SUSINCHAIN
SUSTAINABLE INSECT CHAIN

Microbiological safety of insect products

Effect of transport/storage and processing technologies

Deliverable 6.3

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CHAPTER 1

INTRODUCTION

Chapter 1 Introduction

Eventually, the aim of producing insects as food and feed is to employ them in a wide series of applications. In the SUSINCHAIN project, an array of processing and production technologies, either novel or established, are being evaluated in order to store, stabilise, process or manufacture insects or (semi-finished) insect products. While many of the technologies investigated have been applied widely in food, feed and other industries, their application on insects as a matrix is generally new and un(der)explored. As such, not only the technological aspects of the selected processes were to be assessed, but also the impact on the insect matrix was subject of research. In this regard, both quality aspects as well as safety aspects should be considered.

The quality of insects during or after application of processing technologies can be evaluated in order to obtain insights in e.g. shelf life, nutritional value, further processing potential, consumer/buyer acceptance and especially economic value. While product quality is generally of major importance, insect products produced with any technology should in the first place be safe for consumers. Consumers can, in this case, be humans or (livestock) animals, depending on the application.

The (food) safety of insect products can be approached from a chemical, physical or microbiological point of view. In WP 6 of the SUSINCHAIN project, the chemical as well as the microbiological food safety were considered, but in separate tasks. As a part of Task 6.3, the microbiological safety of insects or insect products that underwent transport and storage or were produced by the processing techniques investigated in WP 3 was analysed. By subjecting samples obtained prior to or during processing as well as after processing to a selected variety of microbiological analyses, the microbiological safety of the technologies applied could be evaluated.

Technologies considered in WP 3 were storage under modified/controlled atmosphere, dielectric drying (microwave or radio frequency drying), low energy electron beam (LEEB) decontamination, high moisture extrusion (HME) and tricanter centrifugation with enzymatic pre-treatment. Finally, black soldier fly

larvae meals from different processing technologies were produced for animal feed. The potential of these processes in the insect industry, including technological and economical aspects, is being reported in Deliverables related to WP 3. In this Deliverable 6.3, the microbiological safety aspects of the five selected technologies are described.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 Materials and methods

2.1 Processing technologies

2.1.1 Controlled atmosphere packaging and storage

In T3.1 of WP 3, storage and transport technologies for black soldier fly (BSF) larvae were investigated, based on the concepts of controlled atmosphere packaging (CAP) and storage (CAS). Eventually, four storage technologies were selected, either for living, (freshly) killed or dried insects. For the living and dried insects, the impact of the applied storage technology on survival or lipid oxidation were the main topics of investigation, respectively. For the freshly killed insects that were stored either with or without vacuum, microbiological analyses were included in order to observe the impact of the CAP on the shelf life and general microbiological quality compared to storage in air. Additionally, the impact of the storage temperature and of the killing method on the microbiology of the BSF larvae were taken into account.

The CAP concept for killed BSF larvae was described in Deliverable 3.1. In brief, living larvae were either killed by freezing or blanching and then packed under vacuum or under air (control). These packages were then stored either at room temperature, at 15 °C or at 4 °C. Microbiological counts were executed on samples taken after killing but before storage, as well as at day 2 and day 6 of storage. Parameters investigated were total viable aerobic counts, Enterobacteriaceae counts, lactic acid bacteria counts and aerobic bacterial endospore counts. Microbiological analyses are described below (see section 2.2).

2.1.2 Dielectric drying

Two types of dielectrical drying, being microwave (µW) and radio frequency (RF) drying were investigated in T3.2 from WP 3. In this task, two insect species, mealworms and BSF larvae, were considered. For both insects, the drying technologies were first optimised in order to obtain high quality and stable (water

activity (a_w) below 0.60) dried insects. To this end, drying parameters such as energy input, layer thickness, treatment time and maximal temperature of the insects, etc. were determined. After optimisation, the impact on the microbiological quality and safety of the mealworms and BSF larvae was investigated during a large-scale dielectrical drying experiment. In this experiment, microwave drying and RF drying were compared to oven drying and freeze drying as benchmark technologies.

Optimisation of the µW drying technology (partner MEAM) could already start at the beginning of the project. For both mealworms and BSF larvae, optimal process conditions were identified in a pilot-scale microwave device. Next, insects with these optimised protocols were subjected to microbiological analyses as described in paragraph 2.2. During optimisation, total viable aerobic counts, Enterobacteriaceae counts, aerobic bacterial endospore counts and yeasts and moulds counts were investigated for microwave drying of both insect species. Finally, the impact of µW drying on the microbiological quality during drying as well as during storage and on microbiological safety was assessed in the large-scale drying experiment. Here, total viable aerobic and anaerobic counts, aerobic and anaerobic bacterial endospore counts, yeasts and moults counts were monitored, along with a selected set of pathogens.

For RF drying (partner Dymotec), a new drying device had to be developed and constructed first (see Deliverable 3.3), before optimisation of drying protocols could start. For the RF drying technology, microbiological assessments of the dried products (quality and safety) were only assessed with optimised drying protocols during the large-scale experiment.

2.1.3 Low energy electron beam

The application of low energy electron beam (LEEB) as decontamination treatment was investigated (partners ETH Zürich and Bühler Insect Technology Solutions) for oven-dried and microwave-dried mealworms and BSF larvae within T3.3 of WP 3. The optimisation of the LEEB conditions that should be applied for these insect species were described in Deliverables 3.5 and 3.6.

Both before and after LEEB treatment as well as during a subsequent storage period of 6 months, mealworms and BSF larvae were subjected to the following microbiological analyses by ETH (as described in paragraph 2.2): total viable aerobic and anaerobic counts, aerobic and anaerobic bacterial endospore counts and yeasts and moulds counts. Results from these analyses provide first insights in the efficacy of the LEEB treatment and the impact on microbiological safety of the end products.

2.1.4 High moisture extrusion

In T3.4 of WP 3, the potential of high moisture extrusion (HME) of plant proteins with 30% insects (mealworms, BSF larvae, house crickets and house fly larvae) was investigated (partner DIL). During and after optimisation of the HME process (see Deliverable 3.7), the impact of HME on the microbiological quality and safety of the insects, intermediate products and extrudates was also evaluated. For example the impact of peak barrel temperature was assessed by DIL (see paragraph 2.2) with regard to the total viable aerobic count, in order to optimise this process parameter. Next, following the optimised HME protocols, samples were taken at various points in the production process of extrudates supplemented with mealworm or BSF larvae and subjected to microbiological analyses, either or not after a frozen storage period. The latter microbiological analyses were executed by KUL, as described below (see paragraph 2.2).

2.1.5 Tricanter centrifugation with enzymatic pre-treatment

A last processing technology applied on insects in WP3 was tricanter centrifugation (partners LEITAT and BioFlyTech). In T3.5, it was investigated whether industrial-scale tricanter centrifugation, either with or without enzymatic pre-treatment, could produce protein-enriched BSF larvae meals. The enzymatic pre-treatment as well as the fractionation of BSF larvae was systematically scaled up from lab scale towards industrial scale, as described in Deliverable 3.8. Finally, insect meals could be produced in a large tricanter centrifuge, both with and without enzymatic pre-treatment. The microbiological impact of this processing technology was assessed for BSF meals produced on industrial scale.

2.2 Microbiological analyses

Microbiological analyses were either executed in microbiology labs from different SUSINCHAIN partners (KU Leuven, ETH Zürich, DIL) or in external accredited laboratories (Eurofins, Schonenwerd, Switzerland or Micro-Smedt, Herentals, Belgium). In most cases, microbiological procedures were based on international standards (e.g. as described in Dijk et al., 2015) or derived alternatives with accreditation. Microbiological analyses that were considered in this Task, together with the method followed, are listed in Table 1. For each microbial count determined, the required dilutions were prepared and plated on the correct agar media as described in the standard methods or as detailed in Table 1. Any alterations to the microbiological analysis standards executed by partners ETH and DIL, are described in Deliverables 3.5, 3.6 and/or 3.7.

Table 1 Microbiological parameters and associated analysis methods considered in Task 6.3.

Microbiological parameter	Method	Executed by
Total viable count (aerobic/anaerobic)	ISO-4833-1	KUL, ETH, Eurofins[1]
	ASU L00.00-88/2	DIL
Total viable psychrotrophic count	ISO 17410	KUL
Bacterial endospore count (aerobic/anaerobic)	ISO-4833-1	KUL, ETH, Eurofins[1]
Yeasts and moulds count	ISO 21527	KUL, ETH
	ASU 01.00-37	DIL
Enterobacteriaceae count	ISO 21528	KUL
	ASU L06.00-24	DIL
Lactic acid bacteria count	ISO 15214	KUL
Coagulase-positive staphylococci count	ISO 6888 with Vogel-Johnson agar	KUL
Clostridium perfringens count	ISO 7937	Micro-Smedt[1]
Staphylococcus aureus count	ISO 6888	Micro-Smedt[1]
Listeria monocytogenes count	ISO 11290-2	Micro-Smedt[1]
Salmonella spp. detection/25 g	ISO 6579	Micro-Smedt[1]
(Presumptive) *Bacillus cereus* count	ISO 7932	Micro-Smedt[1]
Campylobacter spp. count	ISO 10272-2	Micro-Smedt[1]

[1]External accredited microbiological laboratory.

2.3 Physicochemical analyses

Additional to the microbiological analyses performed in T6.3 of WP 6, for a few processes, also some physicochemical analyses were executed. Since intrinsic product parameters such as pH and water activity (a_w) may have an important impact on the microbiota present in the insects or insect products, they were taken into account for certain products.

When applicable, pH was measured using a Portamess 911 digital pH meter (Knick, Germany) with SI-analytics electrode (Germany). Water activity was measured with a water activity meter (Novasina Labmaster-a_w, Switzerland) at 25 °C, after temperature and a_w were both stable for 5 minutes.

CHAPTER 3

MICROBIOLOGICAL SAFETY ASPECTS DURING CONTROLLED ATMOSPHERE PACKAGING OF BSF LARVAE

Chapter 3 Microbiological safety aspects during controlled atmosphere packaging of BSF larvae

3.1 Microbial counts of killed BSF larvae packed under vacuum

During the vacuum packaging experiments of killed BSF larvae, a few microbial counts were monitored. In the first place, it was evaluated whether storage under vacuum could prolong storage time (in terms of microbiological shelf life) of the larvae compared to a control (storage under air). As described in Deliverable 3.1, the vacuum did not prove beneficial compared to packaging without vacuum.

Additionally, also the impact of storage temperature and killing method of the larvae on the insect microbiology were taken into account. Regarding the storage temperature, it was clear that, as expected, storage at 4 °C could reduce microbial activity. The killing method as well had a major impact on the evolution of the microbiology of the packed larvae, since it greatly determined the initial microbiological quality.

The microbiological safety of the killed BSF larvae in the vacuum storage experiment can be evaluated by observing Figures 1 and 2. First, it is clear that without a decontamination treatment such as blanching (i.e. killing by only freezing), total viable counts (TVCs) are very high, even above 10.0 log cfu/g. While this number does not necessarily signifies a microbiological food safety problem, it may indicate that potential risks should be taken into account. Also the other microbial counts (Enterobacteriaceae, up to 8.0 log cfu/g; lactic acid bacteria (LAB), up to 8.0 log cfu/g; and bacterial endospores, up to 5.0 log cfu/g) are high for larvae that were killed by freezing. During a storage period of 6 days, those counts did not change in a meaningful way and remained at a high level, which requires caution in terms of food safety.

