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EFFECT OF KILLING AND TECHNOLOGICAL PROCESSING ON MICROBIOLOGICAL QUALITY OF EDIBLE INSECTS

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ABSTRACT

This study aimed to assess the effect of killing (blanching and freezing) followed by culinary processing on the microbiological quality of *Tenebrio molitor* and *Gryllus assimilis*. These insects were subjected to starvation before killing, which does not ensure sufficient microbiological safety. Therefore, technological or culinary processing of insects is necessary before consumption. For the subsequent processing of insects, boiling, drying, roasting, and microwave treatment were used. Nine categories of microbiological parameters were selected as indicators of hygienic quality. All identification and enumeration were performed using ISO-compliant methods. The results showed that killing insects by blanching or freezing alone does not ensure sufficient microbiological quality, and therefore, further culinary processing is necessary. However, all subsequent culinary treatments applied in this study were able to reduce the microbial load significantly.

Keywords: *Tenebrio molitor*, *Gryllus assimilis*, roasting, boiling, microwave treatment, drying

INTRODUCTION

Due to the increasing population, meat production and consumption are expected to reach unsustainable levels in the coming decades. Therefore, new and sustainable sources of nutrients, especially protein, must be sought **(van Huis and Oonincx, 2017)**. The urgency in finding alternative and sustainable protein sources has triggered an exponential increase in interest in insects as a source of human food or livestock feed over the past decade **(van Huis *et al*., 2021; Shah *et al*., 2022)**.
Compared to conventional livestock, insects have similar nutritional value and a lower environmental impact. However, unlike conventional livestock, their chemical composition is influenced by various factors, including species, developmental stage, sex, diet, rearing and processing methods **(Oonicnx and Finke, 2021)**. Generally, proteins and lipids, the major nutrients in insects, range from 40 to 75 g/100g and 10 to 30 g/100 g dry matter (DM), respectively **(Verkerk *et al*., 2007; Raheem *et al*., 2019)**. Additionally, insects contain significant amounts of vitamins like pantothenic acid, riboflavin, biotin, and folic acid) and minerals, such as iron, copper, magnesium, phosphorus, selenium, manganese, and zinc **(Eswaran *et al*., 2022; Mabelebele *et al*., 2022)**. However, increasing interest in entomophagy has raised concerns about the safety of insect-based products, mainly from a microbiological and chemical perspective **(Imathiu, 2020)**. Insects can carry bacteria dangerous to humans and animals, thus potentially acting as vectors of foodborne pathogens. Therefore, when looking at the microbiology of edible insects, it is essential to consider that insects naturally carry microorganisms and may harbour a complex autochthonous microbial population **(Marshall *et al*., 2016; Garofalo *et al*., 2019; Raheem *et al*., 2019)**.
Freezing is the most common method of killing insects, but it may not guarantee adequate microbiological quality **(Grabowski and Klein, 2017)**. Therefore, it is necessary to heat treat or include another effective technological treatment in insect processing since removing their digestive tracts is impractical on a large scale for farmed edible insects. Moreover, the safety of processed insects varies depending on the species and the production process **(Aguilar-Toalá *et al*., 2022; Pasini *et al*., 2022; Ververis *et al*., 2022; Gałęcki *et al*., 2023)**. From this point of view, the production process should include a heat treatment step that must be optimised with respect to the insect species and the intended use of the harvested insect biomass. For these reasons, investigating the microbiota of edible insects is essential to assessing and addressing potential health risks. Therefore, this study aims to study the effect of various killing methods and subsequent culinary treatments on the microbiological quality of edible insects. The procedures chosen for the experiment (boiling, freezing) were designed to simulate domestic and industrial insect processing. For this, the holometabolous mealworm (*Tenebrio molitor*, Linnaeus, 1758) (TM) and the hemimetabolous Jamaican field cricket (*Gryllus assimilis*, Fabricius, 1775) (GA) were chosen as model organisms. Both species belong to the category of insects that are commercially farmed globally and are widely considered to be highly promising sources of food and feed **(Dourado *et al*., 2020; Oliveira *et al*., 2024)**.

