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Biological contaminants in insects as food and feed

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REVIEW ARTICLE

Abstract

During the last decade, edible insects have successfully taken a meaningful position in the feed and food chain. To expand this position, product safety continuously needs to be warranted. This review focuses on the current knowledge and the future challenges on the prevalence of human foodborne pathogens in edible insects. The top three of the bacterial pathogens associated with insects for food are *Staphylococcus aureus*, pathogenic *Clostridium* spp. and pathogenic species of the *Bacillus cereus* group. Less is known about other types of biological contaminants, the fungi, viruses, protozoa and prions. For insects for feed, even less reports on pathogens are available so far, although the microbiota of *Hermetia illucens* is increasingly being studied in the latest years. In addition to the evaluation of endogenous microorganisms in insects, an overview is given of inoculation experiments to study the fate of specific food pathogens during rearing. Future challenges that are identified mainly relate to the fact that risk assessments directed to specific insect species are needed. Also, more research data are needed on the microbiological quality of substrates and residue, in connection with decontamination treatments. The house flora of rearing facilities has not been investigated before. The insect supply chain can generate insights in the microbiological quality of the integral chain by implementing exhaustive sampling plans and by applying predictive microbiology. Additionally, microbiological methods used in research and quality control require standardisation. Rather unexplored so far is the unculturable fraction of the insect microbial community and its importance in food safety. Last but not least, the most important microbiological challenge may well be situated in the further development of the sector: upscaling in terms of capacity and number of companies will increase the complexity of the sector. That will have implications for monitoring and control of biological contaminants.

Keywords: edible insects, microbiological pathogens, microbiological safety

1. Introduction

In addition to physical and chemical safety, feed and food also have to comply with biological safety. The legal microbiological criteria applicable for the feed and food industry rely on culture-dependent methods, in which a fresh sample is diluted or alternatively resuscitated, and then plated and incubated to determine colony counts, or incubated to observe the absence or presence of a food pathogen, respectively. Hence, depending on the biological contaminant, safety involves either its presence to be below a specific level or its complete absence in a predefined quantity of the matrix (De Loy-Hendrickx *et al.*, 2018). Biological contaminants (hazards) encompass pathogenic strains of micro-organisms (i.e. bacteria, viruses and fungi, which contain both moulds and yeasts), and of parasites (i.e. protozoa and worms), as well as the toxic substances (chemicals) they produce, i.e. bacterial toxins, such as for instance cereulide, histamine, botulin, or mycotoxins (WHO, 1995; Zwietering *et al.*, 2016).

In the last decade, and as shown in this review, increasingly more reports appear that describe the complete microbiome or subgroups of insect species reared for animal or human food, focusing on the rearing stage and/or post-harvest practices. In some of these studies, special attention is paid to – mostly bacterial – pathogens that can cause zoonoses. Conclusions with respect to microbiological

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safety are often difficult to draw. A first reason for this is that legislation on microbiological criteria for insects as feed or food, to be used as a reference for what can be considered as safe, is limited, as also concluded by Garofalo *et al.* (2019). Biological safety is not yet well established for insects and therefore legislation is not extensive. The extrapolation of microbiological criteria from other food types (e.g. included in Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs; EC, 2005) to insects is hardly relevant, since pathogens show a different growth pattern and physiological behaviour, such as sporulation or spore germination, in diverse foods with concomitant intrinsic and extrinsic properties (Jay *et al.*, 2005). Secondly, for some bacterial contaminants criteria exist for insects, but results reported in literature do not always involve the required amount of samples, or the results were not obtained using the methods described in the criteria.

The aim of this review is twofold. In a first part, we present an update of reviews and studies available, describing certain biological contaminants in insects for food and feed. In this review, we consider the possible occurrence of pathogenic bacteria, fungi and viruses, prions and protozoa, but we do not focus on the presence of toxic compounds produced by micro-organisms. In this first part, we make a further distinction: we first describe pathogens in insect species produced for food purposes only or for both food and feed, and secondly we describe pathogens in insects to be (generally) used in feed only. The reason for this structure is that there is a clear distinction in the amount of data available in the two domains. For insects for food or both food and feed, several studies are acquirable, and this work even has been summarised in several reviews. Hence, we will build on the most recent reviews, compare them and add reports that appeared after them, to come to the main state of the art. In contrast, for insects only used for feed, little data are available. Therefore, we provide an overview of original data, collected through searches in PubMed, Google Scholar and Web of Science, by using the search terms 'housefly', '*Musca domestica*', 'black soldier fly', or '*Hermetia illucens*', each combined with 'microbiota' or 'microbiome'. Only papers related to mass rearing of the insects were considered. In the second part, this review also aims at deeply discussing the future challenges in both the research on biological contaminants as well as the practical implementation of measurements to warrant biological safety in the context of the rapidly evolving insect rearing and processing sectors.

2. Biological risks associated with insects to be used in food or in both food and feed

In 2019, four literature reviews were published online that discuss studies performed so far on the microbiological quality and safety of mass reared insects for human food purposes. The reviews have in common that none of them explicitly describes biological risks for producing and processing insects into animal feed. All of them consider the industrial scale production of insects, and three of them also include insects that are wild-harvested at large scale (Table 1). While all reviews do not only cover the rearing phase but also post-harvest processing, they differ somewhat with respect to the mentioned technologies (Table 1). Finally, all reviews discuss results obtained by both culture-independent microbiological analyses (plates counts, presence absence tests), as well as by the mostly used culture-independent approach for microbial community assessment, metagenetics. Metagenetics is based on a polymerase chain reaction (PCR) to amplify and then sequence certain phylum-specific genes, typically the 16S ribosomal RNA gene for bacteria and 18S rRNA gene for fungi, from all DNA extracted from a whole microbial community (Martin *et al.*, 2018). Below, we shortly describe the specificities of each review and then consolidate the main findings.

Murefu *et al.* (2019) reviewed the food safety hazards of both reared and wild-harvested edible insects. The study revealed the lead of Africa in studying food safety of insects until 2016, yet on wild-harvested insects (Figure 1). Later, the majority of studies reporting food safety of edible insects were European, investigating other, reared species. It was concluded that the harvesting type (wild or reared) strongly affects the food safety of the insect. Regarding biological risks, the bacteria *Bacillus (cereus* group), *Clostridium* and *Staphylococcus* as well as the fungi *Aspergillus* and *Penicillium* were regularly mentioned for the five main edible insect species considered in this study (Table 2), as well as for several additional African species.

