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Quantitative characterization of microbial load in wild-harvested edible insects of Nagaland, India

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Abstract

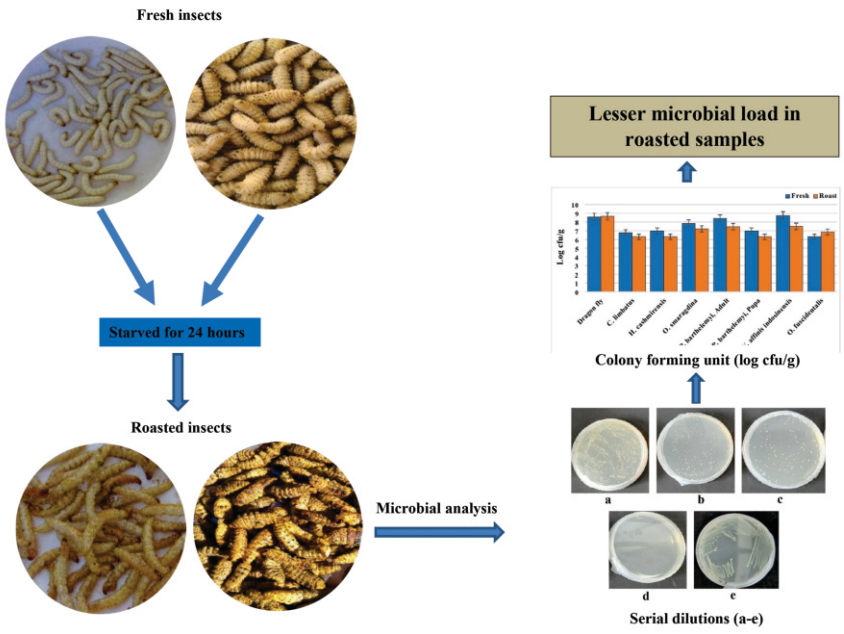
Aim: To analyze the microbial load in raw and roasted insects in an attempt to determine the efficiency of heat treatment in removing the microbial load.

Methodology: Fresh as well as roasted (over low flame for 15 min) insect samples were used to determine the microbial contaminants. Crushed insect samples were suspended in sterile half-strength nutrient broth solution and were further serially diluted 10-fold (up to 10^{-10}) in isotonic half-strength nutrient broth solution. The total number of aerobic mesophilic microorganisms were determined on plate count agar and expressed as log Cfu g^{-1}.

Results: Differences in the number of microbial colonies were observed in fresh and roasted samples. The microbial load ranged from 6.30-8.75 log Cfu g^{-1} and showed that the highest microbial colonies were present in the fresh samples. The average log Cfu g^{-1} in fresh insect samples (7.57±0.87) was significantly higher ($P<0.05$) as compared to roasted samples (7.07±0.76).

Interpretation: Edible insects require proper processing before consumption to reduce microbial contamination and further study is needed to identify specific microbes/food pathogens to develop microbial quality and parameters to ensure consumer safety.

Key words: Alternative food, Edible insects, Food safety, Microbial contaminants, Traditional foods



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Introduction

In recent times, edible insects have garnered much interest for their potential as an alternative food source and feed with comparable nutritional content to the commonly available conventional animal products (Labu *et al.,* 2021). Entomophagy is a common practice in many regions of Asia, Africa, Australia, and Latin America (mainly Mexico). Still, in western countries, insects use is new and exciting food matrix (Stoops *et al.,* 2016). In the context of sustainable development, there is an urgent need to propose and implement new methods for production without affecting the quality of food, natural habits, and biodiversity (Megido *et al.,* 2017). Food safety, processing, and preservation are closely related (FAO, 2013), and there are safety concerns associated with the consumption of insects, such as contaminating chemical and biological agents (Garofalo *et al.,* 2019). The increasing introduction of insects into the human diet imposes increasing attention on their safety concerns. It needs proper evaluation of edible insect safety by monitoring the presence of harmful microorganisms (Baiano, 2020).