MATERIAL AND METHODS

Experimental insects

Insects were obtained from the Department of Zoology and Fisheries rearing facility, Czech University of Life Sciences Prague. Both species were maintained at 27 ± 1°C with a relative humidity of 40–50%, lightning regime 12:12 using the rack system. The house crickets were reared in plastic boxes (56 × 39 × 28 cm, IKEA, Prague, Czech Republic) equipped with egg trays (Schubert Partner, Prague, Czech Republic) and secured using a lid comprising approximately 80% of aluminium anti-insect mesh. Petri dishes containing feed (77.9% wheat, 17.6% soybean meal, 1.8% rapeseed oil, and 2.7% minerals and vitamin premix; particle size < 1 mm) were provided either in case of shortage or every 72 hours with unconsumed substrate removed if present. Petri dishes containing water gel (Oslavan, Náměšť nad Oslavou, Czech Republic) were also provided. The colonies were started with approximately 0.5 grams of 1-day-old pinheads that remained in the same container until harvest at 55±2 days. The containers and Petri dishes were washed with hot water and detergent. The feed substrate was stored hermetically in a container at room temperature. The mealworm colonies were started with approximately 1000 three-week-old larvae, which were reared in the insect breeding trays (60 × 40 × 12.5 cm, Beekenkamp, Maasdijk, Netherlands) using a mixture of wheat bran and the substrate as mentioned above in 4:1 ratio. The water gel was provided directly to the substrate as a source of moisture on a daily basis. The mealworms were harvested when the first pupae occurred in the boxes. All trays were washed in hot tap water with detergent prior to the experiment. The wheat bran was also stored hermetically at room temperature. Before the harvest, the insects were starved for 24 hours.

Sample preparation

The insects were killed using two killing methods: freezing (-18°C for 24 hours) and blanching in hot water (100°C for 1 minute). Following this, the insects

underwent technological processing using the methods outlined in **Table 1**. Once cool, the samples were aseptically wrapped and refrigerated.

Table 1 Technological treatments and their conditions

microwave treatment	TM – 260 g for 10 min, GA 240 g for 10 min, 800 W
boiling	300 g for 30 min in 3 L boiling water
roasting	150 g, roasting without oil for 5 min
drying	60 g GA, 80 g TM, 15 hours, 80°C

Microbiological analysis

Sample preparation involved aseptically homogenised insect biomass by hand in a mixing bowl. One gram was then transferred to 9 ml of dilution medium (9 g of NaCl, 1 g of peptone (Oxoid, England), 1000 ml of distilled water) and serially diluted to a value of up to 10^{-9}. All groups of microorganisms were identified and enumerated using classic culture methods in compliance with the following valid standards and procedures: for total aerobic spores, ISO 4833-1:2013 using Tryptone-Soy Agar (Oxoid, England); for *Bacillus cereus*, ISO 7932:2004 using *B. cereus* agar base (Himedia, USA) enriched with polymyxin B (2 vials/l, Himedia, USA) and yolk emulsion (50 ml/l, Himedia, USA); for *Escherichia coli*, ISO 16649-2:2001 with TBX medium (Oxoid, England); for *Enterobacteriaceae*, ISO 21528-2:2017 using Violet red bile glucose agar (Oxoid, England); for Coagulase-positive staphylococci and *Staphylococcus aureus*, ISO 6888-1:2021 using S Baird-Parker agar (Oxoid, England) with yolk emulsion and potassium tellurite; for *Salmonella* spp. ISO 6579-1:2017 using Rappaport-Vassiliadis (Oxoid, England) and SS agar (Oxoid, England); and for *Listeria monocytogenes* ISO 11290-1:2017 using Brilliance Listeria Agar (Oxoid, England). Coagulase activity was tested using the Staphylase test kit (Oxoid, England).

Statistical analysis

Statistical evaluation was performed using Statgraphics Centurion XV 15.2.05/2007 software (StatPoint Technologies, USA) employing one-way analysis of variance (ANOVA) followed by Fisher's least significant difference as a post-hoc test, with a 95% confidence interval.

RESULTS

Results are presented in **Tables 2** and **3**. For both insect species, it is evident that the method of killing significantly affects the presence of the assessed groups of microorganisms. In GA, there is a significant decrease in aerobic sporulating bacteria from 5.78 ±0.03 log CFU/g in samples killed by freezing compared to 3.53 ±0.09 log CFU/g after blanching. A similar trend is observed for *B. cereus* and other sporulating bacteria. Values of aerobic sporulating bacteria after killing by blanching without other processing method were not significantly different to the values from all types of technological treatment. For TM, the most significant difference between killing methods was observed for *Enterobacteriaceae*, with 4.71 ±0.08 log CFU/g remaining after freezing compared to 1.48 ±0.09 log CFU/g after blanching. Moreover, a significant decrease in the total number of mesophilic aerobic bacteria when blanched was observed, while boiling and microwave treatment gave similar values to blanching (control samples).

