INSECTS AS FOOD AND FEED

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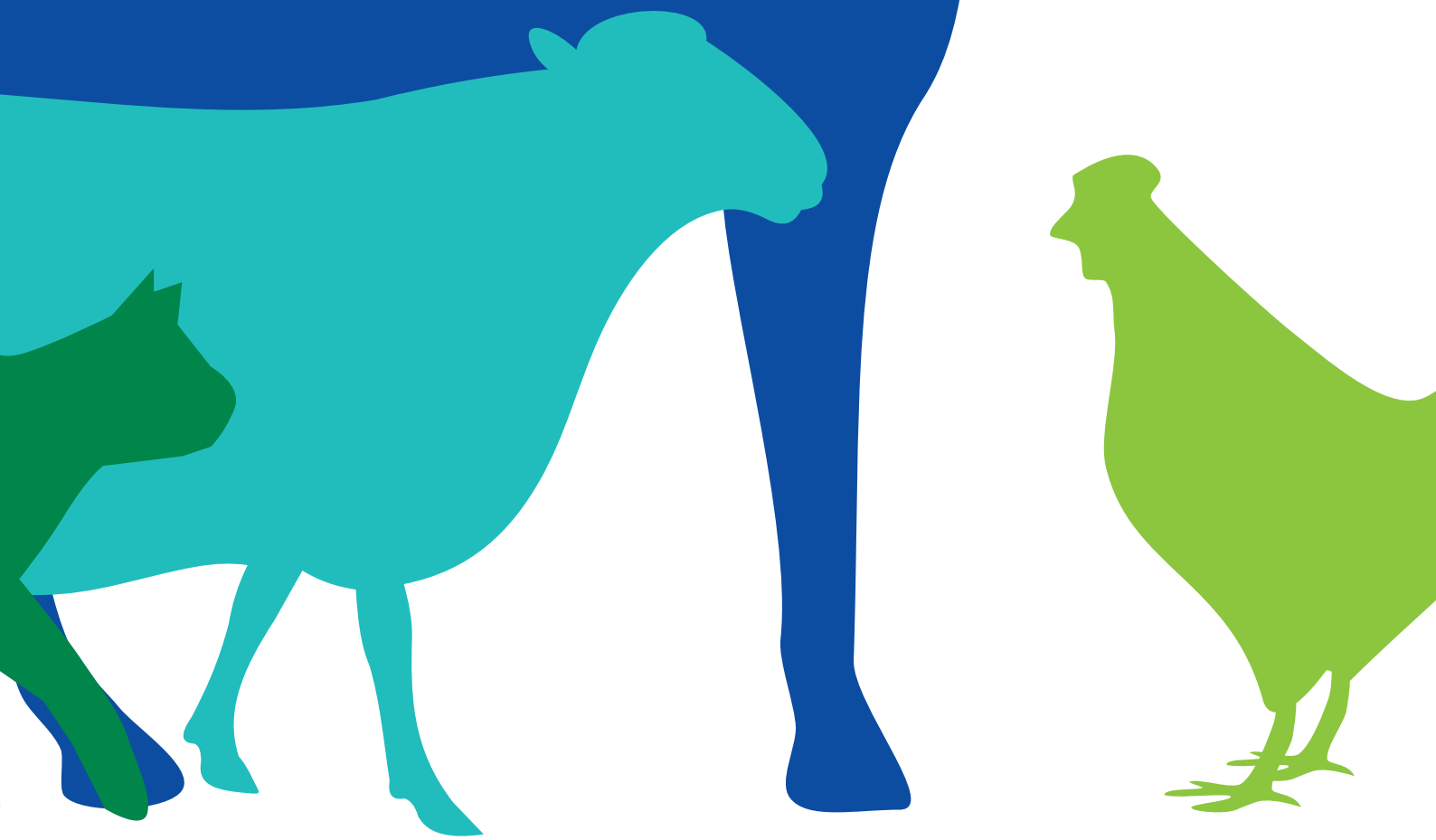

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INSECTS AS FOOD AND FEED

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Table of Contents

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EDITORIAL
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Editorial: Insects as Food and Feed

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Editorial on the Research Topic

Insects as Food and Feed

Insects as a food item for primates generally and humans in particular, have a long history. Just like many species of monkeys even today (1–3), our ancestors since time immemorial have consumed insects, be it accidentally with fruits or be it deliberately after having collected and prepared them for consumption in various way. The widespread use of insects as a food item in different parts of the world and by various cultural and ethnic identities had been reported several times in the past (4–6) and even the Greek and Romans of antiquity appreciated certain species of insects (7). On the other hand, the controlled administration of specific insect species to domestic or pet animals, is something with a more recent origin. However, it was not until Meyer-Rochow (8) suggested that edible insects could help to ease global food shortages and urged the WHO and FAO to support the use of insects as a human food item and as feed for poultry and pigs that the idea caught on (9).

During the last two decades we have witnessed an "explosion" of publications, too many to list, on all aspects of insects as food. Conferences dedicated to identifying edible insects, farming certain species, pointing out their perceived environmental advantages over traditional livestock rearing or highlighting the insects' nutritional benefits were organized; chemical analyses of edible species were carried out; risks and possible dangers of their consumption were examined and improvements to their shelf life, their preparation, marketing, and sale were proposed. Effective means of publicizing edible insects in countries with no recent (or a "forgotten") history of any widespread uses of insects were devised.

Worldwide interest in insects as food and feed is, by necessity, still increasing, for we can expect the global population to exceed 10.9×10^9 people at the end of the century. The demands on food stuffs, let alone water, are daunting and calculations published by the FAO in 2014 show that an increase of the global food production by 70% was needed in order "to feed the world in 2050" (10). This is why insects with their higher reproductive rate, lower requirements for water, superb energetic use of the ingested food (which can be stuff inedible to humans), and their lesser need of space than what conventional livestock requires, need to be studied to meet future food demands and to supplement future food reserves, thereby contributing to reach the United Nations Sustainable Development Goals (SDGs) (11). Since most insects are nutritious, consist of valuable protein and contain easily digestible fatty acids as well as important minerals and vitamins (12–14), it is essential that we discover the best possible ways to breed the most nutritious species under optimal and cost-effective conditions (15, 16).

Toward this end we have brought together the results of nine studies by 62 authors from 11 different countries, demonstrating not only the breadth of the field but also its international

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relevance. Areas covered include risks of accidentally ingested honeybees (Rezende Marinho and Soto-Blanco), of virus loads in crickets reared for feed (de Miranda et al.), of *Hermetia illucens* as protein substitute in pig feed (Jin et al.), and dog food (Penazzi et al.) as well as studies on edible insect diversity in Mexico (Pino Moreno and Ramos-Elorduy Blasquez) and edible insect acceptance in Benin (Ghosh et al.); effects of cooking techniques on the mealworm's nutritional value (Mancini et al.), the development of functional ice-creams containing mealworm larvae (Hernández Toxqui et al.) and a novel molecular authentication for the lesser mealworm *Alphitobius diaperinus* (Marien et al.) round off the selection. We are aware that our Research Topic of papers is incomplete and does not do justice to the full importance of the "*Insects as Food and Feed*" field and that it represents but a relatively small facet of the global research activities. However, it does reflect to some extent where we currently are knowledge-wise, and it helps to show which areas still need more attention paid to in future research efforts.

It has become apparent that three additional aspects need to be addressed. Although insect and food scientists based largely in developed countries, have been making use of the traditional knowledge of people in developing countries, it is the latter that have to become more involved and integrated into the research of insects for food and feed if we want a fair share of the spoils. There is already the trend in some countries (in which insects used to be part of the traditional diet) to lose traditional foods and replace them by what is conceived by the locals as sophisticated "western diet" (17), while at the same time in developed countries non-traditional foods containing insects or insect products are rapidly making inroads.

Another aspect worth looking into is the environmental and ecological aspect of insects for food and feed, especially when uncontrolled harvesting from the wild is involved. The risks related to molecularly modified insects or species reared in countries these insects were not native to (and could possibly escape from and then interact with the local fauna and flora), are not to be dismissed, just like the chances of zoonotic diseases, be it from insect to human or animal, and microbes in ingested insects are (18). The long-term effects of insect consumption regarding general fitness and wellbeing may also have to be monitored.

The third aspect, which in our view, will become increasingly important, has to do with juridical aspects, such as legitimate patent declarations and observances as well as contractual and insurance issues. There have to be internationally accepted guidelines, regulations and safeguards that need to be adhered to.

There is, of course, in addition to these three issues, the problem of the overproduction of conventional foods, especially in developed countries, and the necessity to curb the widespread wastage of such foods. How to reconcile the novel foods and their promotion, exemplified by edible insects, with more typically accepted but over-produced and often wasted food items, would be a crucial but not exactly easy task, to see insects play a role as food and feed in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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Toxicological Risk Assessment of the Accidental Ingestion of a Honeybee (*Apis mellifera* L.) Present in Food

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The aim of the present work was to evaluate the possible risk of toxic effects due to the ingestion of a honeybee (*Apis mellifera* L.) accidentally present in food. The methodology used in this study was a bibliographic survey of studies on the toxic effects related to honeybees, with a critical analysis of the possible risks of accidental ingestion of these insects. The amount of venom present in a bee is considered insufficient to induce detectable toxic effects in a person who ingests it by accident, and various components of the venom are destroyed by gastric secretions. However, despite the rare frequency, there is a risk of the ingestion of a bee, causing an allergic reaction to some components of the venom in sensitized individuals. In addition, pollen carried by a bee may cause an allergic reaction in a sensitive individual. Thus, the accidental ingestion of a bee present in a food does not pose the risk of toxic effects for the majority of the population but may promote allergic reactions in susceptible individuals.

Keywords: accidental ingestion, allergens, honeybee, toxicological hazard, venom, zootoxins

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INTRODUCTION

Honeybees (*Apis mellifera* L.) are social insects bred for the production of honey, pollen, propolis, royal jelly, wax, and poison, and to promote the pollination of various cultivated plant species. These bees have a sting as their defense mechanism, through which their venom is inoculated (1, 2). As honeybees seek products with sugar as their food source, they may end up trapped and incorporated into food (**Figure 1**), and potentially consumed inadvertently. A number of anecdotal reports of accidental ingestion of insects are largely available (3–6), but the actual number remains unknown.

The intentional consumption of insects by humans, known as entomophagy, is a habit of many populations around the world. Entomophagy has been advocated as a way to increase the availability of foods of recognized nutritional value (7). In the case of honeybees, they are traditionally eaten roasted or grilled in countries such as Japan, China, and Indonesia (8). Thus, it is possible that the heat used in the preparation methods may denature any harmful substances present.

However, there is a lack of information in the literature on the toxicological risks arising from the ingestion of raw honeybees. Thus, the aim of the present work was to evaluate the possible risk of toxic effects due to the ingestion of a honeybee accidentally present in food. The methodology used in this study was a bibliographic survey of studies on the toxic effects related to honeybees, with a critical analysis of the possible risks of accidental ingestion of these insects. The possibility that the ingested insect may carry a microorganism with the potential to cause infection was not addressed in this study.

FIGURE 1 | Honeybee trapped in an orange-flavored drink.

THE HONEYBEE VENOM

The honeybee sting is a defensive mechanism against predator attacks on individuals or hives (9–11). The stinger is a modification of the reproductive apparatus and is present only in worker honeybees. The venom, also known as apitoxin, is produced by venom gland cells and is injected at the time of the sting. When the stinger is introduced, it becomes trapped with the venom sac at the sting site and gradually releases the venom (10, 12, 13).

Honeybee venom is composed of peptides, including melittin, apamin, and mast cell degranulating peptide (MCDP), the enzymes phospholipase A_2 and hyaluronidase, and biogenic amines (histamine, dopamine, and noradrenaline) (10, 12, 14, 15) (**Table 1**). Many other compounds were identified in the venom, but they are unlikely to possess toxicological importance.

The peptide melittin is the most abundant toxin in honeybee venom, comprising ∼40 to 60% of the dry weight of this venom, consisting of a basic 26-amino-acid polypeptide (12, 15, 16). Melittin monomers bind to lipid membranes producing pores, exerting a cytotoxic effect (17, 18). Furthermore, it acts synergistically with the enzyme phospholipase A_2, promoting damage to the cellular and mitochondrial membranes of various cell types. Arachidonic acid may be released because of cell damage (10, 15). This peptide is probably the major responsible for the bee venom-induced pain through direct and indirect activation of primary nociceptor cells (16). As melittin has various pharmacological activities, including antitumoral (15, 19), anti-viral (18, 18, 20), antibacterial (15, 17), antifungal (15), anti-arthritis, anti-inflammatory, anti-atherosclerotic, anti-diabetic, and neuro-protective (12) effects, several studies have been conducted to evaluate the safety of the compound when administered orally. The results of these studies indicate that oral ingestion of this peptide results in low toxicity (21–23).

TABLE 1 | Main components of honeybee (*Apis mellifera*) venom (10, 12, 14, 15).

Chemical groups	Compounds
Peptides	Melittin
	Apamin
	Mast cell degranulating peptide (MCDP)
	Other peptides
Enzymes	Phospholipase A_2
	Hyaluronidase
Biogenic amines	Histamine
	Serotonin
	Dopamine
	Noradrenaline

Apamin is a peptide neurotoxin comprising 2 to 3% of dry honeybee venom. This peptide is a specific inhibitor of small-conductance Ca^{2+}-activated K^+ (SK) channels in the central nervous system (13, 15) and activates the M_2 inhibitory muscarinic receptors of the peripheral nervous system (10, 14, 15). Another activity is blockage of the Kv1.3 channel, a potassium channel type found in immune cells (24). Pharmacologically, apamin has antibacterial, antifungal, anti-inflammatory, anti-atherosclerotic, and antitumoral effects (13) and has been tested for treating neurological disturbances, including Parkinson's disease and learning deficit disorder (15).

The main enzymes present in the venom are phospholipases and hyaluronidase Phospholipase A_2, comprises 10% to 12% of dry bee venom, promotes the disruption of the cytoplasmic membrane by the destruction of the constituent phospholipids, resulting in cell lysis (10, 14, 15, 25). This enzyme catalyzes the hydrolysis of glycerophospholipids, releasing fatty acids and lysophospholipids (25). P was also found to have antibacterial, trypanocidal, antitumoral, neuroprotective, and hepaprotective activities (15).

The other enzyme found in honeybee venom is hyaluronidase, which comprises 1 to 3% of venom. This enzyme promotes the fast tissue diffusion of venom through tissue disruption. Hyaluronidase causes the hydrolysis of hyaluronic acid in the extracellular matrix (10, 13–15). Other activities of hyaluronidase include mast cell degranulation and rise in blood vessel permeability (13).

Phospholipases and hyaluronidase are the allergenic proteins in the venom, being responsible for cases of anaphylactic reaction to honeybee venom (13, 15, 26). The enzyme phospholipase A_2, isolated from microorganisms and vertebrate animals, is used in the processing of certain foods, and it has been experimentally verified that the consumption of phospholipase A_2 residues does not represent a toxicological risk (27, 28). However, some cases of allergic reactions to honeybee phospholipase A_2 residues

present in honey consumed by sensitized individuals have been identified (29).

The peptide MCDP acts on mast cells, promoting degranulation releasing histamine, and consequent inflammation (13, 15). Paradoxically, large amounts of MCDP inhibits the release of histamine by mast cells (15). It also blocks fast activation and slowly inactivating K+ channels, resulting in neuronal hyperexcitability (30).

The biogenic amines present in honeybee venom are histamine, dopamine, and noradrenaline. Histamine, which amount present in the venom is smaller than that released by MCDP, promotes vasodilation enhancing the inflammation, whereas noradrenaline and dopamine have a well-known ionotropic effect (10, 13, 15). The ingestion of biogenic amines present in the honeybee venom sac probably does not represent a significant toxicological risk, because these amines are present in amounts that are high enough to impact human health (31, 32).

Honeybees can sting only once, leaving the stinger and venom sac in the sting site. A sting by one or a few honeybees promotes a reaction at the sting site that begins quickly and is characterized by pain, edema, and erythema. These local effects usually last for hours, but may, in some cases, continue for days. Multiple honeybee stings (a minimum of one hundred) are capable of promoting a systemic toxic reaction, characterized by agitation, vomiting, diarrhea, difficulty breathing, seizures, hyperthermia, and shock. Other clinical effects of the systemic toxic reaction are rhabdomyolysis and heart failure. In addition, sensitized individuals, who have been previously exposed to honeybee venom, may exhibit an anaphylactic reaction after only a single sting (33, 34).

A rare effect promoted is myocardial infarction, which usually occurs after multiple honeybee stings (35–38), but has also been observed as a result of only one sting (39). It is likely that myocardial infarction is caused by the spasm or thrombosis of the coronary arteries (39, 40) or is secondary to the hypersensitivity reaction (38).

It has been estimated that the amount of venom from honeybees that is lethal to 50% of humans by injection is 2.8 mg venom per kg of body weight. As a honeybee yields about 160 μg of venom (11), this amount is insufficient to cause detectable toxic effects in a person who has only ingested a single insect. In addition, as reported, various components of the venom are destroyed by gastric secretion. In addition, honeybee venom used for medical purposes is administered only by injection (41–43), rather than orally, most likely because it would lose its activity owing to degradation by the digestive system.

Remarkably, honeybee venom can cause allergic reactions in sensitized individuals (44–46). It has been found that even residual amounts of honeybee venom in honey can induce an allergic reaction, which is a very rare condition (29, 47). These allergic reactions are triggered mainly by the peptide melittin and the enzymes phospholipase A_2, A_1, and hyaluronidase. Allergic reactions to poison can be identified by the production of specific IgE and IgG4 antibodies in the serum of patients (48). Thus, although it is a rare condition, there is the risk that the ingestion of a honeybee may induce an allergic reaction to some venom components in sensitive individuals. In addition, the structural proteins of the honeybee itself may also induce an allergic reaction (47).

POLLEN

Honeybees are pollinating insects that collect pollen grains when visiting the flowers used for their food. The pollen grains collected by honeybees are agglutinated and transported to their colony in structures present in the hind legs named corbicles or pollen baskets. In addition, pollen grains also stick to the bee's body (49–51). In addition, to provide nutrients to bees, pollen serves as a source of enzymes that aid in the digestion of nutrients, such as beta-galactosidase, and helps to establish the beneficial digestive microbiota of these insects (52). For humans, pollen is a bee product known for its pharmacological activities, which include antimicrobial, anti-viral, anti-inflammatory, immunostimulatory, and antioxidant effects (53, 54).

The ingestion of pollen collected by honeybees may be responsible for the development of acute allergic reactions, including anaphylaxis, in sensitized individuals. Honeybee secretions probably do not significantly reduce the allergenic potential of the collected pollen (55). Although relatively rare, allergic reactions to pollen can be quite severe, even lethal (55–60). This reaction occurs after previous exposure to the compound that causes the reaction in the sensitized individual that usually does not occur at first exposure.

A large number of plant species may cause allergic reactions to pollen (**Table 2**). Thus, the pollen from several plant species that is collected by honeybees can cause allergic reactions in humans (60). Importantly, the same patient may have allergic reactions to pollen from more than one plant species simultaneously (58, 61, 62). In addition, patients who have allergic reactions to pollen may not be hypersensitive to honeybee venom components (61).

The allergic reactions to pollen occur after previous sensitization to their allergens. Pollen allergens are trapped and processed by dendritic cells that migrate to lymph nodes and induce the differentiation of naïve T helper cells into Th2 cells. The contact of epithelial barrier organs to pollen allergens can induce epithelial cells to release interleukin (IL) 25, IL-33 thymic stromal lymphopoietin. These factors re-activate Th2 cells that release IL-4, IL-5, and IL-9. IL-4 stimulates B cells to produce and release antigen-specific IgEs, whereas IL-5 activates eosinophils. Furthermore, IL-4 and IL-9 promote mast cell degranulation, releasing a number of compounds, including histamine, leukotrienes, cytokines, and chemotactic molecules, resulting in the clinical signs of allergic reaction (74).

A honeybee can carry more than 15 mg of pollen (75). In addition, pollen is also present within the digestive tract of a bee; a study evaluating two hives found that each honeybee contained, on average, 3.35 and 4.27 mg (76). It was found that one gram of pollen can contain between 400,000 and 6.4 million pollen grains (57). Patients exhibiting an allergic reaction after pollen consumption had a positive skin sensitivity test to 0.1 mg/mL

TABLE 2 | Components of honeybee (*Apis mellifera*) venom.

Common name	Scientific name	Plant family	References
Chrysanthemum	*Chrysanthemum* spp.	Asteraceae	(61)
Dandelion	*Taraxacum* spp.	Asteraceae	(58, 61)
Mugwort	*Artemisia vulgaris*	Asteraceae	(55, 58)
Ragweed	*Ambrosia* spp.	Asteraceae	(61, 62)
Birch	*Betula* spp.	Betulaceae	(63, 64)
Arizona cypress	*Cupressus arizonica*	Cupressaceae	(58, 65, 66)
Japanese cedar	*Cryptomeria japonica*	Cupressaceae	(67)
Oak	*Quercus* spp.	Fagaceae	(68, 69)
Ash	*Fraxinus* spp.	Oleaceae	(69, 70)
Olive	*Olea* spp.	Oleaceae	(58, 71)
Privet	*Ligustrum* spp.	Oleaceae	(71)
Bahiagrass	*Paspalum notatum*	Poaceae	(71, 72)
Bermuda grass	*Cynodon dactylon*	Poaceae	(72, 73)
Corn	*Zea mays*	Poaceae	(73)
Johnson grass	*Sorghum halepense*	Poaceae	(73)
Reed canary grass	*Phalaris arundinacea*	Poaceae	(71)
Tall fescue	*Festuca arundinacea*	Poaceae	(71)
Ryegrass	*Lolium perenne*	Poaceae	(58, 62)
London planetree	*Platanus* × *acerifolia*	Platanus	(58)
Willows	*Salix* spp.	Salicaceae	(69)
Elms	*Ulmus* spp.	Ulmaceae	(69)
Nettle	*Urtica* spp.	Urticaceae	(69)

pollen extracts (53, 54). Thus, pollen carried by a bee may cause an allergic reaction in a sensitized individual.

Pollen may contain toxic substances produced by plants (77–81), notably, pyrrolizidine alkaloids (82–86). These alkaloids have potent hepatotoxic effects and cytotoxic, genotoxic, and oncogenic activities; some compounds also have neurotoxic and nephrotoxic effects (87, 88). A study in Germany revealed that a total of 17 out of 55 pollen samples collected by honeybees and marketed in Europe contained detectable levels of pyrrolizidine alkaloids, with concentrations ranging from 1.08 to 16.35 μg/g (85). On the assumption of the volume of pollen carried by a honeybee as 15 mg (76), the amount of pyrrolizidine alkaloids ranging from 16.2 to 245.25 ng. As the ingestion of pyrrolizidine alkaloids up to 0.1 μg/kg per day is considered safe for humans (84, 86), the probable amounts found in a honeybee should not pose any toxicological risk.

Pollen may also contain pesticide residues used in agriculture (89–92). The highest residual pesticide concentration found in pollen collected by honeybees in a study conducted in the United States was 16.556 μg/g of the pesticide phosmet (89). Again, assuming the volume of pollen carried by a bee as 15 mg (75), this would be a maximum phosmet concentration of 248.34 ng, which would not pose a toxicological risk to humans as the acceptable daily intake for this compound has been set at 5 μg/kg body weight (93).

CONCLUSIONS

The amount of venom present in a honeybee is considered insufficient to cause detectable toxic effects on a person who has accidentally ingested it; moreover, components of the honeybee venom are destroyed by gastric secretion. In contrast, despite the rarity, there is a risk of honeybee ingestion, causing an allergic reaction to some component of the venom in sensitized individuals. In addition, pollen carried by a honeybee may cause an allergic reaction in a sensitized individual. Thus, the accidental ingestion of a honeybee present in food does not carry a risk for the production of toxic effects for the majority of the population but may promote allergic reactions in susceptible individuals.

AUTHOR CONTRIBUTIONS

BS-B conceived the paper. JM and BS-B wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Observations on How People in Two Locations of the Plateau Département of Southeast Benin Perceive Entomophagy: A Study From West Africa

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We surveyed the local populations of Kétou and Pobè in Southeast Benin through interviews and with the aid of a semi-structured questionnaire in order to understand how they currently perceive entomophagy, an age-old tradition in their communities. The study revealed that the majority of the population was familiar with the use of insects as food, and a sizable number of people were still interested in insect consumption. Gender differences were not apparent. Tradition or culture was identified as the most influential factor, followed by taste, as determinants for eating or rejecting insects. However, identifying the edible species and comparing practices how they were prepared for consumption, we found that the knowledge was not homogenous across the society of Benin, with differences depending on ethnicity, culture, respondent's age, and educational background. Awareness and promotion of food insects in the society should help to preserve the practice of entomophagy and in turn could lead to the provision of much needed nutritional supplements to the poorer and disadvantaged sections of the society.

Keywords: tradition, food system, insect as food, nutrition, indigenous traditional knowledge, sustainability

INTRODUCTION

The Republic of Benin, formerly known as Dahomey, is situated in West Africa between latitudes 6 and 13°N, and longitudes 0 and 4°E. The country is bordered by Togo to the west, Nigeria to the east, Burkina Faso to the northwest, and Niger to the northeast. In the surrounding countries and regions further to the south, a large number of insect species are considered as edible by the local inhabitants (1–6). However, compared with these countries, Benin stands out with a seemingly relatively sparse usage of insects as human food [see Table 1 in Kelemu et al. (4)].

Although there could be multiple reasons for this difference, a lack of field studies might well be involved, and insufficient knowledge of the causes why the people of Benin accept or reject edible insects species as human food could also have played a major role.

There is, therefore, a need to question and to interview people. To date, this has not been done, and in a country like Benin with few roads and sometimes difficult access to villages and country areas, it is not exactly easy to organize. Moreover, not everyone is literate, and written questionnaires cannot be used as widely as would be desirable. The need to examine attitudes toward edible insects in the country is there, and the results of an inquiry such as ours, even if somewhat limited, could certainly be useful in comparisons with similar studies involving different regions and their residents in the future.

Food insect acceptance and consumption in Benin are likely to reflect the ethnic composition of the country and different cultural attitudes toward insects in general and food insects in particular. Approximately 42 ethnic communities are known from Benin. The Fon, also known as Dahomey, are the largest ethnic group and constitute ∼39% of the total population of the country. Yoruba, i.e., 19% of the total population, reside mainly in the southeast and Dendi in the northern central part of the country, both having arrived during the twelfth to sixteenth centuries from regions that are now known as Nigeria and Mali, respectively. The Adja people of South Benin migrated from the River Mono to Benin during the twelfth and thirteenth centuries and at present constitute about 15% of the country's population. The northern part of the country is dominated by the Bariba people. The high levels and repeated waves of migration over different periods of time from a variety of regions inevitably led to interactions between different ethnicities. Adding to the wide spectrum of ecological and environmental conditions, it does not come as a surprise that cultural diversity is one of the characteristics of Benin and reflected in food habits and food taboos. To cite an example, 187 traditional leafy vegetables (TLVs) are used as food in Benin, but "the total number of TLVs used, highly varies across ethnic groups" (7).

Although suggested already in the 1970s by Meyer-Rochow (8) that insects could be an alternative sustainable food source to combat global food shortages, backed by the Food and Agriculture Organization of the United Nations (FAO) and WHO, the suggestion was not taken very seriously until a few decades ago. The insects' high nutritional value, their formidable food conversion efficiency, their need for less space and water, and their negligible greenhouse gas emission in comparison with conventional livestock were emphasized repeatedly (9, 10). By developing a food insect industry rather than collecting species in an uncontrolled manner from the wild, countries with a history of entomophagy could reap some benefits. Benin is one such country. Despite its apparently smaller use of edible insects than in the surrounding countries (4), there are several species of insects that have traditionally been accepted as food by the people of Benin (11–14). However, patterns of consumption and preparation processes vary among the different communities, and the concern remains how to preserve indigenous knowledge in the present era of globalization and the tendency of "westernization" (15).

The globalization of food brings two directions into conflict with each other: one is to keep the nutritional potential and ecological benefits of the insect source in mind (16), to proceed toward small-scale insect farming and to establish the legal framework necessary to reduce the pressure on the environment to generate more animal protein (17, 18). On the other hand, in some regions of the world, the availability of non-traditional, foreign-produced food items has become so dominant that consumers are ignoring traditional foods and see them as unfashionable and not sufficiently "modern" or "advanced" [(19), for edible insects: (20)]. This lack of appreciating and integrating tradition with new developments and food products often causes a one-sided dietary shift toward what is regarded as fashionable rather than time-honored (21, 22). Ultimately, the community is in danger of not only losing one important source of nutrition but also losing valuable indigenous knowledge associated with the practice of entomophagy. Benin is currently in the midst of a nutritional transition with an increasingly urban population (23).

The main food items for the people of southern Benin are known locally as *Akassa* (made of cornmeal), *Atassi* or *Watché* (made of bean and rice), and *Gari* (made up of grated cassava) (24). The cereal or cassava-based food is often limiting in amino acids. Inappropriate diets, promoting the onset of metabolic syndromes like cardiovascular disorders and diabetes (23, 25), often have a higher proportion of carbohydrates, less protein, and more saturated fats than foods considered healthy. According to the World Hunger Index, Benin is given a score of 22.4, which implies a serious level of hunger. Wasting, stunting, and micronutrient deficiencies persist among large segments, especially children and pregnant and lactating women, of the population[1]. Low-income regions in particular would benefit from the availability of a diverse diet that should include insects, as they are positively associated with micronutrient adequacy (26, 27).

This paper is a first and somewhat preliminary attempt to examine to what extent urban and rural people in one part of Benin appreciate the consumption of insects. Envisaging future studies of a similar nature, but then focusing on different regions and tribal people in Benin, it is hoped that this first investigation will be a stepping stone to explain why insects as a human food item seem less popular in Benin than in the surrounding countries and regions further south in Africa.

MATERIALS AND METHODS

Study Area and Study Subjects

We surveyed respective rural and urban populations of Kétou and Pobè in the southeastern part of Benin (**Figure 1**). Kétou is a small town of around 22,000 inhabitants in a rural area located in the Plateau Département de Benin. On the other hand, Pobè is a city, located in the same "département," but with a total population of 123,740 according to the 2013 census. The climatic conditions of both places with annual temperature and relative humidity averages of 24°C and 91–92%, respectively, are the same.

Survey and Data Collection

The survey's aim was to obtain some information on how inhabitants of the same "département," but living in different

[1]https://www.globalhungerindex.org/benin.html (accessed January 2, 2021).

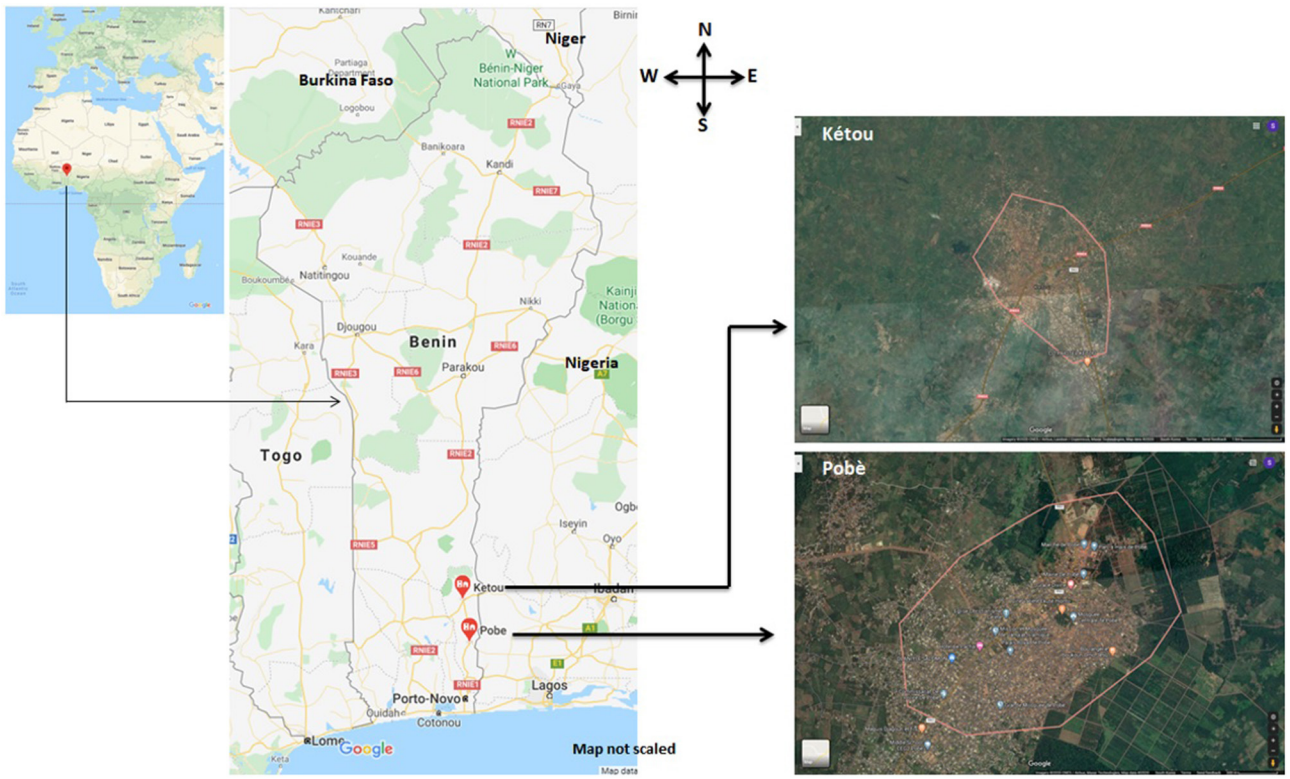

FIGURE 1 | Survey area. The maps are adopted from Google Map.

towns, currently perceive entomophagy, an age-old tradition in their communities. Data from altogether 100 respondents were available. The rural people of Kétou ($n = 50$) that we interacted with belonged to seven ethnic communities, namely, Holli, Nagot, Fon, Yoruba, Adja, Goun, and Mahi. Kétou residents were mostly literate and could be interviewed with the help of a semi-structured questionnaire (**Appendix A**) translated into the common locally used French language. The semi-structured questionnaire and the methods of the interview were adopted from the study by Ghosh et al. (28). The emphasis of the questions was on testing familiarity with insects as a human food item, transmission of ancestral knowledge, and exploring the attitude of the respondents toward the use of insects as a normal part of the human diet. The filled-in questionnaires were collected immediately after the respondents had finished answering the questions or, in some cases, were collected within 48 h. It was inevitable that during contacts with the respondents, some conversation took place, and questions as to why this inquest was carried out had to be answered. However, only the written responses were processed.

On the other hand, due to the respondents' time constraints, impatience, and low literacy level, the 50 inhabitants of Pobè that had consented to be interviewed had to be asked to state their preferences for certain edible insect species verbally. This part of the survey was conducted with the help of photographs and available edible species from the market. The respondents were requested to indicate the edible species according to preference. Their answers were written down and later analyzed together with information on the respondents' background. We recorded information on the ethnic community to which a respondent belonged and the respondent's age, gender, profession, and educational level, but we did not include items like income and religious belief, as these are rather contentious issues in the society. It would have been nice to investigate in more detail also the roles that traditions and emotions play when people are faced with the choice to eat or not to eat insects. However, given the problems of accessibility and gaining the trust of the locals, such a study would have implied a more time-consuming and difficult data collection. As a first study on the topic, we believe that the results, even if limited in scope, will nevertheless be very useful when the project resumes.

The aims of the survey were explained to all rural as well as urban participants, and their consent to use their responses in a future report was obtained, provided no names of the respondents were mentioned. The study complied with the ethical guidelines of the institutions involved but regrettably contained unequal numbers of male and female participants. Traditionally, the women of rural folk are less inclined to speak openly and let the talking be done by the men. Women in an urban setting feel less inhibited and are more willing to answer questions.

Identification of Edible Insects

Specimens of edible insects were collected and identified in the insect taxonomy laboratory of the Center de Recherche pour la Gestion de la Biodiversité (Cotonou, Benin), following standard key morphological characteristics.

Data Analysis

The data obtained from the survey were then analyzed by SPSS (SPSS Inc., Chicago, IL, USA). Because of the relatively small number of respondents, Fisher's exact-tests was seen as superior to the X^2-test (Chi-square test) and used to determine the independence of the responses associated with two nominal (categorical) variables like gender and knowledge system, gender and neophobicity, transmission of ancestral knowledge. It was felt that statistical analyses involving two tests could add support to the validity of our data, given that the number of the respondents was quite small. A Monte Carlo simulation (CI = 95% and sample size 10,000) was carried out wherever it was statistically possible. If the p-value was found to be ≤ 0.05 (CI = 95%), the null hypothesis was rejected.

RESULTS

Socio-Cultural Characteristics of the Respondents

The demographic characteristics of the respondents are presented in **Table 1**. The highest number of respondents from rural area was Holli (36%), followed by Fon, accounting for 22%. The Holli people (a subgroup of the Nagot) live in Pobè, Kétou, and principally the region of the Lama Forest in southern Benin. Buffering the Lama Forest is a comparatively well-developed agro-biodiversity zone, in which people gather wild edible plants. Their diet is primarily based on maize, as also cited in the literature (29). Other ingredients include palm oil, salt, onion, garlic, tomatoes, and dried pepper. TLVs and fish, although not to a great extent, are also part of the food spectrum. For the Holli people of the Lama Forest, the contributions of wild edible plants regarding copper and iron were 13.9 and 4.6%, which are much below the estimated average requirement for women (29). Fruit consumption is also low, and meat is very rare, while eggs, milk, and dairy products are even less common. The Nagot people are dominant in the area of Sakété, Pobè, Kétou, Porto-Novo, and Savè.

The respondents from the rural region of Kétou were aged between 16 and 51. Seventy percent ($n = 35$) of the respondents comprised individuals aged 21–30, followed by 16% of those younger than 21, and 12% of individuals 31–40 years of age. The informants who provided responses to questions on the perception of entomophagy unsurprisingly belonged predominantly to the younger generation. On the other hand, respondents capable of identifying edible insect species ranged from age 5 to 81.

Perception of People Regarding Entomophagy

Results on the perception of the rural inhabitants of Kétou are represented in **Table 2**, and some commonly consumed edible insects are depicted in **Figures 2a–e**. The knowledge of eating insects, i.e., familiarity with entomophagy, was found to be independent of gender (Fisher's exact-test $p = 1.000$). In fact, 90.5% of the male and all female respondents of the interview indicated that they were well-aware of customary practice of eating certain insects. Not all Benin respondents had a desire to eat insects, but 45% of the male and 50% of the female respondents expressed an interest in consuming insects, and a gender difference was not apparent (Fisher's exact-test $p = 1.000$).

In Benin, 50% of the male and only 25% of female respondents declared that it was from their elders that they knew about the tradition of eating insects, while others stated that they were informed about insect-eating from different sources including friends, by seeing in the market the Nigerian people selling edible insects, by observing people collecting them, and by watching reports on insect eating on television, etc. There was no apparent gender bias with regard to the vertical transmission of indigenous knowledge (Fisher's exact-test $p = 0.261$).

In order to understand the nature of traditional knowledge transfer, we categorized the respondents into five different age groups: up to and including those aged 20, those aged 21–30, 31–40, 41–50, and above 50. Although we did not find any significant

TABLE 1 | Demographic characteristics of the respondents from the Plateau Département of southeast Benin.

Variables	Rural		Urban	
	Frequency	Percentage	Frequency	Percentage
Age (years)				
<20	8	16	14	28
21–30	35	70	12	24
31–40	6	12	8	16
41–50	0	0	9	18
>50	1	2	7	14
Gender				
Males	42	84	24	48
Females	8	16	26	52
Ethnic community				
Holli	18	36	31	62
Nagot	4	8	17	34
Fon	11	22	0	0
Yoruba	6	12	0	0
Adja	2	4	0	0
Goun	7	14	0	0
Mahi	2	4	0	0
Bariba	0	0	1	2
Igbo	0	0	1	2
Main profession				
Farmer	2	4	17	34
Businessman	4	8	10	20
Craftsman	5	10	13	26
Student	35	70	10	20
Teacher	4	8	0	0

TABLE 2 | Perception of rural people of Kétou in Southeast Benin regarding entomophagy.

Variables	df	X^2		Fisher's exact-test (2-sided)
		Value	*p*	*p*
Gender × knowledge	1	0.828	0.363	1.000
Gender × interest	1	0.061	0.804	1.000
Gender × knowledge transmission from ancestor	1	1.691	0.193	0.261
Age group × knowledge transmission from ancestor*	3	1.384	0.709	0.900
Gender × feeling	1	0.050	0.823	1.000

**Monte Carlo simulation (CI = 95%, sample no. = 10,000) was conducted for analysis of age group effect on the knowledge transmission, but was not significant (p = 0.900).*

differences on the basis of age (Fisher's exact-test $p = 0.900$), we did find that respondents of the lower age group had received more information from their elders.

In our study, 78.6% of the male and 75% of the female respondents expressed positive feelings about eating insects, and no significant difference was found between the male and female respondents (Fisher's exact-test $p = 1.000$). Seventy-four percent of all respondents were willing to offer edible insects at social parties, while the remainder of 26% did not see this as a positive option.

In Benin, 80% of the respondents identified tradition as an influential factor in the acceptance of insects as a food item, while 70% identified the taste as the primary factor for eating insects, and a few respondents expressed nutrition as the primary factor of insect consumption. Except for tradition and an insect's taste, no respondent mentioned any other factor associated with choosing insects as food.

Preferred Edible Insect Species in the Studied Urban Area

For the urban region, we identified as the most preferred edible insect species the larvae of the beetle *Rhynchophorus phoenicis* (known as *Woiwo* by members of the Nagot tribe and *Hli* by the Fon tribe), followed by crickets (e.g., *Brachytrupes* sp., known as *Hyrè* by the Nago and *Abosaklé* by the Fon) and termites of the species *Macrotermes falciger*, known as *Iba* by the Nago and *Toutou* by the Fon tribe. Other less common edible insect species included the grasshoppers *Zonocerus variegatus*, *Hieroglyphus africanus*, *Kraussaria angulifera*, *Ornithacris turbida*, and *Nomadacris septemfasciata*.

Edible insects are collected mainly for subsistence in times when the harvest is poor. They are washed, fried, and grilled or sometimes directly put into some spicy sauce and consumed by family members (**Figures 2a–e**). For the Nigerien diaspora, a sizable market exists in South Benin where Nigerian food insect enthusiasts import crickets and locusts from Niger and process them to be sold as a food item mainly to members of the Niger community and Nigerian expatriates living in the country (**Figure 2c**). In Pobè, too, some people collect insects to sell them in neighboring Nigeria. Processing of the orthopteran species involves frying pepper and chili powder added. The insects are usually not boiled, and the most popular way to prepare any insect for consumption by humans is to fry, roast, or grill them. Occasionally, insects may be eaten raw or added to a spicy sauce. Generally, both children and adults (men and women) of the family are engaged in collecting the edible species. However, a cricket (or any insect farming) industry, despite the use of *Brachytrupes* sp. as an edible species, does not yet exist in any part of the country.

DISCUSSION

On the Perception of Entomophagy

Regrettably, the number of the people that could be interviewed was rather small, but given the total lack of information on insect consumption by the inhabitants of the southeastern region of Benin, this first survey of ours can still be considered useful, as it provides future studies to be conducted in this region with at least some data. One immediate result that is interesting in the context of familiarity with entomophagy is that by contrast to the present study, we found in an earlier investigation from East Africa that the majority of Ethiopian respondents (from Adama city) were completely unaware of the fact that insects could be regarded as human food (28). The contrasting results also held true in the context of gender and interest. Neophobia, which refers to phobia or fear of anything new, is an important parameter to assess a novel food (28, 30). Female Ethiopian as well as Korean respondents were more neophobic than men (28), but the reverse was found to be true in Benin. Although this is a far guess, as the Dahomey Kingdom had female military regiments, this could possibly have impacted the bravery of women in the country.

In most of the cases, indigenous knowledge plays a critical role in the utilization and conservation of natural resources. This dynamic body of knowledge is often transferred verbally generation after generation, and lack of scientific documentation often leads to the loss of the knowledge. Thus, we examined whether this knowledge of entomophagy remains among the different age groups and how this knowledge is transmitted. However, we did not have a sufficiently large enough database to allow us to draw a firm conclusion. In contrast, one study in Botswana revealed that older people were more knowledgeable and familiar with a wider range of edible species than the younger generation (31), which is in agreement with a survey on edible insects in Northeast India (32). The importance of traditional knowledge in the food culture in the context of entomophagy has been discussed in detail by Sogari et al. (33), who cited comparative accounts between western and eastern societies, while the factors influencing food choice mechanism, particularly in the context of edible insects, have been reviewed by Ghosh et al. (34). Very few respondents expressed nutrition as the factor guiding them in which species of insect to consume. In this context, the rich ethno-entomological wisdom of the

FIGURE 2 | A selection of common edible insects consumed by people of the Plateau Département of Southern Benin. **(a)** Edible orthopteran species collected by children and adults of the rural community. **(b)** Processing of edible locusts by roasting. **(c)** Packaging of dewinged species for selling at the market (small-scale household industry). **(d)** Termites collected by children of the rural community. **(e)** Termites sorted by children of the rural community (photo credit: Séverin Tchibozo).

