c}	2.7 ± 0.1^{b}	<2.0 ± 0.1^{c}
Phase II substrate	LAB 1	3.9 ± 0.3^{d}	<1.0 ± 0.0^{c}	<1.0 ± 0.0^{e}	1.8 ± 0.3^{df}	3.3 ± 0.1^{b}
	LAB 2	7.3 ± 0.1^{b}	2.4 ± 0.3^{bc}	7.1 ± 0.1^{b}	5.2 ± 0.0^{b}	5.5 ± 0.1^{a}
	LAB 3	5.4 ± 0.1^{c}	3.5 ± 0.1^{ab}	3.8 ± 0.1^{d}	1.1 ± 0.3^{f}	2.3 ± 0.1^{c}
	LAB 4	8.5 ± 0.1^{a}	1.5 ± 0.4^{cd}	8.8 ± 0.1^{a}	3.6 ± 0.2^{cd}	4.9 ± 0.9^{abcd}
	EXT-BE°	8.6 ± 0.6^{a}	<1.0 ± 0.0^{c}	8.8 ± 0.3^{a}	6.0 ± 0.2^{a}	5.2 ± 1.3^{abd}
	EXT-NL	6.0 ± 0.1^{c}	<1.4 ± 0.6^{bcd}	6.2 ± 0.0^{c}	3.4 ± 0.0^{d}	4.3 ± 0.1^{d}
	EXT-CH°	8.1 ± 0.4^{ab}	4.1 ± 0.7^{a}	7.5 ± 0.5^{b}	4.3 ± 0.1^{c}	5.5 ± 0.6^{a}
Residue at harvest	LAB 1	9.8 ± 0.2^{abc}	9.5 ± 0.2^{a}	8.2 ± 0.1^{b}	6.3 ± 0.5^{abcd}	5.1 ± 0.2^{a}
	LAB 2	9.2 ± 0.1^{bcd}	9.1 ± 0.1^{ab}	8.8 ± 0.6^{ab}	6.9 ± 0.3^{ad}	6.7 ± 1.3^{abc}
	LAB 3	9.8 ± 0.1^{ab}	8.0 ± 0.7^{abc}	9.8 ± 0.1^{a}	6.2 ± 0.1^{a}	7.8 ± 0.1^{c}
	LAB 4	8.9 ± 0.2^{d}	<5.5 ± 1.3^{abc}	<5.0 ± 0.0^{c}	7.0 ± 0.5^{abd}	<4.0 ± 0.0^{ab}
	EXT-BE†	10.2 ± 0.6^{a}	7.2 ± 1.0^{abc}	6.9 ± 1.0^{abc}	7.0 ± 0.1^{d}	6.3 ± 1.3^{abc}
	EXT-NL	8.5 ± 0.4^{d}	5.7 ± 0.4^{c}	5.7 ± 0.4^{c}	5.8 ± 0.8^{abcd}	3.7 ± 0.1^{b}
	EXT-CH	8.9 ± 0.1^{cd}	8.0 ± 0.4^{ab}	6.1 ± 0.4^{c}	4.2 ± 0.1^{c}	3.6 ± 0.5^{ab}
Larvae at harvest	LAB 1	9.8 ± 0.1^{a}	9.7 ± 0.1^{a}	6.4 ± 0.1^{ae}	6.5 ± 0.6^{abc}	6.1 ± 0.7^{ab}
	LAB 2	9.1 ± 0.1^{b}	8.8 ± 0.2^{b}	7.5 ± 0.5^{ab}	6.1 ± 0.4^{ac}	6.2 ± 0.7^{a}
	LAB 3	8.9 ± 0.3^{b}	8.2 ± 0.4^{bc}	8.5 ± 0.4^{bc}	5.7 ± 0.1^{a}	6.8 ± 0.2^{a}
	LAB 4	8.9 ± 0.1^{b}	8.1 ± 0.3^{bcd}	<4.8 ± 1.3^{acd}	7.5 ± 0.1^{c}	4.0 ± 1.2^{bc}
	EXT-BE†	8.0 ± 0.2^{c}	7.5 ± 0.5^{cd}	5.0 ± 1.3^{acd}	5.8 ± 0.3^{a}	4.9 ± 1.0^{abc}
	EXT-NL	8.1 ± 0.1^{c}	7.3 ± 0.2^{d}	5.4 ± 0.5^{de}	4.5 ± 1.2^{abc}	3.5 ± 0.6^{c}
	EXT-CH	8.0 ± 0.1^{c}	7.4 ± 0.3^{cd}	<4.1 ± 0.4^{d}	3.7 ± 0.0^{b}	<3.1 ± 0.4^{c}

°Results are the average from two substrate batches, each represented by three replicate samples (n = 2 x 3)

†Results are the average of batch 1 and 2 at harvest (day 14) and batch 3 at harvest (day 21).

^{a,b,c,d}Means of one parameter per sample sharing any letter in superscript do not differ significantly ($p \geq 0.05$) between rearing cycles.

Table 2.4 Intrinsic parameters of larvae and residues at subsequent sampling times during rearing at large scale. Results are presented as the average of three replicates ± standard deviation.

Rearing cycle	Sample	Sampling day	Intrinsic parameters		
			pH	Water activity	Moisture content (%)
EXT-BE	larvae	7	N.D.	N.D	N.D.
		14	7.06 ± 0.23	0.98 ± 0.01	79.2 ± 3.0
		21*	7.03	0.97	75.4
	residue	7	7.8 ± 0.0[a]	0.98 ± 0.01[a]	54.5 ± 7.7[a]
		14	7.7 ± 0.1[a]	0.94 ± 0.03[a]	38.0 ± 9.2[a]
		21*	8.43	0.97	52.6
EXT-NL	larvae	7	7.22 ± 0.03[a]	0.97 ± 0.00[a]	83.9 ± 0.9[a]
		14	7.22 ± 0.12[a]	0.97 ± 0.01[a]	73.2 ± 0.6[b]
	residue°	7	6.86 ± 0.19[a]	0.97 ± 0.00[a]	51.1 ± 1.7[a]
		14	7.80 ± 0.13[b]	0.83 ± 0.01[b]	23.2 ± 0.6[b]
EXT-CH	larvae	7	7.18 ± 0.18[a]	0.98 ± 0.00[ab]	79.9 ± 0.5[a]
		13	6.34 ± 0.23[b]	0.97 ± 0.01[a]	75.9 ± 0.5[b]
		19	6.32 ± 0.12[b]	0.96 ± 0.00[b]	72.5 ± 0.4[c]
	residue°	7	7.54 ± 0.30[a]	0.85 ± 0.08[a]	21.4 ± 7.6[a]
		13	6.54 ± 0.12[a]	0.97 ± 0.00[a]	78.53 ± 0.9[b]
		19	8.06 ± 0.11[a]	0.97 ± 0.00[a]	73.8 ± 3.3[b]

*Results of one remaining batch. The other two batches were full-grown and harvest-ready at day 14.

N.D. Not determined due to too small sample size

[a,b,c]Means of one parameter of larvae/residue within one rearing cycle sharing any letter in superscript do not differ significantly ($p \geq 0.05$) between sampling days.

°Larvae were separated from the residues at the first sampling day (= after phase I), resulting in the first residue consisting of leftover phase I substrate, and the residue during the remainder of the cycle consisting of leftover phase II substrate.

Table 2.5 Microbial counts of larvae and residues at subsequent sampling times during rearing at large scale. Results are presented as the average of three replicates ± standard deviation.

Rearing cycle	Sample	Sampling day	Microbial counts (log cfu/g)				
			Total viable count	Enterobacteriaceae	Lactic acid bacteria	Aerobic endospores	Fungi
EXT-BE	larvae	7	8.9 ± 0.3[a]	7.7 ± 0.6[a]	7.6 ± 0.2[a]	5.9 ± 0.0[a]	7.1 ± 0.6[a]
		14	8.1 ± 0.1[b]	7.7 ± 0.2[a]	5.2 ± 1.0[b]	5.7 ± 0.1[b]	5.0 ± 0.9[b]
		21*	7.8	7.0	3.5	6.2	3.8
	residue	7	10.7 ± 0.1[a]	9.2 ± 0.3[a]	8.9 ± 0.2[a]	6.9 ± 0.2[a]	7.6 ± 0.5[a]
		14	10.5 ± 0.3[a]	7.6 ± 0.4[b]	7.4 ± 0.3[b]	7.0 ± 0.1[a]	7.0 ± 0.2[a]
		21*	9.7	6.1	5.7	6.9	4.8
EXT-NL	larvae	7	8.6 ± 0.2[a]	7.1 ± 0.2[a]	5.6 ± 0.2[a]	6.0 ± 0.3[a]	5.4 ± 0.2[a]
		14	8.1 ± 0.1[b]	7.3 ± 0.2[a]	5.4 ± 0.5[a]	4.5 ± 1.2[a]	3.5 ± 0.6[b]
	residue°	7	10.3 ± 0.1[a]	9.5 ± 0.0[a]	7.9 ± 0.1[a]	6.5 ± 0.4[a]	6.2 ± 0.2[a]
		14	8.5 ± 0.4[b]	5.7 ± 0.4[b]	5.7 ± 0.4[b]	5.8 ± 0.8[a]	3.7 ± 0.1[b]
EXT-CH	larvae	7	9.1 ± 0.3[a]	8.4 ± 0.6[a]	7.6 ± 0.2[a]	2.4 ± 0.6[a]	6.6 ± 0.8[a]
		13	8.4 ± 0.2[b]	8.0 ± 0.2[a]	6.6 ± 0.2[b]	3.8 ± 0.2[a]	6.4 ± 0.3[a]
		19	8.0 ± 0.1[b]	7.4 ± 0.3[a]	<4.1 ± 0.4[c]	3.7 ± 0.0[a]	<3.1 ± 0.4[b]
	residue°	7	10.4 ± 0.3[a]	>9.6 ± 0.7[a]	9.0 ± 0.3[a]	3.2 ± 0.1[a]	7.1 ± 0.9[a]
		13	8.5 ± 0.6[b]	7.2 ± 0.9[b]	8.0 ± 0.4[b]	3.8 ± 0.0[b]	6.5 ± 0.4[a]
		19	8.9 ± 0.1[b]	8.0 ± 0.4[ab]	6.1 ± 0.4[c]	4.2 ± 0.1[c]	3.6 ± 0.5[b]

*Results of one remaining batch. The other two batches were full-grown and harvest-ready at day 14.

[a,b,c]Means of one parameter of larvae/residue within one rearing cycle sharing any letter in superscript do not differ significantly ($p \geq 0.05$) between sampling days.

°Larvae were separated from the residues at the first sampling day (= after phase I), resulting in the first residue consisting of leftover phase I substrate, and the residue during the remainder of the cycle consisting of leftover phase II substrate.

2.3.2 Microbial counts

As shown in Table 2.3, the microbial counts - both total viable counts as well as the other microbial groups - differed to a large extent between cycles and locations. The phase I substrate of EXT-BE was higher for all microbial counts except for Enterobacteriaceae when compared to the phase I substrate of other cycles (all grain-based). Furthermore, even though methylparaben (0.1%) was administered with the phase I substrate, fungi were abundantly present (5.9 log cfu/g). Large differences were also observed between microbial counts of phase II substrates, which differed more in intrinsic parameters and likely also differed more in nutritional composition from each other than the phase I substrates did. Indeed, the lowest TVC was observed for the fruit/vegetable waste of rearing cycle LAB 1 (3.9 log cfu/g), whereas the highest TVC was observed for phase II substrate of EXT-BE, being a mixture of DDGS and an apple waste stream (8.6 log cfu/g). Similarly, also for larvae and residues a large variability in microbial counts was observed between cycles, resulting in significant differences. Total viable counts of the larvae ranged on average from 8.0 to 9.8 log cfu/g, whereas those of the residue ranged from 8.5 to 10.2 log cfu/g. Other microbial counts showed even a larger variation between cycles for both residues and larvae. Furthermore, no correlations were observed between average microbial counts of the substrates on the one hand, and of the larvae or residue at the other hand (Figure S2.3, Supporting Information). In contrast, a significant correlation was observed between the larvae and residues for the average number of fungi, lactic acid bacteria and endospores ($p = 0.008$, 0.005 and 0.016, respectively).

Also here, it should be noted that microbial counts for residues and larvae are dependent on the timing in a rearing cycle (Table 2.5). For instance, the larval microbial counts changed during the course of the cycles at EXT-CH, EXT-BE and EXT-NL. TVCs and fungal counts significantly decreased over the course of all cycles. The number of Enterobacteriaceae did not significantly change in any cycle, whereas for lactic acid bacteria and endospores either no significant changes or significant decreases were observed.

2.3.3 Pathogen detection

Listeria monocytogenes and coagulase-positive staphylococci were not detected in any cycle (although it should be noted that the detection limit for the latter at EXT-CH was set from 1000 to 10 000 cfu/g for the residues, due to the large background

microflora during analysis). However, *Salmonella* sp. *enterica* serovar Agona was present in the residue, but not in the larvae, of the one batch harvested at day 21 at EXT-BE. Additionally, presumptive *Bacillus cereus* was detected in quantities of 200 cfu/g in the residue of one batch of EXT-NL, as well as in all samples of larvae and residue from EXT-CH. In the latter samples, the bacterium was observed in quantities up to 6000 cfu/g and 5000 cfu/g, respectively.

2.3.4 16S rRNA gene amplicon sequencing

High-throughput 16S rRNA gene sequencing was used to characterise the bacterial community composition of the substrate, residue and larvae samples. A total of 1306 OTUs was recovered from all samples (Table S2.4, Supporting Information). Relative OTU abundances and diversity indices were averaged over all replicate samples of phase II substrate, larvae and residue of each rearing cycle. Average sample coverage ranged from 81.2% to 99.5% (Table 2.6), indicating that the majority of the community members were recovered.

For the phase II substrates, the richness, equitability and Shannon-Wiener diversity (Table 2.6) were the highest in cycles LAB 2 and 3, followed by LAB 1, which corresponded well to the large percentage of OTUs present in abundances of below 5% in these samples as seen in Figure 2.1a. Furthermore, while the most abundant OTUs in rearing cycles LAB 1-3 were varying, the bacterial community of the other substrates (LAB 4, EXT-BE, EXT-NL and EXT-CH) showed more similarity with large abundances of OTUs belonging to the genus *Lactobacillus* (OTUs 6, 7, 9, 18, 26, 29, 41 and 57). Nevertheless and as expected due to the large differences in origin, great variability was observed in the bacterial community composition of different substrates.

Similarly to the phase II substrate (Figure 2.1a), the bacterial diversity of the residues (Figure 2.1b) and of the larvae (Figure 2.1c) differed largely between cycles and locations, as was also shown from the Chao1, Shannon-Wiener and Equitability diversity indices (Table 2.6). In addition, NMDS analysis did not show clear clustering of phase II substrates, larvae and residues within rearing cycles (Figure 2.2a). Cluster analysis based on the larvae from different cycles also showed that, although LAB cycles 1-3 belonged to one cluster, and the external facilities to the other, LAB cycle 4 was more similar in bacterial community composition to the external facilities. The latter indicates that even within one location, vast differences in larval microbiota were

observed (Figure 2.3). Nevertheless, a total of 48 OTUs were in common for all larvae over all cycles, and most of these OTUs belonged to the phyla Proteobacteria (21 OTUs) and Firmicutes (21 OTUs) (Table 2.7 and Table S2.5, Supporting Information). However, none of them were present in abundances of more than 1% in all cycles. Figure 2.3 shows a heat map based on the OTUs that were present with a relative abundance of at least 1% in any larval sample, thus giving a more detailed overview as compared to Figure 2.1 of the most prevalent OTUs present in the larvae. Also among these most abundant OTUs, some were present in larvae from all cycles. For instance, larvae from LAB 1 and LAB 2 showed a high abundance of a *Morganella* sp. (OTU 1), which was present in average abundances of 62.0% and 52.5%, respectively (Figure 2.1c; Figure 2.3). Also in the other cycles, that OTU was observed in abundances ranging from 0.5% to 2.1%. Other omnipresent OTUs in the larvae in this study were a *Providencia* sp. (OTU 23), ranging from less than 0.1% to 13.8% in abundance, and an *Enterococcus* sp. (OTU 11), with an abundance ranging from 0.9% to 9.9%. In addition, multiple OTUs corresponding to *Pseudomonas* sp. (e.g. OTUs 14, 32 and 46) were identified in larvae from all cycles, be it in abundances of below 2%. Furthermore, in all larvae (and residues) from cycles LAB 4, EXT-BE, EXT-NL and EXT-CH, members of Bacillaceae (OTUs 2, 4, 5, 15, 25, 33 and 110) were recovered in total abundances of more than 10%. A list of all 48 OTUs which were omnipresent in larvae from all cycles is given in Table 2.7, and more in detail in Table S2.5 (Supporting Information).

Some OTUs which were abundant in the larvae (Figure 2.1c) were also abundant in the residue (Figure 2.1b) of the same cycle. For instance, *Morganella* sp. (OTU 1) was also present in abundances higher than 10% in the residues of LAB 1 and 2. In addition, similar to the larvae of LAB 3, the residue of that cycle showed a high abundance of a *Lactobacillus* sp. (OTU 7). Residues of cycles LAB 4, EXT-BE, EXT-NL and EXT-CH were, similar to the larvae of those cycles, characterised by a high abundance in members of Bacillaceae. Nevertheless, the overall bacterial communities of residues were still largely different from those of the larvae of the same rearing cycle.

Table 2.6 Diversity indices for samples subjected to metagenetic analysis in this study[1].

Sample	Rearing cycle	Observed richness	Chao1[2]	Coverage (%)[3]	Shannon-Wiener[4]	Equitability[5]
Phase II substrate	LAB 1	127 ± 5b^{c}	127.7 ± 6.0^{ab}	99.1 ± 0.5	3.05 ± 0.03^{d}	0.63 ± 0.01^{ab}
	LAB 2	287 ± 60^{b}	289.9 ± 59.6^{a}	99.0 ± 0.49	3.69 ± 0.17^{a}	0.65 ± 0.01^{a}
	LAB 3	312 ± 73^{ab}	313.4 ± 73.5^{a}	99.5 ± 0.1	3.65 ± 0.62^{abd}	0.63 ± 0.08^{acd}
	LAB 4	83 ± 7^{d}	88.3 ± 11.4^{cd}	94.1 ± 4.8	2.00 ± 0.31^{c}	0.45 ± 0.06^{bdf}
	EXT-BE	73 ± 56^{cde}	81.0 ± 52.5^{bcd}	87.0 ± 15.3	2.27 ± 0.16^{bc}	0.56 ± 0.04^{af}
	EXT-NL	56 ± 8^{e}	60.6 ± 6.1^{c}	81.2 ± 5.0	0.84 ± 0.09^{e}	0.59 ± 0.05^{e}
	EXT-CH	77 ± 2^{d}	80.7 ± 3.5^{d}	95.2 ± 1.5	1.34 ± 0.53^{ce}	0.31 ± 0.12^{bcef}
Residue at harvest	LAB 1	41 ± 16^{bde}	45.6 ± 15.1^{ac}	88.5 ± 12.5	1.86 ± 0.36^{de}	0.51 ± 0.01^{ab}
	LAB 2	58 ± 53^{bc}	61.2 ± 53.4^{abc}	94.1 ± 6.8	1.97 ± 0.21^{e}	0.52 ± 0.0^{ab}
	LAB 3	61 ± 16^{bc}	69.6 ± 18.3^{abc}	88.0 ± 10.6	2.51 ± 0.08^{cd}	0.62 ± 0.05^{bc}
	LAB 4	95 ± 21^{ac}	96.7 ± 22.4^{b}	98.4 ± 1.11	3.08 ± 0.09^{ab}	0.68 ± 0.03^{c}
	EXT-BE	199 ± 100^{cd}	200.6 ± 99.6^{abc}	99.1 ± 0.9	3.63 ± 0.53^{a}	0.69 ± 0.04^{c}
	EXT-NL	36 ± 1^{bd}	42.1 ± 8.3^{a}	88.1 ± 13.9	1.81 ± 0.15^{e}	0.50 ± 0.04^{a}
	EXT-CH	67 ± 11^{ce}	70.6 ± 10.6^{bc}	94.2 ± 7.3	2.60 ± 0.32^{bc}	0.62 ± 0.05^{bc}
Larvae at harvest	LAB 1	157 ± 125^{a}	160.7 ± 122.7^{a}	94.5 ± 9.9	1.95 ± 1.90^{ab}	0.37 ± 0.32^{ab}
	LAB 2	189 ± 38^{a}	189.6 ± 37.6^{a}	99.4 ± 0.8	2.54 ± 0.62^{a}	0.49 ± 0.12^{a}
	LAB 3	153 ± 136^{a}	156.3 ± 134.1^{a}	95.6 ± 5.9	2.95 ± 0.81^{ab}	0.61 ± 0.08^{ab}
	LAB 4	150 ± 132^{a}	154.8 ± 130.3^{a}	95.1 ± 4.0	2.55 ± 0.99^{ab}	0.52 ± 0.12^{ab}
	EXT-BE	232 ± 65^{a}	233.9 ± 63.6^{a}	99.0 ± 1.3	3.77 ± 0.25^{b}	0.70 ± 0.04^{b}
	EXT-NL	63 ± 9^{a}	64.9 ± 8.2^{a}	96.2 ± 2.0	2.42 ± 0.23^{a}	0.59 ± 0.05^{ab}
	EXT-CH	133 ± 50^{a}	134.4 ± 49.8^{a}	98.9 ± 1.2	2.91 ± 0.44^{a}	0.60 ± 0.08^{ab}

[1]Sequences were grouped into Operational Taxonomic Units (OTUs) defined by 97% sequence identity at the 16S rRNA gene (V4 region, 250 bp). Data are the mean values of two analysed DNA-extracts from two (substrates) to three (residues and larvae) replicate samples ± standard deviation.

[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).

[5]Equitability: index for the evenness in OTU abundances (also called Pielou's evenness (Pielou, 1966)), calculated by dividing entropy (Shannon index) by the logarithm of the number of OTUs. A value of 1 indicates perfectly even (equal abundances), small values indicate a highly skewed abundance distribution.

[a,b,c,d,e,f]Means of one parameter per sample sharing any letter in superscript do not differ significantly between rearing cycles ($p > 0.05$).

2.4 DISCUSSION

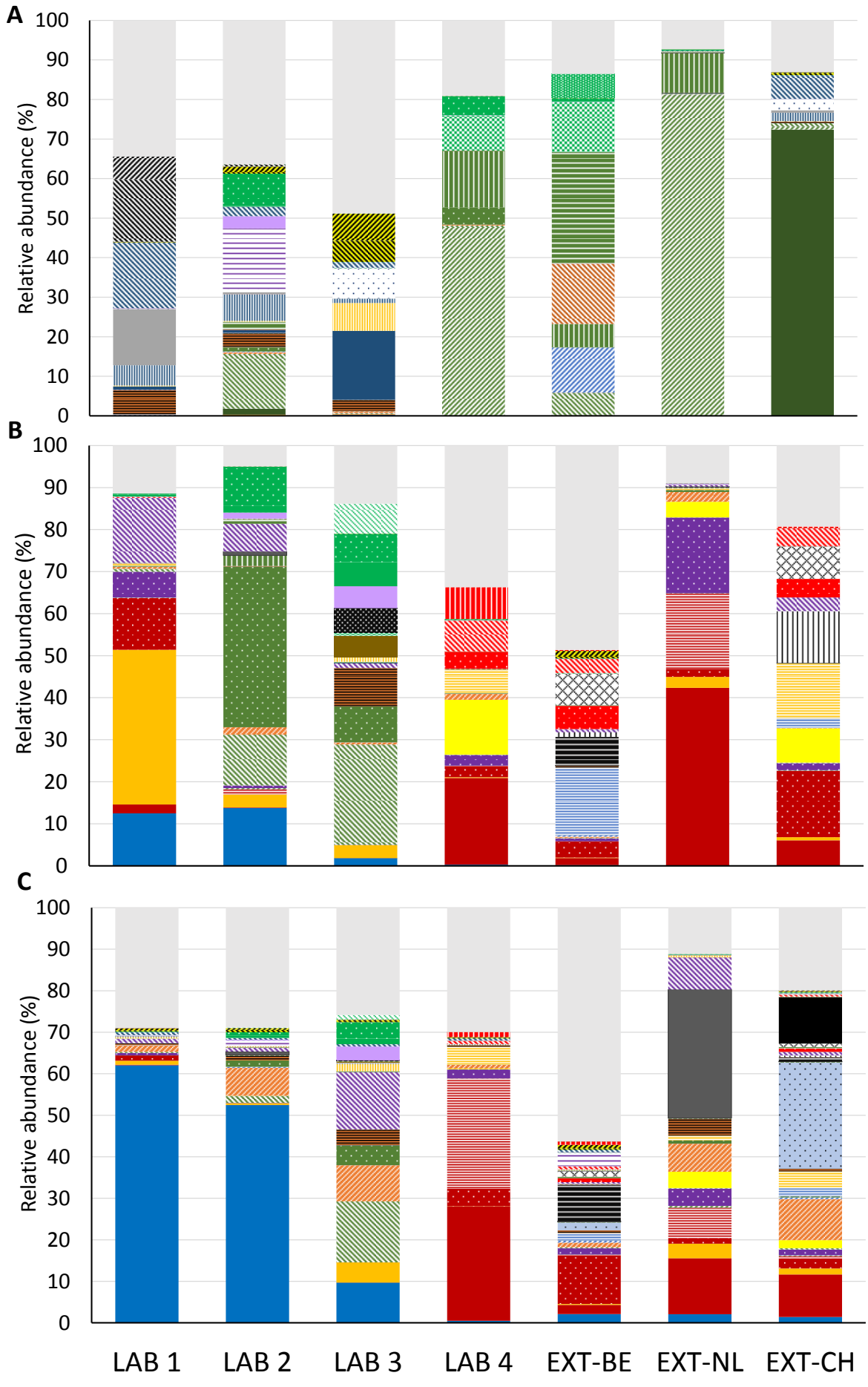
In this study, the microbiota of BSF larvae reared on a variety of waste streams at different locations, each with its own rearing methods and infrastructure, was studied. The aim of this study was (1) to characterise (part of) the variability in waste streams and rearing methods applied at different facilities (2) to assess the variability in microbiota of BSF larvae reared in different facilities and (3) to study the correlation (if any) between the substrates used on the one hand, and the larvae and residues on the other. To this end, samples were taken from substrates, as well as from larvae and residues of seven different rearing cycles. As only one rearing cycle was studied per waste stream, it should be noted that inter-cycle variability for one location is not included in this study. More research is needed in order to assess the consistency in the microbiota when using the same substrate and rearing protocol in different cycles.

2.4.1 Microbial characterisation of waste streams as BSF rearing substrates

A common aspect in all rearing cycles studied is the administration of a phase I substrate containing a grain-based product, such as laying hen/chick feed or wheat bran. The provision of a nutritionally dense substrate of known quality, such as chick feed or laying hen feed, during the first days of the cycle is a common practice in BSF rearing, in order to promote optimal growth during the first life stage. For most cycles, phase I substrates existed of this grain-based product, moisturised with water immediately before administration to the larvae. However, for cycle EXT-BE, the chick feed was already mixed in with the phase II substrate. Because of this, the moisture content was the highest and the pH was the lowest of all phase I substrates (likely due to the apple waste stream ingredient (pH = 3.78 ± 0.16) and DDGS ingredient (pH = 4.88 ± 0.00, data not shown; Table 2.2). Because of the deviating composition of this phase I substrate compared to the others, most microbial counts were higher (Table 2.3). Nevertheless, all phase I substrates showed a water activity of at least 0.96 and their pH ranged between 5.59 and 6.58. These properties make them highly opportune matrices for microbial growth (Adams & Moss, 2008), especially combined with the temperature in the rearing environment (on average between 24.9 and 29.1 °C; Table S2.1, Supporting Information) which was close to the optimal growth temperature for many micro-organisms.

The varying composition of the phase II substrates clearly resulted in large differences in intrinsic parameters, in microbial counts and in composition and

diversity of the bacterial community. Nevertheless, NMDS analysis (Figure 2.2) showed the bacterial community of some substrates to be closer related to each other than to other substrates. For instance, phase II substrates of LAB 1, LAB 2 and EXT-CH, as well as substrates of LAB 4 and EXT-BE, were more similar in bacterial composition to each other than to the other substrates. The substrate of EXT-NL, in contrast, was the least similar to the other substrates, despite the fact that its community was highly abundant in the same *Lactobacillus* sp. (OTU 7) as the substrate of LAB 4. In general, it can be stated that various waste streams used to grow BSF larvae at different locations can highly differ in intrinsic parameters, microbial numbers as well as in bacterial community composition. It should also be noted that the results obtained from the substrates in this study cannot be extrapolated to other substrates which may differ in proportion of the ingredients used, in ingredient types or in the way they were treated, transported, and/or stored.


A
B
C
Relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
LAB 1
LAB 2
LAB 3
LAB 4
EXT-BE
EXT-NL
EXT-CH

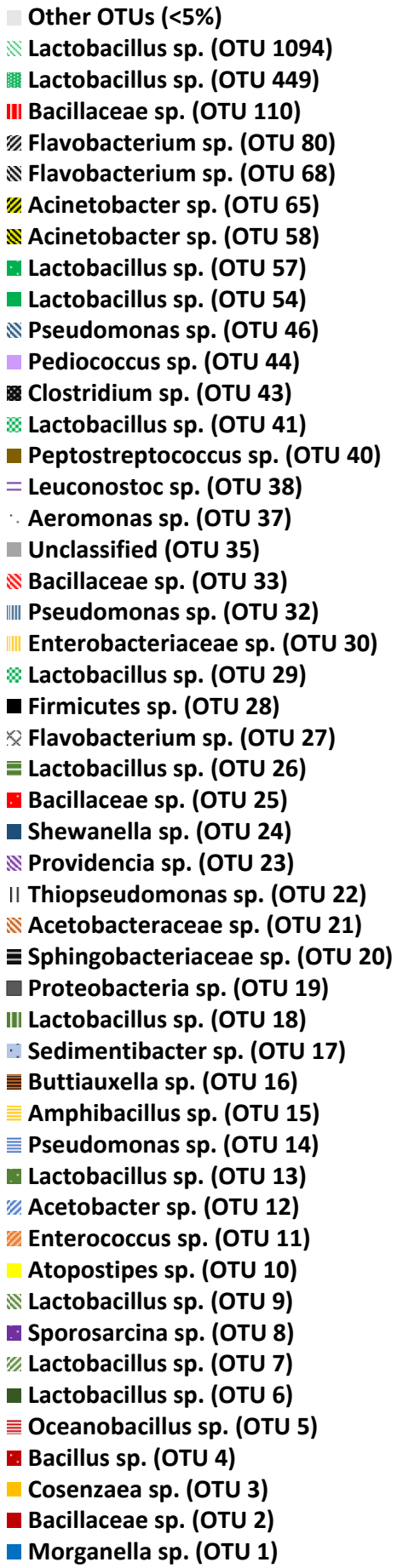


Figure 2.1 Relative abundance (%) of Operational Taxonomic Units (OTUs) present in the samples of **A)** phase II substrates, **B)** residues, and **C)** larvae per rearing cycle. Data are mean values of two extracts per replicate sample (n = 2 x 2 substrates, n = 2 x 3 for residues/larvae). Standard deviations varied between below 0.1% and 40.0%. Only OTUs represented by an average relative abundance of more than 5% of sequences in any sample are shown. OTUs with a mean relative abundance of less than 5% are grouped in "Other OTUs (<5%)".

2.4.2 Microbiota of harvested larvae from different rearing cycles

Microbial numbers (Table 2.3) as well as bacterial communities (Figure 2.1c; Figure 2.3) and diversity indices (Table 2.6) of freshly harvested larvae differed to a large extent between rearing cycles. Given the observed changes in some of the microbial counts during the course of the rearing period (Table 2.5), it can be concluded that besides the selection of the substrate and the rearing methods, the timing of harvest likely influences the microbial numbers of the harvested larvae. Indeed, the timing of harvest determines the age and developmental stage of the harvested larvae, which in turn may affect their intrinsic parameters and microbiota. For instance, larval fat bodies are thought to represent a key tissue for insect humoral immunity and particularly for synthesis of antimicrobial peptides (Park et al., 2015; Zdybicka-Barabas et al., 2017), and it is also suggested that the fat content and accordingly the fat body sizes increase during BSF development (Liu et al., 2017). As a consequence, larvae may express increasing levels of antimicrobial peptides as they mature. In addition, recent evidence exists for dietary effects on both BSF fat body metabolism (Pimentel et al., 2017) and antimicrobial peptide expression profiles (Vogel et al., 2018). Thus, both larval age as well as dietary changes may trigger feedback signals on temporal microbial dynamics.

Besides the possible influence of harvesting age, this study clearly shows that a large variability exists in the microbiological quality of larvae reared at different facilities and on different substrates. The variability seen in this study is generally larger than the variability reported in two studies by Vandeweyer et al. (2017a; 2018), who investigated intrinsic parameters, microbial counts and bacterial community composition of mealworms (*T. molitor*) and crickets (*A. domesticus* and *G. sigillatus*) from different facilities and different production batches. This observation can likely be explained by the fact that substrates and rearing procedures for the latter insect species are generally more comparable between production facilities than for BSF.

Remarkably, a total of 48 OTUs were in common for the larvae from all cycles (although none of them were present in abundances of more than 1% in every cycle; Figure 2.3; Table 2.7; Table S2.5, Supporting Information). Of these OTUs, *Morganella* sp. was also reported in other studies in BSF eggs (Zheng et al., 2013c), in BSF larvae grown on calf forage, food waste and cooked rice (Jeon et al., 2011) and in different gut sections of BSF larvae grown on a standard Diptera diet, a diet containing fruits and vegetables, and a diet based on fish feed (Bruno et al., 2019). All three studies also

mention the presence of a *Providencia* sp., while the latter two also report the presence of *Enterococcus* sp. on all rearing substrates used. Similar to our study, Zheng et al. (2013c), who studied the microbiota in different BSF life stages, reported the presence of *Pseudomonas* sp. in BSF larvae, prepupae and adults as well as the genus *Bacillus* in prepupae only. *Pseudomonas* sp. was also reported in all larval gut samples analysed by Bruno et al. (2019), which also report the genus *Bacillus* sp. in low abundances (< 2%). Except for *Pseudomonas* sp., the aforementioned genera detected in the larvae in this study, were not detected in large abundancies in the phase II substrates (<1%). However, it should be noted that Bacillaceae may have been present in the substrate as endospores, which are more difficult to detect through sequencing due to their resistance to DNA isolation techniques (Filippidou et al., 2015). Possibly, the genera *Morganella, Enterococcus, Pseudomonas* and/or *Providencia,* as well as certain Bacillaceae sp. are part of a group of micro-organisms often recurring in BSF larvae, regardless of substrates or other rearing conditions. In addition, NMDS analysis shows that larvae from different cycles to be positioned more closely together as compared to phase II substrates and residues (Figure 2.2b), suggesting that biotic and abiotic interactions in the larval gut may select for their bacterial community composition to become more alike. In accordance with that, Bruno et al. (2019) found extreme differences in the pH of subsequent gut compartments of BSF larvae. The pH of the middle midgut (pH <3) was much lower as compared to that in the anterior (pH <7) and to that in the posterior midgut (pH >8). Presumably, these pH changes contribute to a selection process inside the gut, explaining the fact that bacterial diversity in that study was highest in the anterior gut and the lowest in the posterior midgut. However, the total bacterial load was highest in the posterior midgut, suggesting that those bacteria surviving the extreme variations in pH are able to reach high numbers in the posterior gut. Whether a true 'core microbiota' is present, according to one of the definitions or approaches given in literature, such as for instance by Astudillo-Garcia et al. (2017), remains to be established. Some genera could also be part of a so-called "house flora" present in rearing facilities, but this cannot be stated with certainty since the microbiota of the production environment in rearing facilities has never been investigated so far, according to our knowledge. Further research may also focus on the exploitation of micro-organisms abundantly present in the BSF gut as a probiotic to enhance biomass production and/or immunity (De Smet et al., 2018). For instance, research already showed that specific *B. subtilis* strains could be isolated from BSF larvae, and when added to the substrate, they were

shown to enhance larval growth, likely due to their aid in substrate digestion (Yu et al., 2011; Xiao et al., 2018). As for *Providencia*, this genus was shown to attract females for oviposition (Zheng et al., 2013b) and is likely transmitted vertically through the haemolymph, although more research on this hypothesis is advised (De Smet et al., 2018). Thus, the microbial community of eggs and hatchlings may already differ between rearing facilities, before they even had contact with the substrate, depending on their parental origin. Such hypothesis was also suggested for mealworm rearing (Osimani et al., 2018a). Although it is difficult to determine the historical origin of each BSF strain studied, each facility has been using a single strain for multiple years and hence multiple generations, before the rearing cycles in this study were conducted. Therefore, it is very likely that BSF reared at different locations harboured a location-specific microbiota. Moreover, specific interactions between the insect's genotype on the one hand, and its microbiota on the other hand, may possibly affect key life history performances such as nutritional physiology and immune defences (Dobson et al., 2015; Early et al., 2017; Näpflin et al., 2018; Vorburger & Perlman, 2018). Furthermore, there is emerging evidence showing that BSF larvae comprise a vast genetic diversity, and possibly even a cryptic species complex (i.e. two or more distinct species classified as a single species (Bickford et al., 2007; Sandrock, personal communication). Concomitantly, variation in overall larval microbial composition may also be fuelled by interactions with the host's genetic background, an aspect that clearly deserves further research. However, even within the same BSF strain that was used for cycles LAB1-4, large variation exists in microbial community between rearing cycles. Indeed, cluster analysis showed that LAB cycle 4 was even more similar to the external rearing facilities than to LAB 1-3 (Figure 2.3), than to the other three lab-scale rearing cycles. The latter indicates that even when those larvae were reared in the same location, using the same rearing methods, the use of a different substrate and possibly other contributing factors (e.g., slightly different environmental conditions, …) will likely have impacted the larval microbiota. Thus, although a given BSF strain could possess a characteristic innate microbiota, it is suggested that the microbial dynamics in the larval gut during rearing are largely determined by other biotic and abiotic factors in the rearing environment. The latter likely encompasses feed composition and quality, other micro-organisms present in the rearing system, and the overall responses of microorganisms to relevant biotic and abiotic changes triggered by the larvae themselves (see also section 2.4.3). Even within one location using the same rearing methods, differences in microbiota of the larvae may exist. It is thus reasonable to assume that even when

the same substrate and rearing techniques are used in a facility, one rearing cycle may even differ from another when conducted at a different point in time.