For GA and TM, all types of treatment could eliminate *Enterobacteriaceae*, which could not be achieved by killing alone. Similar results apply to coagulase-positive staphylococci, while *L. monocytogenes*, *Salmonella* spp. and *E. coli* were not observed in the tested samples. The lowest counts for *B. cereus* in both TM and GA were observed in the boiled samples. However, exact log CFU values could not be determined in the samples due to recurrent overgrowth of the colonies over the whole surface of Petri dishes.

In TM, the lowest significant log CFU/g values for mesophilic aerobic bacteria were detected after roasting in insects killed by blanching. In GA, drying was the most effective method, regardless of the killing method. Roasted samples also produced the same values, but only for blanched insects, while in GA, the lowest significant values of sporulating aerobic bacteria were recorded after roasting frozen and blanched insects. Notably, all treatments significantly reduced the number of aerobic spore-formers in TM, although significantly higher values were recorded for boiled samples after freezing than other treatments.

Table 2 Results of microbiological indicators for *Gryllus assimilis* (log CFU/g). Values with different superscripts indicate significant difference ($p < 0.05$).

	control		drying		boiling		microwave treatment		roasting	
	freezing	blanching	freezing	blanching	freezing	blanching	freezing	blanching	freezing	blanching
Bacillus cereus	<4	<2	<2	<2	<1	<1	<2	<2	<2	<2
Enterobacteriaceae	5.85 ±0.09	4.75 ±0.10	<1	<1	<1	<1	<1	<1	<1	<1
Escherichia coli	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase-positive staphylococci	1.44 ±0.24	<1	<1	<1	<1	<1	<1	<1	<1	<1
Staphylococcus aureus	0	0	0	0	0	0	0	0	0	0
mesophilic aerobic bacteria	7.94 ±0.18^{d}	6.82 ±0.06^{c}	4.72 ±0.19^{a}	4.66 ±0.09^{a}	5.61 ±0.18^{b}	4.69 ±0.17^{a}	5.71 ±0.06^{b}	5.73 ±0.15^{b}	5.66 ±0.06^{b}	4.66 ±0.16^{a}
aerobic sporulating bacteria	5.78 ±0.03^{d}	3.53 ±0.09^{abc}	3.54 ±0.06^{abc}	3.58 ±0.11^{abc}	3.68 ±0.18^{c}	3.48 ±0.10^{ab}	3.62 ±0.07^{bc}	3.61 ±0.10^{bc}	3.44 ±0.05^{a}	3.46 ±0.09^{ab}
Listeria monocytogenes	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g
Salmonella	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g

Table 3 Results of microbiological indicators for *Tenebrio molitor* (log CFU/g). Values with different superscripts indicate significant difference ($p < 0.05$).

	control		drying		boiling		microwave treatment		roasting	
	freezing	blanching	freezing	blanching	freezing	blanching	freezing	blanching	freezing	blanching
Bacillus cereus	3.63 ±0.24	<2	<2	<2	<1	<1	<2	<2	<2	<2
Enterobacteriaceae	4.71 ±0.08	1.48 ±0.09	<1	<1	<1	<1	<1	<1	<1	<1
Escherichia coli	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Coagulase-positive staphylococci	1.33 ±0.15	1.35 ±0.24	<1	<1	<1	<1	<1	<1	<1	<1
Staphylococcus aureus	0	0	0	0	0	0	0	0	0	0
mesophilic aerobic bacteria	7.97 ±0.07^{d}	5.69 ±0.05^{c}	4.67 ±0.14^{b}	4.71 ±0.27^{b}	5.85 ±0.08^{c}	5.79 ±0.12^{c}	5.79 ±0.12^{c}	5.73 ±0.14^{c}	4.71 ±0.09^{b}	3.67 ±0.04^{a}
aerobic sporulating bacteria	5.76 ±0.03^{c}	4.68 ±0.13^{b}	3.59 ±0.15^{a}	3.62 ±0.06^{a}	4.51 ±0.06^{b}	3.63 ±0.14^{a}	3.56 ±0.15^{a}	3.62 ±0.11^{a}	3.62 ±0.21^{a}	3.54 ±0.04^{a}
Listeria monocytogenes	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g
Salmonella	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g	0/25 g

