DISSERTATION

EDIBLE MEALWORMS: CAN FERMENTATION IMPROVE CONSUMER ACCEPTABILITY AND NUTRITIONAL VALUE?

Submitted by

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ABSTRACT

EDIBLE MEALWORMS: CAN FERMENTATION IMPROVE CONSUMER ACCEPTABILITY AND NUTRITIONAL VALUE?

As the global population increases, the demand for animal-based protein is also on the rise. To meet this demand, it is important to identify sustainable sources of animal protein that have a smaller environmental impact than conventional animal protein production. One potential solution to this challenge is the development of consumer-acceptable insect-based protein products utilizing the larva and pupae of *Tenebrio molitor,* a type of darkling beetle whose larval and pupal states are edible.

These beetles can be reared in small spaces, do not require direct sources of fresh water, and convert feed into protein more efficiently than conventional meat production. In addition, their waste (frass) is dry, making it easier to contain than waste from conventional animal rearing operations, reducing the risk of contaminating the surrounding environment. In addition, the larvae and pupae can be eaten in their entirety, eliminating potential waste streams of byproducts associated with conventional meat processing. With less space, less water usage, more efficient feed conversion ratios, and nearly zero waste, the development of an edible mealworm industry in the global West would help relieve some of the pressures on the current animal protein systems and improve global food security.

To accomplish this, it is necessary to generate a consistent demand in the global West for insect protein. Currently, in the United States, edible insects are largely relegated to ground powders designed to “hide” the insects or are placed in novelty products like chocolates

and lollipops to confront people's notion of disgust. To introduce mealworm protein into the mainstream, it must be in a form that is accessible to the average American consumer, be safe to eat, and have comparable nutritional attributes as other protein-based products on the market. Tempeh fermentation techniques may be an appropriate approach to accomplish these goals. Tempeh fermentation uses Rhizopus oligosporus mycelium to knit together legumes into a solid cohesive substrate. The product can then be utilized in a variety of ways that are familiar to American consumers including stir frys, burgers, nuggets, and crumbles. This project seeks to utilize tempeh fermentation techniques to develop an insect-based product that is both consumer-friendly but also capitalizes on the known and emerging nutritional and environmental benefits of edible insects.

In Chapter 1, I examine the safety of tempeh produced with various life stages of the *Tenebrio molitor* beetle. Samples were assessed for water activity (aw), and pH to determine the shelf stability of the products. Pathogenic risk was assessed through testing for coliforms, *Salmonella* and *Listeria*, and samples were analyzed for heavy meatal content via utilized Inductively Coupled Plasma and Mass Spectrometry (ICP-MS). This exploration allows us to determine best storing and cooking methods and helps identify critical control points in production to help minimize the risk to the consumer. In this chapter, I was able to demonstrate that tempeh made with *Tenebrio molitor* was just as safe as conventional soy-based tempeh and requires similar storage and cooking precautions to minimize the risks of consumption.

In Chapter 2 we conducted a nutritional analysis of the insect-based tempeh products in comparison to traditional soy-based tempeh. We utilized ICP-MS to quantify the presence of micronutrients within each example. Samples were also analyzed for vitamins, macronutrients, and amino acid profile. We then calculated the protein digestibility using the

Protein Digestibility-Corrected Amino Acid Score (PDCAAS). Comparison of results with traditional soy-tempeh products helps us to determine if the products provide comparable nutrition to products already on the market. It also helps to determine if the novel products will fill the nutritional space of more conventional sources of protein. This chapter found that the tempeh products made with the *Tenebrio molitor* had nutritional attributes comparable to conventional soy tempeh.

Chapter 3 examines the bioavailability of iron within the sample set and compare the results to conventional bee samples, current on-the-market plant-based meat alternatives, and traditional soy-based tempeh. ICP-MS was utilized to quantify the amounts of iron present in each chemically digested sample. Then, digestates were added to Caco-2 human colonic cells to allow absorption of available iron. Iron absorption rates were then determined by using a human ferritin Eliza kit. This assessment helps us determine if the presence of insect protein improves the bioavailability of iron in a traditionally plant-based food and allows us to compare the availability of the iron in the novel products to conventional beef and current plant-based meat products. The bioavailability of iron in the novel products exceeded that of the conventional beef and the plant-based meat alternative.

In Chapter 4, we conducted a consumer acceptability study to analyze the potential for consumer acceptance of a tempeh product made with 50% mealworms and 50% soybeans when compared to a commercial soy-based tempeh. An online survey was conducted to assess the public's attitudes to entomophagy, their current level of exposure to the practice, and the willingness to consume insects. Next, the mealworm soybean tempeh was evaluated by a trained sensory panel to develop a lexicon that describes the organoleptic attributes of the product. Finally, a blind in-person sensory evaluation was conducted to assess the overall acceptability of the product. During the in-person evaluation, participants were provided with different prompts to determine if details around the environmental impact of insect eating versus conventional meat production would affect the favorability of the product. Participants in this study rated the flavor equal to that of the commercial soy tempeh and majority of participants indicated they were equally or more likely to consume insect-based products again.

The final chapter explores the need for research around branding and availability on insect-based food products to increase acceptance of entomophagy in Western society. This dissertation aims to determine the safety of utilizing edible insects in tempeh fermentation, examine the nutritional attributes of tempeh products made with various life stages of the Tenebrio molitor beetle, determine how effective these products are in filling their intended nutritional niches, and assess the potential for consumer acceptance of insect-based tempeh products. This dissertation provides a strong foundation for the understanding of the safety, nutrition, and acceptability of utilizing T. molitor-based tempeh as an alternative source of protein.

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DEDICATION

This dissertation is dedicated

To my community.

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Chapter 1: Examining the Food Safety of Tempeh Products Utilizing *Tenebrio molitor* as a Substrate.

1.1 Summary

With the global population on the rise, edible insect protein may help meet the nutritional needs of the growing population. To access this protein source, it is important to develop products that can maximize consumer acceptance and still meet safety standards. In this study, we used *Rhizopus oligosporus* spores and two juvenile life stages of the *Tenebrio molitor* beetle to develop four insect-based tempeh products and evaluated the food safety of those products in comparison to a commercially available soy-based tempeh product. To do this we tested each product for water activity (a_W), pH, and microbial counts to assess the inherent risks in preparing and the insect-based product as compared to a commercially available soy tempeh. We also examined the heavy metal content of lab-produced tempeh products using microwave digestion and Inductively Coupled Plasma and Mass Spectrometry (ICP-MS) Analysis and compared the results to unfermented controls of their constituent parts.

A comparison of the product showed no statistically significant differences in a_W of the samples. While significant differences in the pH across the sample set, all pH's detected were still above 4.6, indicating a potential risk for pathogen growth. The pH and water activity of all samples tested were favorable for pathogen growth, and the levels of two toxic metals, cadmium, and cobalt, exceeded the maximum allowable amount per serving. The evidence also suggests that the bioaccumulation of arsenic and cadmium in the insects used in the feed

provided to the insects may be influencing the heavy metal content of the products containing insects. In addition, an association between lead content and the presence of soybeans was observed, suggesting that the soybeans may be a contributing factor to the presence of this metal. Care should be taken in sourcing the raw materials for production to reduce the heavy metal content of the final product. Microbial analysis of the products detected neither *Listeria spp*. nor *Salmonella spp*., but the coliform counts were high for all samples tested, suggesting that there may be a potential risk of pathogenic bacteria. As a result, all the tempeh products tested should be stored refrigerated, and care should be taken during preparation to avoid cross contamination with other foods. In addition, all products should be cooked to a minimum internal temperature of 74°C to minimize the risk of pathogenic bacteria.

1.2 Introduction

Increasing global insect consumption could provide benefits for both human and planetary health. With regard to the nutritional aspects of edible insects, they contain high-quality protein, fiber, polyunsaturated fats (PUFAs), vitamins, and minerals (Aguilar-Toalá et al., 2022). On the environmental side, insects have a more efficient feed conversion ratio, use less water, and require less space than traditional livestock (van Huis, 2022). Therefore, increasing insect production and consumption globally could improve food security, lessen the climate burden of food production through the reduction of greenhouse gases and energy usage, and provide humans with a nutritious food source. However, there are cultural barriers to insect consumption in most Western cultures, but acceptance is growing as insects are being increasingly incorporated as invisible ingredients- such as flours or powders- into various familiar food products like pastas, cookies, and bars (Alhujaili et al., 2023). Adding insects as visible ingredients in familiar products may be the next step in normalizing insect consumption.

However, the safety and stability of insect-based processed food products should be identified. In this chapter, we will explore the food safety of insect consumption in processed foods, with a specific focus on a mealworm-based tempeh product.

During the development of a new food product, it is important to ensure that the product is safe for consumption. To accomplish this, consumers should be aware of any intrinsic risks of consuming a food as well as the requirements for properly storing, preparing, and consuming the food for optimal safety. While both edible insects and soy-based tempeh have a long history of consumption by cultures around the world, there are still risks inherent to their use. Understanding those risks, and the ways to mitigate them are essential to the development of new products that utilize these two food sources. As an insect and soy-based product, mealworm tempeh does have some safety risks, especially potential biological and chemical hazards.

There are more than 2,100 known edible insect species around the world (Zhou et al., 2022). When exploring insects as a novel source of edible protein for mass production, it is important to understand the food safety risks of production and consumption. In general, there are three categories of risk associated with food safety: biological risks, or risks associated with the microbial load of a product or the physiological response to constituent parts inherent to the product; chemical risks, which include the potential toxicity of compounds that may be present in the product; and physical risks or physical hazards that may cause damage to the body upon consumption (Aguiar et al., 2018).

1.2.1 Biological Risks of Entomophagy

Water activity (a_W) and pH measurements are two ways to assess the potential for microbial growth that can cause food spoilage and/or human diseases. Water activity is a measurement of the amount of water available for use by microorganisms. The higher the water activity, the more water is available in the product to facilitate the biological functions of bacteria and fungi that may be present (Sarrette et al., 1992). For example, most human pathogens cannot grow on substrates with a water activity of less than 0.91 (Table 1.1; (Khuntia, 2018)). Therefore, knowing the water activity of a product provides important information about how it should be stored, handled during preparation, and cooked to minimize the risk of foodborne illness (Sarrette et al., 1992).

In combination with water activity, pH is also a critical characteristic in understanding and reducing the risks of foodborne illnesses. Foods with a lower pH are less hospitable to both pathogenic and spoilage organisms. A pH $\leq$ 4.6 can prevent the formation of botulism toxin by *Clostridium botulinum*, which can cause a variety of symptoms including muscle weakness, difficulty breathing, and even death (Lin et al., 2022) (Derman et al., 2015). Foods with a pH above this level typically need to be stored, handed, and cooked in a manner that minimizes the risk of pathogen growth to reduce the potential for foodborne illness.

While water activity and pH can provide information on how hospitable a product is to the growth of microorganisms, the actual microbial load of a product is also relevant. This can be estimated by targeting specific pathogens known to cause serious illness, like *Listeria spp*. and *Salmonella spp*., as well as by monitoring indicator organisms like coliforms. Like many pathogenic foodborne organisms, coliforms are bacteria that are most often found in the gastrointestinal (GI) tract. The presence of coliforms can serve as an indicator that fecal contamination may be present in the food product. The higher the number of coliforms, the greater the risk that other gastrointestinal pathogens may be present (Ruiz-Llacsahuanga et al., 2021). Knowing whether specific pathogens are present and identifying coliform levels is essential to ensuring that the product is handled and cooked properly to minimize the risks of foodborne illness.

Microbial contamination is a potential concern when consuming edible insects. Microbial contamination can be affected by a variety of factors such as rearing conditions, contact with contaminated surfaces, feed sourcing, and handling post-harvest (Garofalo et al., 2019). Pathogenic organisms like *Bacillus ceres, Campylobacter spp., Coxiella spp., Escherichia coli, Klebsiella aerogenes, Pseudomonas aeruginosa, Salmonella spp*., and *Staphylococcus aureus* and numerous commensal organisms have all been associated with edible insects. For wild-sourced insects, environmental contact may also play a role in microbial loads (Garofalo et al., 2019). In addition, the microbial load for fresh, commercially raised mealworms can be high, with observed total viable microbial counts being between 7-8 log Colony Forming Units (cfu)/g (Vandeweyer et al., 2017). Since most of the insect products available for purchase in the global west are dried and packaged, the primary concern for these products is spore-forming bacteria (Fasolato et al., 2018). When stressed, or experiencing sub-optimal conditions like heat or drying, spore-forming bacteria can go dormant, surrounding themselves in a protective polysaccharide case, or spore, until optimal conditions return. Bacterial spores can survive prolonged periods of dryness, exposure to chemicals that would harm vegetative cells, and extreme heat (Koukou et al., 2021). Once optimal conditions return, spore-forming cells can become vegetative and proceed with their typical function. Therefore, insects that are dried and packaged for consumption could potentially harbor viable bacterial spores that may lead to pathogenesis.

The major concern for bacterial contamination in insects is aerobic spore-formers. Aerobic spore formers are found in higher levels in processed insect powders, like crickets or mealworms, than are found associated with the live insects (Klunder et al., 2012). This is likely due to the stress incurred during processing, including the reduction of water activity and induction of heat stress; factors that will induce sporulation of bacterial species (Osimani et al., 2018).One species of particular concern is *Bacillus cereus*. This spore-forming aerobic bacteria is a common cause of foodborne illness. Transmitted through the improper cooking and handling of food, *B. cereus* can cause severe vomiting and diarrhea within one to five hours of consumption. The maximum load of *B. cereus* is 10^4 Colony Forming Units (CFUs) per gram; however, concentrations of up to 6.6 Log^10 CFUs per gram have been observed in processed insect samples, representing a significant risk to consumers if the dried products are not handled correctly (Fasolato et al., 2018).

A survey of dried, shelf-stable insect protein products showed that all had a pH of 5.5 or higher. This pH is above the target value of 4.6, which would prevent spore-forming bacteria like *Clostridium botulinum* for producing botulism toxin. Any product containing a pH above 4.6 runs the risk of botulism toxin production. However, this risk can be mediated by reducing the overall water activity, or water available for use, to below 0.97 (Koukou et al., 2021). Across the samples surveyed, which included dried crickets, silkworms, mole crickets, and mealworms, the water activity was less than 0.70. While this is below the growth for spore formers, there is a potential for them to be dormant at this water activity range, and they may become vegetative if the water activity rises.