Many studies have reported edible insects as a valuable source of proteins, lipids, carbohydrates, and specific vitamins and minerals. However, there are only limited studies on their food safety and shelf life (Stoops *et al.,* 2016). Banjo *et al.* (2006) reported that insects can harbor many pathogenic bacteria and fungi, such as Staphylococcus aureus, *Pseudomonas aeruginosa, Bacillus cereus* and *Aspergillus niger*. Earlier studies have also highlighted the presence of different genera of bacteria and fungi in fresh and processed (ready-to-eat) insects such as *Locusta migratoria, Tenebrio molitor, Acheta domesticus, Gryllodes sigillatus* and *Ruspolia differens* (Stoops *et al.,* 2016; Megido *et al.,* 2017; Vandeweyer *et al.,* 2017; Osimani *et al.,* 2018; Garofalo *et al.,* 2019; Labu *et al.,* 2021). Food safety is influenced by how it is produced, stored, processed, and eaten. The rearing environment, rearing procedures, hygiene measures, and insect feed affect the microbiota of insect species (Wynants *et al.,* 2018). The microbial community of *R. differens* has been reported to be influenced by the environment from which it is harvested and by the post-harvest handling conditions and processes (Ssepuuya *et al.,* 2019). Even though insects can act as biological vectors of pathogenic microorganisms, they are considered to be safe if properly processed and stored (Van Huis *et al.,* 2013). Some bacterial genera such as *Leuconostoc, Lactococcus, Lactobacillus*, and *Streptococcus* identified in *R. differens* are associated with beneficial effects on human health if ingested in sufficient quantly (Ssepuuya *et al.,* 2019).

Edible insects are considered an essential component of traditional food and highly appreciated as alternative protein sources compared to other conventional meat sources (e.g., beef, chicken, fish, and pork). They are essential nutritional food for the people of Nagaland and are prepared for consumption based on palatability. However, studies on microbiological contaminants are essential for food and consumer safety. In Nagaland, edible insects are mainly collected from the wild and made available at local markets. Members of the Naga tribes are reported to consume 106 species of insects and are particular about how an insect species is cleaned/prepared for consumption. Generally, insects are consumed as boiled, cooked, fried, or roasted (Mozhui *et al.,* 2020). The consumption of dragon nymphs, *Cybister limbatus, Hydrophilus caschmirensis, Oecophylla smaragdina, Provespa bartheleymi*, and *Vespa affinis indosinensis*, as boiled, chutney, cooked or fried is reported (Mozhui *et al.,* 2020). While insects are consumed after being well-cooked by the ethnic communities in Nagaland, the quality and safety of insect consumption is a significant concern for consumer safety globally. In view of the above, this study was conducted to analyze freshas well as roasted insect samples for microbial loads in order to determine the efficiency of heat treatment on microbial contaminants.

Materials and Methods

Insect samples: The study was conducted from July 2021 to December 2021. Insect samples (dragonfly nymphs, *C. limbatus, H. caschmirensis, O. smaragdina, P. bartheleymi, V. affinis indosinensis* and *O. fuscidentalis*) for the present study were obtained from different local markets at Dimapur and Kohima (Nagaland, India). Insects were starved for 24 hr before being killed by freezing. Thereafter, the samples were cleaned thoroughly with water. and roasted over low flame (approx. 130°C) for 15 min. Fresh as well as roasted insect samples were used for further analysis.

Plate counts: Insect samples (0.1g) were crushed using a mortar and pestle. The crushing instrument was autoclaved before use. Samples (n = 3) were suspended in 2 ml sterile half-strength nutrient broth solution containing the composition of peptone 15.0 g l^{-1}, yeast extract 3.0 g l^{-1}, Sodium chloride 6.0 g l^{-1}, D(+)-Glucose 1.0 g l^{-1}, final pH 7.5 +/- 0.2 at 25°C temperature and homogenized for 1 min. The suspensions were serially diluted 10-fold (up to 10-10) in isotonic half-strength nutrient broth solution. Growth observed was only visible up to 10-4 dilutions. From the dilutions, 0.1 ml suspension was placed on pre-prepared nutrient agar Petri plates. Total numbers of aerobic mesophilic microorganisms were determined on plate count agar (BioRad, Hercules, CA, USA) after incubation at 30ºC for 72 hr or until colonies appeared on the plate (Megido *et al.,* 2017). After incubation period, the plates were monitored for several aerobic bacterial colonies that appeared on the surface of nutrient agar. All microbial counts were analyzed in triplicates and expressed as log Cfu g^{-1}.

Statistical analyses: Data were analyzed using the software SPSS version 22. Kruskal-Wallis test was performed to determine significant differences (P=0.05) in microbial count between fresh and roasted samples, followed by a pair-wise comparison test.

Results and Discussion

Microbial load in insects was quantified by calculating the colony count in each insect extract. Differences in the number of microbial colonies were observed in fresh and roasted samples.

Table 1: Insect samples tested for presence or absence of microbial load (+ shows the presence of single colony, ++ shows presence of double colonies).