2.4.3 Relation between rearing substrates, residues and larvae

It is reasonable to assume that both intrinsic parameters as well as the microbial composition of the substrate affect the microbial dynamics during the rearing phase. However, despite the high variability in the microbial load of the substrates, the leftover residues (being the (partially) digested substrates, larval faeces and exuviae), showed an average TVC of at least 8.5 log cfu/g in all rearing cycles. In general, most other residue counts were also higher compared to the substrates, indicating the rearing system (i.e. environmental conditions, interactions with larvae, and the nutrient composition of the substrates) to be highly suitable for microbial growth. However, no correlations were observed between microbial counts or intrinsic parameters between the phase I and II substrates on the one hand, and the residue on the other hand (Figures S2.2 and S2.3, supporting information). In all cycles, except for LAB 3, the residue at harvest showed a higher pH value when compared to the substrates administered. Previous studies also detected increases in pH in the residue (Erickson et al., 2004; Popa & Green; 2012; Lalander et al., 2015; Ma et al., 2018). The rise in pH can be explained in the first place by the production of ammonia during the digestion of proteins by the larvae, but the production of ammonia by the indigenous microflora of the substrate also has been hypothesised (Erickson et al., 2004; Lalander et al., 2015). The ammonia produced might have an antimicrobial effect on certain bacteria such as *Salmonella* sp. (Erickson et al., 2004). Nevertheless, even the residue of LAB 4, with pH 9.09, contained an average TVC of 8.9 log cfu/g and showed the highest Shannon-Wiener diversity. Further research is needed in order to unravel the interplay between substrate pH, larvae and the present microbiota.

Previous studies have indicated that the substrate can affect the bacterial (Jeon et al., 2011; Bruno et al., 2019) and fungal (Boccazzi et al., 2017) composition of BSF larvae. However, in our study, also for the larvae, no correlations were observed between intrinsic parameters and microbial counts of the substrates and those of the larvae, respectively. The bacterial communities of the substrates (Figure 2.1a) are clearly represented by a distinct set of bacteria compared to those of the larvae (Figure 2.1c) and residues (Figure 2.1b). Although most of the OTUs which are abundant in the larvae are also present in the phase II substrate, they are generally present in very low

abundances (below 1%) in the latter. Similarly, very little of the OTUs abundant in the phase II substrates reach a high prevalence in the larvae or residues. As suggested by Gold et al. (2018), microorganisms are selectively inactivated in the gut by digestion, gut pH, and by enzymes and antimicrobial proteins. Those microorganisms that survive the selection process are hypothesised to be potential contributors to larval development.

Yet, multiple OTUs are also present in each cycle of this study that are observed in the larvae but were not at all recovered from the substrate. Some of these OTUs may originate from the phase I substrate. Even though phase I substrates were discarded from data analysis due to a too low number of sequence reads, a separate data-analyses of these substrates (rarefied to 250 sequence reads, Figure S2.1, Supporting Information), revealed the most abundant OTUs (more than 5% of sequences) for the laboratory cycles belonged to genera *Erwinia*, *Pedobacter*, *Parabacteroides* and *Hafnia*, and to Microbacteriaceae. The wheat bran of EXT-NL was most abundant in an *Erwinia* sp., a *Massilia* sp. and a *Parabacteroides* sp. The laying hen feed from EXT-CH was highly colonized by an *Erwinia* sp. and two *Pseudomonas* sp. These genera are generally not found in high abundances in the residues or larvae. It should be noted, however, that, given the low number of sequence reads in these analyses, the results should be interpreted with caution. The phase I substrate of EXT-BE, which consisted of chick feed supplemented with methylparaben (0.1%) mixed into the phase II substrate, was as expected highly similar in bacterial composition to the phase II substrate alone (Figure S2.1). Yet, also here no correlating patterns could be discerned between substrates and larvae/residues. It is therefore assumed that other factors besides substrate type also contribute to the bacterial composition of larvae and residues (Zheng et al., 2013c; De Smet et al., 2018). First, bacteria from both the environment inside as well as the environment outside the rearing facility may enter the rearing containers through the air or via personnel (Schneider, 2009). If those bacteria are suited to the environment inside the crates, they might colonise the system and compete with other microorganisms for nutrients. Additionally, the environmental conditions in the rearing containers can be different from those in substrate storage (often in a cool environment), and they were also shown to change during rearing (e.g. pH and water activity). Although micro-organisms themselves are likely among the causative drivers for these environmental changes (Erickson et al., 2004; Lalander et al., 2015), these changes in turn can cause shifts in the microbial composition of the substrate post-administration. Furthermore, some bacterial species

may survive the conditions in the larval gut and even multiply (Dillon & Dillon, 2004; Engel & Moran, 2013). Subsequently, they may be excreted into the residue in high numbers (as suggested by Wynants et al. (2018a) for lesser mealworms). This hypothesis may also explain the correlations observed between larvae and residues for some of the counts (lactic acid bacteria, endospores and fungi; Figure S2.3, Supporting Information), as well as the occurrence of some OTUs abundant in both residues and larvae. Indeed, although NMDS analysis did not show any clustering of substrates, larvae and/or residues within rearing cycles, the residues and larvae of the same cycle were generally positioned slightly closer to each other as compared to the phase II substrate (Figure 2.2a). Finally, the presence of the larvae themselves likely affects the microbiota of the rearing system, as was suggested in previous studies that showed the number of *Salmonella* sp. and *E. coli* to be reduced in the presence of BSF larvae (Sheppard et al., 1994; Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2013; Čičková et al., 2015; Lalander et al., 2015). BSF larvae (and/or members of their gut microbiota) likely produce antimicrobial components, which may influence the microbial community of the matrix by targeting specific bacteria (Choi et al., 2012; Lalander et al., 2015; De Smet et al., 2018; Spranghers et al., 2018). There can even exist interactions between environmental conditions, such as pH, and the stability of these antimicrobial compounds from BSF larvae in the rearing environment (De Smet et al., 2018).

In our study, the microbiota of the substrates prior to administration differed largely from that of the residues. Although no high-quality data were obtained for the bacterial community of the phase I substrate, this at least indicates that the microbiota of initial substrates undergoes microbiological changes due to environmental conditions in the rearing system, spoilage, and/or presence of the larvae. This is in contrast to the study by Bruno et al. (2019), who reared BSF larvae under laboratory conditions on a variety of substrates (standard Diptera diet, vegetables/fruits and fish feed) and analysed the bacterial community of substrate, residue and larvae. To our knowledge, this is the only other study focussing on the bacterial community composition of BSF larvae in relation to that of the original substrate and residue. Those authors observed that the substrate community remained mostly unaltered after being used fed to the larvae, yielding residues highly similar in bacterial community composition as compared to the initial substrate. It should be noted that that study differed to a large extent from the study described here: larvae were reared at laboratory scale, substrate was added at libitum every two days until the end of the

rearing phase, and the bacterial community composition was based on the RNA extract. Nevertheless, it does prove that more research is needed in order to fully understand the interplay between microbial communities of the substrate, BSF larvae and residue.

Table 2.7 Overview of OTUs that were identified in larvae from all rearing cycles studied. The OTU, phylum and genus are shown. A more detailed taxonomy is given in table S2.5. Values between brackets indicate the bootstrap value, i.e. the certainty that the assigned identification is correct. Bootstrap values equal or higher to 0.80 are considered reliable.

OTU	Phylum	Genus	OTU	Phylum	Genus
1	Proteobacteria	*Morganella*(1.00)	**84**	Bacteroidetes	*Parabacteroides*(0.96)
2	Firmicutes	*Gracilibacillus*(0.38)	**85**	Firmicutes	*Lactococcus*(1.00)
3	Proteobacteria	*Cosenzaea*(1.00)	**89**	Proteobacteria	*Enhydrobacter*(1.00)
4	Firmicutes	*Bacillus*(0.98)	**124**	Proteobacteria	*Afipia*(1.00)
7	Firmicutes	*Lactobacillus*(1.00)	**128**	Firmicutes	*Streptococcus*(1.00)
9	Firmicutes	*Lactobacillus*(1.00)	**146**	Proteobacteria	*Methylobacterium*(1.00)
11	Firmicutes	*Enterococcus*(0.94)	**147**	Proteobacteria	*Pragia*(0.39)
13	Firmicutes	*Lactobacillus*(1.00)	**150**	Proteobacteria	*Pseudomonas*(1.00)
16	Proteobacteria	*Buttiauxella*(0.99)	**152**	Actinobacteria	*Corynebacterium*(1.00)
23	Proteobacteria	*Providencia*(0.97)	**154**	Firmicutes	*Streptococcus*(1.00)
26	Firmicutes	*Lactobacillus*(1.00)	**169**	Proteobacteria	*Rhodanobacter*(1.00)
30	Proteobacteria	*Escherichia*(0.69)	**172**	Firmicutes	*Gemella*(1.00)
31	Firmicutes	*Staphylococcus*(0.99)	**180**	Firmicutes	*Aerococcus*(1.00)
35	Cyanobacteria (?)	*Loriellopsis*(0.32)	**201**	Actinobacteria	*Leucobacter*(0.81)
38	Firmicutes	*Leuconostoc*(1.00)	**247**	Proteobacteria	*Herminiimonas*(0.54)
42	Firmicutes	*Oceanobacillus*(1.00)	**253**	Firmicutes	*Anaerofilum*(0.65)
44	Firmicutes	*Pediococcus*(0.99)	**275**	Proteobacteria	*Haemophilus*(0.67)
46	Proteobacteria	*Pseudomonas*(1.00)	**311**	Firmicutes	*Finegoldia*(1.00)
52	Proteobacteria	*Hafnia*(0.88)	**363**	Proteobacteria	*Sphingomonas*(1.00)
57	Firmicutes	*Lactobacillus*(1.00)	**574**	Bacteroidetes	*Parabacteroides*(0.89)
63	Actinobacteria	*Corynebacterium*(1.00)	**591**	Proteobacteria	*Erwinia*(0.79)
64	Firmicutes	*Weissella*(1.00)	**732**	Proteobacteria	*Massilia*(0.98)
65	Proteobacteria	*Acinetobacter*(1.00)	**1010**	Proteobacteria	*Pseudomonas*(0.94)
70	Proteobacteria (?)	*Rhizobium*(0.77)	**1257**	Firmicutes (?)	*Vagococcus*(0.59)

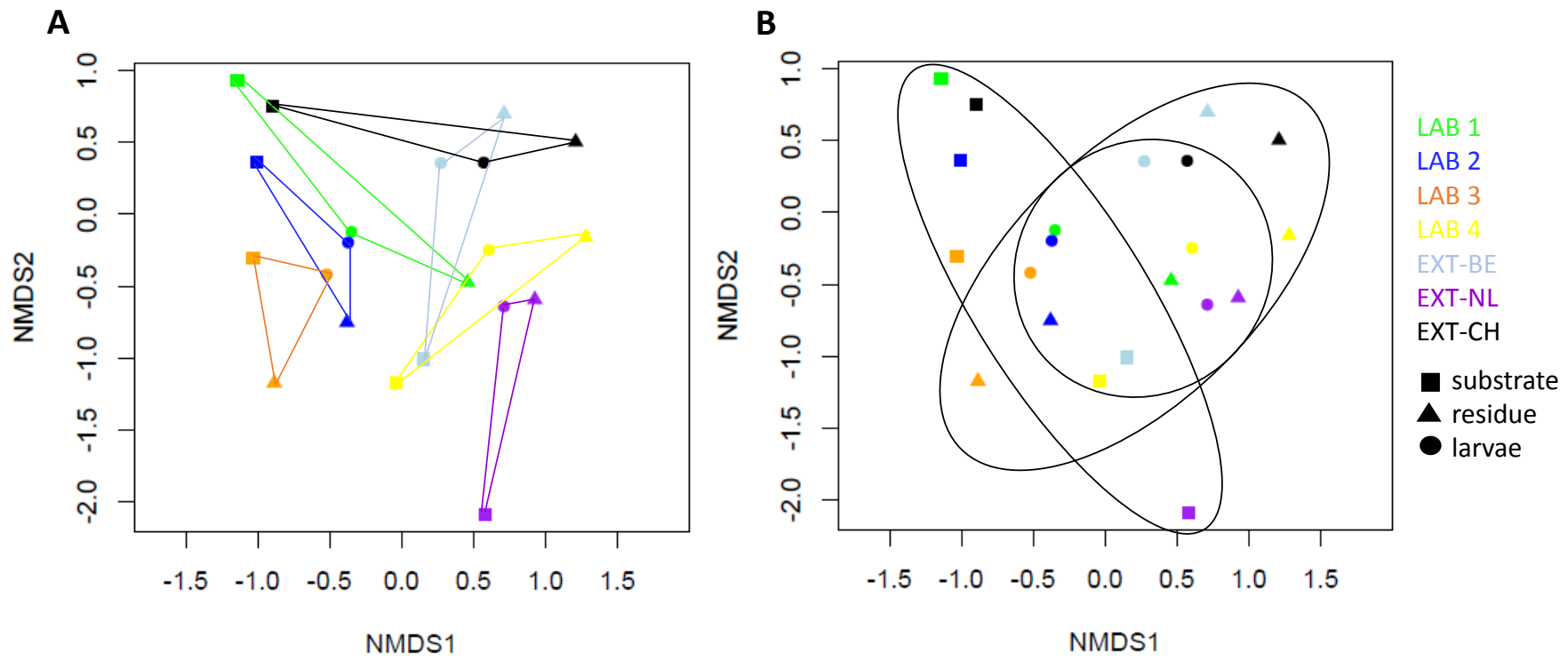


Figure 2.2 Nonmetric multidimensional scaling (NMDS) ordination (2D stress = 0.198), based on the Bray-Curtis similarity matrix, representing the bacterial community composition in the samples subjected to metagenetic analysis. The NMDS analysis was based on the 200 most abundant OTUs in this study. Different rearing cycles are depicted in different colours (LAB 1 = green, LAB 2 = dark blue, LAB 3 = orange, LAB 4 = yellow, EXT-BE = light blue, EXT-NL = purple, EXT-CH = black) and different sample types are depicted in different symbols (squares = phase II substrates, triangles = residues, dots = larvae). The two figures show **A)** visualisation of relation between substrate, residue and larvae within rearing cycles by means of connecting lines and **B)** clustering of all substrates, residues, and larvae, respectively, over the seven rearing cycles.

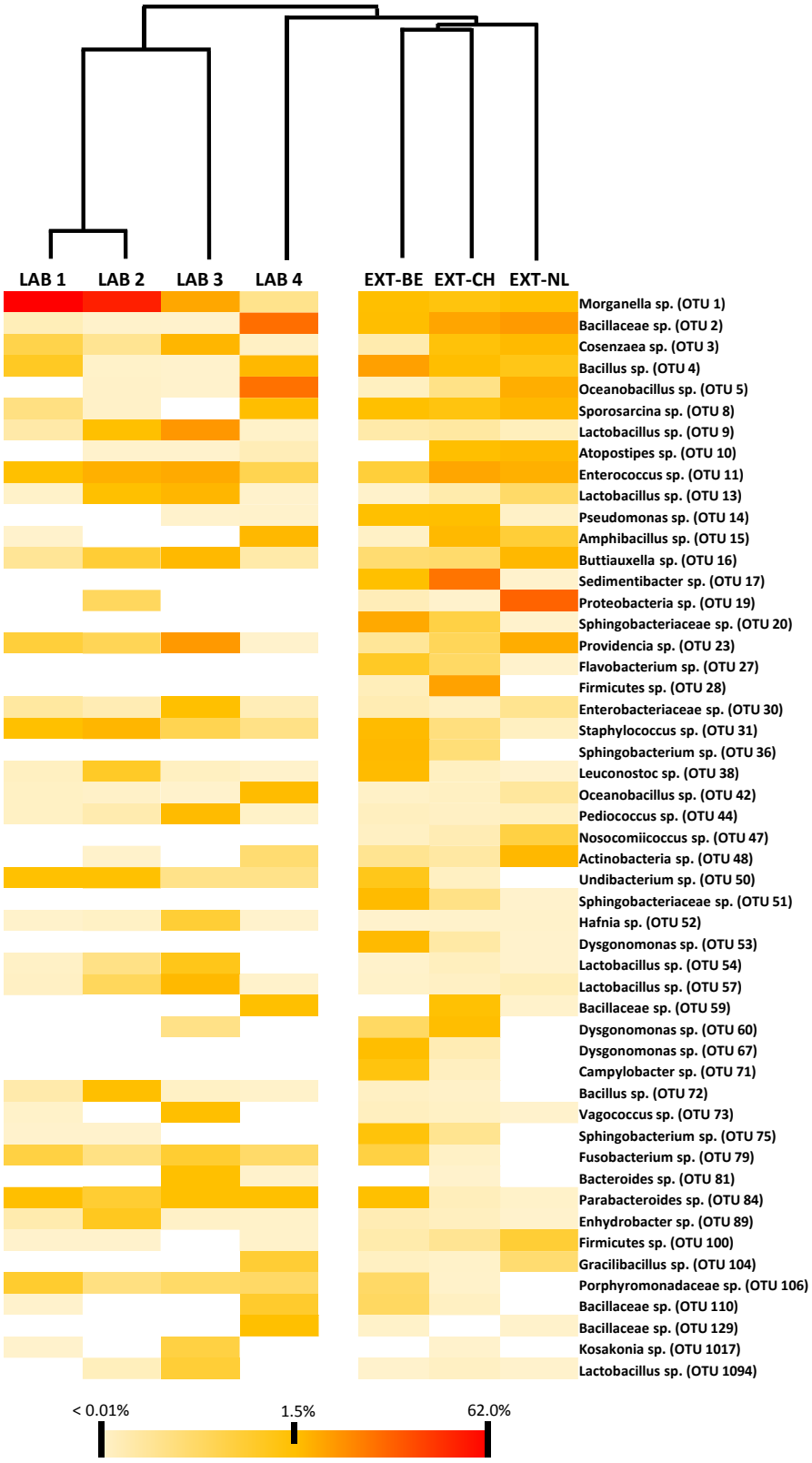


Figure 2.3 Heat map of OTUs in larvae from different rearing locations. Only OTUs with an average relative abundance of at least 1% in any larval sample are shown. The middle value of 1.5% of the colour scheme represents the 75% percentile of relative abundances shown in the figure. OTUs depicted in white were not detected in that location. The dendrogram shows relative similarities in larval bacterial communities from different rearing cycles based on cluster analysis (performed on all OTUs present at any abundance in the larvae).

2.4.4 Microbiological safety aspects

It is unclear whether *Salmonella* sp., which was revealed to be present in the residue - but not in the larvae - of the one batch that was harvested at day 21 at EXT-BE originated from one (or more) of the substrate components, or from the rearing environment. Even though the pathogen was not detected in any of the three larval samples (absent in 25 g), there is no guarantee of its absence in all larvae. As suggested in literature, a heat treatment to eliminate all possible Salmonellae is advised prior to processing of the larvae into feed and other products (Wang & Shelomi, 2017; De Smet et al., 2018). Other decontamination technologies alternative to heat treatment (for instance high hydrostatic pressure or irradiation) may well be suitable too, provided the processing conditions necessary to kill *Salmonella* sp. in the particular matrix of the larvae are well established. Presumptive *Bacillus cereus*, which was detected in one residue sample of EXT-NL as well as in larvae and residue samples of EXT-CH, is widely spread in soil, in water and in plants (Stenfors Arnesen et al., 2008). It could have contaminated the rearing environment via the substrate, as both cycles contained a vegetable component and both substrates from phase I and II contained endospores (Table 2.3). Indeed, *Bacillus cereus* has been identified in edible insects in previous studies (Grabowski & Klein, 2017; Fasolato et al., 2018). It should be noted, however, that the count for *B. cereus* according to ISO-standards does not discriminate between *B. cereus* and other members of the *B. cereus* group (see section 1.8.4), which also includes *B. thuringiensis*. In most food products, the latter species is not frequently encountered. In insects, however, *B. thuringiensis* is a known entomopathogen known to occur rather regularly. Therefore, it cannot be concluded with absolute certainty that the counted colonies during ISO-analyses were *B. cereus* sensu stricto. As shown from Illumina sequencing (Figure 2.1), many Bacillaceae sp. were among the most abundant OTUs in the residues and larvae of rearing cycles LAB 4, EXT-BE, EXT-NL, and EXT-CH, confirming their wide-spread origins in BSF rearing. It is unknown whether the presumptive *B. cereus* cells detected for EXT-NL and EXT-CH were present in the larvae and/or residues as spores or vegetative form. However, given the large quantities recovered at EXT-CH combined with a pH not low enough to prevent spore germination, it can be assumed that the beneficial temperature and water activity and the nutrient rich matrix may have encouraged endospores to germinate and multiply (Wells-Bennik, 2016). On the other hand, the progressive nutrient depletion during the course of the rearing cycle both by larvae and by microorganisms may have triggered

sporulation during rearing (van der Voort, 2013). The specific mechanisms leading to the high numbers of (*B. cereus*) spores in the rearing system should be further established. Nevertheless, the presence of endospores and vegetative *B. cereus* cells may imply risks regarding the microbiological safety of BSF larvae to be used in feed or eventually in food. First, spores are in general very resistant to heat treatments and/or other processing steps (Wells-Bennik et al., 2016). Second, in the current study, presumptive *B. cereus* counts of up to 3.8 log cfu/g were observed. Although the threshold cell density for production of the heat-resistant toxin cereulide is generally considered to be 4 to 5 log cfu/g, some studies also report even lower densities for toxin production (Stenfors Arnesen et al., 2008). As a consequence, the possible production of cereulide cannot completely be excluded. Attention should thus be paid to the presence *B. cereus*, both as endospore or in vegetative state, before and during the processing of BSF larvae into feed/food or other products (Fasolato et al., 2018). Also a *Clostridium* sp. (OTU 43) was identified through Illumina sequencing in large abundancies in the residue of LAB 3. The latter genus also includes sporeforming food pathogens, such as *C. perfringens* and *C. botulinum.* BSF producers are thus advised to monitor *B. cereus* numbers in harvested larvae, and in case of high numbers (higher than 5 log cfu/g), monitor the presence of toxins (e.g. cereulide), as is already described for some products in the list of action limits by the FASFC (see section 1.5.4). Furthermore, rearers should invest in the development and validation of post-harvest treatments ensuring that sporeforming pathogens are reduced and that germination of spores and growth of vegetative cells during storage of the treated larvae is prevented. In addition, when residues are to be used as fertiliser or soil conditioner, the presence of food pathogens such as *Salmonella* sp. or large numbers of *B. cereus* may pose a hazard and the residue should thus be used with care and undergo a decontamination step prior to application.

Noteworthy, high fungal counts were recorded in substrates (up to 5.9 log cfu/g), residues (up to 7.8 log cfu/g) and larvae (up to 6.8 log cfu/g) among BSF rearing cycles. Although no fungal identifications were performed (in contrast to Chapters 3 and 4), these high numbers raise questions on the possible presence of mycotoxins. Further research may identify fungal species present in the rearing crates, not only in the framework of feed safety and the possible presence of mycotoxins, but also related to larval performance.

2.5 CONCLUSIONS

The results from this study unravel a great variability in microbiological quality and bacterial community composition when using different waste streams as a substrate for BSF larvae at laboratory and large scale. Although it was not disproved in this study that the substrates are an important source for bacterial species for the larvae and the residue, they are colonized by a different set of OTUs within each cycle. The results indicate that the microbiological quality and community composition of BSF larvae cannot be explained alone by the microbial composition of the substrate. Furthermore, it is clear that the microbiota of the larvae, both in numbers as well as the bacterial composition, also largely differs between rearing locations. Future research, however, should be dedicated to unravel the inter-batch variability within one location. Differences are likely caused by a multitude of factors, including differences in substrate type and rearing methods, interactions with other microbial community members and with the larvae, and parental origin of the larvae. Nevertheless, a number of OTUs were present in more than one rearing cycle in the current study, be it in varying abundances. Some of these OTUs, such as a *Morganella* sp., an *Enterococcus* sp., *Pseudomonas* spp., a *Providencia* sp., and Bacillaceae, were also reported in BSF in literature. The wide-spread presence of these genera in different BSF larvae from different locations suggest the possible existence of a core microbiota in BSF larvae, although the abundance of these species seems highly variable, depending on the abiotic and biotic factors in the rearing system, and possibly even the BSF strain used.

Two food pathogens, *Salmonella* sp. and presumptive *Bacillus cereus*, were identified in some of the residues and/or larvae. Their presence implies biological risks when BSF larvae are to be used in feed and maybe food. It is advised to apply an adequate heat treatment during processing of BSF larvae to reduce vegetative *Salmonella* sp. and *Bacillus cereus* cells and to elaborate the time-temperature conditions to attain sufficient reduction. However, *Bacillus cereus* spores may eventually survive processing, as well as possible toxins produced during rearing. While one strategy to avoid this is to use only substrates not carrying *Bacillus cereus*, this would hinder the extensive use of food/feed side streams as substrate, in turn jeopardising the economic feasibility and sustainable nature of BSF rearing. Hence more research should focus on how to mitigate these risks to obtain microbiologically safe and toxin-free BSF larvae. The use of classical feed additives or fermentation of

the substrate as strategies to control the microbial community during rearing may contribute to this aim. When exploring the potential of substrate fermentation, an ultimate innovation would be the development of a (mixture of) strain(s) as starter culture that not only secures microbiological safety of the larvae but also promotes their growth.

CHAPTER 3

Microbial dynamics during production of lesser mealworms (*Alphitobius diaperinus*) for human consumption at industrial scale

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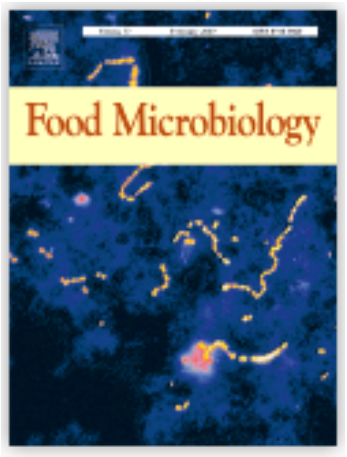

Wynants, E.*, Crauwels, S., Verreth, C., Gianotten, N., Lievens, B., Claes, J., Van Campenhout, L. (2018). Microbial dynamics during production of lesser mealworms (*Alphitobius diaperinus*) for human consumption at industrial scale. *Food microbiology 70*, 181-191.

*The complete content of this paper 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, E.W. contributed to all parts described in this work, from experimental design, to sampling at the rearing facility and subsequent analyses, to the writing of the paper. High-throughput 16S rRNA gene sequencing and bio-informatic analysis of sequencing data were performed in collaboration with the Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), KU Leuven (Prof. B. Lievens).

3.1 INTRODUCTION

Rearing of insects under controlled conditions and with safe diets can yield safe insects of known and constant quality (Hanboonsong et al., 2013; van Huis et al., 2013). The rearing environment, rearing procedures, hygiene measures and insect feed have been suggested to affect the microbiota of insects (Dillon & Dillon, 2004; Schneider, 2009; Klunder et al., 2012; Engel & Moran, 2013; SHC & FASFC, 2014; EFSA Scientific Committee, 2015; Li et al., 2016b; Wynants et al., 2017). Many microorganisms provide beneficial roles to their insect hosts, such as aid in nutrition, pathogen resistance, detoxification of toxic components in the diet, etcetera (Dillon & Dillon, 2004; Engel & Moran, 2013; Li et al., 2016b). Nevertheless, microbial contamination with entomopathogens or food pathogens should be avoided. Research data on the microbiological aspects of insect production are needed, also to support evaluation of insects in the light of the renewed European Novel Food Regulation (EU) N°2015/2283.

The aim of this study was to characterise the microbiological dynamics in the insect, substrate and residue (being the crate content excluding the insect, existing of remaining feed, faeces and exuviae) during a production cycle of lesser mealworms (*Alphitobius diaperinus*). Samples were collected during a production cycle in an industrial rearing company producing lesser mealworms for human consumption. Intrinsic parameters, including pH, water activity and moisture content were determined. Samples were also subjected to culture-dependent microbiological analyses as well as to high-throughput 16S rRNA gene sequencing using the Illumina Miseq platform. Additionally, samples were assessed for the prevalence of four food pathogens (*Salmonella* sp., *Listeria monocytogenes*, *Bacillus cereus,* and coagulase-positive staphylococci). The mycoflora was studied through identification of fungal isolates.

3.2 MATERIALS AND METHODS

3.2.1 Industrial production cycle

The insects investigated in this study were reared at Protifarm BV, a large scale company producing lesser mealworms for human consumption. A schematic overview of the production cycle is given in Figure 3.1a. Briefly, newly hatched larvae were placed into small plastic containers (20 x 10 x 5 cm) that contained a specific formulated dry substrate (standard lesser mealworm feed, based on vegetable raw materials which are suitable and allowed for animal feed, further referred to as “dry

substrate") and a moist substrate (side stream from food industry, also allowed for animal feed). The latter was added to supply moisture to the larvae and is further referred to as "moist substrate". Hatchlings were kept in a small room at room temperature for one week. Then, the content of the containers was transferred into larger crates (60 x 40 x 7 cm) supplemented with the same dry substrate, and placed in a larger rearing hall for four weeks. The rearing hall, which was equipped with a ventilation system, was kept at an average temperature of 30°C with an average relative humidity of 50% and a 8:16 h light:dark cycle. Moist substrate was added daily with bare hands (one handful per crate) during the remaining four weeks of the production cycle. Dry substrate was no longer administered after transfer to the rearing crates. Finally, 35 day old larvae were harvested by emptying all rearing crates over an automated sieving system that separated them from the remaining crate content. The latter existed of the remaining dry substrate, the remaining moist substrate, faeces (frass) and exuviae, and it is further referred to as "residue". Subsequently, larvae were placed in a cooled chamber (16°C) for 24 hours without substrate in order to empty their gut content. Next, a second sieving step was conducted by use of a second automated sieving system in order to remove the residue. The larvae were then heat-treated by submerging them in batches of 8 l in a 50-l kettle containing water of 90°C. Adding the larvae to the water, however, caused the water to cool slightly. Larvae were kept submerged until the water temperature rose to 88°C (which was after approximately five min). The larvae were then rinsed with tap water until their temperature decreased to 15°C, as measured by placing a thermometer (GTH 175/Pt, GHM Greisinger, Germany) in the larval mass. Subsequently, the larvae were treated according to the intended end product (frozen, freeze-dried, etc.). This protocol was chosen by the company based on external advice, microbiological testing (to comply to ready-to-eat food products as described by regulation EC 2073/2005) and effect on taste. After the production cycle, dirty crates were rinsed with water at 60°C containing a hydroxide and sodium hypochlorite disinfecting solution (DM CiD 20%, CID LINES NV, Belgium).

3.2.2 Sampling

Samples were taken aseptically at weekly intervals during the complete production cycle from hatchling to full-grown, harvested larva that is killed by a heat treatment (Figure 3.1b; Table S3.1, Supporting Information). The dry substrate (DS) and moist

substrate (MS; Figure 3.1b) were sampled from storage (three replicate samples from one batch), before they were added to the rearing crates. The dry substrate was sampled at days 0 and 7, being the only days during the production cycle that it was administered to the larvae. The moist substrate, which was administered daily, was sampled at days 0, 7, and 28. The residue (R) and larvae (L; Figure 3.1b) were sampled weekly throughout the cycle.

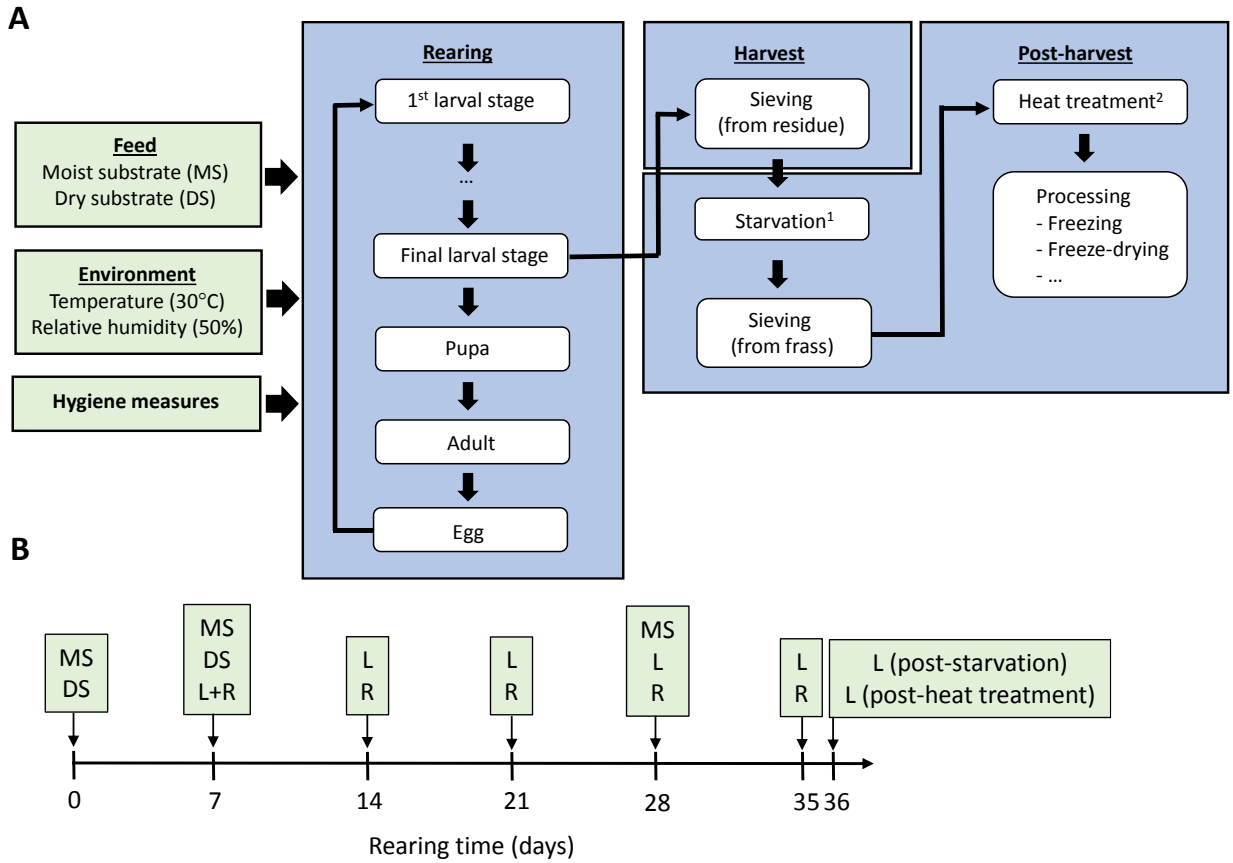

Figure 3.1 A) Schematic representation of the production cycle for lesser mealworms. [1]Starvation was performed for 24 h at 16 °C. [2]The heat treatment existed of submerging larvae in batches of 8 l in a 50 l kettle filled with 90 °C water, and kept submerged until the water reached 88 °C again. **B)** Sampling plan throughout the production cycle, with indication of different samples: MS = moist substrate, DS = dry substrate, L+R = mixture of larvae and residue, L = larvae, R = residue.

Each week, three rearing crates, including larvae and residue, were transported to the laboratory. There, the crate content was homogenised using a sterile spoon and the larvae were aseptically separated from the residue using a manual sieve and/or forceps. However, due to the small larval size at day 7, it was not possible to separate the larvae from their residue. Therefore, a mixture of larvae and residue was sampled and analysed at day 7 (L+R; Figure 3.1b). At the end of the production cycle, three

replicate samples of larvae were collected after harvest (after the first sieving step, day 35), after starvation (after the second sieving step, day 36) and after the heat treatment (day 36). Depending on the intended analyses, between 5 and 100 g of the samples were used for analysis.