DISCUSSION

After comparing many studies, **Garafalo et al. (2019)** concluded that there were significant differences in the microbial load among different species of edible insects. Fresh edible insects generally contain a high microbial load, with mesophilic aerobes ranging from 3.6 to 9.4 log CFU/g, *Enterobacteriaceae* from 4.2-7.8 log CFU/g, and sporulating aerobic bacteria from 0.5-5.8 log CFU/g. In this study, the values for GA and TM also fall within these ranges. Moreover, the different microbial loads between TM and GA are evident, especially in the case of *Enterobacteriaceae*, where GA counts are two orders of magnitude higher than those of TM. TM is typically reared in a feed substrate of wheat bran, supplemented with fresh fruit and vegetables. In addition, they are in close contact with their frass. On the other hand, crickets are mainly fed a dry soy-based substrate and are not in close contact with their frass, which may explain the differences in the composition of the microorganisms. Notably, this study reveals a significant difference in the number of bacteria in insects killed by freezing and blanching. **Speck and Ray (1977)** stated that although some microorganisms may be killed during freezing, many survive in different states of viability. Increased resistance to freezing is usually provided by food components such as proteins, simple and complex carbohydrates, and triacylglycerols. Insects are rich in proteins **(Kouřimská *et al*., 2023)** and can serve as cryoprotectants, thereby protecting the microorganisms present, especially in the digestive tract, since insects are only starved before being killed. From a microbiological perspective, blanching appears more efficient, although, in the case of GA, there is a risk of insects escaping during handling.

After treatment, the lower microbial count is apparent compared to insects killed without any further treatment, which shows their effectiveness in reducing microbial contamination, agreeing with the findings of **Garafalo *et al.* (2019)**, **Aguilar-Toalá *et al.* (2022)**, **Pasini *et al.* (2022)**, **Ververis *et al.* (2022)**, and **Gałęcki *et al.* (2023)**. Furthermore, the present study found a significant decrease in microorganism counts after blanching. This finding agrees with similar results reported by **Vandeweyer *et al.* (2017)**, **Wynants *et al.* (2017)**, **Mancini *et al.* (2019)**, and **Cacchiarelli *et al.* (2022)**. **Nyangena *et al.* (2020)** reported roasting and drying in a hot air oven as the most effective methods for reducing the microbial load, boiling was also a suitable treatment. These findings confirm the results obtained for GA, which, regardless of the killing method, showed the lowest numbers of mesophilic bacteria after drying and TM, which had the lowest values after roasting. Similarly, boiling showed the greatest effect on reducing the amount of *Bacillus cereus*, i.e., by one order of magnitude compared to other types of treatment.

Although *L. monocytogenes*, *Salmonella* spp. and *E. coli* are important microbiological parameters in evaluating food quality, they were not detected in any of our samples, including untreated ones. Similar findings were reported by other studies, such as **Garafalo *et al*. (2019)**, **Kolakowski *et al.* (2021)**, and **Ververis *et al.* (2022)**.

The total number of aerobic bacteria represents one of the microbial guidelines used to assess the quality of fresh foods, with high total aerobic numbers being associated with rapid spoilage and potential health risks **(Ssepuuya *et al*., 2019)**. Specifically, for the house cricket, data on the microbial quality of unprocessed samples showed a high load of total mesophilic bacteria ranging from 7.2 to 10.2 log CFU/g **(Ververis *et al*., 2022)**. For GA, another cricket species, the results of this study produced similar values: 7.94 ±0.18 log CFU/g. **Stoops *et al*. (2016)** found total aerobic numbers for unprocessed TM of 7.7–8.3 log CFU/g, agreeing with the present study's results (7.97 ±0.07 log CFU /g). Similarly, **Klunder *et al*. (2012)** also reported total aerobic bacterial counts for fresh TM and house cricket in the 6.7–7.7 log CFU/g range. However, after technological modifications (boiling, roasting and frying), the total aerobic bacteria counts were <1.7–4.8 log CFU/g. In our case, the samples treated by roasting and drying did not exceed 4.8 log CFU/g, but for boiling and microwave radiation treatment, the total aerobic bacteria count was higher (TM: 5.73-5.85 and for GA: 4.69-5.73 log CFU/g). These findings indicate that the microwave treatment applied could not reduce total aerobic mesophilic bacteria after blanching.

Staphylococcus aureus is a common human pathogen present in edible insects. Contamination can be caused during handling or processing **(Garofalo *et al*., 2017; Milanović *et al*., 2018; Garofalo *et al*., 2019)**. It is also known for producing toxins resistant to heat treatment, freezing, and drying **(Kooh *et al*., 2020)**. However, *S. aureus* is sensitive to heat processing, and processed edible insects are favourable for the growth of this bacterium because of its ability to thrive without competition. Moreover, it is also resistant to low water activities **(Milanović *et al*., 2018; Walia *et al*., 2018; Garofalo *et al*., 2019; Kooh *et al*., 2019)**. In our experiment, although the presence of *S. aureus* was not demonstrated, coagulase-positive staphylococci were detected. However, in TM and GA, they were destroyed by all treatment types, regardless of the killing method. However, in other studies, the presence of *S. aureus* was demonstrated in fresh and processed edible insects: yellow mealworm **(Garofalo *et al*., 2017; Wynants *et al*., 2017; Milanović *et al*., 2018)**, lesser mealworm **(Wynants *et al*., 2018),** house cricket and desert locust **(Garofalo *et al*., 2017; Milanović *et al*., 2018)**. Therefore, evaluating its presence in edible insects is necessary to ensure their safe use in the food industry.