Sub-Saharan Kalahari San people (35) and the sustainable use of insects as food in Sub-Saharan Africa (36) generally are remarkable, but more recent data on present-day attitudes toward entomophagy are not available.

Members of the surveyed populations in Benin were found to have positive feelings about entomophagy, a result that is at variance with findings of a previous study involving an Ethiopian population. A majority of the Ethiopian respondents expressed their unwillingness to accept insects as food and found insect consumption not to be "culturally superior" (28). Similarly, in a study conducted in Italy, the majority of the respondents stated that entomophagy would not be endorsed or supported by family and friends and that this negative opinion would represent a barrier to accepting insects as a novel food (37). Hedonic senses like taste, flavor, texture, and coloration generally play important roles in food selection, but one study with blindfolded and nose-clipped respondents showed that food insects could not be distinguished from non-insect food items by taste alone (38). In Benin, except for tradition and an insect's taste, no respondent mentioned any other factor associated with choosing insects as food. Taste, incidentally, was also found to be a dominant factor followed by nutrient content and environmental issues in the case of Korean and Ethiopian respondents (28).

Although this present study focused on the southeastern part of Benin, it has been noted that different species of insects are consumed by different ethnic communities in North and South Benin (11, 12). Overall, the consumption of insects in Northern Benin appears to be more common than in the southern part of the country, presumably because of different agro-climatic conditions and thus abundance and occurrence of different insects (14). However, diverse ethnic communities, traditional knowledge, presence of natural landscape, and less urbanization in the northern part would also contribute to the difference. Termites of the genus *Macrotermes*, the gryllid *Brachytrupes* sp., and grasshoppers of the genus *Hieroglyphus* are among the most common genera of edible insects in both North and South Benin, but nowadays, none of these species is consumed much.

Possible Role of Insects in Improving Nutrition

We described in the previous introductory section that there is a gap existing between the desired nutritional level and the nutrition obtained from the local food. As innumerable scientific reports have demonstrated, the nutritional value of edible insects is no longer in doubt (39). The consumption of insects can enhance health, as most of the edible species contain high amounts of protein and minerals especially iron, calcium, and zinc, and less total fat but generally a higher proportion of unsaturated fatty acids, e.g., crickets (40, 41), honey bees (42–45), and termites (46, 47). However, as the finding of the present study has confirmed, acceptability of insects as food for humans does not solely depend on the nutritional potential of the resource but includes many other factors (34, 48–50). As with other nutritious but neglected traditional food items, edible insects are getting increasingly shunned in areas where they had been consumed for centuries but have received more and more attention by consumers in countries that are considered developed (31, 51). However, the consumer acceptance is rather a complex issue, and

framework is required to innovate or develop proper strategy and promote edible insects (52).

Further Prospects on Development of Edible Insects in Benin Context

Benin, with an increasing urban share of the population, is in the midst of a nutritional transition (25). Awareness and integration of traditional practices with modern trends may protect those cultural characteristics that are associated with the utilization of local resources including edible insects. However, we do not outrightly advocate the uncontrolled harvesting of edible insects from the wild. We suggest instead that insect farming systems receive the support they need in order to prosper. Herbivorous insects, feeding on plants in the wild, can accumulate several secondary metabolites with anti-nutritional properties that offer protection to plants but are usually not desired in edible species of insects (46, 53).

Farmed insects can be monitored with regard to the amount of anti-nutrients in their bodies (54). Having started barely 20 years ago, as of 2016, there have been 724 registered cricket farms in Korea (55, 56). Farming systems of a similar nature with the necessary and associated legal framework have been developed or are in the process of being set up in many countries of Asia, Europe, Africa, and North America (18, 57). Establishments such as these do not only ensure the systematic supply of products of high nutritional value, but they also generate employment opportunities, which enhance the local economy and serve the well-being of the society. However, at present, there are no insect farming or rearing facilities in Benin.

Some Questions Arising From Our Survey

Based on our survey, safety turned out to be one of the important issues of concern. Traditionally, edible insects are harvested from wild areas by the local people, and they are aware of aposematic coloration that warns them of harmful and toxic individuals. But some insects, seemingly not dangerous in any way, may feed on a variety of herbs that contain allelochemicals like polyphenol and flavonoids, which may have anti-nutrient properties and build up in the insects' body (46, 53). Moreover, pathogenic microorganisms in the insects' gut, fat body, brain, or endocrine organs could affect human health negatively. Among pathogenic bacteria, *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Aeromonas hydrophila*, *Bacillus cereus*, *Clostridium perfringens*, etc., may pose additional potential hazards to human health (58).

A reduction of moisture content would help to avoid microbial contamination and increase shelf life, but drying edible insects prior to their use is rarely practiced in Benin (11). Blanching, freeze-drying, oven drying, cold atmospheric pressure plasma treatment, etc., are methods employed for the reduction of microbial load of edible insects in modern food processing, but not in Benin (59). Suitable processing is also important for the extraction of desired compounds or groups of compounds like chitin, protein, and fat. Although such advanced processing techniques are not found in the surveyed area, techniques that the locals use like frying, grilling, and, on some occasions, boiling, are methods that will to some extent take care of pathogens and may reduce water-soluble anti-nutrient contents like trypsin inhibitor and oxalate etc. present in some insects, as these methods are found effective for some plant foods (60, 61). Scientifically monitored production systems and processing technology now available in many European and Asian countries still need to be developed for Benin (59). Proper maintenance of hygiene and sanitation of any insect farm to avoid contamination are of course as essential as conserving the wild species of interest and the knowledge associated with their roles in nature as a potential source of food and medicines.

CONCLUSION

Most people of Benin are familiar with entomophagy, but the knowledge regarding edible insect species is not homogeneous across the population of the "Plateau Département" of southeastern Benin; noted differences depend on ethnicity, culture, age, etc. Awareness and promotion of food insects in the society should help to preserve the practice of entomophagy and in turn could lead to the provision of much needed nutritional supplements to the poorer and disadvantaged sections of the society. Spin-offs like the establishments of insect-rearing facilities could possibly improve the livelihood of some farmers and enhance the economic growth of certain localities.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SG: design, data curation, analysis, and writing manuscript. ST and EL: field data collection and editing manuscript. VBM-R: design and editing manuscript. CJ: funding, design, analysis and editing manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.637385/full#supplementary-material

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Taxonomic Analysis of Some Edible Insects From the State of Michoacán, Mexico

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In the state of Michoacán, Mexico, 49 genera and 69 species of edible insects were registered, and they belong to the following orders: Orthoptera: (families) Pyrgomorphidae (2 species) and Acrididae (5); Hemiptera-Heteroptera: Coreidae (1), Corixidae (2), Pentatomidae (2), Membracidae (2), and Aethalionidae (3); Coleoptera: Cerambycidae (1), Cicendelidae (2), Curculionidae (2), Dytiscidae (1), Noteridae (1), Gyrinidae (1), Passalidae (1), Scarabaeidae (1), Tenebrionidae (2), Bostrichidae (1), Buprestidae (1), and Melolonthidae (1); Lepidoptera: Cossidae (1), Danaidae (1), Megathymidae (1), Pieridae (1), Bombycidae (1), Sessidae (1), Noctuidae (1), and Nymphalidae (1); Diptera: Stratiomydae (2); and Hymenoptera: Diprionidae (2), Apidae (10), Formicidae (4), and Vespidae (12). The order Coleoptera presents the highest number of families (12), but the order Hymenoptera has the highest number of genera (18) and species (28), 12 of which belong to the family Vespidae. Among the genera, 75.52% are monospecific, 14.28% are bispecific, 4.08% are trispecific, 4.08% are tetraspecific, and 2.04% are pentaspecific. Their distribution by locality is indicated, and these localities correspond to the municipalities of the state sampled so far; likewise, we report various forms in which they are prepared and the economic importance of, for example, the grasshoppers of the genus *Sphenarium* that are widely looked for, collected, accepted as part of the diet, consumed, and marketed.

Keywords: entomophagy, Mexico, Michoacán, taxonomy, gastronomy

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INTRODUCTION

Society currently faces a variety of significant crises. Preexisting quotidian crises (such as currency devaluation, rising product prices, overproduction of processed foodstuffs, drought, floods, etc.) are now accompanied by drastic climate fluctuations caused by global warming, including issues such as rising pollution, unchecked demographic growth, and low crop yield. As a result, to feed an ever-growing world population, insects are an important alimentary resource. Not only do many animals feed on them, but they also have a long culinary history providing food for people in different parts of the world: these animals have been valued by many cultures and for some are the sole protein source available (1). Being a regular component of the diet of many countries, almost 1,900 species are consumed by 3,000 cultural groups in 13 cities; in the order of importance due to the number of species that are widely consumed, lepidopters, hymenopterans, orthopters, bugs, cockroaches, dragonflies, and flies are consumed in the developmental stages known as eggs, larvae, pupae, and adults (2). As our attention mostly focuses on increasing the production of foodstuffs that must be abundant, innocuous, and nutritious, with a good rate of energy turnover, diverse

international agencies such as the FAO (2) have proposed that insects may solve this problem, and there is evidence that people of different social backgrounds look for and consume insects in countries like Argentina, Australia, Brazil, Colombia, Costa Rica, China, Ecuador, Spain, United States, France, India, Kenya, Japan, Mexico, Thailand, and Venezuela (2–7). Insects are the primary alimentary source of all terrestrial and freshwater ecosystems; for example, spiders, scorpions, fish, amphibia, reptiles, birds, and mammals survive in the trophic chains, thanks to insect consumption, and insects are also bred as feed for tarantulae, fish, reptiles, poultry, and diverse types of pets to which these arthropods provide balanced quantities of nitrogen, phosphorus, potassium, calcium, and also calories (1, 8). Insects constitute the largest biomass on the planet, and societies have used them to obtain stains, foodstuffs, medicines, honey, silk, lacquer, fertilizers, etc. throughout history (9–12). At present, it is widely known that insects harbor a high mineral nutritious value. They are relatively high in fat and rich in protein, B group vitamins, and energy (13–17); they also contain biodynamic compounds important for human health (18) and have a caloric yield rate, among other nutritional properties, much higher than that of vertebrates (19).

The state of Michoacán is situated on the volcanic axis in part of the Balsas and the Sierra Madre del Sur basin. Its limits extended to Jalisco and Guanajuato in the north, Querétaro in the northwest, Estado de México in the east, Guerrero in the south and southeast, the Pacific Ocean in the south and southwest, and Colima and Jalisco in the west; it covers an area of 59,928 km^2. The topography of the state include Sierra Tarasca in the north pertaining to the Eje Volcánico along with numerous volcanoes, such as Zacapú; the main mountain ranges such as the ones of Angangueo in the limits of the Estado de México; Ucareo, Mil Cumbres, and Otzumatlán; the Pico de Orizaba; mount Zirate; the Sierra de Patambán; mount Tancítaro; Sierra de Inguarán; the Paricutín and Jorullo volcanoes; and the northern part of the Río Tepalcatepec basin contained the flatlands of Antunez, Lombardia, and Nueva Italia. Sierras Tarasca and Coalcamán have a subhumid temperate climate, and the mountainsides toward the basins of Tepalcatepec and Balsas and toward the Pacífic are hot humid and the lower part of that basin is semi-dry with a summer rainfall regime. The following rivers are present in the state: Coahuayana, Coire, Coalcamán, Nexpa, Carrizal, and Zacatula; the last one has numerous tributaries in the state, such as the Grande, which also receives water from the Cupatitzio or del Marqués and forms the waterfall known as the Tzaráracua at the south of the city of Uruapán, the Tacámbaro, the Carácuaro, the Cutzamala, the Tuzantla, the Tuxpan, the Zitácuaro, and the Temascaltepec. The river Lerma marks boundaries between Michoacán and the states of Querétaro, Guanajuato, and Jalisco; the closed basins include lakes Cuitzeo, Pátzcuaro, and Zirahuén. The state also has numerous springs of thermal and mineral-medicinal waters. In the Sierra Madre del Sur, the soils are poor and undeveloped, such as the regosols and cambisols; in the Sierra Tarasca, the soils are fertile and derive from volcanic ash (andosols); and in the Balsas basin, the soils are clayey lateritic (acrisol and luvisol) alternating with cambisol. The state is divided into 113 municipalities and 7,716 localities. Of the total area of the state, 33.3% is farmland, 30.4% is rainfed, and 4.9% is irrigated, that is, Michoacán is an agricultural state that produces corn, sorghum, rice, beans, wheat, barley, safflower, sesame, sugar cane, cotton, alfalfa, potatoes, and tomatoes and fruits such as strawberries, melons, avocados, lemons, mangos, apples, watermelons, and bananas. Grasslands compromise 6.7% of the total state area. Bovine cattle are the most important type of livestock, significant of which were the porcine, along with goats, sheep, horses, mules, and donkeys being bred. The total forest area is of 4,320,800; 2,052,800 ha are covered in trees, and of these, 1,733,200 ha has temperate and semicold climate forests and 319,000 medium forests, low forests, and mezquital correspond to 867,600 ha, shrubland to 259,200 ha and the areas that have been cleared so as to be used otherwise represent 1,141,200 ha. Pines, oaks, and firs are exploited for their wood; resin is obtained from pines. Fishing activities in its lakes are also important for its economy; the main species are charal, carp, catfish, frog, white fish, huachinango, tilapia, and sardine (20).

Currently, many countries are seeking sustainable ways to use and preserve landscapes. It is very important to make an inventory of edible insects, whilst also respecting conservation and finding ways to ensure the rational management of biodiversity, which is critical for human survival. This is why, in this case, the objective of this study was to carry out exploratory research so as to know, collect, and identify the edible insect diversity in the state of Michoacán, Mexico, as well as discuss the economic and gastronomic importance of some of the species registered.

MATERIALS AND METHODS

Field Work

During 2017 and 2019, diverse visits to 48 localities were carried out so as to survey and collect the edible insects. With this in mind, we developed a questionnaire exclusively aimed at the inhabitants of rural areas in which we asked about the insects included in their diet, their common names and hosts, and their gastronomic importance. Edible insects were collected using tweezers and entomological nets (aerial, aquatic, and sweep nets); they were then placed in plastic jars filled with 70% ethanol for their preservation. In all the samples collected, we included the following data: locality name, collection date, collector's name, common name, and type of host (21). Several insects were bought in some *tianguis* (small markets, mainly the ones that are installed periodically on the street) and/or municipal markets.

Laboratory activities

After their collection, insects were brought to the Entomology Laboratory that is part of the Department of Zoology of the Biology Institute, where they were mounted, labeled (21), and identified by means of the taxonomical keys available for the different orders, for example, Orthoptera: (22–24); Hemiptera-Heteroptera: (25–34); Coleoptera: (35–37); Lepidoptera: (38, 39); Diptera: (40) and Hymenoptera: (41–44). Afterwards, they were ratified by comparing them with the insect collection and the aid of different experts from the Biology Institute of the National Autonomous University of Mexico so as to be finally cataloged

and introduced in the edible insect collection that is part of the National Insect Collection of the same institute. We also report edible insects recorded in the literature of other authors such as Argueta and Castilleja (45), Lagunas (46), Reyes et al. (47), and Reyes et al. (48).

In alphabetical order, the localities sampled were: Ahuiran, Alto Balsas, Angangueo, Aquila, Aranza, Ario de Rosales, Capacuaro, Angahuan, Chauzingo, Charapan, Cherán, Cocucho, Copándaro de Galeana, Cotija, Eronganicuaro, Jerecuaro, Jiquilpan, Juchitán, Jungapeo, La Piedad, Lago de Cuitzeo, Lago de Pátzcuaro, Las Cocinas, Mazamitla, Meseta Tarasca, Morelia, Nahuatzen, Neocupétaro, Pátzcuaro, Pomocuaran, Quinceo, San Francisco Corupo, San Lorenzo, San Pedro Tarimbaro, Sevina, Tacámbaro, Tecomán, Tingambato, Tlalpujahua, Tumbizca, Tupataro, Tuxpan, Uruapan, Zacán, Zamora, Zirahuen, Zitácuaro, and Ziracuaretiro. The collection sites include pine-oak forests, ravines, shrubland, hills, and springs.

RESULTS AND DISCUSSION

Taxonomic Analysis

The edible insects reported by the persons interviewed are presented in **Table 2**.

In this study, we report 6 orders, 31 families, 49 genera, and 69 species.

The order Coleoptera has the highest number of families (13) (Cerambycidae, Cicindelidae, Curculionidae, Dytiscidae, Noteridae, Gyrinidae, Passalidae, Scarabaeidae, Tenebrionidae, Bostrichidae, Buprestidae, and Melolonthidae) (**Graph 1**). In this order, it is mainly the larvae that are consumed in the developmental stage; only in the case of *Trichoderes pini* are both larvae and pupae are eaten, and, in the genus *Cysbister*, the larvae and adults are eaten. It is convenient to point out that the larvae are the most digestible in the developmental stage as they possess the smallest quantity of "raw fiber" and that the adults are consumed in very few cases.

The order Hymenoptera has the highest number of genera (19) and species (28) (**Graphs 2**,**3**), and the family Vespidae holds the largest number of species (13). In hymenopterans, all in the immature developmental stages (eggs, larvae, and pupae) are generally consumed; in bees, both stingless bees and the genus *Polybia*, the consumption of honey is well-known and reproducing adults are consumed in the case of the ants of the genus *Atta*. A taxonomic synthesis is presented in **Table 1**.

TABLE 1 | Taxonomic synthesis of the number of families, genera, and species in order.

	Families	Genera	Species
Orthoptera	1	4	7
Hemiptera-Heteroptera	5	8	10
Coleoptera	12	13	15
Lepidoptera	8	8	8
Diptera	1	1	2
Hymenoptera	4	18	28

We also record common or linguistic names, where the edible stage of development and the localities in which the consumption was recorded (**Table 2**).

Five species have an aquatic habit, that is, 7.24% (*Corisella mercenaria*, *Krizousacorixa femorata*, *Cybister* sp., *Suphisellus* sp., and *Gyrinus parcus*), and 92.76% are terrestrial.

As compared with other states in relation to the number of species recorded, Hidalgo has 99, Oaxaca 79, Guerrero 50, Estado de México 105, Chiapas 178, and Morelos 61 (49, 50). This means that the number of species is higher in Michoacán than in Guerrero and Morelos but lower than in Hidalgo, Estado de México, and Chiapas. This is most probably due to the diverse socio-economic activities carried out in the state, and among them are agriculture, animal husbandry, forestry, and fishing. Agriculture, for example, is the main activity, and it yields 84.9% of the Mexican production of avocado; in this case, this aspect can be seen as a means of diversifying foodstuffs. Insect collection and recording depends on factors such as abundance throughout the year, reproductive capacity, number of generations per year, and ecological traits of the sampled zones. The number of species so far recorded is significant and provides an aid for developing and rescuing and information that will help promote and preserve knowledge about this alimentary habit. The advantages of insect consumption by humans and/or animals are widely known, among them, for example, they form part of traditional habits; they are innocuous; they are rich in proteins, essential and non-essential amino acids, saturated and unsaturated fatty acids, minerals such as calcium, iron, and zinc, and vitamins A, D, and C (14, 51–54); they possess a satisfactory efficiency of food conversion (55) which is being able to feed on organic detritus (11); and they discharge a smaller quantity of greenhouse gases as compared to, for example, poultry production, and other animal husbandry.

Their culture requires a smaller area and a minimum proportion of feed, their collection and culture require a minimum of infrastructure and investment, and they offer employment opportunities for people in both rural and urban areas (2). Even today they are asked for, looked for, collected, produced in the sustainable alimentary systems, commercialized, and prepared by many chefs in diverse countries worldwide as snacks, appetizers, desserts, and in gourmet dishes, all of which is evidence of the acceptance they have and their importance in nutrition and economy, which makes them an alternative for the solution of hunger and malnutrition problems that are common in continents such as Africa (56–60).

Economic and Gastronomic Importance

The gastronomy of Michoacán comprises the foodstuffs, culinary techniques, and the traditional dishes of the state. The diverse ecosystems have enabled the development of a culinary tradition that is varied, abundant, and millenary. It is the heritage of the pre-Hispanic people that lived there, such as the Purépechas, Nahuas, Mazahuas, and Mixtecos. As a result, Michoacán has the reputation of being one of the states with the greatest gastronomic culture in which insects along with corn have been one of the basic foodstuffs of Mexican cuisine since pre-Hispanic times. At present, they are still included in the diet due to the

TABLE 2 | Taxonomic list of the edible insects of the state of Michoacán.

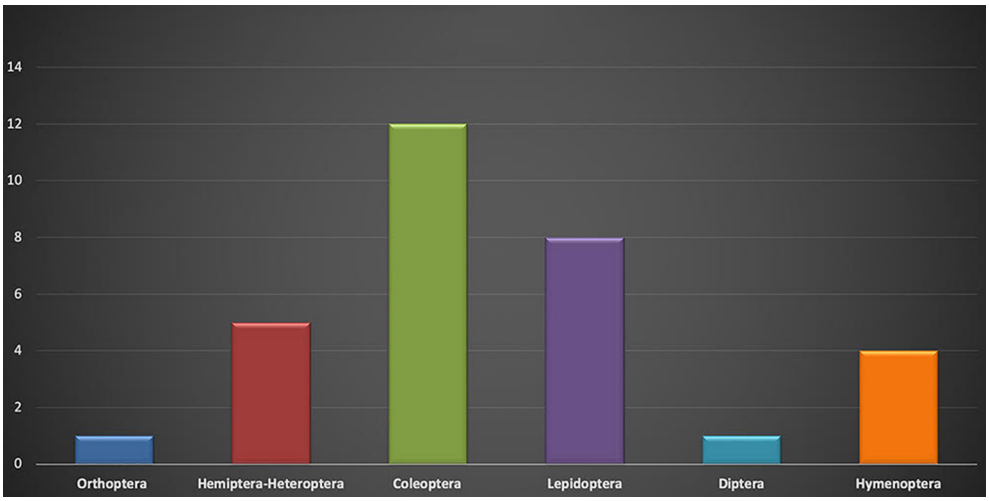
Order–family	Genus	Species	Common or linguistic name	Edible stage	Place of consumption
Orthoptera: Acrididae	*Melanoplus*	*differentialis* (Thomas, 1865)	Grasshopper	N, A	Jungapeo
	Spharagemon	*equale* (Say, 1825)	Grasshopper	N, A	Jungapeo
	Orphulella	*orizabae* (Mcneill, 1897)	Grasshopper	N, A	Jungapeo
	Orphulella	*tolteca* (Saussure, 1861)	Grasshopper	N, A	Jungapeo
	Orphulella	*quiroga* (Otte, 1979)	Grasshopper	N, A	Uruapan
	Sphenarium	sp.	Grasshopper	N, A	Las Cocinas, Charapan, Nahuatzen
	Sphenarium	*purpurascens* (Charpentier, 1842)	Grasshopper	N, A	Charapan
Hemiptera-Heteroptera: Coreidae	*Thasus*	*gigas* (Klug, 1835)	"Xamues," "Cocopaches"	N, A	Copándaro de Galeana
Corixidae	*Corisella*	*mercenaria* (Say, 1832)	"Mosco," "Axayacatl," "Ahuahutle"	E, N, A	Lago de Cuitzeo
	Krizousacorixa	*femorata* (Guerin-Meneville, 1844)	"Mosco," "Axayacatl," "Ahuahutle"	E, N, A	Lago de Cuitzeo
Pentatomidae	*Brochymena (Arcana)*	*tenebrosa* (Walker, 1867)	"Jumil"	N, A	Cotija
	Chlorocoris	sp.	"Jumil"	N, A	Copándaro de Galeana
Membracidae	*Hoplophorion (Metcalfiella)*	*monograma* (Germar, 1835)	"Periquito del aguacate"	N, A	Juchitán, Uruapan, Jiquilpán
	Stictocephala	*bisonia* (Koop Yonke, 1977[1])	–	N, A	–
Aethalionidae	*Aethalion*	*quadripunctatus*	–	N, A	Uruapan
	Aethalion	*nervosum punctatum* (Signoret, 1851)	–	N, A	Jiquilpán
	Aethalion	*quadratum* (Fowler, 1897)	Avocado greenfly	N, A	Tingambato
Coleoptera: Cerambycidae	*Arhopalus*	sp.	Pine worm	L, P	Charapan, San Francisco Corupo, Pomocuaran, Zacán, Aranza
Cicindelidae	*Cicindela (grupo dorsalis)*	*curvata*	–	L	Zitácuaro
	Cicendela (grupo rufiventris)	*roseiventris* (Chevrolat, 1834)	–	L	Tacambaro
Curculionidae	*Rhynchophorus*	*palmarum* (Linnaeus, 1758)	Coconut palm weevil	L	Tecoman
	Sitophilus	sp.	Corn weevil	L	Zacán, Ahuiran
Dytiscidae	*Cybister*	sp.	–	L, A	Pátzcuaro
Noteridae	*Suphisellus*	sp.	–	L, A	Zamora
Gyrinidae	*Gyrinus*	*parcus* (Say, 1834)	Whirlwind beetle	L	Tupataro
Passalidae	*Passalus (Passalus)*	*af. punctiger*	Rotten log worm	L	Mazamitla
Scarabaeidae	*Phyllophaga*	sp.	"Gallina ciega"	L	Quinceo, Pomocuaran Charapan, Tumbizca
Tenebrionidae	*Tenebrio*	sp.	Meal worm	L	Tuxpan
	Tenebrio	*molitor* (Linnaeus, 1758)	Yellow flour worm	L	Tuxpan
Bostrichidae	*Prostephanus*	*truncatus* (Horn, 1878)	Larger grain borer	L	Zacán, Ahuiran
Buprestidae	*Chalcophora*	sp.	Pine log worm	L	Charapan, Capacuaro, San Felipe de los Herreros
Melolonthidae	*Dynastes*	*hyllus* (Chervrolat, 1843)	Avocado trunk worms	L	Cotija, Uruapan
Lepidoptera: Cossidae	*Comadia*	*redtenbacheri* (Hammerschmidt, 1848)	Red maguey worm	L	San Pedro Tarimbaro
Danaidae	*Danaus*	*plexippus* (Linnaeus, 1758)	Monarch butterfly	L	Angangueo
Megathymidae	*Aegiale*	*hesperiaris* (Walker, 1856)	White maguey worm	L	Tlalpujahua, San Pedro Tarimbaro, Capacuaro, San Francisco Corupo, Ario de Rosales
Pieridae	*Eucheira*	*socialis* (Westwood, 1834)	Arbutus tree worm "cupiche"	L	San Pedro Tarimbaro, Aranza, Aquila, Charapan, Pomocuaran, Pátzcuaro, Zitácuaro

(Continued)

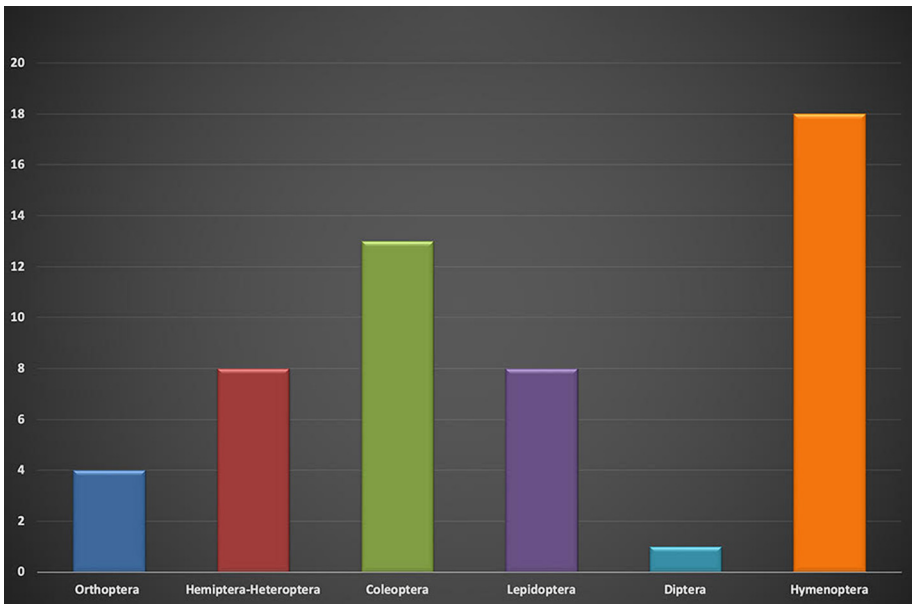
TABLE 2 | Continued

Bombycidae	*Bombyx*	*mori* (Linnaeus, 1758)	Silkworm	L	Ziracuaretiro
Sessidae	*Synanthedon*	*cardinalis* (Damf, 1930)	Resin moth	L	Charapan
Noctuidae	*Helicoverpa*	*zea* (Boddie, 1850)	Corn worm	L	Anganhuan, Zacán
Nymphalidae	*Charaxes*	*jasius* (Linnaeus, 1767)	"Cupiches," "Huenches," "Conduchas," or "Chamas"	P	Zitacuaro, Patzcuaro
Diptera: Stratiomydae	*Hermetia*	*aurata* (Bellardi, 1859)	Soldier fly	L	Morelia
	Hermetia	*ceria* (Williston, 1900)	Soldier fly	L	Morelia
Hymenoptera: Diprionidae	*Neodiprion*	*guilletei*	Saw fly	E, L, P	Charapan, Meseta Tarasca
	Zadiprion	*falsus* (Smith, 1988) = *vallicola*	Saw fly	E, L, P	Charapan, Meseta Tarasca Cocinas, Angahuan, San Lorenzo
Apidae	*Apis*	*mellifera adansonii* (Latreille, 1804)	Honey bee	E, L, P, H	La Piedad
	Lestrimelitta	*chamelensis* (Ayala, 1999[2])	Stingless bee	L, P, A. H[5]	Neocupétaro
	Melipona	*fasciata* (Latreille, 1811[2])	Stingless bee	E, L, P H[4]	Neocupétaro
	Scaptotrigona	*hellwegeri* (Friese, 1900[2])	Vermilion bee	L, P (H[4]) W	Neocupétaro
	Trigona	*nigra* (Cresson, 1878)	Stingless bee	E, L, P	Zacapu
	Geotrigone	*acapulconis* (Strand, 1919)	Ground hive	E, L, P H[5]	Neocupétaro
	Plebeia	*fulvopilosa* (Ayala, 1999[1,2])	"Abeja sapita"	NP	Alto Balsas
	Trigonisca	sp.[2]	"Abeja cepimilla"	H[1,5]	Neocupétaro
	Nannotrigona	*perilampoides* (Cressson, 1878[2])	"Abeja trompetera"	H[4]	Uruapan
	Frisiomelitta	*nigra* (Cresson, 1870[2])	"Abeja zopilota"	H[4]	Neocupétaro
Formicidae	*Liometopum*	*occidentale var. luctuosum* (Emery, 1895)	"Escamol"	E, L, P	Tlalpujahua, San Pedro Tarímbaro
	Liometopum	*apiculatum* (Mayr, 1870)	"Escamol"	E, L, P	Tlapujahua, San Pedro Tarímbaro
	Atta	*mexicana* (Smith, 1858)	"Chicatanas," "Nucú"	RA	Eronganicuaro, Tingambato
	Atta	*cephalotes* (Linnaeus, 1758)	"Chicatanas," "Zampopo"	RA	Eronganicuaro, Tingambato
Vespidae	*Brachygastra*	*lecheguana* (Latreille, 1824)	–	E, L, P	Cheran
	Polistes	*instabilis* (Saussure, 1853)	"Avispa zapatona"	E, L, P	Jungapeo
	Polistes	*major* (Palisot de Beauvois, 1818)	–	E, L, P	Jungapeo
	Polistes (Polisotius)	*major major* (Palisot de Beauvois, 1818)	–	E, L, P	Zamora
	Polistes	sp.		E, L, P	Cherán
	Polybia	*occidentalis nigratella* (Buysson, 1915)	Juniper wasp	E, L, P	Morelia, Jerecuaro, Jungapeo, Lago de Pátzcuaro, Cheran
	Polybia	*occidentalis bohemani* (Holmgren, 1868)	"Avispa rayada"	E, L, P	Chauzingo, Morelia, Jerécuaro, Charapan, Meseta Tarasca, Sevina, Cheran, Aranza. Cocucho
	Polybia	*parvulina* (Richards, 1970)	Black wasp	E, L, P	Lago de Pátzcuaro
	Polybia	sp. (a)	–	E, L, P	San Pedro Tarimbaro, Cherán, Ahuiran, Nahuatzen
	Polybia	sp. (b)[3]	"Uauapu," "moxquito pequeño," "abejitas negras y pequeñas"	L, C, H, Ho	Sierra Tarasca
	Vespula	*pensylvanica* (Saussure, 1857)	–	E, L, P	Lago de Pátcuaro
	Vespula	*squamosa* (Drury, 1773)	Honeycomb ground	E, L, P	Aranza, Zirahuen, Cheran, Zacan Pomocuarán

[1]Reyes et al. (48), [2]Reyes et al. (47), [3]Villamar and Castilleja (45). [4]Alimentary and [5]Medicinal. E, Eggs; L, Larvae; P, Pupae; A, Adults; RA, Reproductive adults; Ho, Honeycomb; H, Honey; W, Wax; NP, Nest products. Finally (a) and (b) are two species of the genus Polybia that are in the process of identification.



Graph 1 | Number of families per order.



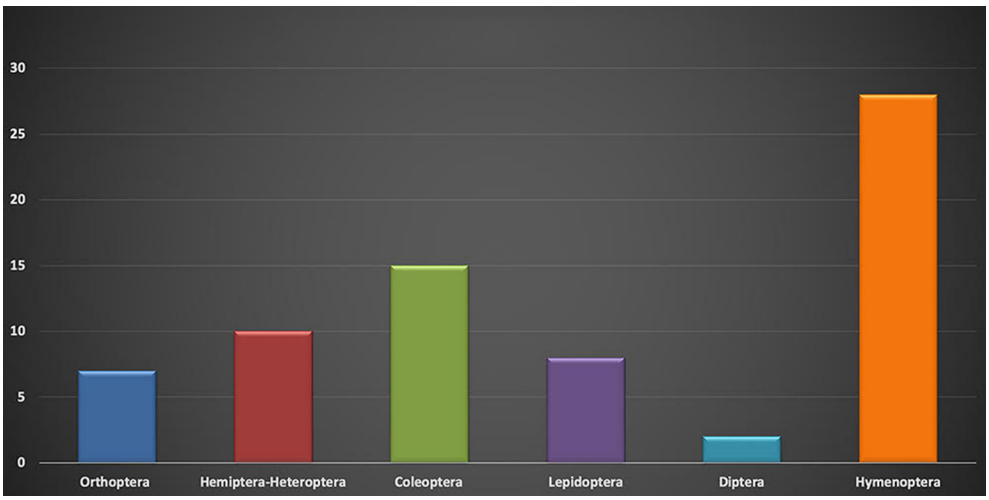
Graph 2 | Number of genera per order.

high protein content they have in diverse stages of development. This state culturally considers entomophagy as beneficial to both nutrition and the economy, with a significant commercial future.

In the state of Michoacán, as a strategy to make known the virtues of edible insects in all social strata, the students of the Biology Faculty of the Universidad Michoacana de San Nicolás de Hidalgo and of the Gastronomy School of the Universidad Vasco de Quiroga carried out a gastronomical exhibition in the city of Morelia, the state's capital, and prepared a great variety of insect-based dishes. This activity was supported both by the government and the productive sector, including the honorable city council of Morelia; the municipal departments of tourism, culture, and rural development; and the state's coordination committee for communication, ecotourism, e-fest extreme productions, and hyperlink publicity, as it is considered a promising commercial activity (46).

This exhibition hosted almost 2,500 visitors, a large-scale event that was widely publicized in Mexico and abroad by diverse television and radio networks and by some newspapers of the United States, Canada, and Latin America. It was visited by persons of different social classes, both male and female, and of different age groups that were able to taste and savor the dishes that were the attraction of the exhibition.

Detailed below are some forms of preparation used in Michoacán of some of the edible insects recorded. Order Orthoptera encompasses many phytophagous species that are



Graph 3 | Number of species per order.

pests, of which some are also predators; in this order, the nymphs and adults of grasshoppers are the Mexican edible insects most sought after, asked for, and commercialized in several states like Tlaxcala and Puebla, and they even have given rise to alimentary industries in Oaxaca. The grasshoppers (*Sphenarium*), before being prepared in diverse dishes, are dehydrated in a microwave oven to eliminate excess water and, if they are used in the preparation of a sweet dish, they are placed in a container with sugar and cinnamon, boiled, and then microwaved. With grasshoppers, they make stuffed sweet crepes with a chocolate covering, cheese pie with kiwi and strawberries, rice and milk, pizza, and *tacos* with tomato sauce. These grasshoppers are also consumed as an appetizer; they are toasted and seasoned with *piquing chili* or they are cooked so as to be eaten in *tacos* accompanied by *guacamole* or sauce. They are also prepared in a sauce made of *morita chili*, garlic, and *tomatillo* and are sold in the streets, fried and seasoned with lemon and chili. They are used in the design of delicious gourmet dishes.

For order Hemiptera, we see the following: The *Jumiles* (from the náhuatl, *xotlimilli*) are bug species pertaining to the family Pentatomidae that are considered to be a delicacy, where both nymphs and adults are consumed. The best known in the state is *Brochyymena tenebrosa*; these organisms can be eaten fried or ground to prepare a sauce and they may also be prepared in *enchiladas* (61). The *axayacatl* (a mix of eggs, nymphs and adults) is considered a delicacy since pre-Hispanic times and it is consumed toasted. The *cocopaches* (nymphs and adults) are eaten prepared in a sauce of chopped greens (tomatoes, onions, chili, and coriander) known as "pico de gallo" (rooster's beak).

For order Coleoptera, we see the following: This order includes the highest number of species in the Class Insecta, the adults of which receive diverse names, such as *mayates*, beetles, ladybirds, weevils, and *picudos*. They feed on both live and dead organic matter and, thus, they are important agricultural and forest pests; nevertheless, some of them are used in pest control. In Mexico, members of 22 families, 66 genera, and 119 species are consumed (11).

In the case of grub worms (*Phyllophaga*), they are cooked and filled with cheddar or mozzarella cheese, wrapped in a piece of bacon and accompanied by a cherry tomato, presented as sweet brochettes with kiwi, strawberry, and pineapple; as chocolate covered larvae; or as salted brochettes with broccoli, carrots, onions, and green pepper with a yellow dip with garlic; and as Chinese rolls with cream cheese and cucumber in a soy sauce. Depending on which flavor is selected when they are prepared, if it is salty they are fried in olive oil with salt and garlic, and if it is sweet with butter and sugar; and it is important to emphasize that the smell perceived during this preparation process is delightful, and it invites its savoring.

For the order Lepidoptera, we see the following: It encompasses the insects known as butterflies and moths, and among them, the edible ones that are most popular are the white and red maguey worms that are distributed in all states of Mexico where this plant grows. The *cupiches* (larvae), *huenches*, *conduchas*, or *chamas* (pupae) are toasted in a *comal* and are eaten in *tacos* accompanied by hot sauce. Red and white maguey worms (larvae) are eaten in *tacos*.

For order Hymenoptera, we see the following: This order is equally one of the most numerous species, which are known by the common names of wasps, bees, and ants, that are beneficial, and some have been domesticated, as is the case of the bee *Apis mellifera*, while others are involved in pollination and biological control.

Bee larvae (*Apis*) are prepared with chantilly cream cheese filled strawberries, the strawberries are cored and filled with the mix of the cheese, cream, and larvae; they can also be added to rum-flamed bananas or to the custard used for filling pies; larvae are added directly to the dishes, giving them a sweet and pine nut like flavor. The reproductive adults of the *chicatana* ants are consumed fried and roasted and in sauces; another preparation form is to toast them and then grind them in a *molcajete* with chili, garlic, and salt so as to be eaten in *tacos*.

The visitors and chefs who tasted these dishes found the diverse gastronomic presentations made with these insects

palatable. They supported the promotion of this type of research and outlined that they would potentially include insects in their diet. They outlined that the aesthetic presentation of the dishes was encouraging, and many proposed that they would be the food of the future, an idea also put forward by Ramos-Elorduy (62).

In relation to insect gastronomy, there exists in Morelia, Michoacán, a catering micro-company called "Bichus Delicious" (natural protein source), whose founder, the entrepreneur Janette Lagunas Rayas, has published that the market of insect consumption is growing slowly, but it is nevertheless growing (63). This company is focused mainly on the distribution of edible insects that are consumed with mescal made in Michoacán. "They are appetizer grasshoppers that are seasoned with salt, lemon, chili and a bit of garlic, also salt that has been seasoned with maguey worms," says the founder, who points out that they also sell salts flavored with grasshoppers and maguey worms (larvae), grasshopper chips, peanuts with grasshoppers, appetizers that accompany drinks like mescal and tequila, a mixed appetizer of peanuts with grasshoppers and *chinicuil* (larvae), grasshopper flour, grasshopper and *chinicuil* chocolates, and caramelized grasshoppers. She says that the acceptance of these foodstuffs has been growing in Morelia, the capital city of the state, especially in the health sector due to the nutritional virtues of insects that, as we have already mentioned, are widely documented in the scientific literature, both in Mexico and worldwide. This firm also organizes training courses in rural zones where they show how to collect, clean, and prepare them for their sale, so as to ensure that they are innocuous in the processed products. This activity has become the source of income of many persons who are now devoted to collecting grasshoppers for human consumption. This is proof that this activity enables them to obtain a significant income (64, 65), for example, a bag of dehydrated grasshoppers containing 20 g costs 35 pesos that are ∼US$1.74; therefore, for the sale of 1 kg, 87 dollars are obtained. They are currently working with some mescal producers, using as a complement the grasshoppers and the salts, and that these commercial activities are profitable. Grasshoppers have an important potential market through products such as "*salpulín*," a mix of salt and ground grasshoppers, a "grasshopper seasoning," which is a combination of salt, spices, and ground grasshoppers that can be added to meats, soups, and other preparations such as the "jumping cookies" made with flour (66). Some farmers of Tarímbaro, Michoacán, have even industrialized grasshoppers as a method of pest control of their crops and as an alternative commercial protein-based food (67). In this case, instead of buying insecticides for pest control, they have opted for training 60 farm hands in the new control methods with alimentary objectives, thus reducing the pollution that arises from applying insecticides.

Other insects that have economic importance in diverse ways include the bees known as Uauapu bees (eggs, larvae, and pupae). Honey is sold in local and regional markets. Honey is considered a very rich foodstuff both for its flavor as well as for its nutritional properties; it is consumed because people like it and it is considered a privileged foodstuff. The honeycomb is also sold commercially and eaten in sliced pieces, and the larvae are consumed as well (45). Honey is used in therapeutic procedures, for example, it is used as an antiseptic and healing agent. Pollination: The importance of the uauapu bee in the pollination and reproduction of both wild and cultivated plants has been widely recognized, for example, in the following localities: Cherán, Urapicho, Angahuan, Zipiajo, Comachué, Uricho, Cheran, Cocucho, Charapan, and Tanaco (45). In brief, the "uauapu" complex is important in the life of the Purépecha communities, especially with respect to their alimentary, medicinal, and agricultural culture (68). Despite the benefits and the potential commercial prospects of this honey, there are a number of challenges, as it is harder to find the bees and honeycombs near villages due to the following: forest fires, forest deterioration due to felling, competition with the European introduced bees, changes in soil vocation, expansion of the farming frontier and the urban footprint, and use of nicotinic insecticides and pesticides; all these factors are related with the bee collapse syndrome that accounts for the disappearance of 40% of the beehives in the state (64, 69).

CONCLUSIONS

In the state of Michoacán, Mexico, 69 species of edible insects in their different stages of development are consumed. The localities in which the greatest number of species are consumed are: Charapan (10 species) Jungapeo (7), Pátzcuaro (6), San Pedro Tarímbaro (6), Neocupétaro (6), and Cherán (6).