3.2.3 Intrinsic parameters

Water activity, moisture content and pH were determined for all samples taken from the dry substrate, the moist substrate and the residue (with exception of the residue at day 14, due to the difficulty to obtain pure residue without the presence of small larvae in the sample). For the larvae, these parameters were determined after harvest, after starvation and after heat treatment (Table S3.1, Supporting Information). To this end, 30 g of larvae were homogenised according to Stoops et al. (2016) prior to analysis. Water activity and moisture content were determined as described in Chapter 2. The pH was measured using a digital pH meter (Portamess 911, Knick, Germany with SI analytics electrode, Germany). For the dry substrate, moist substrate, and residue samples, demineralised water was added to 5 g of the sample before pH measurement in 1:1, 2:1 or 4:1 (demineralised water:sample) ratios, depending on the sample.

3.2.4 Culture-dependent microbiological analysis

Plate counts. All samples were kept at 3°C until analysis (maximum 24 h for larvae and residue samples or 48 h for the substrates). The larvae (30 g) were pulverised prior to analysis as described by Stoops et al. (2016). Plate counts were performed according to the ISO standards for microbiological analyses of food as compiled by Dijk et al. (2015), as described in Chapter 2.

Pathogen detection. Larvae and residue samples collected at days 7, 21, and 35 were analysed for *B. cereus* and coagulase-positive staphylococci. Detection of *B. cereus* was performed according to ISO 7932 (plate count). Prevalence of coagulase-positive staphylococci was studied according to ISO 6888-2 (plate count). Additionally, samples taken at day 35 were analysed for the presence of *Salmonella* sp. and *L. monocytogenes*. Prevalence of *Salmonella* sp. was assessed according to ISO 6579 (absence in 25 g), the occurrence of *L. monocytogenes* according to AFNOR BRD 07/4-09/98 (absence in 25 g; Table S3.1, Supporting Information).

Identification of fungi. To gain insight into the fungal species present in the substrates, the residue, and the larvae, a number of fungal isolates were recovered and identified. A selection of colonies with distinct morphology was picked from the DRBC medium for further identification (five colonies for the dry substrate (day 0 and 7), three for the moist substrate (day 0 and 7), ten for the residue (day 35), and ten for the larvae (day 35, post-harvest)). Colonies were streaked on Potato Dextrose Agar (PDA, Biokar diagnostics) and incubated at 25°C. After 5-7 days of incubation, genomic DNA was extracted from purified strains using the phenol-chloroform extraction method described by Lievens et al. (2003). Genomic DNA was extracted from purified strains using the phenol-chloroform DNA extraction procedure described by Lievens et al. (2003). Identifications were performed by amplifying and sequencing the internal transcribed spacer region (ITS1-5.8S rDNA-ITS2) as described previously (Lievens et al., 2003). Obtained sequences were compared with the nucleotide database in GenBank (Benson et al., 2013; excluding unclassified and environmental entries; Table S3.2, Supporting Information), and isolates were assigned to the highest taxonomic rank possible. Obtained sequences have been deposited in GenBank under the accession numbers MF442392-MF442419.

3.2.5 16S rRNA gene amplicon sequencing

The bacterial community composition of a selection of substrate, residue and larvae samples was characterised by high-throughput 16S rRNA gene sequencing using the MiSeq Illumina platform. Selected samples included the dry substrate and the moist substrate at day 0, the residue at days 14, 28 and 35, and the larvae at days 14, 28, 35 (post-harvest) and 36 (post-starvation; Table S3.1, Supporting Information). Genomic DNA was extracted from two replicate samples (0.2 g) in duplicate using the Powersoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). Next, the two DNA-extracts were pooled and two separate PCR reactions were performed for each pooled DNA extract, thus resulting in two technical replicates per replicate sample. PCR amplification, library preparation, high-throughput Illumina sequencing and data-analyses were performed as described in Chapter 2 (Table S3.3). Due to uneven sequencing depth, the number of sequences was rarefied to 2440 sequences per technical replicate. Replicates that yielded too few sequences were discarded from further analysis. In this way, one replicate sample of starved larvae (day 36), one replicate sample of the residue at day 14 and three technical replicates of the dry

substrate (day 0) were discarded. In addition to identification against the SILVA database (see chapter 2), OTU representative sequences were compared to the nucleotide database in GenBank (excluding uncultured/environmental entries; Table S3.5, Supporting Information). Sequence data have been deposited in the Sequence Read Archive under BioProject accession PRJNA392391 (accession numbers SAMN07298661-SAMN07298689). Representative sequences for each OTU were deposited in GenBank under the accession numbers MF431415-MF431492.

3.2.6 Statistical analyses

Data are presented as averages with their standard deviation. Differences in the intrinsic parameters, microbial counts, and diversity parameters (OTU richness, Chao1 and Shannon-Wiener indices) of the samples at different sampling days were analysed by one-way ANOVA followed by a Games Howell *post hoc* test using SPSS (v. 23, IBM statistics). For the dry substrate, independent t-tests were performed. For all tests, a significance level α of 0.05 was considered. Based on the sequencing data, nonmetric multidimensional scaling (NMDS) was performed and Chao1 and Shannon-Wiener diversity indices were calculated using the R-packages Vegan (v.2.43) and Phyloseq (v.1.19.0) (R Development Core Team, 2013).

3.3 RESULTS

3.3.1 Intrinsic parameters

The results obtained for the intrinsic parameters are presented in Table 3.1. For the moist substrate, pH ranged between 3.14 and 3.29, water activity between 0.97 and 0.98 and moisture content between 72.4% and 75.4%. The moisture content at day 28 was significantly higher than at days 0 and 7 ($p = 0.003$ and $p < 0.001$, respectively). This may be explained by the fact that between day 7 and day 28, a new batch of moist substrate arrived at the insect farm that may have differed from the first one in original moisture content and/or in storage time at the company. For the dry substrate, pH ranged between 5.92 and 6.02, water activity between 0.62 and 0.63 and moisture content between 11.2% and 11.7%. Significant differences were observed between the two sampling days (day 0 and day 7) for pH ($p = 0.011$) and moisture content ($p = 0.011$). The intrinsic parameters of the residue in the crates were close to those for the dry substrate, with slightly lower pH values (between 5.54 and 5.90) but slightly broader ranges for water activity and moisture content (between 0.49 and 0.68, and

between 9.9% and 13.2%, respectively). The latter significantly differed between day 28 and day 35 ($p = 0.004$). The result for moisture content is to be considered as a snapshot, however. It is not known whether or not the moist substrate was already administered before sampling at that specific day. That may of course have influenced the water content of the residue measured and it might explain the statistically significant variation between the sampling days.

For the larvae, intrinsic parameters were only determined at the end of the production cycle. At harvesting, the average pH of the homogenised larvae was 6.17, the water activity 0.98 and the moisture content 70.3%. After starvation, a statistically significant increase in average pH ($p = 0.009$) and water activity ($p = 0.023$) was observed. From a microbial point of view, however, the increase in water activity is expected to have little to no influence on the microbiota. Average values of 6.53 and 0.99, respectively, were observed after starvation. During heat treatment, the pH further increased significantly to 7.30 on average ($p < 0.001$).

Table 3.1 Intrinsic parameters[1] of the samples taken during the production cycle of lesser mealworms.

Sample	Rearing time (days)	pH	Water activity	Moisture content (%)
Moist substrate	0	3.27 ± 0.23[a]	0.97 ± 0.00[a]	72.4 ± 0.4[a]
	7	3.14 ± 0.04[a]	0.97 ± 0.00[a]	72.7 ± 0.2[a]
	28	3.29 ± 0.08[a]	0.98 ± 0.00[a]	75.4± 0.2[b]
Dry substrate	0	5.92 ± 0.02[a]	0.62 ± 0.02[a]	11.7 ± 0.2[a]
	7	6.02 ± 0.04[b]	0.63 ± 0.02[a]	11.2 ± 0.1[b]
Residue	21	5.65 ± 0.06[a]	0.60 ± 0.05[a]	12.2 ± 1.1[a,b]
	28	5.54 ± 0.08[a]	0.68 ± 0.07[a]	13.2 ± 0.5[a]
	35	5.90 ± 0.23[a]	0.49 ± 0.01[a]	9.9 ± 0.6[b]
Larvae	35 (post-harvest)	6.17 ± 0.03[a]	0.98 ± 0.00[a]	70.3 ± 0.2[a]
	36 (post-starvation)	6.53 ± 0.07[b]	0.99 ± 0.00[b]	70.6 ± 0.3[a]
	36 (post-heat treatment)	7.30 ± 0.05[c]	0.99 ± 0.00[a,b]	73.0 ± 0.5[a]

[1]Values are the mean of three replicate samples ± standard deviation.

[abc]Values per parameter for the dry substrate, moist substrate, residue (= remaining substrate, faeces and exuviae) and larvae with the same letter do not differ significantly ($p > 0.05$) between sampling moments.

3.3.2 Culture-dependent microbiological analyses

Plate counts. The dry substrate showed on average a TVC of 5.1 to 5.2 log cfu/g, whereas other counts ranged between 3.1 and 4.3 log cfu/g (Figure 3.2). No significant differences between the two sampling days (day 0 and day 7) were found ($p = 0.447$, 0.585, 0.518, 0.671, 0.770 for total, Enterobacteriaceae, lactic acid bacteria, endospores, and fungal counts, respectively). The TVC of the moist substrate ranged between 6.9 and 8.1 log cfu/g, the number of Enterobacteriaceae between 3.4 and 6.4 log cfu/g, the number of lactic acid bacteria between 7.3 and 8.0 log cfu/g and the number of fungi between 5.8 and 7.7 log cfu/g. In contrast, the counts for bacterial endospores were lower (between 1.6 and 4.1 log cfu/g). For the moist substrate, the highest microbial load during the production cycle was observed for the second batch, at day 28, for all counts. The microbial numbers at that sampling day were significantly higher compared to the other days for the Enterobacteriaceae ($p = 0.006$) and aerobic endospores ($p < 0.001$).

The average TVC of the larvae ranged between 7.8 and 8.3 log cfu/g during rearing, the number of Enterobacteriaceae from 5.9 to 7.6 log cfu/g, lactic acid bacteria from 5.2 to 6.5 log cfu/g, endospores from 5.0 to 5.8 log cfu/g, and fungi from 4.7 to 5.4 log cfu/g (Figure 3.2). None of the counts were significantly higher or lower by harvest time (day 35) when compared to the start of the rearing period (day 14; $p = 0.519$, 0.118, 0.313, 0.154 and 0.346 for the TVC, Enterobacteriaceae, lactic acid bacteria, endospores, and fungi, respectively). The starvation treatment did not significantly alter the microbial numbers of the larvae ($p = 0.175$, 0.360, 0.055, 0.174, and 0.215 for TVC, Enterobacteriaceae, lactic acid bacteria, endospores, and fungi, respectively). After heat treating, however, the number of Enterobacteriaceae, lactic acid bacteria and fungi dropped significantly ($p < 0.001$; $p = 0.004$, and $p < 0.001$, respectively) to close to or below the detection limit (1.0 and 2.0 log cfu/g for bacterial and fungal counts, respectively). This resulted in log reductions ranging from at least 2.5 (fungi) to 6.2 (Enterobacteriaceae). The TVC and aerobic endospores also significantly decreased during heat treatment ($p = 0 < 0.001$ and $p = 0.005$, respectively) but after the treatment, the counts were still 4.1 and 4.0 log cfu/g, respectively. That means that endospores were the main survivors of the heat treatment.

The TVC of the residue ranged between 8.8 and 9.4 log cfu/g throughout the production cycle, which was approximately one log cycle higher compared to the larvae (Figure 3.2). Similar observations were made for the number of aerobic

endospores (between 7.1 and 7.2 log cfu/g) and the number of fungi (between 6.3 and 7.4 log cfu/g). The number of Enterobacteriaceae and lactic acid bacteria in the residue approximated that of the larvae (ranging between 5.6 and 6.9 log cfu/g and between 5.4 and 7.10 log cfu/g, respectively; Figure 3.2). No significant changes in microbial numbers were observed in the residue at day 35 compared to day 14 ($p = 0.435$, 0.996, 0.435, 0.884, and 0.080 for the TVC, Enterobacteriaceae, lactic acid bacteria, endospores, and fungi, respectively). Furthermore, the microbial counts for the mixture of the larvae and the residue at day 7 were generally similar to or higher than those obtained for the residue (Figure 3.2).

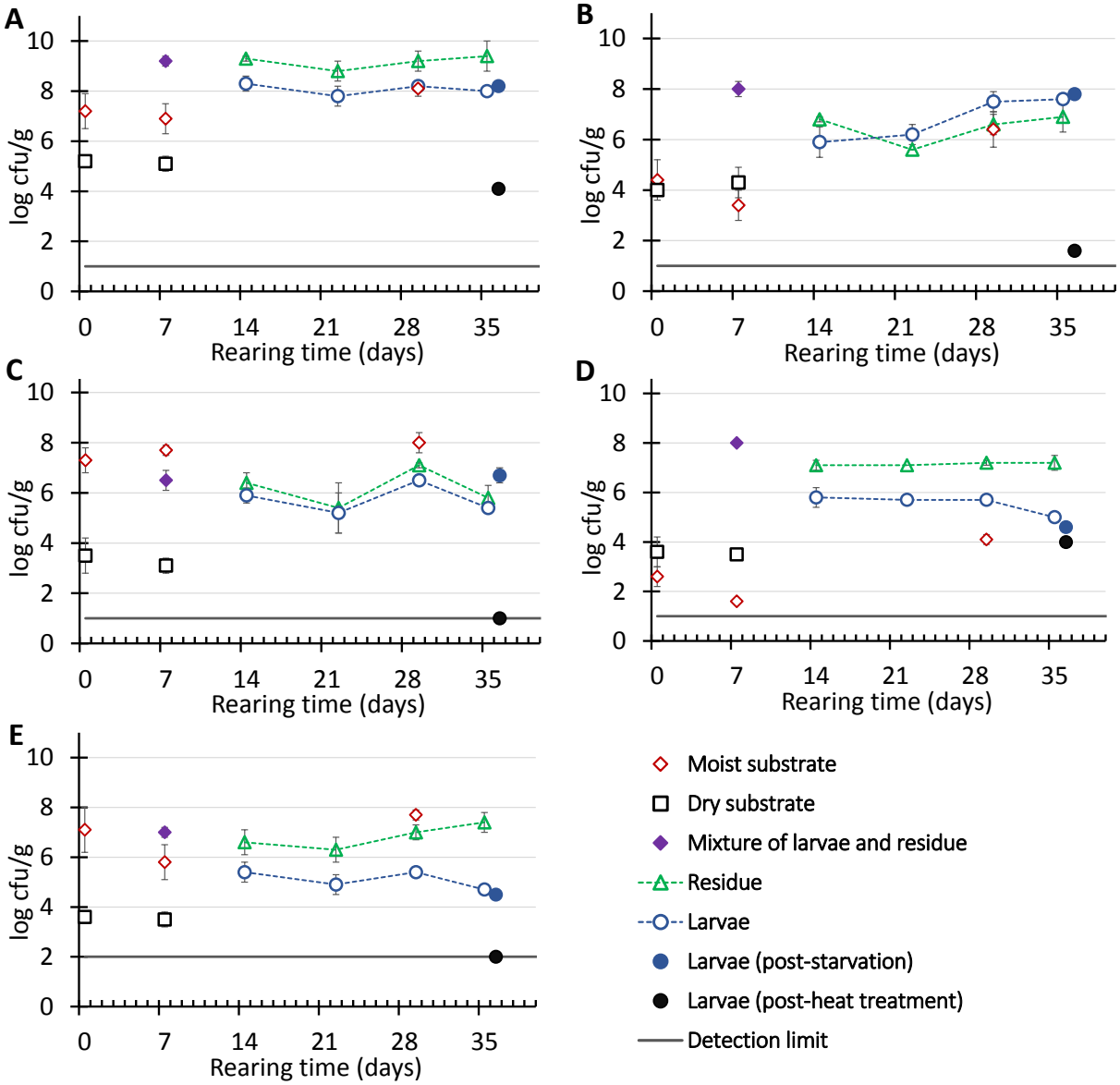

Figure 3.2 Dynamics of the microbial numbers of the dry substrate and moist substrate, residue and larvae during rearing: **A)** Total viable count, **B)** Enterobacteriaceae, **C)** lactic acid bacteria, **D)** aerobic endospores and **E)** fungi. Data are the mean of three replicate samples. Error bars represent the standard deviation.

Pathogen detection. Neither *Salmonella* sp. nor *L. monocytogenes* were detected in the larvae and residue samples (absent in 25 g). Coagulase-positive staphylococci and *B. cereus* were both below the detection limit (<100 cfu/g).

Identification of fungal isolates. For the dry substrate and the moist substrate, respectively five and three isolates were identified (Table S3.2, Supporting Information). According to the highest sequence homology, the isolates belonged to *Candida santamariae*, *Fusarium* sp., and *Purpureocillium liliacinum* for the moist substrate, and to *Wickerhamomyces anomalus*, *Fusarium* sp., *Penicillium cinnamopurpureum,* and *Penicillium solitum* for the dry substrate. The ten isolates from the larvae and the ten from residue showed highest sequence homology with *Aspergillus flavus*, *Diutina rugosa*, *Issatchenkia orientalis, Trichosporon asahii,* and *Pichia sporocuriosa* (the latter two only isolated from the larvae).

3.3.3 16S rRNA gene amplicon sequencing

High-throughput 16S rRNA gene sequencing was used to characterise the microbial communities in the samples. Relative OTU abundances and diversity indices were averaged over all replicate samples, each existing of two technical replicates. The total average coverage, based on Chao1 and calculated over all DNA extracts, was 80.86 ± 12.62% (SD), suggesting that the most abundant community members were recovered. A total of 78 bacterial OTUs was obtained throughout the whole dataset (Tables S3.4-S3.5, Supporting Information). Eight of those OTUs were common for all samples (OTUs 1, 2, 5, 7, 10, 12, and 30).

The observed richness as well as the Chao1 and Shannon-Wiener diversity indices were higher for the dry substrate than for the moist substrate (Table 3.2), suggesting a more diverse bacterial community for the dry substrate. However, it should be noted that only one technical replicate was available for the dry substrate. Diversity indices were lowest for the larvae, especially towards the end of the production cycle. By day 35 (post-harvest), the average number of OTUs had decreased significantly ($p = 0.002$) from 37 to 22 on average. After starvation, a further decrease to 15 OTUs was observed ($p = 0.026$). Chao1 and Shannon-Wiener diversity indices also decreased (marginally) significant from day 14 to day 36 ($p = 0.025$ and 0.090, respectively). Also in the residue a decrease was observed during rearing in the number of OTUs from 46 to 31 on

average (p = 0.024). That decrease was, however, not reflected in the Chao1 and Shannon-Wiener indices.

Table 3.2 Microbial community diversity indices of the samples of the lesser mealworm production cycle subjected to metagenetic analysis[1].

Sample	Rearing time (days)	Observed OTU richness	Chao1[2]	Coverage (%)[3]	Shannon-Wiener[4]
Moist substrate	0	31 ± 10	37.87 ± 10.23	81.89 ± 4.87	1.88 ± 0.24
Dry substrate	0†	57	61.67	92.42	2.78
Residue	14°	46 ± 3^{a}	53.31 ± 3.80^{a}	86.69 ± 11.48	2.26 ± 0.09^{a,b}
	28	40 ± 2^{a,b}	49.25 ± 4.61^{a}	82.14 ± 7.61	2.17 ± 0.09^{a}
	35	31 ± 5^{b}	38.50 ± 11.88^{a}	83.62 ± 17.12	1.98 ± 0.09^{b}
Larvae	14	37 ± 3^{a}	43.99 ± 3.84^{a}	83.13 ± 5.51	1.57 ± 0.17^{a}
	28	22 ± 2^{b}	23.88 ± 3.28^{b}	91.44 ± 4.79	1.51 ± 0.08^{a}
	35 (post-harvest)	22 ± 3^{b}	36.63 ± 12.44^{a,b}	64.49 ± 14.87	1.39 ± 0.08^{a}
	36 (post-starvation)°	15 ± 1^{c}	22.25 ± 3.18^{b}	66.07 ± 12.63	1.20 ± 0.09^{a}

[1]Sequences were grouped in Operational Taxonomic Units (OTUs) defined by 97% sequence identity at the 16S rRNA gene (V4 region, 250 bp). Values are the mean ± standard deviation of analyses performed on two replicate samples, with two technical replicates per sample (n = 2 x 2), except for † (1 sample, 1 technical replicate (n = 1)) and ° (1 sample, 2 technical replicates (n= 2)).
[2]Chao1 richness estimator: the total number of OTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1984).
[3]Observed richness/Chao1 estimate * 100.
[4]Shannon-Wiener diversity index: an index to characterise species diversity based on species richness as well as their relative abundance. A higher value represents more diversity (Shannon, 1948).
[a,b]Values per diversity index for the larvae and residue (= remaining substrate, faeces and exuviae) with the same letter do not differ significantly (p > 0.05) between sampling moments.

The most abundant phyla in the dataset were the Firmicutes and Proteobacteria (Figure S3.1). Whereas the former were more abundant in the moist substrate (91.3%), the latter were more abundant in the dry substrate (60.7%). In the residue, the bacterial community was dominated by Firmicutes throughout the whole production cycle (ranging between 55.0 and 68.8%), followed by Proteobacteria (ranging between 29.3 and 38.5%). In the larvae, Firmicutes dominated at day 14 (79.5% of sequences), but Proteobacteria became dominant from day 28 onwards, representing 67.8% of the sequences at day 28 and finally 77.5% at day 36 (post-starvation). The Actinobacteria, which were present to some extent in the dry substrate (22.2%) and in low abundance in the moist substrate (0.4%), became more abundant in the residue towards the end of the cycle. They increased from 2.1% at day 14 to 10.7% at day 35. In the larvae, the

abundance of the Actinobacteria remained below 5%. Bacteroidetes represented less than 1% of sequences in the samples, except in the dry substrate (5.6% of sequences).

In the moist substrate (Figure 3.3a), the most abundant OTUs corresponded to a *Staphyloccoccus* sp. (OTU 2), and two *Lactobacillus* sp. (OTUs 4 and 6). Together, these OTUs represented approximately 70% of all sequences. The dry substrate sample (Figure 3.3a) was mainly dominated by a *Buttiauxella* sp. (OTU 1) and a *Nocardiopsis* sp. (OTU 14) whereas the other OTUs were present in lower percentages (below 10%). These observations in the substrates can be related to the larvae (Figure 3.3c) because in the larvae, *Staphylococcus* sp. (OTU 2) was highly abundant at day 14. It decreased, however, in abundance by the end of rearing (from 56.8 to 1.6%), whereas *Buttiauxella* sp. (OTU 1) increased in abundance from 10.6 to 57.8% from day 14 onwards. Furthermore, OTUs corresponding to an *Aeromonas* sp. (OTU 3) and an *Enterococcus* sp. (OTU 5) substantially increased in average abundance of below 1% to 19.5% and 16.1%, respectively. In the residue (Figure 3.3b), on the other hand, *Staphylococcus* sp. remained the most dominant community member (ranging between 31.7 and 33.3%), although *Buttiauxella* sp. increased in abundance from 6.6 to 25.9%. Similar to the larvae, the genera *Aeromonas* (OTU 3) and *Enterococcus* (OTU 5) increased in abundance from below 1% to 7.7% and 8.1%, respectively. Of the OTUs that were present in the moist and/or dry substrate (70 OTUs), 50 were also detected at least at one sampling day in the larvae and 57 in the residue (Table S3.4, Supporting Information). On the other hand, eight OTUs (OTUs 26, 33, 38, 43, 62, 71, 73, and 79) that were present in the larvae and/or residue at any sampling day, albeit in low abundances (below 1%), were not recovered from the dry substrate and/or moist substrate.

NMDS ordination showed that the community composition of residue and/or larvae differed more from the community composition of the dry substrate than to that of the moist substrate (Figure 3.4). Furthermore, the larvae at day 14 were similar in bacterial community composition to the residue samples, whereas the community of the larvae during the remainder of the production cycle differed more from that of the residue.

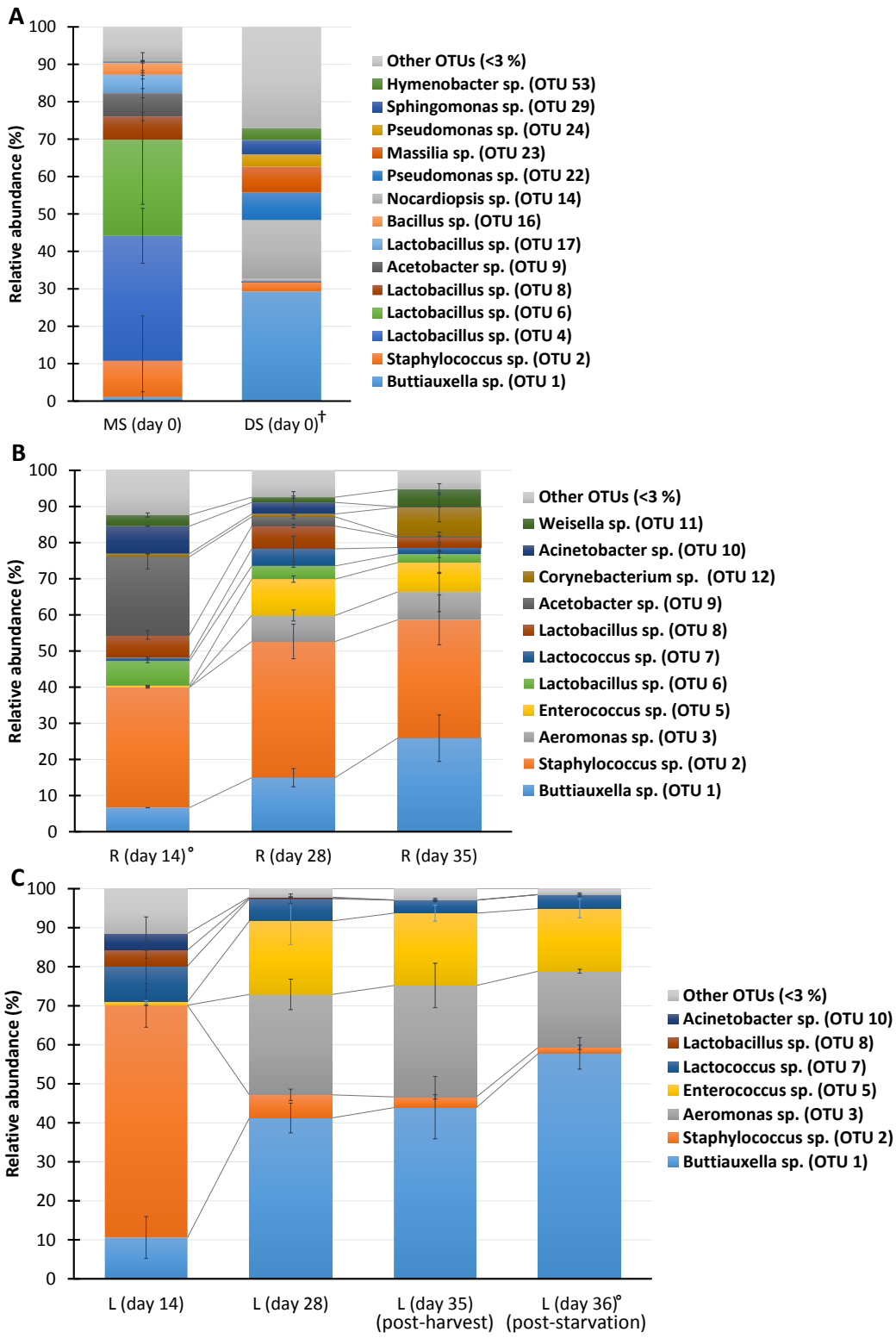

Figure 3.3 Relative abundance (%) of Operational Taxonomic Units (OTUs) present in samples of A) the dry substrate (DS) and moist substrate (MS), B) the residue (R) and C) the larvae (L). Values are the mean of analyses performed on two replicate samples, with two technical replicates per sample (n = 2 x 2) except for † (1 sample, 1 technical replicate (n = 1)) and ° (1 sample, 2 technical replicates (n= 2)). Error bars represent the standard deviation. Only OTUs represented by an average relative abundance of more than 3% of the sequences in any sample are shown. OTUs with an average relative abundance of less than 3% are grouped in "Other OTUs (< 3%)".

3.4 DISCUSSION

3.4.1 The microbiota of lesser mealworms during rearing

The TVCs of the residue during rearing were similar or higher to those of the larvae (Figure 3.2). That is remarkable, given the low water activity and pH of the residue (Table 3.1). The water activity of the residue was close to or even below 0.60, which is the limit for microbial growth (Jay et al., 2005). It is possible that the faeces contributed to the high microbial load of the residue. Preliminary tests (data not shown) on faeces from multiple yellow mealworm (*T. molitor*) batches showed average TVCs (including standard deviation from three replicate samples) ranging between 8.8 ± 0.1 and 10.5 ± 0.8 log cfu/g. Additionally, the environment temperature (30°C) and the daily addition of the moist substrate to the crates may have led to microbial growth. The larvae may not have been able to contain a higher number of micro-organisms, despite the higher load of the residue. Indeed, TVCs of approximately 8 log cfu/g have often been reported for fresh, living insects (Klunder et al., 2012; Stoops et al., 2016; Stoops et al., 2017; Vandeweyer et al., 2017b,c; Wynants et al., 2017). In contrast to the other counts, the number of Enterobacteriaceae and lactic acid bacteria in the residue did not exceed those in the larvae. However, as shown in this study and in other reports, yellow and lesser mealworms contain high numbers of Enterobacteriaceae and lactic acid bacteria (Klunder et al., 2012; Stoops et al., 2016; Stoops et al., 2017; Vandeweyer et al., 2017b,c; Wynants et al., 2017). It can be speculated that the conditions in the insect gut were more favourable for their growth and/or survival than those in the residue.

Approximately 70% percent of the bacterial sequences in the moist substrate consisted of members of the genus *Lactobacillus* (OTUs 4, 6, 8 and 17; Figure 3.3), corresponding to the high counts of lactic acid bacteria (7.3 to 8.0 log cfu/g; Figure 3.2c). The dry substrate showed the lowest TVC in the plate counts (Figure 3.2a), which can be attributed to its low water activity (0.62 – 0.63; Table 3.1). Nevertheless, its bacterial community was more diverse compared to the other samples (Table 3.2). That is also highlighted by the large portion of OTUs represented by less than 3% of the sequences ("Other OTUs"; Figure 3.3). Of the 70 OTUs present in the moist and/or dry substrate, 50 were also detected in the larvae at least at one sampling day, suggesting that the substrates were an important source of bacteria for the larval bacterial community. Except for OTUs corresponding to *Buttiauxella* sp. (OTU 1) and *Staphylococcus* sp. (OTU 2), however, the most abundant OTUs in the larvae were not

found to a large extent in the dry substrate and/or moist substrate (Figure 3.3). Those OTUs show a colonisation potential specific for the larval gut. The abundance of some OTUs that were hardly present in the substrates (below 2%), such as *Aeromonas* sp. (OTU 3) and *Enterococcus* sp. (OTU 5), also increased in the residue during production. Those organisms were likely able to grow inside the larval gut and were subsequently excreted in high numbers into the residue. Additionally, the number of OTUs in the larvae decreased from 37 to 15 OTUs on average during rearing. Likewise, decreases were observed in the average diversity parameters Chao1 and Shannon-Wiener (Table 3.2). As described by Engel & Moran (2013), insect gut communities are not expected to be random assemblages of bacteria from the insect diet or the environment. Although the diet is an important determinant for the community composition, colonisation of insect guts seems to be selective and to depend on the physicochemical conditions of the gut compartments (pH, redox potential, etc.; Dillon & Dillon, 2004; Engel & Moran, 2013). Likewise, the decrease in bacterial diversity and the increase in abundance of few OTUs in the larvae may be attributed to the competitive advantage of some species in the gut environment. Moreover, NMDS ordination showed the microbial community composition of the larvae to differ more from those of the residue and moist substrate towards the end of the production cycle (Figure 3.4). It should be noted, however, that metagenetic results also include dead cells (Cangelosi & Meschke, 2014), which may also have been present in our study.

Eight OTUs which were present in low abundances (below 1%) in the larvae and/or residue during the production cycle, were not found in the dry or moist substrate. They were either not recovered in the substrates through sequencing, or do not originate from them. These OTUs, corresponding to *Listeria* sp., *Brevibacterium* sp., *Clostridium* sp., *Brevibacillus* sp., *Rummeliibacillus* sp., and *Bacillus* sp. (Table S3.4, Supporting Information), are commonly found in soil, on human skin and/or in animal (including human) gastro-intestinal tracts (Dworkin, 2006a, 2006b) and may have ended up in the rearing crates from the production environment or the personnel (Rediers et al., 2008; Schneider, 2009).

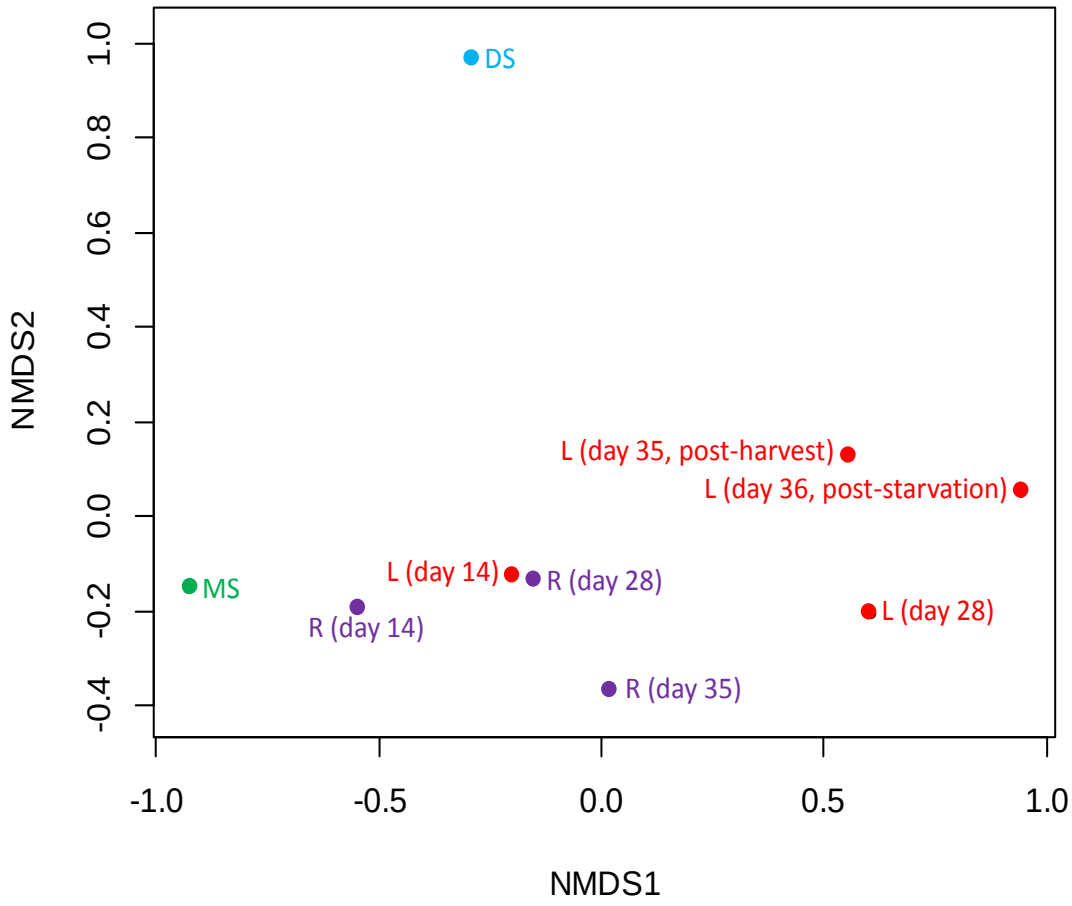

Figure 3.4 Nonmetric multidimensional scaling (NMDS) ordination (stress value = 0.05) representing the bacterial community composition of the moist substrate (MS, green), dry substrate (DS, blue), residue (R, purple) and larvae (L, red) during the production cycle.

3.4.2 The microbiota of harvested lesser mealworms

The intrinsic parameters of the larvae after harvest and starvation (Table 3.1) were comparable to those obtained by Vandeweyer et al. (2017b) for yellow mealworms. An exception on this is pH, which was slightly lower in our study compared to values reported by Vandeweyer et al. (2017b) ranging between 6.61 and 6.76. Nevertheless, the values of all intrinsic parameters are optimal for microbial growth. Because the larvae were homogenised prior to analysis, the results are only valid for homogenised larvae and do not indicate the potential for microbial growth on specific locations in the gastro-intestinal tract (Vandeweyer et al., 2017b).