In the review by Garofalo *et al.* (2019), data originated from over 32 species (7 orders) of both fresh and processed insects, either harvested in the wild or (industrially) reared (Figure 1). Furthermore, also microbiological data from insect-based products were included in the review. Up till 2015, most data were obtained from wild-harvested African insect species, while as from 2016, associated with the renewed novel food regulation (Regulation (EU) No. 2015/2283; EC, 2015), a drastic increase in scientific studies on edible insects reared in Europe was observed (Figure 1). Consequently, the most studied insects for human food nowadays are 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*). With an extensive metadata collection, Garofalo *et al.* (2019) were able to assess and discuss both food hygiene and food safety of edible insects. Additionally, the data provided insight into the microbial profiles associated with different insect species, to evaluate their dynamics during rearing and the effect of commonly applied treatments on those microbial profiles. Pathogenic microorganisms reported in

Table 1. Comparison of the four review publications on the microbiological safety of insects for food published online in 2019.

Review characteristics	Reviews			
	Murefu *et al.* (2019)	**Garofalo *et al.* (2019)**	**Cappelli *et al.* (2020)**	**Kooh *et al.* (2019)**
Online publication date	6 March 2019	25 July 2019	6 September 2019	14 October 2019
Time span covered[1]	1993-2019	2000-2019	2016-2019	1994-2019
Systematic or narrative review[2]	systematic	systematic	systematic	narrative
Including wild-harvested insects?	yes	yes	no	yes
Post-harvest treatments described with respect to effect on microbiological quality	blanching (par)boiling/cooking canning chilling degutting drying[3] fermenting freezing frying milling/grinding/grounding/pulverising packing (vacuum or not) plucking rinsing/washing roasting salting smoking sterilising	blanching boiling/cooking chilling degutting drying[3] extruding fermenting (deep-)frying marinating milling/crushing plucking rinsing/washing roasting salting smoking spicing/condimenting starving sterilising	blanching boiling/(vacuum) cooking chilling cold atmospheric pressure plasma crushing/grinding drying[3] enzymatic hydrolysis fermenting fractionating freezing frying *in vitro* digestion marinating pH change pureeing rinsing/washing roasting smoking starving/fasting sterilising	boiling/cooking grinding drying[3] freezing frying high hydrostatic pressure packing rinsing roasting starving/fasting

[1] Year of oldest and newest publication included in the review.

[2] A systematic review involves a systematic search of the literature while a narrative review tends to be mainly descriptive and does not involve a systematic search of the literature (Ulman, 2011).

[3] Drying techniques can include freeze-drying, oven drying, microwave drying and/or solar drying.

the aforementioned most important insect species for food are listed in Table 2. The list contains both spore-forming and non-spore-forming bacteria, as well as mycotoxin-producing fungi. From the data compiled by Garofalo *et al.* (2019), it can be concluded that the bacterial genera *Bacillus* (including the *B. cereus* group, as discussed further below), *Clostridium* (including *Clostridium perfringens*), and *Staphylococcus* (including *Staphylococcus aureus*) are to be considered as the most relevant risks regarding food safety of edible insects. The genera *Cronobacter* (including *C. sakazakii*), *Pseudomonas* (including *Pseudomonas aeruginosa*), *Vibrio*, *Campylobacter*, *Salmonella* and *Listeria* (including *Listeria monocytogenes*) were concluded to pose a lower risk. More recently, presumptive *Cronobacter* spp. were detected sporadically in freeze-dried lesser mealworms and in house cricket meal (Greenhalgh and Amund, 2019), but also here, safety risks were concluded to be low. Regarding mycotoxin-producing fungi, *Aspergillus* spp. and *Penicillium* spp. were found to involve the highest risks.

Cappelli *et al.* (2020) provided a chemical and microbiological risk assessment applied on the insect families Tenebrionidae (darkling beetles, including *T. molitor* and *A. diaperinus*) and Gryllidae (crickets, including *A. domesticus* and *G. sigillatus*). Regarding the microbiological risks, human foodborne pathogens that were reported per insect species are again included in Table 2. The genera *Bacillus*, *Clostridium* and *Staphylococcus* were mentioned most often and also this study concluded that *Salmonella* spp. and *L. monocytogenes* are low-level