Anthropo-entomaphagy persists in Michoacán due to its rural population that, despite the influence of miscegenation and the introduction of other alimentary habits, has kept its traditional knowledge about the ecology, distribution, management, and consumption of edible insects as these arthropods have been part of culture since pre-Hispanic times. They have been a significant aspect of Mexican cuisine and diet for centuries. Today, many chefs have incorporated entomophagy into Mexican haute cuisine, developing dishes that, although exotic and expensive to certain social strata, are nevertheless increasingly more acceptable and consumed in different restaurants and markets.

However, it must be emphasized that even with the number of species registered in the state, anthropo–entomophagy is not very popular in the big cities and municipal heads; they used to be consumed in rural zones in which they are collected, prepared, and eaten; likewise, we observed that this activity is very much appreciated by the elders and ignored by the young. This is due to the large quantity of modern foodstuffs which the latter tend to consume as part of their diets.

Finally, we can say that this pre-Columbian legacy prevails, and we ascertain that edible insects are an essential part of our alimentary culture and an element of identity that generates nutritional, medicinal, and economic benefits to those that practice entomophagy in the Mexican Republic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

Both authors participated in the field research, writing, and final review of the article.

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Virus Diversity and Loads in Crickets Reared for Feed: Implications for Husbandry

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Insects generally have high reproductive rates leading to rapid population growth and high local densities; ideal conditions for disease epidemics. The parasites and diseases that naturally regulate wild insect populations can also impact when these insects are produced commercially, on farms. While insects produced for human or animal consumption are often reared under high density conditions, very little is known about the microbes associated with these insects, particularly those with pathogenic potential. In this study we used both target-free and targeted screening approaches to explore the virome of two cricket species commonly reared for feed and food, *Acheta domesticus* and *Gryllus bimaculatus*. The target-free screening of DNA and RNA from a single *A. domesticus* frass sample revealed that only 1% of the nucleic acid reads belonged to viruses, including known cricket, insect, bacterial and plant pathogens, as well as a diverse selection of novel viruses. The targeted screening revealed relatively high levels of *Acheta domesticus* densovirus, invertebrate iridovirus 6 and a novel iflavirus, as well as low levels of *Acheta domesticus* volvovirus, in insect and frass samples from several retailers. Our findings highlight the value of multiple screening approaches for a comprehensive and robust cricket disease monitoring and management strategy. This will become particularly relevant as-and-when cricket rearing facilities scale up and transform from producing insects for animal feed to producing insects for human consumption.

Keywords: metagenome, virome, *Acheta domesticus* densovirus, invertebrate iridovirus 6, *Acheta domesticus*, cricket rearing, frass, *Acheta domesticus* iflavirus

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INTRODUCTION

Most insect species have very high reproductive rates, leading to boom-bust population dynamics regulated by a combination of competition, predation and disease. Disease in particular can have dramatic effects on insect reproduction, growth and survival, at both the individual and population level (1). However, compared to other domestic livestock, very little is known about the prevalence and impact of diseases in commercially reared insects, or about the potential for disease management in high density insect cultivation (2). The notable exceptions are beneficial

insects with a long history of commercialization, such as honeybees (3) and silkworms (4, 5). The rapidly emerging insects-as-feed-and-food industry has major knowledge, awareness and research gaps concerning the diseases specific to this industry (6, 7). These are diseases that could significantly impact the production, processing and commercialization of both the insects and their derived products (8). There is consequently an urgent need to develop expertise on insect health and pathology, as the small-scale harvesting of wild insects transitions to commercial insect cultivation, and rearing insects for animal feed develops into rearing for human consumption (7). The feed-food insect rearing industry presently focuses on a few insect species, with Orthopteran insects (grasshoppers and crickets) constituting approximately half the volume of insects reared (9–11). The house cricket (*Acheta domesticus*) and the two-spotted cricket (*Gryllus bimaculatus*) are expected to be important species for the developing insects-as-food market (12). Diseases caused by viruses account for much of the economic impact of the diseases affecting mass-reared insects in historical industries such as apiculture and sericulture (3, 5). This is partly because viruses are particularly well suited to the natural boom-bust population dynamics and high local and temporary densities of many insect species. The vast majority of viruses in the world are asymptomatic (13). Only relatively few viruses are consistently pathogenic, invariably in situations with a high density or continuous supply of susceptible hosts. The risk with viruses lies primarily in their capacity to adapt rapidly to changing circumstances, particularly those governing transmission (13–15). These circumstances are very specific for each individual virus, but the process can be extremely powerful, capable of quickly transforming an insignificant, asymptomatic virus into a major pathogen (16). These are important considerations for the nascent insect feed-food industries, which need to produce large numbers of healthy individuals in the least amount of time and space, i.e., ideal criteria for virus transmission, disease and virulence evolution. This is a reason why viruses have been particularly singled out as a potential threat to the industry (17). Unfortunately, our knowledge of insect viruses is heavily skewed toward those insects that are damaging to human progress, either as vectors of viral diseases to humans or their domesticated plants and animals, or as biocontrol agents of insect pests (13). Very little is known about viruses of insects in general, although major efforts have been made recently to at least catalog the virus diversity in a wide range of insect and invertebrate species (18–26). Orthopteran insects in particular are underrepresented in virological research, despite the pressing need for screening, quarantine and disease management protocols for rearing Orthopteran insects to acceptable animal welfare and food-feed health and safety standards. This study takes a first step toward redressing these imbalances, by characterizing novel and known viruses in commercially reared *A. domesticus* and *G. bimaculatus*. For this we used two complementary approaches: a target-free exploration of cricket frass nucleic acid, for discovering new potential virus hazards, and a targeted screening of a limited set of insect and frass samples from several local Swedish cricket retailers and producers, for detecting and quantifying known viral pathogens of crickets. This targeted screening focused on nine different viruses, of which seven (*Acheta domesticus* densovirus - AdDV, invertebrate iridovirus 6 - IIV-6, *Gryllus bimaculatus* nudivirus - GbNV, *Acheta domesticus* volvovirus - AdVVV, *Acheta domesticus* mini ambidensovirus - AdMADV, *Acheta domesticus* virus - AdV and cricket paralysis dicistrovirus - CrPV) have been previously associated with the rearing of crickets (27–35). The remaining two viruses are slow bee paralysis virus [SBPV, (36)], which was present at high levels in the target-free nucleic acid exploration of the current study, and a novel Iflavirus recently characterized from wild *A. domesticus* (37).

METHODS

Sample Collection and Processing

Samples of commercially reared house crickets (*A. domesticus*) and two-spotted crickets (*G. bimaculatus*) were obtained from six different Swedish retailers (identified anonymously by the letters A–F; **Supplementary Table 1**). The samples were shipped according to the retailers' specifications. Both insects and frass were screened for viruses, since most cricket viruses are acquired orally and shed as particles into the gut lumen (8). Insect homogenates were prepared by pulverizing flash-frozen insects in BioReba meshbags (Bioreba, Reinach, Switzerland) with a pestle and resuspending in 2 mL sterile water per insect. Frass homogenates were prepared in 0.5 mL sterile water per 0.1 g frass using a MixerMill 400 beadmill (Retsch Haan, Germany) and ten 3 mm glass beads shaking at maximum speed for 60 s (38). DNA was extracted from 100 μL homogenate using the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany) following the "Tissues and Rodent tails" protocol and eluting the DNA in 200 μL AE buffer. RNA was extracted from a separate 100 μL aliquot of homogenate using the Qiagen RNeasy Plant Mini kit, following the "Plant" protocol and eluting the RNA in 50 μL RNase Free water. The DNA/RNA concentration was estimated using a NanoDrop 1,000 instrument (NanoDrop, USA), after which the samples were stored at −20°C until further use.

Target-Free Exploration - Sequencing and Bioinformatic Analyses

The target-free virus prospecting study was based on bioinformatic analysis of mass parallel sequencing data of nucleic acid (RNA and DNA) extracted from a single frass sample from commercially reared A. *domesticus*. We chose frass for the target-free screening, since most of the cricket viruses associated with commercial rearing are acquired orally and accumulate in the gut lumen until voided into the environment as part of the frass (8). This means that frass nucleic acid will have a higher proportion of viral genomes than nucleic acids from insect tissues, improving the chances of discovering new, low-abundance viruses. Moreover, the viral nucleic acids in frass will be mostly derived from virus particles shed into the gut lumen, while viral nucleic acids from insect tissues will mostly represent replication or transcription-translation intermediates, rather than infectious units. Around 1.5 μg total RNA was depleted of ribosomal RNA using the Illumina RiboZero rRNA

depletion kit and submitted for Ion Proton S5XL sequencing while around 1.0 μg of total DNA was submitted for PacBio sequencing, both conducted by LifeSciLab in Uppsala Sweden. The RNA reads were converted to FASTQ format using the SamToFastq tool (39). The DNA reads were delivered in FASTQ format as circular consensus sequencing (CCS) reads. Both the RNA and DNA reads were trimmed and checked for quality control using FastQC (40) and the Fastx-Toolkit (41). The reads were compared against a local copy of the NCBI nr database (downloaded on 3 June 2020) and assigned to a taxonomic group using DIAMOND BLASTx v0.9.31 (42). The quantitative and phylogenetic distributions of the reads were visualized using hierarchical pie charts produced with Krona Tools v2.7 (43). The taxonomic data were evaluated for potential viral pathogens and candidate reference genomes were identified and retrieved from GenBank in FASTA format. A more detailed description of the sequencing and bioinformatic analyses can be found in the **Supplementary Material**.

Targeted Screening - PCR-Based Virus Detection and Quantification

The presence and amount of the nine viruses of interest was determined by quantitative PCR in a limited selection of insect and frass samples from commercially reared *A. domesticus* and *G. bimaculatus* (**Supplementary Table 1**). The viruses with a DNA genome (AdDV, IIV-6, GbNV, AdVVV, AdMADV) were assayed directly from about 5–70 ng of frass/cricket DNA template. For the viruses with RNA genomes (CrPV, AdV, AdIV and SBPV), between 60 and 1,800 ng RNA was first converted to cDNA using the InVitrogen SuperScript-III 1st-strand cDNA kits (ThermoFisher Scientific, Waltham, MA, USA) and diluted 5-fold in ultrapure water. The cDNA equivalent of 1–36 ng original RNA was then used as template for qPCR. The forward and reverse primers for each assay were either obtained from the literature or designed *de novo* (**Supplementary Table 2**). All primers and assays are compatible with the thermocycling profile for the AdDV VP gene (38). All assays were run in duplicate using the SsoFast EvaGreen Supermix kits (BioRad, Hercules, CA, USA) with the mean Cq value used for quantitative analyses (44). Only data from the first 35 cycles were used for making detection assessments and quantification, due to the risk of false positive or false negative results beyond 35 cycles of amplification (45). The identity of all amplicons of the expected size (**Supplementary Table 2**) was confirmed by Sanger sequencing (Macrogen Europe, Amsterdam, The Netherlands).

Statistical Analyses

To compare virus titres across samples and viral burden between the *A. domesticus* suppliers (all except supplier "C") we created a series of simple Bayesian linear models (**Supplementary Appendix 1**) to: (1) estimate the viral burden for individuals from each supplier, for each of the five viruses where we detected virus from more than one supplier (AdDV, IIV-6, AdVVV, GbNV and AdIV), and (2) whether the titres of the different viruses were correlated across suppliers.

We used Bayesian models because: (1) the very limited sample sizes preclude any statistical approach other than Bayesian methods, which fit the data to a likelihood function and thus can produce probability estimates from any sized data set, (2) the estimated parameter ranges make it relatively easy to compare the 95% Confidence Intervals of the group-level effects (i.e., comparing estimated viral loads from suppliers) in addition to estimating the probability that the beta parameter >0, which represents correlation between viral titres, and (3) because our hierarchical models use a single distribution to estimate the range of intercepts, this allows parameter estimation for suppliers with little information to "borrow strength" from the entire dataset to maximize the information available and limit the effect of unusually large or small data points when little data exist. A detailed description of the models, equations and statistical packages used can be found in **Supplementary Appendix 1**.

RESULTS

Target-Free Exploration of the *Acheta domesticus* Frass Virome

The target-free exploration of *A. domesticus* frass DNA and RNA showed that this frass sample consisted mostly of bacterial and plant material, with viruses comprising a very small fraction (about 1%) of the RNA metagenome and even less of the DNA metagenome (**Supplementary Figure 1**). The only viruses identified through DNA sequencing were invertebrate iridovirus 6 (IIV-6) and bacteriophages from the Order Caudovirales. The RNA sequencing identified a much larger diversity of viruses, from insects, their microbiome and their (plant) food (**Figures 1**, **2**). Most prominent among these are RNA transcripts of the bacteriophages that were also found in the DNA sequencing, bacteria-infecting RNA viruses from the Order Levivirales, slow bee paralysis virus, an Iflavirus with bumblebees as its suspected primary host (46), a "Thika-like" virus similar to a trio of closely related Drosophila-infecting viruses (Thika virus, Kilifi virus and Machany virus) in a small unassigned clade in the Order Picornavirales (24, 25), and a "BSRV-like" dicistrovirus similar to Big Sioux River virus (47), Bundaberg bee virus 2 (19) and *Aphis gossypii* virus (48). The SBPV reads were >98% identical to the SBPV reference genome sequence (36). Not enough reads were available for the "Thika-like" and "BSRV-like" viruses for detailed genetic characterization or for designing reliable diagnostic assays. In addition to these three groups of relatively abundant reads corresponding to three individual viruses there was also a large group of reads matching a diverse assortment of viruses from a wide range of insects and invertebrates (18), with each virus accounting for just a handful of reads. The next largest category consisted of reads matching viruses that primarily infect plants, mostly belonging to turnip vein-clearing virus and bell pepper mottle virus, 2-well-characterized Tobamoviruses from the Family Virgaviridae (49). Another major group of reads matched a wide range of fungus-infecting viruses, mostly associated with *Cladosporium cladosporioides* (a common mold), *Botryosphaeria dothidea* (a wide-ranging canker-causing plant pathogen) and *Plasmopara viticola* (causative agent of grapevine downy mildew). Finally there were a few reads matching viruses associated with Antarctic

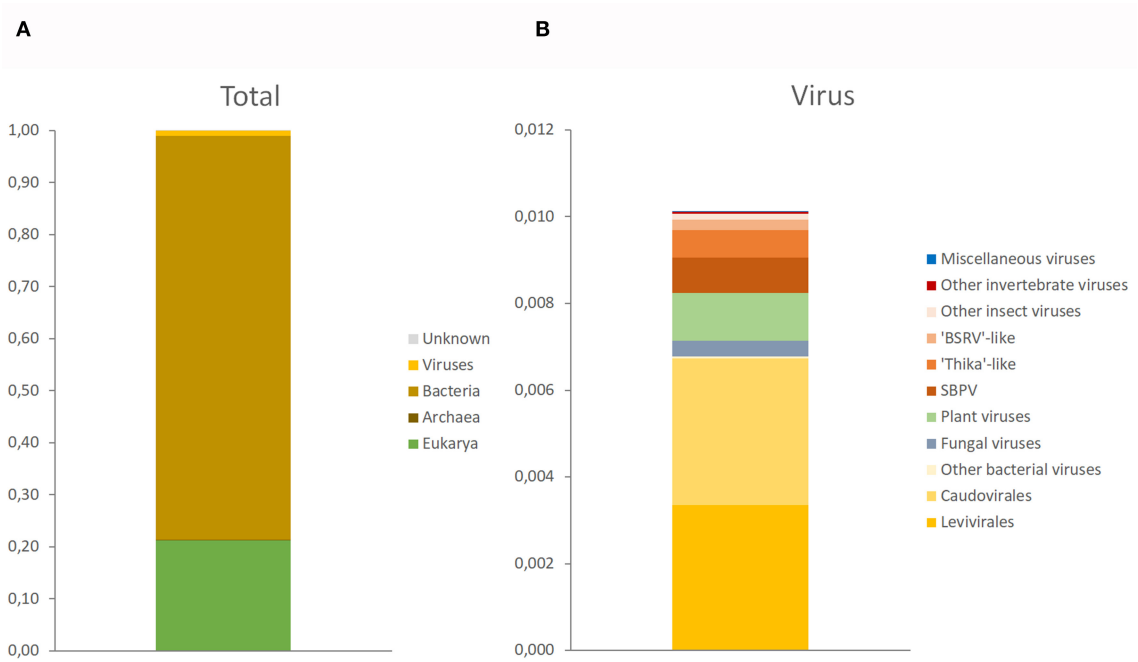

FIGURE 1 | Taxonomic composition of *Acheta domesticus* frass. **(A)** Full composition at the level of domain (Archaea, Bacteria, Eukarya, and Viruses). **(B)** Composition of the 1% virus fraction, separated by primary host: bacteria (yellow), fungi (gray), plants (green), invertebrates (orange-red), and miscellaneous viruses (blue) whose host status is unclear.

penguins and their ticks (26). These data were obtained from just a single frass sample, and can therefore only be used in a descriptive sense, as a case study.

Targeted Screening of Nine Viruses of Interest

Of the seven viruses previously associated with cricket cultivation, AdDV and IIV-6 were relatively common and abundant in this limited survey of Swedish cultivated crickets. AdVVV and GbNV were detected at relatively low levels in just a few samples, while neither AdMADV, CrPV nor AdV were detected in any of the samples (**Figure 3**). Slow bee paralysis virus, which was highly abundant in the target-free exploration screen, was not detected in any of the Swedish cultivated cricket samples by targeted screening, while the novel Iflavirus recently identified in wild *A. domesticus* (37) was both very common and abundant in these samples. None of the viruses were detected in the *G. bimaculatus* cricket or frass samples from supplier "C." Older crickets and their frass tended to have higher levels of AdDV and IIV-6, although there was much variation between individual crickets, while no such tendency was seen for AdIV. There was no significant difference between the five *A. domesticus* suppliers in virus load for AdDV, IIV-6, AdVVV and AdIV (**Supplementary Figure 2**). The suppliers appeared to differ in GbNV load (**Supplementary Figure 2**), although this result would need confirmation with larger sample sizes. There was some evidence of correlation between several of the viral titres across samples. AdDV appeared to be positively correlated with both IIV-6 (beta estimate = 0.99 ± 0.30; with 99.3% probability of the beta estimate >0 based on the Bayesian posterior distribution), AdIV (beta estimate = 0.83 ± 0.39; posterior probability of 97.4%) and AdVVV (beta estimate = 1.27 ± 1.0; posterior probability of 91.5%). There was no evidence of correlation with GbNV. Both the analysis of virus loads between suppliers and the correlation between virus titres across samples should be interpreted with caution because of the extreme paucity of data used to inform the models.

DISCUSSION

Our study revealed that viruses comprised only a very small fraction, around 1%, of the total frass nucleic acid content which was dominated by nucleic acids from bacteria and plants. This is not surprising for a frass sample, which can be expected to consist mostly of intestinal bacteria and the remnants of the food consumed by the crickets. This also applies for the viruses identified in the frass sample, which are linked to either the host, its intestinal bacteria, or the plants consumed. Although the target-free screening approach is comprehensive and ostensibly neutral, its output is contingent on the quality of the genetic databases. These are strongly biased toward organisms with economic significance for human progress, such as our domesticated life forms (e.g., crop plants, production animals (cattle, pigs, poultry, honeybees) and companion animals) and their diseases. Microbial diversity in particular is underrepresented in these databases, and skewed

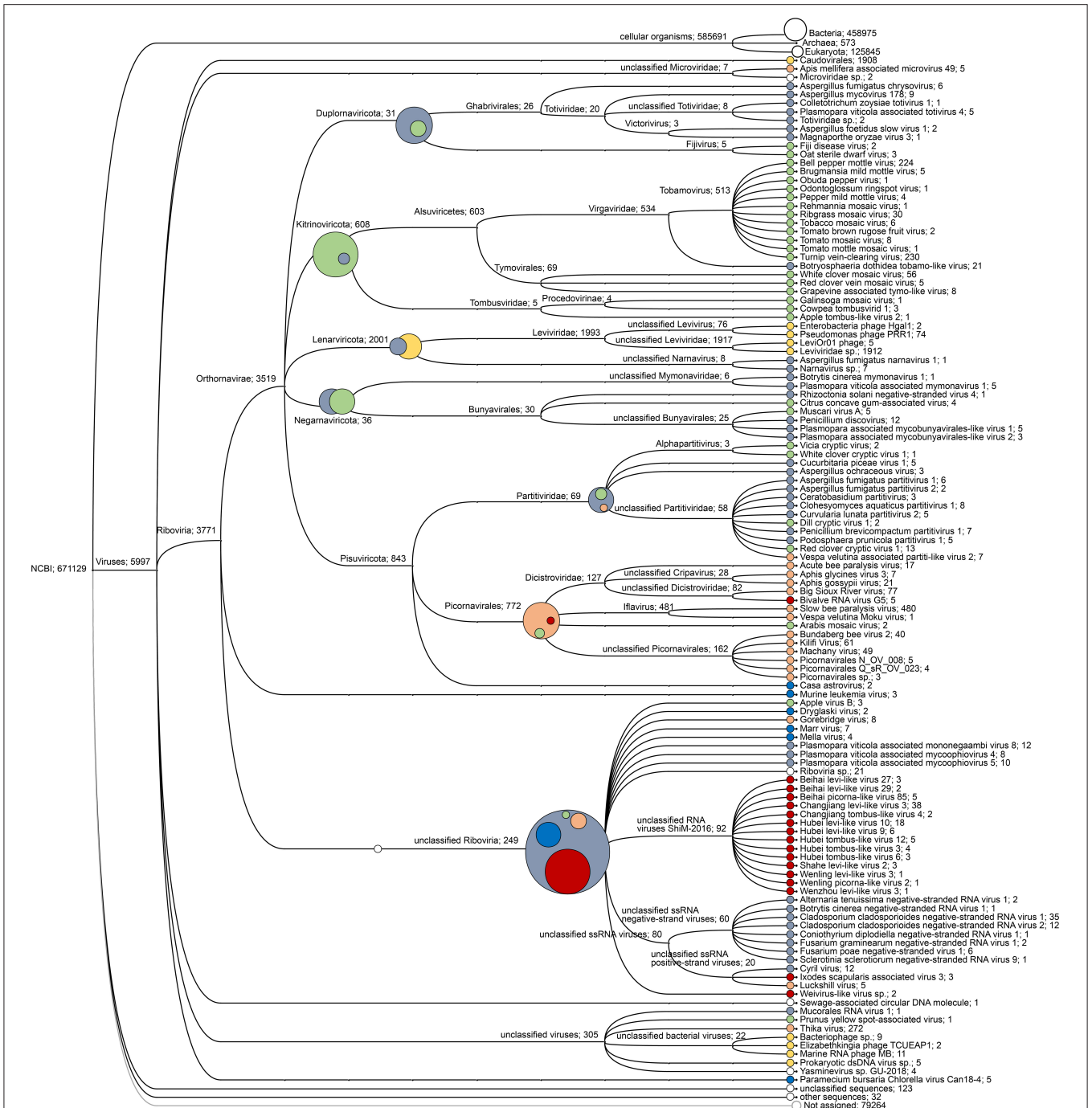

FIGURE 2 | Virus read cladogram and hosts. Cladogram of the viral reads obtained through from the RNA sequencing. The primary hosts of the viruses are indicated by the same colors as for **Figure 1**: bacteria (yellow), fungi (gray), plants (green), invertebrates (orange-red), and miscellaneous viruses (blue). The composite circles represent the host distribution at higher virus taxonomic categories.

toward pathogenic organisms. This affects both the proportion of reads that can be identified and the type of identification made. The target-free screening in our study identified both specific virus and viruses that could be identified at higher taxonomic levels but not at species level, i.e., novel viruses. The fully identified insect-infecting viruses included IIV-6 (in the DNA fraction) and SBPV (in the RNA fraction). The IIV-6 reads matched the lizard strain of IIV-6, which infects both Orthopteran and reptilian hosts (28), and thus represents a direct potential health hazard for reptilian pets. The SBPV reads were closely related, though not identical, to the SBPV type strain (36). SBPV belongs to the Iflaviridae: an exclusively insect-infecting

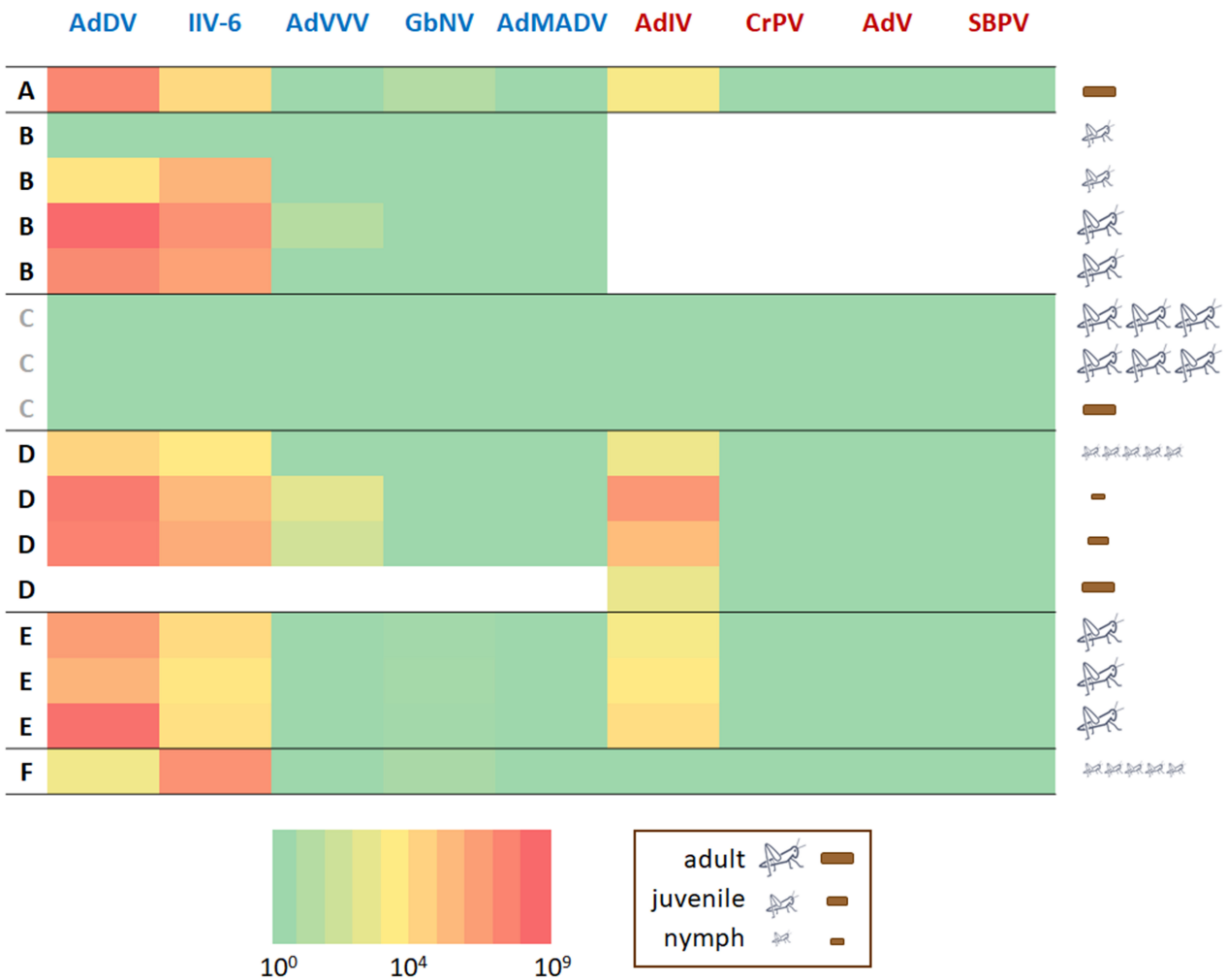

FIGURE 3 | Virus detection & quantification. Heat map of the levels of six viruses in cricket and frass samples from different retailers (A–F). Retailer C (gray font) supplied *Gryllus bimaculatus* crickets. All other retailers (black font) supplied *A. domesticus* crickets. The viruses with a DNA genome: *Acheta domesticus* densovirus (AdDV), Invertebrate iridovirus 6 (IIV-6), *Acheta domesticus* vovovirus (AdVVV), *Gryllus bimaculatus* nudivirus (GbNV), and *Acheta domesticus* mini ambidensovirus (AdMADV) are shown in blue font. The viruses with an RNA genome: *Acheta domesticus* iflavirus (AdIV), Cricket paralysis dicistrovirus (CrPV), *Acheta domesticus* virus (AdV), and slow bee paralysis virus (SBPV) are shown in red font. The legend next to the heat map indicates which sample type (crickets or frass) was analyzed, the developmental stage (adult, juvenile, nymph) the insect or frass sample came from and how many individuals were included in each insect sample (not applicable for frass samples). The heat map is on logarithmic scale and refers to the number of estimated copies of virus detected in the (RT)-qPCR reaction. White fields indicate missing data.

virus family with a single-stranded RNA genome (50), and is so far primarily found in bumblebees (36, 46). We also identified several viruses of the cricket intestinal microbiome, such as bacteriophages (DNA fraction) and Leviviruses (RNA fraction).

The novel viruses identified at higher taxonomic levels included several moderately abundant viruses from insect-specific virus families in the Picornavirales: a very diverse, widespread and successful order of viruses with hosts throughout the plant and animal kingdoms. These virus families include many pathogenic viruses, which makes these new viruses particularly interesting from a cricket health and disease management perspective. However, there were also some unexpected absences from the target-free exploration. The most obvious of these is the absence of AdDV from the frass DNA fraction, despite the high incidence and abundance of AdDV in similar *A. domesticus* samples revealed by the targeted screening. This discrepancy may have a technical origin. PacBio sequencing requires double-stranded DNA. Iridoviruses and bacteriophages have double-stranded DNA genomes and were therefore readily identified by the PacBio screening. However, densoviruses have single-stranded DNA genomes, with positive and negative ssDNA genome copies packaged in separate particles. Even though these ssDNA genomes can hybridize upon extraction and purification (51), the GC-rich terminal palindromic sequences may make these reconstructed dsDNA genomes inaccessible for PacBio sequencing. Other anomalies, and insights, are revealed

by comparing the viral composition of the DNA and RNA fractions. Viruses with RNA genomes [i.e., the vast majority; (18, 19)] will obviously only be recovered in the RNA fraction. However, all viruses with DNA genomes need to transcribe their genes into mRNA prior to translation. They could therefore in theory also be identified in the RNA fraction, and thus provide evidence of both their existence and their replication. This was clearly the case for the bacteriophages, whose sequences were in both the DNA and RNA fractions. Since bacteriophages infect the bacteria of the gut microbiome, this is entirely logical and expected. However, IIV-6 was only identified in the DNA fraction of the frass sample, not in the corresponding RNA fraction. The inference is therefore that only Iridovirus particles were present in the frass, and not any RNA traces of their replication in insect tissues, such as the gut epithelial cells. The same may well also apply to the other DNA viruses detected in similar samples by the targeted screening, such as AdDV, AdVVV, and GbNV.

Comparing the results from the target-free exploration with those of the targeted screening can also be informative. A good example is provided by the target-free and targeted screening results for SBPV and AdIV. In the case of AdIV, the results of the target-free exploration were confirmed by the targeted screening of similar additional samples, clearly establishing this virus as a consistent and abundant part of the *A. domesticus* virome (37). By contrast, SBPV was only identified by target-free sequencing of a single frass sample, and not by the targeted screening of the other insect and frass samples. There are multiple possible explanations for this discrepancy. It is of course entirely possible that the results are real, and that the discrepancy is simply a stochastic consequence of the small sample sizes of the target-free ($n = 1$) and targeted ($n = 12$) screening. Biologically it is certainly not out of the question that an Iflavirus like SBPV could, possibly, be infectious to crickets as well as to bees: this is a simple question of host range and Iflaviruses are very common in all insects that have been screened (18, 19, 50). However, it is also possible that this result is an artifact of the target-free screening workflow. The sheer number of SBPV reads recovered [480] and their even distribution across the SBPV genome argue against either technical or bioinformatic contamination during the sequencing and analysis workflows (52, 53). Although laboratory contamination during RNA extraction cannot be entirely ruled out, it is difficult to envision where and how this could entered the workflow in the amounts required to return the results from the RNA sequencing. Finally, the absence of reads matching other common bee viruses, or bees themselves, argue against passive acquisition of SBPV through feeding commercially reared crickets on contaminated material, e.g., dead bees, bee-collected pollen (54) or plant material contaminated with bee feces (55). In summary, the data presented here is too limited and uncertain to make active determination on the possible status of SBPV as a cricket-infecting virus, which must therefore remain "unproven" until more convincing evidence is obtained.

These insights and logical deductions highlight the value of complementary screening strategies and sample types for a robust holistic assessment of viruses and their potential risks for cricket rearing. The advantage of screening frass samples is that this allows an assessment of the health of the cricket microbiome, such as viral diseases of beneficial bacteria and fungi, as well as the health of the cricket itself. Since the main functions of the microbiome are in food metabolism and protection against diseases (56), the health of the microbiome is directly relevant to the health of the cricket, and thus also relevant to cricket husbandry and health management. Targeted screening is very precise and accurate but only detects what it is being asked to detect. It is therefore useful for monitoring known threats but not for explaining new diseases or identifying potential future threats. A health strategy based exclusively on targeted screening for known pathogenic agents therefore brings a risk of potential misdiagnosis, and consequently an inappropriate management strategy. These strengths and weaknesses are reversed for target-free screening, which can identify all viruses present in a particular sample but not distinguish between those that are benign and those that are pathogenic. It is therefore very good at identifying potential threats and new disease associations, but is less accurate for monitoring actual threats. It is also still subject to a number of workflow and bioinformatic errors, uncertainties and biases, as we also discovered in this study. However, a combination of the two screening approaches maximizes their individual strengths, minimizes their respective weaknesses, and reduces the risk of misinformation and mismanagement, to form a solid basis for a robust disease monitoring and management strategy.

Viruses are a natural part of life. The diversity of viruses detected in these particular cricket samples is, in and of itself, neither unusual nor alarming. The new viruses identified add to the growing list of novel viruses identified from insects and invertebrates, and likely represents only a fraction of the complete cricket virome. It is impossible to predict *a priori* which of these will develop into a biohazard for the cricket industry: that depends on the compatibility of the cricket rearing conditions with the transmission characteristics of each virus and can only be determined experimentally.

However, the frequent detection of high titres of several known pathogenic cricket viruses in just a small selection of commercially sourced orthopterans is alarming. It demonstrates that these pathogenic viruses are probably widespread in the local cricket rearing and retailing facilities. This is especially concerning in the light of the minimal regulation or sanitary control in the extensive trade and movement of Orthopertans (1, 27). These viruses are known to significantly impact both individual and population health, and represent a direct major biological and economic risk for the cricket rearing industry (8, 17, 27, 57). The retailers sampled in this study sold crickets as feed for pets, such as reptiles, amphibians and spiders. The cricket rearing criteria for this market are less stringent that those for rearing crickets for human consumption, whose operations may therefore be less affected. Both the silkworm and bumblebee rearing industries have significantly reduced their overall disease profile and risk for epidemic spread through high hygienic standards, containment measures, regular monitoring and tight control over external inputs into their operations. The current practices for rearing, movement and sale of crickets for feed and food are not yet up to these standards (58, 59), so our

findings are likely of interest for a large part of the rearing sector (60, 61).

In the meantime, the following recommendations can help limit the spread and potential impact of viruses, and other diseases:

(1) Only incorporate new individuals into a population after testing for relevant pathogens
(2) Quarantine individuals before release into the population
(3) Monitor regularly the pathogen status of the population
(4) Quarantine with any change in mortality, reproduction or behavior of animals
(5) Keep informed on new developments concerning the health and pathology of the insect species reared.

DATA AVAILABILITY STATEMENT

The sequence data analyzed in this study have been deposited in the GenBank small read archives, under accession numbers SRR13582031 (DNA) and SRR13582032 (RNA), Study accession SRP303879, Bioproject accession PRJNA697972 and Biosample accession SAMN7703531.

AUTHOR CONTRIBUTIONS

ÅB, AJ, and JMd conceived and designed experiments. ES, PO, and FG performed experiments. FG, ML, JdM, and ES analyzed the data. AJ and ÅB contributed reagents, materials, and analysis tools. JdM and ÅB wrote the initial manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.642085/full#supplementary-material

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Effect of Cooking Techniques on the *in vitro* Protein Digestibility, Fatty Acid Profile, and Oxidative Status of Mealworms (*Tenebrio molitor*)

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Tenebrio molitor (T. molitor) (mealworm) larvae are one of the most promising insects for feed–food purposes. Mealworms are rich in several macro and micro nutritional elements and can be practically reared on side stream substrates. In this study, the effects of seven different cooking techniques were tested on the nutritional value of mealworms focusing the attention on protein digestibility, fatty acid (FA) profile, and oxidative status. Uncooked larvae (UC) were used as control and compared to two combinations of temperature/time in oven cooking (70°C for 30 min, OC70-30, 150°C for 10 min, OC150-10), two methods of frying (mealworms fried in sunflower oil as deep fry, DF, or pan fry, PF), microwaving (MW), boiling (in plastic bag under vacuum, BO), and steaming (ST). Proximate composition, *in vitro* digestibility (gastric and duodenal), FA profile, and oxidative status (tocopherol and tocotrienol, carbonyl, and lipid oxidation) were then tested. Cooking technique affected all the tested parameters. As expected, cooking affected proximate composition in relation to the method applied (dry matter increased after oven cooking and frying; lipids increased by frying). *In vitro* digestion revealed the highest value for the OC70-30 method, followed by UC and ST. Deep frying revealed the worst digestibility percentage. FA profile was deeply affected by the cooking technique, with general decrease in SFA and MUFA. The highest modifications in FA profile were revealed in ST larvae with an increased percentage of linoleic acid linked to the lowering of SFA and MUFA contents. Furthermore, deep frying larvae in sunflower oil increased the relative abundance of PUFAs. Tocols values were higher in DF and MW groups than PF (about 6-fold more) and all other groups (7-fold more). Carbonyls increased with oven cooking (OC150-10 and OC70-30), whereas the values were lower with frying and similar to ST and UC. Lipid oxidation was highest as well in OC150-10 but similar to frying methods (DF and PF). Based on the obtained results, it can be concluded that mealworm larvae surely meet human nutritional requirements, but the cooking method must be carefully chosen to maintain a high nutritional value.

Keywords: protein digestibility, frying, microwave, tocols, carbonyl, TBARS, oven cooking

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INTRODUCTION

In the last few years, attention on edible insects has grown in relation to the increasing request of nutrient foods (1). Alternatives to the ongoing animal productions are required to sustain the increase of human population while lowering environmental impact. Insects could meet human nutrient requirements and increase the production yields without negatively impacting the environment. One of the main intrinsic potential of insect production lies on their capacity to be reared on unemployed feeds, also called substrate, that nowadays are not employed in conventional animal productions or in some cases are even treated as waste materials (2, 3). Indeed, insects could be fed on low-value feed contributing to decrease the environmental negative impact of animal farming and increasing the circular economy, with positive benefits on the entire food chain production (4, 5). Several research studies highlighted the potential of insects to bio convert low-value substrates into high-value nutrient products, with different outcomes in relation to the dualism insect substrate. Despite the increased attention shown by the scientific world, consumers' acceptance of edible insects in Europe is still low (6). Several researches highlighted that acceptability and consumers' awareness could be increased by providing information about insects' farming features, their sustainability and environmental perspectives, along with introducing insects in daily products (7–9). Processing of insects is a crucial step to make them utilizable as food and reach consumers' tables. As one of the last steps before consumption, cooking plays a key role in the nutritional value of food. Safe and healthy cooking methods may contribute to increase availability of edible insects in daily food consumption (10, 11).

Cooking may improve the sensory appeal of products, modifying color, texture, and flavors while affecting bioavailability of certain nutrients in the digestive tract. Heat treatment may also induce oxidations, proteolysis, lipolysis, and losses of susceptible molecules (11, 12).

Among edible insects, mealworm (*Tenebrio molitor* L. 1758; Coleoptera Tenebrionidae) is one of the most studied and produced insects for human consumption in Europe due to its features in rearing management, environmental sustainability, and nutritional value. Mealworm is the first edible insect that has achieved an important milestone for the EU insect sector as it was positively assessed by the European Food Safety Authority (EFSA) as novel food following the Regulation (EU) 2015/2283. Indeed, recently, EFSA released the first scientific opinion about insects as novel food (13) in which the EFSA Panel affirmed that dried mealworm (*T. molitor* larva) could be considered as a safe novel food. Considering this scientific opinion, a considerable expectation arises on mealworms and their potential will be released in the next years following market developments. From a nutritional point of view, mealworm larvae are quite balanced in macronutrients with high level of proteins (about 50% on dry matter basis) and lipids (about 33% on dry matter basis) and a good composition of essential amino acids, vitamins, and minerals (4, 14–17). Rearing substrate could affect development of mealworms (18, 19) as well as partially affect protein concentration and fatty acid (FA) composition (also ratio n6/n3) (4, 20, 21). Similarly, processing (i.e., cooking methods) of the larvae could affect the chemical composition of the products, both nutritional and quality characteristics (11, 22, 23).

In this study, the effects of seven different cooking techniques (two combinations temperature/time in oven cooking, two types of frying methods, microwave, boiling, and steaming) were tested on the nutritional values of mealworms focusing the attention on the *in vitro* protein digestibility, FA profile, and oxidative status.

MATERIALS AND METHODS

Insect Rearing

Tenebrio molitor larvae were fed with brewery spent grains and bread as former foodstuff. Brewery spent grains were directly collected from a local brewery and immediately frozen at −20°C, and bread was collected from a local market shop at the closure of the shop as daily remains. Both breweries spent grains and bread were dried in an oven at 90°C (spent grains were previously thawed at 4°C for 18 h) until the excessive humidity was removed reaching respectively about 95 and 97% in dry matter. Then, spent grains and bread were finely ground and mixed in 1:1 ratio. Larvae were reared in plastic containers (39 × 28 × 14 cm) at the Department of Veterinary Sciences (University of Pisa, Italy) under a laboratory-scale production. Rearing environment was maintained at 25 ± 1°C with 50–60% of relative humidity. Adult beetles (1–2 weeks old) were placed for 1 week to deposit eggs and then were removed to leave larvae to hatch in the substrate. Feeds were added weekly if needed (*ad libitum*, weighted before adding) and potato slides were placed once a week to provide moisture. During the rearing period, three times per week, rearing boxes were visually monitored and eventually dead larvae were removed. Mealworms were then harvested when the first pupa was observed into the box and used as samples for experimental units. Larvae were starved for 48 h in in sterile plastic containers with plastic web on the base in order to separate feces (frass) and avoid fecal contact. Larvae were then killed by freezing at −20°C.

Cooking Parameters

Larvae were cooked with seven different cooking methods as reported below. Two different combinations of temperature/time were selected for the oven cooking method, as well two types of frying were chosen.

- Oven cooking: oven was pre-heated at 70°C or 150°C and larvae were cooked in aluminum trays for 30 min (OC70-30) or 10 min (OC150-10).
- Frying: mealworms were fried in sunflower oil: either deep fry (300 ml of oil per 100 g of larvae, DF) or pan fry (30 ml of oil per 100 g of larvae, PF) for 2 min and dried on a paper towel.
- Microwave: larvae were cooked in a glass bowl at 800 W per 150 s (MW).
- Boiled: larvae were kept in a plastic bag under vacuum and inserted in boiling water for 30 min (BO).
- Steamed: larvae were cooked in a steamer for 10 min (ST).