Some producers also attempt to reduce biological risks by purging or withholding food from the insects for several days prior to processing, to rid the insects of their gut contents. However, the efficacy of this practice needs further research. One study observed no reduction of microbial load in mealworms that were purged in this manner, suggesting that purging may not effectively clear the gut of potential harmful microorganisms, or microbial contamination may not be sourced in the guts of the insects processed. In addition, since insects such as mealworms are typically reared in contact with their most recent eliminations, external contact with the gut contents may contribute to higher microbial loads in the overall population (Fasolato et al., 2018). To reduce these risks, it is recommended that vegetative microbial loads are reduced via heat treatments like blanching or sterilization prior to further processing to reduce the overall concentration of spore-formers in the final product (Vandeweyer et al., 2017).

1.2.2 Allergens and Chemical Risks of Entomophagy

Consumption of edible insects has occasionally been associated with the risk of Immunoglobulin E (IgE) allergic reactions. Foodborne IgE reactions occur when the body produces antibodies against specific foods after exposure. According to the United States Food and Drug Administration (FDA), the main food allergens are milk, eggs, tree nuts, peanuts, sesame, wheat, soy, fish and shellfish (Food and Drug Administration, 2023). Recent studies have indicated a potential cross reactivity of allergens in people with shellfish allergies and people who have allergic responses when consuming insects. Similar reactions have also been observed in people who are allergic to house dust mites, suggesting a cross reactivity between a variety of arthropod species (Ribeiro et al., 2018). Cross reactivity often occurs between species that are taxonomically related. This cross reactivity between shellfish and insect IgE response has been linked to tropomyosin and arginine kinase, allergens that are generally associated with arthropods (Broekman et al., 2017). As a result, it is recommended that people who experience allergic reactions to shellfish and house dust mites avoid eating insects.

Another potential risk associated with entomophagy is exposure to high levels of toxic metals. Toxic metals like lead (Pb), arsenic (As), chromium (Cr), cadmium (Cd), and mercury (Hg) can bioaccumulate in both plant and animal tissues over time. As plants and animals enter the food system to be processed, they are then passed on to humans for consumption. Heavy metal consumption can lead to a variety of adverse effects including disruption of kidney function, disorders of the nervous system, disruption of the immune system, birth defects and cancer (Turkez et al., 2012). For example, acute ingestion of cadmium can lead to short-term gastrointestinal effects like nausea, vomiting, abdominal cramps and diarrhea. Chronic cadmium ingestion can lead to cancer and disrupt the reproductive, cardiovascular, nervous and respiratory systems (Rahimzadeh et al., 2017). Some heavy metals are necessary in small amounts but can become toxic in high concentrations. Cobalt is essential for a variety of functions in the body including gene expression and is an important component of vitamin B12. With toxic levels in blood serum being around 300 ug/L, excessive amounts of cobalt in the system can disrupt thyroid function, causing cobalt-induced goiter, and cardiomyopathy (Chen & Lee, 20203; Leyssens et al., 2017). While the risk of acute heavy metal poisoning is low in the United States, there is still a risk of bioaccumulation of heavy metals over a long period of time. Chronic exposure to heavy metals can result in a variety of symptoms including gastrointestinal distress, nausea, vomiting, neurological disorders, and death. Although plants and animals may not naturally carry high levels of these minerals, some organisms are high bio-accumulators and factors like growth location, feed used, and water sources can lead higher levels of heavy metals in the tissues (Truzzi et al., 2019).

In general, the risk of toxic metal exposure from insect protein is no greater than for other plant or animal foods. While heavy metals like arsenic, cadmium, cobalt, chromium, nickel, lead, tin, and zinc can accumulate from the environment and in feed given to farmed insects, the levels of these metals within edible insects are dependent on the rearing conditions. For example, some research has found that the bioaccumulation of heavy metals in edible insects can pose a significant risk in areas where mining is a major industry (Mwelwa et al., 2023). As a result, there may be a potential for bioaccumulation in human tissue, and more research is needed to determine the long-term risk of consumption.

Another chemical hazard includes environmental contaminants, such as carcinogenic Persistent Organic Pollutants (POPs) like dioxins, which can be found in insect protein (Poma et al., 2017a). Dioxin is a broad category of roughly 75 man-made chemicals seven of which are associated with toxicity (Tuyet-Hanh et al., 2010). They are nonreactive with water and oxygen, allowing them to persist in the environment for a long period of time (Tuyet-Hanh et al., 2010). The environmental persistence of these chemicals allows them to bioaccumulate up the food chain to human food sources (Tuyet-Hanh et al., 2010).

These chemicals are environmental pollutants that accumulate in the fatty tissues of animals. Animal models have demonstrated that dioxin exposure can result in damage to a variety of organ systems including the cardiovascular, immune, reproductive, and nervous systems (Tuyet-Hanh et al., 2010). In a recent study, several species of edible insects including *Galleria mellonella* (greater wax moth), *Locusta migratoria* (migratory locust), *Tenebrio molitor* (mealworm beetle), and *Alphitobius diaperinus* (buffalo worm) were tested for dioxin content along with prepared insect-based food products like buffalo worm balls, cricket croquettes, and buffalo worm burgers. In the study, dioxin compounds were detected in amounts ranging from

0.0001-0.25 pg. In comparison, the maximum allowed concentration of these compounds in beef is 4.0 pg, and in fish 6.5 pg making the risk of exposure to these compounds through insect consumption relatively low. This is likely due to the shorter rearing times for insects, leaving less time for the bioaccumulation of these compounds within the insect tissues (Poma et al., 2017b).

Flame retardants and pesticides have also been observed in processed insect protein products. In a recent study, six different phosphate flame retardants (pFRs) were detected in meal worm and wax moth larvae samples (Poma et al., 2017a). These chemicals were potentially bioaccumulated from rearing substrates and soil. In a recent study analyzing the pesticide content in insects, including *Galleria mellonella* (greater wax moth), *Locusta migratoria* (migratory locust), *Tenebrio molitor* (mealworm beetle), and *Alphitobius diaperinus* (buffalo worm), chemicals like vinyltoluene were ubiquitous in edible insect samples (Poma et al., 2017a). Tributylphosphate and pentafluoropropionic acid were also detected in 75% of the samples analyzed. In addition, methoprene, empenthrine, pirimiphos-methyl, widely used pesticides for the control of a variety of pests across the agricultural sector, were observed in 50% of the samples analyzed. It is believed that these detection levels are the result of bioaccumulation from edible insect feed (Poma et al., 2017a). Therefore, mitigation of these chemicals could be achieved by rearing insects on clean food stocks.

1.2.3 Physical Hazards

While the physical hazards to insect consumption have not been well researched, it is important to recognize the possible risks. Many insect species contain spines, horns, and irritating hairs that may be caught in the throat or cause minor lacerations in the mucus membranes of the body. More research is needed to determine the physical risks inherent in consuming specific insect species. In addition, there are the potential physical hazards inherent in food processing like slivers of metal, shards of glass, pieces of plastic, or other material hazards that may find their way into the final product. While there are legitimate food safety concerns in consuming insect protein, these are generally concerns that are ubiquitous across every product within the food production network. As insect protein grows as a viable food source, best practices continue to be developed to ensure the safety of the consumer.

1.2.4 Food Safety of Traditional Tempeh

The safety of insects is not the only consideration when it comes to developing an insect-based tempeh product. There are also risks associated with traditional tempeh production as well. With a relatively high pH, and a water activity around 0.9, tempeh is a highly perishable food that has the potential to carry pathogens that can cause food-borne illness if not handled and cooked properly (Nout & Kiers, 2005).

As illustrated in Table 1, the water activity of traditional tempeh can facilitate the growth of a range of microorganisms. In addition, the pH of tempeh can be between 6.8 and 8.0, which is within the optimum range of pH for pathogens such as *Clostridium perfringens, Campylobacter spp., Staphylococcus aureus, Listeria monocytogenes*, and *Salmonella spp.*

Table 1.1: Minimum water activity to allow for the growth of spoilage organisms (adapted from Khuntia, 2018).

Minimum water activity For the Growth of Spoilage Organisms	
Spoilage microorganism	**Minimum** aw
Bacteria	0.91
Yeast	0.88
Mold	0.80
Halophillic Bacteria	0.75
Xerophillic Fungi	0.65
Osmophillic Yeast	0.60

In addition, temperature range is optimum for the production of *Staphylococcus* toxin and is well above the target of pH 4.6 to prevent the production of toxins by *Clostridium botulinum* (Lin et al., 2022). This means that tempeh must be consumed fresh within 4 days of production or refrigerated for no longer than one week (Nout & Kiers, 2005). This also means that tempeh must be handled carefully to prevent cross-contamination and cooked thoroughly to eliminate vegetative cells prior to consumption. However, even with those precautions, the presence of heat-stable toxins may still pose a risk to the consumer if the product is not handled correctly, resulting in incidents of foodborne illness.

Consumption of tempeh has been associated with several food-borne outbreaks. In the United States, this was seen during an outbreak of gastroenteritis in North Carolina in

2012. At the time, public health officials investigated an outbreak of *Salmonella enterica* that sickened 89 people. The source of the outbreak was traced to a contaminated *Rhizopus* culture used in the production of tempeh (Griese et al., 2013). In Indonesia, a pathogen identified as *Burkholderia cocovenans* lead to the production of bongkrekic acid, a compound that inhibits adenosine triphosphate and adenosine diphosphate synthesis, within tempeh bongkrek, a type of tempeh that utilizes coconut. Exposure to bongkrekic acid can lead to weakness, dizziness, and jaundice, as well as shock, coma, and death (Ahnan-Winarno et al., 2021).

To identify risks associated with an insect-based tempeh, herein we examined several chemical, physical, and microbiologic factors of different tempeh formulations containing *Tenebrio molitor* (mealworm). It is important to understand the physical and microbial properties of tempeh made with mealworms so that proper cooking and handling guidelines can be established for the consumer. Understanding the water activity, pH, and microbial load of these products when compared to conventional soybean tempeh production is essential to maximizing the impact of safe-handling instructions to prevent food-borne illness.

1.3 Materials and Methods

1.3.1 Sample Production

Five different variations of tempeh were produced in the lab for this project: 100% lab-based soy tempeh, 100% mealworm tempeh (*T. molitor* larvae), 100% pupae tempeh (*T. molitor* pupae), 50/50 mealworm soybean tempeh by weight, and 50/50 pupae soybean tempeh by weight. All mealworms used were between 18 and 25 mm long and were procured from a commercial producer (Rainbow Mealworms, Campton, California) and fed a diet of wheat bran (Star of the West Milling Co. Churchville, NY), lyophilized brewer’s yeast, and whole carrots for a minimum of five days prior to processing. The mealworms were then euthanized with liquid nitrogen and stored at -20 °C until use. Pupae were acquired by allowing the mealworms to enter the pupal stage of their development, separating them from the remaining stock, and euthanizing with liquid nitrogen before storing at -20 °C until use.

<u>100% Soy</u> – 400g of dry soybeans were soaked, boiled, and dehusked as described above for control samples. Once the husks were separated, 2ml of distilled white vinegar was added and the beans were mixed for 60 seconds to incorporate. After the mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

<u>100% Mealworm</u> – 400g of frozen mealworms were blanched in boiling water for 60 seconds. After blanching, the mealworms were allowed to cool for 20 minutes. Once cool, 2ml of distilled white vinegar was added and the worms were mixed for 60 seconds to incorporate. After the mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca),, rice flour, and soy flour was added, and processed in the same manner as the 100% soy tempeh.

<u>50/50 Mealworm Soy</u> – Soybeans were prepared as described and 200g of dehusked and acidified soybeans were added to 200g of frozen mealworms that were blanched in boiling water for 60 seconds, refrozen with liquid nitrogen, and pulverized into rice-sized pieces with a food processor. Next, 2ml of distilled white vinegar was added and the substrate was mixed for 60 seconds to incorporate. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca),, rice flour, and soy flour was added, and the substrate was mixed for an

additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

100% Pupae – 400g of frozen pupae were blanched in boiling water for 60 seconds. After blanching, the pupae were allowed to cool for 20 minutes. Once cool, 2ml of distilled white vinegar was added and the pupae were mixed for 60 seconds to incorporate. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

50/50 Pupae Soy - 200g of cooked, dehusked and acidified soybeans were combined with 200g of frozen pupae that were blanched in boiling water for 60 seconds. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

In addition to the tempeh samples, each formulation was matched with unfermented controls. The raw materials were prepared as follows: soybeans were soaked, cooked, and dehusked as described above. Mealworms and pupae were blanched and chilled as described. The raw ingredients were then mixed in the appropriate portions (200g insect/200g soy) or left separate and heated in a dry pan at medium-high heat for five minutes and chilled for 30 min at

4°C before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

1.3.2 Water Activity

Water activity (aw) was determined using an Aqualab 3AQ20000 1974 water activity meter (Pullman, WA) and SKALAControl software version 2.86.90. Replicate samples (n=6) of tempeh formulations and a commercially available soy tempeh (Lightlife, Turner Falls, MA) were analyzed for aw.

1.3.3 pH

pH was determined using a Foodcare pH meter model H99161 (Smithfield, RI). Replicate samples (n=6) of a commercially available soy tempeh, lab-produced soy tempeh, 100% mealworm tempeh, 100% pupae tempeh, 50/50 mealworm soy tempeh, and 50/50 pupae soy tempeh were analyzed.

1.3.4 Microbial Analysis

N=3 Samples of the 100% lab-based soybean tempeh, 100% mealworm tempeh, 100% pupae tempeh, 50/50 mealworm soybean tempeh, and 50/50 pupae soybean tempeh were analyzed for total coliform count, Listeria, and Salmonella by Anresco Laboratories (San Francisco, CA). Coliform detection was determined by utilizing the Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM) method Chapter 4: Enumeration of Escherchia coli and the Coliform Bacteria (Feng et al., 2020). *Listeria* was tested for using the Association of Official Analytical Chemists (AOAC) method 2013.10 (*Testing*

Methodology for Listeria Species or L. Monocytogenes in Environmental Samples, 2015). Salmonella was tested for using the AOAC 2013.01 Method Salmonella.