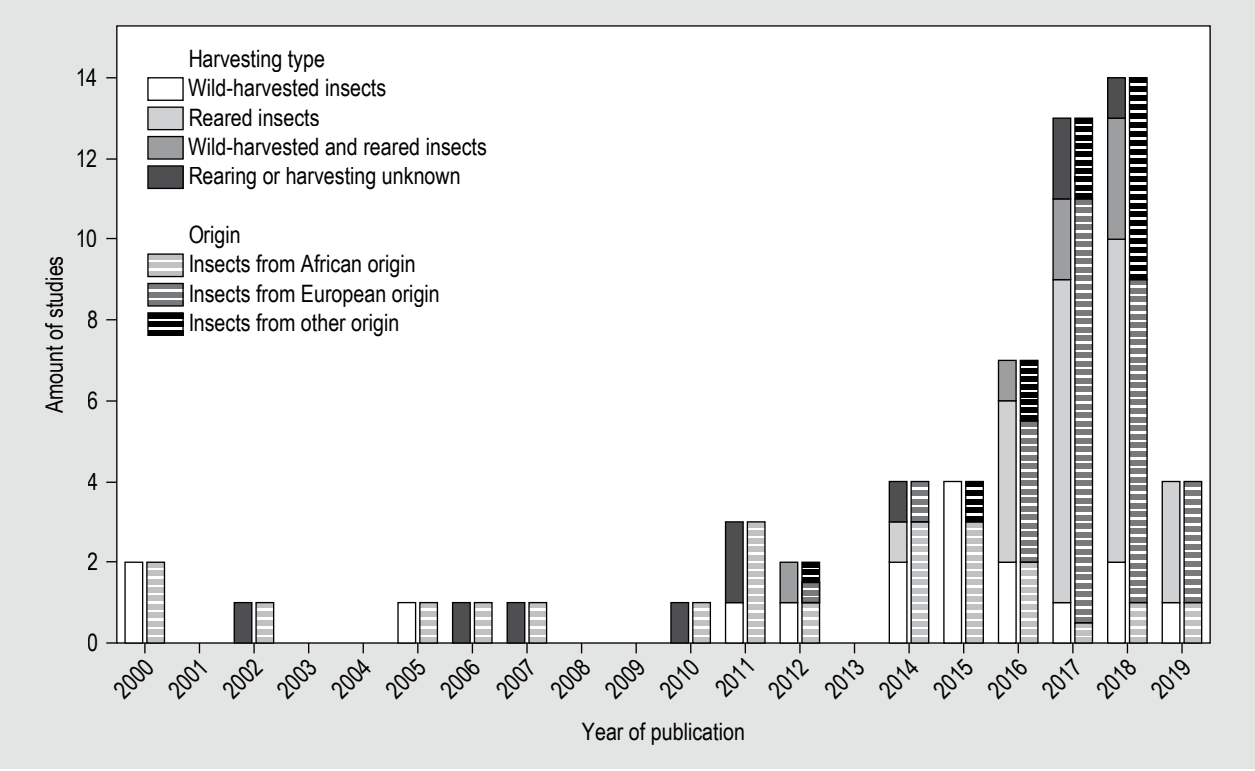

Figure 1. Amount of studies reporting microbiological data for insects for food between 2000 and 2019. Two graphs per year represent the reared or wild-harvested nature of the insects and the geographical origin of the insects, respectively, employed in the studies. Data were compiled from Garofalo *et al.* (2019) and supplemented with data from Kooh *et al.* (2019), Murefu *et al.* (2019) and Cappelli *et al.* (2020).

risks. Further, Cappelli *et al.* (2020) reported that prions and foodborne parasites and viruses can be considered as low risks and are not described yet in reared insects for human consumption.

Finally, a review article by Kooh *et al.* (2019) described the biological health risks associated with entomophagy. Pathogens reported by this study associated with the mentioned main five insect species are also included in Table 2. Using a more general, narrative approach, the authors came to similar conclusions as Garofalo *et al.* (2019), being that edible insects and derived products pose a low risk regarding *Salmonella* spp., *Campylobacter* spp., *Yersinia* spp., *Vibrio* spp., and *L. monocytogenes*. However, *S. aureus* was concluded to be very abundant in edible insects and spore formers such as *Clostridium* spp. and *Bacillus* spp. were reported to be of major concern. Especially members of the *B. cereus* group were presented as a substantial food safety hazard for insects as human food. Information regarding other foodborne biological contaminants (parasites, prions and viruses) was concluded to be scarce.

We can compile the aforementioned reviews and the studies they cover, to make overall conclusions on the state of the art in knowledge on the microbiological safety of insects for food. First, the currently available literature shows that barely any data are available regarding prions and foodborne viruses and parasites in insects farmed for human consumption. While certain microbiological risk assessments on edible insects (EFSA Scientific Committee, 2015; Fernandez-Cassi *et al.*, 2018) claim that those biological contaminants present only a low safety risk, this has not been investigated yet. For foodborne viruses, however, a first effort was made in a very recent study (Vandeweyer *et al.*, 2020a) which investigated the presence of Hepatitis A and E virus and Norovirus genogroup II in several raw insect samples collected from industrial producers. This research could confirm the low risk regarding those three viruses for the samples investigated, since none of the viruses were detected. In view of the recent outbreak of COVID-19, Dicke *et al.* (2020) investigated the transmission potential of edible insects for the zoonotic coronavirus SARS-CoV-2 (which does not appear to be foodborne so far), and concluded that hazard to be extremely low. Nonetheless, further research investigating other foodborne viruses and other insects, as well as research regarding prions and foodborne parasites is still required. Their fate during insect processing has also not been investigated so far.

Table 2. Foodborne biological contaminants (bacteria and fungi) reported in edible insect species that pose a potential food safety risk. In the case only a genus name is mentioned, one or more species within that genus (other than a species that also may be listed) can be pathogenic.

Study	Insect species	Potential foodborne biological safety risks identified	
		Bacteria	**Fungi**
Garofalo *et al*. (2019)	*Alphitobius diaperinus*	*Aeromonas, Bacillus, Pseudomonas*	*Aspergillus flavus, Aspergillus, Penicillium*
	Tenebrio molitor	*Bacillus cereus* group, *Bacillus, Clostridium perfringens, Clostridium, Cronobacter, Escherichia coli, Listeria*[1], *Pseudomonas, Salmonella*[2], *Staphylococcus aureus, Staphylococcus, Vibrio, Yersinia*	*Penicillium*
	Acheta domesticus	*B. cereus* group, *Bacillus, C. perfringens, Clostridium, Listeria*[1], *Pseudomonas, Staphylococcus*	*Aspergillus*
	Locusta migratoria	*Bacillus, C. perfringens, Pseudomonas, Staphylococcus, Yersinia*	*Aspergillus*
Kooh *et al*. (2019)	*A. diaperinus*		*A. flavus*
	T. molitor	*B. cereus* group, *C. perfringens, Clostridium*	
	A. domesticus	*B. cereus* group, *C. perfringens*	
	L. migratoria	*C. perfringens*	
	Edible insects in general	*Bacillus, B. cereus* group, *C. perfringens, S. aureus*	
Murefu *et al*. (2019)	*A. diaperinus*	*Bacillus, Clostridium*	*A. flavus, Aspergillus, Penicillium*
	T. molitor	*Bacillus, B. cereus* group, *Clostridium, Escherichia, Listeria*[1], *Pseudomonas, Staphylococcus*	*Penicillium*
	A. domesticus	*Bacillus, B. cereus group, Clostridium, Listeria*[1], *Staphylococcus*	
	L. migratoria	*Staphylococcus*	
Cappelli *et al*. (2020)	*A. diaperinus*		*Aspergillus*
	T. molitor	*B. cereus* group, *Bacillus, C. perfringens, Clostridium, Listeria*[1], *Staphylococcus*	
	A. domesticus	*B. cereus* group, *Bacillus, C. perfringens, Clostridium, Listeria*[1], *Staphylococcus, Yersinia*	

[1] Only the genus *Listeria* was detected so far. The pathogenic species *Listeria monocytogenes* has never been detected in edible insect species.
[2] *Salmonella* spp. were only detected by means of DNA-based analyses. Viable cells have not been detected so far.

Secondly, it is clear that the three major foodborne bacterial hazards in Europe, *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* (Van Cauteren *et al.*, 2017), are barely reported and consequently pose a low health risk for edible insects. Also *Cronobacter* spp., *Vibrio* spp. and *Yersinia* spp. are very rarely reported. However, the three main bacterial genera/species associated with food safety problems in edible insects are *S. aureus*, *Clostridium* spp., with the species *C. perfringens* and *Clostridium botulinum* being the most relevant food pathogens within this genus, and the *B. cereus* group. The *B. cereus* group, but also *Clostridium* spp., are spore-forming bacteria. This observation is noteworthy, since mitigation of the risks associated with bacterial endospores requires more drastic strategies than those for vegetative cells (Kort *et al.*, 2005). As to mycotoxin-producing fungi, *Aspergillus* spp. and *Penicillium* spp. are noted as the most hazardous species. Also in the most recent studies (Borremans *et al.*, 2019; Fernandez-Cassi *et al.*, 2020; Frigerio *et al.*, 2020; Mancini *et al.*, 2019b), (some of) the same main biological safety hazards were addressed.