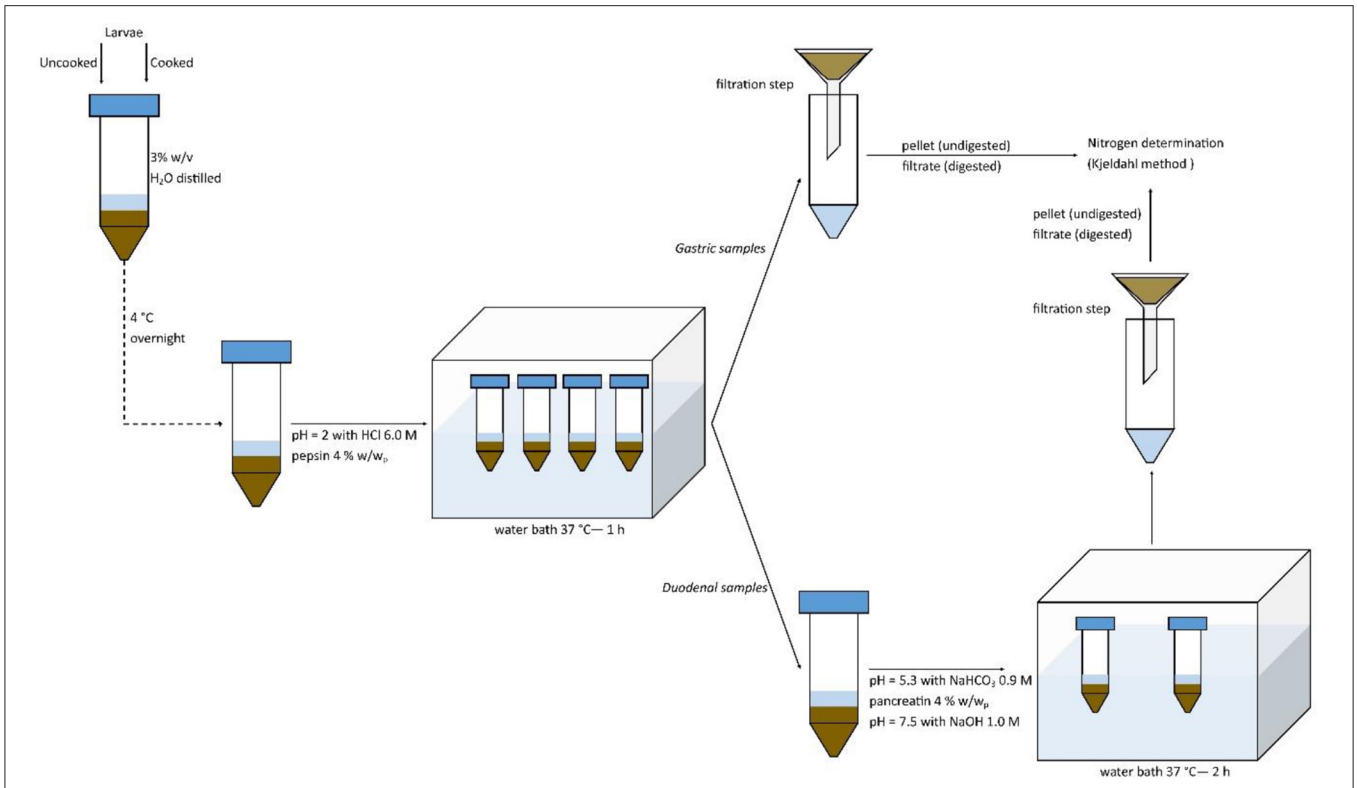

FIGURE 1 | *In vitro* protein digestibility method.

Cooking loss was recorded and calculated as percentage of the decrease of weight before and after cooking. Raw larvae were used as control (uncooked, UC).

All the cooking sessions were performed in triplicate on three different experimental units.

Proximate Composition

Dry matter content was determined by dehydration in a drying oven at 105°C until constant weight. Soxhlet extraction method was used to quantify lipid content using petroleum ether as solvent. Ash content was determined by incineration in a muffle furnace at 550°C. Crude protein content was determined by the Kjeldahl method. For protein-to-nitrogen conversion, two factors were used, 6.25 as normally calculated for meat an unknown samples and 4.76 as suggested by Janssen et al. (24) for insects. Proximate composition analyses were performed in triplicate for each sample.

In vitro Protein Digestibility

In vitro protein digestibility was performed following the method of Lacroix et al. (25) with slight modifications, as reported in **Figure 1**. In detail, ground uncooked or cooked larvae were diluted in distilled water at a concentration of 30 g/L (3% w/v) and solubilized at 4°C overnight. Then, samples were adjusted to pH 2 with HCl 6 M pre-incubated at 37°C. Pepsin was added at the concentration of 4% enzyme: substrate, based on the ratio w/w of protein (determined by the Kjeldahl method right after cooking). Solutions were then incubated in a water bath at 37°C for 1 h in order to simulate gastric digestion. Samples were then split into two ways: the first ones were filtrated (filter paper Whatman 1) in order to obtain the pellet (undigested) and filtrate (digested) fractions after the *in vitro* gastric digestion; the second ones were adjusted to pH 5.3 with $NaHCO_3$ 0.9 M, and then pancreatin was added at the concentration of 4% (w/w of protein basis). Solutions were then adjusted with NaOH 1.0 M at pH 7.5 and incubated at 37°C for 2 h in a water bath in order to simulate an *in vitro* duodenal digestion. Samples were then filtered to obtain digested (filtrate) and undigested (pellet) fractions. All the fractions were subjected to Kjeldahl determination. Digestibility values were calculated as percentage of the nitrogen contents of the fractions on the total sample's nitrogen content. Blanks containing only enzymes were simultaneously run and subtracted to the sample's determinations.

In vitro digestibility was performed in triplicate on each cooking technique and on uncooked larvae.

FA Profile

The lipid extraction from larvae was performed in duplicate on each sample according to Folch et al. (26) and the esterification of FA was performed according to Christie (27). The trans-methylation procedure was conducted using eicosenoic acid methyl esters (Sigma-Aldrich, Germany) as internal standard. The recovery rate of the internal standard was 85% ± 5% in the larvae.

The FA composition was determined using a Varian gas chromatograph (CP-3800) equipped with a flame ionization detector and a capillary column of 100 m length × 0.25 mm × 0.2 μm film (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas with a flow rate of 0.6 ml/min. The split ratio was 1:20. The oven temperature was programmed as reported by Mattioli et al. (28). Individual fatty acid methyl ester (FAME) was identified by comparing the relative retention times of peaks in the sample with those of a standard mixture (FAME Mix Supelco, Sigma-Aldrich, Germany). The FA were expressed as % of total FA. The average amount of each FA was used to calculate the sum of the total saturated (SFA), total monounsaturated (MUFA), and total polyunsaturated (PUFA) FA form n-3 and n-6 series. Even n6/n3 FA ratio was calculated.

In the insect samples, some nutritional indexes of lipids were evaluated as reported below.

The index of peroxidability (IP) was calculated according to Arakawa and Sagai (29):

$$\begin{aligned} IP = {} & (\%monoenoic \times 0.025) + (\%dienoic \times 1) + (\%trienoic \times 2) \\ & + (\%tetraenoic \times 4) + (\%pentaenoic \times 6) + (\%hexaenoic \times 8). \end{aligned}$$

Indexes of atherogenicity (IA) and thrombogenicity (IT) were calculated according to Ulbricht and Southgate (30). In particular:

$$\begin{aligned} IA = {} & (C12:0 + C14:0 \times 4 + C16:0)/[(MUFA + \Sigma n6) \\ & + \Sigma n3)]; \\ IT = {} & (C14:0 + C16:0 + C18:0)/[(MUFA \times 0.5 + \Sigma n6 \times 0.5 \\ & + \Sigma n3 \times 3) + (\Sigma n3/\Sigma n6)]. \end{aligned}$$

Oxidative Status

The tocopherol (δ-, γ-, and α-isoforms) and tocotrienol (γ- and α-isoforms) contents of the samples were quantified by a high-performance liquid chromatography (HPLC) system, according to Zaspel and Csallany (31). About 3 g of larvae was saponified in 60 g/100 ml KOH in ethanol in a thermostat bath at 70°C for 30 min. Then, the content was sonicated and extracted twice with 15 ml of n-hexane/ethyl acetate (9:1, v/v). After collecting the upper phase, the samples were nitrogen dried and then reconstituted in 200 μl of acetonitrile. Fifty microliters was injected into the HPLC system (Perkin Elmer series 200, equipped with an autosampler system, model AS 950–10, Tokyo, Japan) on a Synergy Hydro-RP column (4 μm, 4.6 × 100 mm; Phenomenex, Bologna, Italy). A Fluorometric Detector (FD) (model, FP-1525; Jasco, Tokyo, Japan; excitation and emission wavelengths of 295 and 328 nm, respectively) was used to identify the different isoforms. External calibration curves were used to quantify isoforms by increasing amounts of pure tocopherols in ethanol. The sum of tocopherols and tocotrienols were named tocols.

Carbonyl derivatives of proteins were detected according to Mattioli et al. (32). Briefly, the pellets from trichloroacetic acid (TCA) extracts were mixed with 1 ml of 10 mM DNPH in 2 M HCl. Samples were incubated for 1 h at RT and then centrifuged at 13,000 × *g* for 5 min. Supernatants were discarded and pellets were washed three times with 1 ml of ethanol–butylacetate (1:1, v/v) to remove unreacted DNPH. Pellets were then dissolved in 1.5 ml of 6 M guanidine-HCl and centrifuged as above to pellet insoluble particles. The carbonyl content of the resulting supernatants was evaluated spectrophotometrically at 370 nm using a molar extinction coefficient of 22,000 1/M*cm; values were expressed as nanomoles of carbonyl per milligram of protein in the guanidine chloride solution. Protein concentrations were measured *via* the Bradford method with Coomassie Brilliant Blue G-250 (33), using bovine serum albumin as standard.

Lipid oxidation was evaluated using a spectrophotometer set at 532 nm (Shimadzu Corporation UV-2550, Kyoto, Japan) that measured the absorbance of TBARS and a 1,1,3,3-tetraethoxypropane calibration curve (34). Oxidation products were quantified as malondialdehyde equivalents (μg MDA/g).

Statistical Analysis

Proximate composition, cooking loss, *in vitro* protein digestibility, FA profile, and oxidative status were statistically analyzed using a one-way ANOVA with regard to the cooking technique factor (UC, OC70-30, OC150-10, DF, PF, MW, BO, and ST). Statistical significance was set at 0.05 and 0.001 and differences were assessed using Tukey's test. R free statistical software was used (35).

RESULTS

Proximate Compositions and Cooking Losses

Cooking techniques affected proximate compositions of larvae (**Figure 2**). Dry matters of fried larvae (PF and DF) were higher than the other samples, followed by oven cooked at 150°C (OC150-10) and microwaved (MW), boiled ones (BO) and by oven cooked at 70°C (OC70-30), steamed (ST), and uncooked (UC). These losses of humidity were also detected by cooking losses that were, respectively, detected as 5.05, 35.91, 16.38, 25.27, 27.22, and 3.35% for OC70-30, OC150-10, PF, DF, MW, and BO. Steamed larvae (ST) reported cooking loss as a negative value, −15.39%, as during the cooking step, larvae gained weight instead of losing weight. Cooking losses indeed affected all the proximate compositions as the higher cooking technique in terms of cooking loss, i.e., OC150-10, DF, and MW, reported higher concentration of crude proteins followed by PF. No significant variation to the UC larvae was highlighted for the other cooking techniques (OC70-30, BO, and ST). As expected, frying increased the percentage of lipids in the final products, as revealed by DF and PF ether extract values. As reported for crude protein contents, cooking losses affected the lipid percentages too. Oven cooking at 150°C (OC150-10) and MW larvae reported higher contents in ether extract than UC, OC70-30, and ST. Boiling larvae slightly increased the ether extract concentration in relation to the raw larvae. Ash concentration was mainly affected by the cooking technique, highlighting a higher concentration in OC150-10 followed by PF, DF, and MW, and then by UC,

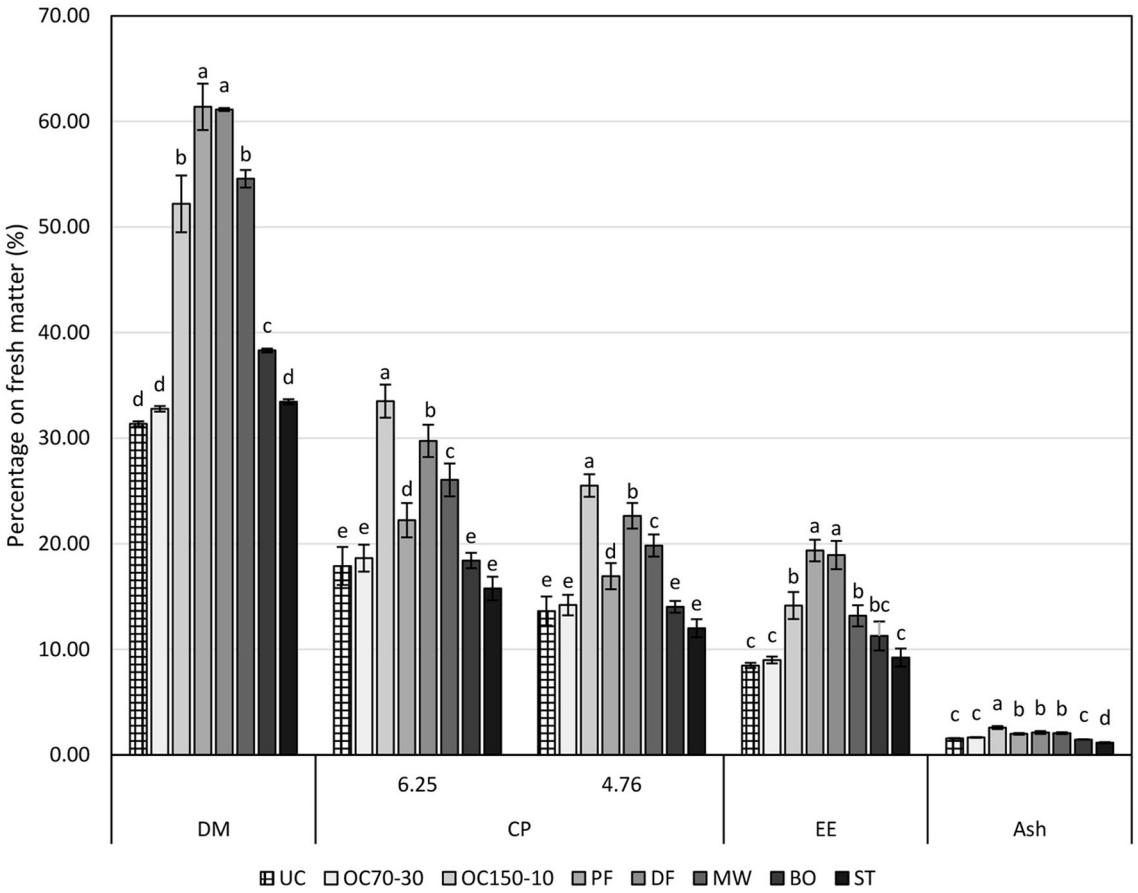

FIGURE 2 | Proximate compositions (on fresh matter, %) of uncooked larvae and larvae cooked with different techniques. DM, dry matter; CP, crude protein (protein-to-nitrogen conversion factors of 6.25 and 4.76); EE, ether extract; UC, uncooked; OC70-30 oven cooked at 70°C for 30 min; OC150-10, oven cooked at 150°C for 10 min; PF, pan fried (30 ml oil per 100 g of larvae, 2 min); DF, deep fried (300 ml of oil per 100 g of larvae, 2 min); MW, cooked in microwave (800 W per 150 s); BO, boiled (plastic bag under vacuum for 30 min); ST, steamed for 10 min. Different letters show statistically significant differences among samples for each determination (DM, CP_6.25, CP_4.76, EE and Ash) at $P < 0.05$.

TABLE 1 | Proximate compositions (on dry matter, %) of uncooked larvae and larvae cooked with different techniques.

	UC	OC70-30	OC150-10	PF	DF	MW	BO	ST	RMSE	*P*
CP_6.25	57.07^{ab}	56.85^{ab}	64.20^{a}	36.21^{c}	48.65^{b}	47.73^{b}	48.07^{b}	47.12^{b}	7.305	<0.001
CP_4.76	43.47^{ab}	43.30^{ab}	48.89^{a}	27.58^{c}	37.05^{b}	36.35^{b}	36.61^{b}	35.89^{b}	5.564	<0.001
EE	27.04^{b}	27.44^{b}	27.11^{b}	31.54^{a}	30.97^{a}	24.14^{b}	29.41^{b}	27.56^{b}	5.384	0.049
Ash	4.95^{a}	5.05^{a}	4.97^{a}	3.25^{c}	3.48^{bc}	3.78^{b}	3.81^{b}	3.46^{bc}	0.173	<0.001

DM, dry matter; CP, crude protein (protein-to-nitrogen conversion factors of 6.25 and 4.76); EE, ether extract.
UC, uncooked; OC70-30 oven cooked at 70°C for 30 min; OC150-10, oven cooked at 150°C for 10 min; PF, pan fried (30 ml oil per 100 g of larvae, 2 min); DF, deep fried (300 ml of oil per 100 g of larvae, 2 min); MW, cooked in microwave (800 W per 150 s); BO, boiled (plastic bag under vacuum for 30 min); ST, steamed for 10 min.
Different letters in the same row show statistically significant differences among samples at $P < 0.05$.

OC70-30, and BO larvae. Steamed larvae (ST) due to humidity increase revealed lower ash content than UC ones.

Proximate composition of the larvae was also reported as percentage on dry matter (DM, **Table 1**). Crude protein content revealed, as percentage on DM, to be higher in oven-cooked larvae at 150°C, as consequence of humidity lost. Raw larvae (UC) and OC70-30 crude protein contents were similarly high and comparable to DF, MW, BO, and ST. The lowest value was shown by PF larvae. Frying indeed increased ether extract as DM percentage; as a consequence, PF and DF showed around one-third of DM as lipids. No statistical differences were highlighted between the other samples. Ash content showed a similar trend to that of crude protein and seems to be mainly affected by cooking losses.

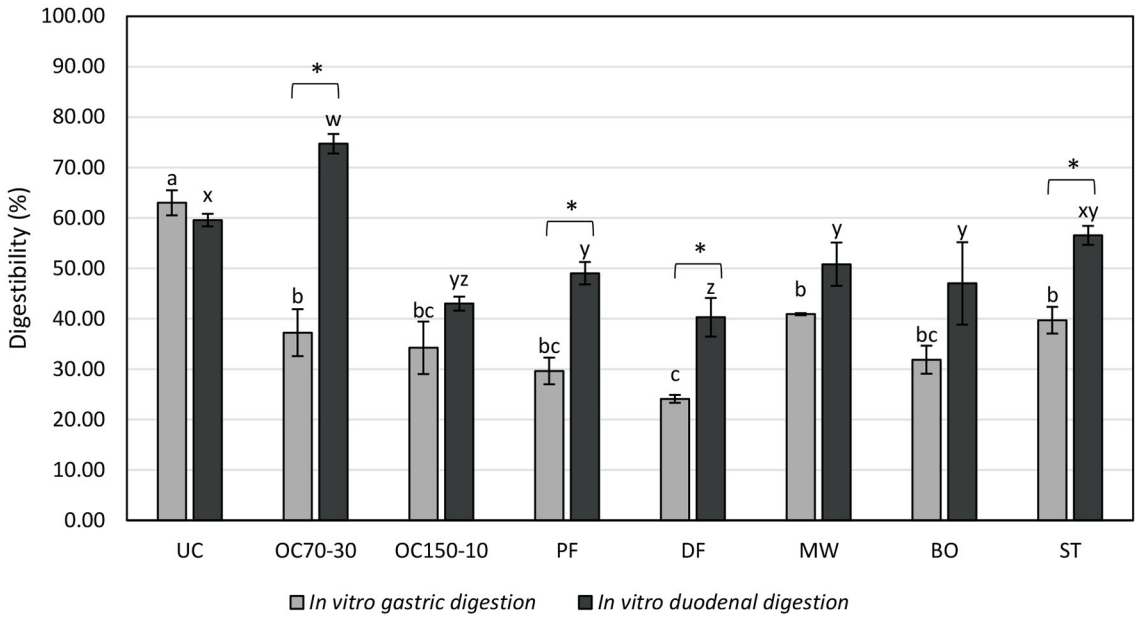

FIGURE 3 | *In vitro* digestibility (filtrate nitrogen content on the total sample nitrogen content, %) of uncooked larvae and larvae cooked with different techniques. UC, uncooked; OC70-30 oven cooked at 70°C for 30 min; OC150-10, oven cooked at 150°C for 10 min; PF, pan fried (30 ml oil per 100 g of larvae, 2 min); DF, deep fried (300 ml of oil per 100 g of larvae, 2 min); MW, cooked in microwave (800 W per 150 s); BO, boiled (plastic bag under vacuum for 30 min); ST, steamed for 10 min. ^{a,b,c}Letters show statistically significant differences among samples for *in vitro* gastric digestion at $P < 0.05$. ^{w,x,y,z}Letters show statistically significant differences among samples for *in vitro* duodenal digestion at $P < 0.05$. *Shows statistically significant differences between *in vitro* gastric and duodenal digestions among each treatment at $P < 0.05$.

In vitro Protein Digestibility

In vitro digestibility values, reported as percentage of nitrogen content of the filtrates on the total samples, are reported in **Figure 3**.

After *in vitro* gastric digestion, uncooked samples revealed the highest digestibility value, followed by OC70-30, MW, and ST samples. Deep fried larvae (DF) showed the lowest digestibility after *in vitro* gastric digestion. After *in vitro* duodenal digestion, the highest value was determined in OC70-30 larvae, followed by UC, ST, and then PF, MW, BO, and OC150-10 samples. As reported before for *in vitro* gastric digestion, DF larvae revealed the worst digestibility percentage. Statistical differences in *in vitro* digestibility between gastric and duodenal samples were not shown by UC, OC150-10, MW, and BO samples even if for all the cooking techniques a numerical increase in digestibility was revealed (not for UC). A statistically significant increase in digestibility of OC70-30, PF, DF, and ST samples was highlighted with values of +37.48%, +19.39%, +16.18%, and +16.83%, respectively. Interestingly, OC70-30 digestibility doubled after *in vitro* duodenal digestion in relation to gastric ones.

FA Profile

Many differences were found on FA proportion of cooked larvae (**Table 2**). All cooking processes decreased the content of SFA (mainly constituted by palmitic acid, C16:0) with respect to the uncooked larvae. The MUFA showed less variations with similar value in UC, OC150-10, PF, MW, and BO ($P > 0.05$); ST showed the lowest value (32.24% of total FA). The main differences were due to the palmitoleic (C16:1) and oleic (C18:1) acid proportion, which were the most representative FA in OC70-30 and UC, respectively (C16:1 was 7.91% in OC70-30 and C18:1 was 40.68% in UC).

The PUFA content followed the rank ST and DF > BO, OC150-10, and PF > OC70-30 > UC, and MW. The most representative FA were the LA for n6 series and ALA for n3 series. Among the long-chain FA (LCP), strong differences were recorded; however, the OC70-30 showed the highest values for both LCP n3 and n6.

The n6/n3 ratio was interestingly lower in OC70-30 (1.53) whereas the highest value was recorded in DF treatment (79.41), mainly due to the higher proportion of LA (2-fold higher with respect to the average of the other treatments). The IP reached the highest value in OC70-30, followed by UC; all the other cooking processes showed lower values. IA and IT were also widely reduced in cooked samples with respect to the fresh larvae.

Oxidative Status

The tocols content were widely affected by moisture of cooked samples and then the values were reported on DM basis (**Table 3**). The α-tocopherol isoform was the most abundant in all samples. Tocols values were higher in DF and MW groups than in PF (about 6-fold more) and all other groups (7-fold more).

Protein oxidative status was significantly different in all groups ($P < 0.001$), following the rank: OC50-10 and OC70-30 > MW > BO > others, whereas the lipid oxidation showed

TABLE 2 | Fatty acid profile (%) of uncooked larvae and larvae cooked with different techniques.

	UC	OC70-30	OC150-10	PF	DF	MW	BO	ST	RMSE	*P*
C12:0	0.24	0.19	0.19	0.35	0.24	0.36	0.24	0.24	0.063	0.470
C14:0	3.53^{A}	2.12^{B}	3.23^{A}	3.53^{A}	0.05^{C}	0.12^{C}	3.07^{A}	0.09^{C}	0.163	<0.001
C16:0	21.42^{A}	19.58^{A}	19.16^{B}	19.05^{B}	9.39^{C}	19.27^{B}	18.81^{B}	6.96^{D}	0.594	<0.001
C18:0	4.54^{a}	3.14^{b}	3.54^{b}	3.53^{b}	3.62^{ab}	3.82^{ab}	3.64^{b}	3.50^{b}	0.128	0.002
C20:0	0.65^{B}	2.33^{A}	0.23^{B}	0.06^{B}	0.47^{B}	0.04^{B}	0.14^{B}	0.03^{B}	0.180	<0.001
SFA	30.37^{A}	27.35^{B}	26.35^{B}	26.52^{B}	13.76^{D}	23.61^{C}	25.90^{BC}	10.82^{D}	0.472	<0.001
C14:1cis9	0.33^{C}	1.04^{A}	0.11^{DE}	0.15^{D}	0.77^{B}	0.14^{D}	0.10^{DE}	0.02^{E}	0.019	<0.001
C16:1cis9	1.68^{B}	7.91^{A}	1.63^{B}	1.84^{B}	0.60^{C}	1.73^{B}	1.74^{B}	0.19^{C}	0.137	<0.001
C18:1cis9 n9	40.68^{A}	25.11^{E}	39.81^{AB}	39.24^{AB}	34.82^{C}	37.99^{B}	38.54^{AB}	32.00^{D}	0.436	<0.001
C20:1cis+trans11	0.15^{B}	0.86^{A}	0.15^{BC}	0.15^{B}	0.05^{D}	0.12^{C}	0.04^{E}	0.03^{E}	0.005	<0.001
MUFA	42.83^{a}	34.92^{c}	41.69^{ab}	41.38^{ab}	36.23^{c}	39.98^{b}	40.42^{ab}	32.24^{d}	0.449	<0.01
C18:2cis n6 LA	14.49^{C}	11.94^{C}	28.11^{B}	27.12^{B}	47.22^{A}	14.20^{C}	28.38^{B}	51.43^{C}	1.267	<0.001
C20:4n6 AA	0.58^{B}	1.73^{A}	0.03^{D}	0.05^{D}	0.18^{C}	0.09^{D}	0.05^{D}	0.24^{C}	0.012	<0.001
C22:5n6	0.20	0.06	0.01	0.05	0.04	0.02	0.00	0.01	0.065	0.437
n6	15.27^{C}	13.73^{C}	28.15^{B}	27.22^{C}	47.44^{A}	14.31^{C}	28.43^{B}	51.68^{A}	1.266	<0.001
C18:3 n3 ALA	0.71^{C}	3.15^{B}	1.37^{B}	1.42^{B}	0.55^{C}	0.15^{D}	1.42^{B}	0.64^{C}	0.058	<0.001
C20:5n3 EPA	1.12^{B}	1.48^{A}	0.03^{C}	0.10^{C}	0.03^{C}	0.05^{C}	0.06^{C}	0.02^{C}	0.036	<0.001
C22:5n3 DPA	0.11^{b}	2.05^{a}	0.03^{d}	0.04^{bc}	0.01^{d}	0.04^{d}	0.04^{bc}	0.01^{d}	0.014	0.091
C22:6n3 DHA	0.84^{B}	2.31^{A}	0.00^{C}	0.05^{C}	0.01^{C}	0.01^{C}	0.05^{C}	0.03^{C}	0.066	<0.001
n3	2.78^{B}	9.00^{A}	1.43^{CD}	1.61^{C}	0.60^{DE}	0.23^{E}	1.57^{C}	0.69^{DE}	0.153	<0.001
PUFA	18.05^{D}	22.73^{C}	29.58^{BC}	28.83^{BC}	48.04^{A}	14.55^{D}	30.00^{B}	52.38^{A}	1.268	<0.001
tot	91.25^{AB}	85.00^{BC}	97.62^{A}	96.73^{A}	98.03^{A}	78.14^{C}	96.33^{A}	95.44^{A}	1.469	<0.001
others	8.75^{BC}	15.00^{AB}	2.38^{C}	3.27^{C}	1.97^{C}	21.86^{A}	3.67^{C}	4.56^{C}	1.469	<0.001
n6/n3	5.49^{DE}	1.53^{E}	19.71^{C}	16.92^{CD}	79.41^{A}	62.46^{B}	18.10^{C}	74.76^{AB}	2.835	<0.001
IP	14.08^{B}	39.67^{A}	0.35^{C}	1.20^{C}	0.32^{C}	0.53^{C}	0.98^{C}	0.37^{C}	0.741	<0.001
IA	0.59^{A}	0.49^{B}	0.45^{B}	0.48^{B}	0.12^{D}	0.37^{C}	0.44^{BC}	0.09^{D}	0.014	<0.001
IT	0.78^{A}	0.48^{C}	0.66^{B}	0.67^{B}	0.30^{D}	0.83^{A}	0.65^{B}	0.24^{D}	0.016	<0.001

UC, uncooked; OC70-30 oven cooked at 70°C for 30 min; OC150-10, oven cooked at 150°C for 10 min; PF, pan fried (30 ml oil per 100 g of larvae. 2 min); DF, deep fried (300 ml of oil per 100 g of larvae. 2 min); MW, cooked in microwave (800 W per 150 s); BO, boiled (plastic bag under vacuum for 30 min); ST, steamed for 10 min.

^{a,b,c,d}Letters show statistically significant differences among samples at $P < 0.01$.

^{A,B,C,D,E}Letters show statistically significant differences among samples at $P < 0.001$.

TABLE 3 | Antioxidant content (μg/g DM) and oxidative status (Thiols, μmol SH-group/g DM; carbonyls, nmol/mg proteins calculated on DM basis; TBARS μg of MDA/g DM) and of uncooked larvae and larvae cooked with different techniques.

	UC	OC70-30	OC150-10	PF	DF	MW	BO	ST	RMSE	*P*
Antioxidants										
γ-Tocotrienol	0.82^{b}	1.11^{a}	1.23^{a}	0.55^{b}	1.27^{a}	0.77^{b}	0.94^{b}	1.14^{a}	0.081	0.038
α-Tocotrienol	0.02	0.03	0.03	0.01	0.03	0.02	0.03	0.03	0.004	0.644
δ-Tocopherol	0.01	0.02	0.00	0.03	0.26	0.24	0.00	0.00	0.064	0.466
γ-Tocopherol	0.15^{b}	0.10^{b}	0.10^{b}	0.17^{b}	1.18^{a}	1.04^{a}	0.09^{b}	0.13^{b}	0.07	0.460
α-Tocopherol	1.55^{C}	1.26^{C}	1.20^{C}	7.79^{B}	17.12^{A}	17.19^{A}	1.86^{C}	1.24^{C}	3.740	<0.001
Tocols	2.56^{C}	2.52^{C}	2.56^{C}	8.55^{B}	17.40^{A}	17.40^{A}	2.93^{C}	2.55^{C}	47.490	<0.001
Oxidative status										
Carbonyls	8.23^{D}	95.38^{A}	117.02^{A}	7.95^{D}	7.91^{D}	55.99^{B}	15.22^{C}	8.87^{D}	5.613	<0.001
TBARS	2.85^{c}	3.97^{b}	5.68^{a}	4.02^{b}	5.70^{a}	2.99^{c}	2.50^{c}	2.38^{d}	0.608	0.018

UC, uncooked; OC70-30, oven cooked at 70°C for 30 min; OC150-10, oven cooked at 150°C for 10 min; PF, pan fried (30 ml oil per 100 g of larvae, 2 min); DF, deep fried (300 ml of oil per 100 g of larvae, 2 min); MW, cooked in microwave (800 W per 150 s); BO, boiled (plastic bag under vacuum for 30 min); ST, steamed for 10 min.

^{a,b,c,d}Letters show statistically significant differences among samples at $P < 0.05$.

^{A,B,C,D,E}Letters show statistically significant differences among samples at $P < 0.001$.

significantly higher values in OC150-10 and DF with respect to the other groups.

DISCUSSION

Cooking may improve the sensory characteristics of food and plays a key role in assuring safety parameters. Noteworthy, it may also affect nutritional value, both positively and negatively. Heat treatments might decrease the amount of thermolabile compounds (such as PUFAs or vitamins) and induce interactions between proteins themselves or with other oxidizing agents [sugars, polyphenols, tannins, or solvents; (36)] affecting the total digestibility. Moreover, high temperatures could denature protein unfolding polypeptide chains increasing susceptibility to enzymes and inactivate antinutritional compounds that may inhibit specific enzymes (36, 37). At the same time, excessive high temperatures can reduce digestibility of proteins inducing amino acid reactions that vane enzyme digestion (37).

Proximate Composition

Mealworms show a high content of proteins (about 20% of fresh matter) and lipids (about 14% of fresh matter). These two parameters make mealworm nutritional value comparable to other conventional animals' products such as meat, eggs, or milk. The obtained ranges of nutrients, beside the cooking technique, are in agreement with other previously published researches on mealworm larvae (4, 5, 24, 38). The proximate results are also in line with the one reported by Caparros Megido et al. (11) on mealworm larvae cooked with household cooking technique.

Dry matter (DM) content is strictly related with the cooking technique and the corresponding water loss. Unprocessed larvae were not modified in their structure and maintained humidity content naturally present into their body; indeed, uncooked larvae revealed the lowest DM contents. Boiling (under vacuum) and steaming preserved the samples from water evaporation and created an environment where water was kept or even added into the larvae, as highlighted by the low or negative values of cooking losses. Moreover, heat treatment could also lead to protein denaturation that may cause higher water losses due to degeneration of protein structures and losses of capillary forces (12). Apparently, oven cooking at 70°C for 30 min did not deeply affect the mealworm structure and then DM was comparable to the one of UC larvae or even to steamed ones (ST). Higher oven temperature–time combination or microwave cooking induced modifications into the larvae structure that caused water losses and increased DM contents. Frying larvae, both in pan or deeply, induced highest water losses and evaporation increased DM contents, also partially affected by the incorporation of lipids from the frying oil [as reported also in other products, (12)]. Indeed, PF and DF larvae showed the highest content in lipids doubling the UC ether extract content. A similar trend was reported by Caparros Megido et al. (11) in mealworms pan fried in olive oil.

Regarding the crude protein contents, the cooking technique deeply modified the nutritional value of the larvae. Oven-cooked larvae for 10 min at 150°C, due to water loss and concentration of the nutrients, revealed the higher protein content followed by DF and MW. Again, as reported before, water replacement with lipids must be taken into consideration in the total nutritional evaluation of DF larvae.

In vitro Protein Digestibility

Cooking conditions may reduce or increase protein digestibility also in relation to the type of organic material (37). *In vitro* determination highlighted a decreasing percentage of protein digestibility after simulated gastric digestion in all the cooked samples in comparison with unprocessed larvae (UC). Cooking may contribute to the formation of complex organic compounds that are difficult to be digested by pepsin. Indeed, enzymatic proteolysis may be affected by modification that occurred during cooking such as formation of amide bonds or disulfide/dityrosine bridges (39). Between the cooking techniques employed, DF showed the highest reduction in simulated protein gastric digestibility followed by OC150-10, PF, and BO with minor differences to OC70-30, MW, and ST.

In vitro protein digestibility increased in all the cooked samples after simulated duodenal digestion, with significant changes in OC70-30, PF, DF, and ST larvae. Uncooked larvae maintained the same percentage during the two *in vitro* steps showing that after gastric digestion, the matrix was not furthermore digested. Increases in cooked larvae digestibility and absence of variation in UC affected the trend in protein digestibility after *in vitro* duodenal digestion with the highest value in OC70-30 followed by UC and ST samples.

Our results were partially in accordance with the one shown by Caparros Megido et al. (11) with some differences related to the methods and cooking techniques employed. Fried larvae decreased protein digestibility mostly in relation to their potential oxidation that occurred during the cooking step. Oxidation products of lipids may create complexes with proteins and modify the chemical structure and functionality of proteins and reduce their enzymatic susceptibility (40).

Protein digestibility is important not only in terms of nutritional value but also in relation to potential harmful risk, mainly associated with the risk of colorectal cancer, as a higher quantity of undigested protein entering the colon could increase potential deleterious effects on colonic epithelial cells (41).

FA Profile

For all cooking methods, a general decrease in SFA and MUFA content was observed. This observation may be explained by the fact that both FA classes are largely represented in neutral lipids and are more prone to migration (42). However, MUFA showed less variations than SFA; the main differences were due to the oleic acid (C18:1) proportion, which was the most representative FA in UC and a similar concentration was found in ST, OC150-10, and PF. Regarding the frying method, Juárez et al. (12) found in buffalo meat the lowest SFA content due to the incorporation of MUFA (C18:1) from oil; conversely, in the present paper, a similar trend was not found regarding MUFA, whereas an increase of PUFA was recorded in the DF group. Furthermore, the PUFA content was also higher in the ST group, followed by BO, OC150-10, and PF with respect to UC and MW. The ST method better preserved the cellular integrity of larvae and then

the membrane FA content. Indeed, the PUFA decrease has been related to triglycerides' unsaturated FA drip losses as reported for the grilled meat (43). Among n3 and n6 FA, great differences were recorded; however, it is not possible to draw a unique trend for each FA. The OC70-30 showed the highest values for both LCP n3 and n6, showing a n6/n3 ratio that is 50- (DF) to 11-fold (UC) lower with respect to the other cooking techniques. Such trend may be modulated by an oxidative process that occurred on unsaturated FA during cooking; accordingly, the IP reached the highest value in OC70-30. Indeed, unsaturated FA are strongly susceptible to oxidation, due to the presence of double bonds. Some authors (44) consider that changes in FA composition that occur during cooking may be overlooked when only total lipid extracts are analyzed. In fact, the thermal hydrolysis, the migration of FA from tissue to other locations, the loss of volatile FA, and the deactivation of enzymes occurred during heating may be responsible for many of the observed changes.

Oxidative Status

The oxidative status of larvae was widely affected by the cooking processes, with strong differences related to the methods used. In the present research, carbonyls increased with oven cooking (OC150-10 and OC70-30), whereas the values were lower with frying and similar to ST and UC. Such trend was probably due to the time/temperature ratio of cooking because frying consisted of a high temperature (350–370°C) for a few seconds (120 s), and then the effect was comparable to the less impactful methods (i.e., ST). Protein oxidation is the covalent modification of a protein induced either directly by reactive oxygen species or indirectly by reaction with secondary products of oxidative stress. Different groups of amino acids are sensitive to oxidation (45): basic amino acids are oxidized into carbonyls, whereas thiol groups of cysteine can be oxidized with the formation of disulfide bridges (46). In agreement with our findings, Gatellier et al. (47) reported that beef meat heated at 207°C for 300 s showed higher carbonylation with respect to heating the same material for a short burst (60 s) at high temperature [(270°C; (48)]. Similarly, cooking larvae *via* microwave showed a negative effect on protein oxidative status.

Concerning the lipid oxidative status, higher values were recorded in DF and OC150-10. It should be noted that a higher lipid and protein oxidation corresponds to a lower antioxidant concentration (tocols). The best oxidative status was recorded in the ST group, although the tocols content was very low (2.55 μg/g DM), probably due to an involvement of such molecules to counteract the oxidative thrust (49). During cooking, antioxidant defense systems are impaired, and contemporary free radicals are produced, leading to protein, and lipid oxidation (50). In agreement, some cooking processes showed a strong antioxidant/pro-oxidant imbalance that negatively affected the oxidative status of the larvae, particularly both oven methods and microwave cooking. A body of literature reported that microwave might affect mainly protein oxidation, whereas frying (both pan and deep) is involved in lipid oxidation (51). Contrary to conventional heating processes, such as oven cooking or pan frying, electromagnetic microwaves have frequencies between 0.3 and 300 GHz and wavelengths between 1 nm and 1 mm that can penetrate food and directly excite specific molecules by ionic conduction and dipole rotation. The overall effects of these movements lead to an increased kinetic energy of the molecules, resulting in increased temperature and cooking rate. Given that this mechanism allows for intimate interaction with the molecules of the food, generation of reactive oxygen species could result due to disruption of cellular compartmentalization.

Contrary to the trend in protein oxidation, deep frying and cooking at 150°C in an oven for 10 min contributed to a greater increase in lipid oxidation (TBARS) compared to microwave cooking. Some studies have, however, reported contrary results with higher lipid oxidation in microwave compared to frying (52). This discrepancy may be due to the nature of the food involved, as well as the length and temperature of the cooking method. In addition, total fat content and FA profile surely played an important role in lipid oxidation (TBARS).

CONCLUSIONS

Tenebrio molitor larvae represent a valid source of nutritional values. Cooking technique could affect (*in vitro*) the protein digestibility, FA profile, and oxidative status of the product. Between the tested cooking techniques, steaming was the less invasive method in terms of nutritional value modifications, lipid–protein oxidations, and *in vitro* protein digestibility. Microwave and oven cooking methods showed mixed effects leading to miscellaneous outcomes. Frying, both in pan and deep frying, induced great modifications in larvae FA profile, with a subsequent decrease in lipid oxidative status. Boiling could also be reported as a mild cooking method despite the fact that some modifications in chemical composition were shown (mainly PUFA and vitamins). Nutritional composition of cooked larvae was also affected by cooking losses that occurred in relation to the method employed, and attention must be paid to maintain certain types of molecules also in relation to the fixed goals. Noteworthy, despite differences highlighted by the cooking techniques, nutritional values of mealworm could meet humans' requirements and increase vitamins and PUFA intakes. The cooking method must be carefully chosen to maintain a high protein digestibility.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SMan and FF contributed to conception and design of the study. SMan, SMat, SP, and FF performed the laboratory analysis. SMan and SMat performed the statistical analysis. SMan wrote the first draft of the manuscript. SMat wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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In vivo and *in vitro* Digestibility of an Extruded Complete Dog Food Containing Black Soldier Fly (*Hermetia illucens*) Larvae Meal as Protein Source

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Growing attention is being directed toward insects as a novel and sustainable source of protein for pet food. The aim of the study was to evaluate nutrient digestibility of a diet containing black soldier fly larvae as its main protein source. Moreover, the purpose of the study was to compare the traditional *in vivo* total collection method with the *in vivo* marker method and *in vitro* digestibility method. Two isonitrogenous and isoenergetic dry diets containing either venison meal (CTRL diet) or black soldier fly larvae meal (BSF diet) as their primary sources of proteins were fed to six adult dogs, according to a Latin square design. The digestibility of nutrients was determined using both *in vivo* ("total collection" and "internal marker" approaches) and *in vitro* methods. The two diets showed similar nutrient digestibility values for dry matter, organic matter, ether extract, ash, and phosphorus. However, a statistical trend ($p = 0.066$) was observed indicating greater protein digestibility in the BSF diet compared with the CTRL diet. Calcium digestibility was higher in the BSF diet compared with the CTRL diet ($p = 0.018$). On the contrary, fiber digestibility was lower in the insect-based diet compared with the venison diet ($p < 0.001$). There was no difference between total collection and internal marker methods in the assessment of *in vivo* digestibility for any of the nutrients considered. The *in vitro* digestibility values for dry matter, organic matter, and crude protein, as well as the estimated *in vivo* digestibility of organic matter and crude protein by the means of the predictive equation, were aligned with the *in vivo* results, although *in vitro* estimations were consistently higher compared with those obtained by *in vivo* analysis. Digestibility analysis of a dog food containing insect meal as the sole source of protein (36.5% inclusion) showed promising results in terms of it presenting similar values as a meat-based diet, indicating its suitability as a sustainable protein source for pet food. Moreover, the study showed that both the *in vivo* marker method and the *in vitro* method could be possible alternatives to the traditional total collection method in digestibility trials.

Keywords: sustainability, pet food, digestibility, protein, novel feed materials, insect meal

INTRODUCTION

With the livestock industry at its limit in terms of sustainable production capacity, and the pet food business in constant growth, new sources of protein are being sought in order to meet the market's demand and the expectations of pet owners (1). Insects may provide a possible solution as an alternative feed, since they can partially replace traditional feed sources, while they also provide a means to bio-converting organic waste (2). Of the various insects being considered, the black soldier fly (*Hermetia illucens*) is showing particular promise due to its immediate potential for large-scale production (3).

The black soldier fly (BSF) has a balanced protein composition and one of the highest amino acid scores compared with other currently reared insects or traditional protein sources (such as fish meal) (4). Compared with crickets and mealworms, BSF boasts a more stable nitrogen and phosphorus composition and has a more advantageous feed conversion ratio (5). It can also be considered a possible sustainable solution due to the possibility of rearing the insects on materials deemed unsuitable for human nutrition, such as alimentary by-products and organic substrates (6).

As pointed out by Böhm et al. (7), insects may constitute an appropriate novel protein source for dogs, presenting cutaneous adverse food reactions. Nevertheless, societal negative opinions about the use of insect meal in pet nutrition have arisen, especially due to insect phobia and concerns about safety. Security aspects about insect consumption were also discussed critically in EFSA Scientific Opinion (8), where uncertainty regarding the risk of non-processed items, due to the lack of data, has been acknowledged. However, EFSA concluded that microbiological risks are expected to be comparable with other food raw materials, provided that insects are fed with allowed feedstuff. Consumers from Western countries still continue to have prejudices regarding the introduction of insects in their diet (9), and, due to the current "humanization trend" (10), this fact could be also translated to their pets. Notwithstanding, public opinion seems to be less concerned about the use of veterinary-prescribed diets based on insects (11). Indeed, veterinarians have expressed interest in hypoallergenic food alternatives prepared using insects (12). According to the Commission Regulation (EU) 2020/354 (March 4, 2020) (13), a product can be claimed to reduce ingredient and nutrient intolerances if it is composed of hydrolyzed proteins or selected and limited protein sources or selected carbohydrate sources. Therefore, according to the current European Regulations, a product composed only of insects as the main source of protein could be considered with the particular purpose of reduction of food intolerance. Concurrently, and reflecting the growing interest in this field of research (14), various recent studies have investigated the possibility of feeding BSF larvae to poultry (15–18), fish (19–21), and swine (22, 23). Recently, a thorough review from Bosch and Swanson (24) explored in depth the palatability, digestibility, and nutritional aspects of the inclusion of insects in dog and cat diet, showing the potential of insects as future pet food products.