1.3.5 Microwave Digestion

Microwave digestion was conducted utilizing a Titan MPS microwave digester prior to ICP analysis. To determine total ferritin content of the gastric digests, samples were prepared by adding 300mg of homogenized and lyophilized sample to the microwave vessel with 10 mL of nitric acid. The samples were allowed to sit for 15 minutes to allow for an initial reaction. Then, the samples were placed in the microwave for 1 hour. Once cooled, the samples were decanted into 50 mL conical tubes and diluted to 20 mL with milliQ water. One mL of this was added to a 15 mL conical and further diluted to 15 mL with milliQ water to prepare them for ICP analysis.

1.3.6 Inductively Coupled Plasma and Mass Spectrometry (ICP-MS) Analysis

Elemental concentrations of Li, Be, B, Cd, Se, As, Na, P, S, Mg, K, Ca, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, Ba, W, and Pb were measured in N=6 samples using a NexION 350D mass spectrometer (PerkinElmer) connected to a Type A quartz MEINHARD® concentric nebulizer and a quartz cyclonic spray chamber. Samples were introduced using a SC-2DX autosampler (ESI). Li, Be, B, Na, P, S, Mg, K, Ca, W, and Pb were measured in standard mode. Se, and As were measured in DRC mode using oxygen as the reactive gas. Al, V, Cd, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, and Ba were measured in DRC mode using ammonia as the reactive gas. Before analysis the torch alignment, nebulizer gas flow and the Quadrupole Ion Deflector (QID) were optimized for maximum Indium signal intensity. A daily performance check was also run which ensured that the instrument was operating properly and minimized oxide and doubly charged species formation by obtaining a $CeO^+:Ce^+$ of <0.025 and a solution made from a mixture of single-element stock standards (Inorganic Ventures, Christiansburg, VA). To correct for instrument drift, a quality control (QC) solution, which consisted of a pooled digested sample prepared by mixing 1 mL of each digested individual sample, was run every 10th sample.

1.3.7 Statistical Analysis

Data were statistically analyzed using Graphpad Prism version 9.5.1 (733). Data were assessed via One-way ANOVA with post-hoc Tukey's multiple comparison analysis. A p-value of <0.05 was considered statistically significant. P-values of 0.080-0.051 were identified as trending significance, indicating that differences may be revealed with a larger sample set. Descriptive statistics were calculated via Microsoft Excel.

1.4 Results

1.4.1 Water Activity

The water activity (a_w) of the samples was largely uniform across the sample set, with the most variability observed within the commercial tempeh product as illustrated in Figure 1.1 and Table 1.2.

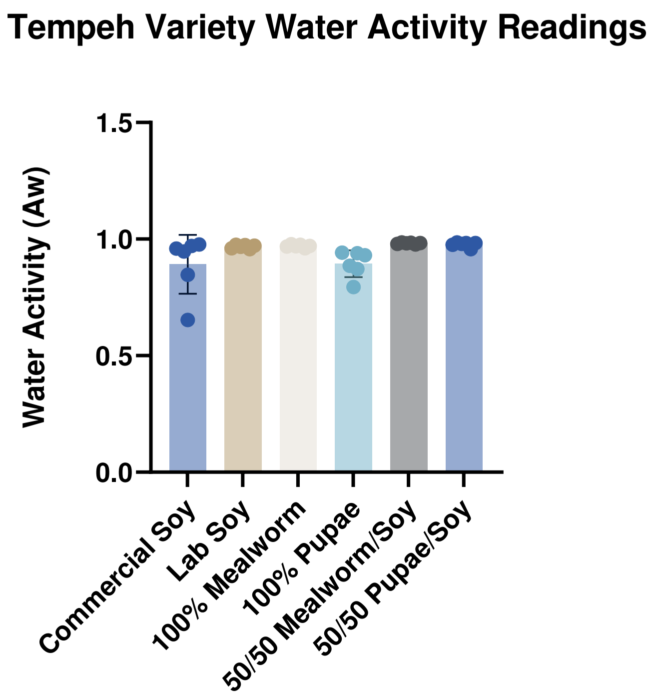

Figure 1.1: Water activity of tempeh samples n=6.

Table 1.2: Average water activity detected across samples n=6.

Average Water Activity		
	Average	Standard Deviation
Commercial Soy Tempeh	0.892	0.126
Lab Soy Tempeh	0.968	0.008
100% Mealworm	0.965	0.015
50/50 Mealworm Soy Tempeh	0.982	0.003
100% Pupae Tempeh	0.894	0.057
50/50 Pupae Soy Tempeh	0.977	0.010

With an a_W range of 0.98-0.89, every formulation shows a high amount of available water to allow microbial growth of most bacteria, molds, and yeasts associated with food spoilage and pathogenesis. Although there is a large range of averages among samples, and small changes in a_w can result in important hurdles for pathogen growth, statistically there were no differences between any of the samples (Figure 1.2).

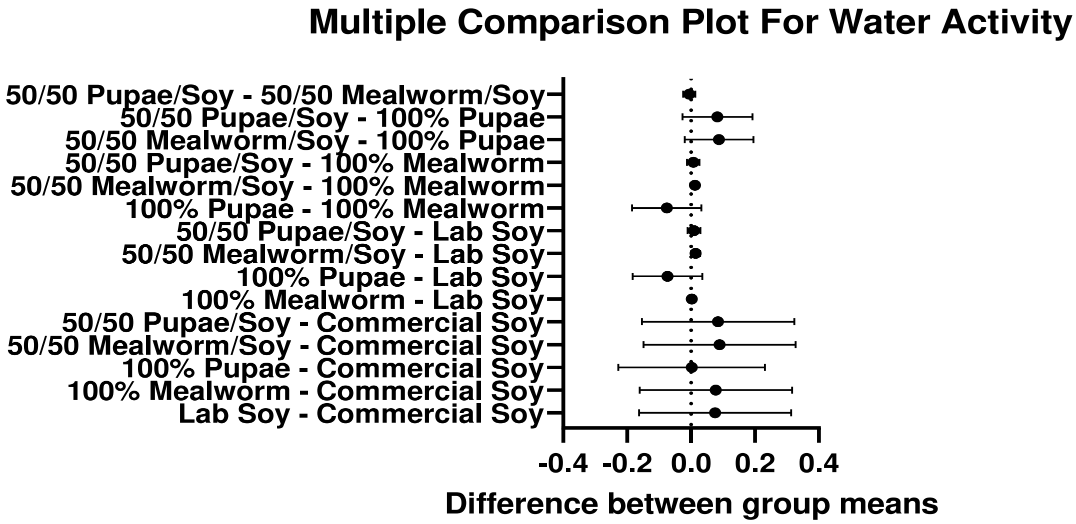

Figure 1.2: Multiple Comparison – One-ay ANOVA showing the differences in average aw present in each sample.

Importantly, the commercial sample was not significantly different from the lab-produced samples containing insects ($P>0.05$). This suggests that a mealworm tempeh product could be stored and prepared in a similar manner to traditional tempeh without introducing additional microbial risks. It is interesting to note that the commercial tempeh average a_w was sufficient to reduce the growth of most bacterial spoilage organisms and pathogens; however, it also had the greatest variability between replicates. It is possible that differences in storage time as well as differences in transport and storage of the products might be the source of this variability. This is supported by the observation that when prepared and stored under the same conditions, the mealworm-containing and lab-prepared soy samples have very little difference in their average water activity. The 100% pupae tempeh also had an average a_w <0.90. This is likely due to the chitin exoskeleton of the pupae. Chitin makes up a large portion of the exoskeleton of insects and is extremely hydrophobic which could help exclude free water (Qian et al., 2023).

1.4.2 pH

Unlike with the water activity, the pH across the sample set were statistically different (p <0.0001; Figure 1.3; Table 1.3). Specifically, the 50/50 pupae soy samples had a lower pH than any of the other lab-produced samples except the 100% mealworm tempeh (Figure 1.4).

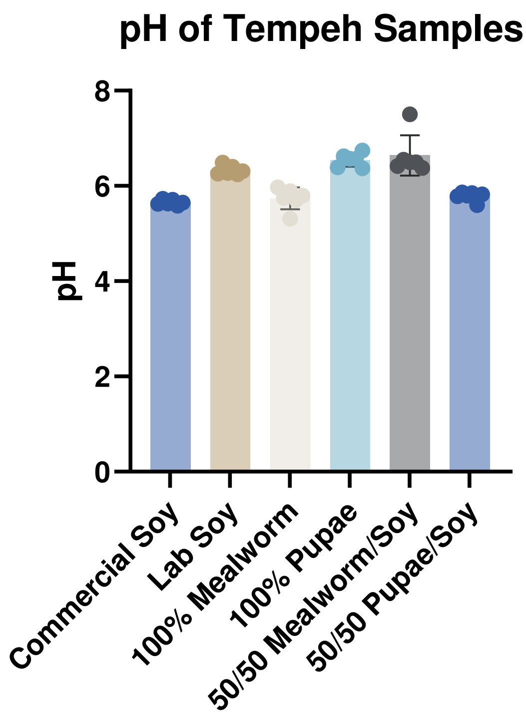

Figure 1.3: pH of Tempeh Samples

Table 1.3: Average pH across samples

Average pH		
	Average	Standard Deviation
Commercial Soy Tempeh	5.7	0.057
Lab Soy Tempeh	6.3	0.097
100% Mealworm	5.4	0.774
50/50 Mealworm Soy Tempeh	6.6	0.425
100% Pupae Tempeh	6.5	0.141
50/50 Pupae Soy Tempeh	5.8	0.095

Table 1.4 shows significant differences in the pH of samples across the sample set, the commercial soy product presenting a significantly different pH from the lab set, 100% pupae, and 50/50 mealworm soybean tempeh.

Table 1.4: Significant differences from a Tukey's multiple comparison analysis of pH detected across the sample set.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Commercial Soy vs. Lab Soy	-1.051 to -0.2988	-0.675	<0.0001
Commercial Soy vs. 100% Pupae	-1.266 to -0.5138	-0.89	<0.0001
Commercial Soy vs. 50/50 Mealworm/Soy	-1.364 to -0.6122	-0.9883	<0.0001
100% Mealworm vs. 100% Pupae	-1.181 to -0.4288	-0.805	<0.0001
100% Mealworm vs. 50/50 Mealworm/Soy	-1.279 to -0.5272	-0.9033	<0.0001
100% Pupae vs. 50/50 Pupae/Soy	0.3822 to 1.134	0.7583	<0.0001
50/50 Mealworm/Soy vs. 50/50 Pupae/Soy	0.4805 to 1.233	0.8567	<0.0001
Lab Soy vs. 100% Pupae	-0.5912 to 0.1612	-0.215	0.5185
Lab Soy vs. 50/50 Mealworm/Soy	-0.6895 to 0.06282	-0.3133	0.1464
Lab Soy vs. 50/50 Pupae/Soy	0.1672 to 0.9195	0.5433	0.0016
Lab Soy vs. 100% Mealworm	0.2138 to 0.9662	0.59	0.0006

In addition, the 100% mealworm tempeh presented a significantly lower pH than the 100% pupae and the 50/50 mealworm soybean tempeh. Finally, the pH of the lab-produced soy tempeh was significantly lower than the 100% pupae and the 50/50 mealworm soybean tempeh and near significantly higher than the 50/50 pupae soybean and the 100% mealworm tempeh.

However, with all the samples containing a pH > 4.6 they all pose a risk of promoting the growth of a variety of pathogenic bacteria including *Bacillus cereus*, *Clostridium botulinum, Listeria monocytogenes*, and *Salmonella spp*., which would require a pH of 4.6 or below to mitigate the risk of forming toxins (FDA, n.d.). All pH ranges observed fall firmly in the middle of the tolerance range for the bacteria most responsible for foodborne outbreaks within the United States, as illustrated in Table 1.5. This suggests that despite statistical differences in pH these tempeh formulations would not warrant different storage, handling, or cooking requirements from the commercial to ensure each sample is safe to consume.

Table 1.5: The minimum and maximum pH ranges for the most common foodborne pathogenic bacteria (Fda & Cfsan, 2011).

pH Ranges for Pathogenic Bacteria		
Group of micro-organisms	**Minimum pH**	**Maximum pH**
Bacillus cereus	4.3	9.3
Campylobacter jejuni	4.9	9.5
Clostridium botulinum	4.6	9
Listeria monocytogenes	4.4	9.4
Salmonella spp.	3.7	9.5
Shigella spp.	4.8	9.3
Staphylococcus aureus	4.0	10

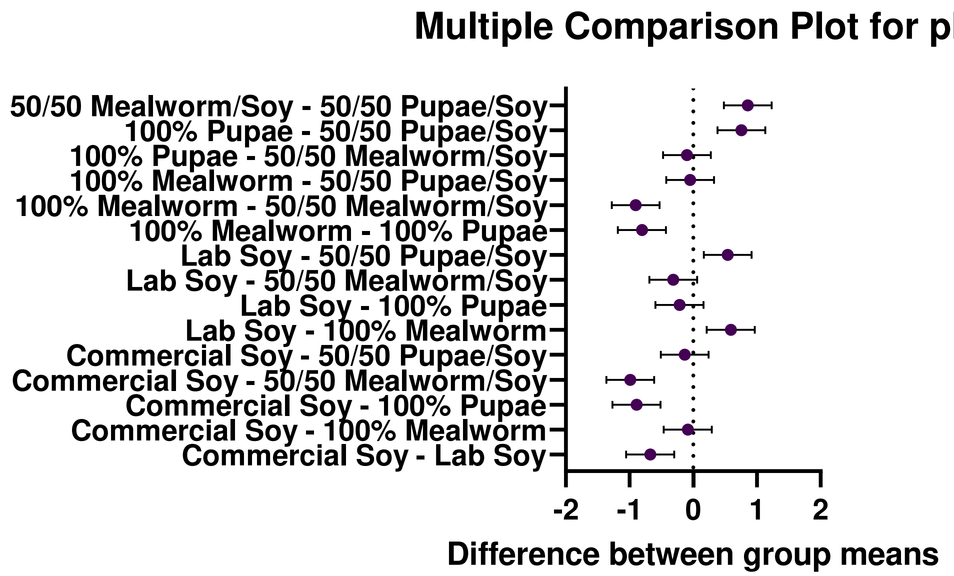

Figure 1.4: Multiple Comparison – One-ay ANOVA showing the differences in average pH present in each sample.