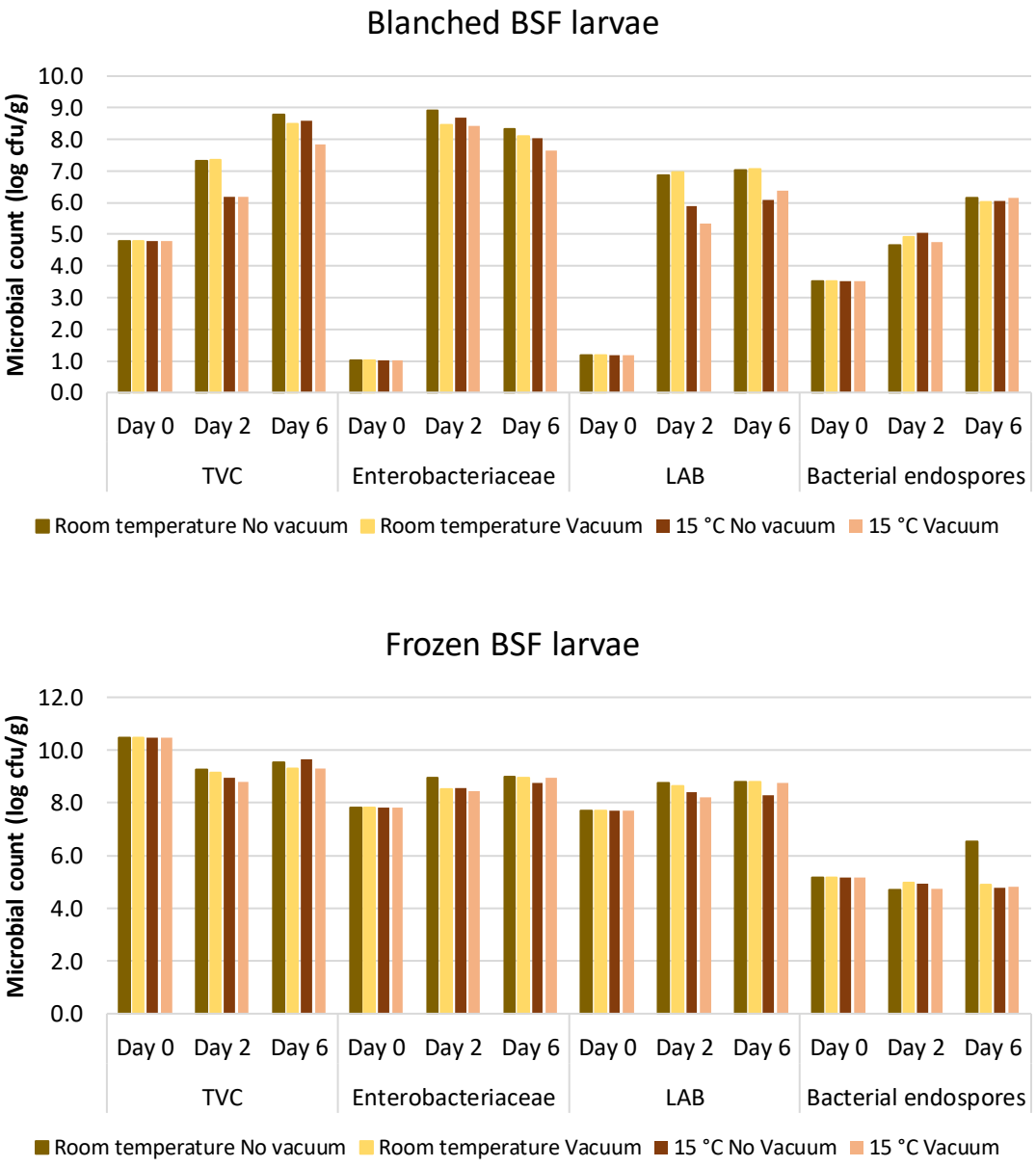

Figure 1 Microbial counts of blanched BSF larvae (top) or BSF larvae killed by freezing (bottom) during storage experiment 1. Larvae were stored at room temperature (gold/yellow graphs) and at 15 °C (brown/orange graphs), packed with (light coloured graphs) or without (only sealed as control; dark coloured graphs) vacuum. Each bar represents 1 package. TVC = Total viable aerobic count, LAB = lactic acid bacteria. Bars displayed with a "<" represent data below the detection limit of 1.0 log cfu/g.

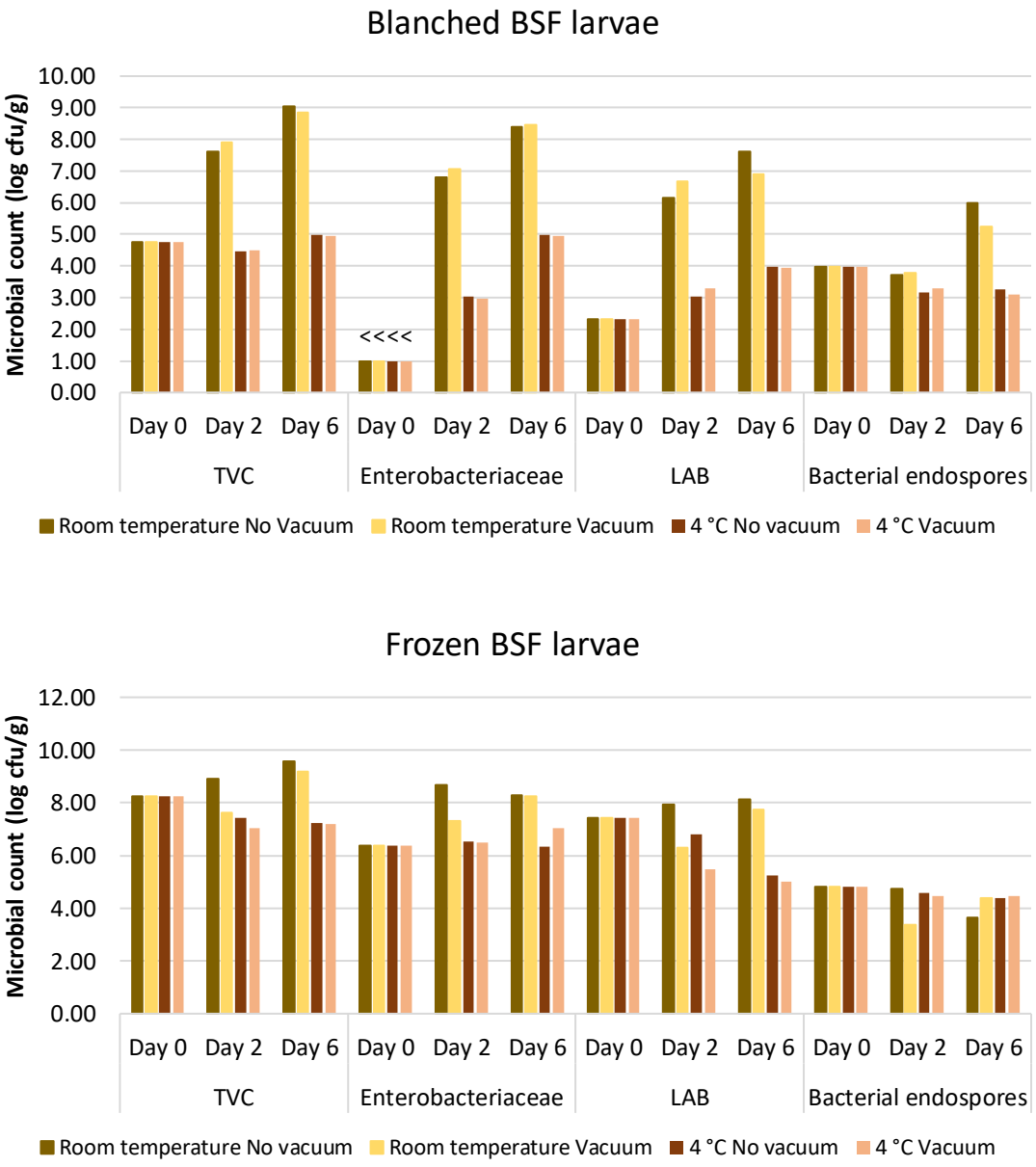

Figure 2 Microbial counts of blanched BSF larvae (top) or BSF larvae killed by freezing (bottom) during storage experiment 2. Larvae were stored at room temperature (gold/yellow graphs) and at 4 °C (brown/orange graphs), packed with (light coloured graphs) or without (only sealed as control; dark coloured graphs) vacuum. Each bar represents 1 package. TVC = Total viable aerobic count, LAB = lactic acid bacteria. Bars displayed with a "<" represent data below the detection limit of 1.0 log cfu/g.

For BSF larvae that were blanched, the situation was somewhat different. The blanching step clearly reduced the amount of microorganisms (TVC) to a number between 4.0 and 5.0 log cfu/g. This number more or less corresponded to the amount of bacterial endospores, which are resistant to pasteurising effect of blanching and were therefore not eliminated. The number of Enterobacteriaceae and LAB was reduced substantially, sometimes even to below their detection limit. During a storage period of 6 days, either with or without vacuum, all microbial counts rose, but the increase was dependent of the storage temperature. At room temperature or at 15 °C, the Enterobacteriaceae and LAB counts increased to their initial levels of 7.0 – 9.0 log cfu/g and also the amount of bacterial endospores rose about 3 log cycles. The general spoilage level of 7.0 log cfu/g TVC, as defined by Sperber & Doyle (2009) was already exceeded after two days of storage at room temperature and slightly later at 15 °C. Also under these storage conditions, attention must be given to the food safety of the insects.

Storage at 4 °C seemed to control the microbial activity and growth in the BSF larvae. While the number of Enterobacteriaceae and LAB increased slowly, TVCs remained constant and never reached the spoilage level of 7.0 log cfu/g. As a result, to retain an acceptable microbiological quality, BSF larvae should preferably be killed by blanching and then stored at 4 °C. Application of vacuum proves no extra benefit in this case. Consequently, no further experiments or microbiological analyses were executed in the scope of vacuum packaging of BSF larvae.

3.2 Conclusions and impact on microbiological food safety

From a microbiological food safety point of view, vacuum packaging of killed BSF larvae is not beneficial. It could not prevent the outgrowth of certain groups of microorganisms, nor did it slow down spoilage compared to storage under air. To start with a reduced initial microbial load and thus a lower safety risk, raw BSF larvae are advised to be subjected to a decontamination step, either as killing method or afterwards. Next, storage at 4 °C was proven to be successful to retain

the reduced microbial load for six days without substantial increase in microbiological food safety risks.

The microbiological food safety of killed BSF larvae were evaluated based on the TVC and numbers of Enterobacteriaceae, LAB and bacterial endospores. The TVCs provide information about the general microbiological quality and impact of processing or storage on the total microbial load. Similarly, the number of LAB may indicate spoilage, but is not related to potential safety risks. The Enterobacteriaceae and bacterial endospore counts provide more insight in the fate of potential food pathogens. The family of Enterobacteriaceae, for example, contains several pathogenic bacteria, for instance *Salmonella* spp., *Shigella* spp. and *Yersinia enterocolitica* and can be considered as an index group for these pathogens. The fact that the Enterobacteriaceae can be eliminated below the detection limit of 1.0 log cfu/g by blanching, indicates that risks related to those pathogens is greatly reduced. The slow increase of Enterobacteriaceae during storage at 4 °C also limits the risks, compared to the higher storage temperatures.

Bacterial endospores are known to be heat-resistant. Since blanching only represents a pasteurisation step, vegetative cells can be (partially) eliminated, but the endospores will survive and potentially even get activated to germinate. Also the bacterial endospore count can include food pathogenic species such as *Bacillus cereus* (group) and *Clostridium perfringens*. While the required concentrations of these bacteria to pose food safety risks are generally determined as 5.0 and 6.0 log cfu/g in the product, respectively (Adams at al., 2015), the numbers of bacterial endospores in BSF larvae are rather high (and sometimes close to 5.0 log cfu/g). In suitable conditions, endospores can germinate to vegetative cells and the number of spore-forming bacteria and eventually of bacterial endospores may increase due to absence of other competing microorganisms (that were eliminated through blanching). It is therefore necessary to retain the amount of bacterial endospores as low as possible.

CHAPTER 4

MICROBIOLOGICAL SAFETY ASPECTS OF DIELECTRIC DRYING OF INSECTS

Chapter 4 Microbiological safety aspects of dielectric drying of insects

4.1 Impact of microwave drying on microbial counts of insects during process optimisation

Optimisation of microwave drying for mealworms and BSF larvae was performed in a modular and explorative microwave dryer (MEAM Explorer, Figure 3) at small scale. The optimised results from this system can be directly translated to a large-scale industrial size microwave dryer. Yet, samples obtained after drying the insects in the Explorer were already subjected to the first microbiological analyses.

Figure 3 MEAM Explorer

4.1.1 Mealworms

For mealworms, the optimal drying protocol turned out to last 20 minutes at a maximum temperature of 75 °C with 2 cm layer thickness. Further details regarding the process parameters for microwave drying of mealworms is described in Deliverable 3.2. Microbiological analyses were performed on raw mealworms and compared to analyses of the microwave-dried mealworms following the optimised protocol. The results are shown in Figure 4.