The aim of the present study was to evaluate the inclusion of defatted BSF larvae meal in extruded dog food in terms of its *in vivo* and *in vitro* digestibility, in order to assess its suitability for the pet food market. Furthermore, the purpose of the study was to evaluate if the *in vivo* marker method and the *in vitro* digestibility method could be comparable to the traditional *in vivo* total collection method also in these particular diets. The estimated *in vivo* digestibility of organic matter and crude protein calculated by means of predictive equations utilizing data obtained by *in vitro* analysis was also assessed.

MATERIALS AND METHODS

All the experimental procedures were approved by the Bioethics Committee of the University of Turin (Italy) (prot. n. 336595).

Animals and Experimental Design

Six clinically healthy West Highland White Terrier adult dogs [three males and three females, 3 ± 1.8 years old, 7.2 ± 0.8 kg BW, BCS ranging between 4.5 and 5.5 on a nine-point scale (25)] were fed two isonitrogenous and isoenergetic dry extruded diets (control vs. insect diet) according to a Latin square design. During the digestibility experiment, the dogs were housed individually in 3 × 3-m kennels and had *ad libitum* access to fresh water. The dogs were allowed to walk freely for 1 h per day in a concrete outside the pen and play with toys during the adaptation periods.

Diets and Digestibility Protocol

Two diets were tested during the trial. The diets were formulated to be isoenergetic and isonitrogenous. In the control diet (CTRL diet), the protein source was provided in the form of processed [rendering process, method III, according to the EU Reg. 142/2011 (26)] deer (*Cervus elaphus*) protein, whereas the insect diet (BSF diet) provided defatted BSF (*H. illucens*) larvae meal as its sole protein source (Hermetia Futtermittel GbR, Baruth/Mark, Germany). The chemical composition, amino acidic profile, and ingredient composition of both diets are shown in **Table 1**. Diets were formulated and balanced in order to meet nutrient requirements in accordance with the FEDIAF (27) nutrient guidelines for dogs.

Venison was chosen as the primary protein source for this trial since it is one of the protein sources usually incorporated in commercial foods for dogs which show adverse food reactions; similarly, insect meal showed a similar potential (7). Nevertheless, venison meal is more expensive than other common sources of proteins as well as insect meal so far and, for these reasons, was deemed eligible for the comparison of the diets.

The trial was conducted according to the guidelines of Carciofi et al. (28) regarding the use of a marker method and the total collection method for assessing *in vivo* total tract apparent

Abbreviations: CTRL diet, venison meal-based diet/control diet; BSF diet, black soldier fly larvae-based diet/insect diet; BSF, black soldier fly; ME, metabolizable energy; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract/crude fat; CF, crude fiber; HPLC, high-performance liquid chromatography; ATTDC, apparent total tract digestibility coefficients; TFC, total fecal collection method; SEM, standard error of the mean; D, diet; M, method; D×M, interaction between diets and methods.

TABLE 1 | Ingredients and nutritional composition of the experimental diets.

	CTRL[a]		BSF[b]	
Ingredients	**(% as fed)**	**(% of DM)**	**(% as fed)**	**(% of DM)**
Potato meal	51.5		54	
Venison meal	40		-	
Black soldier fly meal	-		36.5	
Vitamin and mineral premix	3		3	
Oils and fats[c]	2.5		2	
Yeast (hydrolysate)	2		2	
Calcium carbonate	-		1.5	
Other ingredients[d]	1		1	
Nutrient and chemical composition[e]				
Dry matter	93.80	-	96.04	-
Organic matter	86.11	91.80	90.21	93.93
Crude protein	16.97	18.09	20.70	21.55
Ether extract	17.42	18.57	15.61	16.25
Crude fiber	5.77	6.15	4.09	4.26
Ash	7.69	8.20	5.83	6.07
Calcium	1.03	1.10	0.87	0.91
Phosphorus	0.93	0.99	0.53	0.55
Collagen	2.72	2.90	0.88	0.92
Hydroxyproline	0.34	0.36	0.11	0.11
Amino acidic profile[e]				
Aspartic acid		1.88		2.09
Serine		0.68		0.79
Glutamic acid		1.98		2.19
Glycine		1.14		1.01
Histidine		0.31		0.49
Arginine		0.86		1.02
Threonine		0.60		0.68
Alanine		0.87		1.15
Proline		1.12		1.07
Cysteine		0.15		0.16
Tyrosine		0.40		0.78
Valine		0.71		1.01
Methionine		0.23		0.39
Lysine		0.80		0.97
Isoleucine		0.53		0.69
Leucine		1.03		1.23
Phenylalanine		0.64		0.79
ME (MJ/kg)[f]	15.66		16.44	

[a]CTRL, control diet; [b]BSF, black soldier fly diet; [c]Poultry purified fat, sunflower oil; [d]Digest (hydrolyzed poultry liver), mineral, and vitamin pre-mix; [e]Analyzed; [f]Estimated according to FEDIAF (27).

digestibility. Chromium oxide (Cr_2O_3) was used as digestibility marker. It was added to a final concentration of 2.5 g/kg of diet. A 5-day test diet adaptation period preceded 5 days of feces collection during the experimental trial.

Food was weighed each day, divided into two equal portions, and given to the animals at 9 a.m. and 5 p.m. in stainless-steel bowls. Food quantity was administered considering maintenance energy requirements according to the FEDIAF equation (110 kcal $\times$ $BW^{0.75}$) (27). Bowls were removed before the next meal, and any uneaten food was weighed and recorded. Feces were collected twice daily, weighed, and kept frozen at −20°C until analysis.

Chemical Analyses

At the end of the collection period, pooled individual feces were thawed, homogenized, and freeze-dried. Feces samples were freeze-dried using a laboratory freeze dryer (5Pascal, Trezzano sul Naviglio, Italy). The process of lyophilization consisted of dry sublimation with water evaporation under low pressure (0.200 mbar) until the samples reached room temperature (25°C). Both the foods and freeze-dried feces were ground to pass through a 1-mm sieve and stored in airtight plastic containers for laboratory tests. The dry matter (DM) of the foods was determined by drying the samples at 103°C to constant weight. The foods and feces were analyzed according to the AOAC (29) standard procedures; thus, ash was determined by muffle furnace incineration (section 942.05), crude protein (CP) was ascertained using the Kjeldahl method (section 954.01), and ether extract (EE) was analyzed following acid hydrolysis (section 954.02). In addition, diet crude fiber (CF) was determined using the method described in section 962.09 (29), and amino acid content by HPLC (Waters Alliance System with a Waters 1525 Binary HPLC Pump, Waters 2707 Autosampler, and Waters 2475 Multi λ Fluorescence Detector, Milford, USA) after pre-column derivatization (30) in samples ground to pass a 0.5-mm sieve. The detection limit ranged from 2.9 to 20.1 pmol/μl depending on the amino acid. Tryptophan was not analyzed.

Samples of foods and feces were burnt to ashes and acid-digested in the microwave (31), prior to the determination of chromium concentrate by inductively coupled plasma optical emission spectrometry (ICP-OES). Calcium and phosphorus were also determined by ICP-OES in the absence of the previous incineration.

Hydroxyproline and the related collagen content were assessed according to the colorimetric method adapted by Kolar (32) and described in the AOAC (29) section 990.26. The acid hydrolysis of the sample was performed under heat; an oxidizing agent was added to the sample, and oxidized hydroxyproline was measured photometrically.

In vivo Digestibility Calculations

Apparent total tract digestibility coefficients (ATTDC) of the individual dietary elements of the two diets were calculated as follows:

a) Total fecal collection method (TFC):

$$\text{ATTDC } X_{diet}\ (\%) = [(\text{total } X_{diet} - \text{total} X_{feces})/\text{total } X_{diet}] \times 100$$

where X is the total contents of DM, organic matter (OM), CP, EE, ash, calcium, or phosphorus in the consumed food or feces produced (X_{diet} and X_{feces}, respectively);

b) Marker method (Cr_2O_3):

$$\text{ATTDC } X_{diet}\ (\%) = \{[(X/Cr_2O_3)_{diet} - (X/Cr_2O_3)_{feces}]/(X/Cr_2O_3)_{diet}\} \times 100$$

where X represents the concentrations of DM, OM, CP, EE, ash, calcium, or phosphorus in the diet or feces;

Cr_2O_3 represents the chromium oxide concentration in the diet or feces;

$(X/Cr_2O_3)_{diet}$ = ratio between nutrient (X) and Cr_2O_3 concentration in the diet;

$(X/Cr_2O_3)_{feces}$ = ratio between nutrient (X) and Cr_2O_3 concentration in the feces.

In vitro Digestibility

The *in vitro* digestibility of DM, CP, and OM of the food was determined (in triplets) employing the methods described by Hervera et al. (33, 34). The methods involve two phases: the first entails incubation for 2 h under conditions simulating gastric digestion (pH 2, 39°C, and inclusion of pepsin), whereas the second phase simulates 4 h of post-gastric digestion (pH 6.8, 39°C, and inclusion of a pancreatin preparation for enzymatic digestion). The resulting residue was filtered, dried, and weighed to determine the remaining DM content and incinerated to determine the residual OM content. Residual CP was determined by ascertaining the nitrogen content of the residue (using the Kjeldahl method) and considering a N:P conversion factor of 6.25. The *in vitro* digestibility of DM, OM, and CP was calculated as the difference between the amount of each initial nutrient in the sample vs. the undigested residue, divided by the initial nutrient content of the sample.

Estimated Digestibility

Data from the *in vitro* digestibility analyses were also used to estimate *in vivo* OM and CP digestibility according to the regression equations reported by Hervera et al. (33, 34):

Estimated digestibility of OM (%) $= -9.15 + 1.06 \times$ *in vitro* OM digestibility (%) (33);

Estimated digestibility of CP (%) $= 37.91 + 0.52 \times$ *in vitro* CP digestibility (%) (34).

Statistical Analysis

The statistical unit was the individual dog for *in vivo* digestibility trials, and the diet for *in vitro* digestibility trials. The comparisons between diets (CTRL vs. BSF) and methods (*in vivo* TFC vs. Cr_2O_3) were analyzed using two-way ANOVA, considering the diet (D) and the method (M) of *in vivo* digestibility calculation as the source of variation, respectively. Before testing for group and method differences, the normality of the data distribution and the homogeneity of variance were assessed by the means of the Shapiro–Wilk test and Levene test, respectively. The significance level was set at $p = 0.05$. A statistical trend was considered for $p \leq 0.10$. All statistical analyses were performed using R Software (version 3.6.1) (35).

RESULTS

The foods were well-accepted during all the trial lengths, and no episode of nausea or vomiting has been reported. The *in vivo* ATTDC digestibility results are summarized in **Table 2**. The two methods used to estimate *in vivo* digestibility (TFC and Cr_2O_3) showed similar results between the CTRL and BSF groups in relation to DM, OM, EE, ash, and phosphorus. The ATTDC of CF was significantly lower ($p < 0.001$) in the BSF diet compared with the CTRL diet. On the contrary, the ATTDC of calcium was significantly higher ($p < 0.05$) in the BSF compared with the CTRL diet. A statistical trend ($p = 0.066$) was observed for the ATTDC of CP, being higher in the animals fed the BSF compared with the CTRL diet.

No statistical differences were observed between the two ATTDC methods (TFC vs. Cr_2O_3). Furthermore, no statistical interaction between diets and methods was found.

The *in vitro* digestibility data and estimated *in vivo* digestibility results, obtained utilizing the regression equations described in Hervera et al. (33, 34), are reported in **Table 3**. The digestibility values for DM, OM, and CP obtained using the *in vitro* method were higher for both the CTRL and the BSF diet (by an average of +8.43, +5.25, and +6.08%, respectively) compared with those obtained using *in vivo* methods. The estimations of *in vivo* digestibility of OM and CP (based on *in vitro* data) were consistently higher than the data obtained using *in vivo* ATTDC methods: *in vitro* estimation of *in vivo* digestibility overestimated OM and CP digestibility by up to 4.0% and 9.8%, respectively, compared with the *in vivo* methods.

DISCUSSION

This study evaluated the nutritional quality of defatted BSF larvae meal as a potential sustainable novel raw material for pet food, to be integrated into extruded diets as a protein source. In addition, it explored the suitability of the *in vivo* marker method and the *in vitro* digestibility method with the traditional *in vivo* total collection method.

Although the control (containing venison meal) and insect-based diets were formulated to be isonitrogenous, our analysis showed CP content to be almost 4% lower in the former (16.97 vs. 20.70%, respectively); the discrepancy between the diets was nevertheless within the limits stipulated in the EU regulation 2017/2279 regarding "Tolerances for analytical constituents" (36). It is also important to remember that since chitin is a nitrogen-containing polysaccharide, this could also have led to a mild overestimation of the protein content in the BSF diet (6, 37).

We must also acknowledge that the higher crude protein content of the BSF diet compared with the CTRL diet could be an overestimation due to our use of a nitrogen to protein (N:P) conversion factor of 6.25. In fact, several authors recently pointed out that this conventionally used conversion factor may lead to the overestimation of protein content in a variety of feedstuffs (38, 39), including insect meals (40, 41). Furthermore, although Finke et al. (42) estimated that the amount of nitrogen in insect chitin would not significantly affect the total amount of nitrogen, other authors support the hypothesis that the presence of non-protein nitrogen (NPN) in insect CP could cause the overestimation of CP (40, 41).

In our trial, the ATTDC of DM, OM, and EE were similar in both BSF and CTRL groups, whereas the ATTDC of CP were higher in the BSF vs. CTRL group. A similar result was obtained by Lei et al. (43), where increasing levels of BSF meal inclusion (at 0, 1, and 2%) in Beagle dog rations raised nitrogen digestibility, whereas EE digestibility remained similar to that of the control diet. However, Gariglio et al. (18) observed that up to 9% BSF

TABLE 2 | Comparison of the *in vivo* digestibility using the total fecal collection method (TFC) and *in vivo* digestibility with marker (Cr_2O_3) in six dogs (mean values are presented).

	TFC[a]		Cr_2O_3		SEM	*p*-value		
	CTRL[b]	BSF[c]	CTRL[b]	BSF[c]		D[d]	M[e]	D × M[f]
***In vivo* digestibility (%)**								
Dry matter	82.11	82.17	83.05	83.83	0.52	0.698	0.241	0.740
Organic matter	86.23	85.04	86.98	86.46	0.45	0.358	0.247	0.719
Crude protein	72.41	75.80	74.04	78.22	1.01	0.066	0.311	0.842
Ether extract	96.58	96.40	96.72	96.75	0.14	0.800	0.411	0.717
Crude fiber	43.13	18.83	45.78	23.60	3.18	<0.001	0.393	0.798
Ash	32.73	35.76	35.88	41.39	1.95	0.292	0.280	0.757
Calcium	12.16	24.88	19.19	31.62	2.61	0.018	0.162	0.976
Phosphorus	20.77	21.46	26.17	25.83	2.00	0.946	0.280	0.908

[a]*TFC, total fecal collection;* [b]*CTRL, control diet;* [c]*BSF, black soldier fly diet;* [d]*D, diet;* [e]*M, method;* [f]*D×M, diets and method interaction.*

TABLE 3 | Comparison of the *in vitro* digestibility of the two diets (CTRL vs. BSF) and estimated *in vivo* digestibility based on the *in vitro* results.

	CTRL[a]	BSF[b]
***In vitro* digestibility (%)**		
Dry matter	90.65	91.79
Organic matter	90.82	92.04
Crude protein	80.06	82.33
Estimated *in vivo* digestibility (%) based on the *vitro* results		
Organic matter[c]	87.12	88.41
Crude protein[d]	79.54	80.72

[a]*CTRL, control diet;* [b]*BSF, Black soldier fly diet;* [c]*According to Hervera et al. (33) for OM estimation;* [d]*According to Hervera et al. (34) for CP estimation.*

meal inclusion in the diet of growing Muscovy ducks did not change diet digestibility, with the exception of the ATTDC of EE, which was improved in BSF groups. In line with these data, Biasato et al. (23) observed no change in the ATTDC of BSF diets (up to 10% inclusion) in growing piglets. Similarly, Freel et al. (44) did not notice any difference in ATTDC of DM, CP, and EE in a trial involving 56 Beagle dogs fed with diets containing graded levels of BSF meal (5.0, 10.0, and 20.0%) and BSF oil (1.0, 2.5, 5.0%). Furthermore, in a study where BSF meal completely replaced soybean meal in the diet of laying hens, Cutrignelli et al. (45) found BSF to correlate with lower crude protein digestibility, whereas lipid digestibility remained unaffected. Likewise, Kröger et al. (46), in a study involving 12 Beagles, observed a decrease in ATTDC of CP in the BSF group compared to the control group, while the ATTDC of DM was increased when dogs were fed the diet containing the BSF meal (at 20.0% of inclusion). This result could be explained by differing levels of chitin, which can negatively affect protein digestibility (47). Indeed, the reported difference in fiber digestibility between the diets supports this result and explanation, since chitin gets recognized as part of the crude fiber fraction during the analysis (48). Furthermore, the mean values of crude protein ATTDC (for BSF-based diets) observed in our study were in line with those found in Kröger et al. (46) but below those recovered in Freel et al. (44).

Hydroxyproline can be used as an index of protein quality (49), due to its being a marker of collagen content (50). The levels of collagen and of hydroxyproline were higher in the control diet compared with the BSF diet, probably due to the fact that collagen is limited in insect meal compared to that in vertebrate protein meal. This could also explain the higher level of digestibility of the BSF diet compared with the control diet, at least with regard to crude protein digestibility, since the net protein utilization of collagen is zero (51). Collagen content also influences the N:P ratio of protein sources, and consequently the real CP content of the diets, in particular that of the control diet (39). It may also be speculated that the control diet had a decreased crude protein digestibility due to the higher ash content; however, high levels of crude ash did not appear to decrease protein digestibility, as previously reported by Bockskopf and Kamphues (52).

The difference in calcium digestibility could be due to the use of different ingredients to adjust the calcium level of the diets. Indeed, calcium carbonate was added to the BSF diet to obtain the minimum requirements for dogs, whereas in the CTRL diet the calcium requirements were satisfied by the presence of ground bone in the venison meal (thus avoiding the need for any calcium salt addition), and this could have led to the discrepancy. Interestingly, Lei et al. (43) noticed significant increases in the level of calcium in the blood of beagles as the BSF larvae meal content of their food was increased. This result points toward a potential increase in the bioavailability of this macro-element that depends on the inclusion of BSF larvae meal in the diet; however, further investigations are required to confirm and understand the basis of any possible relationship.

It is important to note that no statistical differences were observed between the ATTDC values determined using the marker method and the total collection method for both CTRL and BSF diets, confirming the validity of the marker method as an alternative to the total collection method (28). The values of *in vitro* DM, OM, and CP digestibility were also similar to the results obtained with the two *in vivo* methods, despite being, in line with the previous literature (33, 34), slightly overestimated in the former. We also evaluated whether the equations for the

estimation of *in vivo* crude protein and OM digestibility, utilizing *in vitro* digestibility data, as described in Hervera et al. (33, 34), fitted with the results obtained in this study (shown in **Table 3**). Since the predictive equations proposed were only used to assess feedstuff based on vertebrates and, to our knowledge, no other study inspected if they could be applicable to invertebrates, we decided to include these findings. For both the venison and insect diet, the predictive equations gave slightly overestimated values compared with the mean of the *in vivo* digestibility results, even though they were substantially similar from a nutritional perspective. Indeed, the discrepancy between the crude protein digestibility estimated using the equation and the *in vivo* crude protein digestibility results ranged from 3.2 to 9.8%, whereas the overestimation of the OM digestibility ranged from 0.2 to 4.0%, with lower deviations and a narrower range. According to these results, predictive equations utilizing *in vitro* digestibility values appear to constitute a valid tool for the analysis of feedstuff digestibility and therefore offer a means to reduce, if not avoid, the use of live animals.

CONCLUSIONS

The present study suggests that the inclusion of BSF in extruded diets for dogs (at 36.5%) offers a promising alternative source of dietary protein for this species, in particular in relation to the digestibility profile of crude protein, crude fat, and OM. Our findings also highlight the need for further studies in order to understand the effect of chitin on fiber digestibility and mineral absorption in a BSF-based diet.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethic Committee of Turin University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

AS, EP, LPe, and LPr conceived and designed the experiment. EP and NR collected the experimental data. EV, FH, JM, JN, and SM carried out the chemical analyses. AS, LPe, and UA performed the statistical analysis. All the authors interpreted the data. AS, LPe, and LPr wrote the first draft of the manuscript. All the authors reviewed the manuscript for intellectual content and gave approval for the final version to be published.

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Dietary *Hermetia illucens* Larvae Replacement Alleviates Diarrhea and Improves Intestinal Barrier Function in Weaned Piglets Challenged With Enterotoxigenic *Escherichia coli* K88

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A high-quality protein substitute, *Hermetia illucens* (black soldier fly) larvae powder, is rich in protein and often used in animal feed. This study aimed to investigate the feasibility and optimal ratio of replacing fish meal with *H. illucens* larvae in weaned piglets and to demonstrate the effects on piglets' growth performance, intestinal microflora and immune performance. Forty-eight female weaned piglets were randomly classified into three groups. Each group consisted of eight pens (replicates), with two piglets per pen. Three groups containing different proportions of *H. illucens* larvae (0, 4, and 8%) were referred to as C, HI4, and HI8. We first designed a 28-day feeding experiment to detect growth performance; after that, the piglets were induced with oral gavage of enterotoxigenic *Escherichia coli* K88 (ETEC K88) and recording diarrhea on day 29 of the experiment. Samples were taken on the 32nd day to detect the effect of *H. illucens* larvae on the immune performance of the weaned piglets. *H. illucens* larvae replacement did not cause any obvious change in the growth performance nether in HI4 nor in HI8 of weaned piglets with 28 d feeding stage. *H. illucens* larvae could improve the intestinal health of weaned piglets by increasing the content of *Lactobacillus* and reducing the content of *Streptococcus*. Compared with C+K88 group, the diarrhea rate was attenuated for the *H. illucens* supplemented group. The integrity of ileum villi in HI4+K88 and HI8+K88 groups was better than that in C+K88 group, and the villi in C+K88 group were severely damaged. The expression of *IL-10*, *Occludin* and *Claudin-3* in the intestinal mucosa of the HI4+K88 group and HI8+K88 group were significantly increased ($P < 0.05$), and the expression of *TNF-α* was significantly decreased ($P < 0.05$) compared with the C+K88 group. The results of immunoblotting also validated that the same ETEC K88 treatment of weaned piglets enhanced the expression of tight junction protein in the intestinal mucosa

of the *H. illucens* addition group. ETEC-induced diarrhea will be reduced by the diet of weaned piglets containing *H. illucens* larvae, ameliorating the immune performance of piglets. Our results indicates that the optimal dosage of *H. illucens* replacement in weaned piglets is 4%.

Keywords: *Hermetia illucens* larvae, weaned piglet, growth performance, intestinal health, immune performance

INTRODUCTION

A saprophytic hydrofidae insect, *Hermetia illucens* (Black soldier fly), can feed on poultry manure and domestic garbage (1). Being rich in protein, amino acids, lauric acid, and minerals, its larvae are a feed material with a high nutritional value. It can be supplemented in livestock and poultry feed production instead of soybean meal (2). Animals exhibited better growth and digestion performances by substituting *H. illucens* larvae meal for soybean meal or fish meal in the feed (3). Triggered by foreign bacteria, *H. illucens* will activate the immune system and produce antimicrobial peptides (AMPs) (4, 5). *H. illucens* larvae contain active antibacterial substances, such as chitin, thereby inhibiting bacterial reproduction and increasing the effect of beneficial bacteria in the intestine (6). A significant impact on the gut microbiota and colon metabolites of finishing pigs was obtained by using *H. illucens* larvae powder as a protein source for pig feed (7). When employed as a source of dietary protein for broiler quails, the saturated fatty acid of quail is increased, and the quail's oxidative emergency state is reduced (8). *H. illucens* larvae are rich in fat, mainly lauric acid (C12:0), which has an inhibitory effect on gram-positive bacteria.

Supplementing *H. illucens* larvae to feed can significantly alter the animal intestinal flora and microbial metabolites (SCFAs), which is beneficial to animal growth (9). Diet modification will also affect the microbiota of the pig's digestive tract. Some bacterial fermentation products such as SCFAs are beneficial to the physiological functions of the intestines, for example, providing energy for epithelial cells, maintaining the morphology and function of colonic epithelial cells, and inhibiting the growth of pathogens (10). Intestinal barrier function genes and protein expression levels are important for intestinal health. ETEC infection affects the expression of aquaporin and ion channel protein (11). Diarrhea in weaned piglets is usually accompanied by intestinal inflammation and barrier damage (12). The changes in cytokine and tight junction protein expression in the intestinal mucosa of weaned piglets are closely related to diarrhea (13). However, information is scarce on whether larvae protects against diarrhea in weaned piglets caused by ETEC.

Similarly, there are limited studies that explore the effect of *H. illucens* larvae powder as a source of feed protein on the growth performance and gut microbes of weaned piglets. Therefore, our experiment employed different proportions of *H. illucens* larvae powder instead of fish meal to investigate its effects on weaned piglets. We first fed the piglets with feed containing *H. illucens* larvae powder, followed by oral ETEC K88. Thereafter, we evaluated the growth performance, immune performance, and intestinal morphology of weaned piglets, explored the effect of ETEC K88 on the expression of aquaporin and ion transporter, and finally, elucidated the positive effects of *H. illucens* feed on the challenge of ETEC K88 to weaned piglets.

Abbreviations: HI, Hermetia illucens; BW, Body weight; Cd, Crypt depth; CP, Crude protein; DE, Digestible energy; DM, Dry matter; EE, Ether extract; ADG, Average daily gain; F/G, Feed/Gain; ADFI, Average daily feed intake; Vh, Villus height; Cd, Crypt depth; Vh/Cd, Villus height to crypt depth ratio; PcoA, Principal coordinate analysis; SCFAs, Short-chain fatty acids.

MATERIALS AND METHODS

This study was approved by the animal ethics committee of Zhongkai University of agricultural and engineering. The protocol was approved by the Medical Experimental Animal Center of Guangdong Province (Permit Number: 12-179).

Analysis of the Chemical Composition of *H. illucens* Larvae Powder

Five-instar dry *H. illucens* larvae were purchased from Guangzhou AnRuiJie Protection Technology Co., Ltd. (Guangzhou, Guangdong, China). The common nutrients (dry matter, DM; crude protein, CP; ether extract, EE; Ash) in the experimental diet and *H. illucens* larvae were analyzed following the procedures of the Official Association of Analytical Chemists (AOAC) (14). The chitin content was estimated according to Finke (15). The larvae powder was digested with 6 mol/L HCl at 110°C for 24 h and amino acid (AA) concentration was determined by HPLC. According to the AOAC protocols, the concentration of methionine was estimated after oxidation with formic acid, whereas the concentration of tryptophan was obtained after alkaline hydrolysis (14). The chemical composition, energy content, mineral content, and amino acid content are listed in detail in **Table 1**.

Bacterial Strains

ETEC K88 was original clinically separated and identified (16), and stored in our laboratory. ETEC K88 strains were streaked inoculated on LB plates, and cultured overnight at 37°C. The next day, a single colony was inoculated into LB (Luria-Bertani) broth medium. K88 strains were diluted with PBS (phosphate buffer solution) to $\sim 10^9$ CFU/mL for the challenge by oral gavage at a dose of 50 mL/pig. (17, 18).

Animal, Diets and Experimental Design

Forty-eight young female weaned piglets (Duroc × Landrace × Large White) with initial body weights (BW) 7.68 ± 0.26 kg were randomly classified into three groups. Each group consisted of eight pens (replicates), with two piglets per pen. The present

TABLE 1 | The main nutrients of *Hermetia illucens* larvae.

Items	*Hermetia illucens* larvae
Analyzed composition	
DM, %	94.90
CP, %	33.39
Gross energy, MJ/Kg	22.40
EE, %	40.60
Ash, %	10.90
Chitin, %	3.20
Mineral composition, %	
Total P	0.76
Ca	2.77
Essential amino acids, %	
Lysine	2.00
Methionine+Cystine	0.62
Isoleucine	1.47
Tryptophan	2.35
Valine	1.87
Threonine	1.27
Arginine	1.63
Phenylalanine	1.46
Histidine	1.00
Alanine	2.96
Non-essential amino acids	
Aspartate	2.71
Glutamate	4.80
Glycine	2.20
Serine	1.40
Tyrosine	1.77

TABLE 2 | Composition and nutrient level of the basal diet (as-fed basis).

Item[a]	C group	HI4 group	HI8 group
Ingredient, %			
Corn	52.98	51.53	50.08
Soybean meal	9.00	9.00	9.00
Hermetia illucens larvae	0	4.00	8.00
Fish meal	4.00	2.00	0
Extruded soybean meal	9.00	9.34	9.68
Soy protein concentrate	6.00	6.00	6.00
Whey powder	10.00	10.00	10.00
Soybean oil	2.00	1.18	0.36
White granulated sugar (sucrose)	2.00	2.00	2.00
DL-Met (99%)	0.36	0.38	0.40
Lys-HCl (78%)	0.80	0.82	0.84
Thr (98%)	0.38	0.38	0.38
Trp (99%)	0.08	0.08	0.08
Stone powder (36%)	0.65	0.44	0.22
Dicalcium phosphate	1.10	1.20	1.30
Choline chloride (50%)	0.20	0.20	0.20
Salt	0.45	0.45	0.45
Premix (multi-dimensional and multi-ore)[b]	1.00	1.00	1.00
Total	100	100	100
Energy and nutrient composition[c]			
DE (MJ/Kg)	14.59	14.59	14.59
CP (%)	19.15	19.15	19.15
Ca (%)	0.82	0.82	0.82
Total P (%)	0.66	0.66	0.66
Lys (%)	1.58	1.58	1.58
Met+Cys (%)	0.90	0.90	0.90
Thr (%)	1.01	1.01	1.01
Trp (%)	0.27	0.27	0.27

[a] *C is the control diet, diets HI4, HI8 contained 4 and 8% Hermetia illucens larvae in an amount providing similar nitrogen to the diet as control diet, respectively.*

[b] *Provided per kilogram of complete diet: vitamin A, 12,400 IU; vitamin D 2,800 IU; vitamin E, 130 mg; vitamin K_3, 5 mg; vitamin B_1, 3 mg; vitamin B_2, 10 mg; vitamin B_3, 40 mg; vitamin B_6, 8 mg; vitamin B_{12}, 0.04 mg; niacin, 45 mg; pantothenic acid, 15 mg; folic acid, 1 mg; biotin, 0.08 mg; Fe ($FeSO_4$), 120 mg; Cu ($CuSO_4$), 16 mg; I (CaI_2O_6), 0.7 mg; Se (Na_2SeO_3), 0.48 mg; Zn ($ZnSO_4$), 120 mg; Mn ($MnSO_4$), 120 mg;.*

[c] *Calculated values unless indicated otherwise.*

study investigated three different feeding patterns containing different proportions of *H. illucens* larvae powder (0, 4, 8%) named C, HI4, HI8. The formulas of all experimental diets met or exceeded the nutritional recommendations of the National Research Council 2012 (**Table 2**). During the feeding period, the piglets were allowed *ad libitum* access to feed and water.

The experiment was carried out for 32 days. For the first 28 days, piglets were fed with different proportions of *H. illucens* larvae powder. The body weight was measured once a week on an empty stomach; the feed intake was recorded every day. Then orally gavage ETEC K88 (50×10^9 CFU/mL) was provided on the 29th day. Eight weaned piglets in each group were randomly selected for the oral gavage experiment.

Growth Performance

Feed consumption was recorded daily during the trial. At 08:00 on day 1, 8, 15, 22 and 28, all piglets were weighed to determine initial body weight (BW) and final BW. Based on these data, the ADG, ADFI, and F/G were calculated.

Diarrhea Score

On the second stage after oral administration of ETEC K88 (d 29–32), diarrhea was observed at 08:30 and 16:30 daily; the standard grade score of diarrhea was as follows: solid = 0, semisolid = 1, semiliquid = 2 and liquid = 3 (19). Diarrhea index is the sum of repeated fecal scores during the trial period / total number of repeated feeding during the trial period. The number of piglets with diarrhea was determined and used to calculate the incidence of diarrhea, i.e., the ratio of the number of piglets with diarrhea to the total number of piglets.

Sampling and Processing

At 08:00 on the 25th day of the trial, the feces of each group were also collected for 16S rRNA detection. Polyvinyl chloride plastic bags were attached to the anus of weaned piglets to prevent fecal contamination and falling off on the ground. Feces were put in centrifuged tubes quickly and saved in liquid nitrogen. On day 28 of the trial, and 5 mL of blood was collected from

the anterior vena cava, and serum was separated for serum biochemical detection.

After fasting for 12 h before slaughter, on the 32nd day of the experiment, 5 mL of blood was collected. Antioxidant enzymes and immunoglobulin in serum were detected by ELISA. Four pigs in each group were euthanized and slaughtered. The intestinal tissue was isolated and washed with PBS, the intestinal tissue was collected, and the intestinal mucosa was scraped (20). A part of the ileum tissue was fixed in 4% formaldehyde for subsequent section analysis. Other samples were then quickly frozen in liquid nitrogen and stored in an ultra-low temperature refrigerator at −80°C for future use.

Serum Parameters

Eight serum samples were collected from each group on day 28. The serum biochemical indicators including TP, ALB, GLB, AST, ALT, TG, TC, UREA, P, and Ca. were evaluated by automatic biochemical analyzer BS-240VET (Mindray Bio, Shenzhen, China).

Analysis of Ileum Morphology

The fixed ileum segment was dehydrated, paraffin embedding was performed, sectioned for intestinal morphology, and the sections were submitted to Hematoxylin & Eosin staining (21). Images were obtained using a Leica microscope (DM500, Leica, Wetzlar, Germany). Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD) was used to measure the villus height (Vh) and crypt depth (Cd). Morphometric measurement of 10 well-oriented and intact villi and 10 crypts selected from the ileum (22).

DNA Extraction and Sequencing

Feces samples from each group of eight piglets were collected for nucleic acid extraction. Total DNA in the samples was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy), following the manufacturer's instructions. The 16S rRNA gene V3–V4 region was amplified by gDNA to evaluate the microbiota (23). PCR products were tested on the Illumina MiSeq platform (Majorbio, Shanghai, China), following the instructions (24).

Antioxidant Enzyme Activity Determination and Immunoglobulin Determination

Antioxidant enzyme activity in the serum was measured using a commercial assay kit (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China), such as CAT (Catalase, A007–1–1), POD (Peroxidase, A084–2–1), strictly following the manufacturer's instructions. The manufacturer's instructions were strictly followed while using Porcine IgG ELISA KIT and Porcine IgA ELISA KIT (LJ-871426, LJ-871428 Lingjiang Biotechnology, Guangzhou, China).

RNA Extraction From Intestinal Mucosa and Quantitative PCR

Of jejunal mucosa, ileum mucosa and colon mucosa, 0.1 g of the sample was taken, and autoclave magnetic beads were added. Tissues were homogenized with a Tissue Homogenizer and total RNA extracted by TRIzol reagent method (Takara Biotechnology, Dalian, China) (25). The quality of RNA was estimated with a microspectrophotometer Q3000 (Quawell Technology, Inc., USA) and Ratio (OD260: OD280) from 1.8 to 2.0. RNA (1 μg) was reverse transcribed into cDNA with the Synthesis Kit (Takara Biotechnology, Dalian, China). Real-time PCR of the target genes and GAPDH was conducted on CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with TB Green™ Premix Ex Taq™ (Takara Biotechnology, Dalian, China). The primers related to the evaluation of the immune status and the tight junctions are detailed in **Table 3**. Reagents were added according to the operating instructions. The circulation parameters used were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 34 s; 95°C for 15 s; 65°C for 5 s; 95°C for 5 s. The expression levels of each target gene were normalized based on the housekeeping gene GAPDH, according to the following formula $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{treatment} - (Ct_{target} - Ct_{GAPDH})_{control}$.

TABLE 3 | Primers for real-time PCR.

Gene	Sequences	Length (bp)
IL-8	Forward: 5′-CCAGTGCATAAATACGCATTCCA −3′	138
	Reverse: 5′-GGGTCCAGGCAGACCTCTTTT −3′	
IL-10	Forward: 5′-GACCAGATGGGCGACTTGTTG −3′	160
	Reverse: 5′-TCGGCTTTGACATTGGCTAC −3′	
TNF-α	Forward: 5′-GCCTCTTCTCCTTCCTCCTG −3′	193
	Reverse: 5′-TCGGCTTTGACATTGGCTAC −3′	
IFN-γ	Forward: 5′-TCCAGCGCAAAGCCATCAGTG−3′	111
	Reverse: 5′-ATGCTCTCTGGCCTTGGAACATAGT−3′	
ZO-1	Forward: 5′-GCCATCCACTCCTGCCTAT−3′	133
	Reverse: 5′-CGGGACCTGCTCATAACTTC−3′	
Occludin	Forward: 5′-TGGCTGCCTTCTGCTTCATTGC−3′	131
	Reverse: 5′-GAACACCATCACACCCAGGATAG−3′	
Claudin-3	Forward: 5′-GCCAAAGCCAAGATCCTCTA−3′	87
	Reverse: 5′-GTAGTCCTTGCGGTCGTAGG−3′	
AQP1	Forward: 5′-GACACCTGCTGGCGATTGACTAC−3′	90
	Reverse: 5′-GGTCCTGGAAGTTGTGCGTGATC−3′	
AQP3	Forward: 5′-TGACCTTCGCTATGTGCTTCC−3′	212
	Reverse: 5′-GTCCAAGTGTCCAGAGGGGTAG−3′	
AQP7	Forward: 5′-CCCGTGCCTCCAAGATGA-3′	58
	Reverse: 5′-CGCATTATTGTTTGCATCTTTGA-3′	
AQP9	Forward: 5′-TGTCATTGGCCTCCTGATTG-3′	62
	Reverse: 5′-TGGCACAGCCACTGTTCATC-3′	
NKCC1	Forward: 5′-CCAATGCTGTTGCAGTTGCT-3′	364
	Reverse: 5′- TGGGCTTCTTGCTCTCCAAG-3′	
NHE3	Forward: 5′-AGCTGGAGATCATAGACCAGGT−3′	147
	Reverse: 5′-CGGTGAAGAAGATGACGATGAG−3′	
CFTR	Forward: 5′-ACTATGGACCCTTCGAGCCT−3′	123
	Reverse: 5′-CGCATTTGGAACCAGCGTAG−3′	
GAPDH	Forward: 5′-GCCATCACTGCCACCCAGAA−3′	153
	Reverse: 5′-GCCAGTGAGCTTCCCGTTGA−3′	

IL, Interleukin; TNF-α, Tumor necrosis factor-α; IFN-γ, Interferon-γ; ZO-1, Zona occludens protein 1; AQP, aquaporins; NKCC1, Na-K-Cl cotransporter; NHE3, Na+/H+ exchanger 3; CFTR, cystic fibrosis transmembrane conductance regulator.

TABLE 4 | The ADG, ADFI, and F/G conditions of weaned piglets fed with different proportions of *Hermetia illucens* larvae for 7, 14, 21, 28 d ($n = 16$).

Items	C group	HI4 group	HI8 group	*P*-value		
				Inter-group	Linear	Quadratic
1–7 d						
ADG/g	209.05 ± 66.8	180.00 ± 60.80	181.91 ± 56.55	0.360	0.234	0.431
ADFI/g	319.81 ± 64.23	277.91 ± 55.81	304.67 ± 55.33	0.417	0.635	0.222
F/G	1.53 ± 0.30	1.61 ± 0.32	1.67 ± 0.30	0.691	0.396	0.972
8–14d						
ADG/g	268.57 ± 83.37	282.86 ± 102.61	240.48 ± 59.86	0.379	0.364	0.291
ADFI/g	480.57 ± 38.29^{a}	452.48 ± 32.79^{ab}	436.67 ± 28.45^{b}	0.070	0.024	0.696
F/G	1.79 ± 0.14^{a}	1.59 ± 0.11^{b}	1.82 ± 0.11^{a}	0.009	0.669	0.003
15–21 d						
ADG/g	337.10 ± 94.46	283.33 ± 96.70	267.14 ± 91.54	0.116	0.048	0.531
ADFI/g	551.14 ± 48.78^{a}	512.76 ± 41.76^{ab}	494.48 ± 26.02^{b}	0.047	0.017	0.589
F/G	1.65 ± 0.14^{b}	1.80 ± 0.14^{a}	1.85 ± 0.09^{a}	0.022	0.009	0.336
22–28d						
ADG/g	287.14 ± 133.66	292.86 ± 79.08	345.20 ± 111.28	0.715	0.444	0.779
ADFI/g	644.00 ± 39.32	597.05 ± 39.45	602.03 ± 40.88	0.079	0.064	0.177
F/G	2.24 ± 0.13^{a}	2.03 ± 0.13^{b}	1.76 ± 0.13^{c}	0.001	0.0001	0.514
1–28 d						
Initial BW/kg	7.68 ± 0.78	7.67 ± 0.83	7.69 ± 0.75	0.999	0.991	0.963
Final BW/kg	15.38 ± 2.89	14.90 ± 2.33	14.90 ± 2.22	0.832	0.604	0.758
ADG/g	274.88 ± 51.99	257.98 ± 56.93	250.88 ± 55.12	0.820	0.550	0.887
ADFI/g	498.88 ± 136.86	460.05 ± 135.13	459.52 ± 123.89	0.891	0.683	0.818
F/G	1.80 ± 0.31	1.76 ± 0.21	1.77 ± 0.08	0.970	0.873	0.756

In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different letter superscripts mean significant difference ($P < 0.05$). Each group consisted of eight pens (replicates), with two piglets per pen.

Western Blotting

The jejunal mucosa, ileum mucosa, and colon mucosa of pigs were homogenized in tissue lysate containing protease inhibitors. The homogenate was incubated at 4°C for 30 min to promote lysis. The lysate was centrifuged at a speed of 12,000 r/min for 20 min, and the supernatant collected served as the total protein for the western blotting. Subsequently, Western blotting was performed according to the standard protocol (26). *β-Actin* antibody (1:1,000; CST, 13E5–4970), *Occludin* (1:1,000; Abcam, ab167161), *Claudin-3* (1:1,000; Abcam, ab15102), goat anti-rabbit IgG (1:5,000, Boster, BA1055) were used.

Statistical Analysis

The data of growth performance, serum biochemical index were analyzed using the IBM SPSS Statistics V25.0 software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for linear and quadratic curve analysis, and Duncan's method was used for multiple comparisons to evaluate growth performance and serum biochemical indicators. The results of qPCR and western blotting were reflected as mean ± standard deviation (SD). Quantitative PCR and western blotting were triplicates, and the representative results were shown. Statistical analysis was done with the help of one-way analysis of variance (ANOVA), analyzed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). The *P*-value < 0.05 was considered statistically significant.

RESULTS

Effect of Dietary *H. illucens* Larvae on Growth Performance of Weaned Piglets

The animals were in good health since the initiation of the experiment. In this experiment, the piglets were growing well without death. The ADG, ADFI, and F/G of each group of piglets are given in **Table 4**. There was no difference among the three groups in ADG, ADFI, F/G for 1–28 days. However, in 8–14 days, the F/G of the HI4 group was significantly lower than that of other groups ($P < 0.05$); a significant difference was also noted, in 22–28 days, between the F/G of the HI4 and HI8 groups from the C group ($P < 0.05$), which showed a linear response to increasing H. illucens larvae levels. All these results indicates that replace fish meal with H. illucens larva meal either with 4 or 8% in weaned piglets diet sustain a similar growth performance.

Effect of Dietary *H. illucens* Larvae on Serum Biochemical Parameters of Weaned Piglets

Table 5 demonstrated non-significant differences in GLB and AST between the three groups; however, the TP content of the HI4 group and HI8 group was significantly different from the C group ($P < 0.05$). *H. illucens* larvae powder significantly increased the content of phosphorus and calcium in serum (*P*

TABLE 5 | Effects of *Hermetia illucens* larvae on serum biochemical parameters of weaned piglets ($n = 8$).