1.4.3 Microbial Analysis

Microbial analysis of the lab-produced tempeh samples shows that all samples tested were negative for both *Listeria spp. and Salmonella spp*. However, the Most Probable Numbers (MPN) of coliforms for all samples tested ranged between 9,300 and > 1.1 million MPN/g (Table 1.6). This is significantly higher than the < 100 CFUs/g required for a food to be safe to eat raw (FSIS, 2018.).

Table 1.6: Most Probable Number (MPN) of bacteria detected in lab-produced tempeh samples.

Sample	Coliforms	Listeria	Salmonella
100% Soybean	> 1.1 million MPN/g	Negative/ 25g	Negative/ 25g
100% Mealworm	11,000 MPN/g	Negative/ 25g	Negative/ 25g
100% Pupae	9,300 MPN/g	Negative/ 25g	Negative/ 25g
50/50 Mealworm Soybeans	240,000 MPN/g	Negative/ 25g	Negative/ 25g
50/50 Pupae Soybeans	> 1.1 million MPN/g	Negative/ 25g	Negative/ 25g

These numbers indicate that none of the lab-based products are safe to consume raw and require cooking to bring them back into safe levels. Within the sample set, the products containing soybeans contained the largest amounts of coliforms detected, suggesting that the addition of the soy increased the coliform counts in the 50/50 insect soy formulations.

1.4.4 Toxic Metals

Most of the samples tested were observed to be well below the allowable limits of heavy metals as determined by the United States Food and Drug Administration (FDA). However, as seen in table 1.7, the mealworm control, mealworm tempeh, pupae control, and pupae tempeh exceeded the daily allowable limit of Cd. In addition, all the samples exceeded the allowable limits of Co with no statistical differences between cobalt content observed (P>0.05).

Table 1.7: Daily heavy metal allowable limits in a 100g sample as compared to average content detected in ug/g across the lab-based tempeh sample set; n=6.

	As	Cd	Co	Pb
	(ug/g)	(ug/g)	(ug/g)	(ug/g)
Allowable limits	0.5 - 2	0.025	0.01	0.5
Sample	(ug/g)	(ug/g)	(ug/g)	(ug/g)
Soy Control	0.002 (SD: 0.003)	0.019 (SD:0.006)	0.033 (SD: 0.011)	0.010 (SD: 0.005)
Soy Tempeh	0.005 (SD: 0.004)	0.016 (SD: 0.004)	0.037 (SD: 0.015)	0.021 (SD: 0.009)
MW Control	0.010 (SD: 0.002)	0.031 (SD: 0.007)	0.057 (SD: 0.071)	0.004 (SD: 0.006)
MW Tempeh	0.033 (SD: 0.003)	0.042 (SD: 0.003)	0.051 (SD: 0.016)	0.011 (SD: 0.007)
Pupae Control	0.012 (SD: 0.002)	0.046 (SD: 0.040)	0.021 (SD: 0.008)	0.002 (SD: 0.001)
Pupae Tempeh	0.011 (SD: 0.005)	0.029 (SD: 0.012)	0.020 (SD: 0.005)	0.013 (SD: 0.005)
MS Control	0.005 (SD: 0.003)	0.017 (SD: 0.011)	0.056 (SD: 0.069)	0.010 (SD: 0.006)
MS Tempeh	0.009 (SD: 0.004)	0.021 (SD: 0.004)	0.029 (SD: 0.004)	0.018 (SD: 0.017)
PS Control	0.006 (SD: 0.002)	0.020 (SD: 0.002)	0.035 (SD: 0.010)	0.006 (SD: 0.001)
PS Tempeh	0.005 (SD: 0.003)	0.014 (SD: 0.002)	0.039 (SD: 0.016)	0.020 (SD: 0.236)

Examination of the lead content, shown in table 1.8, showed that the 50/50 pupae soybean tempeh and control contained significantly less lead than the lab-produced soy (P=0.0427). In addition, the pupae control contained significantly less lead than the than the 50/50 mealworm soybean tempeh (P=0.0148).

Table 1.8: Significant differences from a Tukey's multiple comparison analysis of lead content detected in sample set; n=6.

Sample Comparison -- Lead	Confidence Interval	Mean Difference	P Value
50/50 Pupae/Soy vs. Lab Soy	-0.03131 to 0.002258	-0.01453	0.0427
50/50 Pupae/Soy Control vs. Lab Soy	-0.03131 to 0.002258	-0.01453	0.0427
Pupae Control vs. 50/50 Mealworm/Soy	-0.03298 to 0.0005894	-0.0162	0.0148
Mealworm Control vs. Lab Soy	-0.03326 to 0.0003111	-0.01647	0.0123
Pupae Control vs. Lab Soy	-0.03579 to -0.002224	-0.01901	0.0021

The mealworm control also contained significantly less lead than the lab-produced soybean tempeh (P=0.0123), and significantly less lead was detected in the pupae control than the lab-based soybean tempeh (P=0.0021). These results suggest that the presence of soy may influence the lead content of the products, though no significant differences were observed between the lead content of the soy control and the other products within the sample set (P>0.05).

We also observed some significant differences in the levels of arsenic in several samples. As seen in table 1.9, The 50/50 mealworm soybean control contained lower levels of arsenic with trending significance than the 100% pupae tempeh (P=0.0533) and significantly lower levels than the pupae control.

Table 1.9: Significant differences from a Tukey's multiple comparison analysis of Arsenic content detected in sample set.

Sample Comparison -- Arsnic	Confidence Interval	Mean Difference	P Value
50/50 Mealworm/Soy Control vs. 100% Pupae	-0.01428 to 0.001214	-0.006532	0.0533
Pupae Control vs. 50/50 Pupae/Soy	-0.0007803 to 0.01399	0.006605	0.0318
Pupae Control vs. Lab Soy	-0.0006487 to 0.01412	0.006737	0.0263
50/50 Mealworm/Soy Control vs. Pupae Control	-0.01502 to 0.0004725	-0.007273	0.0197
Soy Control vs. 50/50 Mealworm/Soy	-0.01488 to -0.0001113	-0.007497	0.0084
Mealworm Control vs. Soy Control	0.0004947 to 0.01527	0.00788	0.0046
Soy Control vs. 100% Pupae	-0.01638 to -0.001606	-0.008992	0.0007
Pupae Control vs. Soy Control	0.002348 to 0.01712	0.009733	0.0002

The soy control contained significantly less arsenic than the 50/50 mealworm soybean tempeh (P=0.0084), the 100% pupae tempeh (P=0.0007), and the pupae control (P=0.0002). Lastly, the pupae control contained significantly higher levels of arsenic than the 50/50 pupae soybean tempeh and the lab-produced soybean tempeh. These results suggest that the presence of insects within the products may affect the overall arsenic content within the products, indicating a greater risk of heavy metal exposure.

Examination of the cadmium content of the samples, seen in Table 1.10, indicates the pupae control contains higher amounts of cadmium with trending significance than the soy control (P=0.0539) and the 50/50 mealworm soy control (P=0.0512).

Table 1.10: Significant differences from a Tukey's multiple comparison analysis of lead content detected in sample set.

Sample Comparison -- Cadmium	Confidence Interval	Mean Difference	P Value
Pupae Control vs. Soy Control	-0.005073 to 0.05921	0.02707	0.0539
50/50 Mealworm/Soy Control vs. Pupae Control	-0.06227 to 0.005145	-0.02856	0.0512
50/50 Pupae/Soy vs. 100% Mealworm	-0.06036 to 0.003923	-0.02822	0.0376
Pupae Control vs. Lab Soy	-0.001806 to 0.06247	0.03033	0.0187
Pupae Control vs. 50/50 Pupae/Soy	0.0003270 to 0.06461	0.03247	0.0089

In addition, the pupae control contained significantly higher amounts of cadmium than the lab-produced soy tempeh (P=0.0187) and the 50/50 pupae soy tempeh (P=0.0089). Finally, the 50/50 pupae soy tempeh contained significantly higher amounts of cadmium than the 100% mealworm tempeh (P=0.0376). These results suggest that the presence of the pupal life stage of the *Tenebrio molitor* beetle may significantly affect the overall about of cadmium un a particular product.

1.5 Discussion

With the high a_w and pH, the samples are highly perishable and require special care during storage, handling, and cooking to ensure safety. The samples should be refrigerated prior to cooking and measures like hand washing, separate cutting boards and knives, and separate preparation vessels should be used to avoid cross contamination with other foods. During cooking, the product should be cooked to an internal temperature of 74°C prior to consumption. Though the existing literature is sparse regarding the safe internal cooking temperature of tempeh, 74°C is the minimal safe temperature for ground poultry and exceeds the maximum survivable temperature for most pathogenic organisms (Fda & Cfsan, 2011).

While *Listeria spp*. and *Salmonella spp.* were not detected in the sample set, the level of coliforms present in the samples makes these unsuitable for raw consumption as they may indicate the presence of pathogenic organisms (Tortorello, n.d.). The reasons for the high coliform counts are unclear. There are some processing differences that may account for higher coliform levels. The lab-manufactured samples containing soybeans went through a maceration step where the beans were manually broken apart to split the husks and expose the interior of the beans to the inoculum. While gloves were utilized during this step, they were not sterile and may have been a point of contamination accounting for increased coliforms in the soybean-containing samples. This may be less of a concern in a commercial production facility that is utilizing good manufacturing practices. In addition, the lab-manufactured products had an extended cooling period to allow for the inoculum to be applied without damaging the spores. This cooling period, combined with the mechanical maceration, may have provided additional opportunities for contamination. Lastly, the application of the distilled white vinegar within the process was intended to reduce the external pH of the substrate and provide an additional food safety hurdle during production. However, since the amounts of each constituent ingredient were measured by weight, there was a considerable difference in volume and surface area between the samples containing soybeans and the all-insect samples. This difference in surface area may account for the difference in overall coliform count in the product before entering the incubator and may also have influenced some of the differences in pH. With the temperature and humidity during incubation ideal for microbial growth, any additional contamination during processing may allow for greater growth during fermentation. To help reduce the risk to the consumer, it may be necessary to pasteurize the tempeh prior to distribution to reduce the overall microbial load of the product. This is a common practice in the tempeh industry and doing so can extend the shelf life and reduce the risk to consumers (Griese et al., 2013).

The presence of several heavy metals detected in the samples appear to be associated with the raw materials for each product. For example, within the sample set, the presence of soy appears to be associated with significantly higher levels of lead detection. While this could not be confirmed with a comparison of the soybean control to the other samples in the set, the evidence suggests that the lead is more likely to be present in larger amounts in the soybeans than the other raw materials utilized in the manufacturing of the products. This could be a result of the soil in which the soybeans were grown, as previous research has demonstrated the uptake of lead and other heavy metals from the soil by soybeans (Blanco et al., 2017).

We observed a similar association with the presence of arsenic, particularly in the products that contain the pupal stage of the *T. molitor* beetle. This is most likely due to bioaccumulation from the feed sources. For examples, we observed the mealworm control containing significantly more arsenic than the soy control (P=0.0046). We then observed a significant increase in arsenic content in several of the products containing mealworm pupae. With this significant increase in levels of arsenic in this later life stage, the evidence points to bioaccumulation of the heavy metals from the feed source. To confirm this, we would need to test for arsenic in the adult beetles that developed during the study. Analysis of the feed materials would also help to confirm the possibility of bioaccumulating this metal from the feed, though previous research has confirmed that this is a possibility (Mwelwa et al., 2023). Mealworm pupae are also implicated in significantly elevated levels of cadmium within the sample set, providing additional evidence that the feed may be the source of the elevated levels of heavy metals observed in the insect-based products.

While the content of some heavy metals is above the safe allowable limits, they are well below the levels for acute toxicity. However, heavy metals like cadmium have a long biological half-life and may accumulate in the tissues of the body with regular consumption. In addition, a recent murine model suggests that chronic consumption can result in dysregulation of the microbiota within the gut (Dai et al., 2022). Since there were no statistically significant differences between the control samples and the fermented tempeh samples, the presence of heavy metals is likely derived from the raw materials utilized for production. While the risks of toxicity of these minerals is rare, care should be taken to reduce the content of the minerals within the food product. More thoughtful insect-rearing practices to minimize consumption of these metals of concern will help reduce the overall content accumulated in animal tissues. In addition, the presence of these metals should also be monitored in the soybean sources as they also exceed the maximum levels allowed in the soybean control.

1.6 Conclusion

Taken together, these data suggest that tempeh produced with mealworm pupae and larvae pose no more of a risk to consumers than soy-based tempeh products. Many of the safety parameters, like coliform counts and variability of pH and a_w, could likely be improved by following standardized production protocols in a commercial facility. However, both conventional and insect-based tempeh are perishable products and would need to be transported, stored, handled and cooked in a manner consistent with reducing the risks of transmitting food-borne illnesses. To minimize this risk, these products should be stored at a minimum of 0°C and cooked to an internal temperature of at least 74°C.

The data also suggest that additional care should be taken when sourcing feed materials for the insects. Consistent monitoring of heavy metal content may be necessary to ensure that the levels of these metals in the products do not exceed acceptable limits. In addition, excluding later life stages of the *Tenebrio molitor*, like pupae and adult beetle, in product development may help to mitigate the risks of bioaccumulation of heavy metals.