The TVC of the larvae at the harvesting stage (Figure 3.2) was comparable to earlier reports on fresh edible insects (Caparros Megido et al., 2017; Klunder et al., 2012; Stoops et al., 2016; Stoops et al., 2017; Vandeweyer et al., 2017b,c). To our knowledge, only one study has reported microbial counts for reared, fresh lesser mealworms (Stoops et al., 2017). The microbial numbers of that study were comparable to our

study, except for the number of aerobic endospores, which were approximately 3-4 log units lower in the study by Stoops et al. (2017; ranging between 0.5 and 1.6 log cfu/g), which cannot clearly be explained.

To our knowledge, our study is the first to report sequencing data on the bacterial community composition of fresh lesser mealworms. The number of OTUs in the post-harvest and post-starvation larvae averaged at 22 and 15 OTUs, respectively (Table 3.2). The average Chao1 species richness estimators of the post-harvest and post-starvation samples in our study were 31.7 and 22.3, respectively. This suggests that more OTUs might have been present than identified through sequencing. The larval microbiota was mainly dominated by Proteobacteria and Firmicutes. These phyla are common in insect guts and may play beneficial roles in their hosts (Colman et al., 2012; Engel & Moran, 2013). The plate counts revealed high numbers of Enterobacteriaceae and lactic acid bacteria and that was reflected in the bacterial community composition uncovered by sequencing. For example, the increase in Enterobacteriaceae in the plate counts can be explained by the increase in *Buttiauxella* sp. (OTU 1). The lactic acid bacteria observed on the plates probably were represented to a large extent by *Enterococcus* sp. (OTU 5), which was among the most abundant OTUs in the samples at the harvesting stage. This genus has previously been identified in yellow mealworms (Garofalo et al., 2017; Jung et al., 2014; Li et al., 2016b; Wang & Zhang, 2015) and grasshoppers (Stoops et al., 2016). It comprises species that are highly adapted to the digestive tract of mammals as well as that of insects (Garofalo et al., 2017). Noteworthy, members of this genus are considered opportunistic pathogens in foods and may cause food intoxication (Giraffa, 2002). Besides Enterobacteriaceae and lactic acid bacteria, OTU 3, corresponding to *Aeromonas* sp., represented over 15% of sequences in the harvested larvae. The genus *Aeromonas* is frequently isolated from a diversity of food products, and naturally occurs in a range of habitats such as aquatic habitats, soils, and animals including insects (Janda & Abbot, 2010). It was detected in the moist substrate and/or dry substrate as well, although in low abundances (below 0.1%), but may also have ended up in the larvae through the personnel and/or environment.

3.4.3 Food safety aspects

The presence of four food pathogens (*Salmonella* sp., *L. monocytogenes*, *B. cereus,* and coagulase-positive staphylococci) was assessed both in the residue and in the

larvae during the production cycle. To our knowledge, *Salmonella* sp. and *L. monocytogenes* have not been detected so far in edible insects sold for human consumption in Europe (Giaccone, 2005; Grabowski et al., 2014; Grabowski & Klein, 2016; Vandeweyer et al., 2017b), although *Salmonella* sp. was detected in grasshoppers consumed in Cameroon (Ali et al., 2010). In contrast, presumptive *B. cereus* and *Stapylococcus aureus* have been detected in marketed insect products in Europe and/or Africa based on lesser mealworms, crickets, locusts and/or rhinoceros beetles (Banjo et al., 2006; NVWA, 2014; Grabowski and Klein, 2016; Osimani et al., 2017; Fasolato et al., 2018). In our study, neither of the samples tested positive for any of the four food pathogens. That suggested the absence of those pathogens in the rearing company, or at least in the rearing crates under study. Because only three samples of larvae were taken, sampling did not comply with the criteria for these food pathogens as described in Table 1.2 (section 1.5.4). Furthermore, it is clear that the total aerobic count and the number of Enterobacteriaceae exceed the maximum limit (7 and 5 log cfu/g, respectively) described in the action limits by the FASFC (Table 1.2), while the numbers of the three samples at harvest for fungi (moulds and yeasts) were between the minimum (3.7 log cfu/g) and maximum limit (5 log cfu/g) for fungi. It can however be argued that these criteria may be too strict for freshly harvested insects, as high numbers are generally reported for fresh insects (see section 1.8.1).

Starvation of the larvae did not result in significant reductions of the microbial counts, nor did it cause significant changes in the Chao1 and Shannon-Wiener diversity indices. That may indicate that this procedure is not necessary from a microbiological point of view, as was previously also demonstrated for yellow mealworms (Wynants et al., 2017). The heat treatment significantly reduced all counts, as expected, but a number of 4.0 ± 0.1 log cfu/g for aerobic endospores remained. All counts were below both minimum and maximum limits as proposed in Table 1.2, although it should be noted that only three samples were analysed instead of five. Thus, it cannot be stated that the larvae after the heat treatment comply with these action limits, but it shows that the heat treatment caused a substantial improvement in microbial quality and serves as a pasteurisation process. Nevertheless, the number of spores was unaffected by the treatment, an effect that was also shown earlier for heat-treated yellow mealworms by Vandeweyer et al. (2017b). From the larval samples before heating, genera *Bacillus* (OTUs 16 and 79) and *Clostridium* (OTU 38) were identified with high reliability (bootstrap value > 0.80; Supporting Information Table S3.4) in low abundances (below 0.1%). Although presumptive *B. cereus* could not be detected in

the samples, further research should focus on the large number of bacterial spores that remains in the larvae and their possible associated health hazards.

Among the fungal isolates (Table S3.2, Supporting Information), sequences corresponding to possible mycotoxin-producing species were identified. In the dry substrate and moist substrate, *Fusarium* sp. was identified, whereas in the larvae and the residue, *A. flavus* was found. The presence of mycotoxinogenic species, however, does not imply that their mycotoxins were also present. Still, the combination of high fungal counts in residues as well as in the larvae (at some sampling moments exceeding the upper limit for yeasts or moulds according to the FASFC action limits, see Table 1.2), do suggest that mycotoxins could pose a risk and should be addressed in further research on lesser mealworms reared for consumption. Additionally, some spoilage species were identified among the isolates, such as *W. anomalus* (grain spoilage) and *I. orientalis* (also known as *Candida krusei*, spoilage of diverse food products) (Chan et al., 2012; Passoth et al., 2005). The fungal species *I. orientalis*, *D. rugosa, and T. asahii* are known causes of candidemia (Hautala et al., 2007; Pfaller et al., 2006) or trichosporonosis (Sugita et al., 1998). However, these species are widespread, including habitats such as soil and/or human skin. Generally, health hazards due to these species only apply to immunocompromised patients. Furthermore, the potential of infection due to these species through consumption of the larvae is rather low, as the heat treatment was shown to reduce fungal counts to below the detection limit. *P. lilacinum*, on the other hand, which was isolated from the moist substrate, is known to show entomopathogenic characteristics (Fiedler & Sosnowska, 2007; Marti et al., 2006) and could possibly affect the rearing system.

3.5 CONCLUSIONS

This study characterised the microbial dynamics during the production of lesser mealworms. Microbial loads were high early on in the production cycle. The residue inside the rearing crates generally showed a higher microbial load and bacterial diversity than the larvae. The excretion of faeces and exuviae, in combination with the regular addition of moist substrate and a rearing temperature favourable for microbial growth presumably caused a pronounced microbial growth in the residue. Most of the species that were detected in the larvae or in the crates could be traced back to the diet. That indicates that the diet is an important source of bacteria for the larval gut. However, the larvae were dominated by a select number of species by the end of the

production cycle, pointing towards a competitive advantage of these species in the insect gut. A heat treatment significantly reduced all bacterial counts, but a spore count of 4.0 log cfu/g remained. No typical food pathogens were detected in this study, although some fungi were identified that may produce mycotoxins. Future research is needed on possible hazards caused by the remaining bacterial spores, as well as on the possible presence of mycotoxins.

CHAPTER 4

Microbial dynamics during industrial rearing of the tropical house cricket (*Gryllodes sigillatus*) for human consumption

Modified from:

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 the tropical house cricket (*Gryllodes sigillatus*) for human consumption. *Applied and environmental microbiology*, e00255-18.

*The paper on which Chapter 4 was based, consists of two parts. The first part of the paper, describing microbial dynamics during cricket rearing was the base for Chapter 4. The second part regarding processing and preservation of fully grown crickets was not included in this chapter. As joined first author, E.W. contributed mainly to the experimental work for the first part of the paper, while D.V. was mainly responsible for the second part. Both authors contributed equally to the writing of the paper. High-throughput 16S rRNA gene sequencing and bio-informatic analysis of sequencing data were performed in collaboration with the Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), KU Leuven (Prof. B. Lievens).

4.1 INTRODUCTION

As also indicated in Chapter 3, it is suggested that the insect substrate is an important factor for the development of the insect microbial composition (Dillon & Dillon, 2004; Engel & Moran, 2013; EFSA scientific Committee, 2015). Furthermore, the substrate may constitute a potential source of hazards from a microbiological origin, such as food pathogens and mycotoxins (EFSA scientific Committee, 2015; Charlton et al., 2015; Pinotti et al., 2016). In addition to the insect substrate, the rearing environment and the manual practices by the workers may also contribute to the microbiota of edible insects (Schneider, 2009). In order to fully understand the relationship between these factors and the microbial community composition as well as possible food safety hazards, a general insight into the microbial dynamics in the insect substrate and the insects as such during rearing is required.

The goal of this study was to investigate the microbial dynamics, including changes in microbial numbers and bacterial community composition, from industrial rearing to processing and storage of the tropical house cricket (*Gryllodes sigillatus*) reared for human consumption. For this purpose, the egg deposition medium, the substrate before administration (further referred to as “substrate”), the substrate present in the rearing cages and thus in contact with the crickets (further referred to as “residue”), and the crickets as such were sampled and analysed. To this end, samples were investigated for their intrinsic parameters, microbial numbers, microbial community composition (bacterial and fungal), and the presence of a selection of foodborne pathogens.

4.2 MATERIALS AND METHODS

4.2.1 Industrial rearing cycle

A complete rearing cycle in a Belgian company rearing crickets for human consumption (Little Food, Brussels) was monitored. An overview of the whole industrial cricket production process, including post-harvest treatments, is given in Figure 4.1a. Eggs were laid by adult crickets in the final 1-2 days before harvest into a mixture of peat soil and coconut peel (“peat-peel mix”) in small plastic containers (17.5 x 13 x 5 cm). These plastic containers were then placed in a larger container on top of a pile of egg cardboard, in which the freshly emerged nymphs could reside. The nymphs were removed from the container and placed in a larger cage (approximately 2 x 1 x 1 m), consisting of a wooden skeleton and Perspex walls, which was open on the

upper side. Inside the cage, egg cartons were piled up to create a dark habitat with crevices. All cages were situated in a ventilated room at an average temperature of 31 °C and an average relative humidity of 70%. Artificial light was present on average 8 hours/day except during weekends. A specialised cricket substrate (main components: wheat bran, linseed flakes and sunflower seed flakes) was added on a cardboard plate on top of the egg cartons, and water was presented separately in a plastic dispenser. New substrate was added one to three times a week in quantities ranging from 1 to 2 kg, with both frequency and quantity increasing as the crickets aged. The water bowl was refilled when empty. Two days before harvest, only carrots were provided as feed and water source and, according to the rearing company, to improve reproduction and taste. Additionally, a small plastic container with peat and coconut peel (see above) was placed inside the cage for oviposition. After 40 days, the crickets were harvested and the cages were cleaned with a brush and, if necessary, with water containing disinfectant. Subsequently, the crickets were killed by submersion in hot water (60 °C) and rinsed (5 min) with regular tap water. Afterwards, 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). This heat treatment was chosen to avoid blackening of the crickets due to overprocessing and to reduce microbial numbers (but without comparing with specific criteria), and was based on practices in Thailand. Subsequently, they were further processed into end products, e.g. by freezing, drying, or smoking followed by drying.

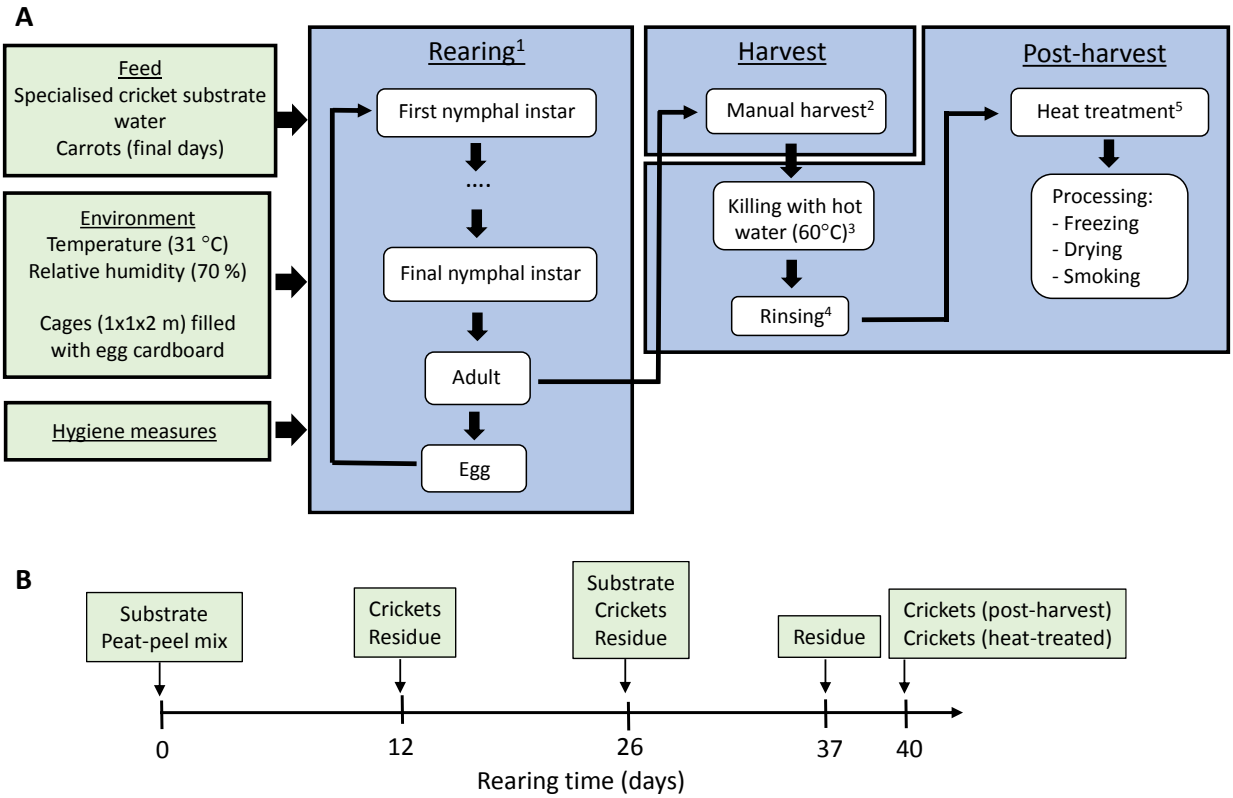

Figure 4.1 A) Schematic representation of the rearing and processing cycle of tropical house crickets. [1]The rearing period, from first instar to harvested adult cricket, took 40 days. [2]Crickets were harvested by shaking them out of the cardboard boxes into a circular plastic container. [3]Crickets were killed by submerging them in hot water inside the container. [4]Crickets were rinsed thoroughly in a colander using running tap water for 5 min per batch. [5]Crickets were submerged in boiling water and heat-treated until the water boiled again, which took 5 to 10 min. **B)** Sampling plan.

4.2.2 Sample collection

During the rearing cycle, samples were taken from the peat soil/coconut peel mixture, the substrate before addition, the residue, and the crickets at two-weekly intervals during the rearing cycle (Figure 4.1b). All samples were collected in threefold, thus resulting in three replicates. For residues and crickets, each replicate was collected from a separate cage. At the end of the cycle, crickets were sampled after harvest and after heat treatment, again in threefold. All samples taken during and at the end of the rearing cycle (fresh samples) as well as the heat-treated samples were kept at 3 °C for maximum 24 hours (cricket samples) or 48 hours (substrate samples) until analysis.

4.2.3 Intrinsic parameters

Water activity (a_w), moisture content, and pH were determined for the egg deposition medium ("peat-peel mix"), the substrate (days 0 and 26) and the residue (days 12, 26 and 37). All selected cricket samples (Table S4.1, Supporting Information) were pulverized prior to analyses as described by Stoops et al. (2016). Peat-peel mix, substrate, and residue samples were analysed without preparation. Water activity and moisture content were measured as described in Chapter 2. The pH was measured directly in the (homogenised) samples using a digital pH meter (Portamess 911, Knick, Berlin, Germany with SI analytics electrode, Mainz, Germany).

4.2.4 Culture-dependent microbiological analyses

Plate counts. All samples collected during rearing (Figure 4.2; Table S4.1, Supporting Information) were analysed for their total viable count (TVC), and the number of Enterobacteriaceae, lactic acid bacteria, aerobic bacterial endospores and fungi. Plate counts were performed according to the ISO standards for microbiological analyses of food and feed as compiled by Dijk et al. (2015), as described in Chapter 2.

Pathogen detection. For three replicate cricket and residue samples at the end of the rearing phase (day 40 and day 37, respectively), the presence of four food pathogens was assessed. The presence of *Salmonella* sp. was assessed according to ISO 6579 (absence in 25 g) and the presence of *L. monocytogenes* according to AFNOR BRD 07/4-09/98 (absence in 25 g). Furthermore, detection of *B. cereus* was performed according to ISO 7932 (plate count) and prevalence of coagulase-positive staphylococci according to AFNOR 3M-01/9-04/03 B (plate count).

Identification of fungal isolates. For the substrate (day 0), the peat-peel mix (day 0), the residue (day 37) and the crickets (day 40) a selection of fungal colonies with distinct morphology was picked from the DRBC medium for further identification (if possible 10 colonies per sample type). Subsequently, isolates were grown on Potato Dextrose Agar (PDA, Biokar Diagnostics) and incubated at 25 °C. After two to seven days of incubation (depending on the growth), genomic DNA was extracted from purified strains and isolates were identified according to the methods described in Chapter 3. Obtained sequences from the identified fungal isolates were deposited in GenBank under the accession numbers MG655272-MG655305.

4.2.5 16S rRNA gene amplicon sequencing

The bacterial community composition of the selected samples (Table S4.1, Supporting Information) 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 pulverized as described for the intrinsic properties and plate count analyses. For each pulverized replicate, genomic DNA was extracted in duplicate, resulting in a total of 4 DNA extracts per sample. DNA extraction, PCR amplification, library preparation, high-throughput Illumina sequencing and data-analyses were performed as described in Chapter 2 (Table S4.2). Downstream diversity analyses used data rarefied to 1700 sequences per DNA extract (Table S4.3). For the harvested (fresh) crickets, only two DNA extractions (of one replicate) delivered useful sequences; the others were not retained for data analysis. 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 S4.4, Supporting Information). Chao1 and Shannon-Wiener diversity indices were calculated using the R package Phyloseq (v.1.19.0) (R Development Core Team, 2013). Sequences obtained from the Illumina Miseq platform were deposited in a Sequence Read Archive (SAMN08032682 - SAMN08032721) under BioProject accession PRJNA418072. Additionally, representative sequences per OTU have been submitted to GenBank under accession numbers MG558004 - MG558332.

4.2.6 Statistical analyses

Differences in the intrinsic parameters, microbial counts and diversity parameters (OTU richness, Chao1, coverage and Shannon-Wiener indices) 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 a 0.05 significance level.

4.3 RESULTS

4.3.1 Intrinsic parameters

The peat-peel mix showed a high a_w and moisture content (average of 0.98 and 76.6%, respectively), whereas pH averaged at 4.99 (Table 4.1). The substrate consisted of a dry material with low a_w (0.59 – 0.61) and moisture content (10.3%). However,

once administered to the crickets in the cage, the water activity and moisture content of the residue gradually increased to 0.78 and 15.5%, respectively, at day 26. Towards the end of the rearing process (day 37), however, when the residue was replenished more frequently, the water activity and the moisture content decreased again ($p = 0.018$ and 0.007, respectively; Table 4.1). Values for pH were not statistically different between substrate and residue, irrespective of the sampling days. 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 4.1).

Following heat treatment (bringing to a boil) of the crickets, mean pH and moisture content significantly increased to 6.84 ($p = 0.031$) and 73.8% ($p = 0.006$), respectively. No difference was observed for water activity (Table 4.1).

Table 4.1 Intrinsic properties during tropical house cricket rearing.[1]

Sample	Sample day	Intrinsic properties		
		pH	a_w	Moisture content (%)
Peat-peel mix	0	4.99 ± 1.09	0.98 ± 0.00	76.6 ± 3.9
Substrate	0	5.51 ± 0.08[a]	0.59 ± 0.02[a]	10.3 ± 0.4[a]
	26	5.59 ± 0.01[a]	0.61 ± 0.01[a]	10.3 ± 0.3[a]
Residue	12	5.57 ± 0.11[a]	0.73 ± 0.02[a]	13.0 ± 1.0[a]
	26	5.41 ± 0.04[a]	0.78 ± 0.04[a]	15.5 ± 1.0[b]
	37	5.49 ± 0.06[a]	0.61 ± 0.04[b]	9.8 ± 0.2[c]
Crickets (post-harvest)	40	6.64 ± 0.10[a]	0.97 ± 0.01[a]	71.5 ± 0.7[a]
Crickets (heat-treated[2])	40	6.84 ± 0.05[b]	0.98 ± 0.00[a]	73.8 ± 0.4[b]

[1]Data are the mean values of three replicates ± standard deviation.
[2]The heat treatment consisted of bringing the crickets to a boil in a kettle with water.
[a,b,c]Means per sample with the same superscript (small letter) within the same column do not differ significantly ($p > 0.05$).

4.3.2 Culture-dependent microbiological analyses

Plate counts. The peat-peel mix showed a high TVC of 8.5 log cfu/g, whereas other counts ranged between 4.3 (lactic acid bacteria) and 6.9 (fungi) log cfu/g (Figure 4.2). The substrate, on the other hand, contained lower numbers of microorganisms. Here, the mean TVC ranged between 5.0 and 5.4 log cfu/g and the other counts between 1.8 (lactic acid bacteria) and 4.5 (Enterobacteriaceae and endospores) log cfu/g. The microbial load of the substrate did not differ significantly between the samples taken

at day 0 and day 26, except for the number of Enterobacteriaceae which was higher at day 0 ($p = 0.017$). Once the substrate was added to the cages (becoming residue), the mean TVC increased and ranged between 6.2 and 6.7 log cfu/g, but did not differ significantly between sampling days 12, 26 and 37. Other average counts for the residue ranged between 3.2 (fungi) and 5.0 (lactic acid bacteria) log cfu/g. Significant decreases in microbial load were detected from day 12 to day 37 for Enterobacteriaceae ($p = 0.018$) and lactic acid bacteria ($p = 0.023$). During the rearing phase, average TVCs for the crickets ranged between 8.2 and 8.5 log cfu/g. Counts of Enterobacteriaceae ranged between averages of 7.2 and 7.5 log cfu/g, lactic acid bacteria between 6.7 and 7.8 log cfu/g, endospores between 3.5 and 3.8 log cfu/g, and fungi between 5.4 and 6.0 log cfu/g. After heat treatment, all microbial counts were reduced, for lactic acid bacteria 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 TVC, Enterobacteriaceae, lactic acid bacteria, endospores and fungi, respectively).

Pathogen detection. At the end of the rearing cycle, harvested crickets (day 40) and residues (day 37) were assessed for the presence of a number of foodborne pathogenic bacteria. Neither *Salmonella* sp. nor *Listeria monocytogenes* were detected in the samples (absent in 25 g). Additionally, *Bacillus cereus* and coagulase-positive staphylococci were below the detection limit of 100 cfu/g.

Identification of fungal isolates. All isolates obtained from the peat-peel mix corresponded to the genus *Trichoderma*. For the substrate, isolates corresponded to the genera *Aspergillus*, *Hyphopichia*, *Lichteimia* and *Penicillium*. With regard to the residue, isolates were identified as *Aspergillus*, *Candida*, *Lichteimia*, *Penicillium* and *Trichoderma*. Isolates from the crickets corresponded to *Aspergillus, Candida*, *Kodamaea*, *Lichtheimia*, *Terapisispora*, *Trichoderma* and *Trichosporon* (Table S4.5, Supporting Information).

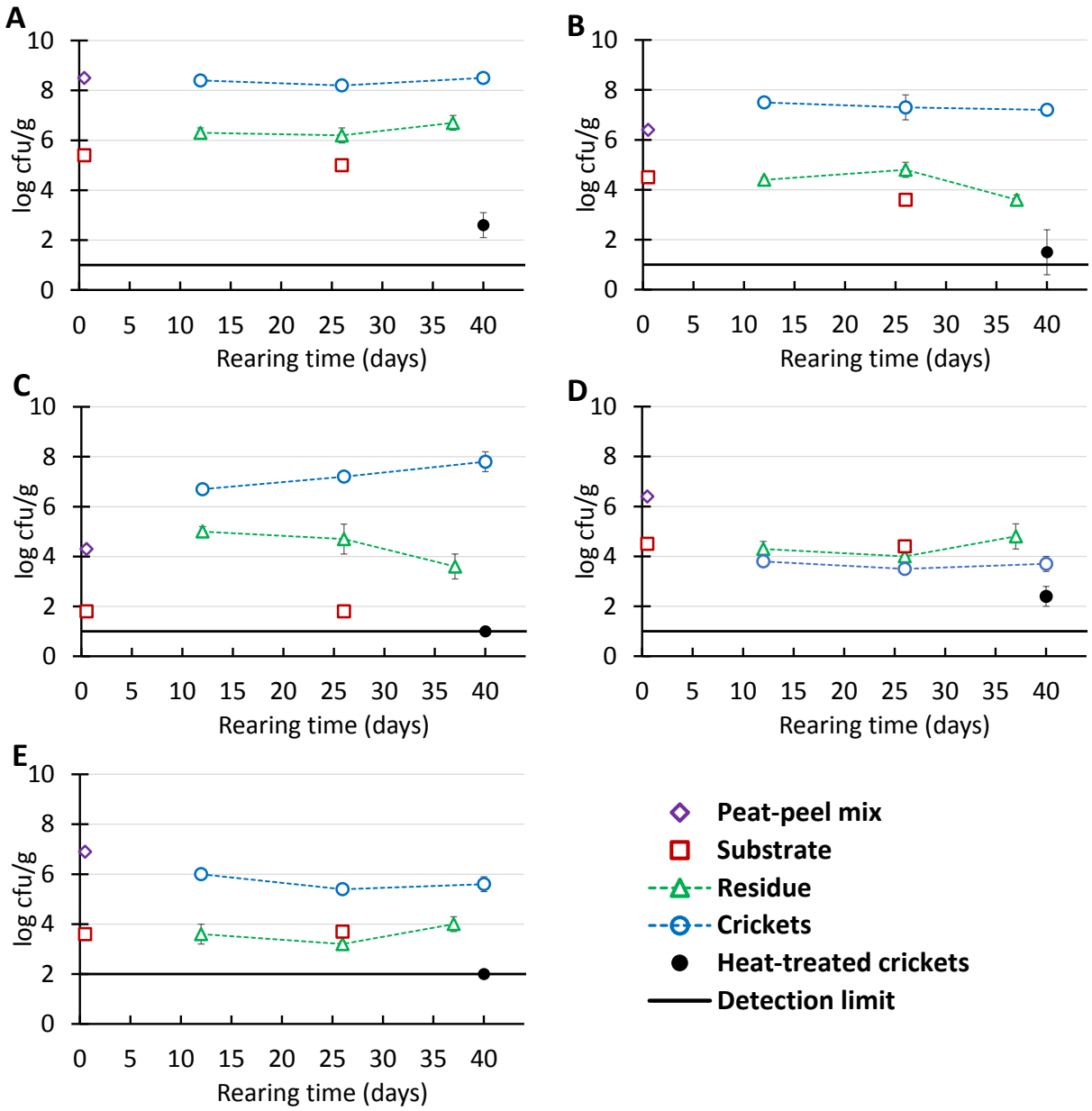

Figure 4.2 Microbial counts of samples of the peat-peel mix, substrate, residue and crickets taken during the rearing cycle: **A)** total viable count, **B)** Enterobacteriaceae, **C)** lactic acid bacteria, **D)** endospores and **E)** fungi. Error bars represent the standard deviation.

4.3.3 16S RNA gene amplicon sequencing

A selection of samples (Table S4.1, Supporting Information), including samples from the peat-peel mix (day 0), the substrate (day 0), the residue (days 12 and 26) and crickets during the rearing phase (days 12 and 26), as well as crickets after harvest (Figure 4.3), were subjected to high-throughput, amplicon-based 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 99.1%, indicating that the majority of bacterial community members was recovered (Table 4.2). Indices for species richness (observed richness and Chao1 (Chao, 1984) showed that the substrate and residue contained most bacterial species, while the least diversity was observed in the peat-peel mix, although its mean Shannon index (Shannon, 1948) was within the range of the other samples (Table 4.2).

Identification of the operational taxonomic units (OTUs) (Table S4.3 and S4.4, Supporting Information) revealed that the most abundant phyla in the peat-peel mix and substrate were Proteobacteria (47.7 ± 11.9% and 39.6 ± 13.0%, respectively) and Bacteroidetes (38.0 ± 13.7% and 43.3 ± 10.2%, respectively). In the substrate, the phylum Firmicutes was also among the most abundant phyla (12.6 ± 0.6%). Once the substrate had been administered inside the cage, the latter phylum became more abundant (17.7 ± 2.6% at day 12 and 20.2 ± 1.7% at day 26) alongside Bacteroidetes and Proteobacteria (65.8 ± 5.6% and 14.1 ± 2.7%, respectively, at day 12; 47.4 ± 9.9% and 29.2 ± 11.7%, respectively, at day 26). During rearing, Bacteroidetes remained the most abundant phylum in the crickets (ranging from 48.5 ± 22.6% in harvested crickets to 68.3 ± 2.7% in heat-treated crickets), followed by Firmicutes (ranging from 6.0 ± 0.7% in heat-treated crickets to 20.2 ± 0.8% for 12-day old crickets) and Proteobacteria (ranging from 14.1 ± 4.6 for 26-day old crickets to 28.4 ± 23.2% in heat-treated crickets). Other phyla were present in abundances below 10% in any sample of the dataset. The peat-peel mix (Figure 4.3a) was dominated (average OTU abundance of more than 5% in any sample) by OTUs corresponding to members of Chitinophagaceae (OTUs 15, 19 and 36), a *Chryseobacterium* sp. (OTU 22), a *Dyella* sp. (OTU 23), a *Burkholderia* sp. (OTU 28), and a *Rhodanobacter* sp. (OTU 44). The substrate and residue samples (Figure 4.3a) were all dominated by the same group of OTUs, consisting of members of the Porphyromonadaceae family (OTUs 1, 2 and 4), an *Erwinia* sp. (OTU 10), a Rhizobiales sp. (OTU 17), and a *Pseudomonas* sp. (OTU 49). More than 40% of sequences recovered belonged to OTUs represented by less than 3% of sequences in all samples. Cricket samples (Figure 4.3b) were abundant in OTUs corresponding to members of Porphyromonadaceae (OTUs 2 and 4) and Enterobacteriaceae (OTU 8), a *Bacteroides* sp. (OTU 3), a *Fusobacterium* sp. (OTU 5), a *Parabacteroides* sp. (OTU 6), and an *Erwinia* sp. (OTU 10). During rearing, OTU 1, (a Porphyromonadaceae sp.), decreased in average relative abundance from 5% to below 1%. After harvest, crickets showed a high abundance of two *Acinetobacter* sp. (OTUs 34 and 52). Also here, a large percentage of OTUs showed a relative abundance below 3% in any sample.

Table 4.2 Diversity indices for samples subjected to metagenetic analysis in this study.[1]

Sample	Sampling moment	Diversity indices			
		Observed richness	Chao1[2]	Coverage (%)[3]	Shannon[4]
Peat-peel mix	Start rearing (day 0)	90 ± 5	92.70 ± 7.99	96.74 ± 3.45	3.22 ± 0.20
Substrate	Start rearing (day 0)	128 ± 12	131.30 ± 7.92	97.01 ± 3.30	3.45 ± 0.28
Residue	Rearing day 12	127 ± 12^{a}	128.28 ± 12.44^{a}	99.00 ± 0.56^{a}	3.39 ± 0.22^{a}
	Rearing day 26	130 ± 4^{a}	131.75 ± 4.76^{a}	98.31 ± 1.24^{a}	3.59 ± 0.02^{a}
Crickets	Rearing day 12	115 ± 7^{a,b,c}	116.33 ± 7.14^{a,b,c}	99.08 ± 0.33^{a}	3.45 ± 0.02^{a}
	Rearing day 26	120 ± 1^{b}	122.53 ± 0.83^{b}	97.94 ± 0.58^{a}	3.48 ± 0.06^{a}
	Harvest day 40	115 ± 11^{a,b,c}	117.05 ± 8.27^{a,b,c,d}	97.75 ± 2.15^{a}	3.53 ± 0.01^{a}
	Heat-treated	101 ± 4^{a,c}	104.43 ± 4.77^{c,d}	96.52 ± 1.47^{a}	3.18 ± 0.02^{b}

[1]Sequences were grouped into Operational Taxonomic Units (OTUs) defined by 97% sequence identity at the 16S rRNA gene (V4 region, 250 bp).
[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).
[a,b,c,d]Means per sample with the same superscript within the same column do not differ significantly ($p > 0.05$). Data are the mean values of two analysed DNA-extracts from two replicates per sampling moment ± standard deviations.

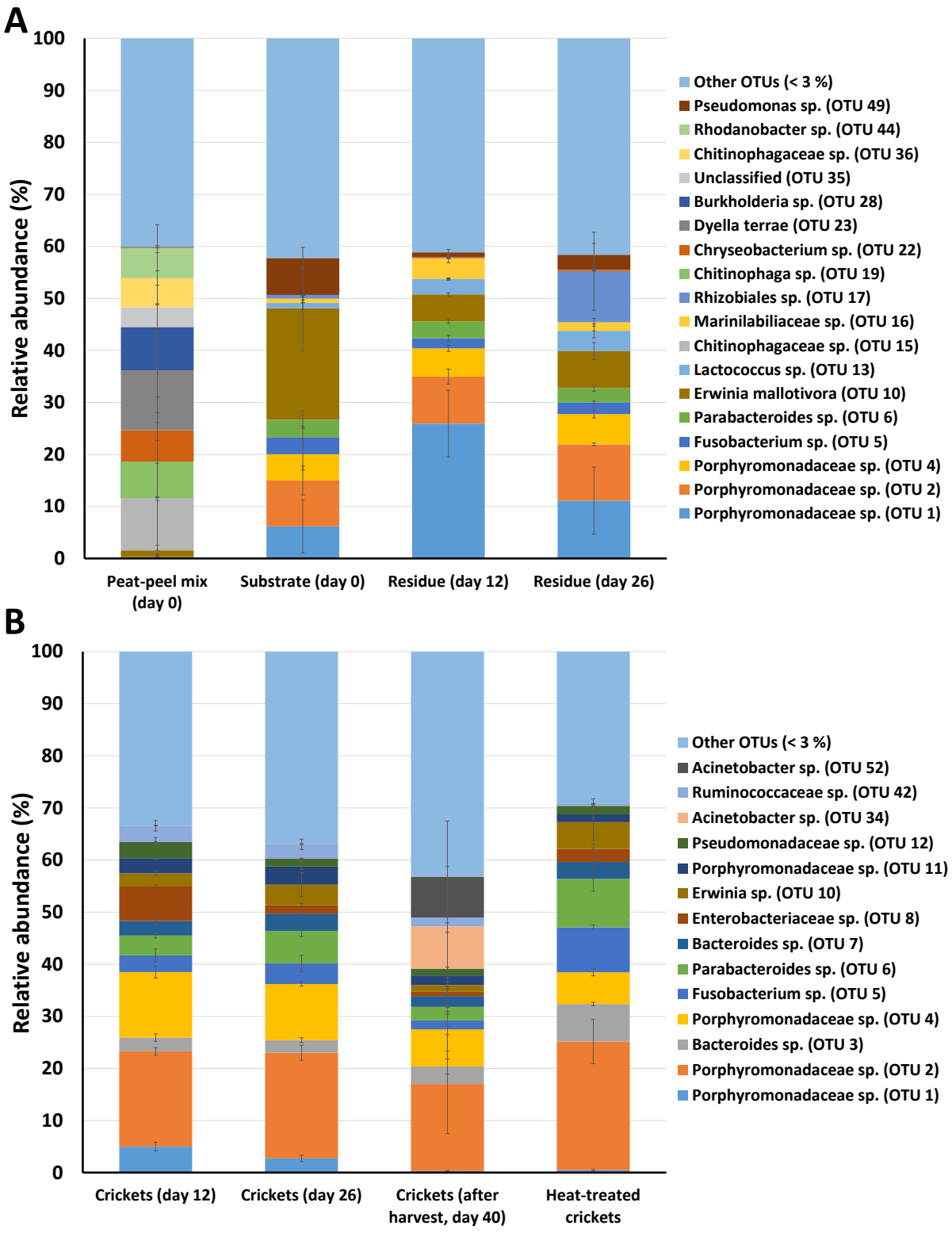

Figure 4.3 Relative abundance (%) of Operational Taxonomic Units (OTUs) present in the **A)** peat-peel mix, substrate, and residue and **B)** crickets during rearing and after harvest and heat treatment. Error bars represent the standard deviation. Only OTUs represented by an average relative abundance 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%)".