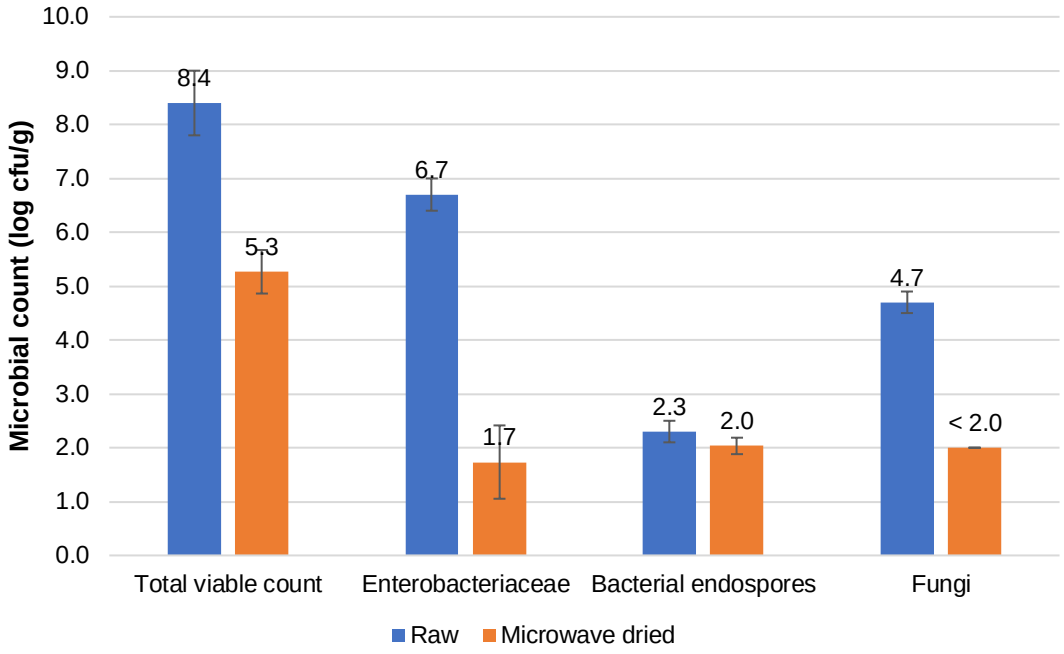

Figure 4 Microbial counts of mealworms before and after microwave drying. Results are expressed as mean value ± standard deviation (n = 3).

Microwave drying started with raw, untreated larvae that were killed by freezing. The initial TVC of the starting larvae was 8.4 log cfu/g, which is very comparable to what has been observed previously (Vandeweyer et al., 2017). After drying, which resulted in a dried product with a water activity of 0.48 ± 0.03 (n = 3), the TVC of the mealworms has dropped to 5.3 log cfu/g. Here it is clear that apart from drying (i.e. removing water from the matrix), also an impact on the microbiota can be observed. Since temperatures above 70 °C are applied during drying, this will have caused elimination of certain microorganisms in the mealworms. Also for the number of Enterobacteriaceae and yeasts and moulds (fungi), a strong

decrease was noticed. Only the heat-resistant bacterial endospores, present in low amounts, remained unaffected. Rather than only stabilising the insect matrix below a water activity (a_w) of 0.60, also the microbial load, present in the dried insects, was reduced. Of course, this has an important and positive effect on the microbiological food safety risks.

4.1.2 BSF larvae

Optimal drying conditions for BSF larvae are 15 minutes at 85 °C with a layer thickness of 1.5 cm. Further details of the drying process will be described in Deliverable 3.2. Microbiological analyses were performed on raw BSF larvae and compared to analyses of the microwave-dried BSF larvae following the optimised protocol. The results are shown in Figure 5.

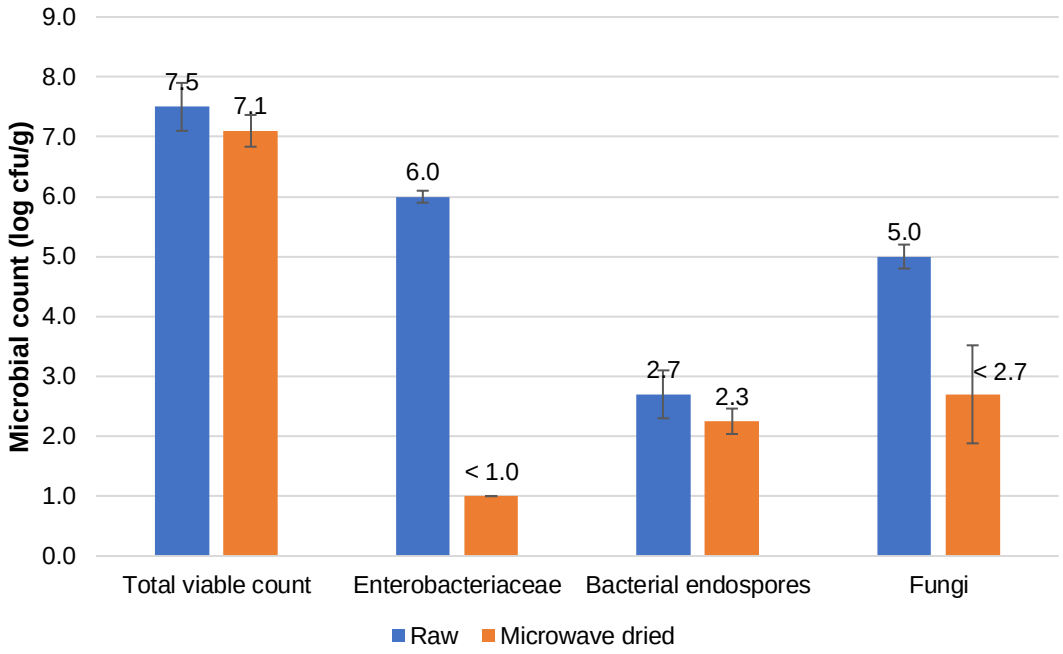

Figure 5 Microbial counts of BSF larvae before and after microwave drying. Results are expressed as mean value ± standard deviation (n = 3).

The high microbial counts present in the raw BSF larvae were also partially reduced by application of microwave drying. The drying process produced BSF larvae with a a_w of 0.60 ± 0.03 (n = 3). In contrast to the results for mealworms, here, the TVC was not substantially reduced, but the numbers of

Enterobacteriaceae and yeasts and moulds were. As expected, also here, the amount of bacterial endospores remained the same. Consequently, the microwave treatment did have an impact on the microbiota in the BSF larvae, but to a lesser extent than for mealworms, despite the higher drying temperature applied. This observation may be related to the slightly different process parameters, the different dimensions of the larvae, a different penetration of microwaves in BSF larvae, etc.

4.2 Large-scale comparison of dielectric drying, oven drying and freeze-drying with regards to microbiological safety of insects

Subsequent to the optimisation trials for microwave and RF drying, a large-scale comparison experiment to compare the microbiological impact of the two dielectric drying methods for mealworms and BSF larvae was executed. Next to industrial microwave and RF drying, also oven drying and freeze-drying were considered in the comparison as two benchmark drying techniques. One large batch of living BSF larvae was killed by blanching and subsequently dried with each of the four technologies selected. The technologies were compared with regard to chemical and nutritional quality and microbiological parameters of the insects before and after drying. Microbiological parameters considered were TVC (aerobic and anaerobic), aerobic and anaerobic bacterial endospore count, yeasts and moulds count, *Clostridium perfringens* count, *Staphylococcus aureus* count, *Listeria monocytogenes* count, Presumptive *Bacillus cereus* count, *Campylobacter* spp. count and detection of *Salmonella* spp. After drying, the products were subjected to a shelf life study for 6 months, following up lipid oxidation and microbiological quality. Results of these comparative experiments were extensively reported in Deliverables 3.2 and 3.4. Only the microbiological results are described in short again in this Deliverable.

In summary, dielectric drying can be considered as a successful technology to produce high-quality and stable dried BSF larvae and mealworms. With water activity levels close to or even below 0.60, microbiological activity can be halted

and products with good initial microbiological quality can retain that quality. Hence, the initial microbiological quality of the insects prior to drying is an important characteristic with regard to food safety.

For example, the total viable aerobic count, an indicator for general microbiological quality, for raw, untreated insects is very high, as shown in Figure 6. However, an important fraction of the TVC can be eliminated already by blanching the insects prior to drying. As can be concluded from Figure 7, the remaining fraction of microorganisms is generally present in the form of bacterial endospores, which are highly resistant to moderate decontamination technologies like blanching. Next, as seen in Figure 6 and Figure 7, the application of dielectric drying may have a slight to substantial effect on the TVC and bacterial endospore counts of the blanched insects. This indicates an additional effect of the dielectric drying on the microorganisms present and this decontaminating effect was not observed for oven or freeze drying.

Since general microbial counts do not directly indicate microbiological food safety, specific analyses for selected pathogens were included as part of the microbiological food safety assessment of dielectric drying. For all samples analysed during the drying processes of both BSF larvae and mealworms, the only pathogen that could be detected in a few cases was *Bacillus cereus*. The pathogen was detected in blanched, oven-dried and freeze-dried BSF larvae and in blanched and freeze-dried mealworms, as shown in Table 2. While this observation requires some caution regarding food safety of the insects, the values obtained were all below the generally accepted food safety risk threshold value of 5.0 log cfu/g for *B. cereus* (EFSA Panel on Biological Hazards, 2016).

B. cereus is a pathogen commonly associated with insects (Vandeweyer et al., 2021). Since it is a spore-forming bacterium, its endospores may survive the blanching step prior to drying. By drying the insects to a stable a_w, the outgrowth of these endospores is prevented and the safety risk related to the (low) presence of *B. cereus* in the dried insects can be classified as low. However, good monitoring and proper storage of the insects after drying are important to warrant microbiological safety. Since no *B. cereus* was detected in any of samples obtained after dielectric drying, these processing technologies may, as was the case for TVC and aerobic

bacterial endospores, also have had an effect on the *B. cereus* counts. Still, further research is required to specifically investigate the effect of processing technologies, including dielectric drying, on the survival and behaviour of *B. cereus* in insect matrices.

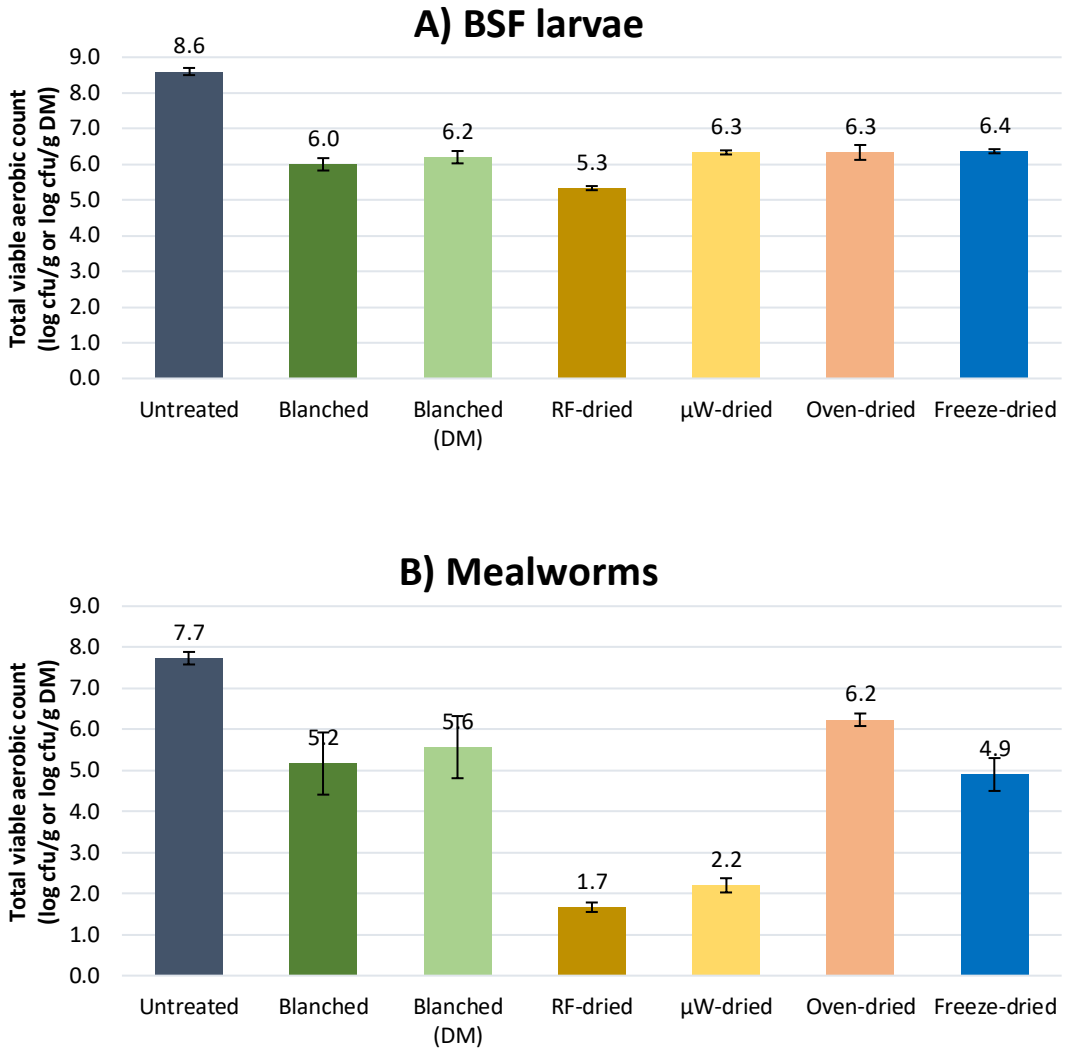

Figure 6 Total viable aerobic counts (TVC) of BSF larvae (A) and mealworms (B) before and after drying with different drying technologies. Results are mean values (n=3) ± standard deviations and expressed as log cfu/g for untreated and blanched BSF larvae and as log cfu/g dry matter (DM) for dried larvae. Blanched (DM) represents a TVC of blanched insects expressed as log cfu/g DM for proper comparison with dried larvae.