From all those risks, the presence of the *B. cereus* group can probably be ranked as the highest food safety risk

for insects for human consumption. As demonstrated by multiple studies (Fasolato *et al.*, 2018; Vandeweyer *et al.*, 2018, 2020a,b), the *B. cereus* group can be encountered in several insect species and derived products, sometimes in high amounts. The *B. cereus* group, or *B. cereus sensu lato (s.l.)*, consists of a number of recognised species, including *B. cereus sensu strictu (s.s.)* and *Bacillus thuringiensis* (EFSA BIOHAZ Panel, 2016). All group members are genetically (and phenotypically) closely related to each other, and therefore no perfect distinction can be made between the members based on the 16S ribosomal RNA gene (Ehling-Schulz *et al.*, 2019). Moreover, their virulence for humans is very difficult to assess, since not all virulence genes (that could be used as marker gene for virulence) are known yet. Also, virulence appears to be very dependent on strain and environmental conditions (Jeβberger *et al.*, 2015) and, as the known virulence genes are situated on (possibly mobile) plasmids, they can potentially be exchanged between members (SciCom, 2018). *B. thuringiensis* possesses a similar portfolio of potential virulence genes as *B. cereus s.s.* strains and is able to express them (EFSA BIOHAZ Panel, 2016). Therefore, *B. thuringiensis*, which is typically known as entomopathogen and can be a threat for the insect yield in mass production, can also be hazardous for humans.

When we look at the insect species that have been investigated with respect to microbiological food safety so far, the most important ones are *T. molitor* and *A. domesticus*. The other mealworm and cricket species, as well as *L. migratoria* are also being considered for human food (and novel food dossiers are submitted), but they were subjected to biological risk assessments to a much lesser extent. Hence, increased efforts to fill the knowledge gap for these particular species are encouraged. Moreover, also for the black soldier fly (*H. illucens*), a novel food dossier was submitted. While rarely considered as a food source in Europe (in contrast to certain Asian and African regions (Wang and Shelomi, 2017) and the discussion of its potential as food for the Pacific Small Islands Developing States (Shelomi, 2020)), biological safety of this species has not been described regularly in the context of human food. For animal feed, however, several studies have been performed, as detailed below.

3. Biological risks associated with insects to be used in feed only

In the EU, insects are considered farm animals and hence they are subjected to the EU 'feed ban' (Regulation (EC) No 999/2001; EC, 2001), which prohibits the use of farmed animal-derived proteins in feed for ruminant and monogastric animals. This effectively limited the use of insect proteins for a long time to applications in pet food or feed for fur animals. Since the 1st of July 2017, the use of insect proteins from seven insect species in feed for aquaculture animals has been authorised (Regulation (EU) No 2017/893; EC, 2017). The exact species are black soldier fly, common housefly (*M. domestica*), yellow mealworm, lesser mealworm, house cricket, tropical house cricket and field cricket (*Gryllus assimilis*). From these seven, only the common housefly and the black soldier fly are not commonly considered as human food and thus have not yet been discussed earlier in this review. Furthermore, these two species (together with the yellow mealworm) are the most generally used insects as protein source for animal feed globally. Hence, we will focus on the microbiological risks associated with these two species to assess the biological risks for insects as feed.

To identify which bacteria or fungi are most likely to cause problems, a clear view on the insect microbiota is needed. For the black soldier fly, this was long underexplored (De Smet *et al.*, 2018), but a number of recent publications expand our understanding of the microbiota in the larvae on a variety of diets (Bruno *et al.*, 2019; Jiang *et al.*, 2019; Klammsteiner *et al.*, 2020; Shelomi *et al.*, 2020; Wu *et al.*, 2020; Wynants *et al.*, 2018; Zhan *et al.*, 2020). These studies are more and more revealing the composition of the present microbiota in the larvae and the impact of substrate on its composition. Wynants *et al.* (2018) reported distinct sets of bacteria in the larvae and residue compared to the fed substrate. The same conclusion was drawn by Klammsteiner *et al.* (2020), who even more clearly hinted at the existence of a core set of microbes that make up the gut microbiota in the larvae, which may be established early on in larval development. Other studies show that microbes from the substrate can enter the gut and, if they are able to proliferate, become part of the gut microbiota, but they also state that these microbes never fully take control of this ecological niche (Bruno *et al.*, 2019; Jiang *et al.*, 2019). Bruno *et al.* (2019) for example reported variation in the bacterial species present in distinct regions of the gut, which is most likely due to the fact that each region represents a different ecological niche with other conditions. At the same time, the consensus is growing that a set of species is present in the larva, albeit to varying extent, during rearing on most of the tested diets to date, making them the core of the black soldier fly larvae (BSFL) gut microbiota. This core appears to include *Actinomyces* sp., *Dysgonomonas* sp., *Enterococcus* sp. and *Morganella* sp. These species have been found in other insects as well and could aid in the degradation of organic substances (Klammsteiner *et al.*, 2020).

While the microbiota of the common housefly has been studied for individuals captured in the wild (Bahrndorff *et al.*, 2017; De Jonge *et al.*, 2020), to our knowledge no studies have been performed to assess its microbiota during an industrial rearing cycle, and therefore the microbiological quality cannot be compared to that of other insects for feed.

When exploring specifically the presence of food pathogens, some studies do report potential risks in BSFL. Wynants *et al.* (2018) reported no detection of *L. monocytogenes* or coagulase-positive staphylococci. In contrast, they observed one contamination with *Salmonella enterica* serovar Agona in the rearing residue, but not in the larvae. Presumptive *B. cereus* was found in the larvae with counts up to 6,000 cfu/g. Other studies report the presence of *Campylobacter* (Wu *et al.*, 2020) or *Clostridium* (Jiang *et al.*, 2019) species in their metagenetics data but they did not quantify the actual pathogen load. Hence, the *B. cereus* group seems to be a major risk for insects for feed as well. With respect to other biological risks, e.g. prions and viruses, no data could be found that address safety questions for neither of both insect species. Only one report deals with the presence of a virus (be it not a food safety issue) in the BSFL gut: Chen *et al.* (2019) isolated a novel temperate *Escherichia* phage from the BSFL gut, and different substrates caused differences in phage induction. Hence, substrates may not only shape the gut microbiota directly, but also indirectly via their impact on lytic/lysogenic switches of phages infecting gut bacteria. For foodborne viruses, no information is available to our knowledge. Also, insects are, to date, not found to be able to produce prions, but prions can enter non-processed insects from contaminated substrates, for example slaughterhouse waste (EFSA Scientific Committee, 2015). This is a low level risk, that can be avoided by substrate quality control or preventing the feeding of these larvae to the same animal species as the one present in the substrate.