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CHAPTER 2: Nutritional Analysis of Tempeh Products Using *Tenebrio molitor* as a Substrate

2.1 Summary

Tempeh has traditionally been used as an inexpensive source of protein around the world. Its pleasant flavor and aroma are relatively neutral, allowing it to be utilized in a variety of applications. In this study, we used texture analysis and observation during fermentation to determine the optimal fermentation time required to achieve full mycelial penetration and substrate cohesion while using *Rhizopus oligosporus* to ferment tempeh utilizing two life stages of the *Tenebrio molitor* beetle. In addition, we used Inductively Coupled Plasma and Mass Spectrometry (IPC-MS) on five different substrate controls and their fermented tempeh counterparts to determine if there were any significant differences in essential mineral content as a result of fermentation. A vitamin and macronutrient analysis were conducted on a 50:50 mealworm/soy control and corresponding fermented tempeh, and the results were compared to a commercially available tempeh sample. Finally, an amino acid analysis was conducted on the 50:50 mealworm/soy control, 50:50 mealworm/soy tempeh, and the commercial soy tempeh to determine if the addition of insect protein affected the digestibility of the overall product. The digestibility was determined by calculating the Protein Digestibility Amino Acid Score (PDCAAS). In this study, we found that the addition of insects had no significant effect on the mineral content of the overall products when compared to traditional soy tempeh. However, the addition of soybeans can increase calcium and manganese content in the final product. The macronutrient analysis showed that the addition of insects lowered the total protein content, carbohydrates, and the calories per serving, while raising the overall dietary fiber content. The protein digestibility analysis showed that while the limiting amino acids differed between the

conventional soy tempeh and the 50:50 mealworm/ soy control and tempeh samples, the commercial soy tempeh had higher protein digestibility than the experimental samples, though more testing is needed to establish statistical significance. Overall, the addition of edible insects only minimally alters the nutritional profile compared to traditional tempeh and provides a nutritionally acceptable option for introducing edible insects to a broader audience.

2.2 Introduction

With the global demand for animal protein on the rise, it is important to identify alternative sources of protein to ensure the security of the current food system and reduce the agricultural impact from large-scale conventional animal protein production. Edible insects have been identified as an alternative source of animal protein due to their high protein by weight, efficient feed conversion ratio, and smaller space requirements when compared to conventional meat (van Huis, 2022). However, one challenge to consumer acceptance in the global west is a lack of familiarity with the practice of eating insects. In the United States, insect eating is not mainstream, with products designed predominantly to hide the presence of insects. To encourage adoption of entomophagy, or insect eating, it may be helpful to present the insects in a form that is accessible or familiar to the population. Fermentation may help transform insect protein into a state that is more familiar to the public, like burgers, nuggets, and crumbles, while also modifying its nutritional profile.

Fermentation is a practice that has been an integral part of human civilization for centuries. It allows humans to preserve foods for long periods of time, while altering flavor, reducing the risks of pathogenic organisms, changing the nutritional qualities of the original food, and detoxifying substrates (Nout & Kiers, 2005) (Dimidi et al., 2019). One fermented food product that significantly improves the digestibility and nutrition of its substrate is tempeh.

Tempeh is a food product from Indonesia, traditionally consisting of soybeans that have been boiled and fermented with *Rhizopus spp.,* a mold that forms a dense mycelial network that binds the beans into a sliceable cake. While a variety of *Rhizpopus* species can be used in the production of tempeh, *Rhizopus oligosporus* is the most common. Other strains of *Rhizopus spp*., such as *Rhizopus oryzae*, are associated with sour flavors that are not desirable in tempeh production (Nout & Kiers, 2005).

Tempeh has typically served as an inexpensive source of protein, and while soybeans are the traditional substrate for the product, the technique has been applied to a variety of substrates including various legumes, vegetables, and coconut waste (Romulo & Surya, 2021). Tempeh is a versatile product that can be fried in slices, boiled in soups, prepared as kebobs, or ground into pastes (Nout & Kiers, 2005). In the United States, tempeh is made from a variety of substrates including soy, barley, brown rice, flaxseed, and a variety of legumes (Ahnan-Winarno et al., 2021). The nutritional quality of tempeh is dependent on the substrates being utilized. For example, tempehs made with soy have higher levels of protein than tempehs made with other legumes, but tempeh produced with black beans have higher levels of sugars, carbohydrates, and iron (Ahnan-Winarno et al., 2021).

Tempeh's ability to be utilized with a variety of substrates makes it particularly suited for the development of new products. In tempeh production, the thick mycelial network formed by *Rhizopus oligosporus* results in a solid product out of a substrate made of many constituent parts. This allows a loose pile of beans and grains to be integrated into a single solid food product. As *Rhizopus spp.* is not particularly discriminating about its source of macronutrients, and produces no known toxins, it can be utilized in novel applications, such as the integration of insects into a cohesive food product.

Using tempeh-producing techniques to develop insect-based food products would allow developers to place edible insect protein into a substrate that has a variety of applications, making it an ideal process for converting edible insects, a protein source not readily accepted in the United States, into more familiar food forms to the American public. Tempeh can be cut into burgers, stamped into nuggets, or ground into crumbles. Its relatively neutral flavor makes it a versatile source of protein that can be adapted to the palates of different target consumer groups. By placing edible insects into forms that are more recognizable to the American public, we aim to increase the acceptance of insects as a source of edible protein and reduce the ethical and environmental impacts imposed by the growing demand for animal protein.

While the nutritional qualities of traditional tempeh are well known, adding insects to this traditional product will undoubtedly change the nutritional profile relative to the original product. Since tempeh is known primarily as a source of protein, understanding how insect protein will impact the protein content and digestibility is essential to ensuring consumers are fully informed when choosing to add it to their diets.

2.2.1 Nutrition of Traditional Soy Tempeh

At 30-40% protein by dry weight, tempeh serves as an excellent source of protein for the diet (Nout & Kiers, 2005). In addition, *Rhizopus spp.* secrete exogenous enzymes that break down macronutrients into constituent parts, making them more digestible (Nurwahidah & Arbianingsih, 2019). Tempeh fermentation is also associated with the reduction of antinutrients, like phytates, which are chelating agents that bind to micronutrients such as iron, calcium, magnesium, and zinc and prevent them from being absorbed by the body (Romulo & Surya, 2021). Phytases secreted by the *Rhizopus spp.* mycelia break down these phytates, making the minerals more bioavailable to the consumer (Azeke et al., 2011).

Vitamin B12 is an essential vitamin that helps in neurological function, facilitates the synthesis of energy in mitochondria, and helps with the production of red blood cells within bone marrow. B12 is produced by microorganisms, making animal sources the primary source of this vitamin in most food chains. Vegetarians and vegans are particularly susceptible to B12 deficiencies as they have little to no intake of animal sources of nutrition (Pawlak et al., 2013). Vitamin B12 deficiency can result in a variety of neurological symptoms like paresthesia, sensory ataxia, anemia, weakness in the lower limbs, dementia, and degradation within the optic nerve (Derin et al., 2016). However, tempeh is thought to be a good plant-based source of vitamin B12, mainly due to the processing of the substrate prior to fermentation. The first step in tempeh productions is soaking the soybeans in water. This allows for the beans to soften and initiates a primary bacterial fermentation. Traditional soaking of soybeans promotes the growth of *Klebsiella pneumoniae* and *Citrobacter freundii*, bacteria associated with the production of vitamin B12 (Kustyawati et al., 2020). While tempeh may serve as an acceptable source of B12 in vegetarian diets, the levels of B12 reported in traditional tempeh vary considerably and may not always be a reliable source of this vitamin (Kustyawati et al., 2020).

There is also growing evidence for the use of tempeh as a functional food. Tempeh contains isoflavones, bioactive compounds that act as antioxidants to reduce oxidative stress associated with chronic diseases like diabetes, neurodegenerative diseases, and cancer. Isoflavones improve the metabolism of cholesterol, suppressing the development of arteriosclerosis. In addition, isoflavones inhibit estrogen function, which can help mitigate the symptoms of post-menopausal osteoporosis (Nakajima et al., 2005). Tempeh fermentation breaks down the conjugated isoflavones endogenous to soybeans into free isoflavones, maximizing the impact of these compounds and making them more bioavailable (Romulo & Surya, 2021).

In A recent study on the effects of tempeh and diarrheal disease, researchers observed that providing tempeh to people experiencing frequent stools reduced the duration of frequent stools and shortened the recovery time from morbidity due to diarrheal disease. The researchers determined that the fermentation process of tempeh, which hydrolyzes the lipids, proteins, and complex carbohydrates in the substrate, makes these macronutrients easier to digest, allowing for more efficient absorption and faster recovery (Nurwahidah & Arbianingsih, 2019). In addition, in vitro studies of tempeh have also shown it to have antibacterial attributes as it inhibits the adhesion of enterotoxigenic *E. coli* (Kuligowski et al., 2013).

Lastly, tempeh consumption has been associated with modulation of the gut microbiota. In a recent study, human consumption of tempeh resulted in higher *Akkermansia muciniphila* abundance in the stool and higher secretory immunoglobulin A concentrations (Dimidi et al., 2019). *Akkermansia muciniphila is* a bacterium associated with reduced bowel inflammation and is often observed in the gut microbiota of healthy adults (Jayachandran et al., 2020). Recently, it has been introduced commercially as a next generation probiotic.

2.2.2 Nutrition of Edible Insects

Edible insects have been a part of the human diet for millennia, providing protein, fiber, and polyunsaturated fatty acids (PUFAS) linolenic and linoleic acids, which are omega-3 and omega-6 fatty acids, respectively. In addition, they also serve as a source of trace minerals like iron and zinc, making them a potential functional food to reduce mineral deficiencies in target populations (Manditsera et al., 2019) (Nowakowski et al., 2022). Insects are also a rich source of protein. Insects have a higher protein content than beans (23% protein), lentils (26%), and soybeans (41%) (Zielińska et al., 2015). In mealworms (*Tenebrio molitor*), the protein content is around 65% of their dry weight. Along with high protein content, mealworms' amino acid profile has been shown to have higher levels of isoleucine, leucine, valine, phenylalanine and tyrosine than conventional beef, most of which are essential amino acids that cannot be synthesized by the human body (Kowalski et al., 2022).

In addition, some insect species can contain substantial amounts of essential minerals like K, Na, Ca, Cu, Fe, Zn, Mn, and P, as well as B vitamins, and Vitamins A, C, D, E, and K (Dürr & Ratompoarison, 2021). Insects also contain a variety of bioactive compounds that can improve hypertension, inflammation, immune function, and oxidative stress, making them not only a good source of human nutrition, but potential nutraceutical or functional foods as well (Aguilar-Toalá et al., 2022). Mealworms are also a good source of vitamin B12, a vitamin often deficient in people choosing to restrict or eliminate consumption of commercial animal protein. One gram of mealworms contains ~1.8 ug of B12, meaning that fewer than two grams of mealworms are needed to reach the recommended daily allowance of 2.4 ug (Schmidt et al., 2019).

Edible insects are also a good source of dietary fiber, largely due to the mix of chitin and chitosan in their exoskeletons (Ibitoye et al., 2018a). Chitin itself serves a type of insoluble fiber, but chitin can also be digested into chitosan, a compound recognized as a functional food that can aid in immune function (Ibitoye et al., 2018b). Chitin binds to cholesterol, preventing its absorption and reducing overall levels in the blood stream (Singh et al., 2018). There is also some evidence that chitin consumption can aid in wound healing, and chronic disease (Kipkoech, 2023). In a recent human feeding study, participants were fed 25 grams of dried and roasted cricket powder a day for 14 days. Researchers observed an increase in the probiotic bacterium *Bifidobacterium animalis* and a decrease in the tumor necrosis factor (TNF) -α (Stull et al., 2018) .Given the high protein and fiber that regular consumption of both tempeh and insects can provide, a product utilizing whole insects in a tempeh-based food product may provide better overall nutrition to the consumer. To understand the quality and nutrition of tempeh produced with the juvenile life stages of the *Tenebrio molitor* beetle, we first needed to determine if the insect-based substrate would be able to support vigorous mycelial growth. We measured this via observation of mycelial growth and analysis of firmness via texture analyzer over a 24-hour period. The mineral content was determined via Inductively Coupled Plasma and Mass Spectrometry (ICP-MS) Analysis. Vitamin, macronutrient, and amino acid analyses were also conducted, and the protein digestibility was determined by calculating the Protein Digestibility Amino Acid Score (PDCAAS) on a formulation containing both *T. molitor* larvae and soybeans compared to non-fermented controls and a commercially produced traditional soybean tempeh. We hypothesized that the tempeh process incorporating edible insects would result in a versatile food product with comparable nutritional qualities of traditional tempeh.

2.3 Materials and Methods

2.3.1 Sample Preparation – Non-fermented Controls

Five different variations of non-fermented controls were produced in the lab for this project: 100% soy, 100% mealworm (*Tenebrio molitor* larvae), 100% pupae (*Tenebrio molitor* pupae), 50/50 mealworm soy by weight, and 50/50 pupae soy by weight All mealworms used were between 18 and 25 mm long and were procured from a commercial producer (Rainbow Mealworms, Campton, California) and fed a diet of wheat bran (Star of the West Milling Co. Churchville, NY), lyophilized brewer's yeast, and carrots for a minimum of five days prior to processing. The mealworms were then euthanized with liquid nitrogen and stored at -20 °C until use. Pupae were acquired by allowing the mealworms to enter the pupal stage of their development, separating them from the remaining stock, and euthanizing with liquid nitrogen before storing at -20 °C until use.

100% Soy Control– 400g of dry soybeans were soaked in deionized (DI) water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool, the beans were manually broken apart to separate the husk of the bean from the inner flesh. The soybeans were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

100% Mealworm Control – 400g of frozen mealworms were blanched in boiling water for 60 seconds. After blanching, the mealworms were allowed to cool for 20 minutes. The mealworms were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

100% Pupae Control – 400g of frozen pupae were blanched in boiling water for 60 seconds. After blanching, the pupae were allowed to cool for 20 minutes. The pupae were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

50/50 Mealworm Soy Control– 200g of dry soybeans were soaked in DI water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool, the beans were manually broken apart to separate the husk of the bean from the inner flesh. Then 200g of frozen mealworms were blanched in boiling water for 60 seconds. The mealworms were added to the soybeans and mixed. The soybeans and mealworms were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

50/50 Pupae Soy Control – 200g of dry soybeans were soaked in DI water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool, the beans were manually broken apart to separate the husk of the bean from the inner flesh. Then 200g of frozen pupae were blanched in boiling water for 60 seconds. The pupae were added to the soybeans and mixed. The soybeans and pupae were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

2.3.2 Sample Preparation -- Tempeh

Five different variations of tempeh were produced in the lab for this project: 100% soy, 100% mealworm (*Tenebrio molitor* larvae), 100% pupae (*Tenebrio molitor* pupae), 50/50 mealworm soy by weight, and 50/50 pupae soy by weight. All mealworms used were between 18 and 25 mm long and were procured from a commercial producer (Rainbow Mealworms, Campton, California) and fed a diet of wheat bran (Star of the West Milling Co. Churchville, NY), lyophilized brewer's yeast, and carrots for a minimum of five days prior to processing. The mealworms were then euthanized with liquid nitrogen and stored at -20 °C until use. Pupae were acquired by allowing the mealworms to enter the pupal stage of their development, separating them from the remaining stock, and euthanizing with liquid nitrogen before storing at -20 °C until use.