4.4 DISCUSSION

4.4.1 Microbiota of crickets during rearing

The peat-peel mix contained high numbers of microorganisms of which the most abundant were typical soilborne bacteria such as *Dyella* (OTU 23), *Burkholderia* (OTU 28), *Rhodanobacter* (OTU 44), and members of Chitinophagaceae (OTU 36) and Rhizobiales (OTU 17) (Brenner et al., 2005; Krieg et al., 2010). In contrast, none of these OTUs was recovered in substantial abundance in the other samples. This suggests that the hatchlings do not transfer these bacteria to the rearing cages, nor that they retain these bacteria within their gastrointestinal tract. The substrate, on the other hand, contained lower microbial numbers, which greatly increased after the substrate was placed inside the cage. That effect may be attributed to the increase in water activity and moisture content due to absorption from the environment and thereby facilitating microbial growth and/or cross-contamination from contact with crickets and possible defecation into the residue. As the crickets grew, the residue was replenished more frequently towards the end of the rearing cycle to provide them with sufficient resources. This “dilution” may not only explain the decrease in water activity and moisture content towards day 37 in the residue, but also the observed decrease in the number of lactic acid bacteria and Enterobacteriaceae. The microbial numbers of the residue were consistently lower than those of the crickets for all counts, except for the endospores, suggesting that the crickets are a better matrix for microbial growth than the residue. This is in contrast to results obtained for the rearing of lesser mealworms (*A. diaperinus*) (Wynants et al., 2018a), where the larvae are reared within the residue, which also contains their excreted feces and exuviae.

Even though the microbial numbers of the substrate and residue are generally lower than those of the crickets, a striking similarity was observed in community composition between the substrate, the residue and the crickets. Indeed, for example, OTUs corresponding to Porphyromonadaceae (OTUs 1, 2 and 4), a *Fusobacterium* sp. (OTU 5), a *Parabacteroides* sp. (OTU 6) and an *Erwinia* sp. (OTU 10) were abundant in all samples. The OTU most abundant in the substrate belonged to the genus *Erwinia* (OTU 10). Most members of this genus are plant-associated and plant-pathogenic bacteria (Brenner et al., 2005), which could explain its high relative abundance in the plant-based substrate. These results suggest that the substrate is an important source for the development of the cricket microbiota. Similar results were obtained during a previous study on lesser mealworms, where 50 out of 70 bacterial OTUs identified from

the substrate were also detected in the larvae (Wynants et al., 2018a). In that study, however, the microbial diversity of the larvae decreased during rearing, with a number of 22 OTUs obtained for harvested larvae. In the present study, the cricket microbial community composition was highly diverse and did not significantly change during rearing, as indicated by the diversity indices as well as by the large portion of OTUs represented by less than 3% of sequences. This observation was also reported by Vandeweyer et al. (2017a), 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 harbor a remarkably more complex bacterial community than mealworms, both during and at the end of their rearing period, with also more dominating organisms.

4.4.2 Microbiota of crickets after harvest

Considering community composition, similarities were observed between the crickets in the present study and those analysed by Vandeweyer et al. (2017a). Indeed, those authors also report the presence of OTUs corresponding to the families Enterobacteriaceae and Pseudomonadaceae, and the genera *Bacteroides, Parabacteroides*, *Erwinia,* and *Fusobacterium* in fresh crickets. Altogether, it is reasonable to assume that *Bacteroides* spp. and *Parabacteroides* spp. (among other Porphyromonadaceae spp.) are typical members of the endogenous intestinal bacterial community of crickets. Also *Fusobacterium* sp. was already observed as member of the tropical house cricket microbiome (Vandeweyer et al., 2017a). 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* sp. 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 et al., 2011), which may explain their appearance in the harvested crickets, since exclusively carrots were administered the final days before harvest.

Considering the microbiological quality of fresh, harvested crickets, plate count numbers obtained in our study were comparable to those obtained by Vandeweyer et al. (2017b) for tropical house crickets and house crickets (*A. domesticus*). Since the beginning of the rearing process, most microbial counts remained stable within the

crickets, except for the lactic acid bacteria, which showed a significant increase in numbers over time. This significant rise may be explained by the good growth of (a few) lactic acid bacteria species which may be well adapted to the cricket gut environment. The retrieved *Enterococcus* sp. (OTU 21) in this study is a possible candidate for this hypothesis, because the abundance of this OTU in the crickets during the rearing phase rose since day 12 (albeit below 3% relative abundance). Moreover, *Enterococcus* species have been frequently detected in other fresh edible cricket samples before (Vandeweyer et al., 2017a). In addition, many genera observed in this study were also encountered in previous research on processed crickets performed by Garofalo et al. (2017).

4.4.3 Food safety aspects

None of the four investigated foodborne pathogens were recovered from the crickets after harvest or from the residue. Although it cannot be stated that this complies with the microbiological criteria as mentioned in Table 1.2 as only three samples were investigated instead of five, it does suggest that these pathogens were absent in the rearing cycle under study. To our knowledge, *L. monocytogenes* has not yet been detected in edible insects for human consumption (Giaccone, 2005; Grabowski & Klein, 2016; Osimani et al., 2017; Vandeweyer et al., 2017b; Wynants et al., 2018a). However, *Salmonella* sp., *B. cereus* and *Staphylococcus aureus* have been reported in products including rhinoceros beetles and grasshoppers consumed in Africa (Banjo et al., 2006; Ali et al., 2010) and in mealworms and/or house crickets consumed in (Grabowski & Klein, 2016; Osimani et al., 2017). Noteworthy, it remains to be investigated whether a pathogen possibly present in the insect substrate may contaminate the insect (Templeton et al., 2006; Zheng et al., 2012b) and thus pose a hazard for food safety of the end product. Although no food pathogens were detected, some possible mycotoxin forming fungi, more specifically *Aspergillus* spp. and *Penicillium* spp., were recovered from the substrate, residue, and/or crickets. High fungal counts were obtained for crickets in this study, exceeding the action limits as described by the FASFC (Table 1.2). Although a high number of fungi does not necessarily mean that mycotoxins are present, these results suggest that the risk is possible. Future research should therefore focus on the presence of mycotoxins in harvested crickets. Mycotoxins can be very heat-resistant (Magan & Olsen, 2004) and, if present, will likely not be reduced by heat treatment and further processing, and be

able to cause mycotoxicosis. Fungal infections (mycoses) caused by e.g. *Aspergillus* or *Candida,* via consumption of the crickets, on the other hand, are highly unlikely, as the heat treatment was shown to reduce fungi to below the detection limit (< 2 log cfu/g).

Counts for fresh crickets (total viable count, Enterobacteriaceae, and fungi) exceeded the maximum limits of the action limits for human consumption as described in Table 1.2. As was already mentioned in Chapter 3 (section 3.4.3), it can be argued that these limits are too strict for fresh insects, as the numbers achieved in this study were similar to numbers obtained in Chapter 3, and comparable to numbers for fresh insects found in literature. Importantly, the application of heat caused all counts except those of the endospores to decline significantly, to values lower than the minimum limits of the action limits in Table 1.2. The heat treatment thus served as a pasteurisation. It should however be noted that only three samples were investigated instead of five, so it cannot be stated that the heat-treated crickets comply with these action limits. Still, and identical to lesser mealworms in Chapter 2, a substantial number of endospores remained, as was expected based on previous research on (lesser) mealworms and crickets (Stoops et al., 2017; Vandeweyer et al., 2017c; Wynants et al., 2018a). Heat-treated crickets had a similar bacterial community composition compared to the crickets during the rearing phase. 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. The presence of endospores in heat-treated crickets may entail a risk when they are further processed into products for consumption, especially since many processing steps for crickets include subsequent freezing/thawing cycles (personal communication with rearers). Rearers are thus advised to monitor the presence of pathogenic sporeforming bacteria and their possible toxin production in harvested crickets. In addition, further research is advised to focus on processing methods able to reduce endospores as well as on prevention of spore germination and outgrowth of vegetative cells in cricket products during processing and storage.

4.5 CONCLUSIONS

In this study, the microbial dynamics during rearing of tropical house crickets were investigated. The most abundant bacterial community members identified from the substrate (e.g. Porphyromonadaceae spp., *Bacteroides, Parabacteroides, Erwinia* and *Fusobacterium*) were also recovered from cricket samples, suggesting the substrate to

be an important source for the cricket microbiota. High microbial numbers of crickets during the rearing phase were significantly reduced by a heat treatment, although endospores survived. Neither *Salmonella* sp., *Listeria monocytogenes*, coagulase-positive staphylococci or *Bacillus cereus* were recovered from the crickets, but some mycotoxin-producing fungal genera were isolated. Further research on the possible presence of mycotoxins in insects is therefore advised, as well as research in order to mitigate the risks regarding the presence of endospores in harvested crickets and their derived products.

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CHAPTER 5

Risks related to the presence of *Salmonella* sp. during rearing of mealworms (*Tenebrio molitor*) for food or feed: survival in the substrate and transmission to the larvae

Modified from:

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*The complete content of this paper was included in Chapter 5, with only small alterations to keep the information provided up to date and to follow the logic course of this dissertation. As first author, E.W. contributed to all parts described in this work, from experimental design, conducting laboratory analyses, to the writing of the paper.

5.1 INTRODUCTION

As for other food and feed products, it is important to monitor possible safety hazards during the production process of mealworms sold for human consumption or for use in animal feed. One of the potential risks associated with the consumption of mealworms is the possible presence of microbial food pathogens, such as – among others – *Salmonella* sp. (EFSA Scientific Committee, 2015). Thus far, to our knowledge, studies conducting classical *Salmonella* sp. presence-absence tests on fresh or processed mealworms reared for human consumption, did not yield *Salmonella* sp.-positive samples (Giaccone, 2005; Osimani et al., 2017; Garofalo et al., 2017; Vandeweyer et al., 2017b). In those studies, however, no analyses were performed on the substrate used, which most often consists of wheat bran, supplemented with carrots, cucumbers, chicory, or other moisture-rich components. Indeed, for reared insects, it has been suggested frequently that the substrate, along with the rearing techniques, rearing environment, and hygiene affect the insect microbiota (Dillon et al., 2010; Klunder et al., 2012; Engel & Moran, 2013; SHC & FASFC, 2014; EFSA Scientific Committee, 2015; Li et al., 2016b; Osimani et al., 2018a). Several studies suggest the substrate to be an important source for the gut microbiota of edible insects (EFSA scientific committee, 2015; Boccazzi et al., 2017; Wynants et al., 2018a, Vandeweyer et al., 2018; Osimani et al., 2018a). Therefore, it is reasonable to assume that when a food pathogen is present in the substrate, it might contaminate the insects, resulting in a health risk when they are harvested, processed and consumed by humans or animals. For other species of darkling beetles such as *A. diaperinus*, research has previously shown that *Salmonella* sp. may be taken up in the gastro-intestinal tract of both larvae and adults (McAllister, et al., 1994; Hazeleger et al., 2008; Roche et al., 2009; Crippen et al., 2009; Leffer et al., 2010; Zheng et al., 2012b), and may be retained for a prolonged period of time, even after metamorphosis and eclosion (Crippen et al., 2012). In those studies, however, the aim was to assess the vector potential of this insect species for *Salmonella* sp., when the insect is present as a pest in traditional farm animal rearing facilities, such as in poultry houses. In those studies, *Salmonella* sp. was either administered in a suspension or agar directly to the larvae (Hazeleger et al., 2008; Crippen et al., 2009; Zheng et al., 2012b), or inoculated in very high numbers in a small amount of feed (McAllister et al., 1994; Roche et al., 2009; Leffer et al., 2010) in order to study the retention of the bacterium in the insect. In contrast, the aim of this study was to investigate transmission of *Salmonella* sp. during rearing of the yellow

mealworm (*Tenebrio molitor*) for food and feed purposes, in particular the transmission from wheat bran as substrate to the larvae.

5.2 MATERIALS AND METHODS

5.2.1 Overview of consecutive experiments

In this study, wheat bran was inoculated with a *Salmonella* sp. culture to obtain a variety of contamination levels, and the presence of *Salmonella* sp. in the bran (both in bran containing larvae and in bran without larvae) and its transmission to and colonisation of the mealworms was studied. This study consists of three different experimental designs. Within each design, three identical experiments were performed, each using a different batch of mealworms, i.e. mealworms from a different production cycle and produced at a different time. This paragraph aims to give a general overview of the set-up of this study, with experimental details described in the subsequent paragraphs. The differences between designs are depicted in Table 5.1 and Figure S5.1 (Supporting Information). The set-up of individual experiments within each design is visualised in Figure 5.1. In the first design, conducted on mealworm batches 1 to 3, transmission was studied when *Salmonella* sp. was inoculated in a way to attain two different contamination levels in the bran; aiming for either 4 or 7 log *Salmonella* sp. per g of wheat bran (4 and 7 log cfu/g "contamination levels"). In addition, the *Salmonella* sp. contamination was distributed in a homogeneous way in the bran. While likely not being representative for *Salmonella* sp. contamination in the insect rearing industry, this design with high contamination levels was included in the experimental set-up in order to validate the protocol, i.e. as a "worst case" scenario. It was hypothesized that at high levels, the dynamics of *Salmonella* sp. in bran and larvae would be easier to monitor throughout the experiment than at lower levels. In this design, larvae were given a surface disinfection treatment prior to microbial analysis in order to focus on the interior microbiota. In the second design, conducted on mealworm batches 4 to 6, scenarios that are likely to be more representative for industrial rearing were investigated, with inoculation to reach levels of 4 and 2 log cfu/g bran combined with a heterogeneous contamination. As in the first design, larvae were disinfected prior to microbial analysis, in order to compare the data obtained in the second design with that of the first. However, when mealworms are reared and sold for consumption, the industrial practise does not include a disinfection step after harvest. In addition, it cannot be stated with certainty

that the ethanol treatment did not affect the gut microbiota. Therefore, a third design was included in the study, conducted on mealworm batches 7 to 9 involving the same inoculation and heterogeneous contamination as in the second design, but without disinfecting the larvae prior to analysis. As mentioned, for each of the designs, three experiments were performed using three different batches of mealworms. Moreover, each experiment comprised at least three experimental conditions, i.e. wheat bran without larvae but with *Salmonella* sp. at the levels described above (to study survival of *Salmonella* sp. in wheat bran), wheat bran with larvae but without *Salmonella* sp. (negative control with larvae) and wheat bran with larvae and with *Salmonella* sp. In designs 2 and 3, a fourth condition consisting of wheat bran without larvae and without *Salmonella* sp. (negative control without larvae) was also included in order to confirm that no cross-contamination between trays (= replicates) occurred. In all of nine individual experiments, two replicates were included for each condition.

Table 5.1 Overview of experimental set-up for all three designs. Each design consisted of three identical experiments, each time using a different batch of mealworms. The number of replicates (trays) for different conditions within one experiment is shown.

Design number	Mealworm batches	Inoculation method	Sampling days	Larval treatment	Analyses[1]	Target *Salmonella* sp. contamination level in wheat bran (log cfu/g)	Number of replicates without larvae (per sampling day)	Number of replicates with larvae (per sampling day)
1	1, 2 and 3	20 x 50 µl	1, 3 and 7	Disinfection[2]	TVC, *Salmonella* sp. count	Control[3]	0[4]	2
						4 log cfu/g	2	2
						7 log cfu/g	2	2
2	4, 5 and 6	2 x 100 µl	1 and 7	Disinfection[2]	TVC, *Salmonella* sp. presence/absence	Control[3]	2	2
						2 log cfu/g	2	2
						4 log cfu/g	2	2
3	7, 8 and 9	2 x 100 µl	1 and 7	No treatment	TVC, *Salmonella* sp. presence/absence	Control[3]	2	2
						2 log cfu/g	2	2
						4 log cfu/g	2	2

[1]Total viable counts (TVC) and *Salmonella* sp. counts were determined on one 5-g sample of bran/larvae per replicate. *Salmonella* sp. presence/absence was determined on three 5-g samples of bran/larvae per replicate.

[2]Disinfection treatments were applied immediately before microbial analysis.

[3]Non-inoculated replicates.

[4]In design 1, no control replicates without *Salmonella* sp. and without larvae were included.

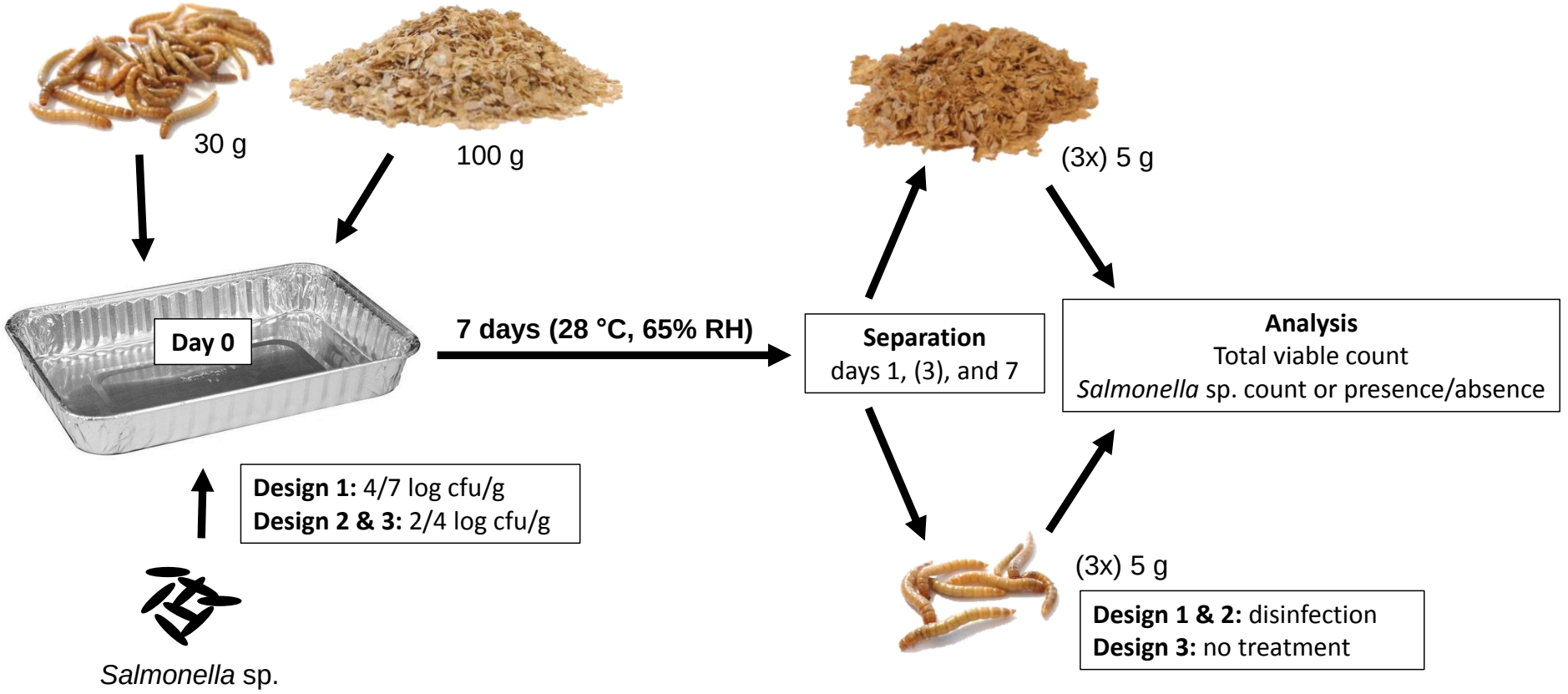

Figure 5.1 Visualisation of the general experimental design conducted for all batches. For each experiment, control groups including bran without larvae, bran without *Salmonella* sp., and/or bran without larvae and without *Salmonella* sp. were also included.

5.2.2 Cultivation of *Salmonella* sp. and inoculation of wheat bran

The *Salmonella* sp. culture used in this study consisted of a mixture of three *Salmonella* sp. strains, being *Salmonella* sp. *enterica* serovar Enteritidis (LMG 18735), *Salmonella* sp. *enterica* serovar Typhimurium (LMG 18732) and *Salmonella* sp. *enterica* serovar Infantis (LMG 18746), all purchased from the Belgian Coordinated Collection of Microorganisms (BCCM). Those strains are among the most common *Salmonella* sp. strains to cause food-associated illness in humans (Adams & Moss, 2008). The use of a mixture of three strains allows to account for variations in growth characteristics among different strains. The strains were cultivated overnight at 37 °C in nutrient broth (VWR international, Radnor, USA) and, after incubation, the broth was centrifuged (3060 rcf, 5 minutes, Multifuge 3S, Heraeus, Hanau, Germany). The supernatant was then discarded and the cells in the pellet were diluted in peptone physiological salt solution (0.85% NaCl and 0.1% peptone). Each *Salmonella* sp. strain was diluted until 3.0 MacFarland units were reached as measured by densitometry (DEN-1 McFarland Densitometer, Grant instruments, Cambridge, UK). This value was shown in a preliminary experiment by means of plate counting (according to the methods described in section 5.2.4) to approach a cell concentration of 9 log cfu/ml. Next, strains were combined in equal volumes and the inoculum was further diluted until the desired concentration for inoculation was obtained. The inoculum was added to 100 g of wheat bran (Tarwezemelen 734/3, AVEVE, Leuven, Belgium) which were placed into sterile aluminium trays (2 l, approximately 5 x 17 x 24 cm). In design 1, which was conducted on batches 1 to 3 and which represented a “worst case” contamination level of the wheat bran, the *Salmonella* sp. culture was inoculated to reach 4 and 7 log colony forming units per gram of wheat bran, respectively, at day 0 (Table 5.1): inoculation of the bran was performed by adding 20 droplets of 50 µl with the desired concentration (by diluting the initial inoculum) to the bran in each replicate, followed by homogenisation using a sterile spoon. In designs 2 and 3, conducted on batches 4 to 9, contamination levels of *Salmonella* sp. of 2 and 4 log cfu/g were used, since these numbers may be more likely to occur in rearing facilities. Furthermore, the inoculum was not added homogeneously as in design 1, but in two droplets (2x 100 µl; Table 5.1) of the desired concentration, as contamination in wheat bran in rearing facilities can be expected to occur in a heterogeneous way.

5.2.3 Mealworm rearing and sampling

In order to study whether *Salmonella* sp. survives in the wheat bran without larvae, replicates without the addition of mealworms were included for experiments of all three designs. For the other replicates, 30 g of mealworms were added to each tray at day 0. Larvae with an age of 9 to 11 weeks were used for the study, as fully grown larvae are easier to handle compared to young larvae. Mealworms used for this study were reared by RADIUS (Thomas More University College, Geel, Belgium) on a diet consisting of the same wheat bran (AVEVE Tarwezemelen 734/3), supplemented with either carrots or chicory, and sieved from the substrate before use. All replicates, both with and without larvae, were then placed in a climate chamber (28 °C, 65% RH; Memmert HPP260, Memmert, Eeklo, Belgium) for seven days. At days 0, 2, 4, and 6, carrot slices were added in quantities of 10 g to each replicate containing larvae, but not in control replicates. Ideally, carrots would also be added to the control trays, but this would have led to misleading results as it was shown in a preliminary experiment that the addition of carrots in the absence of larvae led to excessive moulding of the wheat bran. For design 1, samples were taken at 1, 3 and 7 days after the start of the experiment. More specifically, 5 g of wheat bran and 5 g of larvae were separated under aseptic conditions from each replicate and further analysed. In this first design, at each sampling day, samples were taken from the same replicates. For designs 2 and 3, three samples of 5 g of wheat bran and three samples of 5 g of larvae were sampled from each replicate at days 1 and 7. Due to the relatively large sampling size, by removing approximately half of mealworms per replicate, the sampling of the same replicates at day 7 would not be representative due to the distortion of the ratio of larvae to bran. Therefore, four replicates instead of two replicates for each condition were set up at day 0, two of which were discarded after sampling at day 1, and the two other were sampled at day 7. In all of three experimental designs, the wheat bran was analysed without prior manipulation. Larvae, on the other hand, were disinfected in designs 1 and 2 by washing them (5 g) in 100 ml of 70% ethanol, followed by two subsequent washing steps in 100 ml of sterile demineralised water. Each washing step was performed for 1 min on a laboratory shaker (Unimax 1010, Heidolph, Germany) at 200 rpm. In design 3, larvae were analysed without prior disinfection for the reasons described in section 5.2.1. Prior to each microbial analysis, larval samples were diluted tenfold in either peptone physiological salt solution or buffered peptone water

(depending on the analysis). Next, they were homogenised together with the solution using a home type mixer according to Stoops et al. (2016).

5.2.4 Microbial analyses

For each separate experiment, the inoculum was plated onto RAPID' *Salmonella* sp. agar (BioRad Laboratories, Hercules, USA) and incubated for 24 h at 37 °C in order to confirm whether it contained 9 log cfu/ml of *Salmonella* sp. cells in the undiluted inoculum, as aimed for during inoculum preparation. This procedure revealed that *Salmonella* sp. numbers in the undiluted inoculum ranged between 8.6 and 9.0 log cfu/ml. The actual concentration of *Salmonella* sp. in the wheat bran could not be measured immediately after contamination, but only after one day. Therefore, the contamination levels obtained immediately after inoculation, i.e. 2, 4 or 7 log cfu/g are in the following paragraphs referred to as the "target contamination levels".

Total viable counts were determined for all three designs on one 5-g sample of bran and larvae (if present) per replicate, according to the ISO-standards as described by Dijk et al. (2015), by plating onto plate count agar (PCA, Biokar diagnostics, Beauvais, France) and incubating at 30 °C for 72 h. In addition, in design 1, the number of *Salmonella* sp. was determined for each sample by preparing a ten-fold dilution series in peptone physiological salt solution (0.85% NaCl, 0.01% peptone), plating onto the chromogenic RAPID' *Salmonella* sp. agar (BioRad Laboratories) and incubating at 37 °C for 24 h. Presumptive *Salmonella* sp. colonies were regularly confirmed throughout the experiment (30 confirmation tests in total) using a *Salmonella* sp. latex kit (BioRad Laboratories), never revealing presumptive colonies to be non-Salmonellae. In designs 2 and 3, the presence or absence of *Salmonella* sp. was determined on all three samples of wheat bran and all three samples of larvae (if present) per replicate, using the RAPID' *Salmonella* sp. Agar short protocol (certificated by NF validation according to ISO 16140 N° 07/11-12/05, BioRad Laboratories). Briefly, the sample was diluted tenfold in buffered peptone water (BioRad Laboratories), supplemented with a *Salmonella* sp. specific capsule supplement (BioRad Laboratories), homogenised and incubated at 41.5 °C for 18 h. Next, the enriched solution was streaked onto RAPID' *Salmonella* sp. agar and incubated at 37 °C for 24 h, after which presumptive *Salmonella* sp. colonies (at least one colony per replicate) were confirmed using the latex agglutination kit.

5.2.5 Additional analyses: larval growth and water activity of the wheat bran

For each design, larval growth per replicate was determined by measuring the combined weight of 30 *ad random*-picked larvae at days 1 and 7. Furthermore, the water activity of wheat bran, prior to inoculation, was determined in triplicate according to the methods described in Chapter 2.

5.2.6 Statistical analyses

For design 1, total viable counts as well as *Salmonella* sp. counts of larvae and wheat bran samples for each condition were compared between sampling moments using repeated measures ANOVA in SPSS (IBM SPSS statistics v.23; Table 5.2). For designs 2 and 3, pair-wise t-tests were conducted in order to compare total viable counts between day 1 and 7 for wheat bran and larvae of each condition (Tables 5.3 and 5.4). When counts of a sample were below the detection limit, the detection limit itself was chosen as value to be included for statistical analysis. For each test, a significance level of $\alpha = 0.05$ was considered.

5.3 RESULTS

5.3.1 Design 1: high and homogeneous contamination and disinfected larvae

In the first design, the aim was to contaminate bran to reach a contamination level of 7 and 4 log cfu of *Salmonella* sp. per g bran. *Salmonella* sp. counts obtained at day 1 were close to the target contamination levels, being 6.5 and 3.3 on average for all samples treated, respectively (Table 5.2).

In the wheat bran without larvae, total viable counts did not significantly change during the 7-day rearing interval considered (Table 5.2). Already at day 1, the bran with larvae contained higher total viable counts compared to the wheat bran without larvae. Here, further increases were observed towards day 7 ($p = 0.007$, $p < 0.001$, and $p < 0.001$ for control, 4 log cfu/g and 7 log cfu/g contamination levels, respectively). These observations are most likely due to the excretion of faeces into the bran when larvae are present. The larvae did not show significant changes with respect to the total viable count in their interior microbial load, which ranged on average from 8.1 to 8.3 log cfu/g. The number of *Salmonella* sp. did not significantly change during the course of the experiment when larvae were absent (Table 5.2). For the replicates including larvae, not only contaminated replicates were included but also non-inoculated replicates as a control. Both the bran as well as the larvae of control

replicates showed *Salmonella* sp. counts below the detection limit. This is an important observation, as it proves that no cross contamination took place between inoculated and non-inoculated trays despite the fact that they were placed in the same climate chamber. This is not evident, since indications of airborne *Salmonella* transmission exist but needed to be absent in our set-up (Gast, Mitchell, & Holt, 1998; Holt, Mitchell, & Gast, 1998; Oliveira, Carvalho, & Garcia, 2006).

When the bran was inoculated and when larvae were present, the *Salmonella* sp. count in the bran decreased during the rearing interval considered. This effect was significant for the highest contamination level, where counts decreased from 5.8 to 3.7 log cfu/g on average ($p = 0.016$). For the lower contamination level, *Salmonella* sp. was present at 2.9 log cfu/g at day 1, but it was detected in only some bran samples analysed at day 7. This indicates that a decrease did occur, although this effect was not significant ($p = 0.099$). In the larvae, the *Salmonella* sp. counts did not significantly change towards day 7 for both contamination levels. For the 7 log cfu/g contamination level, the pathogen remained present in numbers of 4.1 log cfu/g on average at day 7. In contrast, in the 4 log cfu/g contamination level, *Salmonella* sp. was below the detection limit of 1 log cfu/g for all larval samples at day 7, suggesting that a decrease – although not significant – did occur.

5.3.2 Design 2: lower and heterogeneous contamination and disinfected larvae

For both target contamination levels of 2 and 4 log cfu/g, all samples of wheat bran - both with and without larvae - tested positive after one day (Table 5.3). The latter indicates that, even though the first sampling occurred one day after the start of the experiment, inoculation was successful for both experimental conditions.

Total viable counts of the bran without larvae were not significantly different at day 7 compared to day 1. Since lower numbers of *Salmonella* sp. were inoculated as compared to design 1, the average total viable count was lower and ranged between 4.3 and 4.6 log cfu/g, which was lower compared to design 1, presumably due to the difference in inoculation quantity and method. When larvae were present, the total viable count of the bran significantly increased with approximately 1 log unit from day 1 to day 7 ($p = 0.003$, 0.002 and 0.004 for control, 2 log cfu/g and 4 log cfu/g contamination levels, respectively), and counts were comparable to those obtained in design 1. Also for the larvae, the total viable counts were comparable to those in

design 1, ranging on average from 8.2 to 8.3 log cfu/g, and remaining stable during the one week experimental period.

For wheat bran without larvae and for the larvae themselves, all samples from all replicates that were not inoculated tested negative for the presence of *Salmonella* sp., showing that no cross contamination occurred. All samples of wheat bran without larvae from all inoculated replicates tested positive for *Salmonella* sp. at both days 1 and 7. When larvae were present, all samples remained contaminated with *Salmonella* sp. for the 4 log cfu/g contamination level. In contrast, all wheat bran samples of the lower contamination level of 2 log cfu/g tested positive for *Salmonella* sp. at day 1, but only one sample was positive at day 7 (no data were obtained for batch 4). Finally, in the larvae retrieved from the 4 log cfu/g contamination level, every sample tested positive for *Salmonella* sp. at day 1, but only four out of the eighteen samples remained positive at day 7. In the lower 2 log cfu/g contamination level, in contrast, already at day 1 only five of eighteen samples were positive for *Salmonella* sp., and at day 7, even none of the samples tested positive.

5.3.3 Design 3: lower and heterogeneous contamination and non-disinfected larvae

Similar as to design 2, all wheat bran samples inoculated with *Salmonella* sp. in design 3 were still positive for *Salmonella* sp. at day 1 (Table 5.4), indicating that inoculation was successful.

Results for total viable counts are very similar to design 2. Total viable counts remained constant for the wheat bran without larvae, except for a statistically significant decrease for the 4 log cfu/g contamination level. However, from a microbiological point of view, the difference of 0.4 log cfu/g is likely due to inherent variation in microbial counts. For wheat bran with larvae, the total viable counts increased in the same way as in design 2 by approximately one log unit, and the increase was significant for all treatments. Similar to design 2, larval counts remained constant and varied from 8.1 to 8.2 log cfu/g on average.

Also in design 3, all non-inoculated control samples were negative for *Salmonella* sp. at both sampling days. While all inoculated wheat bran samples, both with and without larvae, were positive for *Salmonella* sp. at day 1, this was not the case at day 7. At the latter sampling day, while all bran samples of the 4 log contamination level remained positive for *Salmonella* sp., this was only the case for fifteen out of eighteen

samples for bran without larvae, and four out of eighteen samples for bran with larvae of the 2 log contamination level. With respect to the larvae, the presence of *Salmonella* sp. also differed between contamination levels. For the 4 log contamination level, *Salmonella* sp. was detected in all samples at day 1, but only in three out of eighteen samples at day 7. For the 2 log cfu/g contamination level, on the other hand and similar to design 2, only eleven larval samples were contaminated at day 1, but *Salmonella* sp. was detected in none of the samples after seven days.

Table 5.2 Total viable counts and *Salmonella* sp. counts from wheat bran and larvae of design 1 involving high and homogeneous contamination and disinfected larvae. Values represent the mean (± standard deviation) of three batches (batches 1 – 3), each with two replicates per batch (n = 3 x 2).

Sample	Target *Salmonella* sp. contamination level in wheat bran (log cfu/g)	Total viable count (log cfu/g)			*Salmonella* sp. count (log cfu/g)		
		Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Wheat bran without larvae°	4	5.6 ± 0.3[a]	5.2 ± 0.2[a]	5.6 ± 0.3[a]	3.3 ± 0.1[a]	2.9 ± 0.4[a]	2.6 ± 0.8[a]
	7	6.8 ± 0.3[a]	6.4 ± 0.2[a]	6.2 ± 0.4[a]	6.5 ± 0.3[a]	5.4 ± 1.1[a]	5.5 ± 0.8[a]
Wheat bran with larvae	Control*	7.2 ± 0.2[a]	7.6 ± 0.2[ab]	8.0 ± 0.3[b]	<1.0[a]†	<1.0[a]†	<1.0[a]†
	4	7.2 ± 0.1[a]	7.7 ± 0.1[b]	8.0 ± 0.1[c]	2.9 ± 0.4[a]	<1.9 ± 0.6[a‡]	<1.5 ± 1.1[a‡]
	7	7.5 ± 0.2[a]	7.5 ± 0.1[a]	8.0 ± 0.1[b]	5.8 ± 0.2[a]	3.9 ± 1.3[ab]	3.7 ± 0.4[b]
Larvae, disinfected	Control*	8.2 ± 0.1[a]	8.3 ± 0.1[a]	8.3 ± 0.1[a]	<1.0[a]†	<1.0[a]†	<1.0[a]†
	4	8.1 ± 0.1[a]	8.3 ± 0.2[a]	8.1 ± 0.4[a]	<2.7 ± 1.5[a‡]	<1.3 ± 1.5[a‡]	<1.0[a]†
	7	8.2 ± 0.1[a]	8.2 ± 0.2[a]	8.2 ± 0.3[a]	4.4 ± 0.8[a]	4.4 ± 1.3[a]	4.1 ± 1.1[a]

[abc]Average values for total viable counts and *Salmonella* sp. counts within each row that share a letter in superscript did not significantly ($p \geq 0.05$) increase or decrease between sampling days, as was shown from repeated measures ANOVA.

*Non-inoculated replicates.

°In design 1, no control replicates without *Salmonella* sp. and without larvae were included.

†"<1.0" indicates that *Salmonella* sp. was below the detection limit (1 log cfu/g) in every sample.

‡"<" followed by a value higher than 1.0 log cfu/g indicates that *Salmonella* sp. was below the detection limit in at least one, but not all samples.