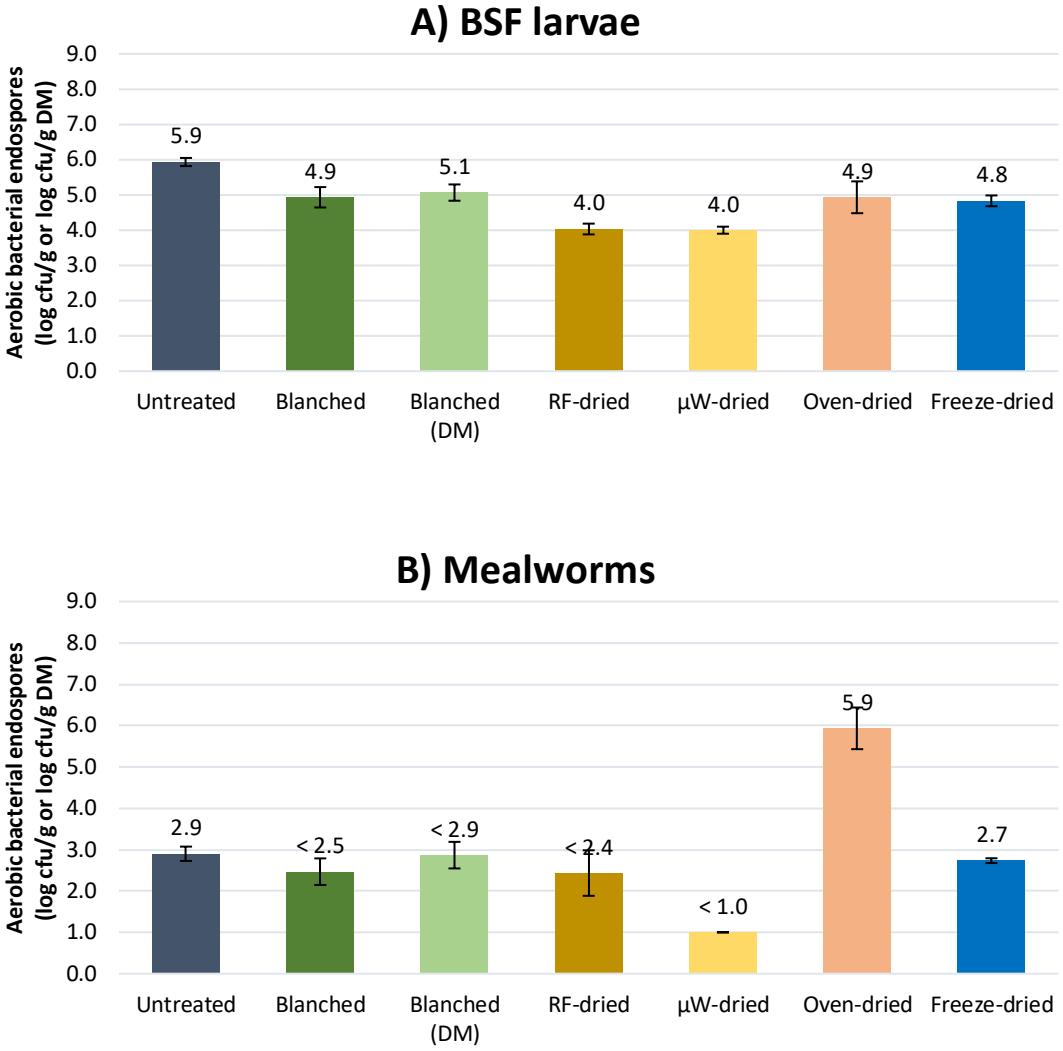

Figure 7 Aerobic bacterial endospore counts (AES) of BSF larvae (A) and mealworms (B) before and after drying with different drying technologies. Results are mean values (n=3) ± standard deviations and expressed as log cfu/g for untreated and blanched BSF larvae and as log cfu/g dry matter (DM) for dried larvae. Blanched (DM) represents a TVC of blanched insects expressed as log cfu/g DM for proper comparison with dried larvae.

Table 2 Occurrence and counts of (presumptive) *B. cereus* for BSF larvae and mealworm before and after drying with different drying technologies. Results are mean values (n=3) ± standard deviations and expressed as log cfu/g. For values with a "<", the *B. cereus* count was below the detection limit of 2.0 log cfu/g for at least one sample.

Insect	Treatment	Mean *B. cereus* count (log cfu/g)	Number of positive samples
BSF larvae	Untreated	< 2.0 ± 0.0	0
	Blanched	< 2.0 ± 0.0	1
	RF-dried	< 2.0 ± 0.0	0
	µW-dried	< 2.0 ± 0.0	0
	Oven-dried	< 2.3 ± 0.4	1
	Freeze-dried	< 2.0 ± 0.0	2
Mealworms	Untreated	< 2.0 ± 0.0	0
	Blanched	3.3 ± 0.2	3
	RF-dried	< 2.0 ± 0.0	0
	µW-dried	< 2.0 ± 0.0	0
	Oven-dried	< 2.0 ± 0.0	0
	Freeze-dried	< 3.1 ± 1.0	2

4.3 Conclusions and impact on microbiological food safety

While it is clear that a dielectric drying process may have a reducing effect on the microorganisms in the insects investigated (mealworms and BSF larvae), only drying is not sufficient to exclude microbiological food safety risks related to the untreated insects. Without a decontamination step prior to drying for example BSF larvae, TVC remains higher than 7.0 log cfu/g, which is the TVC action limit (upper limit M) for dried insect products as adopted by the Belgian Federal

Agency for the Safety of the Food Chain (FASFC, 2021). Also, while the number of Enterobacteriaceae was reduced towards or below the detection limit by application of microwave drying on raw mealworms and BSF larvae, the number of bacterial endospores remained unaffected.

In the first place, it is advised to include a decontamination step such as blanching for the raw insects prior to drying them, in order to obtain stable insects with low initial microbial counts. As is clear from the microbiological analyses, this is not sufficient to eliminate bacterial endospores. Since the insects are dried to a_w values near or below 0.60, these remaining endospores (which may belong to pathogenic species such as *B. cereus*) only pose a low food safety risk, since they are not able to germinate at such low a_w. However, during further storage of the dried insects, it is important to retain the a_w below 0.60 to avoid outgrowth of remaining bacterial spores.

Further research comparing microwave drying with RF drying and other drying technologies with regards to microbiology will provide further insights in the effects of the dielectric drying technologies on the microbiota of the insects.

CHAPTER 5

MICROBIOLOGICAL SAFETY ASPECTS OF LEEB TREATMENT OF INSECTS

Chapter 5 Microbiological safety aspects of low energy electron beam treatment of insects

5.1 Impact of LEEB on microbial counts of insects

Prior to applying a LEEB treatment on insects, they were first blanched and subsequently oven-dried or microwave dried (without blanching step), according to Table 2. Next, they were subjected to microbiological analyses before and after the LEEB treatment, as well as during 6 months of storage afterwards (only for the oven-dried and LEEB-treated samples).

Table 3 Pre-treatment conditions applied for mealworms and BSF larvae prior to LEEB treatment.

Pre-treatment conditions	Mealworms	BSF larvae
Blanching parameters (before oven drying)	3 min at 95 °C	2 min at 75 - 85 °C
Oven drying parameters	3.5 h at 90 °C	27 h at 80 °C
Microwave drying parameters	20 min at 85 °C	

5.1.1 Oven-dried insects

While both insect species were blanched at over 90 °C and dried in an oven at more than 80 °C, a TVC of 4 to 6 log cfu/g still remained, as shown in Figure 6. A comparison with the amount of bacterial endospores (Figure 7) indicates that, especially for mealworms, not only heat-resistant endospores remained after the pre-treatments. As such, an additional decontamination treatment such as LEEB could prove useful. As shown in Figures 6 and 7, the LEEB treatment clearly had an impact on the microbial load still present in and on the dried insects. For mealworms, the TVC could be reduced to below the detection limit of 2.0 log cfu/g (4 log reduction), while the TVC of BSF larvae, which was initially already lower, could be reduced by an extra log cycle. Remarkably, also the number of bacterial

spores was reduced by 1 to 1.5 log cycle after the LEEB treatment for both insect species.

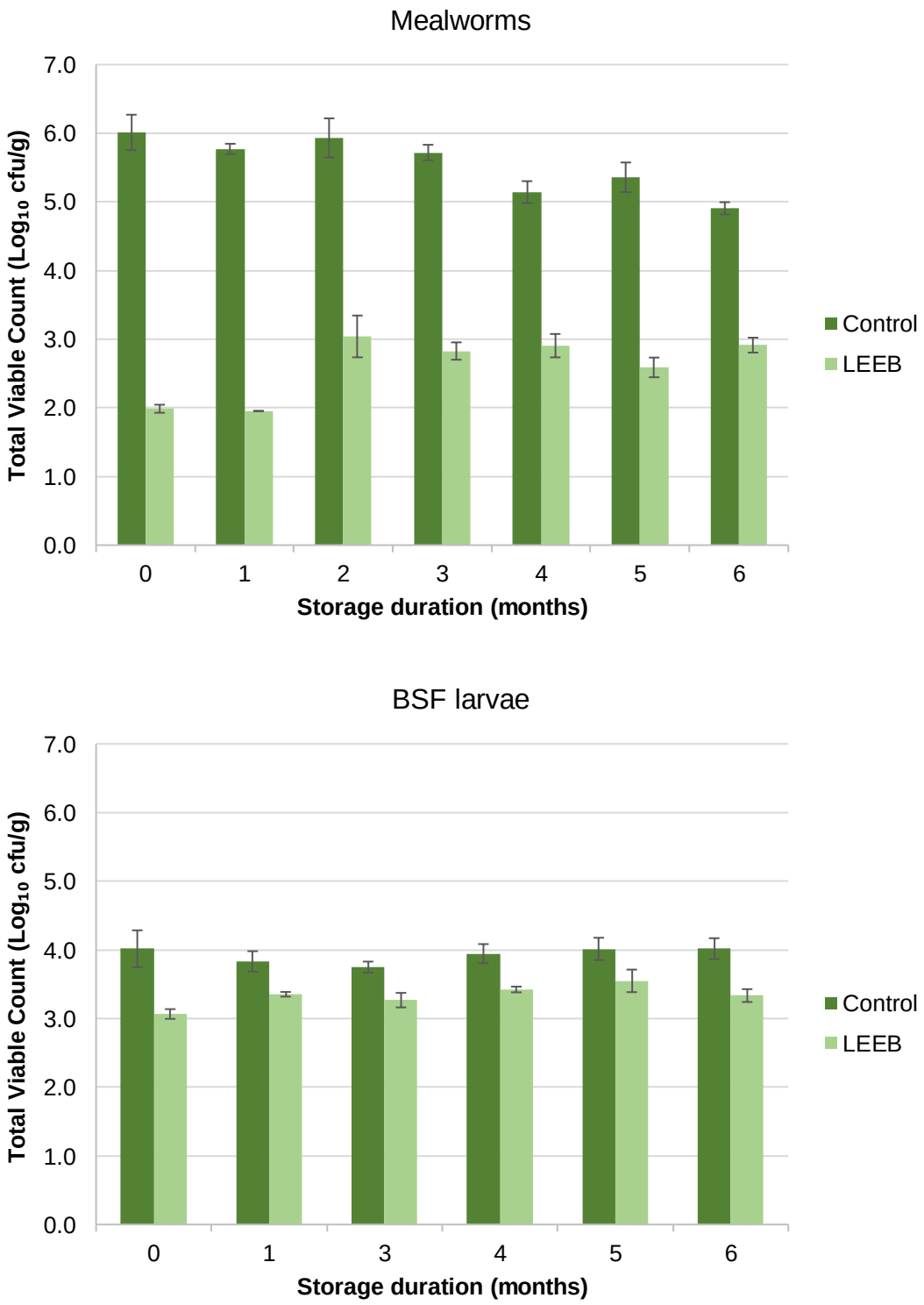

Figure 8 Total viable counts (TVCs) of oven-dried mealworms (top) and BSF larvae (bottom) before and after LEEB treatment as well as during 6 months storage. Results are expressed as mean value ± standard deviation (n = 3).