Insect sample	Edible stage	Mode of sample preparation	Presence/ absence of microbes
Dragon fly	nymph	fresh	+
		roast	+
C. limbatus	adult	fresh	++
		roast	+
H. caschmirensis	adult	fresh	++
		roast	+
O. smaragdina	larva	fresh	+
		roast	+
P. barthelemyi	adult	fresh	+
		roast	++
	pupa	fresh	++
		roast	++
V. affinis indosinensis	larva	fresh	+
	roast	+	
O. fuscidentalis	larva		
	fresh	++	
		roast	+

Microbial colonies were more in fresh samples than in roasted samples. (Table 1), which could be due to the heat treatment applied. The moisture content was high in fresh samples and more susceptible to microbial contamination. Similar observations with a higher microbial count in fresh insects such as *Tenebrio molitor, Acheta domesticus* and *Gryllodes sigillatus* was reported by Murefu *et al.* (2019). The insect samples were further analyzed for the aerobic microbial load. After serial dilution, most of the samples with 10^{-1} dilution showed confluent growth in aerobic bacteria, following which the population density decreased up to 10^{-4} dilution where only a countable amount of colony forming units was observed in all the insect samples. However no or less microbial contamination was observed in roasted samples as most of the microbes were killed due to heat during roasting. The aerobic microbial colonies were morphologically characterized based on their size, color, texture, margin, and surface. Each microorganism followed a particular pattern when grown in the form of culture on plates.

Out of all the colony forming units, some colonies were selected having prominently different morphological features and purified by streaking on nutrient agar Petri plates (Table 2). Microbial counts of fresh and roasted samples of different insect species are presented in Table 3. The microbial load ranged from 6.30-8.75 log Cfu g^{-1} (Fig. 1). The average log Cfu g^{-1} in fresh insets samples (7.57±0.87) was significantly higher ($t_{(23)}$=4.47, P<0.05) as compared to roasted samples (7.07±0.76). From the present study, it is plausible to say that even after roasting, edible insects should be kept in a proper storage facility to avoid any risk of further contamination. A short blanching process before roasting is more effective in reducing the microbial load than dry heat treatment (Murefu *et al.,* 2019). The reason for the high range in a total viable count of fresh and roasted samples could lie in the fact that certain bacteria that are commonly present in insect samples can be inactivated by proper heat treatment. However, spore-forming bacteria cannot be entirely eliminated by boiling/ roasting (Klunder *et al.,* 2012). In insects such as Bombyx mori, Omphisa fuscidentalis, and Gryllus bimaculatus moderate amount of microbial load in frozen forms were also reported (Kurdi *et al.*, 2021). Re-contamination or cross-contamination risks also arise if roasted or fried edible insects are not handled or stored in hygiene condition before consumption. Edible insects are likely to be contaminated with both pathogenic and spoilage microorganisms during harvesting, packaging, and or storage. Therefore, proper storage of heat-treated edible insects is essential to reduce the risks associated with consumption. Processing methods such as boiling, followed by open-pan roasting and hot-ash roasting can reduce microbial contamination. Such reduction in microbial load was observed in *Gonimbrasia belina* (Mujuru *et al.,* 2014; Murefu *et al.,* 2019). Also, simple interventions like drying and acidifying insects with vinegar to pH 4.5 prevents rapid spoilage during storage at room temperature (Klunder *et al.,* 2012). In different household, heat treatments on fresh insects (*T. molitor*), total microbial counts reduced with levels ranging from 2 log Cfu after oven cooking to 4/5 log Cfu after vacuum cooking and frying until 7 log Cfu reduction after boiling (Megido *et al.,* 2018).

However, considering cultural preferences and organoleptic aspects of edible insects, further investigation of these promising interventions is needed. The presence of contaminants even after roasting shows that roasting may not entirely kill the microbiota and needs further heating treatment. Therefore, the effectiveness of various traditional ways of preparation and decontamination of an insect as food, such as drying, frying, boiling, and smoking, needs to be assessed for microbial safety as most of the contamination occurs during sun drying and poor storage conditions (Mpuchane *et al.,* 1996;

Table 2: Morphological characters of microflora extracted from different insect samples