Table 5.3 Total viable counts and number of *Salmonella* sp. positive samples from wheat bran and larvae of design 2 involving lower and heterogeneous contamination and disinfected larvae. Total viable counts are represented by the mean of three batches (batches 4 – 6), each with two replicates per batch (n = 3 x 2). The number of samples that tested positive for *Salmonella* sp. (out of three samples) are shown for individual replicates per batch (replicate 1 + replicate 2).

Sample	Target *Salmonella* sp. contamination level in wheat bran (log cfu/g)	Total viable count (log cfu/g)		Number of *Salmonella* sp. positive samples per three samples taken for each replicate (replicate 1 + replicate 2)					
				Day 1			Day 7		
		Day 1	Day 7	Batch 4	Batch 5	Batch 6	Batch 4	Batch 5	Batch 6
Wheat bran without larvae	Control*	4.3 ± 0.3[a]	4.4 ± 0.4[a]	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0
	2	4.5 ± 0.4[a]	4.5 ± 0.6[a]	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3
	4	4.6 ± 0.2[a]	4.4 ± 0.5[a]	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3
Wheat bran with larvae	Control*	6.9 ± 0.5[a]	8.2 ± 0.2[b]	0 + 0	0 + 0	0 + 0	N.D.	0 + 0	0 + 0
	2	7.1 ± 0.3[a]	8.2 ± 0.2[b]	3 + 3	3 + 3	3 + 3	N.D.	1 + 0	0 + 0
	4	7.2 ± 0.5[a]	8.3 ± 0.2[b]	3 + 3	3 + 3	3 + 3	N.D.	3 + 3	3 + 3
Larvae, disinfected	Control*	8.3 ± 0.1[a]	8.3 ± 0.2[a]	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0
	2	8.3 ± 0.2[a]	8.2 ± 0.1[a]	2 + 2	1 + 0	0 + 0	0 + 0	0 + 0	0 + 0
	4	8.3 ± 0.1[a]	8.2 ± 0.2[a]	3 + 3	3 + 3	3 + 3	0 + 0	2 + 0	2 + 0

[ab]Average values for total viable counts within each row that share a letter in superscript did not significantly ($p \geq 0.05$) increase or decrease from day 1 to day 7 as was shown from pair-wise t-test.

*Non-inoculated replicates.

N.D. not determined.

Table 5.4 Total viable counts and number of *Salmonella* sp. positive samples from wheat bran and larvae of design 3 involving lower and heterogeneous contamination and non-disinfected larvae. Total viable counts are represented by the mean of three batches (batches 7 – 9), each with two replicates per batch (n = 3 x 2). The number of samples that tested positive for *Salmonella* sp. (out of three samples) are shown for individual replicates per batch (replicate 1 + replicate 2).

Sample	Target *Salmonella* sp. contamination level in wheat bran (log cfu/g)	Total viable count (log cfu/g)		Number of *Salmonella* sp. positive samples per three samples taken for each replicate (replicate 1 + replicate 2)					
				Day 1			Day 7		
		Day 1	Day 7	Batch 7	Batch 8	Batch 9	Batch 7	Batch 8	Batch 9
Wheat bran without larvae	Control*	4.5 ± 0.3[a]	4.5 ± 0.3[a]	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0
	2	4.8 ± 0.1[a]	4.5 ± 0.2[a]	3 + 3	3 + 3	3 + 3	2 + 3	1 + 3	3 + 3
	4	4.8 ± 0.2[a]	4.4 ± 0.1[b]	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3
Wheat bran with larvae	Control*	6.9 ± 0.1[a]	7.8 ± 0.2[b]	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0
	2	7.1 ± 0.5[a]	7.9 ± 0.1[b]	3 + 3	3 + 3	3 + 3	2 + 0	2 + 0	0 + 0
	4	7.0 ± 0.6[a]	7.8 ± 0.2[b]	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3	3 + 3
Larvae, not disinfected	Control*	8.2 ± 0.2[a]	8.2 ± 0.2[a]	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0	0 + 0
	2	8.1 ± 0.1[a]	8.2 ± 0.1[a]	1 + 2	1 + 3	1 + 3	0 + 0	0 + 0	0 + 0
	4	8.1 ± 0.2[a]	8.1 ± 0.2[a]	3 + 3	3 + 3	3 + 3	2 + 0	1 + 0	0 + 0

[ab]Average values for total viable counts within each row that share a letter in superscript did not significantly ($p \geq 0.05$) increase or decrease from day 1 to day 7 as was shown from pair-wise t-test.

*Non-inoculated replicates.

5.3.4 Larval growth and water activity of the wheat bran

During the 7-day rearing interval considered, larvae increased in weight on average between 16.5% and 56.2% (Table 5.5). It should be noted that weight increases can only be compared within one batch, as larval age slightly differed between batches. The results show that growth of larvae was not systematically less or more pronounced with increasing numbers of *Salmonella* sp. in the wheat bran. In the same way, dead larvae were only rarely observed in the replicates for all conditions. Water activity of the wheat bran prior to inoculation was 0.56 ± 0.01.

Table 5.5 Relative increase of larval weight at day 7 compared to day 1. Each value is the average increase in weight of larvae from two replicates (n = 2), measured as the weight of 30 larvae per replicate.

Design	Batch	Relative weight increase of larvae at different target *Salmonella* sp. contamination levels in wheat bran			
		Control*	2 log cfu/g	4 log cfu/g	7 log cfu/g
Design 1	1	22.2%	N.A.	42.9%	28.0%
	2	33.4%	N.A.	49.6%	36.5%
	3	33.0%	N.A.	36.5%	42.9%
Design 2	4	29.4%	26.2%	25.0%	N.A.
	5	16.5%	22.2%	24.4%	N.A.
	6	29.9%	37.7%	33.0%	N.A.
Design 3	7	33.8%	22.8%	22.9%	N.A.
	8	56.2%	46.2%	40.0%	N.A.
	9	32.8%	45.5%	34.1%	N.A.

N.A. Not applicable since the contamination level was not included in that specific experimental design.
*Non-inoculated replicates.

5.4 DISCUSSION

It is generally known that traditional livestock animals may become infected with *Salmonella* sp. when their feed is contaminated with the food pathogen. For many animals, this results in asymptomatic carriage which can remain unnoticed until the animals are slaughtered and their carcasses analysed. Depending on the way of processing of the meat, transmission may even occur up to the food products derived from the infected animals (EFSA Panel on Biological Hazards, 2008). Although edible insects highly differ in their physiology from vertebrate livestock, it is reasonable to assume that such phenomenon may also take place when the insect substrate is contaminated. *Salmonella* sp. may be present in wheat bran of a mealworm rearing

facility through a variety of routes. For instance, cereals may be infected during growth on the field through fertilisation with manure, leading to infected bran even before the bran reaches the mealworm rearing facility. Indeed, previous studies in Australia and the USA have reported a small percentage of wheat flour samples to be contaminated with *Salmonella* sp. (Berghofer et al., 2003; Richter et al., 1993; Eglezos, 2010). Since *Salmonella* sp. is likely to be present on the outer surface of the grain kernel, its numbers may even be higher in the bran after separation, as compared to the endosperm further milled into flour (Berghofer et al., 2003). In addition, wheat bran may become infected in the insect rearing facility itself. For instance, personnel might contaminate the bran before/during rearing, since the feed is usually added to the crates by hand (Schneider, 2009). So far, no obligate hygiene requirements exist in industrial insect rearing, e.g. prescribing the use of gloves and/or mouth masks. Furthermore, as was shown from multiple studies, both insects as well as birds or rodents may act as a vector for *Salmonella* sp. (Meerburg & Kijlstra, 2007; McAllister et al., 1994; Reed et al., 2003; Crippen et al., 2012; Zheng et al., 2012b). In that way, infected vermin may enter the rearing system and contaminate the bran during storage or in the rearing crates. Finally, the larvae and beetles themselves may infect other batches, e.g. when individuals escape from one batch and enter another. Especially for the lesser mealworm (*A. diaperinus*), another species of the darkling beetle, its vector potential for *Salmonella* sp. in the poultry industry was extensively investigated. Studies have shown that both larvae and adults can carry *Salmonella* sp. in their gastro-intestinal tract, possibly for a prolonged period of time after exposure (McAllister et al., 1994; Roche et al., 2009; Hazeleger et al., 2008; Crippen et al., 2009; Leffer et al., 2010; Crippen et al., 2012; Zheng et al., 2012b). Hence, it is reasonable to assume that both feral Tenebrionid larvae or beetles, as well as those being reared in production facilities, may cause cross-contamination within a facility. In addition, as was suggested in a study by Osimani et al. (2018), vertical transmission of microorganisms from the parent beetles likely contributes to development of the offspring microbiota. In this way, an infected generation might possibly result in contaminated offspring, when *Salmonella* sp. is present in or on the eggs. In this study, we aimed to assess the fate of *Salmonella* sp. when present in wheat bran, including its survival in the bran and its potential transmission to the larvae.

The results of this study show that *Salmonella* sp. remained viable during the 7-day test period in wheat bran without larvae or carrots. This suggests that when wheat bran becomes infected with *Salmonella* sp. and when it is stored under the conditions

applied in this study, the pathogen will survive for at least one week. Although the water activity of the wheat bran prior to inoculation was low, as indicated by the average measured water activity of 0.56, *Salmonella* sp. easily survives in dry environments (Adams & Moss, 2008). Moreover, inoculation by adding droplets of the *Salmonella* sp. suspension may have increased the availability of water at those locations in the bran. Furthermore, due to the addition of moisture-rich components such as carrots to rearing crates and the excretion of faeces by the larvae, the availability of moisture to the microbiota present may increase. That can allow microbial growth, as shown in this study by the increase in total viable count of the bran in replicates with larvae. However, the results of this study show that when larvae and carrots were present, *Salmonella* sp. counts in both bran and larvae decreased in design 1, for both 4 and 7 log cfu/g contamination levels, and so did the number of *Salmonella* sp. positive samples in the 2 log cfu/g (for both bran and larvae) and 4 log cfu/g (for larvae) contamination levels of designs 2 and 3. Noteworthy, the disinfection treatment could have affected the microbiota in the insect gut. Still, similar results were obtained for designs 2 and 3, either with or without disinfection. These results indicate that when wheat bran is used for mealworm rearing, the larvae, carrots and/or the rearing conditions have a reducing effect on *Salmonella* sp. It cannot be stated with certainty that the reduction of *Salmonella* sp. in the rearing trays with larvae was caused solely by the larvae, or by the carrots or by a combination of both. Nevertheless, mealworm rearing using wheat bran will in practice always include the addition of moist components as water source for the larvae (e.g. carrots, cucumbers, chicory, moist brewer's grains, …).

In the following paragraph, a number of hypotheses are listed which may contribute to the reduction of *Salmonella* sp. during mealworm rearing. First, when larvae and carrots were present, microbial numbers in the rearing trays highly increase to values op to 8.3 log cfu/g, indicating a high number of background flora. It is known that bacteria can outcompete others via exploitative competition (depletion of nutrients to limit growth) or via interference competition (production of antagonistic factors to inhibit growth) (Stubbendieck & Straight, 2016). Another possibility might be digestion of the *Salmonella* sp. cells in the gastro-intestinal tract of the larvae (Lemos & Terra, 1991), or the production of anti-microbial peptides (AMPs) by the larvae. To date, many AMPs have been identified from insects as part of their innate immune response (Yi, Chowdhury, Huang, & Yu, 2014). Some compounds may inhibit specific bacterial groups. This was demonstrated for extracts of the housefly (*Musca domestica)* and

certain peptides from the Carolina sphinx moth (*Manduca sexta*), that showed antimicrobial activity against *Salmonella* Typhimurium (Hou et al., 2007; Rao et al., 2012). Similarly, extracts of black soldier fly larvae (*H. illucens*) were found to exhibit a specific antibacterial effect against gram-negative bacteria (Choi & Jiang, 2014). Furthermore, multiple studies have shown a decrease of *Salmonella* sp. in various substrates in the presence of housefly or black soldier fly larvae (Erickson et al., 2004; Lalander et al., 2013; Lalander et al., 2015; Nguyen et al., 2015a; Nordentoft et al., 2017). In yellow mealworms, an AMP named Tenecin 4 has been identified which was shown to possess properties against gram-negative bacteria (Chae et al., 2012). Possibly, AMPs exert their effect(s) during rearing of mealworms causing the reduction of *Salmonella* sp., but this hypothesis needs further investigation. A final explanation may be the presence of arabinoxylooligosaccharides in the wheat bran, which were shown to decrease the number of *Salmonella* sp. in the gut of poultry (Eeckhaut et al., 2008). Furthermore, when the bran is of fine particle size, it was shown to lower cecal *Salmonella* sp. colonisation in broilers due to the production of short chain fatty acids - such as butyric and propionic acid - by the gut microflora of the broilers, which in turn reduced the presence of *Salmonella* sp. in the cecae (Vermeulen et al., 2017). Similarly, consumption of the bran by the larvae might contribute to the fermentation of the bran by bacteria in the larval gut, since also for mealworms the gut microbiota is shown to have a functional role in digestion (Genta et al., 2006). The reduction of *Salmonella* sp. may also be caused by a combination of the above mentioned mechanisms, which clearly deserves to be unravelled by further research.

Nevertheless, no reduction of *Salmonella* sp. was observed in larvae when the bran was contaminated with *Salmonella* sp. at a target number of 7 log cfu/g. Possibly, the 7-day experimental period was too short for *Salmonella* sp. to be reduced when present in such high numbers or it was simply too abundant for any reduction. In contrast and remarkably, for the lowest contamination level of 2 log cfu/g, no single larval sample -either disinfected or not- was still positive for *Salmonella* sp. after 7 days. These results suggest that the colonisation potential of *Salmonella* sp. in a mealworm rearing system is highly dependent on the original contamination level of the bran. This complies to a statement by NVWA (2014), stating that when *Salmonella* sp. contamination of insects occurs, this would likely be the result of environmental contamination, and that the bacterium is unlikely to reach high numbers in reared insects. In the EU legislation (EC 2073/2005), no specific criteria for *Salmonella* sp., or any microbiological criteria in general, for mealworms sold for consumption exist to

date. According to a local criterion in Belgium, however, insect producers must guarantee the absence of *Salmonella* sp. in five samples of 10 g for mealworms that will receive a further heat treatment, or in five samples of 25 g of harvested mealworms for which further heat treatment is not guaranteed and in mealworm products ready for consumption or (FASFC, 2018a). In our study, presence/absence was only determined on three samples of 5 g, due to availability of larvae. However, this represented 50% and 15% of the total initial amount per replicate of larvae and bran, respectively, which was considered as a reliable sample size. It appears from this study that the microbial risk may be low when *Salmonella* sp. is present in wheat bran at contamination levels of maximally 2 log cfu/g, and when larvae are further reared for at least seven days without further addition of contaminated wheat bran to the rearing crates.

Still, many factors may influence the fate of *Salmonella* sp. in a mealworm rearing system. For instance, transmission potential may be different if larvae are younger as compared to those used in this study. Younger larvae consume a lower amount of bran in a given time as compared to older larvae, thereby lowering chances of taking up *Salmonella* sp. On the other hand, younger larvae may have not yet fully developed a stable gut microbiota, that may exhibit less efficient colonisation resistance to newly encountered microorganisms as compared to the gut microbiota of older larvae. For instance, it was shown that the gut microbiota of 2-weeks old industrially reared *A. diaperinus* larvae differs to a large extent from that of 6-weeks old larvae (Wynants et al., 2018a). In addition, the bran may already contain a background microbiota at the timing contamination, in contrast to the current study during which fresh wheat bran was used. A higher background flora might impede the long-term colonisation of the bran by *Salmonella* sp. Transmission potential may also depend on larval density and ratio of larvae to wheat bran, environmental conditions, etcetera. Furthermore, a mixture of three *Salmonella* sp. strains was used here, but it is not known whether the results would differ depending on the strains used. Further research is needed, covering the variability in the above mentioned factors, in order to obtain a full risk assessment for *Salmonella* sp. in mealworms. Nevertheless, this study demonstrates that *Salmonella* sp. easily survives in wheat bran for at least one week before administration to the larvae, and that mealworms can become contaminated with *Salmonella* sp. Moreover, as no inhibition of growth or increased mortality was observed in the larvae when *Salmonella* sp. was present (Table 5.5), its presence cannot be deduced from the condition of the larvae. Regular microbiological control of

the wheat bran and of the harvested larvae for the presence of *Salmonella* sp. is thus advised. In addition, research is needed on the survival of *Salmonella* sp. through subsequent processing steps applied on the larvae.

5.5 CONCLUSIONS

The results from this study demonstrate in the first place that wheat bran as a substrate for mealworms can remain contaminated for at least seven days once infected with *Salmonella* sp. However, when wheat bran was used for mealworm rearing, including the presence of larvae and carrots, a reduction of *Salmonella* sp. in the bran was observed, which may be explained by a variety of factors. Nevertheless, *Salmonella* sp. was not completely eradicated in the bran after one week. However, the retention of *Salmonella* sp. in the larvae was shown to depend on the contamination level in the bran. Low numbers of maximally 2 log cfu/g *Salmonella* sp. in the bran did not result in contaminated larvae after seven days. However, caution is needed when interpreting the results obtained, as they should not directly be extrapolated to other rearing conditions. Frequent testing of the wheat bran, and especially of the harvested mealworms for *Salmonella* sp. is therefore advised.

CHAPTER 6

Effect of post-harvest starvation and rinsing on the microbial numbers and the bacterial community composition of mealworms (*Tenebrio molitor*)

Modified from:

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*The complete content of this paper was included in Chapter 6, with only small alterations to keep the information provided up to date and to follow the logic course of this dissertation. As first author, E.W. contributed to all parts described in this work, from experimental design, conducting the laboratory analyses, to the writing of the paper. High-throughput 16S rRNA gene sequencing and bio-informatic analysis of sequencing data were performed in collaboration with the Laboratory for Process Microbial Ecology and Bioinspirational Management (PME&BIM), KU Leuven (Prof. B. Lievens).

6.1 INTRODUCTION

Rearing techniques, rearing environment, substrate, hygiene measures and specific handling procedures, such as starvation and rinsing after harvest, are suggested to affect the microbiota of insects (Dillon et al., 2010; Klunder et al., 2012; Engel & Moran, 2013; SHC & FASFC, 2014; EFSA Scientific Committee, 2015; Li et al., 2016b), but no specific information exists. According to a risk analysis of the NVWA (Netherlands Food and Consumer Product Safety Authority), mealworms (*Tenebrio molitor*) are reared in industrial rearing companies at a temperature of 28 to 30 °C and a relative humidity of 60%. The substrate generally consists of bran mixed with flour or ground chicken feed, supplemented with carrots, potatoes and water. After eight to ten weeks of rearing, the last larval stage of the mealworm is harvested by sieving. The larvae are then often starved for one or two days in order to empty their gut. Then they are rinsed with lukewarm to warm water and killed by freezing (NVWA, 2014, and personal communication with insect farmers). When they would be unnecessary, however, these procedures would imply a loss of time by adding extra steps to the rearing cycle, and starvation causes a weight loss in the larvae and hence a loss in produced biomass weight. Some rearing companies assume that the emptying of the gut and rinsing of the larvae enhance the microbiological quality of the larvae. Indeed, it is known that the gut microbiota of insects can harbour a diversity of parasites, fungi and other microorganisms (SHC & FASFC, 2014). Rumpold et al. (2014) observed that the overall microbial load of the mealworms was generally higher (approximately one log cycle) than the surface contamination, which was suggested to be due to the gut microbiota. However, although applied by several companies, the impact of these practices on the microbiological quality of insects as a feed and food matrix has never been investigated. More information is needed for insect farmers in order to optimise rearing practices and also to support the evaluation of insects as Novel Food as they will receive the Novel Food status as from 1 January 2018, according to the renewed European Novel Food Regulation (EU) N° 2015/2283. Research on the effect of starvation of insects for consumption on their food safety was also recommended in an advisory report by the Belgian Superior Health Council (SHC) and Federal Agency for the Safety of the Food Chain (FASFC) (2014).

The goal of this study was to examine whether two specific industrial practices performed at the end of the rearing cycle of mealworms, i.e. starvation and rinsing, have an impact on the microbiota of freshly harvested larvae. In a first experiment,

starvation was investigated under different conditions with respect to duration, temperature and contact with faeces. Both culture-dependent plate counts as well as high-troughput 16S rRNA gene sequencing based community profiling (based on the Illumina Miseq platform) were used to evaluate the microbiota. In a second experiment, the effect of rinsing on the microbial load of both starved and non-starved larvae was assessed by means of plate counts.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design

Final instar mealworms were obtained from an industrial rearing company in Belgium. The larvae were kept for maximum 24 h in the substrate, which consisted of wheat bran supplemented with carrot pieces as supplied by the company, in a disinfected plastic container (39.5 x 34 x 19.1 cm) until use. Starvation was performed under four different conditions. In particular, larvae were starved either at 10 °C or at 30 °C, representing the two temperatures that are commonly used in industry (NVWA, 2014). For both temperatures, starvation was carried out for larvae in contact with their faeces (as is the case in industrial rearing) as well as for larvae that could not take up their faeces (to examine whether a more stringent way of starvation would make a difference). For each condition, a control group of non-starved larvae was included. The experiment was performed on three different batches for each of the four conditions: 30 °C with faecal contact (batch 1 to 3), 30 °C without faecal contact (batch 4 to 6), 10 °C with faecal contact (batch 7 to 9) and 10 °C without faecal contact (batch 10 to 12).

In a second series of experiments, the effect of rinsing was studied for both non-starved and starved larvae. Microbial counts were determined and compared to those of a non-rinsed control group. The microbial load of the tap water before rinsing and of the residual rinsing water was also determined. These experiments were performed with three batches of larvae.

Starvation. For each batch, three samples of 30 g of larvae were sieved out of the substrate and analysed (counts and metagenetics, see below). Then, 800 g of larvae were sieved out of the substrate. Four hundred grams of larvae were placed back in the substrate as control group and kept in a first container (see section 6.2.1), while the remaining 400 g were transferred into a second, empty container for starvation.

That container was, depending on the batch, either or not equipped with a sieve consisting of a plastic mosquito net (mesh size 1 mm). The sieve allowed the faeces to fall through during starvation, while the larvae were kept on the sieve. When faecal contact (and thus possible consumption of the faeces) was allowed, the larvae were placed directly, without sieve, into the container. Subsequently, both the control and starvation group were placed, depending on the batch, in an incubator (Heratherm, Thermo Scientific, Waltham, Massachusets, USA) with set point at 30 °C and ranging between 28 and 32 °C, or in a refrigerator (DynaCool, Miele, Gütersloh, Germany) with set point at 10 °C and ranging between 8 and 12°C. From each group, three replicate samples of 30 g of larvae were taken after 24 and 48 h for analysis.

Rinsing. Each batch of mealworms was divided into a control group of non-starved larvae and larvae that were starved for 48 h at room temperature and without faecal contact as described above. Subsequently, both groups of larvae were subjected to a rinsing procedure: aliquots of 30 g of larvae were transferred into a sterile 250-ml flask containing 100 ml of tap water and shaken for 1 min at 200 rpm on a laboratory shaker (HS501 Digital, IKA Labortechnik, Staufen, Germany). Then, the larvae were drained over a disinfected sieve and the rinsing water was collected. Microbial counts of the non-rinsed larvae, the rinsed larvae and the tap water before and after rinsing were determined. For each batch, samples were analysed in two- or threefold, resulting in a total of eight replicates per condition over all batches.

6.2.2 Classical microbiological analyses

Each larvae sample was kept at 3 °C for approximately one hour for sedation, after which it was pulverised prior to analysis as described by Stoops et al. (2016). Water samples from the rinsing experiment were kept at 3 °C until analysis. Plate counts were performed according to the ISO standards for microbiological analyses of food as compiled by Dijk et al. (2015), except for fungi which were determined according to Dijk et al. (2007). Total viable counts, Enterobacteriaceae and aerobic bacterial endospores were determined as described in Chapter 2. Yeasts and moulds were determined on Oxytetracycline Glucose Agar (OGA, Biokar Diagnostics) supplemented with oxytetracycline (90.91 mg/l, Biokar Diagnostics) incubated at 25 °C for five days. Psychrotrophic aerobic counts were determined by plating onto PCA and incubating at 6.5 °C for ten days.

6.2.3 Metagenetic analyses

In order to study possible changes in the bacterial community composition, 25 g of the pulverised larvae samples taken at 0 h and at 48 h (see section 6.2.4) were subjected to high-throughput 16S ribosomal RNA gene sequencing using the MiSeq Illumina platform. DNA extraction was performed on each sample in duplicate as described in Chapter 2. The PCR amplification differed slightly from those of the previous chapters, consisting of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 45 s, primer annealing at 60 °C for 45 s and primer extension at 72 °C for 45 s, and a final extension of 10 min at 72 °C (Table S6.1). After amplification, duplicate PCR products were combined and resolved using agarose gel electrophoresis. The amplicons within the expected size range were excised and extracted/purified from the gel using the Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Purified dsDNA amplicons were then quantified with the Qubit fluorometer with the high-sensitivity reagent kit according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). Subsequently, all samples were pooled in equimolar concentrations, and the library was diluted to 2 nM. Finally, the library was sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) using 2 x 250 bp chemistry at the Center of Medical Genetics Antwerp (University of Antwerp, Antwerp, Belgium) according to the principle outlined in Kozich et al. (2013).

Data analysis differed in several aspects from that applied in the previous chapters. Resulting sequences were received in the format of a de-multiplexed FASTQ file. Paired-end reads were merged using USEARCH (v.8.1) (Edgar, 2013) to form consensus sequences originating from each sample with a maximum number of 10 mismatches allowed in the overlap region. Subsequently, reads with a total expected error threshold above 1.0 for all the bases in the read were discarded. Next, remaining sequences with a minimum abundance of two, were grouped into species-level operational taxonomic units (OTUs) based on a 3% sequence dissimilarity cut-off and discarding chimeric sequences using the UPARSE greedy algorithm implemented in USEARCH (Edgar, 2013). Global singletons (i.e. OTUs representing only a single unique sequence in the entire dataset) were removed after UPARSE clustering in order to minimize the risk of retaining sequences from sequencing errors (Brown et al., 2015; Waud et al., 2014). Due to uneven sequencing depth the number of sequences was rarefied to 9000 sequences per sample. Two DNA-extracts that rendered too few sequences were omitted from further analysis, leaving one DNA-extract for those

samples (batch 3, non-starved (control) larvae (48 h) and batch 11, starved larvae (48 h)). Next, 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 (Quast et al., 2013) and taxonomic assignments were considered reliable when ≥ 0.80 score value was found. DNA-sequences originating from chloroplasts or mitochondria were eliminated with Mothurs "remove lineage" command. Additionally, OTU representative sequences (selected by UPARSE) were subjected to a BLAST (Altschul et al., 1990) search against GenBank (Benson et al., 2013), excluding uncultured/environmental entries. Nonmetric multidimensional scaling (NMDS), Chao1 and Shannon-Wiener diversity indices calculations were performed on the microbial communities of the samples using R-packages (R Development Core Team, 2013) Vegan (v.2.41) and Phyloseq (v. 1.19.0). Two DNA extracts that rendered a coverage, based on Chao1, of below 50% were omitted from the dataset, leaving one replicate DNA extract for those samples (batch 1 non-starved (control) larvae (0 h) and batch 11 non-starved (control) larvae (48 h)).

6.2.4 Statistical analyses

Statistical analyses were performed using SPSS (IBM SPSS Statistics version 23, New York, USA). For starvation, data were compared per condition and per microbial count by one-way ANOVA. Hence, statistical differences could be detected not only between the control and the starvation group at 24 and 48 h, but also between the time intervals. In addition, one-way ANOVA was performed on the Chao1 and Shannon-Wiener diversity indices of each condition after 0 and 48 h. One-way ANOVA was also performed for each microbial count of the rinsing experiment, comparing larvae subjected to the starvation (48 hours) procedure, the rinsing (1 min) procedure, both procedures or neither procedure (control). For all analyses, multiple comparisons were performed using Tukey's *post hoc* test, while considering a 0.05 significance level.

6.3 RESULTS

6.3.1 Starvation

Classical microbiological analyses. Tables 6.1 and 6.2 show the microbial counts for each condition, averaged over all batches investigated. Initially, at the start of each experiment, total viable counts ranged from 7.9 to 8.4 log cfu/g (Tables 6.1 and 6.2), Enterobacteriaceae counts from 6.9 to 7.6 log cfu/g, endospore counts from 1.5 to

2.0 log cfu/g, fungal counts from 5.6 to 6.5 log cfu/g, and psychrotrophic counts from 6.7 to 7.0 log cfu/g (Tables 6.1 and 6.2). After 24 and 48 hours, no considerable changes were observed in microbial numbers of mealworms during the starvation period for any of the conditions investigated (Tables 6.1 and 6.2). Furthermore, no statistical differences were observed between starved and non-starved larvae for any of the counts in any of the conditions (all p-values > 0.05). Some statistically significant differences could be detected between numbers at different moments within a condition. For example, at 30 °C and with faecal contact (Table 6.1), the psychrotrophic aerobic count of the larvae was statistically lower ($p = 0.046$) after 48 h starvation (6.1 log cfu/g) than at the start of the experiment (6.7 log cfu/g). At the same temperature but without faecal contact (Table 6.1), a significant increase ($p = 0.031$) from 5.7 to 6.7 log cfu/g in fungal counts was observed between 24 and 48 h starvation. Those results, though having statistical significance, are not considered to indicate notable changes from a microbiological point of view. Only numbers differing by one or more log cycles would be of microbial significance in this context. However, in whatever way the larvae were starved, difference between starved larvae and control and differences between time intervals were always below one log cycle.

Metagenetic analyses. Relative OTU abundances and diversity indices were averaged over two DNA extracts per sample. The average coverage per sample, based on Chao1, ranged from 68.18% to 95.32%, suggesting that the most abundant community members were covered in our study. The average number of recovered OTUs ranged from 21 to 57 (average 36.7 ± 9.2 (SD)) per sample. Table 6.3 shows the diversity indices, averaged over all batches, that were obtained per condition for non-starved and starved larvae after 0 and 48 h. The main phyla present were Proteobacteria and Firmicutes, followed by Tenericutes (67.86%, 24.67% and 5.67% of all sequences, respectively). The most abundant OTUs, represented by more than 5% of the sequences in any sample (Figures 6.1 and 6.2), all belonged to those phyla. The Bacteroidetes and Actinobacteria were low in abundance (0.76% and 0.74%, respectively). Some of the most abundant OTUs (i.e. OTUs 2, 10 and 12) could not be identified reliably to the genus level (score value < 0.80) (Table S6.2, Supporting Information). Therefore, these OTUs were further refined to taxonomic ranks by a BLAST search against the GenBank nucleotide database (Table S6.3, Supporting Information). In most samples, Enterobacteriaceae (OTUs 2, 6 and 10), a member of the Gammaproteobacteria (OTU 1), a number of lactic acid bacteria (OTUs 4, 5 and 7),

and a *Spiroplasma* member (OTU 3) represented more than 80% of all sequences (Figures 6.1 and 6.2). Some samples also contained a considerable relative abundance of the genus *Pseudomonas* (OTU 11 and 12). It is clear, when considering the relative OTU abundances in our study, that no consistent changes in the microbial community composition occurred during starvation (Figure 6.1 and 6.2). Statistical analysis showed no difference ($p > 0.05$) in the Chao1 index between non-starved and starved larvae after 0 and 48 h, indicating that starvation does not affect the bacterial species richness of the larvae. However, the Shannon-Wiener diversity indices, which also take into account species evenness, were significantly higher ($p = 0.020$) for non-starved larvae after 48 h as compared to starved larvae that were kept at 30 °C without faecal contact.

Some genera were identified among the most abundant taxa that contain food pathogens, such as *Cronobacter* sp. and *Staphylococcus* sp. (Figures 6.1 and 6.2). Furthermore, in some batches, OTUs belonging to genera *Listeria*, *Clostridium, Shigella/Escherichia, Bacillus* and/or *Vibrio* were identified, albeit in relatively low abundances (0.23%, 0.32%, 0.18, 0.05% and <0.01% of all sequences, respectively).

6.3.2 Rinsing

The initial counts of non-starved, non-rinsed larvae (Table 6.4) were comparable to those observed in the starvation experiment (Tables 6.1 and 6.2). Additionally, no significant differences were observed between non-rinsed and rinsed larvae, for any type of larvae (starved or not) and for any microbial count.

Table 6.1 Microbial counts for non-starved (control) and starved mealworms after 0, 24 and 48 h at 30 °C and with or without faecal contact during starvation. Data are the mean of two to three replicates from each of three different batches ± standard deviation (n = 2 x 3 or 3 x 3).

Microbial counts (log cfu/g)		With faecal contact			Without faecal contact		
		0 h	24 h	48 h	0 h	24 h	48 h
Total viable aerobic count	Control	7.9 ± 0.2[a]	8.0 ± 0.3[a]	8.0 ± 0.3[a]	7.9 ± 0.2[a]	8.0 ± 0.3[a]	8.0 ± 0.2[a]
	Starvation		7.9 ± 0.4[a]	7.9 ± 0.5[a]		7.8 ± 0.4[a]	7.8 ± 0.3[a]
Enterobacteriaceae	Control	7.0 ± 0.3[a]	7.0 ± 0.3[a]	6.8 ± 0.7[a]	6.9 ± 0.3[a]	6.9 ± 0.4[a]	7.0 ± 0.3[a]
	Starvation		7.1 ± 0.3[a]	7.0 ± 0.5[a]		7.1 ± 0.5[a]	7.1 ± 0.3[a]
Aerobic bacterial endospores	Control	1.5 ± 0.6[a]	1.5 ± 0.3[a]	1.2 ± 0.3[a]	1.9 ± 0.2[a]	1.9 ± 0.3[a]	2.0 ± 0.3[a]
	Starvation		1.4 ± 0.7[a]	1.2 ± 0.2[a]		1.7 ± 0.3[a]	1.6 ± 0.4[a]
Psychrotrophic aerobic count	Control	6.7 ± 0.4[a]	6.4 ± 0.6[ab]	6.5 ± 0.5[ab]	7.0 ± 0.3[a]	6.8 ± 0.2[a]	6.9 ± 0.3[a]
	Starvation		6.5 ± 0.4[ab]	6.1 ± 0.3[b]		6.8 ± 0.2[a]	7.1 ± 0.3[a]
Fungi	Control	5.6 ± 0.8[a]	5.4 ± 0.6[a]	5.6 ± 0.8[a]	6.2 ± 0.4[ab]	6.3 ± 0.2[ab]	6.2 ± 0.3[ab]
	Starvation		5.3 ± 1.1[a]	5.8 ± 0.5[a]		5.7 ± 0.4[a]	6.4 ± 1.0[b]

[a,b]Means for one microbial count within one condition (with or without faecal contact) with the same superscript are not significantly different ($p > 0.05$).

Table 6.2 Microbial counts for non-starved (control) and starved mealworms after 0, 24 and 48 h at 10 °C and with or without faecal contact during starvation. Data are the mean of two to three replicates from each of three different batches ± standard deviation (n = 2 x 3 or 3 x 3).

Microbial counts (log cfu/g)		With faecal contact			Without faecal contact		
		0 h	24 h	48 h	0 h	24 h	48 h
Total viable aerobic count	Control	8.4 ± 0.4[a]	8.2 ± 0.2[a]	8.4 ± 0.5[a]	8.0 ± 0.2[a]	7.8 ± 0.2[a]	7.9 ± 0.3[a]
	Starvation		8.2 ± 0.2[a]	8.3 ± 0.4[a]		7.7 ± 0.1[a]	7.7 ± 0.3[a]
Enterobacteriaceae	Control	7.6 ± 0.4[a]	7.5 ± 0.3[a]	7.9 ± 0.6[a]	7.2 ± 0.2[a]	7.0 ± 0.3[a]	7.1 ± 0.4[a]
	Starvation		7.5 ± 0.4[a]	7.7 ± 0.5[a]		6.9 ± 0.2[a]	7.0 ± 0.6[a]
Aerobic bacterial endospores	Control	2.0 ± 0.8[a]	2.2 ± 1.0[a]	1.7 ± 0.5[a]	1.5 ± 0.4[a]	1.7 ± 0.4[a]	1.5 ± 0.4[a]
	Starvation		1.8 ± 0.5[a]	1.6 ± 0.3[a]		1.4 ± 0.5[a]	1.8 ± 0.9[a]
Psychrotrophic aerobic count	Control	7.0 ± 0.3[a]	7.3 ± 0.2[ab]	7.6 ± 0.3[b]	6.8 ± 0.4[a]	7.0 ± 0.5[a]	7.1 ± 0.7[a]
	Starvation		7.1 ± 0.3[a]	7.5 ± 0.6[ab]		6.8 ± 0.3[a]	7.0 ± 0.6[a]
Fungi	Control	6.5 ± 0.8[a]	6.1 ± 0.5[a]	5.8 ± 0.5[a]	6.1 ± 1.0[a]	5.6 ± 0.9[a]	5.7 ± 0.4[a]
	Starvation		6.1 ± 0.6[a]	5.9 ± 0.6[a]		5.7 ± 0.9[a]	5.3 ± 0.9[a]

[a,b]Means for one microbial count within one condition (with or without faecal contact) with the same superscript are not significantly different ($p > 0.05$)

Table 6.3 Microbial community diversity indices for samples of non-starved and starved larvae after 0 and 48 h incubation under different conditions. Values are the mean ± standard deviation of two analyses performed on two DNA extracts from the same sample from each of three different batches (n = 2 x 3).