Items	C group	HI4 group	HI8 group	*P*-value		
				Inter-group	Linear	Quadratic
TP (g/L)	45.96 ± 3.91^{b}	53.50 ± 3.69^{a}	54.56 ± 7.18^{a}	0.006	0.003	0.164
ALB (g/L)	26.07 ± 3.54^{b}	29.69 ± 3.52^{ab}	30.76 ± 3.91^{a}	0.046	0.018	0.133
GLB (g/L)	19.88 ± 3.58	23.81 ± 3.85	23.80 ± 5.81	0.161	0.099	0.327
AST (U/L)	44.66 ± 3.67	44.63 ± 3.59	45.05 ± 3.48	0.424	0.225	0.642
ALT (U/L)	44.76 ± 9.25^{b}	51.71 ± 8.83^{ab}	69.10 ± 26.44^{a}	0.026	0.009	0.485
TG (mmol/L)	0.25 ± 0.06^{b}	0.27 ± 0.07^{b}	0.37 ± 0.11^{a}	0.026	0.014	0.226
TC (mmol/L)	1.70 ± 0.26^{b}	1.96 ± 0.14^{ab}	2.18 ± 0.41^{a}	0.013	0.003	0.903
UREA (mmol/L)	2.53 ± 0.61^{b}	2.74 ± 0.86^{ab}	3.42 ± 0.87^{a}	0.086	0.036	0.490
P (mmol/L)	2.36 ± 0.44^{b}	3.07 ± 0.26^{a}	2.90 ± 0.39^{a}	0.003	0.009	0.013
Ca (mmol/L)	2.00 ± 0.23^{b}	2.32 ± 0.15^{a}	2.36 ± 0.22^{a}	0.004	0.002	0.129

In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different letter superscripts mean significant difference ($P < 0.05$). TP (Crude protein), ALB (Albumin), GLB (Globulin), AST (Aspartate aminotransferase), ALT (alanine aminotransferase), TG (Triglyceride), TC (Total cholesterol), P (Phosphorus), Ca (Calcium), and UREA.

< 0.05) compared with the C group. TP, ALB, ALT, TG, TC, UREA, and Ca, which showed a linear response, respectively, to increasing *H. illucens* meal levels ($P < 0.05$, with the maximum corresponding to the inclusion of 8% of *H. illucens* meal). *P* which showed a linear and quadratic response to increasing *H. illucens* meal levels ($P < 0.05$, with the maximum corresponding to the inclusion of 4% *H. illucens* meal).

Effects of Dietary *H. illucens* Larvae on the Composition and Diversity of Fecal Microorganisms in Weaned Piglets

16S rRNA Illumina MiSeq sequencing revealed the microbial composition in the feces of the piglets treated by *H. illucens* larvae. In this study, 65863 effective sequences from 24 samples were screened for subsequent analysis, with an average of 482 operation taxonomic units (OTUs) per sample in fecal. As illustrated in **Figure 1A**, there was no significant difference in Shannon, Ace, Chao1, Simpson. However, the PCoA with the binary-Jaccard distance results validated that the HI4 group and HI8 group were separate from the C group (**Figure 1B**). The outcome of the analysis between the groups using the PERMANOVA test revealed that $R^2 = 0.17$, $P = 0.001$.

At the phylum level (**Figure 1C**), Firmicutes and Bacteroidetes are two advantageous categories, the contents of Firmicutes in the C group, HI4 group, and HI8 group were 67.21, 69.34, and 70.92%, respectively. The contents of Bacteroidetes in the C group, HI4 group, and HI8 group were 26.55, 23.94, and 23.07%, respectively. The next two most dominant phyla, Proteobacteria and Actinobacteria, accounted for 2.41 and 2.63% in the C group, 1.84 and 3.52% in the HI4 group, and 1.68 and 2.48% in the HI8 group, respectively.

At the family level, the content of Lactobacillaceae in the HI4 group and HI8 group was higher than that in the C group; the most significant difference was reflected between HI8 and C groups ($P < 0.05$). On the other hand, significantly lower content of both Streptococcaceae ($P < 0.05$) and Staphylococcaceae ($P < 0.01$) was evident in the HI4 group and HI8 group than that in the C group (**Figure 2**).

The 10 most predominant genera at the genus level were *Lactobacillus*, *Megasphaera*, *Prevotella*, uncultured_Muribaculaceae, *Streptococcus*, *Agathobacter*, *Subdoligranulum*, *Prevotella_7*, uncultured_Prevotellaceae, uncultured_Veillonellaceae. The levels of *Lactobacillus* in the HI4 group and HI8 group were higher than those in the C group. In contrast, the levels of *Streptococcus* were significantly lower than those in the C group ($P < 0.05$). The relative abundance of 16S rRNA gene sequence in pig feces was represented in **Supplementary Figure 1**, and the heat map portrayed the 20 species of bacteria most dominant at the genus level in the feces.

Effect of Dietary *H. illucens* Larvae on Diarrhea Rate of Weaned Piglets Challenged With ETEC K88

After inoculating piglets with ETEC K88, each group manifested different rates of diarrhea. As depicted in **Table 6**, the incidence of diarrhea was 50% in the C+K88 group, 37.5% in the HI4+K88 group, and 25% in the HI8+K88 group.

Effect of Dietary *H. illucens* Larvae on Ileum Morphology of Weaned Piglets Challenged With ETEC K88

The morphology of the ileum is sketched in **Figure 3** and **Table 7**. We made paraffin sections of the ileum and found that the intestinal morphological integrity of the HI4+K88 and HI8+K88 groups was superior to that of the C+K88 group. As observed in **Figure 3**, the ileum villi in the C+K88 group witnessed the most severe damage with shortened and blunt, deep crypts. However, the integrity of ileum villi in the HI4+K88 and HI8+K88 groups was significantly different from that in the C+K88 group. As described in **Table 7**, comparing the Vh (villus height) and Cd (Crypt depth) of the ileum in the C+K88 group, the Vh was found to be significantly increased in HI4+K88 and HI8+K88

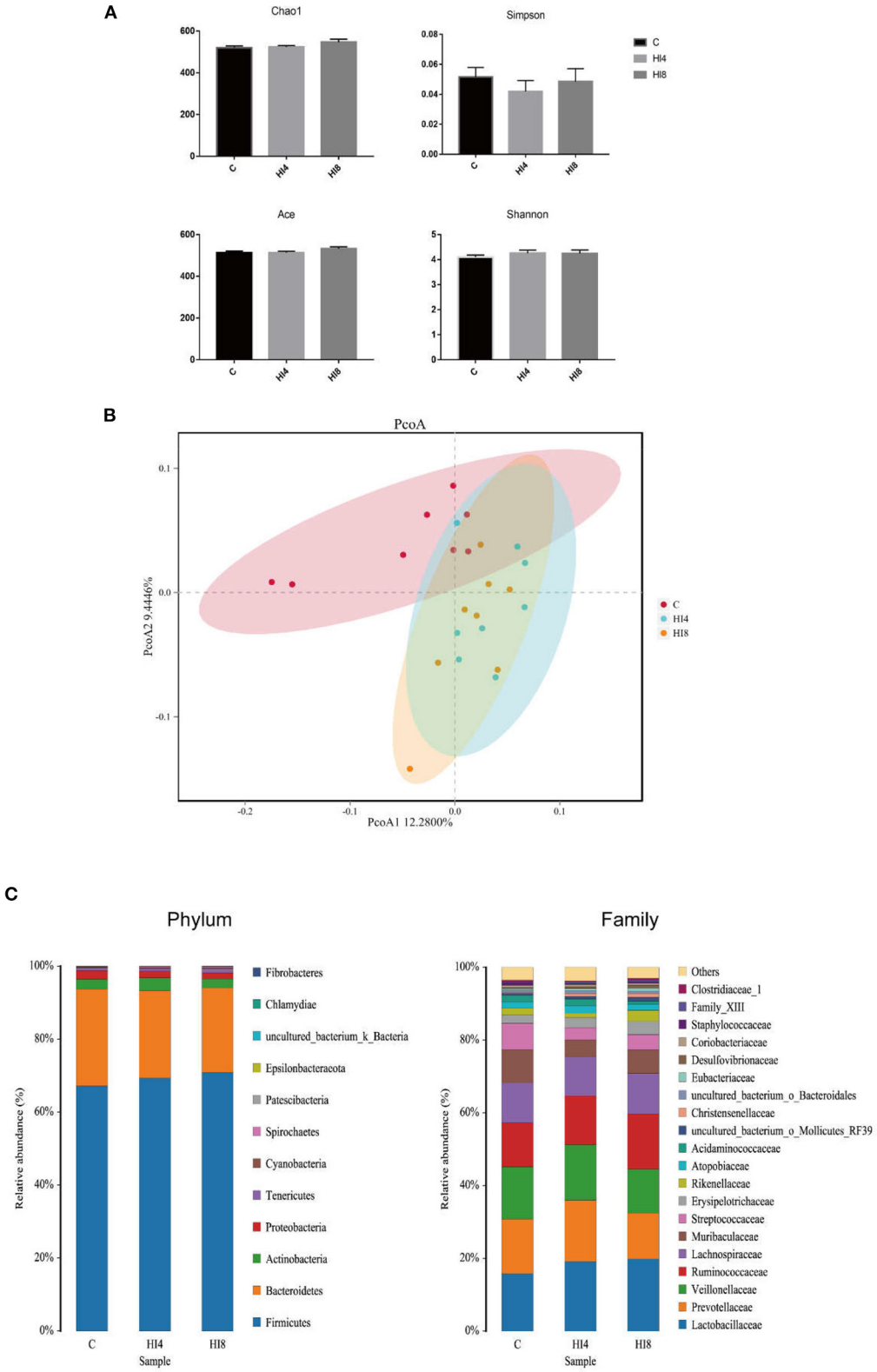

FIGURE 1 | Richness and diversity of fecal. **(A)** Chao1 index, Simpson index, Ace, Shannon. The values are means ± SEM ($n = 8$). **(B)** Principal coordinates analysis (PcoA) of bacterial communities in the fecal of pigs (based on the Binary-Jaccard). ($n = 8$). **(C)** Phylum-level and Family-level relative abundance of 16S rRNA gene sequences from the fecal of pigs ($n = 8$). The figure provided is an average of the three analyses.

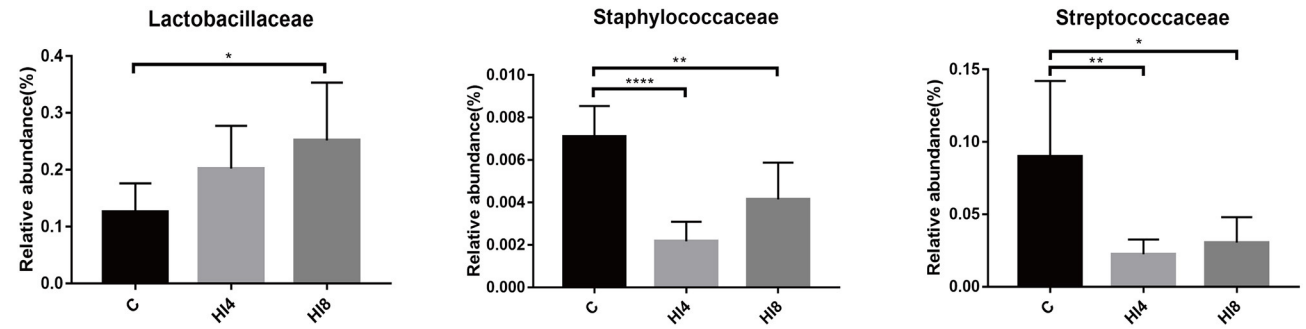

FIGURE 2 | Significantly changed bacterial family. The values were expressed as the mean ± SD (One-way ANOVA with a Tukey *post-hoc* test): $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ($n = 8$). The figure provided is an average of three analyses.

TABLE 6 | Effect of *Hermetia illucens* larvae on diarrhea rate and diarrhea index of weaned piglets after intragastric administration of ETEC K88.

Item	C+K88	HI4+K88	HI8+K88
Incidence of diarrhea (%)	50%	37.5%	25%
Diarrhea Index	1.67	1.25	0.83

groups ($P < 0.05$), whereas there was no significant difference in the Cd. Moreover, the villus height to crypt depth ratios in the ileum in the HI4+K88 and HI8+K88 groups were significantly higher than that in the C+K88 group ($P < 0.05$).

Effect of Dietary *H. illucens* Larvae on the Expression of Ion Transporter and Aquaporin in the Ileum Mucosa of Weaned Piglets Challenged With ETEC K88

As substantiated in **Figure 4A**, the mRNA expression of *NHE3* and *CFTR* was increased significantly in the ileum mucosa of the ETEC K88 challenged piglets belonging to the HI4+K88 and HI8+K88 groups ($P < 0.05$) as compared to the C+K88 group. Among these, the difference in the HI4+K88 group was the most significant.

The results of AQP1 and AQP3 are demonstrated in **Figure 4A**. Compared with the C+K88 group, the expression of *AQP1* and *AQP3* in the HI4+K88 group was significantly different ($P < 0.05$); however, the HI8+K88 group was insignificant. Nevertheless, there is no difference between the three groups of *AQP7*, *AQP9*, and *NKCCL*.

Effect of Dietary *H. illucens* Larvae Powder on the Antioxidant Enzyme Activity and Immunoglobulin Content of Weaned Piglets Challenged With ETEC K88

We estimated the activities of peroxidase and catalase in serum on day 32 of the experiment. As shown in **Figure 4B**, the CAT activity of the HI4+K88 and HI8+K88 groups significantly differed from the C+K88 group ($P < 0.05$). The result of POD activity was parallel to that of CAT; both the *H. illucens* larvae addition group was significantly higher than the control group ($P < 0.05$). Compared with the C group, the IgG and IgA concentrations of the HI4+K88 group exhibited significant differences ($P < 0.05$). Though there was no significant difference in the HI8+K88 group, there was a distinguished tendency to increase.

Effect of Dietary *H. illucens* Larvae on Gene Expression of Inflammatory Factors in the Intestinal Mucosa of Weaned Piglets Challenged With ETEC K88

To evaluate the impact of feeding *H. illucens* larvae on intestinal immune function, we elucidated the mRNA expression of several inflammatory factors.

As shown in **Figure 4C**, compared with C+K88 group, the expression of *IL-10* in the jejunum, ileum, and colon mucosa of HI4 +K88 group and HI8 +K88 group was significantly increased ($P < 0.05$). Compared with C+K88 group, the expression of *TNF-α* in the jejunum mucosa was significantly decreased in HI4 +K88 group ($P < 0.05$). The expression of *TNF-α* in colon mucosa of HI4+K88 group and HI8+K88 group was significantly decreased compared with that of C+K88 group ($P < 0.05$). However, there were no significant differences in the expression of *IL-8* and *IFN-γ* in the jejunum, ileum, and colon mucosa of the three groups of piglets.

Effect of Dietary *H. illucens* Larvae on the Expression of Tight Junction Proteins in the Intestinal Mucosa of Weaned Piglets Challenged With ETEC K88

As evident in **Figure 5A**, a significant increase in the mRNA expression of *Occludin* and *Claudin-3* in the jejunum mucosa ($P < 0.05$) of the HI4+K88 group compared with the C+K88 group was observed. However, in the HI8+K88 group, the mRNA expression level of *Occludin* was significantly increased than that in the C+K88 group ($P < 0.05$). The expression levels of tight junction proteins *Occludin*, *ZO-1*, and *Claudin-3* in the ileum mucosa of the HI4+K88 group manifested a significant increase than those in the C+K88 group ($P < 0.05$) (**Figure 5B**). The *Occludin* and *Claudin-3* expressions were significantly increased

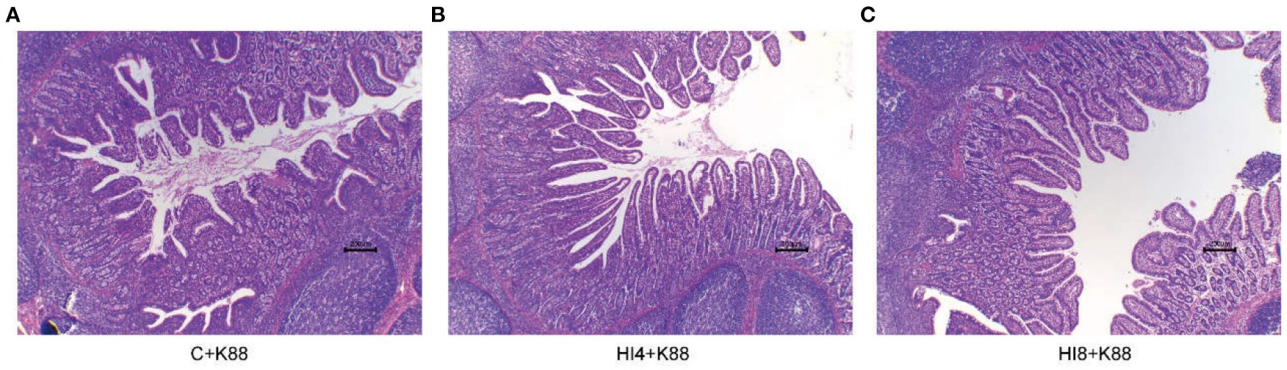

FIGURE 3 | Representative ileum morphology of different group piglets. **(A)** Morphology of ileum villi in C+K88 group. **(B)** Morphology of ileum villi in C+K88 group. **(C)** Morphology of ileum villi in C+K88 group. Representative optical micrographs of intestinal cross-sections. Scale bar = 200 μm. $n = 3$.

TABLE 7 | Effect of *Hermetia illucens* larvae on ileum morphology of piglets attacked by ETEC K88 ($n = 4$).

Item	C+K88	HI4+K88	HI8+K88	*P*-value
Vh, μm	258.75 ± 38.50^{b}	451.79 ± 80.79^{a}	427.79 ± 129.06^{a}	0.001
Cd, μm	405.38 ± 56.47	381.87 ± 83.74	428.68 ± 76.11	0.372
Vh/Cd, μm	0.65 ± 0.14^{b}	1.22 ± 0.31^{a}	1.03 ± 0.37^{a}	0.001

In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different letter superscripts mean significant difference ($P < 0.05$). The data presented are the average of three independent experiments.

in the HI8+K88 group when compared with that in the C+K88 group ($P < 0.05$) (**Figure 5B**). Results from the colon mucosa revealed a significant increase ($P < 0.05$) in the tight junction proteins *Occludin*, *Claudin-3*, and *ZO-1* in the HI4+K88 group, whereas, a significant increase ($P < 0.05$) in only *Occludin* in the HI8+K88 group, in comparison to the C+K88 group (**Figure 5C**).

The results of western blotting substantiated the results of fluorescence quantification. *Claudin-3* and *Occludin* were significantly increased in the jejunum mucosa, ileum mucosa, and colon mucosa in the HI4+K88 group and HI8+K88 group compared with the C+K88 group ($P < 0.05$).

DISCUSSION

With a worldwide shortage of resources, *H. illucens* larvae can evolve as a novel feed protein source, widely applied in animal diets. This can make an important contribution to the sustainable development of the livestock industry, facilitating the protection of the ecological environment (27–29).

This study provides certain guiding significance for production and application. It shows promise to completely replace fish meal with *H. illucens* larva meal in weaned piglets diet after weighing a possible positive effect on gut-barrier functionality as it sustain a similar growth performance. However, there is a tendency to decrease F/G in a later stage. The digestibility and absorption rate of P and Ca of weaned piglets in *H. illucens* larval powder supplemental group were enhanced, which may be related to the presence of calcium and phosphorus in the larval powder of *H. illucens* (30). The results of this experiment showed that *H. illucens* larvae powder could enrich the protein absorption capacity of piglets, but this is contrary to the results of Biasato (30). The result agrees with Spranghers's study and provides a reference for the future application of insect protein (31).

The presence of antibacterial peptides and chitosan in *H. illucens* larvae powder ameliorates the resistance of weaned piglets to disease. Marino's research has shown that *H. illucens* larvae powder improves the antioxidant capacity of fish (32). On encountering pathogenic microorganisms, the piglet's immune system will be activated and produce corresponding antibodies. Thus, a higher concentration of IgG and IgA reflects the improved performance of the piglet's immune system. The intestinal morphology findings signify that improved resistance to ETEC K88 and protection to intestinal health may be attributed to the early feeding of *H. illucens* larvae powder.

Enterotoxigenic *Escherichia coli* is the predominant cause of diarrhea in weaned piglets. Oral gavage of ETEC K88 was used to verify the immunity and disease resistance of piglets and the ability to resist the inflammatory response of ETEC K88 infection. The increase in enterotoxigenic *E. coli* affects intestinal permeability, alters the diversity and composition of the microbiota, and induces inflammation by regulating the expression of inflammatory factors, resulting in intestinal inflammation (33, 34). The pathophysiology of ETEC-mediated diarrhea recognized reduced absorptive surface epithelial cells, destruction of tight junction barrier function, impairment of ion transport, coupled with induction of inflammation (35). Severe damage in the ileum villi of the C+K88 group indicated that ETEC K88 is detrimental to the villus structure of the small intestine (36, 37). Vh and Vh/Cd were usually used as criteria for evaluating intestinal mucosal barrier function and intestinal health (38).

The Vh and the Vh/Cd of ileum in the HI4+K88 and HI8+K88 groups were significantly higher than that in the

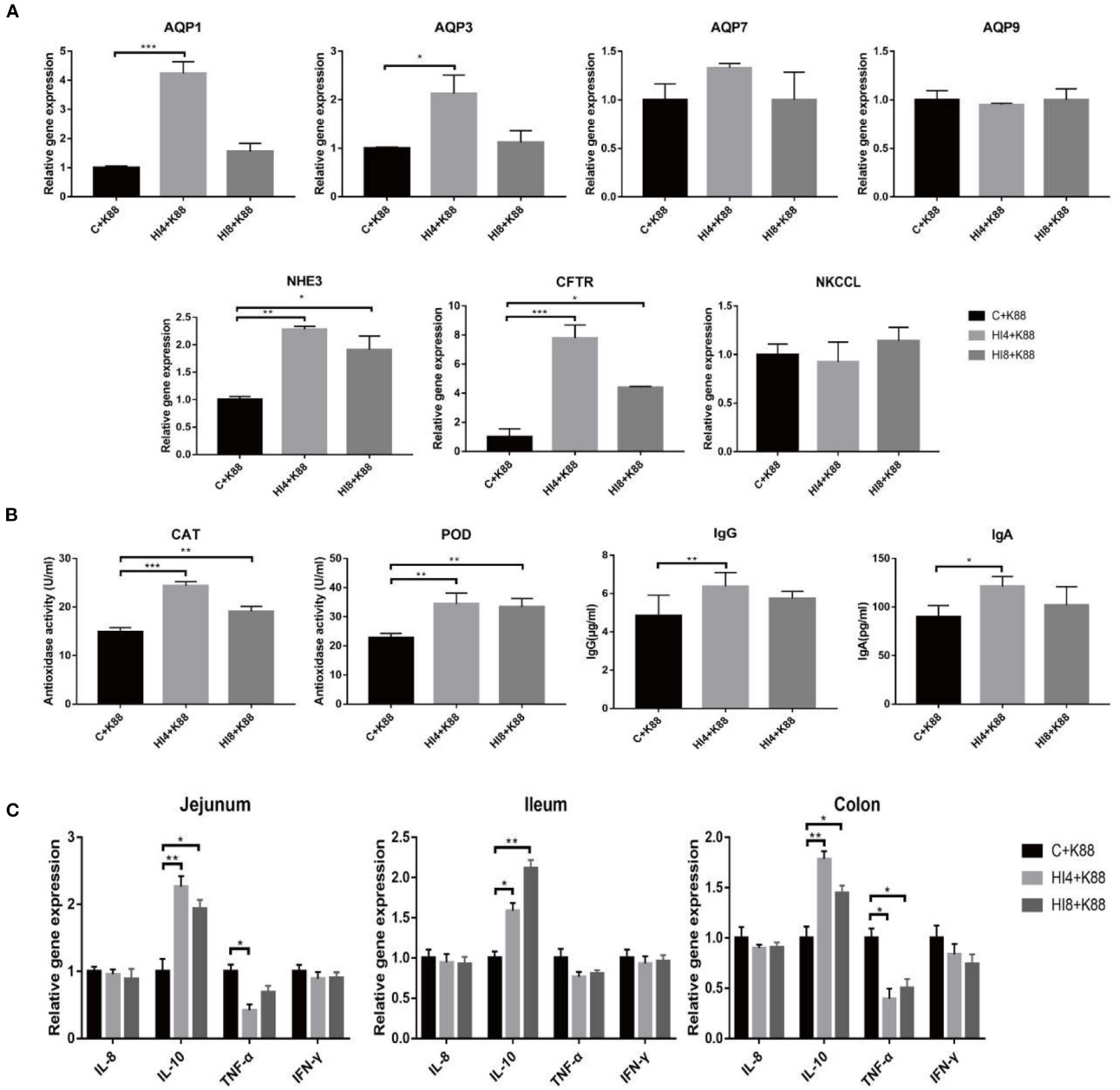

FIGURE 4 | The challenge of ETEC affects the expression of ion transporter and aquaporin in the ileal mucosa of piglets, the content of antioxidant enzymes and immunoglobulins in serum, and the expression of inflammatory factors in the intestinal mucosa. **(A)** The relative mRNA expression of intestinal ion transporters and aquaporins (*AQP*) in ileum mucosa. *AQP*, aquaporins; *CFTR*, cystic fibrosis transmembrane conductance regulator; *NHE3*, Na+/H+ exchanger 3; *NKCC1*, Na-K-Cl cotransporter. Results are mean ± SD ($n = 4$). **(B)** Antioxidant enzyme activity and immunoglobulin concentration in serum of challenged weaned piglets (One-way ANOVA with a Tukey *post-hoc* test) ($n = 4$). **(C)** Effects of dietary supplementation of *Hermetia illucens* larvae on real-time mRNA expression of cytokines and barrier function genes in jejunal mucosa, ileum mucosa, colon mucosa (One-way ANOVA with a Tukey *post-hoc* test) ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ($n = 4$). The presented data are the average of three independent experiments.

C+K88 group. This may be attributed to the beneficial biomass, such as chitosan and lauric acid, resisting the invasion of ETEC K88. (39, 40). *Occludin* and *Claudin-3* play an important regulatory role in the intestinal barrier (41). Henceforth, up-regulation or down-regulation of these tight junction proteins in the body is closely associated with the intestinal barrier (42, 43). Our results also indicated that the *H. illucens* larval feeding group might have a counteracting effect on ETEC K88's inhibition of *AQP* and ion transporter expression (11, 44). This study indicates that the early feeding

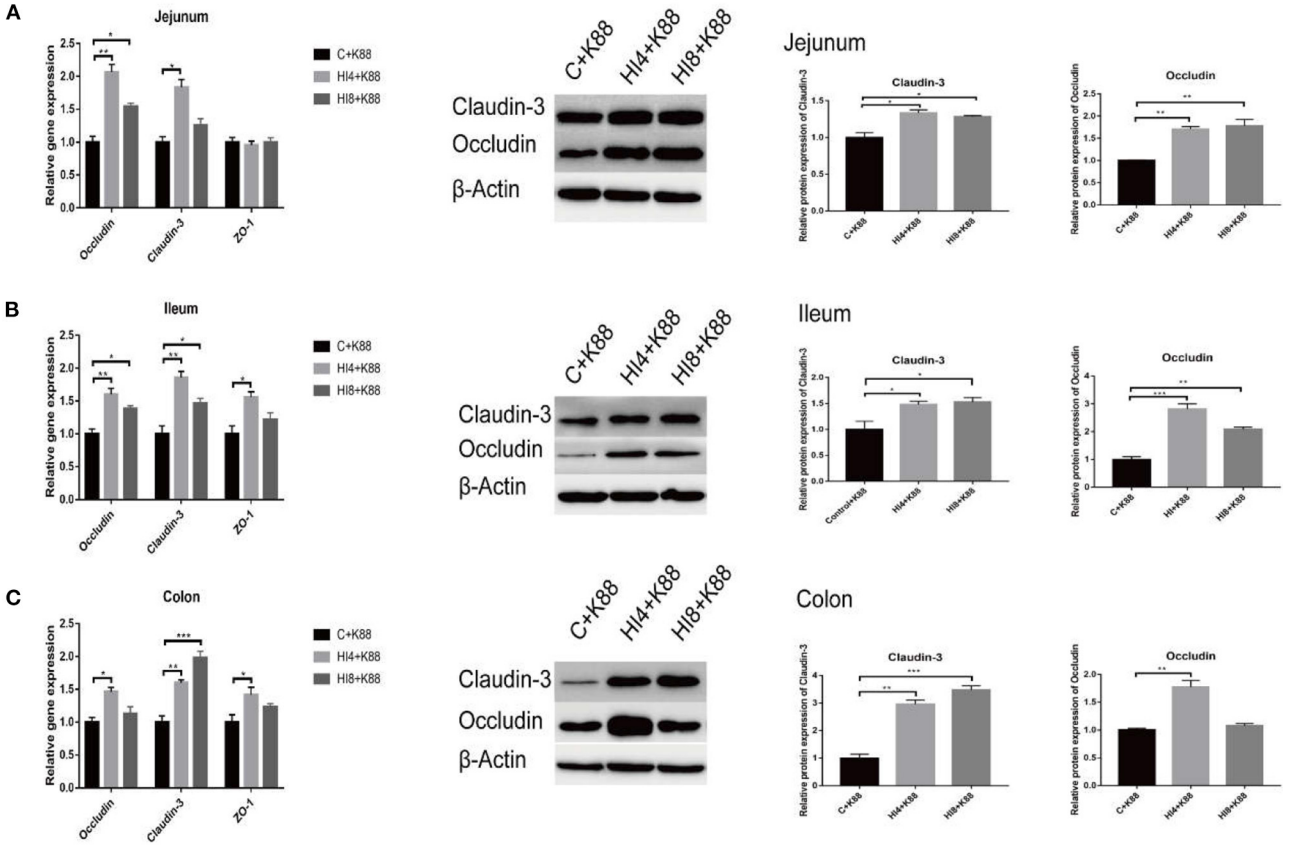

FIGURE 5 | Effects of dietary supplementation of *Hermetia illucens* larvae on real-time mRNA expression and protein of barrier function genes in jejunal mucosa, ileum mucosa, colon mucosa. **(A)** Representative immunoblots and quantification of *Claudin-3*, and *Occludin* expression in the jejunum ($n = 3$). **(B)** Representative immunoblots and quantification of *Claudin-3*, and *Occludin* expression in the ileum. **(C)** Representative immunoblots and quantification of *Claudin-3*, and *Occludin* expression in the colon. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ($n = 3$). Data are based on an average of three independent experiments.

of *H. illucens* larvae powder feed may protect intestinal health by stimulating the expression of tight junction proteins in the mucosa when invaded by pathogenic bacteria. This study also demonstrated a positive correlation between the *Claudin-3* and *Occludin* expression with the intestinal epithelial barrier function.

As feeding nutrition is one of the critical key factors that shape the gut microbial ecology, it increases *Lactobacillus*. In contrast, reduced *Streptococcus* was observed in piglets fed with *H. illucens* feed for 4 weeks in this experiment. This was in accordance with the results of Miaoyu's research in finishing pigs (7). Such dietary-induced shifts in gut microbial ecology may affect interactions among major phyla and lead to potential implications in piglets physiology. *Lactobacillus* imparts a protective effect on the intestinal epithelial barrier damage of IPEC-1 cells caused by ETEC and escalates the expression of tight junction proteins *ZO-1* and *Occludin* (45). As expected, compared with the control group, the diarrhea rate in the HI4 and HI8 groups was reduced in this experiment, the intestinal morphology integrity was better, the activity or concentration of CAT, POD, IgG, IgA in the serum was elevated, the expression of anti-inflammatory factors, tight junction proteins increased. The pro-inflammatory factors decreased, intestinal health and immune performance were ameliorated, and barrier function was better maintained.

H. illucens larvae are also a potential source of antimicrobial peptides (AMPs), with antibacterial activity against both Gram-positive and Gram-negative bacteria (46, 47). As probiotics, their physiological functions include enhancing digestion, regulating the balance of intestinal flora, improving immunity, and inhibiting the growth of harmful bacteria (48, 49). The alteration in intestinal bacteria populations and associated metabolites can affect the immune status of the host. The correlation analysis demonstrated that the changes in inflammatory cytokines, tight junction protein genes, and the SIgA concentration are associated with metabolites and specific bacteria, for example larvae as a potential dietary protein source altered the microbiota and modulated mucosal immune status in the colon of finishing pigs (7). Therefore, down-regulation of the expression levels of pro-inflammatory cytokines and up-regulation of the expression levels of anti-inflammatory cytokines and tight junction proteins confer the best form of resistance to ETEC K88.

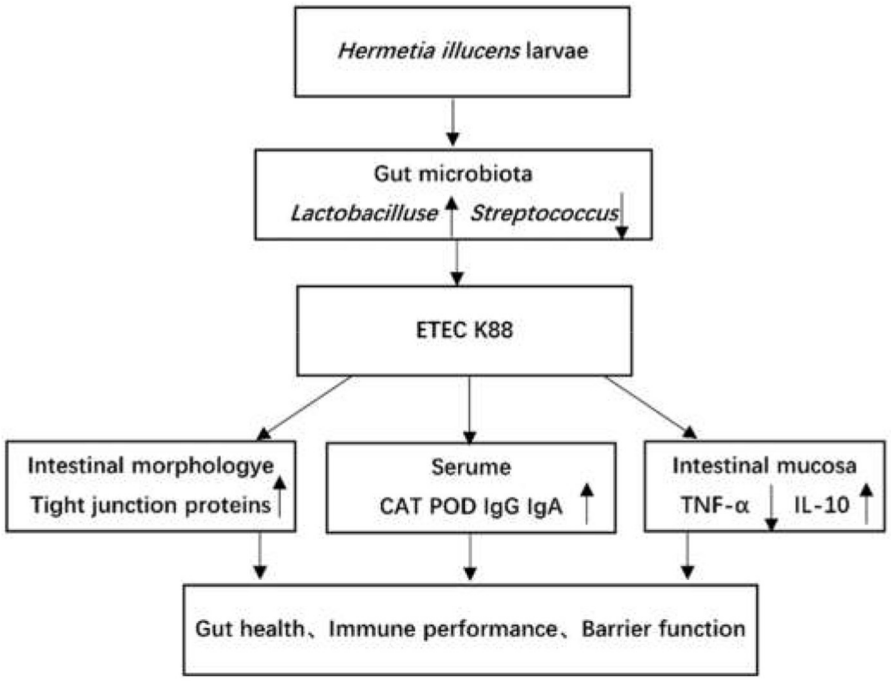

FIGURE 6 | The overall picture shows the main results obtained in the current work. The up arrows (↑) indicate increasing effects, and the down arrow (↓) indicates decreasing effects.

Figure 6 summarizes the the main results obtained from this study. Nevertheless, the mechanism of *H. illucens* larvae powder on the altered intestinal flora and the influence on the intestinal barrier still needs further investigation.

CONCLUSION

This study demonstrates that dietary supplementation of 4 and 8% *H. illucens* larvae did not affect the growth performance of weaned piglets. Notably, it can change the distribution of gut microbiota, increase the relative abundance of *lactobacillus*, and improve intestinal health. Adding 4% *H. illucens* larval to the piglets diet can increase the expression of tight junction proteins *Claudin-3* and *Occludin* and *IL-10* in the intestinal mucosa and maintain the ileum morphology's integrity, and portray a better immune status, improve intestinal barrier function. These studies provide a novel perspective for insect meals as a sustainable protein source for pig feed; the optimal dosage is 4%.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI; PRJNA750611.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of Zhongkai University of Agricultural and Engineering. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

WWa, YL, and YH designed research. XJ, WWu, and MZ conducted the pig trial. WWa, XJ, ML, BY, MZ, and HX prepared the diets. XJ, BY, ML, MZ, GX, and ZW performed research. WWa, XJ, ML, and XZ analyzed data. XJ, WWa, and YL wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Development of Nutraceutical Ice Creams Using Flour Yellow Worm Larvae (*Tenebrio molitor*), Chia (*Salvia hispanica*), and Quinoa (*Chenopodium quinoa*)

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Functional ice creams were developed by adding larvae of the insect *Tenebrio molitor* mixed with a seed (*Salvia hispanica*) and a pseudocereal (*Chenopodium quinoa*) to strawberry–cranberry ice cream. The objective was to increase micronutrients, macronutrients, and antioxidants, thus rendering the product a food complement. Four ice cream formulations were manufactured: the control strawberry–cranberry ice cream and three experimental mixtures, one of them with an addition of *Tenebrio* larvae (HT) and two others with a combination of *Tenebrio* larvae, chia (HTC), and quinoa (HTQ). The ice creams were submitted to proximate chemical analysis: mineral, fatty acid, vitamin, and one antioxidant (cyanidin 3 glucoside) determination. The strawberry–cranberry ice cream was used as a control formulation to evaluate if there were significant differences among nutrients, to which a Dunnett test with a critical value of $\alpha = 0.05\%$ was applied. The three formulations that were studied showed a significant increase in the analyzed micronutrients and macronutrients compared to the control formulation. We observed increases of up to 62% in lipid content in the HTC formulation, while an increase of 41% in the protein content of the HT formulation was observed. We quantified an increase and enrichment of vitamins and minerals in the manufactured products, so that their nutritional value was significantly enhanced. In the determination of cyanidin 3 glucoside, we found that the formulation to which chia had been added showed an increase of 74% as compared to the control ice cream; this is important because anthocyanins are a group of flavonoids that stand out for their antioxidant and antimutagenic capabilities.

Keywords: edible insects, *Tenebrio*, larvae, nutritional value, functional foodstuff

INTRODUCTION

Nutrition relies on different factors, such as place of residence, surrounding habitat, available alimentary resources, religion, ethnic group, education, migration, colonization, trade, and the ways in which food is cooked or preserved and stored, which have their origins in ethnic footprints (1). Besides, in the last decades, there has been an increasing interest among some population sectors not only in favoring a sustainable and healthy diet but also in learning the nutritional value of foodstuffs and their effects on the human body. In the industrialized world, the concepts in nutrition are changing significantly. From a former emphasis on survival, through hunger satisfaction and, more recently, food safety, food sciences now aim at developing foods to promote well-being and health while reducing the risk of some major diseases (2). It is because of this that the food industry currently faces the challenge of developing functional foodstuffs that respond to consumer tastes, needs, and demands (3) as well as their need for learning the nutritional function of the ingredients used in their manufacture (4).

In this context, the search for functional foodstuffs appears to be urgent. Bello (5) defines a functional product as "any product that can be considered as a foodstuff, or part of a foodstuff, that is capable of providing health benefits, includ[ing] disease prevention and treatment." The benefits could be either maintenance or promotion of a state of well-being or health and a reduction of the risk of a pathologic process or a disease. Among functional foodstuffs, different compounds of macronutrients and micronutrients can be found, such as carbohydrates, fats, amino acids, minerals, and vitamins.

Anthropoentomophagy refers to the consumption of edible insects. It is the human consumption of insects, an activity that has been practiced since the rise of mankind in countries such as China and Mexico, whereas in other countries—particularly in Europe—it has recently been adopted and valued (6). The importance of the role of insects as functional foodstuffs has been quantified in some respects. For example, several chemical compounds have been quantified, such as pigments, sterols, coumarins, terpenoids, iridoids, prostaglandins, pterines, alkaloids, and quinines (7–9). Because of this, insects are an important source of these compounds in the research carried out inside the alimentary industry. Nonetheless, they have received scarce attention in the scientific arena (10). For the reasons aforementioned, we considered of the manufacture of functional ice creams using the larvae of *Tenebrio molitor*, the mealworm, as the protein ingredient of interest, in which the presence of good-quality nutrients has been demonstrated (11) and also because insects represent a source of functional compounds yet to be discovered (9).

It is important to point out that due to the current demand for new sources of protein for human and animal nutrition, the insect *T. molitor* has been analyzed quite thoroughly to assess its nutritional value; thus, the state of the art in this respect is very detailed: for instance, we know that the larvae harbor 53.13% protein, 36.65% fat, 3.19% total ash, 5.10% crude fiber, and 1.90% nitrogen-free extract (12).

Ravzanaadii et al. (13) published an excellent research paper that allows us to explain this aspect in a brief way: *T. molitor* larvae, adults, exuviae, and excreta contained 46.44, 63.34, 32.87, and 18.51% protein, respectively, and Aguilar-Miranda et al. (14) reported higher protein content (58.4% for larvae).

This protein was rich in amino acids such as isoleucine, leucine, and lysine. The results showed that amino acid composition met the requirements of not only domestic animals but also human beings: cysteine + methionine (1.18 g/100 g protein) and phenylalanine + tyrosine (5.21 g/100 g protein). Overall, insects contain higher amounts of lysine and threonine, but lower amounts of methionine/cysteine (15).

The total fat content was 32.7, 7.59, 3.59, and 1.3% for *T. molitor* larvae, adults, exuviae, and excreta, respectively. The mealworm larvae presented an average fat content of 32.7%, a quantity that is greater than that of locusts (21.5%) and grasshoppers (3.8%) but less than that of termites (61.1%) (16).

In fatty acid composition, they have a high component of oleic acid (C18:1), linoleic acid (C18:2), and palmitic acid (C16) in larvae, with values of 43.17, 30.23, and 16.72%, respectively. Also, the amount of ω-6 was determined to be significant.

These essential fatty acids demonstrate that insects can be used for many other purposes such as feeding of domestic animals and as food supplements for human beings. Besides, it has been reported that insects' caloric value is 50% higher than that of soybeans, corn, and beef (15). Considerably high amounts of unsaturated fatty acids in larvae, adult, exuviae, and excreta have a similar composition to those of poultry and fish.

Furthermore, larvae have among their fats lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, and linoleic acid (17).

In mineral composition, as in other insects, mealworm contained poor sources of calcium which were 434.59, 484.39, 801.14, and 1,537.97 mg/kg for larvae, adult, exuviae, and excreta, respectively. Even when the calcium content is considerably low in mealworm, it contains instead high levels of P (18).

Therefore, in terms of nutritional content, edible insects (grasshoppers, crickets, termites, ants, beetle larvae, moth caterpillars, and pupae) have been stated to have more of it when compared to other conventional foods.

There will be a huge economical change involved if insects become more commonly considered as an acceptable food source for both human beings and domestic livestock in industrial countries (15).

Insects have potentially been used as a human nutrient source in traditional food among indigenous people throughout history (15). Insects represent 5–10% of animal protein source and also supply fats, calories, vitamins, and minerals among some ethnic groups (12, 19). Furthermore, as an attractive and important natural food source, insects have been used by various kinds of animals, such as birds, lizards, snakes, amphibians, fish, insectivores, and other mammals (20). They are normally encouraged to be sold alive, yet they are likewise sold canned, dried, or powdered; powdered larva is a high-grade item considered as an enhancement to conventional foods (21).

The production and innovation of food based on insects have become a trend in recent years, and in many parts of the

world, it is something completely normal; such is the case in Germany, Belgium, Brussels, Costa Rica, Spain, and Holland, among others. In the Netherlands and China, insects are used as food ingredients and are progressively viewed as an option for meat (22).

Insects have an excellent nutritional value, and their flavor is also fabulous; that is to say, they are even a delicacy; mealworm is widely used in various types of foods, for example, with the larvae, ice creams were prepared at the insect experience festival in Wageningen, The Netherlands (23).

Also, with *Tenebrio*, van Huis et al. (23) reported a wide variety of recipes among which include "triangles," bitterbug bites, bugsitgoreng, nutty mealworms, quick meatballs, flower power salad, pumpkin soup, minestrone, tagliatelle with creamy herb sauce, ravioli, hakunamatata, chukli con carne, chop suey, jambalaya, insect burgers, vol-au-vent, quiche, chebugschichiu, pizza, buglasva, and tarte tatin.

For the aforementioned reasons, in this aspect, it is clear that the future of food lies in insects and in the innovation of new, healthy, nutritious, and delicious products.

As regards ice cream and its importance, we can begin by pointing out that ice cream is defined by its composition and structure: it is a frozen foodstuff that, in general, is manufactured using dairy products such as milk or cream mixed with fruit or other ingredients, along with sugar, flavorings, sweeteners or honey, and stabilizing substances (24). To manufacture high-quality ice cream, it is essential to have excellent ingredients and a mixture that is formulated and well-balanced to provide an adequate function between each ingredient in order to obtain organoleptic properties that may be to the liking of the consumers. All of this is fundamental for the physicochemical properties of the product. On the other hand, ice cream has been considered as a dessert, that is, with minimal nutritional value. However, it has recently been reported that milk and fruit-based ice cream has a fundamental alimentary value (25).