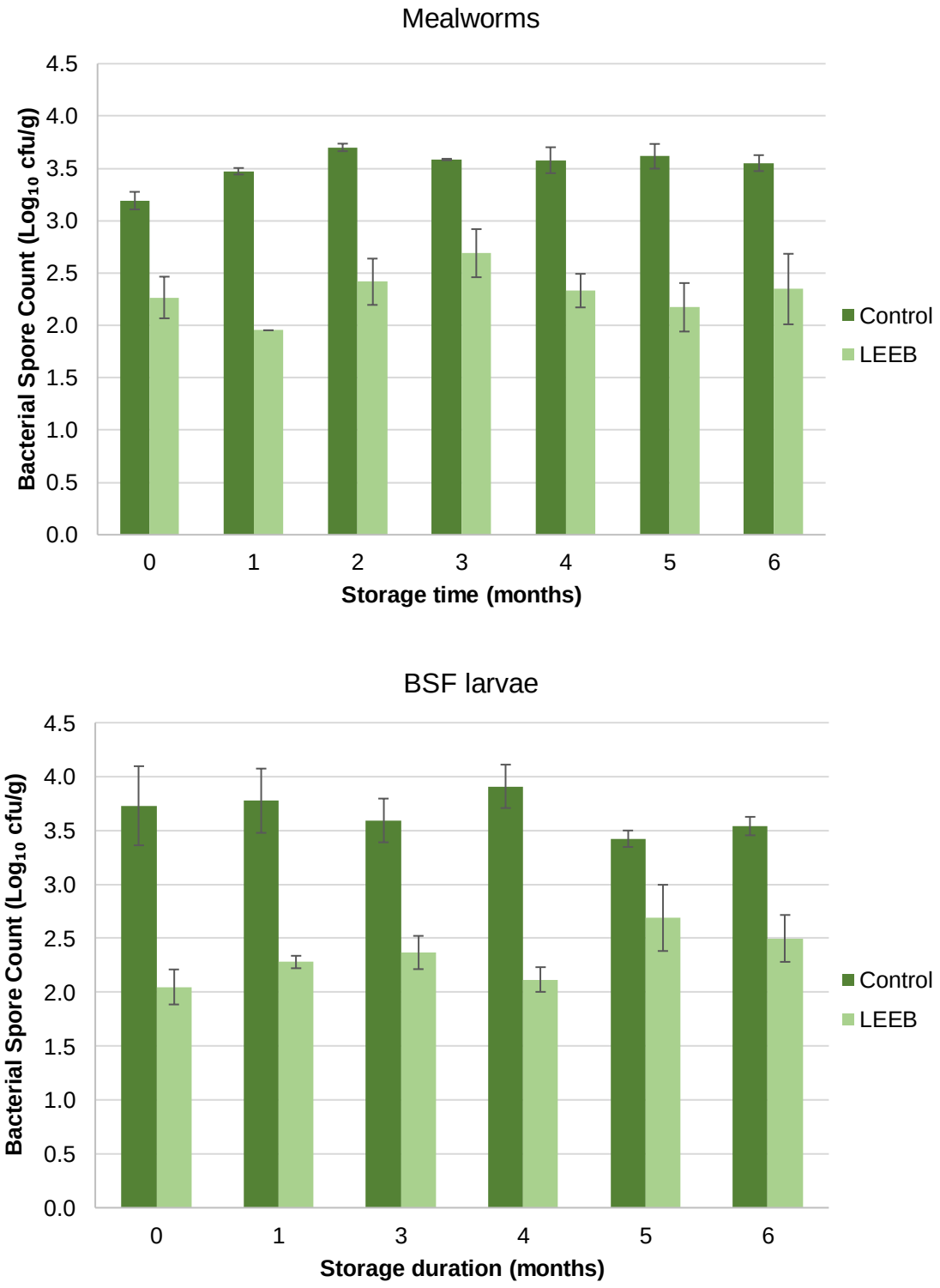

Figure 9 Aerobic bacterial endospore counts of oven-dried mealworms (top) and BSF larvae (bottom) before and after LEEB treatment as well as during 6 months storage. Results are expressed as mean value ± standard deviation (n = 3).

Next, during a storage period of 6 months, the TVC and number of bacterial endospores remained more or less stable for the LEEB-treated samples, with no changes larger than 1 log cycle compared to the samples at the start of the storage period (month 0). Also the control samples remained stable with regards to their microbial counts, but at a higher level. This indicates that the drying process will have stabilised the insects at a a_w value low enough to avoid substantial microbial growth and that the storage was executed in a good way. Comparable results were observed for the anaerobic TVC and bacterial endospore counts (data not shown).

For the yeasts and moulds counts, the results were slightly different. After pre-treatment, the counts were reduced to below the detection limit of 2.0 log cfu/g for both insect species. No impact of the LEEB treatment could therefore be seen in the fungi counts. For BSF larvae, the yeasts and moulds counts remained below the detection limit for the whole storage period. For mealworms, on the other hand, a striking difference between the LEEB-treated and control samples was observed, as shown in Figure 8. As from 2 months of storage, fungi were detected in levels above the detection limit. The LEEB-treated samples showed values between the detection limit and 3.0 log cfu/g, thus still indicating a generally stable storage process. However, the control samples displayed values from 3.0 to over 5.0 log cfu/g. Here, the storage conditions were suitable for yeasts and moulds to be able to grow, e.g. water activity, initial yeasts and moulds count, etc.

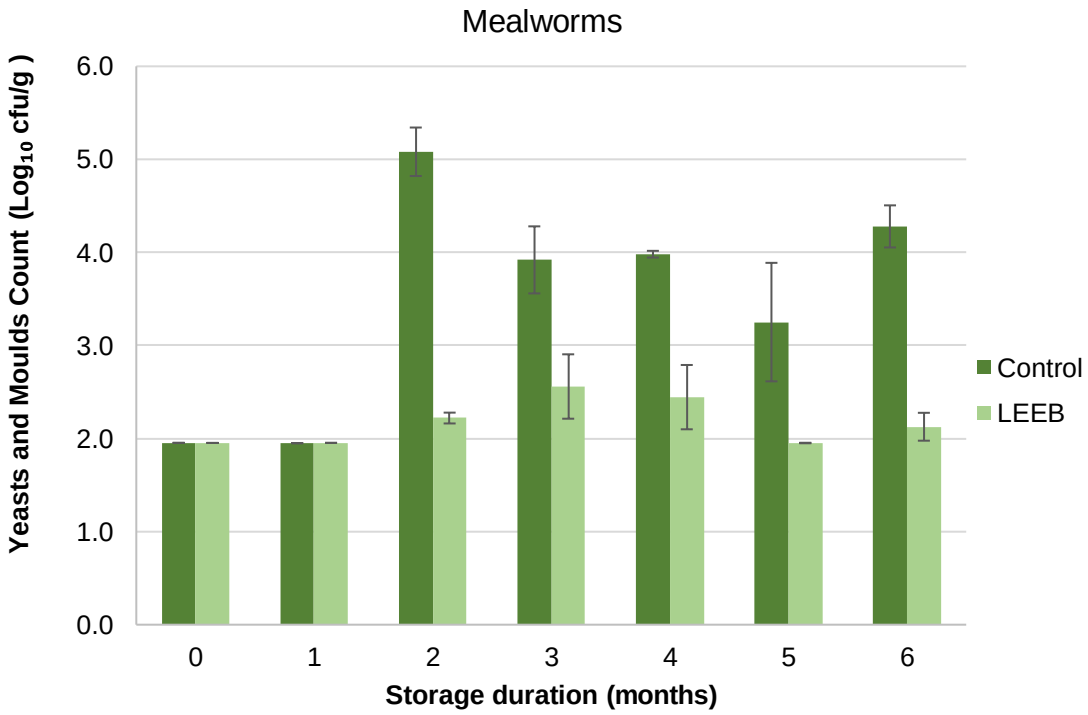

Figure 10 Yeasts and moulds counts of oven-dried mealworms before and after LEEB treatment as well as during 6 months storage. Results are expressed as mean value ± standard deviation (n = 3).

5.1.2 Microwave-dried insects

In a second experiment, insects were dried by application of microwaves instead of hot air. While the initial microbial load was different for these insect samples, again, the application of a LEEB-treatment could considerably reduce the microbial load (Figure 9), for mealworms even below the detection limit of 2.0 log cfu/g. For BSF larvae, also the amount of bacterial endospores was reduced by almost 2 log cycles (data not shown). The number of endospores for mealworms was below the detection limit for both control and LEEB-treated samples, so no impact of the LEEB could be observed.

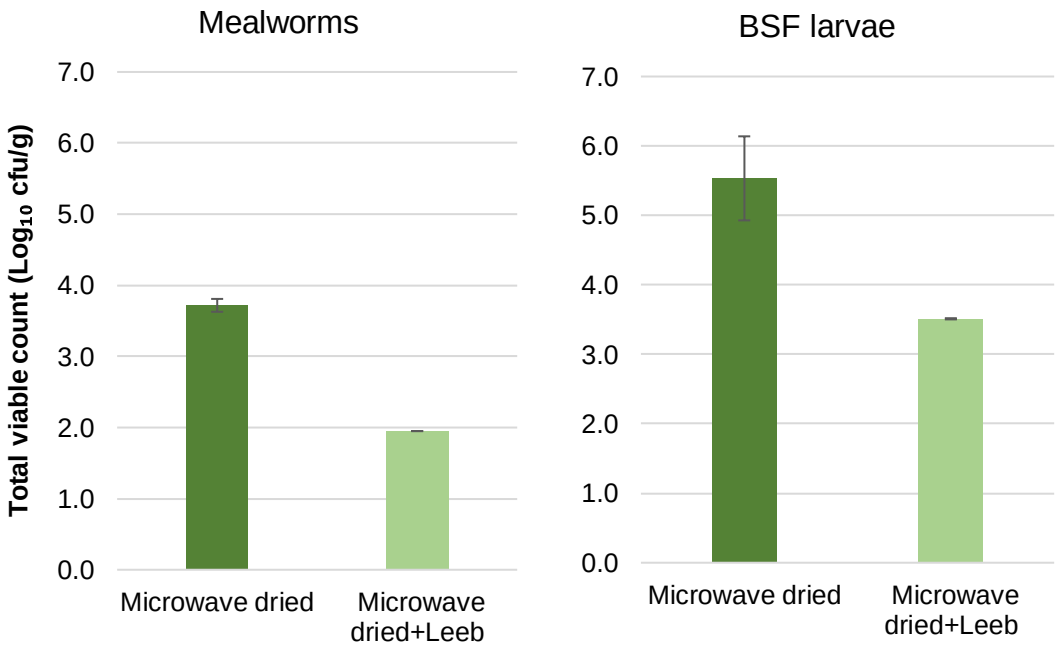

Figure 11 Total viable counts (TVCs) of microwave-dried mealworms (left) and BSF larvae (right) before and after LEEB treatment. Results are expressed as mean value ± standard deviation (n = 3).

5.2 Conclusions and impact on microbiological food safety

As observed from microbiological analyses on dried insect samples from several tasks from WP 3, drying alone is not sufficient to reduce the microbial load to an acceptable level. After applying a decontamination step such as blanching prior to the drying process, microbiological quality can be improved substantially. However, blanching may sometimes not sufficiently reduce the microbial load, and especially bacterial endospores, while low levels of microorganisms (and endospores) may sometimes be required for certain applications. In other cases, a decontamination step after the drying technology may be preferred.