4. Risk assessments on the transfer of biological contaminants during rearing of insects

Biological safety risks are not only defined by the intrinsic presence or absence of pathogenic microorganisms. It is also important to estimate the risks for coincidental contamination during insect rearing or processing by a certain pathogen (EFSA Scientific Committee, 2015; Wynants *et al.*, 2018). Studies that monitor the dynamics of a specific food pathogen when present in the substrate by inoculating the pathogen in the substrate (so-called challenge tests) are emerging in recent years (Belleggia *et al.*, 2020; Mancini *et al.*, 2019c; Wynants *et al.*, 2019).

Almost all the available studies are about *T. molitor* and only some information on other species is available. Mancini *et al.* (2019c) detected a transmission of 3.6-4.6 log cfu/g of *L. monocytogenes* to mealworm larvae since the first day in the substrate at a load of 8 log cfu/g. This contamination seemed to persist for at least seven days. According to Belleggia *et al.* (2020), *Listeria* spp. can even multiply in the gut of the mealworm larvae. Because washing of the larvae did not cause a reduction in microbial counts, the bacteria were assumed to be present in the interior part of the larvae (Mancini *et al.*, 2019c). This can cause considerable health risks when using the larvae, as a whole or after processing without degutting, as food or feed. Also *Salmonella* sp. can be transmitted to *T. molitor* already after one day and, if inoculated at 7 log cfu/g in the substrate, the contamination was still present after seven days (Wynants *et al.*, 2019). BSFL have also been subjected to several challenge studies. Defilippo *et al.* (2018) determined that larvae contain the same concentration of *S. enterica* serovar Typhimurium (3-4 log cfu/g) and *L. monocytogenes* (5-6 log cfu/g) as the inoculated substrate, but they observed a 2-log decreased concentration when reaching the pupal stage. No hypothesis for this observation was given. Swinscoe *et al.* (2020) observed a stable concentration of *L. monocytogenes* and *Vibrio parahaemolyticus* (6 log cfu/g) and a slightly decreasing concentration of *Escherichia coli* and *E. coli* O157:H7 (from 7 log cfu/g to 5 log cfu/g) over a period of seven days after exposure to a contaminated substrate. The authors mention that a selective inactivation (i.e. some strains are inhibited while others are not) of the pathogens by the larvae occurs via exposure to antimicrobials (showing a certain selectivity). In particular, the expression of antimicrobial peptides by BSFL is substantial in protein-rich diets, as the seaweed-based diet used in their study. Although pathogen transfer does not necessarily affect the viability of the larvae in a negative way (Mancini *et al.*, 2019c), it can be a risk when using these contaminated larvae for food or feed purposes. However, it is worth noting that the results observed seem to depend on numerous factors, such as pathogen level in the substrate and stage in the cycle of the insect, and thus general conclusions cannot be extrapolated to other combinations of insect and pathogen species.

Even though a number of challenge tests were performed for bacteria, transfer of other biological risks, such as prions, viruses, fungi and parasites, is much less investigated. One recent study on transmission of parasites via BSFL feed (Müller *et al.*, 2019) revealed a transfer of less than 1% of the parasital oocysts of *Eimeria nieschulzi*, *Eimeria tenella* or eggs of *Ascaris suum* in the larval gut. No or very low contamination was found in the prepupal stages. Nevertheless, it is still too early to neglect the potential risk of parasite transmission when using BSFL as animal feed. Next to parasites, a study from Varotto Boccazzi *et al.* (2017) investigated the transfer of fungi to BSFL reared on chicken feed and vegetable waste, with both substrates causing a different fungal community in the larvae. Larvae grown on chicken feed contained mainly *Trichosporon*, *Rhodotorula* and *Geotrichum* species, whereas in vegetable waste fed larvae, *Pichia* was the most abundant genus.

Besides challenge tests with contaminated substrates, also indirect exposure of insects to human pathogens has been studied. For the housefly (*M. domestica*), studies exist that focus on wild flies. One such study confirmed the transfer of different pathogens by exposure to facilities containing

contaminated farm animals. About 20% of the flies caught on broiler farms were positive for *Salmonella* (Bailey *et al.*, 2001), and even more than 50% tested positive after 4 to 7 days exposure to environments housing *S. enterica* serovar Enteritidis challenged hens (Holt *et al.*, 2007). In another study, *Campylobacter* sp. could be isolated from about 40 to 50% of the flies sampled on chicken farms and piggeries (Rosef and Kapperud, 1983). These studies also demonstrate that rearing of the common housefly should take place in clean conditions as contaminations of the feed are very likely to enter the insect.

Despite the fact that various biological risks can be transferred to insects, some species seem to be able to reduce or even eliminate specific bacteria in their substrate. They can therefore be reasoned to decrease the biological risk. For instance, BSFL have shown to be able to reduce the amount of Enterobacteriaceae. A reduction of *S. enterica* serovar Enteritidis (3.5-5.5 log reduction after two days) and *E. coli* O157:H7 (1.5-5 log reduction after two days and even below detection limit after three days) in chicken manure has been observed (Erickson *et al.*, 2004). Also Liu *et al.* (2008) and Lalander *et al.* (2013) found a reduction of respectively *E. coli* by 5-7 log cycles in dairy manure and of *Salmonella* spp. by 7 log cycles in eight days in faecal sludge. Lalander *et al.* (2015) studied the reduction of *Salmonella* spp. and *Enterococcus* spp. in a mixture of pig manure, human faeces and dog food. For *Salmonella* spp., the concentration in the substrate was reduced from approximately 7 to less than 1 cfu/g after two weeks, while *Enterococcus* contamination remained unchanged. Those experiments could be interpreted as promising perspectives for a safe recycling of animal or human manure by BSFL. Nevertheless, according to (EC) No. 1069/2009, the use as substrate of manure, catering waste and former foodstuff containing meat or fish is forbidden for farmed animals, including insects destined for food and feed purposes. So far, the pathogen-reducing effects of BSFL have not been studied and confirmed enough to alter legislation. It is not known, for instance, whether the effects are substrate-dependent or not. More data may help to fine-tune legislation on this aspect in the future.