100% Soy – 400g of dry soybeans were soaked, boiled, and dehusked as described above for control samples. Once the husks were separated, 2ml of distilled white vinegar was added and the beans were mixed for 60 seconds to incorporate. After the mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

100% Mealworm – 400g of frozen mealworms were blanched in boiling water for 60 seconds. After blanching, the mealworms were allowed to cool for 20 minutes. Once cool, 2ml of distilled white vinegar was added and the worms were mixed for 60 seconds to incorporate. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and processed in the same manner as the 100% soy tempeh.

50/50 Mealworm Soy – Soybeans were prepared as described and 200g of dehusked and acidified soybeans were added to 200g of frozen mealworms that were blanched in boiling water for 60 seconds, refrozen with liquid nitrogen, and pulverized into rice-sized pieces with a food processor. Then, 2ml of distilled white vinegar was added and the pupae were mixed for 60 seconds to incorporate. After the mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

<u>100% Pupae –</u> 400g of frozen pupae were blanched in boiling water for 60 seconds. After blanching, the pupae were allowed to cool for 20 minutes. Once cool, 2ml of distilled white vinegar was added and the pupae were mixed for 60 seconds to incorporate. After the mixing, 20g of inoculum *containing Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

<u>50/50 Pupae Soy -</u> 200g of cooked, dehusked and acidified soybeans were combined with 200g of frozen pupae that were blanched in boiling water for 60 seconds. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

2.3.3 Sample Cooking and Homogenization

All tempeh products were cut into 25mm cubes and cooked on each side in a dry pan set to medium-high to an internal temperature of 73 ˚C. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -20˚C until further processing.

2.3.4 Texture Analysis

Raw, previously frozen samples were thawed at 0°C in a refrigerator prior to analysis. Sample texture was analyzed utilizing a Stable Micro Systems TA-XT2 texture analyzer and Stable Micro Systems Texture Expert Exceed software version 2.64. Fermentation time for the samples was determined by analyzing the texture of N=3 50/50 mealworm soybean tempeh at 12, 15, 18, 21, and 24 hours and compared to samples of commercial soy tempeh (n=3 per sample type). All other samples were analyzed after 24 hours of fermentation. Texture analyzer was set to determine the number of grams of force it takes to compress the sample 5mm.

2.3.5 Inductively Coupled Plasma and Mass Spectrometry (ICP-MS) Analysis

Elemental concentrations of Li, Be, B, Cd, Se, As, Na, P, S, Mg, K, Ca, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, Ba, W, and Pb were measured using a NexION 350D mass spectrometer (PerkinElmer, City, State) connected to a Type A quartz MEINHARD® concentric nebulizer (supplier, scity, state- or is this a component of the other machine?) and a quartz cyclonic spray chamber. Samples were introduced using a SC-2DX autosampler (ESI). Li, Be, B, Na, P, S, Mg, K, Ca, W, and Pb were measured in standard mode. Se, and As were measured in Dynamic Reaction Cell (DRC) mode using oxygen as the reactive gas. Al, V, Cd, Cr, Mn, Fe, Co, Ni, Cu, Zn, Sr, Mo, and Ba were measured in DRC mode using ammonia as the reactive gas. Before analysis, the torch alignment, nebulizer gas flow and the Quadrupole Ion Deflector (QID) were optimized for maximum Indium signal intensity. A daily performance check was also run, which ensured that the instrument was operating properly and minimized oxide and doubly charged species formation by obtaining a $CeO^+:Ce^+$ of <0.025 and a solution made from a mixture of single-element stock standards (Inorganic Ventures Christiansburg, VA). To correct for instrument drift, a quality control (QC) solution, which consisted of a pooled digested sample prepared by mixing 1 mL of each digested individual sample, was run every 10th sample.

2.3.6 Macronutrient and Vitamin Analysis

Nutritional analysis of the commercial soy tempeh, 50/50 mealworm tempeh, and 50/50 mealworm soy control were conducted by IEH Warren Analytical Laboratory (Greeley, CO). Fat content was determined by Fat-Acid Hydrolysis AOAC method 945.44. Dietary fiber was determined by AOAC method 991.43. Carbohydrates and total calories were determined via calculation, ash was determined vis AOAC method 920.153, Vitamin B6 was determined by the Vitamin B6 Pyridoxine Hydrochloride Method (AOAC?), Vitamin B1 (Thiamine) was determined by AOAC method 942.23, and Vitamin B2 (Riboflavin) was determined by AOAC method 970.65. Any nutrient that meets or exceeds 20% of the Recommended Daily Allowance (RDA) was labeled a high source of that nutrient, in accordance with the Food and Drug Administration (FDA) Federal Code of Regulations Title 21, Volume 2, 101.54. All RDAs were taken from the National Institutes of Health's Office of Dietary Supplements (add website info).

Macronutrient calculations were based on a 68kg individual on a 2000 kcal per day diet. Protein requirements are based on 0.8 g of protein per kg per day for a total of 54.4g of protein per day. Fiber calculations used 34.8 g of dietary fiber per day.

2.3.7 Calculation of the Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

PDCASS was calculated by identifying the limiting amino acid, amino acid score, and multiplying that by the recipe protein digestibility. The amino acid profile was provided my IEH Warren Analytical Laboratory (Greely, CO): Test Method:, Valine = Valine METHOD, Tyrosine = Tyrosine METHOD, Tryptophan = Tryptophan METHOD, Threonine = Threonine METHOD, Serine = Serine METHOD, Protein = Protein AOAC 990.03/992.23/992.15 (LECO). Recipe protein digestibility is calculated by multiplying the percent of protein in each ingredient by their corresponding protein digestibility scores and adding the products from all the ingredients.

2.3.8 Vitamin B12 Analysis

B12 analysis was conducted via Enzyme Immunoassay ELIZA for the Quantitative Determination of Vitamin B12 in food from Gold Standard Diagnostics, Kassel, Germany.

2.3.9 Statistical Analysis

Data were statistically analyzed using Graphpad Prism version 9.5.1 (733). Data were assessed via One-way ANOVA with post-hoc Tukey's multiple comparison analysis. A p-value of <0.05 was considered statistically significant. P-values of 0.080-0.051 were identified as trending significance, indicating that differences may be revealed with a larger sample set. Descriptive statistics were calculated via Microsoft Excel.

2.4 Results

2.4.1 Determination of optimal insect-based tempeh recipe formulation

Initial fermentation experiments examined four different tempeh products (100% mealworm, 100% pupae, 50/50 mealworm soy and 50/50 pupae soy) after a 24-hour fermentation. Full mycelial coverage was observed across the surface of each sample, seen in Figure 2.1.

Figure 2.1: Top view of 50/50 pupae soy tempeh, 50/50 mealworm soy tempeh, 100% pupae tempeh, and 100% mealworm tempeh after 24 hours of fermentation.

Observations of the cross sections show full mycelial penetration of the substrate in the 50/50 soybean mealworm, 50/50 soybean pupae, and 100% mealworm samples. Examination of the 100% pupae sample shows that mycelium did not fully penetrate the substrate during the 24-hour fermentation time (Figure 2.2).

While we did achieve full mycelial penetration and coverage of most of the lab-based samples, none of the samples tested were as firm as a commercial soy sample when analyzed for texture (Table 2.1). In addition, the standard deviation of the compression of the commercial soy tempeh samples is high, suggesting a wide variability in the overall texture of those samples.

Table 2.1: Comparison of average grams of pressure to compress samples 5 mm between commercial soy tempeh and the insect-based tempeh samples.

Sample	Average Grams of Pressure
Commercial Soy	9,142 [SD: 900.8]
100% Mealworm	1,526 [SD:313.4]
100% Pupae	845 [SD:161.4]
50/50 Mealworm Soy	1,843 [SD:251.7]
50/50 Pupae Soy	3,078 [SD:448.2]

While the 50/50 pupae soy tempeh was the firmest of the lab-based tempehs tested, the 50/50 mealworm soy tempeh formed the most cohesive mycelial network, allowing the product to be sliced without its constituent parts crumbling away or falling apart from the main body. As a result, this product meets the expectations of a cohesive body more than the other insect-based tempehs made in the lab and was used for further analyses to explore optimum fermentation time. Variations in final product can be observed in figures 2.1 and 2.2.

Figure 2.2: Cross section of 50/50 pupae soy tempeh, 50/50 mealworm soy tempeh, 100% pupae tempeh, and 100% mealworm tempeh after 24 hours of fermentation.

Samples of the 50:50 mealworm tempeh were analyzed for mycelial coverage and, when possible, texture at 12, 15, 18, 21, and 24-hour fermentation times. After 12 hours of fermentation, samples of the 50/50 mealworm soybean tempeh were removed from the incubator and examined for mycelial penetration and overall cohesiveness of the product. As seen in Figure 2.3a there is minimal mycelial growth on the substrate. The bean and insect material are still separate, and there is no cohesiveness to the overall product.

Figure 2.3a: 50/50 mealworm soybean tempeh after 12 hours of fermentation.

At 15 hours, we began to see some mycelial growth around the edges of the substrate, but a cross section of the sample, seen in Figure 2.3b, demonstrated minimal penetration. While the edges of the product are beginning to show signs of cohesion, the product is still too loose to be recognized as a tempeh product.

Figure 2.3b: 50/50 mealworm soybean tempeh after 15 hours of fermentation.

After 18 hours of fermentation, we observed clear formation of a mycelial network in the substrate, including some penetration into the interior of the product. While penetration is not significant enough to form a fully cohesive network inside the substrate, the product is now sliceable, which can be seen in Figure 2.3c.

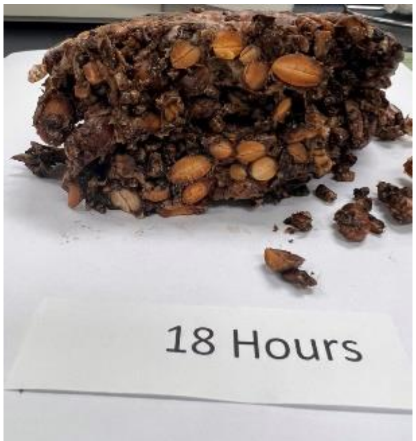

Figure 2.3c: 50/50 mealworm soybean tempeh after 18 hours of fermentation.

Twenty-one hours of fermentation resulted in visible formation of mycelial network throughout the substrate, with visible penetration into the center of the product. The product is cohesive, however, gaps in the penetration can be observed in Figure 2.3d, indicating that additional time is necessary to achieve the desired mycelial growth.

Figure 2.3d: 50/50 mealworm soybean tempeh after 21 hours of fermentation.

Twenty-four hours of fermentation resulted in full mycelial penetration of the substrate. The product is a single, cohesive product that is sliceable. As seen in Figure 2.3e, the mycelial network is dense on the exterior of the product and penetrates through the interior of the substrate. Based on the mycelial penetration, 24 hours was determined to be the optimum time for fermentation for this model.

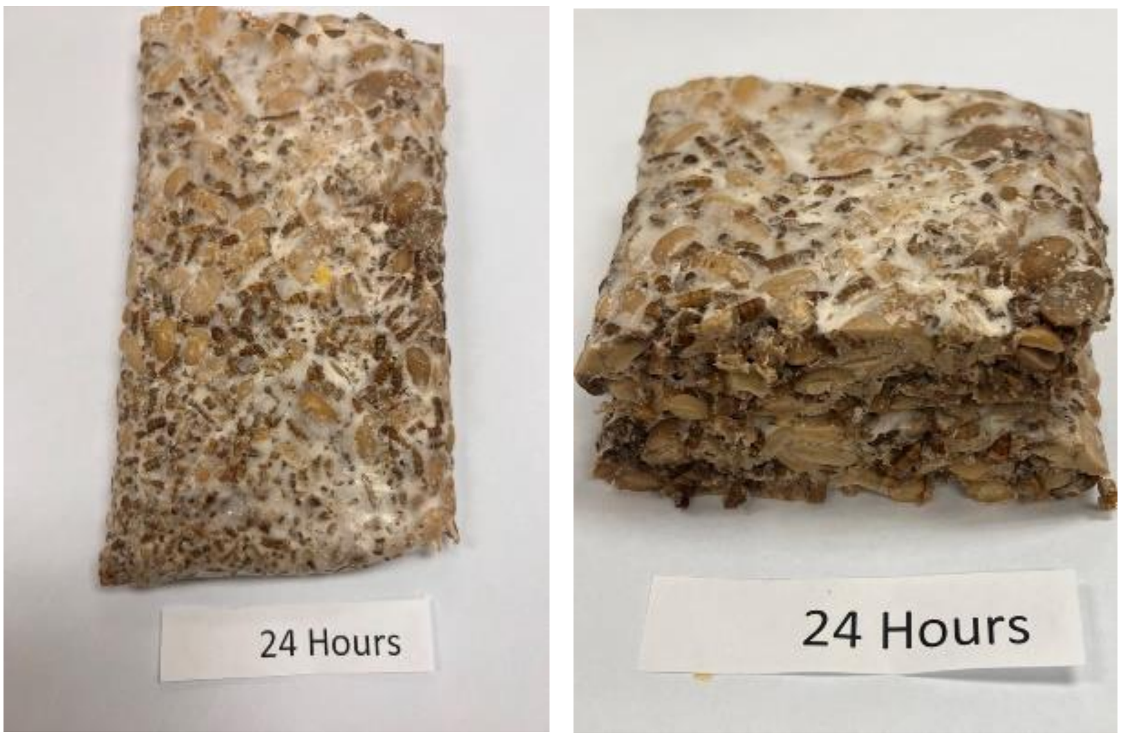

Figure 2.3e: 50/50 mealworm soybean tempeh after 24 hours of fermentation.