The application of a LEEB treatment on dried insects shows to be a useful technology to improve the microbiological quality after drying. While the impact

of LEEB is limited to the surface of the insects, it was shown that the TVCs and bacterial endospore counts of either oven- or microwave-dried mealworms and BSF larvae could be reduced effectively. For insects, especially endospore-forming bacteria such as *Bacillus* spp. and *Clostridium* spp. are considered as relevant microbiological food safety risks (Vandeweyer et al., 2021). In this view, the potential of LEEB to also reduce the amount of bacterial spores on dried insects is a major advantage regarding the microbiological food safety of the products. Moreover, during storage of the LEEB-treated dried insects, it was observed that the microbial load remained stable, while for control samples without LEEB-treatment, fungal growth was noticed. Hence, application of LEEB may also contribute to an extension of the shelf life of dried insect products, as well as to increased food safety related to mycotoxin production by those fungi.

CHAPTER 6

MICROBIOLOGICAL SAFETY ASPECTS OF HME WITH INSECTS

Chapter 6 Microbiological safety aspects of HME with insects

6.1 Microbiological impact of barrel peak temperatures for HME with soy and mealworms

During the optimisation of the HME process, a few peak barrel temperatures were tested. Resulting extrudates from those process temperatures were subjected to a total viable count analysis at DIL to assess the temperature impact on the general microbiology of the insect-supplemented extrudates. Figure 10 displays the total plate counts (TVC or TPC in the figure) of raw, intermediate and end products, the latter obtained from 4 different extrusion barrel peak temperatures, from the HME with mealworms. The microbiological data clearly show that the extrusion causes a 4 to 5 log reduction for each temperature in total count compared to the raw insects and the pre-extrusion mixture. While 180 °C caused the highest reduction of countable microorganisms, the other temperatures already had a large, significant effect on the total counts and as such improved the microbial quality of the product greatly. Based on these results, as well as on the technological implications, a temperature of 160 °C was adopted for HME with soy and mealworms. Similar trials were performed for other plant protein x insect species combinations to determine HME process parameters.

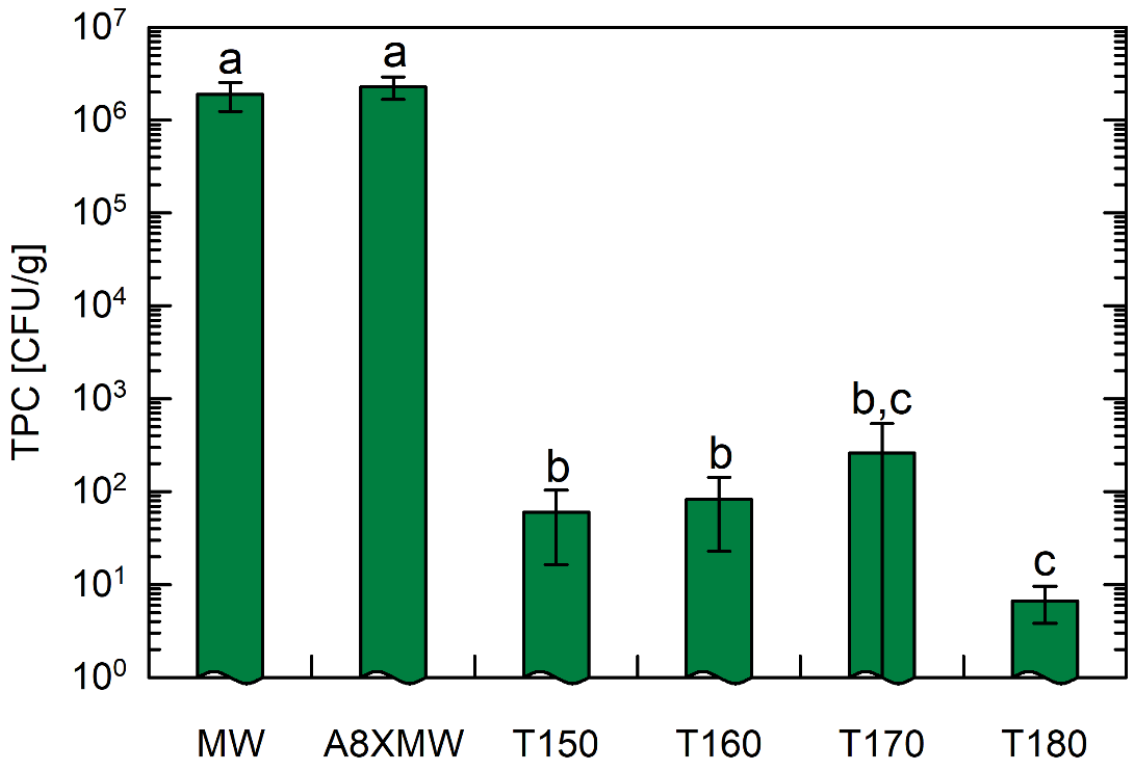

Figure 12 Total plate count (TPC, also TVC) of raw materials before extrusion (mealworms (MW) and a mixture of soy protein concentrate with mealworm (A8XMW)) and after processing at 150 – 180 °C peak barrel temperature (T150 – T180); different letters indicate significant differences of mean values (n = 3; α = 0.05).

6.2 Microbiological impact of optimised HME with soy and mealworms or BSF larvae

To evaluate the microbiological impact of HME on insect-supplemented extrudates, microbiological analyses were performed on samples obtained before, during and after the optimal HME process at DIL. Extrudates were produced by mixing soy protein concentrate with 30 g/100 g raw insects and then extruded by inclusion of 52.9 g/100 g tap water at a barrel peak temperature of 160 °C. Since the final extrudates are generally stored frozen, also samples that were frozen and transported to KUL were subjected to a more extensive set of microbiological analyses. While freezing, storage and transport may all have influenced the microbiology in the samples, this method closely resembles the usual way of handling the end product.

The results from the microbial counts before and immediately after HME are shown in Figure 11. Comparable to what was observed during the optimisation of the HME (Figure 10), the TVC (here TPC) could be reduced by 4 to 5 log cycles for both mealworm and BSF larvae-supplemented extrudates. A very similar observation was seen for Enterobacteriaceae, with decreases in counts between 3 and 4 log cycles. Yeasts were even eliminated below their detection limit after HME. Compared to the control (pure plant protein HME, A8), the remaining microbial load was higher when insects were included.

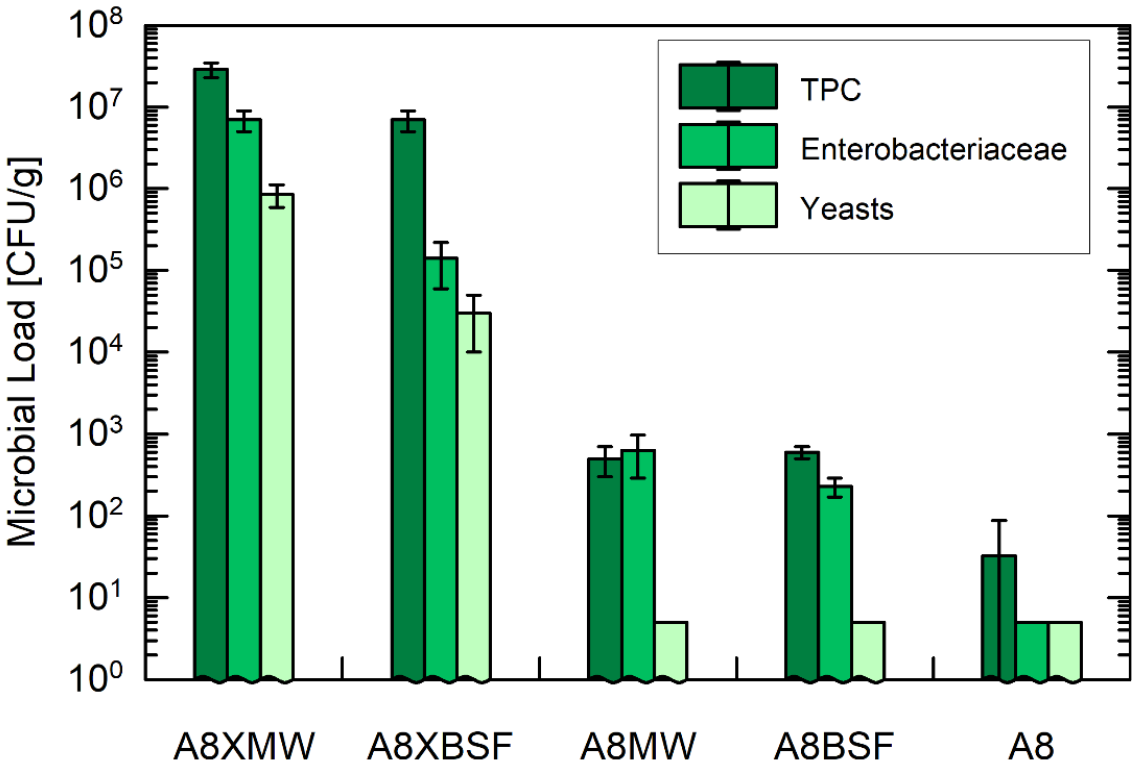

Figure 13 Microbial counts of a mixture of soy protein concentrate with mealworm (A8XMW) and black soldier fly (A8XBSF) before extrusion and after processing at 160 °C peak barrel temperature; pure soy based HME (A8) as reference. Results are expressed as mean value ± standard deviation (n = 3).

To further investigate the impact of the HME on more microbiological parameters, additional analyses were performed on extrudates after frozen transport to KUL. Figures 12 and 13 show the results of the seven microbiological parameters determined for each sample obtained during HME of mealworms and BSF larvae, respectively. Again, the impact of the HME (at 160 °C) on the microbial counts is clear, this time on all seven counts performed for both extrudates. However, there seems to be a difference in magnitude of the reduction after HME for certain counts between the two insect species. For the BSF larvae extrudate, for example, all microbial counts were observed to be near or below detection limit (which means e.g. a 5 log reduction for TVC), while for the mealworm extrudate, TVC and the amount of LAB and coagulase positive staphylococci were still countable (2.8 to 4.1 log cfu/g). Interestingly, bacterial endospores were found to be eliminated (not detected) in the extrudates of both insect species, whether the initial amount of spores was very high (BSF larvae, 4.5-4.8 log cfu/g) or lower (mealworms, 2.8-3.7 log cfu/g).

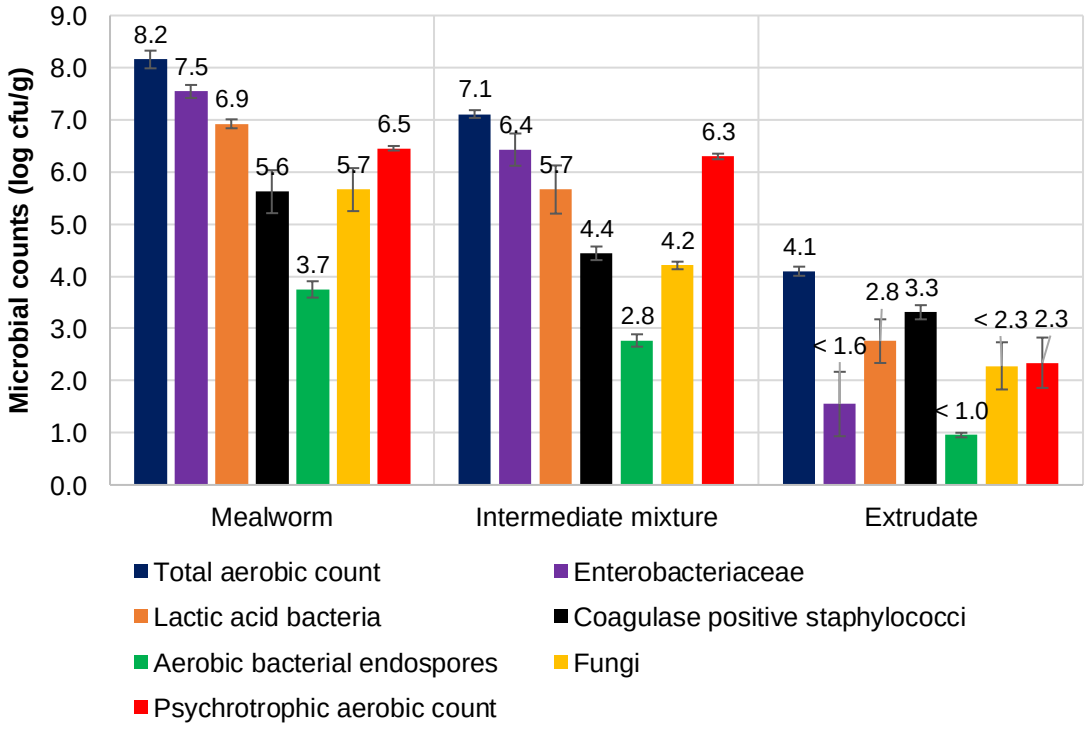

Figure 14 Microbial counts of raw, intermediate (pre-extrusion mixture with soy concentrate and mealworms) and end HME mealworm products after freezing and transport from DIL to KUL. Results are expressed as mean value ± standard deviation (n = 3).