5. Mitigation strategies to reduce food and feed safety risks

As is clear from literature, the high microbial counts associated with untreated edible insects and the possible occurrence of certain pathogenic microorganisms require suitable mitigation strategies, i.e. strategies to reduce the microbiological risks, to be applied on the insects in order to ensure safety. The four reviews on insects for food referred to before discuss studies that report on treatments to reduce microbiological safety risks (Table 1). Altogether, thermal treatments such as boiling, roasting, (deep-)frying and blanching, even for short times, have proven to be very effective to reduce the amount of vegetative bacteria and fungi. Alternative treatments such as fermentation, cold plasma treatments, microwave drying may also reduce microbiological food safety risks. For example, a recent study investigated the efficacy of high hydrostatic pressure (HHP) to reduce certain microbiological risks in a meal derived from dried BSFL (Kashiri *et al.*, 2018). They reported that *Listeria* was not present in the extract, but *Salmonella* and *E. coli* were found at around 5 to 6 log cfu/g. The killing efficacy of a HHP treatment at 400 MPa for 7 minutes was the highest for yeasts and moulds (no survivors), but had hardly any effect on the count of naturally present total aerobic mesophilic bacteria (0.35 log reduction). No data were provided on the reduction of *Salmonella*. However, the treatment did reduce the load of inoculated *E. coli* with 6.56 log cycles (Kashiri *et al.*, 2018). The fact that this technology was less effective against naturally present bacteria than against inoculated *E. coli* is hypothesised to be due to the presence of bacterial endospores. This is an important factor, as the *B. cereus* group is considered as a high risk. Non-thermal drying methods such as freeze-drying, which is commonly used in the insect industry, only have a microbiostatic effect and are therefore not sufficient to assure microbiological food safety. Starvation of the insects at the end of the rearing cycle may be thought to have the effect of emptying the gut and hence clearing the insects from pathogens as well, but starvation should not be considered as a mitigation strategy since the effect on the microbiota appears to vary for different studies (Mancini *et al.*, 2019a,b; Wynants *et al.*, 2017).

6. Future challenges and research needs for biological safety of edible insects

Focus needed on individual insect species

In the same way as the gut microbiome is not equal for all traditional farm animals, it is not accurate to make general statements on the microbiota of edible insects altogether. Risk profiles and assessments should envisage individual species, such as the risk profile for *A. domesticus* by Fernandez-Cassi *et al.* in 2018 and in 2019, in order to identify and implement species-specific hygiene measures during rearing. When organising studies that focus on an individual insect species, care should be taken to include samples obtained from industrial producers rather than from insects reared in laboratory conditions. To obtain a representative view on the microbiota and on food pathogens during mass rearing of insects, it is necessary to involve several producers, hence covering differences in substrates and production processes applied on different locations, as done for instance by Wynants *et al.* (2019). In addition, per producer several batches or rearing cycles need to be sampled using proper sampling plans, as detailed below. Batches are ideally produced over a time span of several months to capture possible variations over time in

substrate quality and seasonality, environmental conditions and rearing procedures. Such studies are extensive, but allow to describe possible correlations between the microbiota including the occurrence of biological contaminants on the one side and rearing conditions (feed, environmental conditions, hygiene practices and house flora, etc.) on the other side.

To supplement and deepen species-specific risk assessments, the behaviour of certain biological contaminants during the life cycle of an insect can be studied as mentioned before by challenge tests. Deliberately infecting insects at a certain stage in their development with a known and standardised concentration of the pathogen allows to investigate if and how fast the contaminant is taken up by insect specimens (horizontal transfer). Also, it can be monitored where it is located in the gut and how passage through the gut occurs, how fast it is spread between specimens, whether it can grow in the substrate and/or insect or whether it naturally dies or maybe is actively eradicated by the insect. As discussed above, some combinations of insect and pathogen species have been investigated in this way, but a number of relevant insect-pathogen combinations have not been investigated so far. Especially for the main biological risks identified, *B. cereus*, *Clostridium* spp. and *S. aureus*, such data are still lacking for insects for food and feed. Overall, there is still a gap in the knowledge in the behaviour of biological contaminants that can be present in side or waste streams or that can be transmitted via personnel, i.e. *Bacillus*, *Campylobacter*, *Clostridium*, *Listeria*, *Salmonella*, and *Staphylococcus* during rearing of mass produced insects. *Bacillus* and *Clostridium* are two spore-forming bacterial species, and studies ideally involve both vegetative cells as well as spores. To the best of our knowledge, studies on vertical transmission of food pathogens, i.e. from one cycle to the next over the egg phase, are not yet available. It is not known so far whether the eggs can be a route by which food pathogens are introduced in a batch of larvae or nymphs. At least for *H. illucens*, it is thinkable that food pathogens may follow this transmission route, because bacterial communities including *Bacillus* sp. have been shown to be stimulating for oviposition (Yu *et al.*, 2011; Zheng *et al.*, 2013).

Also post-harvest decontamination and preservation strategies should be designed specifically for each individual species. A heat treatment comprises both a time and a temperature for the slowest heating point in the material to be treated. Inactivation kinetics cannot simply be extrapolated from one insect(-based product) to another, because: (1) heat transfer in a matrix depends on the composition (water content, fat content, etc.) and structure (whole insects, finely mixed paste or powder, or roughly grinded and hence containing air pockets) of the matrix; and (2) because individual insect species are characterised by other levels and types of target microorganisms. In the end, the time-temperature combination is to be determined to reduce the load of the most significant target organism in an insect matrix in a sufficient way, which is in the food industry often a reduction with 6, 8 or 12 log cycles. For inactivation strategies that do not use heat, also process parameters need to be defined for the matrix to be treated, i.e. a specific insect species, to obtain the same required reduction. Research to unravel the survival of spore-forming bacteria in post-harvest treatments preferably includes both vegetative cells as well as spores. While it may not be possible due to too much quality deterioration to eliminate all spores, heat treatments should not have the side effect of activating the spores, and if spores are observed to survive a treatment, then care should be taken to provide conditions during further storage and transport of the treated insect(-based product) that impede spore germination, such as (sufficient) refrigeration and/or acidification. As described before, a number of studies exist for particular species investigating the effect of heat or other treatments, but no general recommendations exist. Likely, knowledge and expertise is established in insect-processing companies and not (yet) publicly available.