As to the general characteristics of the ingredients, the nutritional value of edible insects in Mexico has been widely studied. Edible insects have been the source of 10–81% of protein (26); essential and non-essential amino acids, for instance, leucine (5.20–8.46) (g/100 g of protein) (27); vitamins, especially the ones from group B such as thiamine (0.08–6.110) and riboflavin (0.050–3.230) (mg/100 g^{-1}) (28); and minerals such as sodium (0.020–1.608), potassium (0.014–2.912), and calcium (0.040–0.224) (g/100 g) (29).

In certain cases, they are rich in fats (12, 17); for example, the beetle known as *T. molitor* has been used as a source of protein with different alimentary purposes throughout the world (30). The larvae harbor up to 58 g of protein, for every 100 g of dry weight, and 38.29 g of fats with a high content of unsaturated fatty acids (12).

Chenopodium quinoa is an annual plant with a height between 1 and 2 whose seeds have a 2-mm diameter. These seeds can be white, red, or black and are edible when consumed whole, having a high nutritional value (31). Even the FAO (32) has furthered it as a crop with the potential to guarantee alimentary food security in the 21st century (33).

Salvia hispanica is an herbaceous, annual plant with a height of 1 or 1.5 m whose seeds measure a mean of 2-mm length and of 1.5-mm width (34). The oleaginous seed of this plant has a significant quantity of ω-3 fatty acids (α-linolenic acid), fiber (+30%), high-biological-value proteins, and natural antioxidants. It also has essential and non-essential amino acids, vitamins, minerals, and dietary fiber (35).

An antioxidant is defined as a substance that is found in foodstuffs in our everyday life and that can prevent the adverse effects of reactive species on the normal physiological functions of human beings; they also delay the aging process (36). Quinoa seeds possess a high antioxidant activity as they contain anthocyanins (37). Given the aforementioned facts, the objective of this research was to manufacture functional ice creams with both animal and plant products so as to enhance their nutritional (proteins, fatty acids, vitamins, minerals, and antioxidants) value through the use of non-conventional ingredients such as the larvae of *T. molitor*, chia, and quinoa.

MATERIALS AND METHODS

This research comprised three phases.

The first one consisted of (a) the purchase of the ingredients for the ice cream foundation: water (Bonafont, Mexico City), pasteurized cow's milk cream, powdered whole milk (Cuautitlan Izcalli, State of Mexico), sugar (Trademark Zulka, Monte Caucaso 915, Lomas de Chapultepec, Miguel Hidalgo, C.P. 11100 Mexico City, México), gums (carob bean, carboxymethyl cellulose, and carrageenan) (Cosmopolitan Drugstore, Avenida Revolución 1080 Col. Mixcoac, Benito Juárez, Mexico City, Mexico), and the raw materials to provide it with flavor and functionality (strawberry, cranberry, chia and quinoa seeds); the ice cream foundation was then left to mature for 24 h at 4°C; and (b) the selection and cleansing of the insect larvae: these were obtained from the Institute of Biology, UNAM, National Autonomous University of Mexico, where they are cultured in controlled conditions at a temperature of 26–27°C and a relative humidity of 60%, being fed on wheat bran for a period of 90 days; the larvae were dipped in boiling water for 5 min—this was done in order to avoid them changing color during the procedure—and afterwards they were stored in freezers at −4°C; and (c) quinoa's preparation: seed conditioning achieved by boiling it for 10 min and drying it in a stove at 80°C for 6 h.

In the second phase, we developed four formulations for strawberry–cranberry ice cream: HCOo, which was used as a control; HT with *T. molitor* larvae; HTC, larvae mixed with chia; and HTQ, larvae mixed with quinoa. These were manufactured according to the specifications contained in NMX-F-714-COFOCALEC-2012 (38). Three control batches of ice cream HCOo of 1 kg each were made; the same was done for the experimental formulations HT, HTC, and HTQ; all the formulations were prepared on the same day and in triplicate.

Also, we chose an experimental design to determine if there existed significant differences among the experimental ice

cream formulations (as a whole, not by their components) and the conventional ice cream (control ice cream) whose basic formulation was used to prepare all the ice creams, as well as to verify if there was an increase in the nutrients when these were assessed by means of diverse types of chemical analysis.

The substitution and inclusion of the seed (chia), pseudocereal (quinoa), and *Tenebrio* larvae ingredients were done with respect to the control formulation to observe how the nutrients varied and to verify that the objective of the investigation was achieved.

In this case, we considered the amount of matured ice cream base (for the preparation of that quantity, the percentages expressed in the table cover the 500 g of matured ice cream base, to make 1 kg of total ice cream) and the adjustment of solids with respect to the NMX-F-714-COFOCALEC-2014 standard to consider it milk ice cream.

In the third phase, we carried out the chemical analyses to elicit the chemical composition of each of the ice creams: (a) proximate chemical analyses (fats, protein, dietary fiber, reducing carbohydrates, ash, and carbohydrates), (b) liposoluble and hydrosoluble vitamin determination, (c) fatty acid quantification, and (d) assessment of the antioxidant known as cyanidin chloride. Strawberries, cranberries, chia, and quinoa were not analyzed; nevertheless, for chia and quinoa, we used the data from several reliable bibliographic sources published in the United States or even by the FAO (39). The larvae of *T. molitor* were in fact analyzed.

The general process of the ice cream manufacture was as follows. The ingredients were weighed, mixed, and pasteurized at 68°C for 30 min. The addition of the strawberry–cranberry pulp, seeds, and larvae compromised the aging of the foundation at 4°C/24 h, which was then whipped for 10 min and frozen at −4°C.

To carry out chemical determinations, official published and standardized methods were used: for humidity (40), we determined humidity of foodstuffs by thermal treatment (the sand or gauze method); by means of the AOAC (41) procedures, we quantified fat in ice cream and frozen desserts (952.06), protein in ice cream and frozen desserts (930.33), total dietary fiber in foods (985.29); by the enzymatic-gravimetric method, we determined ash of milk (945.46); by proximate analysis of milk-based infant formulation, we determined carbohydrates (986.25); and finally, to determine reducing carbohydrates, we used the 3,5-dinitrosalicylic method.

The methods employed for the quantification of micronutrients in the manufactured formulations were the following. The mineral profile was quantified by the AOAC 985.3 method that assesses minerals in ready-to-feed milk-based infant formulations by atomic absorption spectrophotometry (Perkin Elmer Model 3110 Waltham, Massachusetts, USA). For the fatty acid profile, we applied the gas chromatography method GC-FID (Perkin Elmer Model Auto System XL Waltham, Massachusetts USA) (AOAC 969.33).

To assess the liposoluble vitamin profile, the method of Chotyakul et al. (42) was used. For identification and quantification, we used a vitamin A, E, D, and K stock solution with a concentration of 500 μg/ml. The calibration curve was constructed from the mix in which the vitamins ranged in an interval of 50–500 μg/ml. The vitamin composition analysis of the formulations was carried out by means of chromatogram interpretation.

To quantify hydrosoluble vitamins, we used the method from Gliszczynska and Rybicka (43) and Rokayya et al. (44). For identification and quantification, we used a vitamin B1, B2, B3, B6, B9, and B12 stock solution with a concentration of 500 μg/ml. The calibration curve was constructed from the mix in which the vitamins ranged in an interval of 50–500 μg/ml.

For the mineral profile, we used AOAC 985.35. And for cyanidin quantification, we applied a modified method from Martínez-Cruz et al. (45); we interpreted the chromatograms and constructed the corresponding calibration curves.

To evaluate if there were significant statistical differences between the nutrients thus determined, we carried out a one-factor analysis of variance (ANOVA) between the four formulations, as well as a Dunnett test to identify which macronutrients or micronutrients show variations with respect to the control ice cream. The Dunnett test is used to create confidence intervals between the mean of each factor level and the mean of the control group. In such a way, this test compares each formulation finding if there are any or no differences between each of them, but also with respect to the control with a confidence level of 0.05 (46).

We must initially point out that all the results presented in our tables are the mean obtained from three repetitions (that mean had a standard deviation and a variation coefficient or error lower than 5%) and that the values in the rows with different letters signal that between them there exists a significant difference; only the values of carbohydrates were obtained by difference, and in each table, the highest quantified values are indicated in bold.

RESULTS AND DISCUSSION

Proximate Chemical Analysis

According to NMX-F-714-COFOCALEC-2012 (38), the products we manufactured were classified as "milk ice cream" as they comply with the minimal physicochemical specifications required. In **Table 1**, the results of the proximate chemical analysis are presented, upon a dry basis of g/100 g of dry sample, since it is human food and it is reported in accordance with the labeling regulations in force in Mexico, NOM-051-SCFI/SSA1-2010 (47).

We observed significant differences in composition between the control ice cream and the functional formulations: these are due to the addition of chia, quinoa, and the insect larvae. In this case, the fiber content corresponds to the formulations of elaborate ice cream, that is, to the total mixture of all the ingredients. Consequently, it does not refer to *Tenebrio*. In the same table, reducing carbohydrates or reducing sugars are defined as sugars that can be oxidized by weak oxidizing agents; for example, all monosaccharides are reducing sugars.

Lipids

The experimental samples (HT, HTC, and HTQ) have significant differences in their lipid contents that enhance their nutritional value and consistency, with the HTC formulation being the

TABLE 1 | Results of the proximate chemical analyses of the ice cream upon a dry basis (g/100 g of sample).

Sample	*HCOo	HT	HTC	HTQ
Fat	$11.07^{a} \pm 0.46$	$14.34^{b} \pm 0.10$	**15.55**$^{b} \pm 0.16$	$12.01^{b} \pm 0.08$
Protein	$9.36^{a} \pm 0.25$	**11.81**$^{b} \pm 0.25$	$11.36^{b} \pm 0.26$	$10.95^{b} \pm 0.25$
Dietary fiber	$15.01^{a} \pm 0.93$	$13.89^{a} \pm 0.51$	**21.22**$^{b} \pm 0.69$	$15.59^{a} \pm 0.34$
Reducing carbohydrates	$26.91^{a} \pm 0.61$	$25.87^{b} \pm 0.26$	**27.09**$^{a} \pm 0.14$	$26.76^{a} \pm 0.28$
Ash	$1.88^{a} \pm 0.05$	$2.11^{b} \pm 0.08$	**2.24**$^{b} \pm 0.00$	$2.02^{a} \pm 0.07$
Carbohydrates	**35.77** ± 0.00	31.98 ± 0.00	22.54 ± 0.00	32.67 ± 0.00

*HCOo, control ice cream; HT, Tenebrio molitor ice cream; HTC, Tenebrio and chia ice cream; HTQ, Tenebrio and quinoa ice cream. *a,b The values in the rows with a different letter indicate that there exists a significant difference among them. Coefficient of variation (CV), known as relative standard deviation (RSD), corresponds to the average value of the three samples processed, that is, less than 3% and with an alpha level of significance equal to 0.05 (α = 0.05). The highest values quantified in each determination made in bold.*

one that shows the highest proportion by the addition of such ingredients. Ramos-Elorduy et al. (11) mentioned that the larvae possess a lipid content of 38.29%, and chia, according to the USDA (48), is a seed with a high lipid content of 30.74% upon a dry basis. In this case, the FAO (49) recommends that adults consume a minimum of 15% of their calories as lipids (33 g of lipids for a 2,000 kcal/day diet) and a maximum of 35% (78 g of lipids for a 2,000 kcal/day diet) to avoid disease.

As **Table 1** shows, no ice cream provides more than 9% of what is recommended by the FAO (49); that is, ice cream ingestion could complement the diet so as to reach the suggested fat percentage. When the types of lipids found in ice cream were analyzed, it was observed in **Table 2** that although 65–70% of the total lipids were saturated, no formulation surpassed the energy of 10% (22 g of saturated lipids for a 2,000 kcal/day diet) that the FAO (49) defines as the maximum limit. Furthermore, all of the experimental formulations provided a significant contribution of unsaturated fatty acids thanks to its ingredients.

When carrying out the study, the lipids quantified by gas chromatography are classified as saturated or unsaturated, and these are simultaneously reclassified into monounsaturated and polyunsaturated; they are added, and these are the values reported in **Table 2**.

In **Table 2**, we also show the results of the type of fatty acids found in each formulation (g FA/100 g of fresh sample). The experimental formulations have a different profile to that of the control ice cream, but we must point out that the addition of *T. molitor*, chia, and quinoa—being ingredients with high levels of unsaturated fatty acids—diminished the percentage of the saturated ones. The predominant fatty acids in the ice creams were palmitic acid, which varied between 29 and 32% and was the most abundant fatty acid in the HT and HTC formulations, and oleic acid, which ranged from 22 to 25% and was the only unsaturated acid present in important quantities in the experimental formulations.

The latter is due to the fact that the foundation to manufacture the ice cream is made up of dairy products in which saturated fats make up 70% of the total fat weight, with palmitic acid being the most common (50) in the results of the fatty acid profile of the formulations. On the other hand, milk lipids are very poor in polyunsaturated fatty acids (linoleic and α-linolenic) (51). That is, the control ice cream has a very similar lipid profile to that of milk, so when quinoa, chia, and larvae are added, a significant increase was shown by linoleic acid (C18:2ω-6); this is why it is important to include it in the diet. **Table 2** also shows that the HT formulation was characterized by having a very favorable proportion of ω-6/ω-3 fatty acids. This composition can be considered an indicator of its high nutritional value (52).

The adequate proportion of these fatty acids, ω-6/ω-3, can be considered as another determining factor of the high quality and nutritional value maintained in the investigated formulations (52); for example, ω-3 and ω-6 help to reduce LDL cholesterol in blood, and AGP ω-3 reduces triglyceride levels and platelet aggregation and promotes the immune response. The greatest beneficial effect of this type of polyunsaturated fatty acids resides in their antiarrhythmic mechanism that improves cardiovascular diseases. In addition, recent studies have suggested that they also play a fundamental role in reducing the risks derived from diseases such as type 2 diabetes or hypertension (53).

Table 1 shows the proportions of protein, carbohydrates, reducing carbohydrates, dietary fiber, and ash.

Protein

This is a macronutrient, and the ice cream with added chia and quinoa showed significant change with respect to HCOo. Nevertheless, the addition of larvae to the formulations had a positive impact, as it increased its content due to the fact that larvae have a high protein content (58%). When combining larvae with the seed or pseudocereal, a decrease was seen in the protein percentage, as these last two ingredients provided a higher quantity of dietary fiber, fat, and/or carbohydrates. All of the manufactured ice creams (except HCOo, 9.36%) had a greater protein content than commercial ice cream. Our manufactured ice cream provided 7% to 10% of the protein required daily, so it could be considered a good dietary complement.

Carbohydrates

The addition of larvae and its combination with the seed and pseudocereal diminished the proportion of carbohydrates; HTQ ice cream was the exception. This harbored the greatest quantities of carbohydrates even in comparison to HCOo, which is due to the fact that quinoa has a proportion of starch of 53.1% (54).

TABLE 2 | Results of the types of fatty acids in each formulation manufactured (g FA/100 g of fresh sample).

Fatty acids	HCOo	HT	HTC	HTQ
Butyric C4:0	**3.73*** ± 0.00	3.15^{b} ± 0.00	2.86^{b} ± 0.00	2.37^{b} ± 0.03
Caproic C6:0	1.21^{a} ± 0.00	0.62^{b} ± 0.01	**1.22**^{a} ± 0.02	0.88^{b} ± 0.00
Caprylic C8:0	**1.43**^{a} ± 0.01	0.77^{b} ± 0.02	1.37^{b} ± 0.02	0.97^{b} ± 0.00
Capric C10:0	2.61^{a} ± 0.02	2.43^{b} ± 0.01	**2.77**^{b} ± 0.03	2.16^{b} ± 0.01
Lauric C12:0	4.36^{a} ± 0.01	4.06^{b} ± 0.01	3.88^{b} ± 0.03	**3.76**^{b} ± 0.05
Tridecanoic C13:0	**0.91** ± 0.00	0.13 ± 0.00	—	—
Myristic C14:0	**11.23**^{a} ± 0.05	10.75^{b} ± 0.00	8.89^{b} ± 0.03	10.52^{b} ± 0.1
Myristoleic C14:1	**1.49**^{a} ± 0.00	0.96^{b} ± 0.00	1.35^{b} ± 0.04	1.01^{b} ± 0.01
Pentadecanoic C15:0	**1.67**^{a} ± 0.02	1.03^{b} ± 0.00	1.33^{b} ± 0.02	1.39^{b} ± 0.02
Palmitic C16:0	29.29^{a} ± 0.19	**32.96**^{b} ± 0.77	30.66^{b} ± 0.02	31.96^{b} ± 0.51
Palmitoleic C16:1n7	1.59^{a} ± 0.02	1.41^{b} ± 0.00	**1.72**^{b} ± 0.07	1.53^{a} ± 0.01
Heptadecanoic C17:0	**0.83**^{a} ± 0.01	0.43^{b} ± 0.00	0.72^{b} ± 0.01	0.61^{b} ± 0.00
Stearic C18:0	10.60^{a} ± 0.32	**10.91**^{a} ± 0.10	10.03^{b} ± 0.08	10.64^{a} ± 0.12
Oleic C18:1n9	22.00^{a} ± 0.17	**25.30**^{b} ± 0.61	23.61^{b} ± 0.03	23.59^{b} ± 0.17
Linoleic C18:2n6	2.89^{a} ± 0.02	3.37^{b} ± 0.01	**3.80**^{b} ± 0.01	**4.95**^{b} ± 0.02
γ-Linolenic C18:3n6	—	—	0.28 ± 0.01	—
α-Linolenic C18:3n3	0.35^{a} ± 0.00	0.06^{b} ± 0.00	**1.78**^{b} ± 0.00	0.21^{b} ± 0.00
Arachidonic C20:0	**2.36**^{a} ± 0.01	1.49^{b} ± 0.09	2.20^{b} ± 0.01	2.22^{b} ± 0.01
Eicosenoic C20:1	**0.98**^{a} ± 0.02	0.09^{b} ± 0.01	0.82^{b} ± 0.00	0.717^{b} ± 0.00
Behenic C22:0 and Erucic C22:1	**0.78**^{a} ± 0.00	—	0.66^{b} ± 0.00	0.458^{b} ± 0.00
Total Lipids	4.25	5.427	**6.924**	4.943
Saturated**	2.977	3.733	**4.567**	3.337
Unsaturated***	1.275	1.694	**2.357**	1.606
Monounsaturated	1.137	1.508	**1.950**	1.351
Polyunsaturated	0.138	0.187	**0.407**	0.255

*HCOo, control ice cream; HT, Tenebrio molitor ice cream; HTC, Tenebrio and chia ice cream; HTQ, Tenebrio and quinoa ice cream. *The values in the rows with a different letter indicate that there exists a significant difference among them. Coefficient of variation (CV), known as relative standard deviation (RSD), corresponds to the average value of the three samples processed, that is, <3% and with an alpha level of significance equal to 0.05 (α = 0.05).*

*Saturated Fatty Acid **$\sum SFA = (C4:0 + C6:0 + C8:0 + C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0)$. Unsaturated Fatty Acid classification ***$\sum MUFA = (C14:1 + C16:1 + C18:1 + C20:1 + C22:1)$. $\sum PUFA = (C18:2 + C18:3n6 + C18:3n3)$. The highest values quantified in each determination made in bold.*

Reducing Carbohydrates

In dry basis analyses, it was observed that only the HT formulation had significant differences compared to the control, as these sugars found in the ice cream derive from strawberries, cranberries, and milk.

Dietary Fiber

As **Table 1** shows, only the HTC formulation showed a significant difference with respect to the control ice cream, which is due to the fact that the seed provides this component and in doing so its consumption favors good health and prevents diseases (55). The ice cream we manufactured provided 15–25% of the RDI (Recommended Daily Ingestion) for men and 16–30% for women. That is, consuming 100 g of ice cream covers an important quantity of required fiber per day.

In **Table 3**, we present a comparative analysis of some foods prepared with insects and examples of foods made with insects. It can be observed that *Bunaeopsis aurantiaca* and *Imbrasia oyemensis* are rich in calories; *B. aurantiaca*, *I. oyemensis*, and *Rhynchophorus phoenicis* have similar protein amounts to beef; *Cirina forda*, wheat flour, and the different formulations of ice cream are high in nitrogen-free extract, and with respect to ash, the highest amount corresponds to *C. forda*.

It is especially observed that cookies made with *R. phoenicis* and wheat flour are enriched in proteins by 130%, since wheat flour only has 10.89. In addition, there are published data that point out that the nutritional value of mealworm is comparable to that of beef (23); therefore, *Tenebrio* is ideal to enrich various types of conventional foods such as ice cream. Furthermore, the possibility of using *Tenebrio* larvae as commercial food has been demonstrated as well as evaluated to enrich various foods since they provide a high amount of trace elements, fatty acids, and vitamins.

We must emphasize the nutritional aspects, because this is an important reason for eating insects, and according to numerous edible insect publications around the world, we know that enriching foods high in carbohydrates with proteins of insect origin with a quality comparable to those of chicken, pork, or beef will be a success in the future, as in Tokyo, where they already sell them in special dispensers, and so we must focus on

TABLE 3 | Comparative analysis of the nutritional value of ice cream with other foods made from insects and beef at a g/100 g dry basis.

Scientific name	Prepared as	Energy (kcal/100 g)	Protein (g)	Fat (g)	Nitrogen-free extract (g)	Ash (g)	R
Bunaeopsis aurantiaca	Flour[1]	433.00	24.20	49.00	4.50	3.20	a
Imbrasia oyemensis	Flour[2]	477.00	23.70	57.70	11.01	2.60	a
Cirina forda	Flour[3]	410.00	12.50	20.00	54.30	8.70	a
Rhynchophorus phoenicis	Cookies[4]	477	25.06	26.47	34.83	3.08	b
Wheat flour		474.98	10.89	19.82	63.26	2.54	b
HCOo	Ice cream	388.07	9.36	11.07	62.75	1.8	
HT	Ice cream	407.66	11.81	14.34	57.84	2.11	
HTC	Ice cream	383.75	11.36	15.55	49.59	2.24	
HTQ	Ice cream	389.13	10.90	12.01	59.36	2.02	
Beef		277.00	25.60	18.70			c

a, Ombeni and Munyuli (56); b, Adedayo et al. (57); c, Schockley and Dossey (58); R, Reference; 1 = contains sorghum flour, maize flour, liquid oil, and sugar; 2 = contains maize flour, liquid oil, and sugar; 3 = contains sorghum flour maize flour, oil, sugar, and salt; and 4 = contains 50% wheat flour and 50% R. phoenicis.

their production without destroying ecosystems. In the case of Mexico, numerous industries have been created, among which the following stand out: Abeja reina, Acetta, Becrikets, Bicho, Brinkos, Criks, Engrillo, Fertifrass, Gran Mitla, Gricha, In insect nutrition, Okuilli, Once chance, Optiprotein, Packs, Q-Kis, Sal de aquí, Santa colmena, Smart bites, Tanti, Totolines, and Zofo, which currently commercialize various products made from insects such as snacks, flours, salts, sauces, fertilizers, "totolines," and "mescal." For example, the company called Okuilli markets *T. molitor* larvae flour at a price of $1,150.00 per kilo, which at the exchange rate corresponds to 54 American dollars; this indicates that this activity is a business in continuous growth, both in our country and abroad, whose net earnings are significant. And in Europe, the world's largest producer of *Tenebrio* is called Ynsect. This is a French company located in Dole, near Dijon in eastern France, which breeds and processes yellow mealworms, transforming them into proteins, fats, and chitin that are used in feeding fish and domestic animals and as fertilizers.

Products made from insects also offer great expectations for the pharmaceutical, chemical, and food industries.

Minerals

Table 4 shows the mineral profile (expressed as mg/100 g of dry sample) of the manufactured formulations.

Calcium (Ca)

As can be observed, all of the formulations have significant differences in concentrations, and comparatively, HTQ showed a decrease of this mineral. In this respect, Vilcacundo and Hernández-Ledesma (59) pointed out that quinoa has a high calcium concentration, but during the "popping" process which it was submitted to, it could have lost calcium. The inclusion of chia in the HTC ice cream had positive effects as it increased its calcium content; this is understandable as this seed contains six times more calcium than milk (60). The experimental HTQ and HTC ice creams only present 239.058–332.048 mg of calcium; that is, none surpasses the 90 mg of the mineral indicated by the NOM-051-SCFI/SSA1-2010 (47). This is why it is suggested that the diet must include other foodstuffs rich in such a mineral to comply with this requirement.

Phosphorus (P)

As shown in **Table 4**, with the addition of larvae, chia, and quinoa, the experimental ice cream had a significant increase in its phosphorus concentration. The highest values were present in the HTC and HTQ formulations since phosphorus is the second most abundant mineral (706 mg/100 g) in their composition. The individual inclusion of larvae within the ice cream had a favorable response, but in the combinations larvae/seeds and larvae/pseudocereal, the concentrations found were higher because the combination of two ingredients high in phosphorus renders a foodstuff that is rich in such a mineral. Nevertheless, even with an increase of phosphorus in the product, the consumption of a 100-g portion provides only 9–12% of the RDI according to the NOM-051-SCFI/SSA1-2010. This is why we recommend the consumption of this product in the diet only as a phosphorus complement.

Sodium (Na)

In the HTC formulation, a significant difference can be observed as compared to the control ice cream. Even when the literature mentions that the seed's sodium content is very low (61), when added to the product, a significant increase is observed, and this can be explained by the variability of the seed composition that depends on the type of soil and climate it is cultivated in. With the aim of reducing cardiovascular disease, the WHO (62) recommends lowering sodium consumption: for adults, it suggests a daily intake below 2% (5 g of salt). As **Table 4** shows, no ice cream surpasses the daily intake limits established by this association.

Potassium (K)

The addition of larvae, chia, and quinoa significantly increased the potassium content in all of the formulations. HTC is, among all of the formulations, the ice cream that provides the highest quantity of this mineral; to this respect, Muñoz et al. (60) pointed out that potassium is the third most abundant microelement in the seed (407 mg/100 g). On the other hand, even when the

TABLE 4 | Profiles of minerals and vitamins quantified in the manufactured formulations.

Minerals (mg/100 g of dry sample)	**HCO**	**HT**	**HTC**	**HTQ**
Calcium	254.70* ± 1.11	265.42^{b} ± 0.60	**332.04**** ± 2.77	239.05^{b} ± 1.58
Phosphorus	156.36^{a} ± 4.39	185.07^{b} ± 4.34	**224.50**^{b} ± 0.34	198.98^{b} ± 2.06
Sodium	202.04^{a} ± 8.14	215.27^{a} ± 3.14	**232.78**^{b} ± 6.37	188.66^{a} ± 4.99
Potassium	337.93^{a} ± 8.43	403.97^{b} ± 1.57	**408.21**^{b} ± 7.35	365.43^{b} ± 6.99
Magnesium	23.92^{a} ± 0.83	32.11^{b} ± 0.89	**50.01**^{b} ± 0.58	42.07^{b} ± 0.77
Iron	1.05^{a} ± 0.07	1.15^{a} ± 0.07	1.58^{b} ± 0.06	2.23^{b} ± 0.12
Liposoluble vitamins (mg/100 g of dry sample)				
A, retinol	0.97^{a} ± 0.01	1.04^{b} ± 0.01	**1.89**^{b} ± 0.00	1.50^{b} ± 0.00
D, calciferol (μg/100 g)	479.74^{a} ± 8.87	584.34^{b} ± 11.5	**949.23**^{b} ± 9.77	679.30^{b} ± 14.9
E, α-tocopherol	4.87^{a} ± 0.09	5.93^{b} ± 0.08	**9.73**^{b} ± 0.14	6.34^{b} ± 0.10
K, phylloquinone (μg/100 g)	N/D	N/D	**997.0**^{b} ± 18.55	554.75^{b} ± 24.3
Hydrosoluble vitamins (mg/100 g of dry sample)				
Thiamine, B1	21.53^{a} ± 0.20	22.60^{b} ± 0.61	**27.34**^{b} ± 0.31	25.03^{b} ± 0.12
Riboflavin, B2	30.58^{a} ± 0.14	33.74^{b} ± 0.02	33.35^{b} ± 0.32	**35.02**^{b} ± 0.32
Niacin, B3	74.55^{a} ± 1.07	86.69^{b} ± 0.42	**102.95**^{b} ± 0.58	90.10^{b} ± 0.92
Folic acid, B9	17.50^{a} ± 0.1	36.82^{b} ± 0.58	**39.97**^{b} ± 0.41	38.73^{b} ± 0.58
Cobalamin, B12	4.35^{a} ± 0.02	**4.39**^{a} ± 0.02	4.26^{b} ± 0.01	4.17^{b} ± 0.01

*HCOo, control ice cream; HT, Tenebrio molitor ice cream; HTC, Tenebrio and chia ice cream; HTQ, Tenebrio and quinoa ice cream; N/D, not determined. *The values in the rows with a different letter indicate that there exists a significant difference among them. Coefficient of variation (CV), known as relative standard deviation (RSD), corresponds to the average value of the three samples processed, that is, <3% and with an alpha level of significance equal to 0.05 (α = 0.05). The highest values quantified in each determination made in bold.*

HTQ formulation presented no evident potassium increase, the important fact is that such a mineral is present in a bioavailable form (59); therefore it is considered essential in the diet. For this mineral, the WHO (63) recommends a daily intake of 351 mg; since our ice cream only provides 3–5% of this quantity, the consumption of foodstuffs rich in this mineral is recommended to meet this need.

Magnesium (Mg)

Magnesium is found in a very low concentration in the control ice cream HCOo. Nevertheless, the addition of larvae, chia, and quinoa significantly increased this mineral's levels as compared to HCOo. In the HTC ice cream, chia was the ingredient that significantly provided the highest quantity of Mg, since this seed has a rough content of 335 mg/100 g. The NOM-051-SCFId/SSA1-2010 points out that the RDI for magnesium is 248 mg; our ice cream provides only 3–8% of this mineral, which is why they are recommended only as an alimentary complement.

Iron (Fe)

Iron was the mineral quantified in the lowest concentration in all of the ice creams; it is found in trace quantities, and because of this, its levels were minimal. It was observed that just with the addition of larvae, there was no difference with respect to HCOo, but in HTC and HTQ, a significant increase was found. This is due, for example, to chia having a high content of this mineral (7.7 mg/100 g). HTQ ice cream was the one that had the highest Fe concentration; this is because quinoa has 1,416.7 mg Fe/100 g upon a dry basis. However, its content does not reach a whole milligram in the final product; that is, ice cream provides 25% of the RDI according to NOM-051-SCFI/SSA1-2010, 17 mg. Therefore, we recommend the consumption of iron-rich foodstuffs to comply with this requirement.

Liposoluble Vitamins

Table 4 shows the results obtained in relation to the levels of liposoluble vitamins in the formulations upon a dry basis.

Vitamin A (Retinol)

In this case, it was observed that all of the formulations had an increase due to the addition of the ingredients. Nonetheless, the highest value was found in the HTC formulation, due to the fact that this vitamin is the most abundant in the seed with 44 μg/100 g (64). That is, the combination larvae/seed achieved a synergic effect since larvae contain this vitamin but in a lower quantity than that in chia, which has 29 UI/100 g. A significant increase has been shown by the HTQ ice cream; it is minimal when compared to HTC, but better than HCOo.

According to the FAO (49), quinoa is composed of retinol in a range of 0.12–0.53 mg/100 g. However, before the addition of quinoa to the ice cream, it was submitted to two thermal processes, "popping" and drying, which could have had an effect on the vitamin's stability, causing its oxidation. The NOM-051-SCFI/SSA1-2010 points out that the RDI for vitamin A is 568 μg; as observed in **Table 4**, the HTC product complies with such a requirement, compared to the HCOo, HT, and HTQ formulations, which only provided 66–69% of retinol.

Vitamin D (Calciferol)

In this research, the highest concentrations were found in the ice cream to which chia had been added (HTC). Nevertheless, for the ice cream added with quinoa, a high content of vitamin D was also obtained. The NOM-051-SCFI/SSA1-2010 indicates an RDI of 5.6 μg; as can be seen, all of the ice creams surpassed this figure, but the occasional consumption of these ice creams would not affect consumers' health.

Vitamin E (α-Tocopherol)

It was quantified in a high proportion in the formulations, as they showed a higher quantity than the control ice cream. Nonetheless, when only larvae were added, the increase of vitamin E was minimal, which is due to the fact that larvae have very low concentrations of this vitamin (1.9 mg/100 g) as compared to chia and quinoa (65). On the other hand, when chia was added, we saw a higher increase of vitamin E in HTC, because of the large amount of this vitamin in chia (23,842.7 mg/100 g) (60).

Quinoa is also an abundant source of vitamin E, whose concentration ranges from 3.7 to 6.0 mg/100 g; however, when the pseudocereal is mixed with the larvae, vitamin E concentration diminishes because—as both ingredients have low levels of tocopherols—the combination of both dilutes them. The NOM-051-SCFI/SSA1-2010 points out that according to the RDI of vitamin E, one must consume 11 mg of it. As **Table 4** shows, our ice cream provides 9–36% of that vitamin, and therefore, the consumption of complementary foodstuffs with tocopherol content is recommended.

Vitamin K (Phylloquinone)

In **Table 4**, it is observed that the quantity of vitamin K that HTC and HTQ provide is due to the addition of chia and quinoa and that comparatively the larvae provide a minimal proportion, given that when HT was analyzed, its concentration could not be quantified. NOM-051-SCFI/SSA1-2010 indicates an RDI of 78 μg. As **Table 4** shows, the ice cream in which this vitamin was quantified complies with such a requirement, but—since these products are recommended as dietary complements—excess consumption does not pose any health risks.

Hydrosoluble Vitamins

In **Table 4**, we present the concentration of hydrosoluble vitamins found in the formulations upon a sample of mg/100 g dry basis.

Thiamine (B1)

All of the experimental formulations showed a significant increase. However, HTC is the one that provides the highest quantity of this vitamin, because, as Muñoz et al. (60) have reported, chia has an important thiamine content (0.62 mg/100 g). We must point out that even when larvae were the ones that contributed less to this enrichment, they acted in synergy with the chia seed. On the other hand, the addition of quinoa also favored a thiamine increase in the ice cream of up to 15%, which is due to the fact that quinoa harbors a concentration of 0.4 mg/100 g of vitamin B1 (66). All of the ice creams comply with the RDI established by the NOM-051-SCFI/SSA1-2010: 800 μg; that is, the consumption of a portion of 100 g of any of them will meet the daily requirement of this vitamin.

Riboflavin (B2)

The results in **Table 4** show that a significant increase occurred in all of the formulations. In the same way, it was observed that larvae are the ones that provide the highest quantity of B2 in the products, and when combined with quinoa, the formulation's enrichment is even higher. This is due to the fact that the larvae have 1.21 mg/100 g of riboflavin, and quinoa has 0.39 mg/100 g (65, 66), so that, when the two ingredients are combined, a synergy occurs resulting in an increase of vitamin B2 of up to 14% as compared to the control formulation. Meanwhile, in the HTC sample, we quantified a lower level, since chia only provides 0.17 mg/100 g of this vitamin.

Niacin (B3)

Niacin was the vitamin that had the highest concentrations in the ice cream, even in the control formulation, since milk contains tryptophan—which is considered niacin's precursor—in a significant quantity: 7 mg/g protein. According to **Table 4**, when larvae and the seed were mixed in the HTC, this content was reinforced up to 38%, given that chia is considered a rich source of this vitamin with a content of 8.83 mg/100 g (60), and larvae contain 4.10 mg/100 g. In such a way, when combined, they present a synergy, with a product rich in niacin being the result. When the larvae and the pseudocereal were combined, the increase was minimal, but still higher than the quantity found in HCOo.

Folic Acid (B9)

In all of the experimental formulations, significant increases were obtained when compared with HCOo. The fortification of this vitamin in the ice creams can be appreciated, as when an extra ingredient or their combination is incorporated, an increase of up to 87% is observed. As **Table 4** shows, larvae provide the highest quantity of vitamin B9, because, as Novak et al. (65) have mentioned, these possess a concentration of 137 mg/100 g. In the same way, it can be observed that when larvae are combined with the seed, the amount of folic acid increases up to 128% with respect to the control ice cream.

Cobalamin (B12)

Table 4 shows that cobalamin was the vitamin quantified in the lowest concentration in the ice cream, HT being the one that had the highest quantity. On the other hand, when larvae were included in HTC and HTQ, since its content is minimal (0.30 μg 100 g^{-1}), there was a decrease as compared to that of the HCOo; that is, the larvae favored only a dilution of this vitamin in the formulations. Unfortunately, there are no data about the quantity of this vitamin in these ingredients that would enable us to draw a quantitative comparison. Notwithstanding this situation, the quantities of B12 in the ice creams effectively cover the RDI of 2.1 μg (0.0021 mg) that the NOM-051-SCFI/SSA1-2010 recommends.

TABLE 5 | Composition of the four formulations (%).

Ingredient	Formulations			
	Control ice cream, HCOo	***Tenebrio molitor* ice cream, HT**	***Tenebrio* and chia ice cream, HTC**	***Tenebrio* and quinoa ice cream, HTQ**
Ice cream base*	40.0	31.5	21.5	21.5
Strawberry	30.0	30.0	30.0	30.0
Cranberry	30.0	30.0	30.0	30.0
Tenebrio molitor larvae	–	8.5	8.5	8.5
Chia	–	–	10.0	–
Quinoa	–	–	–	10.0

**The components of the control ice cream are the following: water, powdered whole milk, sugar, carob bean gum, carboxymethyl cellulose (CMC), carrageenan gum, strawberry, and cranberry in 1 kg.*

TABLE 6 | Cyanidin chloride concentration upon a dry basis of the formulations.

Sample	Concentration (mg/100 g of dry sample)
Control ice cream HCOo	$33.39^{a} \pm 0.00$
Tenebrio ice cream HT	$36.04^{b} \pm 0.00$
Tenebrio chia ice cream HTC	$42.35^{b} \pm 0.00$
Tenebrio quinoa ice cream HTQ	$31.57^{b} \pm 0.00$

**HCOo, control ice cream; HT, Tenebrio molitor ice cream; HTC, Tenebrio and chia ice cream; and HTQ, Tenebrio and quinoa ice cream. *The values in the rows with a different letter indicate that there exists a significant difference among them. Coefficient of variation (CV), known as relative standard deviation (RSD), corresponds to the average value of the three samples processed, that is, <3% and with an alpha level of significance equal to 0.05 (α = 0.05).*

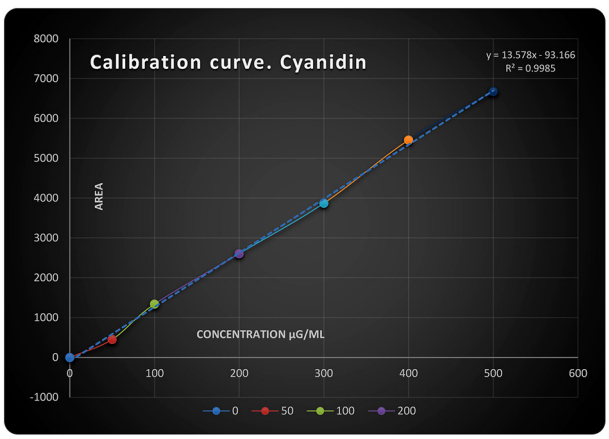

FIGURE 1 | Cyanidin chloride calibration curve.

Anthocyanins

In **Tables 5**, **6** and **Figure 1**, we present the concentrations of cyanidin chloride quantified in the different ice creams upon a dry basis. The content of cyanidin in the four formulations derives from the strawberries and cranberries that give the ice cream their flavor. The table shows that there were significant differences as compared to HCOo. The formulations with the highest concentrations of anthocyanins were HT and HTC. The latter had the highest levels of anthocyanins, which could be due to the quantity of antioxidants found in the seed, caffeic acid (6.6 mg/100 g), and chlorogenic acid (7.1 mg/100 g), which, in turn, increases cyanidin's stability (64). On the other hand, even when the pseudocereal is an abundant source of antioxidants (66), HTQ had a lower concentration of cyanidin. This is due to the process of "popping" and drying to which quinoa was submitted and which made it lose its antioxidant activity and, thus, its cyanidin concentration, given that it becomes unstable in the presence of oxygen.

Strengths and Weaknesses

In this case, the nutritional value of the ice creams prepared with insect addition was improved in macronutrients and micronutrients, as compared to the control batch. Therefore, in this research, we focus on the potential application of ice creams with diverse functional properties.

The results shown with the experimental batches of ice cream are an important contribution in the elaboration of nutritious and functional foods in the future (67).

Besides, as the yellow mealworm is an organism with a short life cycle whose cultivation is simple, it has been developed on an industrial scale, for example, in Europe. It is also widely known that various foods prepared with the larvae of the yellow mealworm are consumed directly after their culinary preparation; for example, technological innovations include the development of nutritional bars and bread, and diverse high-quality foodstuffs can be obtained to meet various needs of both man and pet animals such as dogs and cats, being also beneficial to the growth and development of poultry and livestock. It is generally believed that it can provide a good deal of comprehensive and rich nutrients (68) and can also be made into common and processed foods. Predictably, new deep processed foods, such as jam, beverages, vinegar, and wine, along with powder, capsules, and tablets, will be available in the market, as has been done with *Clanis bilineata tsingtauica* (67).

Moreover, due to the antioxidant and anti-aging activity of the larvae, corresponding components can be extracted to develop

more functional foods and could be a suitable functional food resource for humans due to its composition of active substances.

However, further research is required to assess the effects of preparation on foodstuffs when the larvae of this beetle are added; it is necessary to carry out statistical sensory analyses, compare shelf life characteristics, and, in the case of ice creams, prepare them according to the dietary needs of the people who consume them, for example, diabetics, considering their age, weight, gender, height, occupational activity, etc. It would also be necessary to establish a business plan and increase production on a larger scale to be able to commercialize the ice creams at the national and international markets.

Besides, to know the nutritional value of some edible insects and several conventional foods, statistical models called Ofcom and the Nutrient Value Score (NVS) have been used, as well as non-parametric tests and Bonferroni adjustments to verify nutritional differences. Of the available insects studied, the ones that are commercially important are *Bombyx mori*, *Imbrasia belina*, *R. phoenicis* (Fabricius, 1801), *T. molitor* Linnaeus, 1758, *Acheta domesticu*s (Linnaeus, 1758), and *Apis mellifera*; they were compared with beef, pork, and chicken. Details of the methods are found in Payne et al. (69).

According to the Ofcom model, insects are not healthier than meat products, and with the results of the NVS method, crickets and yellow worms are significantly healthier than beef and chicken.

In this publication, it is reported that crickets and bees are richer in iron compared to beef, pork, and chicken and higher in calcium and riboflavin than meat and meat offal, but there is no indication that the insects investigated are statistically healthier than meat. However, their results are not conclusive at this time and should therefore be treated with reserve.

In any case, the nutritional quality of the insects studied is "relatively" unimportant since in the daily diet through the use of mixed diets, the phenomenon of complementation/supplementation between different proteins occurs. Food supplementation is a concept used to design diets or foods in which different sources of protein are mixed in order to improve the quality of the resulting combination.

CONCLUSIONS

The three formulations—HT, HTC, and HTQ—showed a noteworthy augmentation in the previously examined macronutrients and micronutrients in comparison to the control formulation. After a thorough examination, it could be observed that the HTC formulation has proven to be the best one due to containing the upmost values of essential nutrients such as protein, fat, dietary fiber, ash, minerals, cyanidin, and vitamins. According to the formulations of the ice creams made, the amount of protein increased by 41%; that is, these larvae are ideal to enrich various types of conventional foods such as ice cream, and this will be a success in the future. Likewise, ice creams improved significantly in fatty acids, vitamins (A, E, B2, B3, and B9), and minerals (phosphorus, potassium, magnesium, and antioxidants); therefore, the larvae have a high potential to supplement conventional foods.

To sum up, we can conclude by saying that the importance of creating new formulations of functional food ice cream lies not only in its nutritional value but also in the functional macronutrients and micronutrients, which, all in all, effectively prevent any sort of disease.

For the aforementioned reasons, the food industry is looking for alternative nutritional sources such as insects, for the elaboration of products; such is the case of the nutraceutical ice creams analyzed and the protein enrichment of various conventional foods, among which we have "jungle bars," cookies, tortillas, cakes, chocolates, and sausages. At present, various foods made with *T. molitor* larvae such as ice cream are a novel and emerging food industry. Therefore, it may be developed as a sustainable source of alternative resources for human consumption and widely used in many other investigations such as animal feeding and the elaboration of some functional foods.