Condition		Observed richness	Chao1[1]	Coverage (%)[2]	Shannon-Wiener[3]
30°C, faecal contact	0 h	35.33 ± 4.37	47.91 ± 8.05[a]	74.92 ± 8.95	1.48 ± 0.35[a]
	Control (48 h)	36.00 ± 8.76	41.19 ± 8.29[a]	87.57 ± 4.44	1.60 ± 0.29[a]
	Starvation (48 h)	28.33 ± 6.43	35.10 ± 9.49[a]	82.10 ± 6.41	1.36 ± 0.26[a]
30°C, no faecal contact	0 h	37.00 ± 2.60	43.67 ± 2.98[a]	86.07 ± 2.71	2.03 ± 0.02[ab]
	Control (48 h)	35.33 ± 8.50	42.67 ± 6.68[a]	81.56 ± 6.09	2.20 ± 0.21[a]
	Starvation (48 h)	29.67 ± 3.82	33.90 ± 5.69[a]	88.12 ± 7.14	1.74 ± 0.16[b]
10°C, faecal contact	0 h	42.67 ± 5.80	57.04 ± 16.29[a]	77.63 ± 14.29	2.02 ± 0.24[a]
	Control (48 h)	36.83 ± 7.85	44.76 ± 10.22[a]	82.95 ± 3.55	2.01 ± 0.10[a]
	Starvation (48 h)	36.83 ± 4.65	46.25 ± 1.47[a]	81.26 ± 10.43	1.92 ± 0.07[a]
10°C, no faecal contact	0 h	46.00 ± 9.73	51.63 ± 12.45[a]	89.68 ± 4.52	2.18 ± 0.23[a]
	Control (48 h)	36.50 ± 4.33	46.78 ± 3.73[a]	80.62 ± 4.70	1.82 ± 0.34[a]
	Starvation (48 h)	42.17 ± 9.41	48.30 ± 12.72[a]	89.60 ± 5.07	2.11 ± 0.18[a]

[1]Chao1 richness estimator: the total number of OTUs estimated by infinite sampling. A higher number indicates a higher richness (Chao, 1948).
[2]Observed richness/Chao1 estimate * 100
[3]Shannon-Wiener diversity index: an index to characterise species diversity based on species richness as well as their relative abundances. A higher value represents more diversity (Shannon, 1948).
[a,b]Means for the Chao1 or Shannon-Wiener diversity indices within one condition with the same superscript are not significantly different ($p > 0.05$).

Table 6.4 Microbial counts of mealworms that were (1) non-starved and non-rinsed, (2) starved and non-rinsed, (3) non-starved and rinsed, and (4) starved and rinsed. Additionally, microbial counts of the tap water before rinsing and of the residual water after rinsing are shown. Data are the mean values of two to three replicates originating from each of three different batches ± standard deviation (n = 2 x 3 or 3 x 3).

		Microbial counts (log cfu/g)			
Parameter	**Type of larvae**	**Non-rinsed larvae**	**Rinsed larvae**	**Tap water**	**Residual water**
Total viable aerobic count	Non-starved	8.0 ± 0.2[a]	7.9 ± 0.2[a]	1.5 ± 0.4	6.0 ± 0.7
	Starved	7.8 ± 0.4[a]	8.0 ± 0.3[a]	1.6 ± 0.6	5.5 ± 0.6
Enterobacteriaceae	Non-starved	7.0 ± 0.2[a]	7.1 ± 0.2[a]	<0.0 ± 0.0	4.0 ± 0.6
	Starved	7.0 ± 0.4[a]	7.2 ± 0.5[a]	<0.0 ± 0.0	4.2 ± 0.9
Aerobic bacterial endospores	Non-starved	1.7 ± 0.3[a]	1.6 ± 0.3[a]	0.0 ± 0.1	0.7 ± 0.3
	Starved	1.7 ± 0.5[a]	1.7 ± 0.4[a]	<0.0 ± 0.0	0.5 ± 0.3

[a]Means for the larvae for one parameter with the same superscript are not significantly different ($p > 0.05$).

6.4 DISCUSSION

6.4.1 Microbiological quality and bacterial community composition of the larvae

The initial total viable counts, initial Enterobacteriaceae counts, and the initial psychrotrophic counts of the larvae examined in this study were comparable to numbers found for fresh mealworms in literature (Klunder et al., 2012; Stoops et al., 2016; Vandeweyer et al., 2017b,c). The average endospore counts however, were generally lower compared to those reported in literature, which generally range from 1.7 to 5.0 log cfu/g (Klunder et al., 2012; Stoops et al., 2016; Vandeweyer et al., 2017a,b). The average initial counts for fungi were comparable to results obtained by Stoops et al. (2016) and Vandeweyer et al. (2017b). Additionally, the latter counts were comparable to results obtained by Vandeweyer et al. (2017c) for those batches that were obtained from the same rearing company as in our research (4.8 – 5.0 log cfu/g), whereas the counts obtained from another rearing company in that study were lower (ranging from 3.5 to 3.8 log cfu/g).

High-throughput 16S rRNA gene sequencing was used to unravel possible changes in the microbial community composition upon starvation. The main phyla present were Proteobacteria and Firmicutes, followed by Tenericutes, while Bacteroidetes and Actinobacteria were low in abundance. This is in contrast to Stoops et al. (2016), who reported higher abundances for the latter groups (26.9% for Actinobacteria and 2.9% for Bacteroidetes), but did not report the presence of Tenericutes in fresh mealworms. On the other hand, Garofalo et al. (2017) reported a large abundance of Tenericutes (44.2%), Proteobacteria (39.22%) and Firmicutes (13.09%), and a low abundance of Bacteroidetes (0.13%) and Actinobacteria (0.06%) in dried mealworms. In most samples, Enterobacteriaceae (OTUs 2, 6 and 10), a member of the Gammaproteobacteria (OTU 1), a number of lactic acid bacteria (OTUs 4, 5 and 7), and a *Spiroplasma* member (OTU 3) represented more than 80% of all sequences (Figures 6.1 and 6.2). These findings correspond to the large amount of Enterobacteriaceae obtained in the plate counts, as well as to the large amount of lactic acid bacteria that was found for mealworms in other studies (Stoops et al., 2016; Vandeweyer et al., 2017b,c). Notably, these microbes may contain possible spoilage organisms (Sperber & Doyle, 2009), but they are easily reduced in numbers by a heat treatment before consumption (Vandeweyer et al., 2017c). Some samples contained a considerable relative abundance of the genus *Pseudomonas* (OTU 11 and 12), which also contains important spoilage organisms (Sperber & Doyle, 2009). The genus *Spiroplasma,* on the

other hand, is known to harbour insect pathogens. It is, however, thought to not affect mealworms, since the genus has been detected in the mealworm gut in several previous studies (Jung et al., 2014; Wang & Zhang, 2015; Garofalo et al., 2017). The composition of the most abundant OTUs in our samples largely differs from that reported by Stoops et al. (2016), who found fresh mealworms to contain mostly Enterobacteriaceae species, *Haemophilus*, *Lactobacillus*, *Pseudomonas*, *Propionibacterium*, *Staphylococcus* and *Streptococcus.* Other studies also report large numbers of Enterobacteriaceae and *Spiroplasma* for dried mealworms (Garofalo et al., 2017) and the gut of fresh mealworms (Jung et al., 2014). The reason for these differences between studies may be the difference in rearing company or rearing techniques from which the larvae were obtained. The substrate and the rearing environment are believed to determine the microbial community inside the insect gut (SHC & FASFC, 2014; Li et al., 2016b).

In addition, some genera were identified, both among the most abundant taxa as well as present in low abundances, that contain food pathogens. However, this does not necessarily mean food pathogens were present. Still, our results indicate the possible presence of spore-forming food pathogens of the genera *Bacillus* and *Clostridium*. Endospores generally survive heat treatments applied so far for insects, such as blanching and boiling (Klunder et al., 2012; Vandeweyer et al., 2017c). Therefore, further studies should be conducted to characterise the risks related to the occurrence of spore-forming pathogens in or on edible insects such as mealworms.

6.4.2 Effect of starvation and rinsing on microbiological quality

Several insect-rearing companies believe that the emptying of the gut content of mealworms, as stated by the NVWA (2014), may have a positive impact on the microbiological quality and reduce microbial numbers. Furthermore, preliminary experiments showed that mealworm faeces excreted after 24 and 48 h of starvation contain very high microbial numbers, with a total viable aerobic count ranging from 8.8 to 11.4 log cfu/g (data not shown). Our data, however, do not show considerable changes in microbial numbers of mealworms during the starvation period for any of the conditions investigated (Tables 6.1 and 6.2). The results strongly indicate that starvation of mealworms does not reduce their microbial load as often assumed, neither when they are starved at rearing temperature nor chilled, and neither with nor without the ability to consume their faeces. The opposite hypothesis, being that the

gut microbiota can multiply intensively during starvation, in the absence of a plug flow of substrate through the gut, can be rejected as well.

This statement was also supported by the second part of this study, where it was shown that neither starvation, nor rinsing, nor a combination of both procedures significantly affected the microbiological quality of the larvae. Those findings correspond very well with results obtained in preliminary experiments in which sterile demineralised water was used for rinsing (Wynants et al., 2016). Decontamination using water is a technique that is often industrially applied for products such as fresh fruit, vegetables and meat. However, in these cases, the water is often enriched with disinfecting chemicals or it is heated (e.g. more than 74 °C in the case of meat carcasses) (Beuchat, 1998; Huffman, 2002). For lesser mealworm beetles, Crippen & Sheffield (2006) tested multiple chemical washes as external disinfectants, including combinations of 70-95% EtOH, NaOCl, H_2O_2 and H_2O. They found that only a 95% EtOH condition followed by a 20% H_2O_2 wash resulted in total external disinfection of all beetles. Given the results of those authors, a low disinfection efficiency of water without the addition of disinfecting agents can be speculated. However, the total viable aerobic counts of the rinsing water increased with 4.9 to 5.5 log cfu/ml during rinsing, indicating that the procedure removes a considerable amount of microorganisms from the larvae. Rumpold et al. (2014) showed an indirect plasma treatment to be effective for surface decontamination, as the technology could not eradicate the total microbiota. It illustrates the large share of gut microorganisms in the total microbiota.

From our study it can be concluded that rinsing of larvae, without the addition of chemicals or without the use of higher water temperatures, does not reduce the microbial numbers on larvae. Furthermore, even a combined approach of both rinsing and starvation was shown to not affect the microbiological quality of the larvae. It should be noted that the starvation period in our study only lasted for 48 h, which was selected based on practices in rearing companies. This may indicate that 48 h is not enough to eradicate certain pathogens, or by extension any microorganism that may be present in the larval gut. Starvation for a period that exceeds 48 h is, however, not practiced by rearing companies, as the larvae would either dehydrate or start pupation. Importantly, it has to be noted that the effect of starvation and rinsing on the chemical quality (i.e. with respect to chemical contaminants such as pesticide residues, heavy metals, mycotoxins, veterinary substances, …) remains to be established as well. Those procedures may be useful to eliminate chemical contaminants, but this has not been

demonstrated so far. It is known, for aquatic insects, that the gut content can contribute significantly to the total body load of chemical pollutants (Cain et al., 1995). Furthermore, faeces of mealworms that were fed with a deoxynivalenol (DON)-contaminated substrate were found to contain the mycotoxin (van Broekhoven, 2014 in EFSA scientific committee, 2015). Therefore, the question remains from a chemical point of view whether starvation or rinsing should be incorporated as a necessary procedure into guidelines for insect-rearing companies. Nevertheless, mealworm rearers will likely hold on to the rinsing practice, as it yields clean larvae free from any substrate or exuviae. The advice is, as for any food product that is rinsed, to use clean water with a low microbial load in order not to contaminate the larvae during this step.

6.4.3 Effect of starvation on the bacterial community composition

Studies have shown that dietary changes in some insect species, such as crickets, cockroaches and fruit flies, affect the composition of the gut microbiota (Kane & Breznak, 1991; Domingo et al., 1998; Broderick et al., 2004; Broderick & Lemaitre, 2012). Furthermore, Dillon & Dillon (2004) suggested that an insect that is constantly fed is likely to possess a different gut bacterial community as compared to a starved insect due to the food transit in the gut of the former. This was confirmed in a study by Dillon *et al.* (2010), where the microbial diversity in the gut of desert locusts (*Schistocerca gregaria*) increased after a 5-day starvation period. It should be noted, however, that the gut microbial community composition differs between insect species (Yun et al., 2014; Stoops et al., 2016; Garofalo et al., 2017). It is clear, when considering the relative OTU abundances in our study, that no consistent changes in the microbial community composition occurred during starvation (Figure 6.1 and 6.2). Furthermore, analyses on the diversity indices (Table 6.3) showed that, in general, it seems variation between samples was more prominent than a shift in the community composition due to starvation. Furthermore, NMDS (Figure 6.3) showed no visual clustering of samples, neither of the four conditions, nor of samples of non-starved versus starved larvae, confirming that starvation under whatsoever conditions does not influence the bacterial community composition.

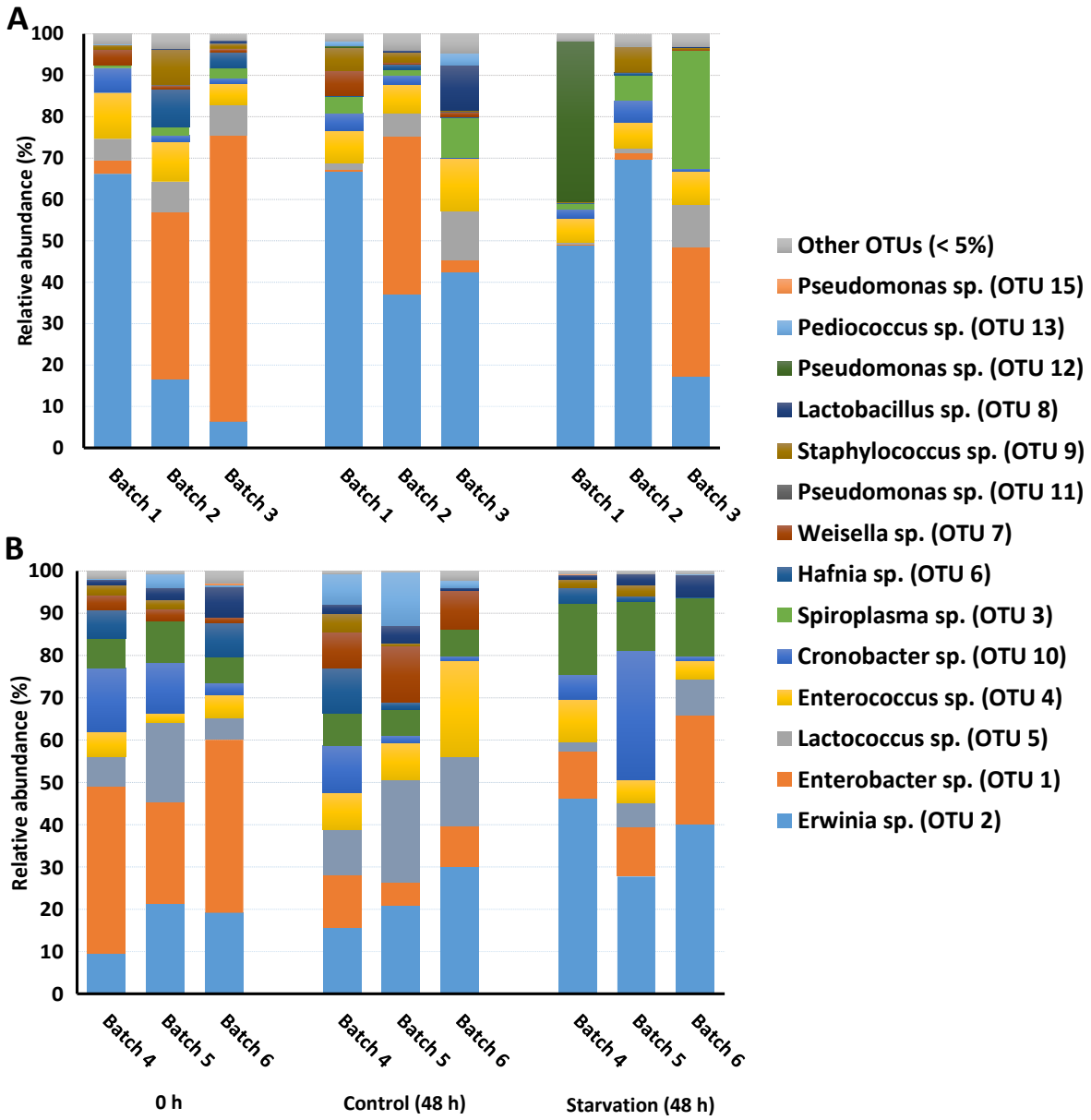

Figure 6.1 Relative abundance (%) of the most abundant bacterial operational taxonomic units (OTUs) in non-starved (control) and starved mealworms after 0 and 48 h incubation at 30 °C with **(A)** or without **(B)** faecal contact. OTUs with a maximum abundance below 5% in all samples were grouped in "Other OTUs". Identifications were performed using the SILVA reference database and taxonomic assignments were considered reliable when a score value ≥ 0.80 was found. When OTUs could not be reliably identified to the genus level, OTUs were further refined by a BLAST analysis against the GenBank nucleotide database (uncultured/environmental sample sequences excluded).

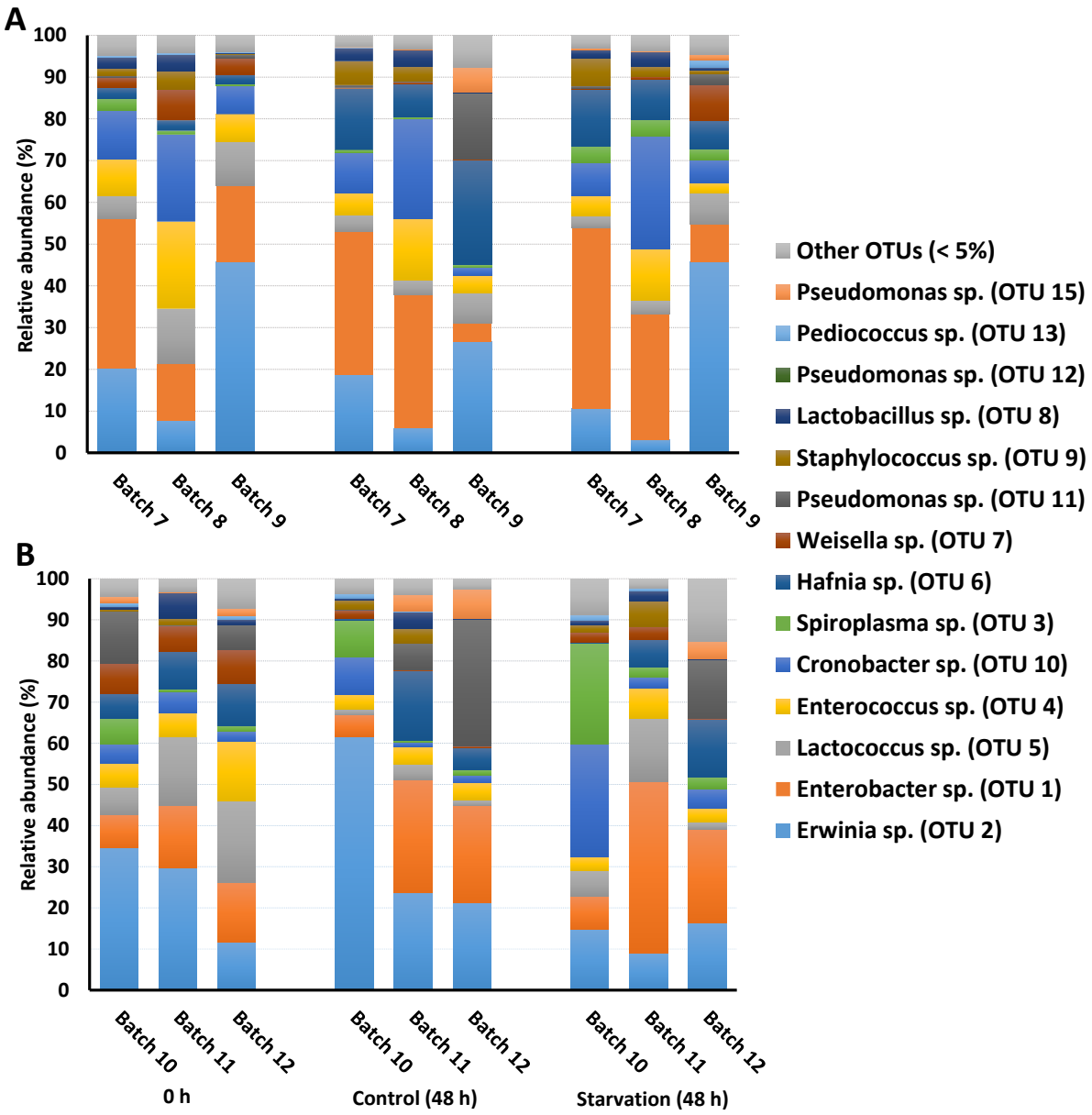

Figure 6.2 Relative abundance (%) of the most abundant bacterial operational taxonomic units (OTUs) in non-starved (control) and starved mealworms after 0 and 48 h incubation at 10 °C with **(A)** or without **(B)** faecal contact. OTUs with a maximum abundance below 5% in all samples were grouped in "Other OTUs". Identifications were performed using the SILVA reference database and taxonomic assignments were considered reliable when a score value ≥ 0.80 was found. When OTUs could not be reliably identified to the genus level, OTUs were further refined by a BLAST analysis against the GenBank nucleotide database (uncultured/environmental sample sequences excluded).

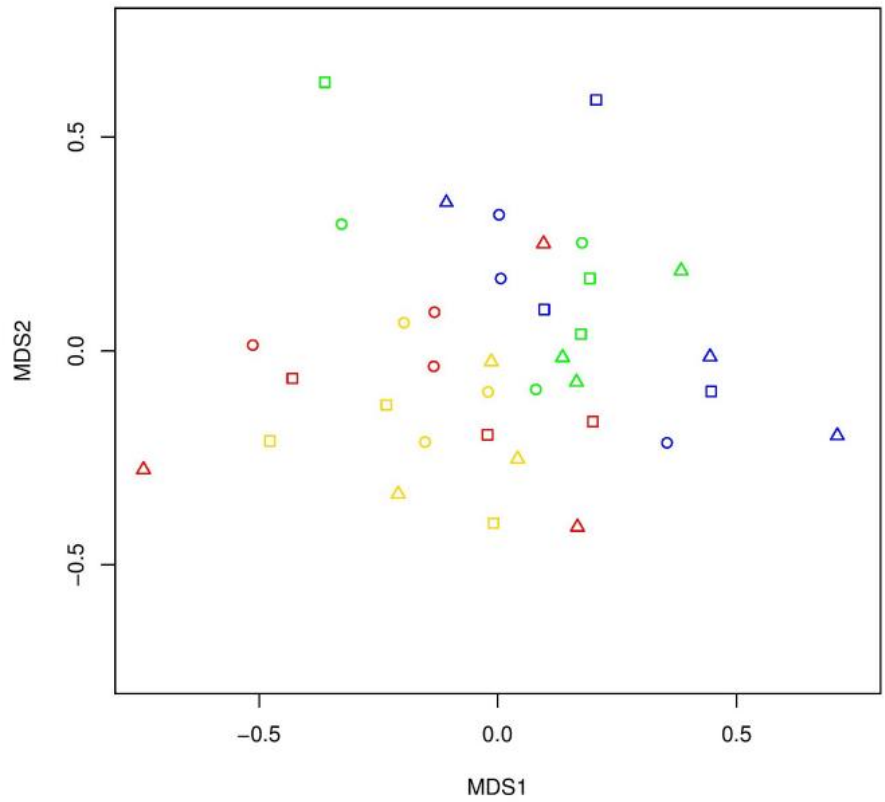

Figure 6.3 Nonmetric multidimensional scaling (NMDS) ordination (stress value = 0.24) representing the bacterial community composition of mealworms after 0 h (circles), non-starved larvae (control) after 48 h (squares) and starved larvae after 48 h (triangles). Different colours represent different treatments: incubation at 30 °C with faecal contact (blue) or without faecal contact (green), or at 10 °C with faecal contact (yellow) or without faecal contact (red).

6.5 CONCLUSIONS

Starvation and rinsing of mealworms are procedures typically included at the end of the rearing process of mealworms for human consumption. These practices are often assumed to enhance the microbiological quality of the edible insects. However, the results of this study show no differences in microbial numbers between larvae that were starved, rinsed or were subjected to a combination of both procedures. Furthermore, a starvation period of 48 h does not bring about a systematic shift in the composition of the bacterial community. Further research on the impact of these practices on the chemical quality of the insects, and on the impact of other rearing practices, hygiene measures and the substrate on the microbiota of edible insects is necessary in order to provide additional guidelines for the insect-rearing industry to ensure food safety of their end products.

CHAPTER 7: CONCLUSIONS AND FUTURE PROSPECTS

7.1 OUTCOME OF RESEARCH OBJECTIVES

7.1.1 Objective 1: Characterisation of the microbial dynamics during industrial rearing of insects, in particular of *H. illucens*, *A. diaperinus* and *G. sigillatus*

Very little studies have investigated the endogenous microbiota and its dynamics during the rearing process of insects thus far. To our knowledge, no such studies exist for insects reared at industrial scale. Yet the scale of rearing, and concomitantly the environmental hygiene and the practices applied likely differ from rearing at laboratory scale, and hence may affect the microbiological results. Therefore, in this dissertation, the aim was to characterise the endogenous microbiota of substrates, insects and residues during rearing at industrial and/or large scale and to search for possible relations. To this end, in Chapter 2, the microbiota of BSF larvae was studied at three external rearing facilities (and for four lab-scale rearing cycles for comparison). In Chapters 3 and 4, similar analyses were conducted for the lesser mealworm and the tropical house cricket, respectively. Based on the three studies, a number of general conclusions can be drawn:

1) In general, the microbial load of residues and insects was higher than those of the substrates before administering. For species residing inside the substrate (i.e. lesser mealworms and BSF larvae), high counts for the residue were observed which is likely caused by the excretion of faeces and exuviae by the insects.
2) For the lesser mealworm, bacterial diversity of the larvae was shown to decrease during rearing. A few OTUs, which are presumably more adapted to a life in the lesser mealworm gut, became more dominant towards the end of rearing. This suggests that during development, the microbiota of the lesser mealworm needs time to be established. For the tropical house cricket, in contrast, such a mechanism was not observed.
3) For all studied cycles, many OTUs recovered in the substrate prior to administration were also observed in the insect, suggesting the substrate to be an important source of bacteria for the insect microbiota. Nevertheless, microbial numbers as well as bacterial community composition largely differed between substrates and insect for all three species investigated. This indicates that the microbiota of insects is not merely a reflection of the substrate microbiota, but

that complex mechanisms may contribute to the establishment of the gut microbiota. In addition, for black soldier fly larvae, great variation in microbial composition was observed even between larvae reared at different rearing facilities, as well as larvae reared within one facility but on a different substrate. It is suggested that many factors, both biotic and abiotic, determine the final bacterial community composition of the harvested insect. Monitoring of the microbial safety of the substrate is likely valuable in assuring food safety of the harvested insects.

7.1.2 Objective 2: Studying selected microbiological safety risks during industrial rearing of *H. illucens*, *A. diaperinus* and *G. sigillatus* for feed and food

During the sampling at industrial scale, special attention was paid to food safety aspects. Larvae and residues at harvest were screened for a selection of food pathogens. For insects reared for human consumption, i.e. the lesser mealworm and tropical house cricket, a selection of fungal isolates was also identified in order to gain insight into the most abundant members of the fungal community. This led to the following conclusions:

1) Of the four food pathogens investigated during large scale rearing (*Salmonella* sp., *Listeria monocytogenes*, *Bacillus cereus* and coagulase-positive staphylococci), none were detected in residue and insect samples for the rearing of the lesser mealworm and tropical house cricket. For BSF, in contrast, *Salmonella* sp. was detected in one residue sample of one external rearing facility, whereas presumptive *B. cereus* was detected in residue and/or larvae samples at the two other external facilities. Caution is thus advised when BSF larvae are used in feed, as *B. cereus* spores may survive processing steps. Indeed, as was shown for lesser mealworms and crickets, spores were reduced to a limited extent during post-harvest heat treatments. Thus, post-harvest treatments that minimise the risk of endospores from pathogenic species being present in commercialised insects are of utmost importance.
2) Fungal isolates were obtained from substrates, residues and larvae of lesser mealworm and tropical house cricket rearing cycles. For both insects, possible mycotoxinogenic fungi such as *Aspergillus* sp., *Fusarium* sp., and/or *Penicillium* sp. were identified. The high fungal counts observed in these insects indicate that mycotoxins may be produced and pose a risk during insect rearing.

Increasing knowledge on possible microbial health hazards during insect rearing will contribute to the establishment of risk assessments for insects reared for food or feed. For instance, results obtained in Chapter 4 for the tropical house cricket, were included in a recently-published risk assessment for the house cricket (Fernandez-Cassi et al., 2019).

Furthermore, results from this study and from other studies on fresh insects suggest that action limits as proposed by the FASFC in Belgium (Table 2.1) may be too strict for fresh insects. As insects are living organisms with a gut microbiota, high numbers of microorganisms are inherent to this matrix and are difficult to control. On the other hand, it can be speculated that for processed insects, these limits may not be strict enough. For *Salmonella* sp., a distinction is already made in the Belgian legislation between insects that need further treatment (absence in 10 g) and insects ready for consumption (absence in 25 g). Similarly, the creation of separate microbiological action limits and criteria for fresh insects on the one hand, and ready-to-eat insects on the other hand, would lead to a better monitoring of the microbiological risks.

7.1.3 Objective 3: Assessing the horizontal transmission of a food pathogen to the insect during rearing, with focus on the case study of transmission of *Salmonella* sp. from wheat bran to mealworms (*T. molitor*)

In this research, as well as in literature, *Salmonella* sp. was never detected during rearing of insects for feed or food. In the cases investigated so far, nor the substrate, the production environment or the personnel likely represented a source of *Salmonella* sp. However, in order to assess the consequences in a situation where a source of *Salmonella* sp. is effectively present, in Chapter 5 survival of *Salmonella* sp. inoculated into wheat bran and transmission to mealworms was studied at laboratory scale (since inoculation cannot be performed at industrial scale). It was not surprising to observe that *Salmonella* sp. can survive in the bran (as it is wel known to survive in for instance animal feeds with more or less the same intrinsic parameters). Contamination of the larvae with the pathogen was observed, but some of the observations pointed towards the elimination of *Salmonella* sp. during the experiment, suggesting some antimicrobial activity of the larvae against *Salmonella* sp. While this may sound promising in terms of food safety, caution is advised while extrapolating these findings to the industry. Regular testing for the presence of *Salmonella* sp. in the

bran, for instance for each batch that arrives at the facility, as well as in the harvested larvae is therefore advised.

7.1.4 Objective 4: Assessing the effect of post-harvest practices on the microbial load and the bacterial community, with focus on the case study of starvation and rinsing of mealworms (*T. molitor*)

In Chapter 6, late-instar mealworms were obtained from a commercial rearer, starved using a variety of time-temperature combinations, and/or rinsed after harvest. Neither procedure, or combination of procedures, proved valuable reducing the microbial load of the larvae. If the redundance of these procedure is also confirmed from a chemical point of view (samples from this study were also assessed for their chemical contaminants, results to be published), omitting these steps at the end of the rearing process will represent an economic benefit for insect producers. Noteworthy, although starvation did not enhance overall microbial quality, the effect of starvation on specific food pathogens present in the gut was not yet assessed. It could be hypothesised that, when a food pathogen is taken up by the larvae but is unable to long-term colonise the gut (as was suggested for *Salmonella* sp.), the emptying of the gut may eliminate the pathogen.

7.2 FUTURE RESEARCH AND VALORISATION OPPORTUNITIES

This PhD was conducted in the framework of a research project titled “EDINCO” on request of the Federal Public Service Health, Food Chain Safety and Environment to investigate, as it was formulated originally, “*the food safety risks in the industrial context of insect rearing*”. The dissertation delivered a number of conclusions that are merely practice-oriented and simultaneously yielded new questions or suggestions for more in-depth study. Future research objectives will be further elaborated below. For each objective, an approach will be suggested and possible valorisation of the results will be discussed (Figure 7.1).

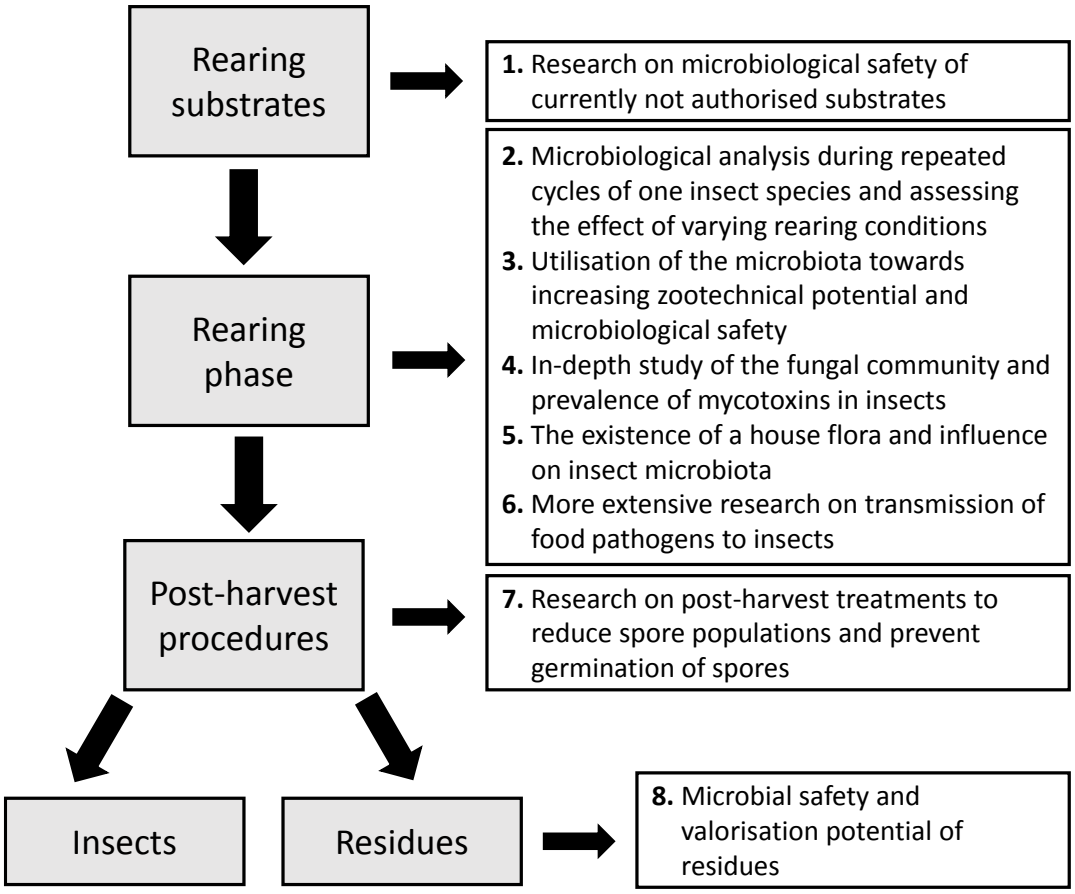

Figure 7.1 Overview of suggestions for further research based on the results of this dissertation.

7.2.1 Research on microbiological safety of currently not authorised substrates

Research suggestion. This dissertation suggested the substrate to be an important source of bacterial species for reared insects, which likely impacts their microbiological safety. Further research is needed on the microbiological safety of rearing substrates that are currently not allowed by legislation, such as post-consumer food waste (category 3) and category 2 animal by-products (e.g. manure, sewage sludge, …).

Approach. Future studies should assess the microbiological quality of a diversity of potentially high-risk substrates, and subsequently assess the resulting contamination in the reared insects by means of feeding trials. A future research project could be titled "*Assessing the risks related to post-consumer waste and category 3 animal by-products during the rearing of (insect species) for feed or food*". If certain substrates are then found to pose a risk with regard to microbiological safety, further research may be conducted on treatments to reduce these risks (e.g. heat-treating, inoculation with beneficial microorganisms, fermentation, irradiation, …) without reducing nutritional quality and larval performance.

Valorisation opportunities. If sufficient monitoring (and perhaps treatment) is established for microbial and chemical contaminants in substrates, as is advised by several national guidance documents (ANSES, 2014; NVWA, 2014; SHC & FASFC, 2014), the allowance in legislation of more substrates in insect diets would allow for a more sustainable and economic insect rearing.