Along with the physical observations, the increase in substrate cohesiveness can be seen in the texture analysis in Table 2.2.

Table 2.2: Average grams of pressure necessary to compress the sample 5mm. ND indicates that the sample was not cohesive enough to be analyzed.

Sample	Average Grams of Pressure
12 hrs	ND
15 hrs	540 [SD:85.9]
18 hrs	654 [SD:48.1]
21 hrs	1,661 [SD:489.7]
24 hrs	1843 [SD:251.3]

With the 12-hour sample lacking enough cohesion to be analyzed, we were unable to record an accurate reading with the method. The subsequent readings show a consistent rise in the amount of pressure necessary to compress the samples, with the biggest increase occurring between 18 and 21 hours. This suggests that the most vigorous mycelial growth occurred during this time.

Table 2.3 shows a significant difference between the 15 hours samples and the 21-hour samples ($P=0.0262$). We also observed a significant difference in texture between the 18-hour samples and the 24-hour samples ($P=0.020$), indicating that the most significant textural changes occur between 15 and 24 hours ($P=0.020$). There were no significant differences observed between the compression pressures of the 21-hour ferment and the 24-hour ferment. Therefore, based on both the qualitative and quantitative assessment, 24-hours was selected as the optimum fermentation time for the 50:50 mealworm soybean tempeh.

Table 2.3: Significant differences from a Tukey's multiple comparison analysis of significantly different force required to compress the samples 5mm.

Sample Comparison	Confidence Interval	Mean Difference	P Value
15 hours vs. 21 hours	-2082 to -159.7	-1121	0.0262
18 hours vs. 21 hours	-1867 to -147.8	-1008	0.0257
15 hours vs. 24 hours	-2355 to -249.4	-1302	0.0202
18 hours vs. 24 hours	-2150 to -227.8	-1189	0.0202

This time is confirmed with an examination of the 24 fermentation times across the sample variables. Table 2.4 shows only two significant differences between samples within the sample set. The 50/50 pupae soy tempeh required significantly less pressure to compress when compared to the 100% mealworm tempeh (P=0.0292). In contrast, the 50/50 pupae soy tempeh required significantly more pressure to compress than the 100% pupae tempeh (P=0.0032). These results suggest that 24-hour fermentation is adequate to achieve consistent and comparable texture across the variations within the sample set.

Table 2.4: Significant differences from a Tukey's multiple comparison analysis compression pressure of samples after 24 hours of fermentation.

Sample Comparison	Confidence Interval	Mean Difference	P Value
50/50 Pupae Soy Tempeh vs. 100% Mealworm Tempeh	-314.9 to 3420	1552	0.0292
50/50 Pupae Soy Tempeh vs. 100% Pupae Tempeh	356.6 to 4091	2224	0.0032

2.4.2 Essential Mineral Analysis of Tempeh Samples

The mineral content of each of the original tempeh products was analyzed to determine whether they differ from traditional tempeh. To reduce variables, such as food additives, that could impact the micronutrient analysis, we used a lab-produced soy tempeh for comparison. One way ANOVA across samples with a Dunnet's post hoc analysis revealed that the only micronutrients and minerals that differed between insect-based samples and the soy control were calcium ($p<0.001$), manganese ($p<0.001$), selenium ($p<0.001$), and zinc ($p<0.001$). These data, represented in Figure 2.3, suggest that the presence of soy is driving the significant differences in the amount of calcium and manganese among formulas. Calcium was higher in the soy compared to both the 100% mealworm ($p<0.001$) and the 100% pupae ($p=0.001$). The insect/soy mixed formulas were not significantly different from the soy control. Manganese showed a similar trend with both the 100% mealworm and pupae samples having less of this trace mineral than soy ($p<0.001$). In contrast, both selenium and zinc were higher in the samples that only contained insects (mealworm, $p<0.001$; pupae, $p=0.057$ for selenium and $p<0.001$ for both mealworm and pupae for zinc) (Figure 2.3 and Table 2.4).

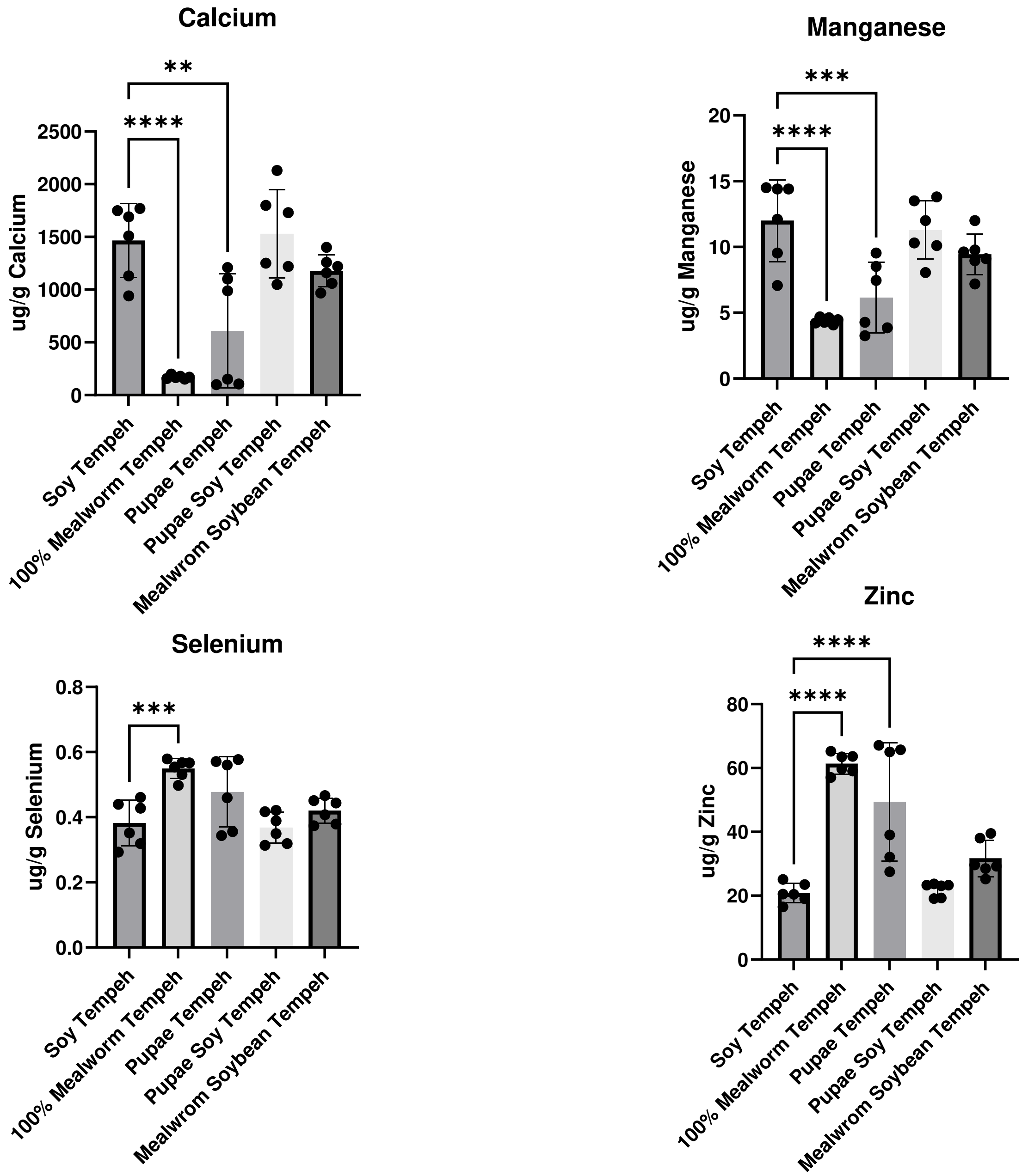

Figure 2.3: Micronutrients that significantly differed between soy-based controls and formulations containing insects.

Further comparisons of the data were done to determine whether the fermentation process resulted in significant changes to any micronutrients. Copper was significantly higher in the soy control compared to the fermented soy ($p=0.002$), and magnesium was significantly higher in the mealworm control compared to the mealworm tempeh ($p=0.006$). However, none of the other comparisons between the fermented and unfermented version of the raw materials differed (Table 2.4).

Table 2.4: Average essential mineral content of each tempeh sample as compared to their constituent controls.

	Ca	Cu	Fe	K	Mg	Mn	P	S	Se	Zn
	(ug/g)	(ug/g)	(ug/g)	(ug/g)	(ug/g)	(ug/g)	(ug/g)	(ug/g)	(ug/g)	(ug/g)
Soy Control	1485 [SD: 718.4]	177 [SD:47.5]	35 [SD:4.6]	7,488 [SD:1,546]	1,295 [SD:165.1]	11 [SD:3.5]	3,578 [SD:319.7]	2,072 [SD:158.4]	0.34 [SD:0.05]	24 [SD:16.0]
Soy Tempeh	1465 [SD:350.6]	90 [SD:15.4]	40 [SD:6.3]	6,152 [SD:2,025]	1,236 [SD:238.4]	12 [SD:3.1]	3,408 [SD:649.0]	2,030 [SD:271.9]	0.38 [SD:0.07]	21 [SD:3.1]
MW Control	282.2 [SD:214.8]	108 [SD:8.1]	35 [SD:2.6]	4,780 [SD:287.2]	1,808 [SD:75.4]	5 [SD:3.0]	3,432 [SD:104.2]	1,943 [SD:88.5]	0.43 [SD:0.02]	55 [SD:15.6]
MW Tempeh	170 [SD: 17.3]	94 [SD:6.5]	38 [SD:8.7]	4,603 [SD:185.2]	1,403 [SD:41.3]	4 [SD:0.2]	3,725 [SD:77.9]	2,085 [SD:122.1]	0.55 [SD:0.03]	61 [SD:3.2]
Pupae Control	68 [SD:96.9]	105 [SD:7.4]	34 [SD:2.5]	5,025 [SD:173.4]	1,089 [SD:41.2]	3 [SD:0.4]	3,633 [SD:244.5]	2,047 [SD:149.4]	0.52 [SD:0.05]	60 [SD:5.6]
Pupae Tempeh	609 [SD:542.1]	117 [SD:26.6]	40 [SD:2.5]	5,242 [SD:2,300.8]	1,144 [SD:234.1]	6 [SD:2.7]	3,592 [SD:258.3]	2,215 [SD:163.8]	0.48 [SD:0.05]	49 [SD:2.2]
MS Control	877 [SD:277.0]	111 [SD:38.3]	30 [SD:1.0]	5,294 [SD:334.1]	996 [SD:2.0]	7 [SD:70.0]	2,852 [SD:151.2]	1,738 [SD:841.2]	0.34 [SD:0.02]	28 [SD:11.7]
MS Tempeh	1,178[SD:17.3]	112 [SD:31.0]	39 [SD:3.6]	5,163 [SD:1041.1]	1,187 [SD:100.9]	9 [SD:1.6]	3,437 [SD:270.3]	2,068 [SD:103.4]	0.42 [SD:0.04]	32 [SD:5.7]
PS Control	990 [SD:96.9]	155 [SD:7.4]	37 [SD:1.8]	6,750 [SD:173.4]	1,262 [SD:41.2]	8 [SD:0.5]	3,755 [SD:130.7]	2,157 [SD:135.4]	0.46 [SD:0.05]	41 [SD:1.9]
PS Tempeh	1592 [SD:419]	118 [SD:56.7]	40 [SD:6.5]	5,970 [SD:2,300.8]	1,263 [SD:234.1]	12 [SD:2.2]	3,486 [SD:514.9]	2,134 [SD:292.2]	0.38 [SD:0.05]	22 [SD:2.2]

From a nutritional perspective, calculating the % Daily Value (DV) supplied by each formula suggests that all of the samples (fermented and raw) were high (containing more than 20% of RDA) in copper, manganese, phosphorus, and selenium. In addition, all the fermented tempeh samples were also high in iron (Table 2.5).

Table 2.5: The average %RDA of essential minerals contained in 100g of each product.

	Ca	Cu	Fe	K	Mg	Mn	P	S	Se	Zn
	% RDA	% RDA	% RDA	% RDA	% RDA	% RDA	% RDA	% RDA	% RDA	% RDA
Soy Control	7%	1970%	45%	16%	37%	34%	51%	NA	62%	26%
Soy Tempeh	7%	998%	50%	13%	35%	38%	49%	NA	69%	22%
MW Control	1%	1200%	44%	10%	31%	16%	49%	NA	79%	57%
MW Tempeh	1%	1074%	48%	10%	40%	14%	53%	NA	100%	65%
Pupae Control	0%	1162%	42%	11%	31%	10%	52%	NA	95%	63%
Pupae Tempeh	3%	1300%	50%	11%	33%	19%	51%	NA	87%	52%
MS Control	5%	1545%	47%	14%	36%	28%	51%	NA	78%	37%
MS Tempeh	6%	1239%	48%	11%	34%	30%	49%	NA	76%	33%
PS Control	5%	1722%	47%	14%	36%	26%	54%	NA	83%	43%
PS Tempeh	8%	1309%	50%	13%	36%	36%	50%	NA	69%	23%

Since the 50/50 mealworm soybean tempeh was not significantly different from soybean tempeh regarding micronutrient content and it showed a favorable texture and sensory attributes (Chapter 4), it was selected for macronutrient analysis, protein digestibility, and vitamin testing.

2.4.3 Macronutrient and Vitamin Analysis

Macronutrient analysis of n=4 of the 50/50 mealworm soy tempeh, one 50/50 mealworm soy control, and one commercial soy tempeh sample suggests the macronutrient content of the insect-containing product is comparable to that of a soy-based tempeh. As seen in Table 2.6, the macronutrient values for the commercial tempeh sample fell within the range of values reported for the 50/50 mealworm soy tempeh. Likewise, fermentation seemed to have minimal impact on these values as evidenced by the data from the unfermented control.

Table 2.6: Average macronutrients for 50/50 mealworm soy tempeh (n=4), an unfermented control of the raw ingredients, and a commercial tempeh. Standard deviations for the lab-produced tempeh are in parentheses.