With some exceptions, bacteria belonging to the family of *Enterobacteriaceae* are relatively sensitive to heat treatment **(Stoops *et al*., 2017)**. For example, **Fröhling *et al.* (2020)** found that enterobacteria prevailed in untreated and treated house crickets and **Messina *et al.* (2019)** in house crickets and yellow mealworms. In the present study, untreated TM samples also had relatively high values of *Enterobacteriaceae* (4.71 ±0.08 log CFU/g). Nonetheless, blanching alone resulted in a significant reduction of *Enterobacteriaceae* (1.48 ±0.09 log CFU/g). For GA, the killing without further processing did not have such significant impact as for TM, resulting in values of 6.85 ±0.09 to 4.75 ±0.10 log CFU/g. **Osimani *et al.* (2017)** also found viable counts of *Enterobacteriaceae* (<1 log CFU/g) in dried house cricket and yellow mealworm, while **Klunder *et al.* (2012)** and **Stoops *et al.* (2017)** found that boiling for 10 minutes is sufficient to inactivate *Enterobacteriaceae*. Although the present study confirms these results, it also shows that all technological treatments can reduce *Enterobacteriaceae* to <1 log CFU/g in both species of edible insects and meet the specifications proposed by **EFSA (2021a, 2021b)**.

A potential risk associated with edible insects is the presence of spore-forming bacteria, which are likely introduced by contact with the soil, such as from the vegetables used as feed, which cannot be eliminated by boiling or drying **(Stoops *et al*., 2017; Walia *et al*., 2018; Garofalo *et al*., 2019)**. **Fasolato *et al*. (2018**) and **Osimani and Aquilanti (2021)** also confirmed that drying and freeze-drying were ineffective against microbial spores. In our study, the most successful method of *B. cereus* reduction was processing by boiling. Also, blanching was a significantly more efficient killing method for reducing total aerobic spore count than freezing, but with further treatment (all tested treatments), a significant reduction in spore formers could be achieved. It has also been shown that heat treatment can reduce the number of bacterial spores and thus increase the possible shelf life, but at the expense of the nutritional and sensory quality of the final product **(Klunder *et al*., 2012; Grabowski and Klein, 2017; Vandeweyer *et al*., 2017)**. **Osimani *et al.* (2017)** and **Fasolato *et al*. (2018)** pointed out that *B. cereus* was found across the species of edible insects. In our study, its presence was found in both two monitored species. *Bacillus cereus* is a foodborne pathogen, and a value of 5 log CFU/g was determined as microbial load that can cause gastrointestinal disease after ingestion **(Kramer and Gilbert, 1989; Garafalo *et al*., 2019)**. **Osimani *et al*. (2017)** reported *B. cereus* counts above the critical level of 5 log CFU/g in processed insects, that are now commercially available in Europe, including house cricket meal samples. In the present study, untreated samples showed >5 log CFU/g in both species.

Based on the results of this study, we agree with the conclusions of **Ververis *et al*. (2022)**, who stated that technological processing is essential to ensure the microbiological safety of edible insects. Importantly, the quality of unprocessed insects needs to be monitored at least in terms of the following microbiological criteria: total numbers of mesophilic bacteria, spore-forming bacteria, *B. cereus*, *S. aureus*, *Enterobacteriaceae* and coagulase-positive staphylococci. These microorganisms were the most prevalent in untreated samples of TM and GA in our study.

CONCLUSION

Based on the analyses conducted in this study, the presence of *B. cereus*, mesophilic aerobic bacteria, aerobic sporulating bacteria and bacteria of the genus *Enterobacteriaceae* were detected in the samples GA and TM. Pathogenic bacteria such as *S. aureus*, *Salmonella* spp., and *L. monocytogenes* remained undetected in any of samples. After the killing of the edible insects, the microbial load remained relatively high. Killing by blanching in hot water helps to reduce the microbial load more than by freezing, but even so, additional culinary treatment did show an effect and is recommended for consumer safety to prevent potential exposure to pathogenic bacteria. In summary, insects killed by blanching or freezing without further technological processing should not be considered microbiologically safe for consumption.

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