Insect sample	Edible stage	Processing mode	Morphological features
Dragon fly	nymph	fresh	Two types of colonies 1. Medium in size, opaque with smooth edges. 2. Small, opaque in the center, and translucent at the edges.
		roast	Two types of colonies 1. Medium size, white, smooth 2. Large smear of orange texture, wavy margin
C. limbatus	adult	fresh	Small, translucent, convex surface and smooth margin
		roast	Single large colony, cream, smooth margin, and flat surface
H. caschmirensis	adult	fresh	Two types of colonies observed 1. Medium, cream with slightly rough margin, convex surface. 2. Small, cream smooth margin, convex surface.
		roast	Single small cream colony, smooth margin, convex surface
O. smaragdina	larva	fresh	Two types of colonies 1. Very large, cream, flat surface, smooth margin 2. Small, pale yellow, convex surface, smooth edges.
		roast	Two types of colonies 1. Large, opaque, smooth margin 2. Small, opaque, smooth
P. barthelemyi	adult	fresh	Two colonies observed 1. Medium size, creamy texture colony with smooth surface and margin 2. Small colonies, smooth surface, and convex margin
		roast	Medium size, cream texture, translucent margin
	pupa	fresh	Two types of colonies observed 1. Large, creamy pink with a slightly rough margin and flat surface 2. Creamy in the center and translucent margin, convex surface.
		roast	Single large white colony with a flat surface and rough margin
V. affinis indosinensis	larva	fresh	Heavy load of bacteria with two types of colonies. 1. Medium, cream texture, flat surface 2. Medium, cream texture, convex surface
		roast	Small colonies, cream color, smooth surface, soft margin.
O. fuscidentalis	larva	fresh	Single large colony with rough and wavy edges and flat surface
		roast	Small colonies, creamy texture, convex surface, and opaque

Gashe *et al.,* 1997; Simpanya *et al.,* 2000; Abu-Ghannam and Crowley, 2006; Rumpold *et al.,* 2014). After spreading insects' cultures at different dilutions, a change in the number of colony, counts was observed. Treating insects up to 100°C removed most bacterial contaminations from insects, however, results showed that temperature treatment was insufficient to kill the microbiota. The persistence of microbial load in insects could be due to microorganisms in the insects' gut. While insects are consumed after proper heat treatment, consumers should avoid consuming the insects' digestive tracts as different microbes are found in their gut. Despite proper heat treatment, wrong methods of storage conditions can cause food borne illness and higher risks of microbial contamination (Klunder *et al.,* 2012). Generally, hyalinization by sterilization, pasteurization, blanching (*i.e.,* immersion in hot water in a temperature range of 80–100°C), or roasting is recommended for consumer safety (Abu-Ghannam and Crowley, 2006). In a study conducted by Adamek *et al.* (2018), the processing of insects by boiling in water, drying at 103°C for 12 hr, and hermetic packaging proved to be promising for long-term storage of *T. molitor*, *Gryllus assimilis*, *L. migratoria* and *Alphitobius diaperinus*. As observed in the present study, fresh and roasted insects require further treatment for consumer safety. Drying or dehydration by sun drying, roasting, freeze-drying, smoke drying, and oven-drying of insects is done to increase the shelf life of insect products (Hernández-Álvarez *et al.*, 2021). Fresh insects should freezed at -20°C, while for dried and powdered insects, refrigeration is reported to be the best way to prevent oxidative and microbial degradation (Grabowski and Klein, 2017). Additionally, vacuum storage conditions for ready-to-eat *Ruspolia nitidula* helped to maintain microbial, sensory, and chemical properties for 22 weeks, similar to low-temperature storage (Ssepuuya *et al.,* 2017). A species-specific processing step with anti-microbial effect and precise characterization of insects' microbiota is required to avoid or minimize risks involved with edible insects' consumption and ensure that the product is harmless (Megido *et al.,* 2017; Vandeweyer *et al.,* 2017).

Several studies have suggested that microbes present in a specific particular matrix or ecological niche represent only a tiny fraction of the total microbial community. Food uncultivable

Table 3: Microbial counts (log cfu g^{-1}) from insect samples

Insect samples	Stage analyzed	Processing mode	Microbial counts (log cfu g^{-1})
Dragonfly	nymph	fresh	8.57 ± 0.03^{ab}
		roast	8.65 ± 0.01^{a}
C. limbatus	adult	fresh	6.78 ± 0.00^{cd}
		roast	6.30 ± 0.00^{c}
H. caschmirensis	adult	fresh	6.97 ± 0.06^{bcd}
		roast	6.30 ± 0.00^{de}
O. smaragdina	larva	fresh	7.84 ± 0.03^{abc}
		roast	7.20 ± 0.05^{abcde}
P. barthelemyi	adult	fresh	8.40 ± 0.00^{abc}
		roast	7.46 ± 0.02^{ab}
	pupa	fresh	6.97 ± 0.06^{bc}
		roast	6.30 ± 0.00^{cde}
V. affinisisindosinensis	larva	fresh	8.75 ± 0.01^{a}
		roast	7.50 ± 0.03^{ab}
O. fuscidentalis	larva	fresh	6.30 ± 0.00^{a}
		roast	6.86 ± 0.07^{bcde}

#Data are mean of three replicates ± S.D. Mean values with different letters in the column differe significantly at P=0.05.