Specific attention on substrate, residue and house flora

Not only the insects need further microbiological characterisation, also substrates should be characterised for their typical microbial profile, their core microbiota, if any, and their so-called specific spoilage organisms (SSO; Man and Jones, 2000). In the context of side stream storage, SSOs are those (subgroups of) microorganisms (bacteria, yeasts, moulds) that will dominate the microbiota when these organic streams are stored in insect production facilities and by their metabolism cause spoilage phenomena as off-odours and flavours. Side streams are often given one or more pre-treatment steps prior to be fed to the insects, such as mixing, concentration by a heat or alternative treatment, milling, acidification and so on. Those are all unit operations that can have an effect on the microbial load of the substrate, by either a killing effect or conversely improving conditions for microbial growth, and in turn on the insects reared on them. The impact on food pathogens that can occur in (mixtures of) organic side streams of the currently used preparation technologies is not yet thoroughly investigated. The effect of time-temperature conditions on survival of food pathogens inoculated in the substrate matrices at several contamination levels should be documented. Similar as to insect processing, this may have been studied to some extend by companies, but information in literature is missing.

The residue remaining after harvesting insects, containing unconsumed substrate, insect faeces or frass, and/or exuviae, offers the potential to be upcycled as soil fertiliser or plant growth supplement (Houben *et al.*, 2020). At least in the EU, legislation is not harmonised yet, and different

member states impose other inactivation conditions. Several (microbiological) questions are being discussed by researchers, authorities and stakeholders. According to Regulation (EU) No 142/2011 (EC, 2011), so-called 'Method 1 to 5' (describing time, temperature and pressure combinations related to specific particle sizes of the material to be treated) or 'Method 7' may be used for the hygiene of residual fractions from insect rearing. Methods 1 to 5 apply to materials with a certain particle size, but in insect rearing the particle size of the residual fraction can vary or is not always known by producers. Method 7 allows the development of a treatment that can be shown to result in the achievement of certain microbiological criteria for *C. perfringens*, *Salmonella* and Enterobacteriaceae. While Method 7 leaves room for insect producers to optimise their own processing method, in Europe there is a general interest to equalise the processing conditions for insect residue with those valid for animal manure, i.e. heat treatment of 70 °C for one hour (IPIFF, 2019a). A next step could then be to assess whether less stringent parameters and non-thermal inactivation strategies (that better preserve the chemical quality of the residue) can still warrant compliance with the safety standards. This requires inactivation trials with inoculated residues and results will be insect-specific, since the composition of the residue is also species-specific.

In the insect sector, the impact of the house flora in a rearing and/or processing company on the microbiological safety of the end product is pretty much uncharted terrain. Numbers of microorganisms present on food contact surfaces in food companies are known to increase during production and to be reduced during proper cleaning and disinfection (Holah, 2014). While these organisms were first only thought to be responsible for spoilage problems, they are now also known to be related to safety issues. Pathogens can interact with and grow in biofilms, and in a production environment a persistent house flora that is pathogenic can develop (Holah, 2014). For example, it has been demonstrated that the composition of the resident microorganisms on a surface, or the house flora, can either promote or inhibit the growth of *L. monocytogenes* in the biofilm, and hence determine whether a surface can be a contamination route for a pathogen or not (Carpentier and Chassaing, 2004). In the insect industry, research is needed on the potential of food pathogens to reside in the production environment or not, and whether the environment can in this way be part of the transmission routes for a pathogen or not. Investigating the house flora in an insect production plant can be of support in the implementation of a hazard analysis and critical control points (HACCP) plan, which will also be discussed below. The application of a HACCP plan is based on 7 principles and consists of 12 steps (FAO/WHO, 2009). In step 6, all potential hazards should be listed that may reasonably be expected to occur in the whole process line. If the composition of the house flora is known for several surfaces in the production site, it is also known whether these communities can harbour pathogens (and which ones, at what level and at what specific locations). Next, potential transfer from the environment to the insect(-products) can be assessed. If relevant, the transfer can be identified as a hazard and implemented in the determination of critical control points, in particular related to the cleaning and disinfection practices (step 7).

Proper sampling plans and predictive microbiology for knowledge extension

Whether it is in the investigation of microbiological quality of insects themselves, or of substrates, residue or the production environment, a central question is always how to construct a proper sampling plan. Insect producers are responsible for the microbiological safety of their products and have to make sure their products fulfil national and international compulsory microbiological criteria, as summarised in the IPIFF guide on good hygiene practices (IPIFF, 2019b). While these criteria may still be refined in the future, the challenge behind these targets for insect producers is how to convert those requirements (legal, or maybe even more stringent Business-to-Business agreements between producer and buyer) into practices, measures and interventions, that determine the microbiological quality during production (and hence also may evolve and be fine-tuned) in order to warrant the achievement of the targets for the end product. In the context of the food industry, Jacxsens *et al.* (2009) defined such 'company specific set of control and assurance activities to realise and guarantee food safety' as a food safety management system (FSMS). An FSMS should translate good hygienic practices and the HACCP system in the specific context of the company (De Loy-Hendrickx *et al.*, 2018). Jacxsens *et al.* (2009) also described a microbial assessment scheme (MAS) to investigate the microbial performance of such FSMS, i.e. to find out whether the correct sampling locations are identified, whether the most relevant microbiological parameters are selected, and to assess the sampling and analytical methods. A MAS has been applied to assess the microbiological performance of integral production and processing chains of e.g. lamb (Osés *et al.*, 2012), pangasius (Tong Thi *et al.*, 2014) and in Kenyan fresh produce processing and export companies (Kamau Njage *et al.*, 2017), but this exercise would also be useful for insect companies. Moreover, the subdomain in food microbiology of predictive microbiology, in which mathematical models predict the growth, survival, spore germination and toxin production of pathogens in a certain food matrix, is completely unexplored so far for insects, but can certainly bring new insights.

Microbiological methods: standardisation and dealing with the unculturable fraction

As one of its future tasks, the Commission on Insects of the European Federation for Animal Science (formerly European Association for Animal Production or EAAP) has decided to work on the standardisation of research methods. This is also pertinent for microbiological methods. Not only the media and incubation conditions used influence the result, but for insects it was shown that whether or not pulverising a sample prior to preparing a dilution series can make a difference in the count of even 1.6 to 2.2 log cfu/g (Vandeweyer *et al.*, 2017). Also, for culture-dependent microbial counts, it is a prerequisite that samples are investigated immediately after sampling or at the latest after one day storage under refrigerated conditions (rather than after frozen storage of samples). This is a practice that is unequivocal in microbiological food analysis, but it may not yet be understood and followed by all insect producers.