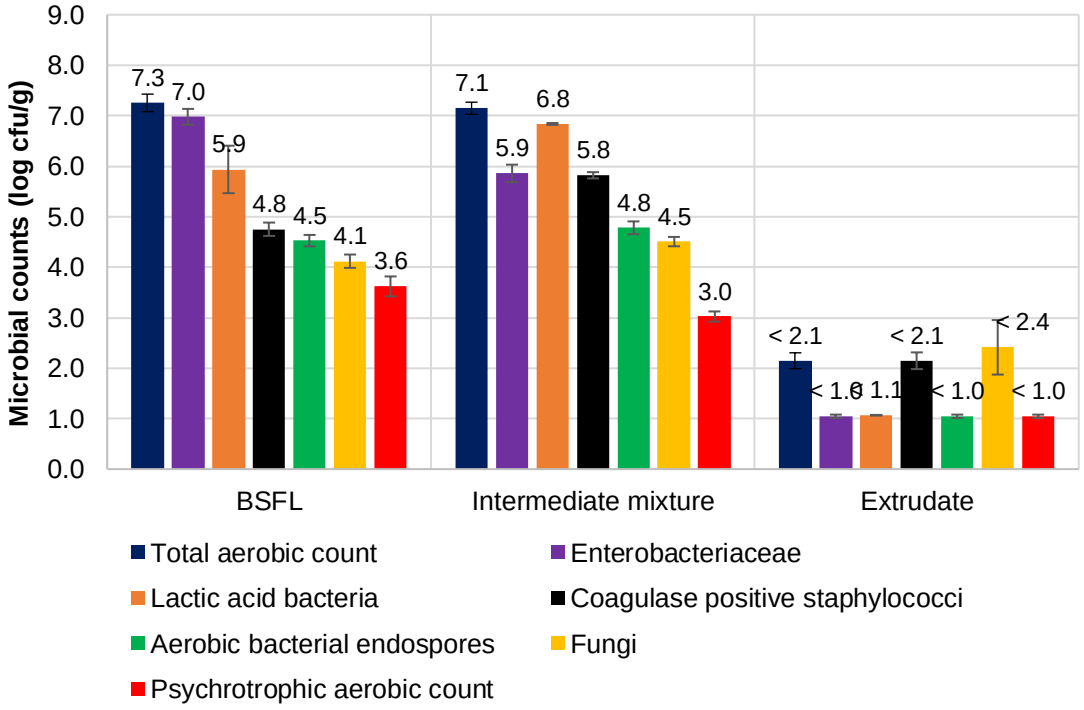

Figure 15 Microbial counts of raw, intermediate (pre-extrusion mixture with soy concentrate and BSF larvae) and end HME BSF larvae products after freezing and transport from DIL to KUL. Results are expressed as mean value ± standard deviation (n = 3).

Compared to the results of the first microbiological assessment at DIL (Figure 11), the total counts for the mealworm HME products after transport were found to be higher for all samples. The magnitude of reduction between raw and extruded products is similar (3-4 log cfu/g), however. As such, it may be possible that a post contamination has occurred during handling, freezing, storage and/or transport here. Also, while the total counts between the raw insects and the intermediate pre-mixture did not differ in the first analyses (Figure 11) for mealworm HME samples, here it was observed that certain counts were different between both stages. For mealworm-soy pre-mixes, a lower count was observed for all counts but the psychrotrophic count, for BSF larvae-soy pre-mixes, only the amounts of Enterobacteriaceae (lower), lactic acid bacteria (higher) and coagulase positive staphylococci (higher) were different from the raw insect counts. It may be possible that the addition of soy has altered the microbial counts and composition of the pre-mix and/or that post contamination (see above) have influenced these results.

Figure 14 presents the intrinsic parameters pH and water activity (a_w), which both have a major impact on the survival and growth of microorganisms in a certain product. During the whole logistic chain, these parameters may or may not influence how microbiologically stable a product is. It should be noted, however, that the intrinsic parameters may have been (slightly) influenced by the freezing step prior to transportation.

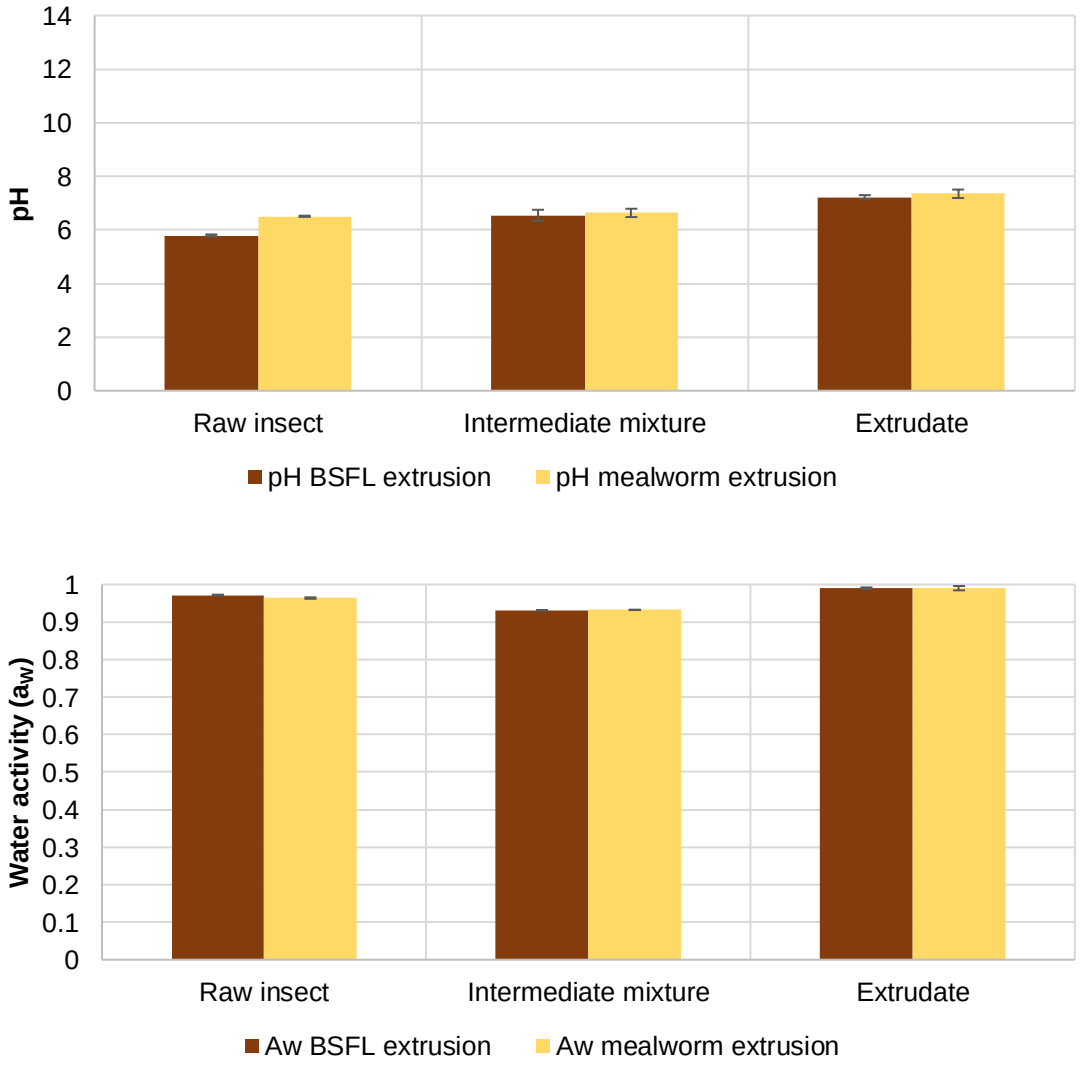

Figure 16 Intrinsic properties of raw, intermediate (pre-extrusion mixture with soy concentrate and fresh insects) and end HME products obtained from Black soldier fly larvae (BSFL) and mealworms after freezing and transport from DIL to KUL. Top: pH, bottom: water activity (a_w). Results are expressed as mean value ± standard deviation (n = 3).

It was observed that both pH and a_w of the HME products change during the HME process. While the pH (Figure 14, top) of raw insects was found to be 5.8 (BSF larvae) and 6.5 (mealworms), it rises after addition of soy and again after the extrusion step, moving towards a near-neutral pH that is optimal for microbial growth and survival. For a_w, a slightly different pattern was observed. The high a_w of the raw insects in both cases was lowered by addition of soy (a concentrate) and rose again after extrusion (introduction of water). In all cases, however, the water activity remained far above 0.60, the threshold for microbial activity. The a_w value of 0.93 in the pre-mixture may have negatively influenced certain bacteria already (especially gram negatives such as Enterobacteriaceae) and might therefore partially explain the differences in microbial counts between raw insects and pre-mixture.

After HME, it is clear that both intrinsic parameters are very well suited for microbial growth. While the HME has indeed reduced all or several microbial counts for BSF larvae and mealworm extrudates, respectively, it should be noted that a proper preservation strategy (e.g. retaining the frozen chain) is necessary in order to prevent outgrowth of microorganisms and spoilage of the products.

6.3 Microbiological impact of HME with mealworms or BSF larvae and different plant protein sources

After the inclusion of mealworms and BSF larvae in soy-based extrudates, also pea protein concentrate was investigated as co-product for HME with insects. Similar to the combinations with soy, the HME process was optimised (here, a peak barrel temperature of 150 °C was adopted). After production, the impact of the HME on the microbiological parameters TVC and bacterial endospore counts before and after processing was investigated again at KUL (after frozen transport).

As displayed in Figure 15, the observations from previous experiments could be confirmed for HME with all combinations of both plant proteins and both insect species. While the process parameters were slightly different, the impact of the HME on the TVC (5 to 6 log reduction) and bacterial endospore counts (3 to 4 log reduction) were very similar for all extrusion combinations. Again, the impact on the bacterial endospores was striking. No differences were observed with HME of only plant proteins for TVC or bacterial endospore counts.