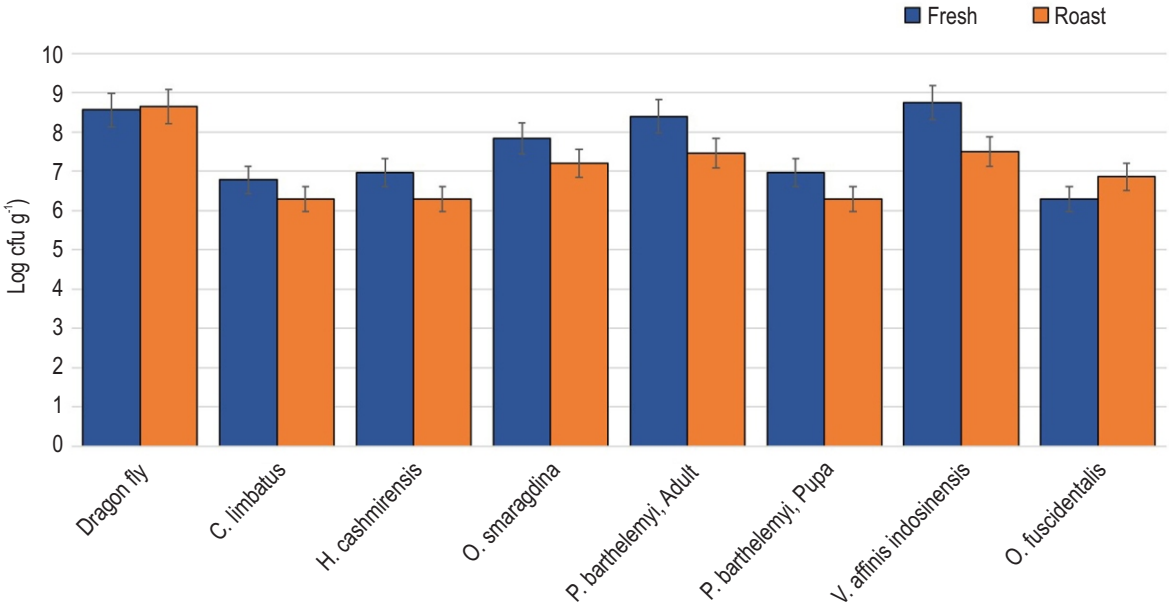

Fig. 1: Microbial count (log cfu g^{-1}) in fresh and roasted insect samples.

food pathogens need to be addressed. Therefore, the limited data on the microbial community structure of edible insects could be incomplete and requires next-generation sequencing technologies to study microbial communities in diverse environments (Stoops *et al.,* 2016). The potential presence of microbiological hazards for human health in insects is affected by a combination of the substrates used and the processing steps applied between farming and consumption (van der Fels-Klerx *et al.,* 2018). Wynants *et al.* (2019) investigated the transmission of *Salmonella* sp. to mealworms (*T. molitor*), from contaminated wheat bran as substrate and reported that the survival of *Salmonella* sp. in larvae and bran depended on the contamination level. At a starting contamination level of 2 log Cfu g^{-1}, *Salmonella* sp. was not detected in the larval samples. However, it was higher in bran initially contaminated with 7 log Cfu g^{-1}. Insects can remain free from pathogens contamination if the farms are properly

managed with no contact of wild insects that are pathogens carriers (Belluco *et al*., 2013). Therefore, possible contamination with microbiological hazards is likely influenced by the nature of the substrate, the hygienic conditions, and the farming environment (Van der Fels-Klerx *et al.,* 2018). As the study shows that more microbial colonies are found in fresh insects as compared to heat-treated samples, for some insect species, roasting is sufficient to inactivate/eliminate contamination. However, most edible insects need more processing to deem them safe for consumption. Even after freezing or heat treatment, contamination occurs due to improper handling of the insect samples, which leads to re-contamination. The preferable edible insects are frequently sold in a dried form packed in plastic film, with or without oxygen and/or moisture absorber (Fasolato *et al*., 2018). Further research needs to be undertaken to identify specific microbes/food pathogens to develop microbial quality and parameters to ensure consumer safety as well as safe production of edible insects, devising better insect species-specific processing methods, proper storage of processed insects for consumer safety of the given product, as well as transport of insects and insect-based products to successfully facilitate the introduction of such products into the global market. Human food safety has to be considered before opening the insect's product to the global market. Therefore, the present study highlights the efficiency of roasting edible insects in reducing or eliminating microbial contamination that could help preserve the product for a longer period making it safer for consumers

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