In colony counting, the incubation step entails a limitation since only a fraction of the microbiota is known to be culturable. Barcina and Irana (2009), as cited by Fakruddin *et al*. (2013), state that in water and soil samples from nature, less than 1% of the microorganisms is culturable. For insects used in food and feed, the share of the culturable fraction in the total microbiota has not been estimated so far. Moreover, within the field of food microbiology, special attention is paid to food pathogens that can enter a viable but nonculturable (VBNC) state, implying that their cells cannot grow on culture media but they still show metabolic activity as described for the first time by Xu *et al.* (1982). Recently, Zhao *et al.* (2017) presented a list of 35 foodborne pathogens that were proven to show this state, many species of which have already been identified on edible insects. From the review by Zhao *et al.* (2017), it appears that the VBNC state can be induced by bringing viable cells in stress conditions, such as in several food treatments relevant for insect processing as well (heating, cooling, drying). Based on their literature review, the authors also conclude that some pathogens retain their pathogenicity (as shown in animal models) while others are avirulent. Microscopic counting and molecular technologies are under development to assess VBNC food pathogens, and this is a new field in the microbiological safety of edible insects, too.

Culture-independent methodologies do not comprise an incubation step in which microorganisms are required to grow in order to quantify or detect them in a next step. Culture-independent analyses can be either a metagenetic analysis, as defined earlier, or they accomplish the amplification (and subsequent detection or quantification) of target sequences of DNA (or RNA) of particular microbial species, such as food pathogens (i.e. PCR technology). Metagenetics provides a more comprehensive overview of the microbial composition of an insect sample than plate counting does, and, as reviewed by Garofalo *et al.* (2019), the technology is increasingly being incorporated in studies characterising the insect microbiota during rearing and processing. Mostly metagenetics is DNA-based, although Bruno *et al.* (2019) worked on the RNA level (i.e. metatranscriptomics). Since RNA is known to disintegrate faster after cell death in contrast to DNA, it is considered as a good target to selectively focus on living cells. In terms of interpretation of microbiological safety, (DNA-based) metagenetics is limited in that it only demonstrates the presence of DNA, rather than the presence of viable and virulent food pathogens. DNA of dead cells is also detected by sequencing analyses, yet it does not always point towards a food safety problem. Conversely, processing of insects may destroy DNA, thereby eliminating the possibility to detect pathogens, which, even though the viable cells may have been inactivated by processing, may have produced toxic metabolites that were resistant to the processing conditions applied (such as the cereulide by *B. cereus*). In addition, Filippidou *et al.* (2015) mentioned that bacterial endospores can resist to DNA extraction and remain under-detected, even when applying methods specially developed to tackle spores. Hence, metagenetics proves its value to some extent in delivering a general overview of the microbial community composition in an insect sample. Meaningful detection (in terms of food safety assessment) of particular food pathogens can also be based on PCR techniques, preferably either Reverse Transcriptase-PCR starting from RNA, or PCR targeting DNA from living cells by first blocking DNA from dead cells using propidium monoazide (PMA) or ethidium monoazide (EMA). For example, Abd El-Aziz *et al.* (2018) examined three hundred fresh and processed samples of traditional meat purchased in Egyptian supermarkets for the presence of a variety of food pathogens. By applying PMA quantitative real-time PCR, they discovered that 90.48% of the culture-negative meat samples contained a high load of a range of VBNC food pathogens, being a strong threat to public health. To the best of our knowledge, such approach has not been applied to evaluate the microbiological safety of insect(-based feed or food)s, but as in previous PMA/EMA studies, a lot of interference of the matrix can be expected.

Microbiological challenges related to the further development of the sector

Edible insects are increasingly being proposed as alternative protein source, with the term 'alternative' often alluding to 'more sustainable' than traditional sources. The sector wants to maximise the sustainability of rearing and processing steps, as can be evaluated in life cycle assessment studies. One aspect in this context is the limitation or avoidance of energy consuming unit operations, such as conventional thermal treatments or freeze-drying, and their replacement by innovative thermal technologies

such as microwave or ohmic heating or non-thermal treatments (high hydrostatic pressure, pulsed electric fields, low energy electron beam, intense light pulses, cold plasma, etc.). From a microbiological perspective, those technologies can involve the risk of a reduced food safety when not properly investigated and applied. Not only should process parameters be established that sufficiently reduce the target pathogens that are known so far to be relevant for insect(-product)s, but also the emergence of new microbiological food safety problems should be avoided. The design of a treatment to ensure minimal processing often comprises the combination of multiple decontamination actions to achieve the same reduction as a single lethal stress (Rosnes *et al.*, 2011). For traditional foods, during the past decades many cases are elaborated based on this combination strategy, but insects are a new matrix to be studied.

In its aim to maximally contribute to upcycling and to a circular economy, the sector hopes for green light to grow insects on low value substrates, such as unprocessed former foodstuff (preconsumer waste) containing meat and fish, postconsumer waste (i.e. catering waste), slaughterhouse waste and, for certain continents, manure. This is reflected for instance in the advice recently formulated by the Netherlands Food and Consumer Product Safety Authority (Anonymous, 2020) to rear insects on former foodstuffs containing meat, which is not allowed today. It goes without saying that feeding these lower value substrates eventually may be linked to (new) biological safety risks, when feeding these streams to insects, fresh or – implying an even larger risk – after storage. Taking this next step requires making an inventory of the food pathogens that can occur in these streams and an investigation of their behaviour in these matrices and of the efficiency of hygienisation practices.

Finally, the insect sector is rapidly growing, both in terms of number of companies as in terms of average company scale. This is demonstrated for Europe in a Factsheet of June 2020 composed by IPIFF (2020): in Europe 6,000 tonnes of insect proteins were produced by its members in 2019 and production is estimated to be around 3 million tonnes in 2030. Hence, the insect supply chain becomes more complex, involving more suppliers, intermediate B2B activities, and with an increasing intermediate and finished product portfolio, a more complex distribution network due to an increasing number and type of clients. For the traditional food industry, Martins *et al.* (2014) exhaustively elaborated on how an increasing complexity and upscaling of food production impacts the prevalence of zoonoses and complicates safety control. It is valid to extrapolate this challenge to the insect sector as well. A sector producing higher volumes and more diverse products will go hand in hand with more comprehensive sampling plans, new critical control points, a thorough traceability, longer transport and storage times, and these are all aspects to be considered from a microbiological viewpoint. This challenge may even be the most important from all challenges, since the relatively young sector definitely needs to avoid the occurrence of food crises and to show the expertise is present to accomplish the safety goals, in order to maintain and even further establish its position.

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Conflict of interest

The authors declare no conflict of interest.

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