The benefits of using insects as human food are diverse; moreover, based upon this premise, we can claim that insects have a high potential of becoming a new sector in the constantly innovative food industry of Mexico.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.629180/full#supplementary-material

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METHODS
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Detection of *Alphitobius diaperinus* by Real-Time Polymerase Chain Reaction With a Single-Copy Gene Target

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Use of edible insects as an alternative source of proteins in food and feed is increasing. These last years, numerous companies in Europe have started producing insects for food and feed purposes. In the European Union, the use of edible insects for human consumption falls within Regulation (EU) No. 2015/2283 on novel foods. For feed, Commission Regulation (EU) 2017/893 authorizes seven insect species as processed animal proteins for aquaculture. Methods of authentication are required to check the conformity of the products. In this study, we propose a real-time polymerase chain reaction (PCR) method for the specific detection of the lesser mealworm (*Alphitobius diaperinus*), one of the species included in the shortlist of authorized insects. The selected target is the cadherin gene with a single-copy (per haploid genome) illustrated by our experimental evidence. The PCR test amplified a 134-bp fragment of the cadherin gene. The qualitative method was assessed toward several performance criteria. Specificity was checked against 54 insect species next to other animal and plant species. The sensitivity, efficiency, robustness, and transferability of the PCR assay were also successfully tested. Finally, the applicability of the test was assessed on real-life processed samples (industrial meals) of *A. diaperinus*. The study also showed that there seems to be a huge confusion on the correct labeling of the marketed mealworms. We did not succeed to get *Alphitobius laevigatus* samples. They all appeared to belong to the *A. diaperinus* taxon.

Keywords: insect, *Alphitobius diaperinus*, lesser mealworm, Coleoptera, detection, real-time PCR, cadherin, feed

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INTRODUCTION

In recent years, edible insects are becoming an increasing alternative source of proteins. The main reason for it is that the food production rate is expected to be lower than the population growth (1). However, the insect consumption in European countries is limited because of strong impeding barriers associated with texture and appearance, much more than the taste (2, 3). Therefore, the

incorporation of insects as ingredients in common food items such as sausages, protein bars, pâté, buns, and pastas is a way to overcome this problem (3).

Insects represent a promising strategy for enriching food in some nutrients, thereby achieving a better nutritional balance (4). In fact, the positive nutritional features of edible insects are the presence of high quantities of proteins, essential and non-essential amino acids, lipids, fibers, vitamins, and minerals (4, 5). Roncolini et al. (4) conducted a study to evaluate the use of lesser mealworm powder to replace a part of wheat flour as a means to enhance the protein and mineral content of crunchy snacks. The technological, microbiological, nutritional, and sensory characteristics of the fortified rusks were also evaluated in their study. A publication of Lacroix et al. (6) suggested the potential of lesser mealworm protein hydrolysates to serve as functional food ingredients to help improve glycemic regulation. Many insect species have been shown to have a high concentration not only of iron but also of zinc (3, 7). The rearing substrate can then modulate the insect nutritional quality (3). Insect growth rates, production efficiencies, and protein quality are influenced by substrates used to feed the insects but also depend on entomological species raised and rearing conditions (8, 9).

In animal production, the main protein source comes from either soybean (to feed cattle) or from fishmeal (to feed fish). However, soybean is associated with deforestation, use of genetically modified plant breeds, and massive use of pesticides, whereas industrial fishing to produce fishmeal depletes fish stocks (10). Insects are an ideal feed alternative because they are a good source of nutrients. Compared with conventional livestock, insects require less space for production (11), are expected to use less water (12), and emit less greenhouse gases (13), making them a more sustainable source of animal proteins (8).

Recently, seven insect species were authorized as processed animal proteins in aquafeed (14) and in pig and poultry feed (15): black soldier fly (*Hermetia illucens* L.), yellow mealworm (*Tenebrio molitor* L.), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus* L.), banded cricket [*Gryllodes sigillatus* (Walker)], field cricket (*Gryllus assimilis* F.), and common house fly (*Musca domestica* L.). Among these species, *A. diaperinus* can easily be reared to obtain protein flour for human consumption, as well as for feed material (16). In the meantime, the list of authorized insects as processed animal proteins was extended to silkworm (17).

Human consumption of edible insects in the European Union falls within Regulation (EU) No. 2015/2283 on novel foods (18). This means that food containing insects and their derived products must be subject to authorization once European Food Safety Authority (EFSA) has performed the risk assessments on their safety (4). In 2015, EFSA published a list of insect species potentially usable as food (19). Lesser mealworm (*A. diaperinus*) is included in this list. *T. molitor* larvae and *A. domesticus* are now assessed as safe novel food by EFSA (20–22), and dried larvae of the yellow mealworm are already authorized for placing on the market (23) with appropriate labeling of the food in which it is contained.

A. diaperinus is therefore used as feed for rearing animals and might be used for human consumption, once EFSA has given its approval for it as a safe novel food. A second species of the *Alphitobius* genus, *Alphitobius laevigatus* F., not listed by EFSA, is also marketed but it is only intended to feed house pets, including reptiles and amphibians.

Methods are required to check the conformity of these novel products. These last years, different methods of insect detection were developed. Mass spectrometry approaches were proposed for the detection of *T. molitor* and *G. assimilis* (24), *H. illucens* (25), *A. domesticus*, *T. molitor*, *Locusta migratoria* L., and *A. diaperinus* (25, 26), as well as the *Drosophila* genus (Fallén) (27). Mass spectrometry may also help to differentiate insect meals according to their taxonomic groups (28). However, an accurate identification by proteomic methods will only be achievable after more intensive sequencing efforts, given the obvious lack of proteomic data for insect species (24). An adapted sedimentation protocol concentrating the insect particles for their detection in feed by light microscopy was proposed by Veys and Baeten (29).

At present, real-time polymerase chain reaction (PCR) remains the reference technique for DNA detection in food or feed products (30–34). Therefore, detection methods by real-time PCR were also proposed for insects. Most PCR tests published were developed on mitochondrial DNA. This facilitates detection in processed food and feed in which the DNA may be degraded, as a mitochondrion contains several copies of its genome, and several mitochondria can be present in a single cell (35). This is applied for *H. illucens* detection in feed (36, 37), *Oxya chinensis* (Thunberg) in food (38), *Bombyx mori L.* in food (1) and feed (39), and *G. sigillatus* in food and feed (40). However, this multicopy characteristic is a disadvantage for quantitation purposes as the copy number per cell will be variable, depending on the considered tissue (36). This is why other publications focused on single-copy gene tests such as the real-time PCR test for *T. molitor* detection in food and feed (41) and *B. mori* in feed (39). In this study, we chose to focus on a chromosomal target that is a single copy per haploid genome.

This study proposes the first real-time PCR method for specific detection of lesser mealworm (*A. diaperinus*). The target used is the cadherin gene, which for *A. diaperinus* was characterized by Hua et al. (42). Its equivalent in *T. molitor* was already used as a target for the development of a real-time PCR test (41).

MATERIALS AND METHODS

Samples

Insects were either collected in natural environment by trained entomologists, purchased from specialized companies, or provided by the Functional and Evolutionary Entomology laboratory of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium). Insects were selected in order to cover several taxonomic groups or to have close relatives with a practical interest to the species considered. All insects were dead at the arrival at the laboratory. DNA extraction was mostly performed on a single individual, except for smaller insects for which several individuals were required.

TABLE 1 | Primers and probe used within this study.

Target	Name	Sequences 5′-3′	Amplicon size (bp)
Alphitobius diaperinus specific cadherin target	Alphi-Dia-Cad-F	CCAAGTGACTCTCATCATTCAGGAT	134
	Alphi-Dia-Cad-R	CTGAAACCGTAATGTCTAGTTCACCTA	
	Alphi-Dia-Cad-P	FAM- CCATTGCAGATCCAAGTCCCCGAAA -TAMRA	
A. diaperinus large cadherin target	Alphi-Cad-Seq-F	GAAGTGCCTGATCCCAGTGC	208
	Alphi-Cad-Seq-R	TGAGTTCTGCTGTGTAAAGTGCG	
A. diaperinus COI targets	COI-Alphi-F	CGTAGATAAATTTACAGTTTATTGCC	760
	COI-Alphi-R	CAGGATGTCCAAAAAATCAAAATAA	
	COI-Alphi-F2	CAGGATTCGGAATAATTTCTCA	758
	COI-Alphi-R2	TGCAGGAGGAGTTCTTT	

Real-life processed samples (four industrial meals from different EU-based companies) of *A. diaperinus* were obtained through the International Producers of Insects for Food and Feed (IPIFF), but the origin of samples must remain anonymous.

For each *A. diaperinus* industrial meal, a mix containing 0.1% (in mass fraction) of *A. diaperinus* in a commercial fish feed (composition: fishmeal, fish oil, wheat gluten, protein concentrate extracted from pea, maize starch, yeast, lecithin, vitamins, and minerals) was prepared.

DNA Extraction

Genomic DNA was extracted and purified from all samples following the CTAB-based method described in Annex A.3.1 of the international standard ISO 21571:2005 (43). Plasmid DNA was isolated from bacterial cultures with the help of the Genopure Plasmid Maxi Kit (Roche Diagnostics GmbH, Mannheim, Germany). The quality and quantity of DNA extracted from samples were estimated spectrophotometrically using a Nanodrop ND-1000 spectrophotometer at 260 nm (A260) and 280 nm (A280) absorbance. DNA purity was determined using the A260/A280 ratio. The amplifiability of the DNA extract was successfully checked by real-time PCR with the 18S target for insects (41), rbcL (44) for plants, and with a generic fish target (45) for fish. Other species were tested with targets developed or evaluated within the European Union Reference Laboratory for Animal Proteins in Feedingstuff[1] (EURL-AP, 2017) (32) or with the 18S target (46, 47). Ten nanograms of DNA was used in the PCRs.

The industrial meals and the feed mixes at 0.1% of *A. diaperinus* were extracted following the method recommended by EURL-AP and based on the adaptation of the protocol of the Wizard Magnetic DNA Purification System for Food kit (Promega, Madison, WI, USA). This method is described in the EURL-AP Standard Operating Procedure[2] (EURL-AP, 2014). The quantities tested for this purpose are also in line with the EURL-AP SOP.

[1]www.eurl.craw.eu/wp-content/uploads/2021/01/EURL-AP-SOP-Ruminant-PCR-FINAL-V1.2.pdf

[2]www.eurl.craw.eu/wp-content/uploads/2021/01/EURL-AP-SOP-DNA-extraction-V1.1.pdf

Primers and Probe for the Real-Time PCR

Eurogentec (Seraing, Belgium) synthesized the oligonucleotides. The primers and probe sequences developed for the detection of *A. diaperinus* are presented in **Table 1**. The probe for this latter method was labeled with the reporter dye FAM™ (6-carboxyfluorescein) at the 5′ end and the quencher dye TAMRA™ (tetramethyl-6-carboxyrhodamine) at the 3′ end. **Table 1** also lists the primers used for the purpose of differentiating *A. diaperinus* from *A. laevigatus*.

Real-Time PCR Method

Real-time PCR (total reaction volume of 25 μL) was performed on a Lightcycler 480 (Roche Diagnostics Ltd., Rotkreuz, Switzerland) thermocycler using the Universal Mastermix provided by Diagenode (Seraing, Belgium). The reaction mixture included 12.5 μL of Master Mix, 1.7 μL of each primer (5 μM), 1.5 μL of probe (9 μM), 2.6 μL of bidistilled water, and 5 μL of DNA and distributed on 96-well reaction plates (Roche Diagnostics) for specific thermocyclers. Wells were covered with an adhesive film and centrifuged (2 min at 500 revolutions/min) to eliminate any air bubble in the well-bottoms. The thermal program was applied as follows: 2 min at 50°C; 10 min at 95°C; 50 cycles of 15 s at 95°C and 1 min at 60°C.

Specificity of the PCR Method

The specificity of the method was checked on 54 insect species of different taxonomic orders including 19 Coleoptera taxa other than *A. diaperinus*, 6 Diptera, 10 Orthoptera, 7 Hemiptera, 4 Hymenoptera, 5 Lepidoptera, 1 Neuroptera, and 1 Blattodea. One sample of larvae labeled buffalo worm and another sample of larvae considered as *A. laevigatus* were purchased from specialized companies. However, a Sanger sequencing trial (see Results) shows that these two samples belong to the *A. diaperinus* species and are therefore considered as such for the specificity test. The specificity tests were performed on two arachnids and six crustaceans, which like insects belong to the Arthropoda phylum, 1 mollusk and 33 species of vertebrates (12 terrestrial mammals, 6 sea mammals, 8 birds, 7 fish). The possibility of a cross-reaction with human DNA was also envisaged. Seven plant species frequently used in feed and food were included in the experimental setup (**Table 2**). Ten nanograms of DNA was used in the PCRs. Each DNA extract was tested at least in duplicate.

TABLE 2 | Specificity of *Alphitobius diaperinus* PCR test on animal and plant species ($n = 2$).

Taxonomic classification		Latin name or family	Common name	Origin	Results
INSECTS	Coleoptera	*Alphitobius diaperinus* (Panzer)	Lesser mealworm	a	+ ($m = 25.30$, $\sigma = 0.03$)
		Alphitobius diaperinus (Panzer)	Lesser mealworm	b	+ ($m = 31.66$, $\sigma = 0.01$)
		Alphitobius diaperinus (Panzer)*	Lesser mealworm	b	+ ($m = 32.23$, $\sigma = 0.02$)
		Alphitobius diaperinus (Panzer)**	Lesser mealworm	b	+ ($m = 29.11$, $\sigma = 0.02$)
		Pachnoda sp. (Burmeister)	Dola's worm	b	–
		Tenebrio molitor L.	Mealworm	b	–
		Zophobas morio F.	Superworm	b	–
		Carabus sp. L.	Beetle	a	–
		Staphylinidae (Latreille)	Rove beetles	a	–
		Curculionidae/Scolytidae (Latreille)	True weevils	a	–
		Coccinellidae sp. (Latreille)	Ladybird	a	–
		Scarabidae sp. (Latreille)	Scarab beetles	a	–
		Oxythyrea funesta (Poda)	White-spotted rose beetle	a	–
		Melolontha melolontha L.	Cockchafer	c	–
		Leptinotarsa decemlineata (Say)	Colorado potato beetle	c	–
		Cassida viridis L.	Green tortoise beetle	c	–
		Cicindela campestris L.	Green tiger beetle	c	–
		Nicrophorus humator (Gleditsch)	Black sexton beetle	c	–
		Nicrophorus vespillo L.	Common burying beetle	c	–
		Cetonia aurata L.	Rose chafer	a	–
		Lucanus cervus L.***	Stag beetle	a	–
		Rhynchophorus ferrugineus (Olivier)	Red palm weevil	b	–
		Cybister limbatus F.	Diving beetle	b	–
	Diptera	*Hermetia illucens* L.	Black soldier fly	b	–
		Tabanus sp. L.	Horse fly	a	–
		Sarcophaga carnaria L.	Common fresh fly	c	–
		Bombylius major L.	Large bee-fly	c	–
		Syrphidae (Latreille)	Hover fly	a	–
		Musca domestica L.	House fly	a	–
	Orthoptera	*Locusta migratoria* L.	Migratory locust	c	–
		Acheta domesticus L.	House cricket	b	–
		Gryllus bimaculatus (De Geer).	Mediterranean field cricket	b	–
		Gryllus assimilis F.	Jamaican field cricket	b	–
		Gryllus sp. L.	Cricket	a	–
		Locusta sp. L.	Locust	b	–
		Acheta sp. L.	Cricket	a	–
		Patanga succincta (Johannson)	Bombay locust	b	–
		Schistocerca sp. (Forsskål)	Sold as ≪ small grasshopper ≫	b	–
		Brachytrupes portentosus (Lichtenstein)	Giant cricket	b	–
	Hemiptera	Aphididae (Latreille)	Aphid	a	–
		Anthocoridae (Fieber)	Bugs	a	–
		Palomena prasina L.	Green shield bug	a	–
		Pyrrhocoris apterus L.	Firebug	a	–
		Belostomatidae sp. (Leach)	Giant water bug	b	–
		Psyllus sp. (Latreille)	Jumping plant louse	a	–
		Cicadidae sp. (Latreille)	Cicada	b	–
	Hymenoptera	*Apis* sp. L.	Bee	a	–
		Bombus terrestris L.	Buff-tailed bumblebee	a	–
		Vespula sp. (Thomson)	Wasp	a	–

(Continued)

TABLE 2 | Continued

Taxonomic classification		Latin name or family	Common name	Origin	Results
		Oecophylla smaragdina F.	Weaver ant	b	–
	Lepidoptera	*Biston betularia* L.	Peppered moth	a	–
		Tineola sp. (Latreille)	Moth	a	–
		Bombyx mori L.	Silkworm	b	–
		Galleria mellonella L.	Greater wax moth	a	–
		Omphisa fuscidentalis (Hampson)	Bamboo worm	b	–
	Neuroptera	*Chrysoperla carnea* (Stephens)	Green lacewing	a	–
	Blattodea	*Blatta orientalis* L.	Oriental cockroach	c	–
Arachnida		*Heterometrus longimanus* (Herbst)	Black scorpion		–
		Haplopelma albostriatum (Simon)	Tarantulas		–
Crustacean		*Euphausia superba* (Dana)	Antartic krill		–
		Penaeus vannamei (Boone)	Whiteleg shrimp		–
		Crangon crangon L.	Common shrimp		–
		Nephrops norvegicus L.	Langoustine		–
		Homarus gammarus L.	European lobster		–
		Paralithodes camtschatieus (Tilesius)	Red king crab		–
Mollusca		Teuthida sp. (Naef)	Squid		–
Terrestrial mammals		*Bos taurus* L.	Beef		–
		Sus scrofa domesticus (Erxleben).	Pork		–
		Sus scrofa scrofa L.	Wild boar		–
		Ovis aries L.	Sheep		–
		Capra hircus L.	Goat		–
		Equus caballus L.	Horse		–
		Equus asinus L.	Donkey		–
		Lepus europaeus (Pallas)	Hare		–
		Capreolus capreolus L.	Roe deer		–
		Cervus elaphus L.	Stag		–
		Rattus rattus L.	Rat		–
		Homo sapiens L.	Human		–
Sea mammals		*Stenella coeruleoalba* (Meyen)	Striped dolphin		–
		Tursiops truncatus (Montagu)	Bottlenose dolphin		–
		Grampus griseus (G. Cuvier)	Risso's dolphin		–
		Ziphius cavirostris (G. Cuvier)	Cuvier's beaked whale		–
		Phocoena phocoena L.	Harbor porpoise		–
		Phocidae (Gray)	Seals		–
Fish		*Salmo salar* L.	Salmon		–
		Gadus morhua L.	Atlantic cod		–
		Scomber scombrus L.	Atlantic mackerel		–
		Clupea harengus L.	Atlantic herring		–
		Mallotus villosus (Müller)	Capelin		–
		Sprattus sprattus L.	Sprat		–
		Engraulis encrasicolus L.	European anchovy		–
Birds		*Gallus gallus* L.	Chicken		–
		Meleagris gallopavo L.	Turkey		–
		Numida meleagris L.	Guinea fowl		–
		Cairina moschata L.	Muscovy duck		–
		Anser sp. L.	Goose		–
		Coturnix japonica (Temminck and Schlegel)	Quail		–
		Struthio camelus L.	Ostrich		–
		Turdus merula L.	Blackbird		–

(Continued)

TABLE 2 | Continued

Taxonomic classification	Latin name or family	Common name	Origin	Results
Plants	*Glycine max* (Merr)	Soybean		–
	Zea mays L.	Maize		–
	Brassica napus L.	Rapeseed		–
	Triticum aestivum L.	Wheat		–
	Oryza sativa L.	Rice		–
	Solanum lycopersicum L.	Tomato		–
	Beta vulgaris L.	Sugar beet		–

+ = Positive signal, − = No signal.

**Sample labeled buffalo worm was purchased from a specialized company but identified as A. diaperinus by Sanger sequencing.*

***Sample labeled A. laevigatus was purchased from a specialized company but identified as A. diaperinus by Sanger sequencing.*

****The Lucanus cervus (protected species) was not collected in the environment but obtained from an old insect box coming from a private collection.*

For positive samples, mean Cq values (m) and standard deviations (σ) are given in brackets. Origin of the insect samples is specified with "a" for insects collected by trained entomologists, "b" for insects purchased from specialized companies and "c" for the insects provided by the Functional and Evolutionary Entomology lab of Gembloux Agro-Bio Tech (ULiège, Gembloux, Belgium).

Cloning of the Target, Copy Number Determination of the Plasmid DNA, and Dilutions

The 134-bp target from the *A. diaperinus* cadherin gene was ligated into the 3.9-kb pCR® 2.1-TOPO plasmid vector (Invitrogen, Merelbeke, Belgium) following the TOPO® TA Cloning® kit instructions (Invitrogen). Plasmid DNA isolated from bacterial cultures was linearized with the *Hin*dIII restriction enzyme (Promega) and then purified using phenol–chloroform–isoamyl alcohol.

The quantity of recovered plasmid DNA was converted into copy numbers as usual (36, 48, 49), taking into consideration that (i) 1 unit of absorbance at 260 nm corresponds to a concentration of 50 μg/mL of double-stranded DNA, and (ii) the mean molar weight of one base pair is set at 635 Da (50).

The sensitivity, efficiency, and robustness of the PCR test were determined on diluted plasmid DNA. These dilutions were performed in water until an estimated copy number of 10,000 copies/5 μL was reached. Higher dilutions of the target DNA were prepared in a solution containing 50 ng/μL of salmon sperm DNA as background DNA. Low binding tubes were chosen to minimize DNA losses.

Copy Number Determination of *A. diaperinus* Genomic DNA and Dilutions

The quantity of genomic DNA corresponding to 36,000 target copies was estimated at 10.08 ng for *A. diaperinus* based on data from the animal genome size database (https://www.genome.size.com/results.php?page=3) at the University of Guelph (Ontario, Canada). The sensitivity (limit of detection [LOD]) and efficiency of the PCR test were determined on diluted genomic DNA. Genomic DNA dilutions were performed in a similar way as described for the cloned target.

Limit of Detection

Target sensitivity was evaluated following the recommendations of the former AFNOR XP V03-020-2 standard (51). This standard no longer exists, but the principles detailed in it are still valid. The absolute LOD was determined for the PCR assay (primers + probe + amplification program) on dilutions of plasmid material and on dilutions of genomic material.

The subsequent dilutions had to contain 50, 20, 10, 5, 2, 1, and 0.1 copies of the target. Six PCRs had to be achieved for each dilution. The method's LOD_6 was the smallest copy number for which the six PCRs were positive, but only if the highest dilution supposed to contain the 0.1 copy per reaction generated a maximum of one positive PCR signal on six replicates. If more than one positive signal was observed for the 0.1 copy, the DNA quantities had to be revised. The copy number corresponding to LOD_6 was then tested 60 times on the same plate (determination of the $LOD_{95\%}$). The $LOD_{95\%}$ is validated as equal or below a given copy number if at least 95% of positive signals are recorded out of the 60 replicates. The highest acceptable copy number for LOD_6 and $LOD_{95\%}$ is 20 copies.

Efficiency

The efficiency of the PCR assay was calculated with a dilution series of genomic DNA and plasmid material at target levels of 5,000, 2,500, 1,000, 500, and 100 copies. Each dilution was analyzed in six replicates and on four runs. The efficiency has to be between 90 and 110% (52).

Digital PCR

The number of copies of the nuclear and plasmid DNA dilutions at approximately 500 copies/5 μL (5 μL being the volume of the DNA extracts added in the real-time PCR mix) were checked by digital PCR. Digital PCR was performed on the Biomark™ HD system (Fluidigm Corporation, South San Francisco, CA, USA) using the 12.765 Digital Array™. These digital arrays comprise 12 panels (12 wells, thus 12 samples), each of which is partitioned into 765 individual PCR of 6 nL. The reaction mixture included 4 μL of Universal Master Mix with passive reference (Diagenode), 0.15 μL of each primer (18.1 μM), 0.15 μL of probe (28.8 μM), 0.4 μL GE sample loading reagent (Fluidigm), and 3.15 μL of plasmid DNA. Eight microliters of reaction mix was dispensed into each sample inlet, and ∼4.6 μL of this reaction mix was distributed throughout the partitions

TABLE 3 | Experimental conditions tested to evaluate the robustness of the described *Alphitobius diaperinus* PCR test.

PCR machine	Lightcycler 480 (Roche Diagnostics Ltd) and QuantStudio 5 (Applied Biosystems)				
PCR reagent kit	Universal Mastermix (Diagenode s.a.) and ABI Taqman 2x Universal PCR Master mix (Applied Biosystems)				
Annealing temperature	59 and 61°C				
Primer concentration	Minus 30%	Standard	Standard	Standard	Standard
Probe concentration	Standard	Minus 30%	Standard	Standard	Standard
PCR volume	Standard (20 µL mix + 5 µL DNA)	Standard (20 µL mix + 5 µL DNA)	Standard (20 µL mix + 5 µL DNA)	Standard + 1 µL Mastermix (21 µL mix + 5 µL DNA)	Standard – 1 µL Mastermix (19 µL mix + 5 µL DNA)

within each panel using an automated NanoFlex IFC Controller (Fluidigm Corporation) (53). Two arrays were analyzed with for each, 11 replicates of plasmid dilution and one no template control. The thermal program was as follows: 10-min activation step at 95°C, 50 cycles of 15 s at 95°C for denaturation and 60 s at 60°C for annealing and extension.

The number of target molecules per panel was determined using the BioMark HD Digital PCR software.

Robustness of the PCR Method

The method robustness was tested by introducing some slight deviations to the standard experimental conditions (54). Parameters considered were as usual (36, 52, 55): the annealing temperature (60 ± 1°C), the primer concentrations (standard or reduced by 30%), the probe concentration (standard or reduced by 30%), and the real-time PCR Master Mix volume (standard or ± 1 µL), which involves a final reaction volume of 25 ± 1 µL. Six replicates of the plasmid borne target at 20 copies/5 µL were tested in the conditions described in **Table 3**. The robustness was performed on two real-time PCR platforms: thermocycler Lightcycler 480 (Roche Diagnostics Ltd.) with Universal Mastermix by Diagenode and thermocycler QuantStudio™ 5 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) with ABI TaqMan 2x Universal PCR Master Mix No AmpErase UNG (Applied Biosystems). The acceptance criterion is that all deviations to the standard protocol must give a positive result at a level of 20 copies of the target in the reaction (52).

Applicability of the PCR Method

The applicability of the PCR method was checked in duplicate on four real-life processed samples (industrial meals) of *A. diaperinus* produced in the EU and on a fish feed containing 0.1% in mass fraction of *A. diaperinus* industrial meal. Two DNA extracts with two dilutions were tested by PCR in duplicate.

Transferability of the PCR Method

The efficiency and the LOD (LOD_6 and $LOD_{95\%}$) of the PCR assay were tested on genomic DNA in the laboratory of Eurofins Biologie Moléculaire France with conditions similar to those of the developer's laboratory (CRA-W, Gembloux, Belgium). The dilution series for the LOD test were carried out by this second laboratory starting from the solution at 500 copies/5 µL checked by digital PCR.

Real-time PCR was performed on thermocycler CFX96 Deep Well Real-time PCR Detection Systems (Bio-rad, Hercules, CA, USA) using the Applied Biosystems™ TaqMan™ Universal PCR Master Mix (Applied Biosystems). Reaction mixtures were distributed on Hard-Shell® 96-Well PCR Plates (Bio-rad) developed for the CFX96 thermocyclers. Wells were covered with adhesive film, and the plates were centrifuged to eliminate any air bubble in the well-bottoms.

Amplicon Preparation for Sanger Sequencing

The PCRs to generate amplicons that had to be checked by Sanger sequencing were done as follows. For the large cadherin target, the PCR contained 1 µL of DNA extract from the sample to be checked, 6 µL of 5× GoTaq® Flexi Buffer (Promega), 3 µL of 2 mM dNTP mix (Thermo Scientific, Waltham, MA, USA), 3 µL of bovine serum albumin (BSA; Roth, Karlsruhe, Germany), 1.8 µL of 25 mM $MgCl_2$ solution (Promega), 3 µL of 5 µM forward and reverse primers, 0.15 µL of 5 U/µL GoTaq® G2 Flexi DNA Polymerase (Promega), and nuclease-free water to 30 µL. The thermal cycling conditions were set as follows: initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min.

To generate amplicons focused on the cytochrome c oxidase subunit I (COI) targets (**Table 1**), PCRs were set up in at similar way and contained 1 µL of DNA extract from the sample to be checked, 6 µL of 5x GoTaq® Flexi Buffer (Promega), 3 µL of 2 mM dNTP mix (Thermo Scientific), 3 µL of BSA (Roth), 1.8 µL of 25 mM $MgCl_2$ solution (Promega), 3 µL of 5 µM forward and reverse primers, 0.15 µL of 5 U/µL GoTaq® G2 Flexi DNA Polymerase (Promega), and nuclease-free water to 30 µL. The thermal cycling conditions consisted in an initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 47°C (COI_Alphi_F/R) or 52°C (COI_Alphi_F2/R2) for 30 s and 72°C for 1 min, and a final extension at 72°C for 10 min.

Five microliters of PCR products were run on a 1.2% agarose gel to check amplicon quality. The remaining 25 µL of PCR products was sent to Eurofins Genomics (Constance, Germany) for Sanger sequencing.

RESULTS

The cadherin gene of *A. diaperinus* was used to select a piece of DNA that is specific to the considered insect species. Appropriate primers and probe were designed to amplify a 134-bp fragment of the cadherin gene (**Table 1**). The latter is considered as a single-copy gene in several insect species such as the Lepidopteran *Ostrinia nubilalis* (Hübner) (56) or the Coleoptera *Diabrotica virgifera virgifera* (Le Conte) (57), which is an advantage for quantitation purposes.

The specificity was first investigated *in silico* using the Blast tool available on the National Center for Biotechnology Information (NCBI) database. The different blasts and the alignments of DNA sequences with other Coleopteran (58) performed indicated that the PCR test should be specific to the target species *A. diaperinus*. The alignment with the closest relative within the NCBI database is the *T. molitor* sequence. It shows some similarity but not enough for allowing primers and probes to hybridize (**Supplementary File 1**). Specificity was also experimentally tested on DNA from *A. diaperinus* of various origins as well as on 53 other non-target insect species, including 19 Coleoptera. Positive results were obtained only with all samples of *A. diaperinus*. No signal was obtained with the 53 other insect species, the 42 other animal species (arachnids, crustaceans, mollusk and vertebrates), and the seven plant species tested (**Table 2**).

A difficulty that appeared was to know if the test enables to distinguish *A. diaperinus* from *A. laevigatus*, a closely related species commercialized as feed product for non-farmed animals. Products labeled as *A. laevigatus* or as buffalo worm were tested, and a clear positive signal with the PCR test for *A. diaperinus* was observed. A doubt existed, however, if these samples really belonged to the *A. laevigatus* species. To check this hypothesis, Sanger sequencing was applied on PCR products of eight collected samples of *Alphitobius* (**Supplementary File 2**). Three targets were considered. The first one focused on a larger cadherin target (**Table 1**), which completely contains the smaller target of 134 bp. The two other targets focused on COI regions known as more variable and considered as suitable to allow a distinction between *A. diaperinus* and *A. laevigatus* according to published data. All Sanger sequencings were successful and showed identical sequences for the eight samples (**Supplementary Files 2–5**).

The sequences obtained for the cadherin target corresponded to the *A. diaperinus* sequence published by Hua et al. (2014-KC470207.1)[3] (**Supplementary File 3**). The alignment of sequenced COI fragments showed that all analyzed specimens belong to the *A. diaperinus* species when compared with *A. diaperinus* and *A. laevigatus* reference sequences. The first COI portion corresponded to the *A. diaperinus* sequence published by Hong et al. (2020-NC_049092.1)[4] (**Supplementary File 4**); this COI region is, however, not available for *A. laevigatus*. Reference sequences are available for both *Alphitobius* species for the second COI region considered. Results for that target showed that all samples of *Alphitobius* tested belonged to the *A. diaperinus* species (**Supplementary File 5**).

TABLE 4 | Copy numbers obtained on dilution of genomic DNA at ∼500 copies/5 μL by digital PCR on a Biomark™ HD system.

Copy number of target/5 μL	Copy number mean of target/5 μL ± SD (σ)	Coefficient of variation
343	339 ± 32.01	9.45%
357		
365		
396		
374		
349		
346		
329		
357		
271		

Ten replicates were analyzed (n = 10).

The amplification efficiency, LOD, and robustness were evaluated on plasmid DNA. The efficiency and LOD were also determined on genomic DNA.

To check that the number of copies in the dilutions used to assess the performance criteria was correct, the dilutions at approximately 500 copies/5 μL were estimated by digital PCR on a Biomark™ HD system. For the genomic DNA, the average obtained over the 10 measurements by digital PCR was 339 copies/5 μL with a variation coefficient at 9.45% (**Table 4**). This mean copy number measured was slightly lower than the expected value (based on the genome size of *A. diaperinus* and considering the cadherin gene as a single-copy gene). The difference between these values did not exceed a factor of 2, corresponding to the subsequent dilution. These results therefore confirm that the cadherin gene is a single-copy gene per haploid genome as mentioned in other studies (56, 57). However, in order to be closer to the expected values to evaluate the efficiency and LOD, new dilutions on the genomic DNA were carried out, taking into account the results obtained in digital PCR. On these new dilutions, the average obtained over the 22 measurements by digital PCR was 498 copies/5 μL with a variation coefficient at 9.21% (**Table 5**). The mean copy number measured was estimated at the expected value and it is from this dilution series that the efficiency and sensitivity were evaluated.

The copy number of the plasmid material (linearized) was also checked, and the average copy obtained over the 22 measurements by digital PCR was 446 copies/5 μL with a variation coefficient at 9.26% (**Table 6**). The mean copy number measured was estimated at the expected value, and it is from this dilution series that the efficiency, sensitivity, and robustness were evaluated.

The PCR efficiency was evaluated at 102.5% on plasmid DNA and 100.0% on genomic DNA. This was calculated, taking into account the mean Cq (quantification cycle) values obtained at the different copy numbers tested (from 5,000 to 10), and no outliers were encountered (**Tables 7**, **8**). When calculated per plate, the efficiency was always higher than 90% and therefore met the acceptance criterion proposed by Broeders et al. (52).

[3]https://www.ncbi.nlm.nih.gov/nuccore/KC470207.1
[4]https://www.ncbi.nlm.nih.gov/nuccore/NC_049092.1

TABLE 5 | Copy numbers obtained on dilution slightly adapted of genomic DNA at ∼500 copies/5 μL by digital PCR on a Biomark™ HD system.

Array	Copy number of target/5 μL	Copy number mean of target/5 μL ± SD (σ)	Coefficient of variation
1	523	498 ± 8.18	9.21%
	515		
	540		
	528		
	515		
	512		
	584		
	531		
	451		
	556		
	526		
2	501		
	401		
	479		
	526		
	445		
	470		
	470		
	470		
	484		
	526		
	404		

Twenty-two replicates was analyzed on 12.765 Digital Array™ (n = 22).

TABLE 6 | Copy numbers obtained on dilution of plasmid DNA at ∼500 copies/5 μL by digital PCR on a Biomark™ HD system.

Array	Copy number of target/5 μL	Copy number mean of target/5 μL ± SD (σ)	Coefficient of variation
1	443	446 ± 8.25	9.26%
	398		
	487		
	484		
	445		
	451		
	531		
	473		
	365		
	418		
	390		
2	434		
	421		
	493		
	448		
	432		
	487		
	434		
	451		
	473		
	476		
	374		

Twenty-two replicates were analyzed on 12.765 Digital Array™ (n = 22).

Concerning the sensitivity testing, the LOD_6 was estimated at five copies following the AFNOR XP V03-020-2 standard approach (51) and also at five copies for the $LOD_{95\%}$ with 57/60 positive signals on plasmid DNA and 58/60 positive signals on genomic DNA. Sixty of 60 positive signals were obtained at the level of 10 copies with a mean Cq value of 36.14 cycles on plasmid DNA and 36.78 cycles on genomic DNA. Therefore, the PCR test easily reaches the recommended performance criterion ($\leq$ 20 copies).

The PCR method robustness was also evaluated on plasmid DNA, with success. All tested deviations to the standard protocol delivered positive results at the level of 20 copies in the PCR.

Positive signals were obtained on industrial samples (PAPs of *A. diaperinus*) showing the applicability of the PCR test on real-life samples (**Table 9**).

The applicability was tested on a commercial fish feed adulterated with a low content of processed *A. diaperinus.* The commercial fish feed was first tested as free of *A. diaperinus* and the amplifiability of the DNA extracts obtained from this fish feed was checked with a 18S rDNA target (46, 47).

The four mixes of fish feed containing 0.1% of different processed *A. diaperinus* meals were tested and gave positive results with the cadherin PCR test (**Table 9**). The 10-fold dilutions provided evidence that there was no inhibitory effect of the feed matrix on the amplification of the *A. diaperinus* target.

TABLE 7 | Cq values obtained on dilutions of plasmid material used for efficiency calculation and for $LOD_{95\%}$.

Copy number of target	Cq (mean value) ± SD (σ) and (*n*)
5,000	26.83 ± 0.06 (24)
2,500	27.85 ± 0.11 (24)
1,000	29.08 ± 0.09 (24)
500	30.08 ± 0.11 (24)
100	32.39 ± 0.23 (24)
10	36.14 ± 0.50 (60)

For efficiency, each concentration was analyzed in six replicates and on four PCR plates (n = 24). For $LOD_{95\%}$, the concentration at 10 copies was analyzed in 60 replicates on 1 PCR plate (n = 60).

Concerning the transferability, the PCR efficiency was evaluated at 90.0% on genomic DNA in a second laboratory and therefore met the acceptance criterion proposed by Broeders et al. (52). **Table 10** indicates the mean Cq values obtained with the different copy numbers tested (from 5,000 to 10), and no outliers were encountered. When calculated per plate, the efficiency was higher than 90% for three plates and slightly below for the fourth one with an efficiency at 87.7%. The LOD_6 was estimated at five copies following the former AFNOR XP V03-020-2 standard approach (51) and at 10 copies for the $LOD_{95\%}$ with 60/60 positive signals. The mean Cq value at 10 copies is of 37.08 cycles.

TABLE 8 | Cq values obtained on dilutions of genomic material used for efficiency calculation and for $LOD_{95\%}$.

Copy number of target	Cq (mean value) ± SD (σ) and (*n*)
5,000	27.55 ± 0.07 (24)
2,500	28.60 ± 0.07 (24)
1,000	29.87 ± 0.09 (24)
500	30.89 ± 0.12 (24)
100	33.21 ± 0.19 (24)
10	36.78 ± 0.48 (60)

For efficiency, each concentration was analyzed in six replicates and on four PCR plates (n = 24). For $LOD_{95\%}$, the concentration at 10 copies was analyzed in 60 replicates on 1 PCR plate (n = 60).

TABLE 9 | Mean Cq obtained with the *Alphitobius diaperinus* PCR test on processed samples from *A. diaperinus* and on mixes containing 0.1% in mass fraction of *A. diaperinus* in a commercial fish feed (*n* = 2).

Identification of samples			Mean Cq obtained with *Alphitobius diaperinus* PCR test	
			Dilution 1×	Dilution 10×
Pure industrial meals of *A. diaperinus* produced in the EU	n°1	Extract 1	21.38	24.87
		Extract 2	21.03	24.39
	n°2	Extract 1	19.69	23.17
		Extract 2	19.55	23.05
	n°3	Extract 1	23.65	27.09
		Extract 2	22.94	26.52
	n°4	Extract 1	22.52	26.04
		Extract 2	22.09	25.59
Fish feed containing 0.1% of *A. diaperinus* from the industrial meal n°1	Extract 1		32.62	35.59
	Extract 2		32.79	36.10
Fish feed containing 0.1% of *A. diaperinus* from the industrial meal n°2	Extract 1		29.97	33.53
	Extract 2		29.78	33.15
Fish feed containing 0.1% of *A. diaperinus* from the industrial meal n°3	Extract 1		33.13	36.38
	Extract 2		33.20	36.56
Fish feed containing 0.1% of *A. diaperinus* from the industrial meal n°4	Extract 1		32.74	36.41
	Extract 2		32.46	35.93

The PCR test easily reached the recommended performance criterion ($\leq$ 20 copies). The transferability of the method was therefore demonstrated.

TABLE 10 | Cq values obtained on dilutions of genomic material used for efficiency calculation and for $LOD_{95\%}$ for the transferability test.

Copy number of target	Cq (mean value) ± SD (σ) and (*n*)
5,000	27.26 ± 0.11 (24)
2,500	28.47 ± 0.16 (24)
1,000	29.92 ± 0.11 (24)
500	31.05 ± 0.14 (24)
100	33.37 ± 0.23 (24)
10	37.08 ± 0.80 (60)

For efficiency, each concentration was analyzed in 6 replicates and on 4 PCR plates (n = 24). For $LOD_{95\%}$, the concentration at 10 copies was analyzed in 60 replicates on 1 PCR plate (n = 60).

DISCUSSION

The study describes a specific, sensitive, and robust test to detect *A. diaperinus*. With the recent authorization in the EU legislation to use eight insect species, among which lesser mealworm, in aquafeed, pig, and poultry feed, the interest of such a PCR test is increasing.

During the search for the target and the validation study, special care was taken to be able to distinguish lesser mealworm from other representatives of the *Tenebrionidae* family (**Supplementary File 1**). Blasting the sequences of the developed primers and of the targeted fragment against the NCBI nucleotide database showed that the PCR test is specific to the target species *A. diaperinus*. Unfortunately, cadherin gene sequences for other *Alphitobius* species are not available in the NCBI and DNA Data Bank of Japan databases. Sequence alignments were therefore not possible. The other *Alphitobius* species are, however, not produced nor marketed, with the exception of *A. laevigatus*.

It seems that there is an important confusion at commercial level between *A. diaperinus* and *A. laevigatus*. Indeed, *A. diaperinus* is known under the common names of lesser mealworm for the larvae (12, 14, 25, 42, 59) and darkling beetle at adult stage (19, 60, 61), whereas *A. laevigatus* is known as black fungus beetle[5] (37, 62, 63). Especially as larvae, the name of buffalo worm can be used to designate both species (7, 9, 64–70). *A. laevigatus* looks like *A. diaperinus* in size and shape[3] (62, 63). In a review on *Tenebrionidae* in France, Bonneau (71) also clearly points out that there are confusions between *A. diaperinus*, *A. laevigatus*, *Alphitobius piceus* Olivier, and *Alphitobius ovatus* Herbst.

This confusion seems also to occur at the level of the sequences available in the NCBI database. Two COI sequences published by (72, 73) (accession no. KM435102.1 and KM652640.1) attributed to *A. diaperinus* show 99% identity with *A. laevigatus* [KP410252.1, (74)] but only 88% with *A. diaperinus* [NC_049092.1, (75)].

This probably also explains that the ordered samples of *A. laevigatus* were wrongly labeled by their providers. The Sanger sequencing revealed that these samples were in reality *A. diaperinus* samples. No real commercial sample of *A. laevigatus* was found, and even if we did not check with all possible sources, it seems that it is not that easy to find marketed *A. laevigatus* larvae. That is the reason why from a merely practical point of view we consider that the 134-bp cadherin target published in this study is specific to *A. diaperinus*. Nevertheless, from a scientific viewpoint, it is not impossible that the target is specific

[5]https://www.ams.usda.gov/sites/default/files/media/StoredGrainInsectsReference2017.pdf

only at genus level. It could not be checked because of a lack of appropriate samples to do so.

The applicability of the PCR method was also successfully tested on real-world samples. This validates the implementation of the method on industrial samples.

Finally, it should be stressed that the target is present only once per haploid genome, which makes it a suitable target for possible quantification purposes. This would require to have a general insect target, present only once per haploid genome. To our knowledge, such a target has unfortunately not yet been identified.

CONCLUSIONS

The developed PCR method based on the cadherin gene fits for the purpose of detection of *A. diaperinus* in feed. Indeed, the efficiency met the required criterion, the specificity gave good results, and only *A. diaperinus* was detected with respect to the insect, animal, and plant species tested. The acceptance criteria were also reached for sensitivity (LOD_6 and $LOD_{95\%}$) and robustness. The PCR method was applicable on real-life samples from industry even when *A. diaperinus* was present at 0.1% in mass fraction in a fish feed. Finally, the transferability of the method in a second laboratory was also demonstrated by testing the efficiency and LOD.

The developed method was primarily aimed for application on feed. However, it is not excluded that several of the PCR tests developed in the study might be helpful to taxonomists in a better delineation of the several taxons within the genus *Alphitobius*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AM, HS, BD, and FD contributed to the design and implementation of the research. AM, HS, BD, JM, and FD designed and performed the experiments. SG and J-FM participated to the transferability study. AM, JM, BD, SG, J-FM, and FD analyzed the data and interpreted the results. AM wrote the manuscript with the help of FD. GB, BD, FF, SG, J-FM, and OF provided valuable comments to improve the quality of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.718806/full#supplementary-material

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