7.2.2 Microbiological analysis during repeated cycles of one insect species and assessing the effect of varying rearing conditions

Research suggestion. Due to the industrial scale and hence the size of the rearing process and number of samples to investigate, only one cycle could be monitored in this study for the lesser mealworm and tropical house cricket, and only one cycle per substrate for BSF larvae. This led to first insights into the microbiota and its dynamics during rearing. Yet, no conclusions can be made with respect to the repeatability of the microbiological quality. Further research is needed in order exploit the variability in the microbiota of insects reared in repeated cycles.

Approach. Repeated cycles for each species investigated under identical conditions, but also under conditions where specific process parameters (e.g. substrate storage, feeding regime, insect genotype, temperature, …) are varied, should be monitored in order to assess the impact of those conditions on the microbiota. This research would require a tremendous amount of time and consumables, as well as a major cooperation with the industry. Such a project could, for instance, be entitled *"Rearing of (insect species) at industrial scale: variation in the endogenous insect microbiota under constant and varying conditions".*

Valorisation opportunities. When the factors affecting the microbiota of insects are better understood, this can lead to the development of practices that ensure the best (and a constant) microbial quality and safety. Those practices may then be a valuable addition to the sector guide, as was recently published by the IPIFF (2019). More detailed advice on practices included in the guide will provide insect producers with strategies to monitor quality, safety and legislative compliance of their product, by incorporating good hygiene practices (GHP), HACCP systems, methods for monitoring food safety by means of sampling plans and subsequent analyses, and so on.

7.2.3 Utilisation of the microbiota towards increasing zootechnical potential and microbiological safety

Research suggestion. The repetition of different rearing cycles, as described in the previous research suggestion, can also contribute to the possible identification of a "core microbiota" (Astudillo-Garcia et al., 2017). Then, research could be conducted to exploit these potential symbionts in order to improve zootechnical performance or specific benefits of the insects (De Smet et al., 2018).

Approach. By artificially adding core microbiota members to the substrate, and subsequently studying larval performance and microbiota, the beneficial effect of these microorganisms can be assessed. Further research may then even include fermentation of substrates by these symbionts prior to administration, or assessing the impact on resistance to colonisation by pathogens. Based on the results of this PhD, research on exploiting the endogenous microbiota of BSF towards increasing zootechnical performance and controlling microbiological safety risks has started at Lab4Food, but it may also be extended in the future for other species.

Valorisation opportunities. If proven useful, the addition of symbionts to the substrate may provide an easy way for insect rearers to increase both larval growth performance as well as microbiological safety. Those impacts will in turn lead to economic benefits to the rearer due to better feed conversion ratios, faster biomass gain, avoidance of losses due to safety issues and/or relaxation of the processing steps required to assure safety.

7.2.4 In-depth study of the fungal community and prevalence of mycotoxins in insects

Research suggestion. The results from Chapters 3 and 4 show that mycotoxinogenic fungi were among the most prominent fungal community members in substrates, residues and/or harvested insects. Considering that fungal counts were also high in residues and larvae in both cycles, this provides evidence for the possible presence of mycotoxins in edible insects. Nevertheless, only a small selection of isolates was identified, and no fungal isolates were analysed for BSF rearing, pointing towards the value of an in-depth follow-up study. Research recommendations with regard to fungi are twofold: (1) performing a more in-depth characterisation of the fungal community of edible insects, and (2) to assess the prevalence of mycotoxins in industrially reared insects.

Approach. Insect samples obtained in this PhD dissertation were also assessed for the presence of mycotoxins in the light of the project EDINCO (results to be published). However, also here, sampling was only limited to one cycle per substrate for BSF, and to only one rearing facility for crickets and lesser mealworms. A characterisation of the fungal community (e.g. by high-throughput sequencing) of multiple batches from multiple rearing facilities, as well as a thorough screening for mycotoxins could be performed in a project titled "*Assessment of the fungal communities and prevalence of mycotoxins in industrially reared edible insects from different facilities and production batches*".

Valorisation opportunities. Based on such a research project, the most relevant mycotoxins may be determined per insect species. If certain insect species appear frequently contaminated with certain mycotoxins, follow-up research should then focus on ways to mitigate mycotoxin contamination levels in the insects. That will eventually lead to recommendations for the insect sector, which may also be included in sector guidelines, as discussed in the previous research suggestion.

7.2.5 The existence of a house flora and influence on insect microbiota

Research suggestion. In this PhD dissertation, the microbiota of the insects was assessed in relation to that of the substrates and residues. However, as frequently suggested in this dissertation, other factors may also contribute to the microbiota of the insect. Futures studies may confirm the existence of and characterise the "house flora" of a rearing facility and its influence on the insect microbiota.

Approach. Such a study can be conducted by swabbing the rearing environment as is typically performed for microbial evaluation of the production environment in the food industry and by simultaneously investigating the insect microbiota.

Valorisation opportunities. Results of the suggested research will give insight into the potential contamination risks or, on the other hand, benefits related to the house flora. Based on this, the usefulness of thorough disinfection and cleaning of the rearing infrastructure can be assessed and incorporated into guidelines for the sector.

7.2.6 More extensive research on transmission of food pathogens to insects

Research suggestion. In this study, the transmission potential of food pathogens to insects was only assessed for the combination of *Salmonella* sp. and yellow mealworms. The study we present in this PhD was the first in its kind, but a number of

extensions can be envisaged in order to obtain a full risk assessment. In addition, similar risk assessments are advised with other insect species and other food pathogens. *Bacillus cereus* is presumably the most relevant pathogen, as (1) the bacterium was found to be present in insects in previous studies as well as in BSF larvae in this dissertation, and (2) its endospores will likely survive most heat treatments.

Approach. For both pathogens, experiments should be repeated for a variety of strains and with different rearing parameters, such as larval age, feed administration regimes, etcetera. A few preliminary trials were conducted during this PhD on inoculating *B. cereus* into wheat bran and studying possible transmission to mealworms. However, the medium used appeared to be not selective enough. This can be solved in future research by using (a) genetically modified *B. cereus* strain(s) with for instance an antibiotic resistance marker, reducing the need for a highly selective medium.

Valorisation opportunities. When transmission is investigated under a variety of conditions as suggested above, full risk assessments can be conducted with regard to the presence of *Salmonella* sp. and *B. cereus* in the substrate fed to insects. In addition, the effect or post-harvest treatments (e.g. starvation, heat treatments) on the presence of these food pathogens should be evaluated. This can eventually lead to more specific guidelines for the insect sector with regard to hygiene measures, necessity and frequency of microbiological analyses of substrates and/or insects, and so on. As also mentioned for research suggestions 2 and 3, these insights may then be included in a sector guide. Apart from guidelines, extensive risk assessments for *Salmonella* sp. and *B. cereus* may also lead to the inclusion in current EU legislation of microbiological criteria specific for these food pathogens in insects, both for feed and food.

7.2.7 Research on post-harvest treatments to reduce spore populations and prevent germination of spores

Research suggestion. Heat treatments in this study were shown to reduce most microbial counts in the lesser mealworm and tropical house cricket, but left spore counts unaffected, which corresponded with previous studies (Klunder et al., 2012; Vandeweyer et al., 2017c). In addition, during BSF rearing, high numbers of *B. cereus* up to 3.8 log cfu/g were observed. Therefore, we advise the development of techniques to reduce spore populations while still maintaining a high quality product,

as well as preventing germination of spores and growth of vegetative cells during storage.

Approach. Valuable alternatives to traditional boiling and/or blanching may include high pressure decontamination combined with heating (Herdegen & Vogel, 1998; Cenkowski et al., 2007) or (gamma) irradiation (Farkas, 2006). A study of these techniques for different insect species, in comparison to the traditional heat treatments, will provide valuable results. In such research, focus should be not only on the microbial quality but also on the effect of these procedures on nutritional quality and texture. In addition, spore germination potential should be assessed during processing, transport and storage of different end products with specific characteristics (e.g. water activity, pH, …) and for different environmental conditions (temperature, humidity, …).

Valorisation opportunities. A cost-efficient technique that reduces spore populations and prevents their germination in the end product, while maintaining quality, would be highly valuable for insect producers in ensuring that risks associated with spore-forming pathogens, such as *B. cereus* group members and *Clostridium* spp., are eliminated. This way, safety of the insects prior to using them in human food or animal feed can be guaranteed while still maintaining nutritional quality.

7.2.8 Microbial safety and valorisation potential of residues

Research suggestion. Currently, residues from insect rearing are discarded and subsequently incinerated. However, as the residue exists for a large part of insect faeces, it could be used for soil conditioner or as fertiliser. Because of its composition, consisting of faeces, insect particles and unconsumed substrate, unclarity exists with regard to legislation and valorisation of this product in the EU, as well as on the processing methods required prior to its utilisation.

Approach. Research may focus, for instance, on determining minimally required processing parameters (e.g. heat treatments) to provide the microbiological safety that is needed for the residues to be used as soil conditioner or fertiliser.

Valorisation opportunities. Research on processing methods of the residue will provide insights into the valorisation options of the substrate, while providing the most cost-efficient option for the rearing facility to ensure microbiological safety of the residues prior to further applications. Results of such as study would also allow for a

further clarification of the current legislative framework with regard to the use of insect rearing residues.

7.2.9 Concluding remarks

A final note important for all future research projects is that high-throughput sequencing technologies have their limitations. The most abundant sequence reads do not always correspond to the most abundant species. This limitation is caused, for instance, by small differences in efficiency during PCR reactions and by differences in 16S gene copy number between species. However, sequencing technologies and data processing are constantly being improved. For further research, these developments may lead to more accurate descriptions of microbial communities. Examples of valuable developments that were not yet applied in this study, include the use of mock communities (i.e. multiple micro-organisms that are cultivated individually, or their DNA, and combined at known abundances to form a community that serves as a reference in sequencing analyses, Nguyen et al., 2015b) and combining relative abundances as obtained by sequencing with measurements of absolute bacterial cell numbers in order to obtain a more accurate view on abundancies (Zhang et al., 2017).

This dissertation provided a number of valuable insights into the microbiological characteristics and changes during the rearing of insects for food and feed. It is clear that research on this topic is still in its infancy, and that many research opportunities still exist. Expanding the knowledge on the microbiota of insects in relation to rearing practices will help the insect industry in becoming a well-established sector, with clear regulations and advisory measures, providing microbiologically safe and sustainable insect products of high nutritional quality.

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SUPPORTING INFORMATION

Supporting Information for Chapter 2

Table S2.1 Detailed overview of the rearing procedures for each rearing cycle.

➔ corresponds to **Online Resource 1** in Wynants, E., Frooninckx, L., Crauwels, S., Verreth, C., De Smet, J., Sandrock, C., Wohlfahrt, J., Van Schelt, J., Depraetere, S., Lievens, B., Van Miert, S., Claes, J., Van Campenhout, L. (2019). Assessing the microbiota of black soldier fly larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. *Microbial Ecology* (in press). https://doi.org/10.1007/s00248-018-1286-x

Table S2.2 Details of the feeding regimes during all rearing cycles.

➔ corresponds to **Online Resource 2** in Wynants et al. (2018), the same paper as Table S2.1.

Table S2.3 Primer design and sample-specific barcodes.

➔ corresponds to **Online Resource 3** in Wynants et al. (2018), the same paper as Table S2.1.

Table S2.4 Abundance and identification of operational taxonomic units (OTUs) according to the SILVA reference database. Taxonomic assignments with highest bootstrap conficence value are shown and can be considered reliable when a confidence value > 0.80 was found. The first section of each sample description indicates the rearing cycle, the second indicates the sample type (sub = phase II substrate; larv=larvae; res=residue), the third indicates replicate sample (1-3) and duplicate DNA extract (A-B).

➔ corresponds to **Online Resource 4** in Wynants et al. (2018), the same paper as Table S2.1.

Table S2.5 List of OTUs which were present in larvae from all seven rearing cycles. Values between brackets indicate the bootstrap value, i.e. the certainty that the

classification is correct. Bootstrap values can be considered reliable when equal or higher to 0.80. Phyla indicated with (?) could not be reliably accredited due to a too low bootstrap value (<0.80).

➔ corresponds to **Online Resource 7** in Wynants et al. (2018), the same paper as Table S2.1.

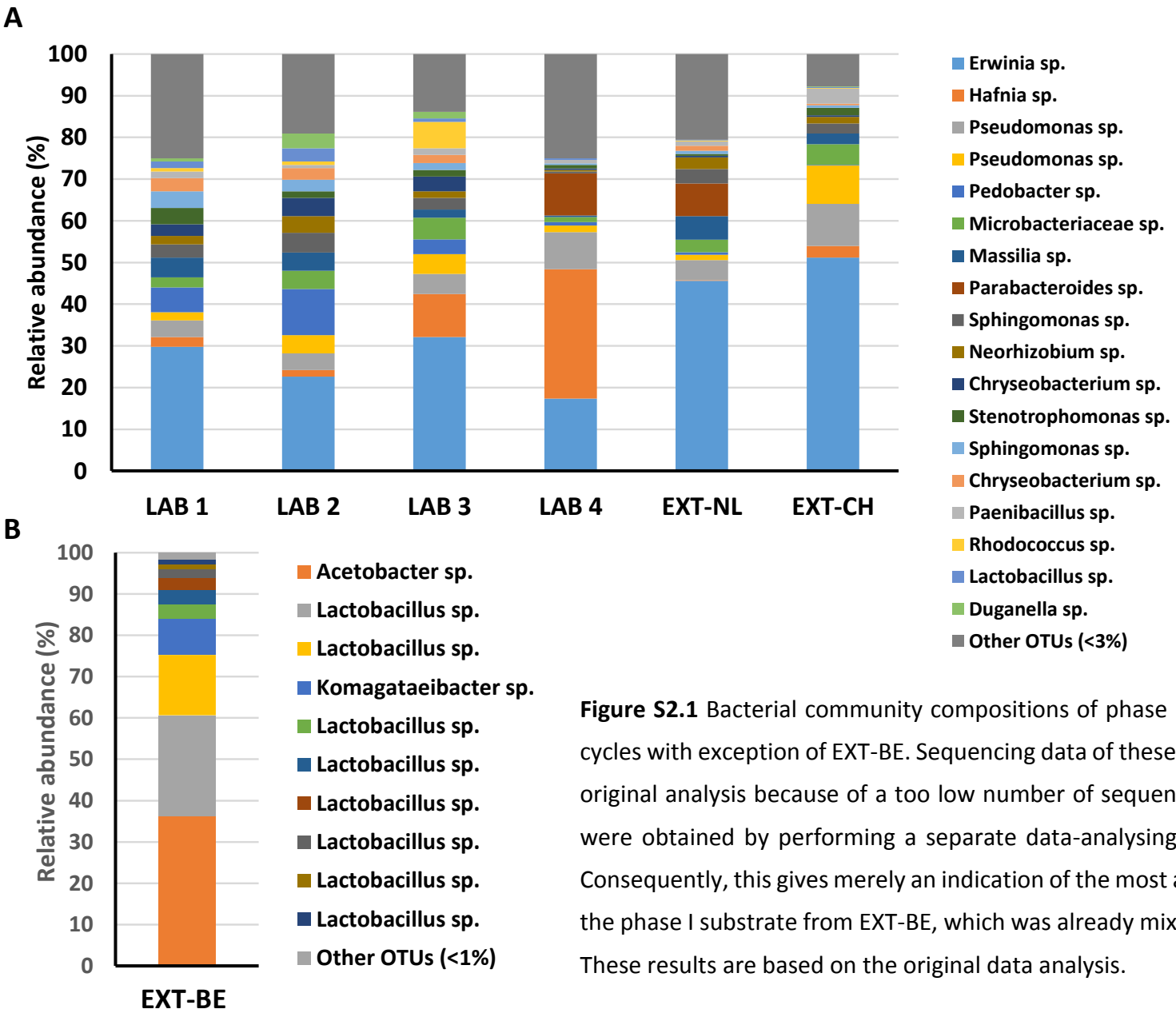


Figure S2.1 Bacterial community compositions of phase I substrates. **A)** for all rearing cycles with exception of EXT-BE. Sequencing data of these samples were discarded from original analysis because of a too low number of sequence reads. Results shown here were obtained by performing a separate data-analysing process, rarefied to 250 bp. Consequently, this gives merely an indication of the most abundant taxa. **B)** results from the phase I substrate from EXT-BE, which was already mixed into the phase II substrate. These results are based on the original data analysis.

Figure S2.2 Correlations of pH (A), water activity (B), and moisture content (C,%) between phase I substrates, phase II substrates, larvae (at harvest) and residues (at harvest). Plots with p-value indicate a significant correlation.

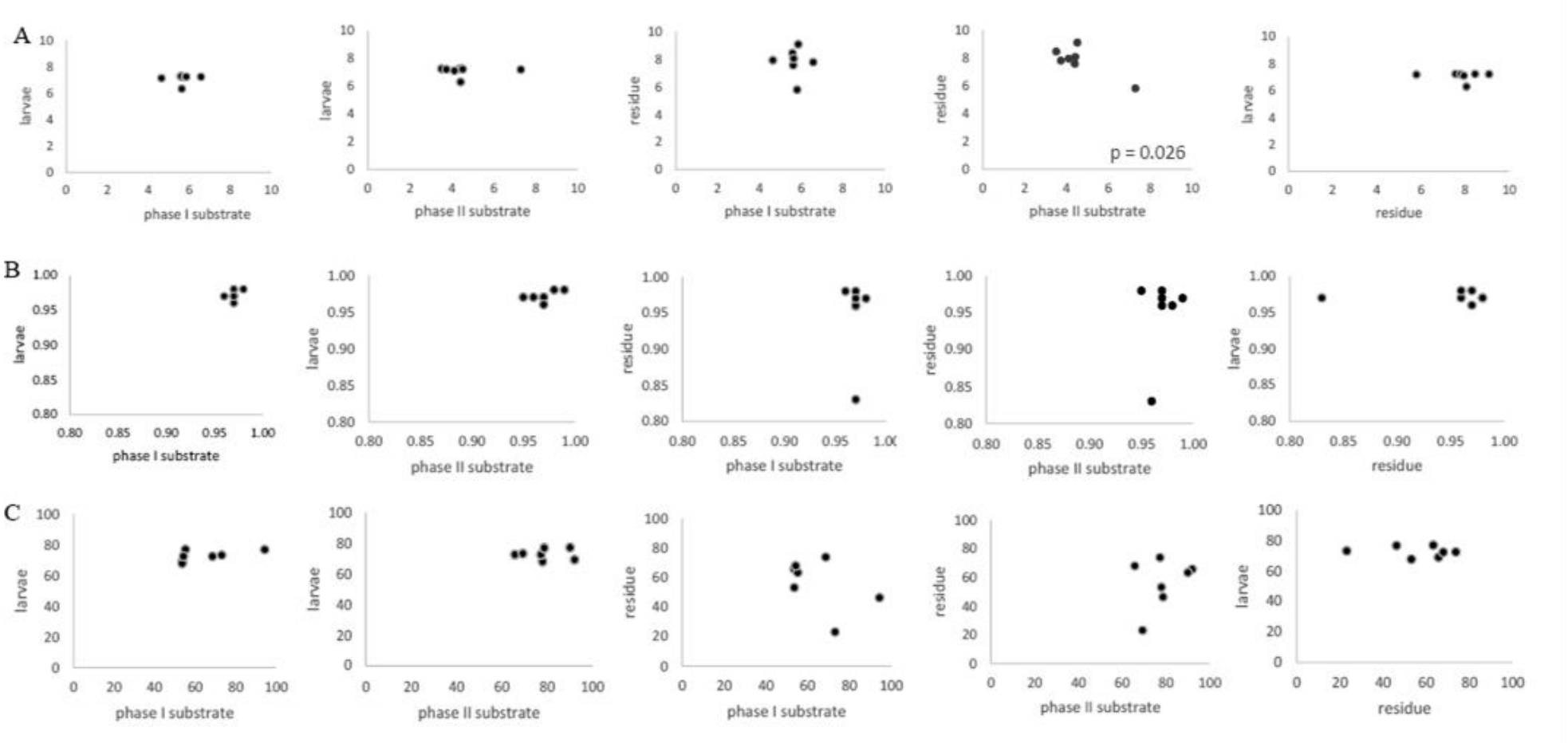


Figure S2.3 Correlations between average counts (in log cfu/g) of phase I substrates, phase II substrates, residues (at harvest) and larvae (at harvest) of all seven rearing cycles. Plots with p-value indicate a significant correlation. A) total viable counts, B) Enterobacteriaceae, C) lactic acid bacteria, D) bacterial endospores and E) fungi.

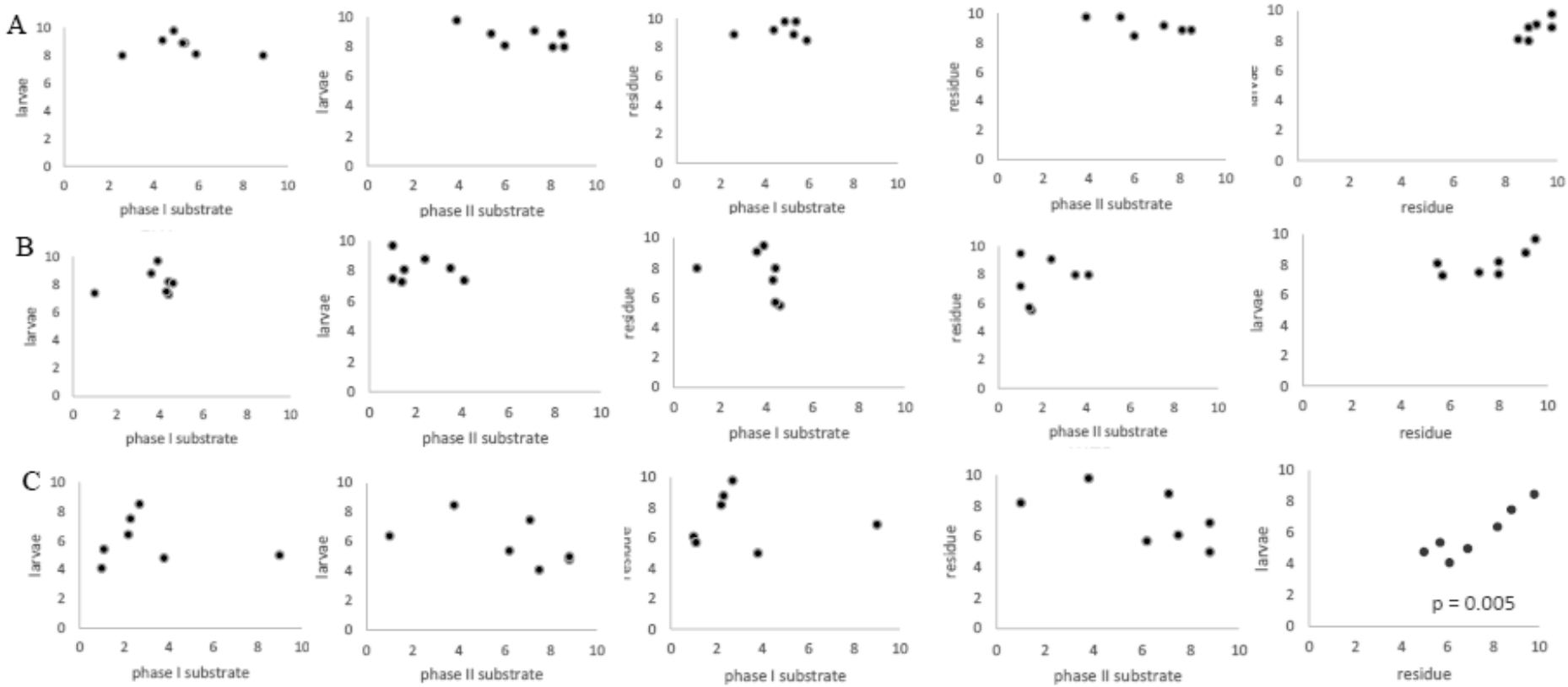
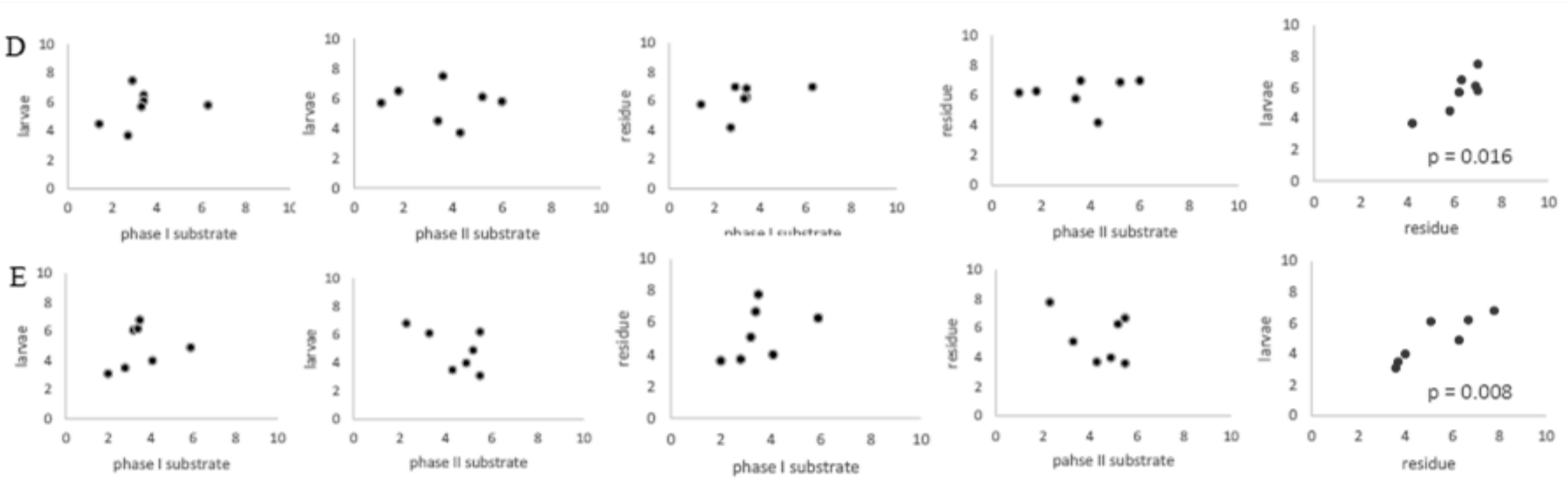


Figure S2.3 (continuation)



Supporting Information for Chapter 3

Table S3.1 Overview of the samples taken during the production cycle of lesser mealworms and corresponding analyses.

➔ corresponds to **Table S1** in 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. https://doi.org/10.1016/j.fm.2017.09.012

Table S3.2 Identification of fungal isolates by BLAST search against the GenBank nucleotide (nt) reference database.

A) Overview of fungal isolates and identification based on a BLAST search of the ITS1-5.8S rDNA-ITS2 region of the large subunit ribosomal RNA gene (October 2016). Only highest matches to named species in GenBank are reported. The numbers between brackets indicate the number of isolates belonging to the corresponding species.

Sample	Number of isolates selected	Identification
Dry substrate (day 0)	5	*Wickerhamomyces anomalus* (2) *Fusarium* sp. *Penicillium cinnamopurpureum* *Penicillium solitum*
Moist substrate (day 0)	3	*Purpureocillium lilacinum* *Fusarium* sp./*Gibberella* sp. *Candida santamariae*
Larvae (day 35, post-harvest)	10	*Aspergillus flavus* (4) *Diutina rugosa* (3) *Trichosporon asahii* *Issatchenkia orientalis* *Pichia sporocuriosa*
Residue (day 35)	10	*Diutina rugosa* (6) *Aspergillus flavus* (3) *Issatchenkia orientalis*

B) Top 5 hits against the GenBank nucleotide (nt) reference database including score, e-value and sequence identity. Uncultured/environmental entries were excluded.

➔ corresponds to **Table S2B** in Wynants et al. (2018), the same paper as S3.1.

Table S3.3 Primer design and sample-specific barcodes.

➔ corresponds to **Table S3** in Wynants et al. (2018), the same paper as S3.1.

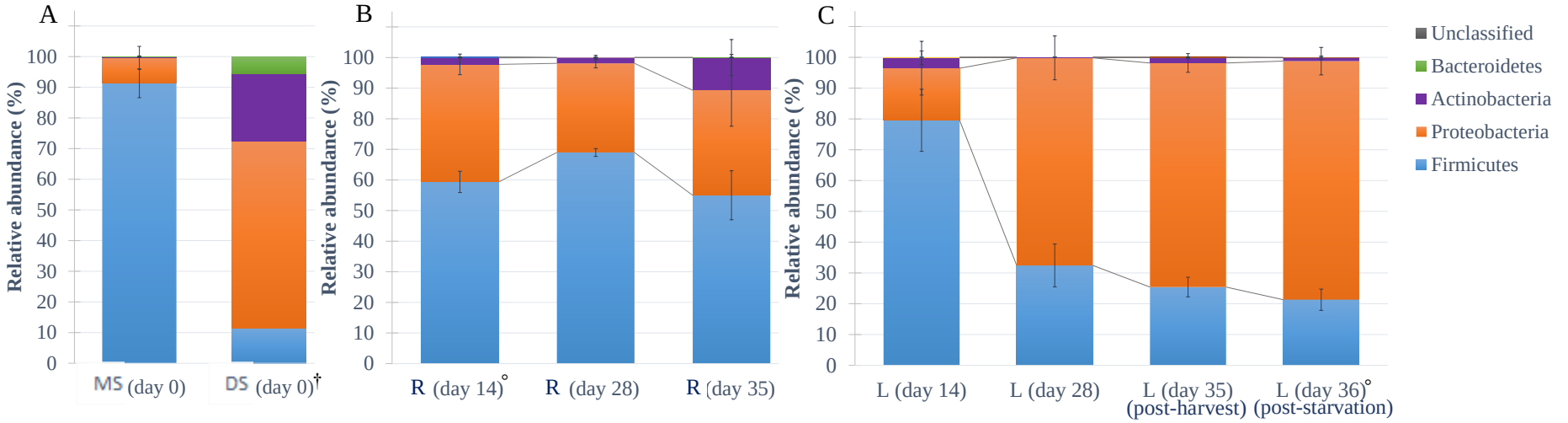
Table S3.4 Abundance and identification of operational taxonomic units (OTUs) according to the SILVA reference database. The numbers between brackets indicate the replicate sample (1 or 2), the letters between brackets indicate the technical replicate (a or b). Taxonomic assignments with highest bootstrap conficence value are shown and can be considered reliable when a confidence value > 0.80 was found (indicated in bold).

➔ corresponds to **Table S4** in Wynants et al. (2018), the same paper as S3.1.

Table S3.5 Identification of operational taxonomic units (OTUs) by BLAST search against the GenBank nucleotide (nt) reference database.

➔ corresponds to **Table S5**, in Wynants et al. (2018), the same paper as S3.1.

Figure S3.1 Relative abundance (%) of phyla present in samples of **A)** the dry substrate (DS) and moist substrate (MS), **B)** the residue (R) and **C)** the larvae (L). Values are the mean of analyses performed on two replicate samples, with two technical replicates per sample (n = 2 x 2) except for † (1 sample, 1 technical replicate (n = 1)) and ° (1 sample, 2 technical replicates (n= 2)). Error bars represent the standard deviation. "Unclassified" sequences are sequences that could not be assigned to phylum level against the Silva or Genbank databases.



Supporting Information for Chapter 4

Table S4.1 Overview of the samples taken during the production and storage of banded crickets and corresponding analyses.

➔ corresponds to **Table S1** 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 the tropical house cricket (*Gryllodes sigillatus*) for human consumption. *Applied and environmental microbiology*, AEM-00255. https://doi.org/10.1128/AEM.00255-18.

Table S4.2 Primer design and sample-specific barcodes.

➔ corresponds to **Table S5** in Vandeweyer et al. (2018), same paper as S4.1

Table S4.3 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 conficence value are shown and can be considered reliable when a confidence value > 0.80 was found (indicated in bold).

➔ corresponds to **dataset S3** in Vandeweyer et al. (2018), same paper as S4.1.

Table S4.4 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.

➔ corresponds to **Table S4** in Vandeweyer et al. (2018), same paper as S4.1.

Table S4.5 Identification of fungal isolates by BLAST search against the GenBank nucleotide (nt) reference database.

A) Overview of fungal isolates and identification based on a BLAST search of the ITS region of the large subunit ribosomal RNA gene (April 2017). Only highest matches to named species in GenBank are reported. The numbers between brackets indicate the number of isolates belonging to the corresponding species.

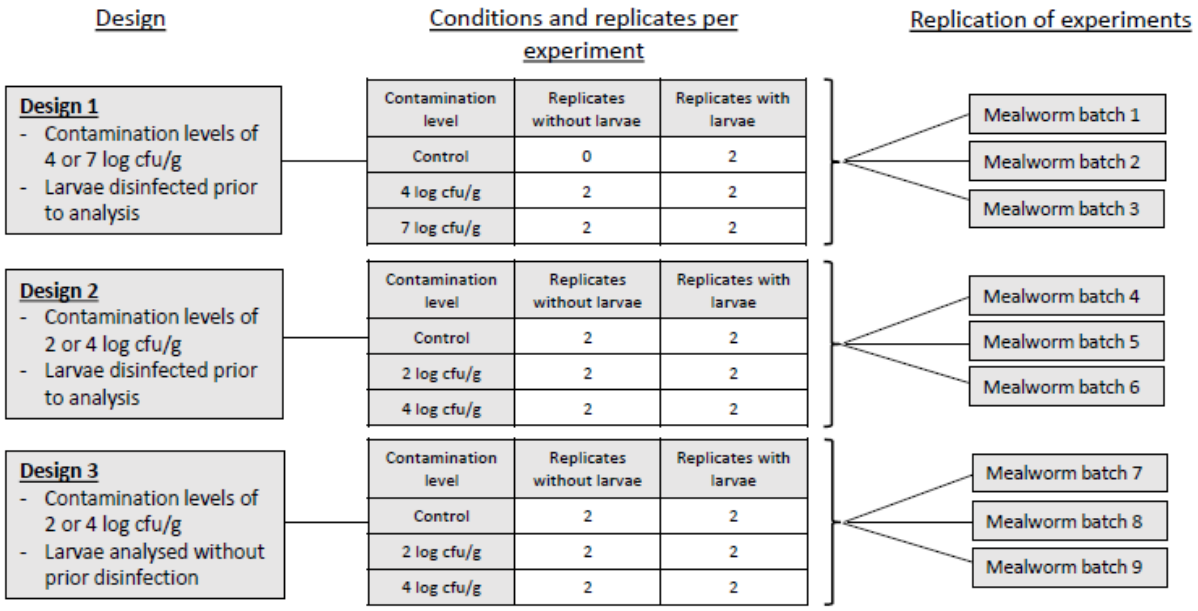
Sample	Number of isolates selected	Identification
Peat soil + coconut peel	4	*Trichoderma* sp. (4x) *Aspergillus* sp. (4x) *Aspergillus ostianus*
Substrate	10	*Aspergillus flavus* *Aspergillus versicolor* *Hyphopichia burtonii* *Lichtheimia ornata/corymbifera* *Penicillium* sp.
Residue (day 37)	10	*Aspergillus* sp./*Davidiella* sp. *Aspergillus clavatus* *Aspergillus amstelodami* *Candida palmioleophila* (2x) *Candida palmioleophila/manassasensis* *Lichtheimia corymbifera/ramosa* *Penicillium* sp. (2x) *Trichoderma asperellum*
Crickets (day 40, after harvest)	10	*Aspergillus* sp. (2x) *Candida allociferrii* (2x) *Kodamaea ohmeri* *Lichtheimia hyalospora* *Lichteimia corymbifera/ramosa* *Tetrapisispora fleetii* *Trichosporon asahii* *Trichoderma asperellum*

B) Top 5 hits against the GenBank nucleotide (nt) reference database including score, e-value and sequence identity. Uncultured/environmental entries were excluded.

➔ corresponds to **Table S2** in Vandeweyer et al. (2018), same paper as S4.1.

Supporting Information for Chapter 5

Figure S5.1 Scheme of the general setup of this study. Three designs were conducted, each design on three separate batches of mealworms, thus resulting in a total of nine individual experiments.



Supporting Information for Chapter 6

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

➔ corresponds to Table S1 in Wynants, E., Crauwels, S., Lievens, B., Luca, S., Claes, J., Borremans, A., Bruyninckx, L., 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 & Emerging Technologies*, *42*, 8-15. https://doi.org/10.1016/j.ifset.2017.06.004

Table S6.2 Identification of operational taxonomic units (OTUs) according to the SILVA reference database. Taxonomic assignments were considered reliable when a score value > 0.80 was found. "Unclassified" refers to a score value <0.80.

➔ corresponds to **Table S2** in Wynants et al. (2017), same paper as Table S2.1.

Table S6.3. Identification of operational taxonomic units (OTUs) by BLAST search against the GenBank nucleotide (nt) reference database.

➔ corresponds to **Table S3** in Wynants et al. (2017), same paper as Table S2.1.