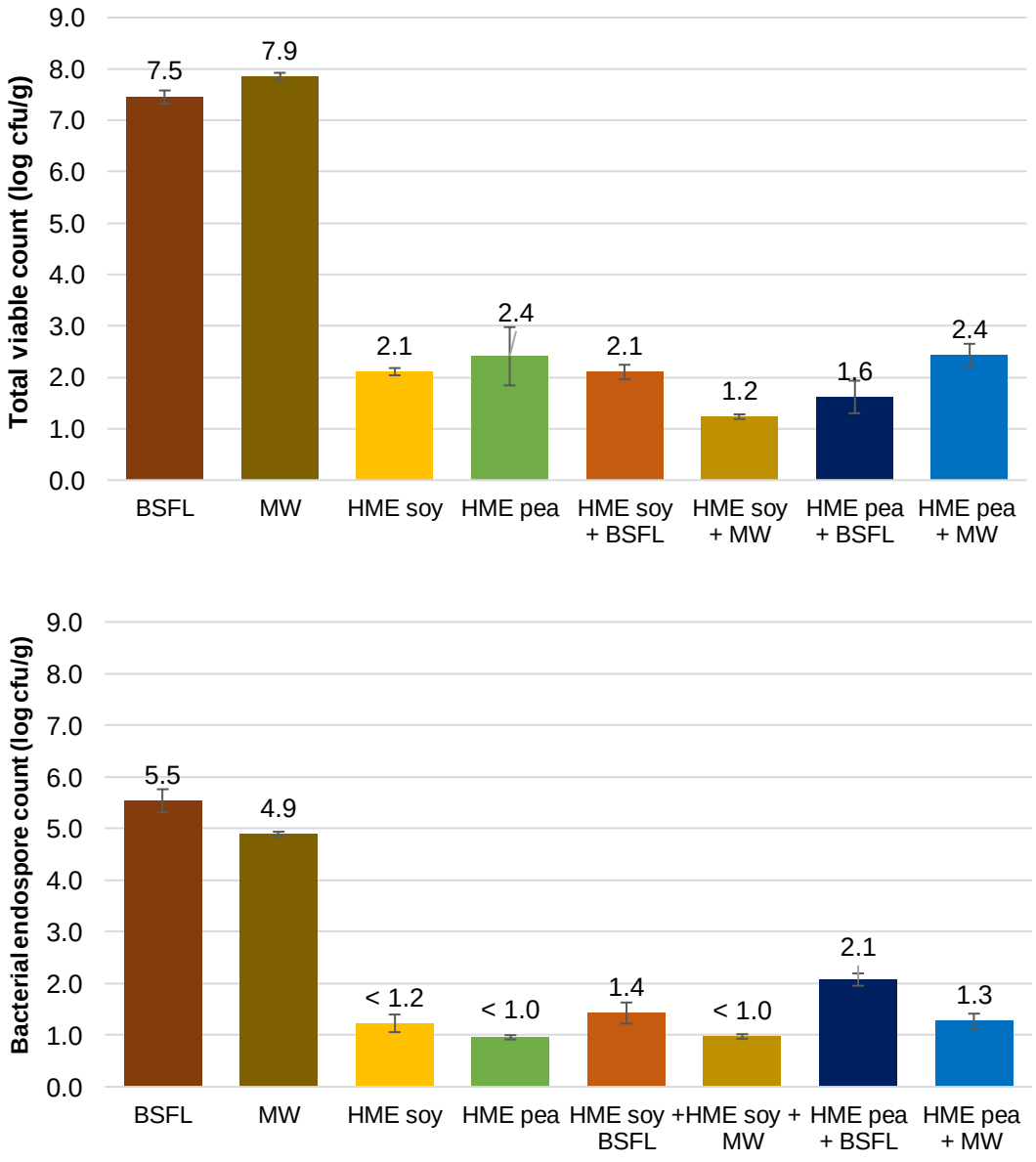

Figure 17 Total viable counts (top) and bacterial endospore counts (bottom) of raw insects and extrusion products with either soy or pea protein concentrate mixed with BSF larva or MW. Extrudates with only soy and pea (yellow and green graphs, respectively) as control. Results are expressed as mean value ± standard deviation (n = 3).

6.4 Conclusions and impact on microbiological food safety

Mealworms and black soldier fly larvae were introduced in high moisture extrusion together with plant protein concentrates. After assessing the microbiological quality of raw, pre-mixture (mixing with soy/pea concentrate) or extruded samples as well as of frozen, stored and transported samples, it was observed that the HME was capable of drastically reducing (5 log cfu/g and more) the microbial counts of the extrusion products for both mealworms and BSF larvae and can even eliminate certain counts below detection limit (including bacterial endospores!). This is promising for the HME technique, since bacterial endospores are considered to be an important microbiological parameter in insect matrices (containing human pathogens such as *Bacillus cereus* and *Clostridium perfringens*) and are typically hard to eliminate. Here, it was observed that the raw insects before HME can contain even over 5 log cfu/g bacterial endospores. The fact that HME can eliminate these endospores (almost) completely is a large asset to use this processing technique to produce microbiologically safe insect products.

Additionally, it was also observed that the addition of soy may alter the microbial load and composition, for example for Enterobacteriaceae, lactic acid bacteria and coagulase positive staphylococci. For mealworm HME, it was observed that after transportation, the total counts were higher, indicating the possibility of post contamination of the samples during handling, storage and/or transport. In addition to the well-suited intrinsic parameters (pH and a_w) of the samples for microbial growth and survival, this observation indicates that proper preservation strategies should be implemented for HME end products.

CHAPTER 7

MICROBIOLOGICAL SAFETY ASPECTS OF TRICANTER CENTRIFUGATION OF BSF LARVAE, WITH OR WITHOUT ENZYMATIC PRE-TREATMENT

Chapter 7 Microbiological safety aspects of tricanter centrifugation of BSF larvae with or without enzymatic pre-treatment

After optimisation of the tricanter centrifugation process to produce protein-rich BSF larvae meals, large batches of insect meals were produced for animal trials in WP 4. During the large scale production of these meals (for details, see Deliverable 3.8), samples were taken to be subjected to microbiological analyses in order to assess the impact of the tricanter centrifugation process on the microbiological quality and safety of the BSF larvae. Samples included (1) a BSF meal produced after tricanter centrifugation without enzyme treatment, (2) a BSF meal produced after tricanter centrifugation with enzyme treatment, (3) whole microwave-dried BSF larvae and (4) whole oven-dried BSF larvae. The latter two batches were used to produce two BSF meals to compare with the meals produced after tricanter centrifugation.

The samples were analyses for TVC (aerobic), aerobic bacterial endospore count, *Clostridium perfringens* count, *Staphylococcus aureus* count, *Listeria monocytogenes* count, Presumptive *Bacillus cereus* count and detection of *Salmonella* spp.

The results of the general microbial counts aerobic TVC and aerobic bacterial endospores are shown in Figure 18. Since the values of the TVC and endospore counts are equal, it can be concluded that after processing the BSF larvae, only bacterial endospores remained in the sample. As such, the high microbial counts from untreated BSF larvae (as reported in previous chapters and in Deliverable 3.8) can be successfully reduced by tricanter centrifugation and the drying processes. This is comparable to a pasteurisation treatment. Still, a considerable amount of bacterial endospores remains present, which urges for good monitoring of the end products in further applications or during storage.

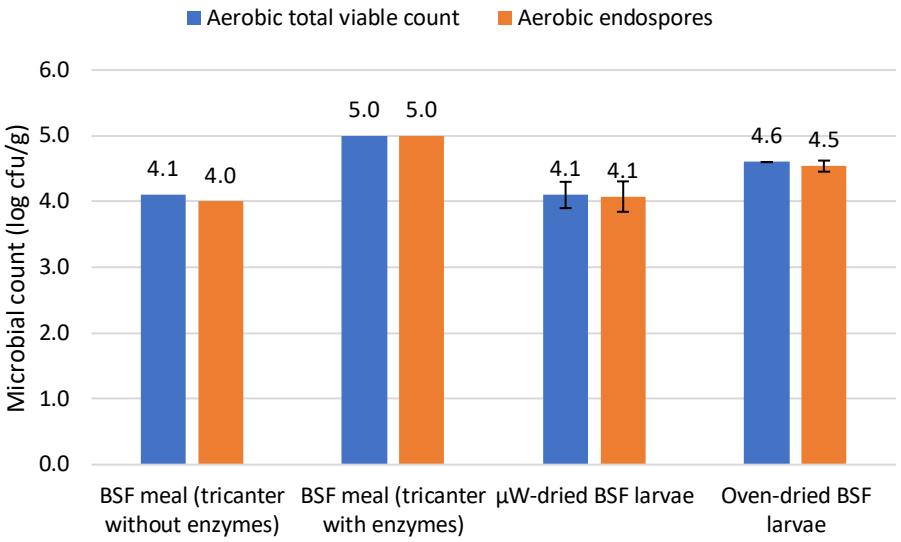

Figure 18 Microbial counts (aerobic TVC and aerobic endospores count) of two BSF larvae meals produced after tricanter centrifugation processing (with or without enzyme treatment) and two dried BSF larvae samples. Results are expressed as mean value ± standard deviation (n = 3) for the dried BSF larvae or as single values (n = 1) for the BSF larvae meals.

With regard to the presence of food pathogens, comparable to what was observed in Chapter 4, only presumptive *Bacillus cereus* was detected in the oven-dried BSF larvae (2.3 log cfu/g) and in both BSF larvae meals (2.8 ± 0.0 log cfu/g without enzyme treatment and 2.5 ± 0.2 log cfu/g with enzyme treatment). Again, in the sample that underwent a dielectric drying treatment, no *B. cereus* could be detected, confirming the potential of reducing certain food safety risks via this technology (see Chapter 4). In the other samples, all *B. cereus* counts remained (far) below the food safety risk limits (EFSA Panel on Biological Hazards, 2016). Still, the frequent detection of B. cereus, either in untreated or treated samples, highlights the importance of monitoring this specific microorganism in the whole insect value chain, as suggested by Vandeweyer et al. (2021).

CHAPTER 8

GENERAL CONCLUSIONS

Chapter 8 General conclusions

For Deliverable 6.3, the selected processing technologies applied on insects for food and feed as investigated in WP 3 were subjected to microbiological analyses. By monitoring certain microbiological parameters before, during and/or after the processing steps, the impact of those processes on the microbiological quality and safety of insects and insect products could be studied.

First, the impact of vacuum storage of killed BSF larvae was considered. It became clear that vacuum storage was not an advantageous preservation strategy in terms of microbiological quality and safety. Yet, the experiments related to this technology allowed to confirm the importance of the killing method and a decontaminating pre-treatment such as blanching in order to reduce the initial microbial counts before storing killed insects. Additionally, the value of refrigerated storage for fresh insects has again become clear, since it can slow down microbial processes and as such increase shelf life and microbiological safety.

The second processing technology investigated was dielectric drying. Firstly, was observed that the application of microwave drying could substantially decrease certain microbial counts (e.g. Enterobacteriaceae, including several pathogenic species), even without decontamination step prior to drying. Yet, also depending on the insect species, other microbial counts remained (partially) unaffected. Secondly, microwave and RF drying of BSF larvae and mealworms were performed in a large-scale experiment. Here, a blanching step was first introduced as a killing step, which had an important first decontaminating effect, comparable to what was observed during the vacuum packaging experiments. Since a high initial presence of microorganisms (including certain pathogens) impacts the microbiological quality and safety of the dried products, it is strongly advised to include this decontamination step prior to the drying process. Furthermore, it was observed that dielectric drying may have an additional reducing effect for certain microorganisms, even including bacterial endospores. Comparing dielectric drying with oven drying and freeze drying, it was observed that the additional decontaminating effect could also impact the pathogenic

bacterium *B. cereus*, which was found in certain samples during the drying process. All values of *B. cereus*, however, remained below the food safety limits during the drying processes. A low risk should be taken into account regarding this microorganism, especially during further applications or during storage of the dried products.

Apart from blanching insects prior to drying them, it is possible to treat the dried insects afterwards with low energy electron beam (LEEB). From the microbiological results in this Deliverable, it is apparent that a LEEB treatment can reduce not only the number of vegetative cells, but also the amount of bacterial endospores at the surface of dried insects. It is known that endospores can survive mild heat treatments such as blanching and dielectric drying (as confirmed in the experiments described here). As such, the observation that LEEB can effectively eliminate those endospores (which may belong to pathogenic species) can prove very valuable in terms of microbiological food safety of dried insects.

Another processing technology investigated in WP 3 and in this deliverable was high moisture extrusion (HME). The production of plant protein extrudates with inclusion of insects was proven to be successful from a technological point of view. Here, it was investigated which impact the HME had on the microbiological quality and safety of the final products. From experiments with BSF larvae and mealworms, incorporated in soy and pea protein extrudates, it was observed that, due to the high temperatures employed in the production process, a large set of microbial counts could be reduced to low or even undetectable levels. Especially of interest were the bacterial endospores again, since also by application of this processing technology, their counts could be decreased substantially.

Finally, also tricanter centrifugation either or not combined with an enzymatic treatment to produce BSF larvae meals is subject of WP 3 and this deliverable. Here, similar results as for the dielectric drying process were observed. The tricanter centrifugation process, either or not including an enzyme treatment, as well as the oven and microwave drying processes, resulted in end products mainly containing bacterial endospores. The processes therefore act as

pasteurisation treatment. Also here, low amounts of *B. cereus* were detected in all samples, except for the microwave-dried BSF larvae.

As a conclusion, it can be stated that, apart from the vacuum packaging, all processing technologies investigated in this Deliverable had a substantial (positive) effect on the microbiological quality and safety of the insects. In general, a dedicated decontamination step is required either prior to, during or after processing the insects into an intermediate or end product to improve the microbiological quality of insects, by preference also impacting the amount of bacterial endospores. Furthermore, it is important to monitor for specific pathogens during insect processing and afterwards. Especially *B. cereus* is an important microorganism to take into account when assessing microbiological food safety of insects as food and feed.

CHAPTER 9

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