Nutrition Facts	Mealworm Soy Tempeh	Mealworm Soy Control	Commercial Soy Tempeh
Carbohydrates (g/100g)	10.25 (2.2)	12	10
Dietary Fiber (g/100g)	9.68 (1.62)	11.1	10
Protein (g/100g)	18.45 (1.17)	17.56	19.74
Fat (g/100g)	6.99 (1.31)	5.26	7.04
Calories (kcal/100g)	178.25 (8.02)	166	182
Ash (g/100g)	1.35 (0.12)	1.34	1.33

Unsurprisingly, when the total recommended daily allowance of a 68kg individual on a 2,000 calorie per day diet is calculated, all three samples provided similar percentages of the recommended daily allowance for protein and fiber (Table 2.7).

Table 2.7: Percent recommended daily allowance of macronutrients provided by experimental and commercial tempehs, based on a 68kg individual consuming a 2,000-calorie diet.

	Commercial Tempeh	50/50 Mealworm Soy Control	50/50 Mealworm Soybean Tempeh
%DV	%DV	%DV	%DV
Carbohydrates	8%	9%	7%
Dietary Fiber	29%	32%	26%
Protein	36%	32%	32%
Calories	9%	8%	9%

In addition, all three samples meet the standard of a good source of protein. At more than 15g per serving, all samples within the set exceed the amount to be considered a good source. All samples also qualify as high in dietary fiber.

Analysis of B-vitamins B1, B2, B6, and B12 were also performed. Except for vitamin B12, the B-vitamin analysis was conducted at Warren Analytical Lab (Greeley, CO) on n=4 of the 50/50 mealworm soy tempeh and compared with the U.S. Department of Agriculture's Food Data Central's nutritional data on tempeh. Although it is not possible to conduct statistical comparisons, the content of B6 and B1 were comparable between the insect-containing tempeh and the commercial sample. On the other hand, riboflavin (B2) was about 5x higher in the 50/50 mealworm soy tempeh compared to the commercial control. Vitamin B12 was assessed by ELISA and the 50/50 mealworm soy tempeh was compared to a lab-produced soy tempeh. There were no significant differences between the insect-based and traditional soy -based tempeh samples (n=4/sample; p=0.997).

Table 2.8: Comparison of the average amount of B-vitamins detected in the 50/50 mealworm soybean tempeh compared with the U.S Department of Agriculture's Food Data Central's nutritional data on tempeh (USDA ARS , 2019). Vitamin B12 levels were determined using ELISA on lab-produced tempeh samples.

B-Vitamin	50/50 Mealworm Soy Tempeh	% DV	Soy Tempeh	% DV
	mg/100g		mg/100g	
Pyridoxine Hydrochloride (B6)	0.206 (SD:0.014)	16%	0.215	17%
Riboflavin (B2)	0.187 (SD:0.149)	14%	0.0358	3%

Thiamine (B1)	0.075 (SD:0.008)	6%	0.078	7%
Vitamin B12	0.223 (SD:0.283)	9,708 %	0.37 (SD:0.447)	15,417 %

2.4.4 Protein Digestibility

The benefits of protein content are dependent on the digestibility of the protein. Protein digestibility is determined by the least abundant essential amino acid within the food. Since essential amino acids cannot be synthesized within the body, they must be obtained from external sources. The degree to which a protein source can meet these essential amino acid requirements is calculated using the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), which is calculated as follows: (% of first ingredient's protein contribution x protein digestibility of first ingredient) + (% of second ingredient's protein contribution x protein digestibility of second ingredient) (Meshulam-Pascoviche et al., 2022).

Table 2.9 lists the full amino acid profiles of the commercial soy tempeh, the 50/50 mealworm soy control, and the 50/50 mealworm soybean tempeh. The commercial tempeh samples contained higher amounts of every essential amino acid than both the 50/50 mealworm soy control and the 50/50 mealworm soybean tempeh.

Table 2.9: Amino acid profiles of the commercial tempeh, 50/50 mealworm soy control, and the 50/50 mealworm soybean tempeh with the essential amino acids highlighted.

	Commercial Tempeh	50/50 Mealworm Soy Control	50/50 Mealworm Soybean Tempeh
	mg/100g	mg/100g	mg/100g
Valine	890	630	560
Tryptophan	200	90	120
Lysine	860	800	910
Isoleucine	870	830	830
Histidine	450	320	260
Threonine	740	710	530
Phenylalanine	920	850	520
Methionine	180	70	80
Leucine	1,710	950	820
Serine	1,070	880	740
Proline	1,290	920	880
Glutamic Acid	3,660	3,210	3,480
Aspartic Acid	2,430	2,870	3,060
Arginine	1,400	1,340	1,080
Tyrosine	630	640	600
Hydroxyprolin	<100	<100	
Glycine	880	990	1,120
Cystine	170	310	370
Alanine	1,150	1,190	1,240

As seen in Table 2.10 tryptophan is the limiting amino acid in both the 50/50 mealworm soy control and the 50/50 Mealworm soy tempeh. In this table, methionine and cystine are paired together as methionine is a metabolic precursor to cystine.

Table 2.10: Amino acid scores indicating the limiting amino acids of the 50/50 mealworm soy control, the 50/50 mealworm soy tempeh, and the commercial soy tempeh. Highlights indicate the limiting amino acid for each sample.

	Ideal Ratio	50/50 Mealworm Soy Control		50/50 Mealworm Soy Temph		Commercial Tempeh	
Amino Acid		mg/g crude protein	Amino Acid Score	mg/g crude protein	Amino Acid Score	mg/g crude protein	Amino Acid Score
Histidine	19	18.8	99%	13.4	70%	21.2	112%
Inoleucine	28	48.7	174%	42.8	153%	41.0	147%
Leucine	66	55.7	84%	42.2	64%	80.7	122%
Lysine	58	46.9	81%	46.9	81%	40.6	70%
Methionine + Cysteine	25	22.3	89%	19.6	78%	16.5	66%
Phenylalanine + Tyrosine	63	87.3	139%	57.7	92%	73.1	116%
Threonine	34	41.6	122%	27.3	80%	34.9	103%
Tryptophan	11	5.3	48%	6.2	56%	9.4	86%
Valine	35	36.9	106%	28.8	82%	42.0	120%

In addition, phenylalanine and tyrosine are paired together since tyrosine can be produced in the body when phenylalanine is present (Matthews, n.d.; Wirtz & Droux, 2005). While we do see an increase in the amount of tryptophan during the fermentation, without statistical replication it is not possible to conclude that the fermentation process is the cause of this increase. In the commercial soy tempeh, the Methionine+Cysteine amino acid pair is the limiting factor.

To calculate the recipe protein digestibility of the 50/50 mealworm soy control, we used the formula (% of total protein provided by soy x the protein digestibility of soy) + (% of total protein provided by the mealworms x the protein digestibility of the mealworms). The protein digestibility of soy is 0.91 (Hess & Slavin, 2016). The protein digestibility of mealworms is between 0.69 and 0.84, depending on processing and storage (Meshulam-Pascoviche et al., 2022). For our calculations, we used the average of 0.765. Within the 50/50 mealworm soy control, the soy contributes roughly 64% of the protein, and the mealworms contribute the remaining 36%. With these values, the recipe digestibility calculation for the mealworm soy control is as follows:

> *Soybeans: [0.64 (% of the protein from the soybeans) x 0.91 (soybean protein digestibility score)]+ Mealworms: [0.36 (% of the protein from the mealworms) x 0.756 (mealworm protein digestibility score) = 0.88]*

If we apply that same calculation to a soybean control, the recipe digestibility score for soybeans would be 0.91, making the digestibility between the two controls similar. If, like the 50/50 mealworm soy tempeh, the limiting amino acid content is not significantly changed between control and the fermented product, then one could expect a proportional difference between the PDCAAS score of the 50/50 mealworm soybean control and the 50/50 mealworm soybean tempeh.

PDCAAS for 50/50 mealworm soy control = recipe protein digestibility (0.88) x amino acid score (0.48) = 0.42. In contrast, assuming the recipe digestibility of the 50/50 mealworm soybean tempeh is similar, then the PDCASS = recipe digestibility (0.88) x amino acid score (0.56) = 0.49, or a potential 7% increase in protein digestibility in the fermented product. While there is improved protein digestibility in the tempeh sample, sufficient replication is lacking to determine whether this is statistically significant. In comparison, the PDCASS of the soybean tempeh = recipe protein digestibility (0.91) x amino acid score (0.66) = 0.60. This is higher than the digestibility of both the control and the insect tempeh samples.

2.5 Discussion

Overall, the application of traditional tempeh techniques is effective in producing a cohesive tempeh product utilizing the juvenile life stages of the *Tenebrio molitor* beetle. We were able to achieve full mycelia penetration within 24 of incubation in three of the four experimental groups, and while texture analysis of these samples was divergent from the commercial soy tempeh sample, the evidence suggests that this may be a result of commercial processing methods and packaging rather than the ability of the substrate to mimic similar texture. While the reasons for the differences in texture are unclear, variations in the texture of the substrates may be due to the presence of insect protein, compression of the commercial substrate prior to fermentation, or age of the product as the commercial product was purchased from a local vendor and the lab-based tempehs were processed fresh. Despite these differences in texture, all the lab-based samples were sliceable, cohesive, and functional as a solid substrate product. From here, we can punch them into nuggets, cut them into hamburgers, or present them as cubes and crumbles for ground applications.

2.5.1 Micronutrient content of soy and insect-based tempeh

The mineral content of the tempeh samples did not change significantly due to the fermentation process. A previous study showed that spontaneous fermentation of mung bean flour had higher calcium and iron than the raw materials, but lower zinc (Onwurafor et al., 2014). Another study reported increases in both iron and zinc in black pea flour after a solid-state fermentation with *Aspergillus oryzae* (Chawla et al., 2017). Finally, fermentation of soybeans by *Bacillus amyloliquefaciens* was associated with higher levels of antioxidants, phenolic content, isoflavones, and total amino acids in addition, to higher levels of Fe, K, Mg, Mn, Na, and Zn (Shahzad et al., 2020). However, in all these reports the fermenting organisms differed from the *Rhizopus oligosporus* used in this study and there were variable soaking and fermentation times.

These studies suggest that the micronutrient content may be more dependent on process and/or types of fermentation organisms. Importantly, another aspect to consider in combination with actual mineral content is bioavailability. Fermentation may significantly impact micronutrient bioavailability through reduction of chelating agents, oxidizers, and polyphenols that may be present in the plant or insect material. Therefore, determining the differences in mineral bioavailability of insect-based tempeh products should be a target of future analyses.

Although there were no changes in micronutrient content with fermentation, there were some differences between the various substrates used. Specifically, calcium and manganese were higher in the samples containing soy than those that had only insects, while zinc and selenium were higher in the 100% insect-containing samples. Although edible insects are often cited as a source of calcium, there is considerable variability in calcium content across insects tested, with one report showing calcium levels at the lower end of all insects tested in *Tenebrio molitor* (Adámková et al., 2014). It has also been reported that there is very little calcium in invertebrates without a hard exoskeleton (de Castro et al., 2018), suggesting that the life stage of the insect may be important in determining final calcium content.

Soybeans are not traditionally considered an ideal source for calcium due to the presence of chelating agents like phytates in the beans, fermentation by *Rhizopus spp.* can help reduce the presence of phytates in the product and make the calcium more bioavailable (Sudarmadji & Markakis, 1977). In a recent study on calcium absorption in rats, the animals were fed a diet of soybeans, conventional tempeh fermented with *Rhizopus spp.* microspores, and an anaerobic tempeh. The rats that consumed the traditional tempeh experienced significantly higher calcium absorption than the animals in the other two groups. While the *Rhizopus spp.* fermentation may have made the calcium more bioavailable, the researchers also noted that the presence of nondigestible oligosaccharides and common food ingredients like casein phosphopeptide are also associated with improved calcium absorption (Watanabe et al., 2008). In addition, lactic acid bacteria have been associated with the reduction of phytate in plant materials as well. Since tempeh fermentation begins with a lactic acid fermentation step during the soaking of the beans, this may also help reduce the amounts of phytates in the material, making the calcium more bioavailable (Damayanti et al., 2017). Therefore, as mentioned, this should be a focus of further study in insect-based tempeh products.

Selenium is an element that has a narrow range from essentiality to toxicity in mammalian species (Mechora, 2019a). Selenium content in insects is not well characterized, but it is known that plant selenium content is strongly influenced by the levels of this element in soil. Therefore, while we show higher selenium in the insect-containing samples relative to the soybean tempeh controls, this could be influenced by the selenium content in the environment. Therefore, soybean levels may vary based on selenium soil content during growth and the mealworms may vary based on the amount and type of selenium accumulated in the food they are supplied.

Zinc, which is important in human metabolism and immune function, was also higher in insect-based tempeh formulations. Like calcium, soybean is not generally considered a good source for zinc because of the high levels of phytic acid that can interfere with zinc absorption. That said, zinc levels in soybean are reportedly higher than levels found in other cereal crops like maize and sorghum (Akomo et al., 2016; Mechora, 2019b). In contrast, insects are also considered to be high in zinc (Mabelebele et al., 2023) and lack phytates that can prevent absorption. Furthermore, rearing conditions and processing can also be manipulated to alter the zinc content of edible insects to optimize them for use in preventing zinc-related nutritional deficiencies (Mabelebele et al., 2023). As with Calcium and iron, future studies should focus on identifying the bioavailability of these micronutrients in humans to establish their true nutritional value.

2.5.2 Macronutrient analysis of the 50/50 mealworm soybean tempeh

According to the Food and Drug Administration, foods containing 20% or more of the daily value of a nutrient are considered high sources of that nutrient. For a food to be considered high in fiber, it must contain 20% of the daily value of fiber, or 6 or more grams of fiber per serving. All the tested samples exceeded that level and could be considered as good sources of fiber. This is particularly important because animal-sourced foods typically have negligible fiber content, and it might be expected that replacing plant protein with animal protein would negatively affect fiber. However, insects are unique among animal proteins as they contain the polymer chitin, which serves as a source of dietary fiber (Stull & Weir, 2023). In addition, the fat, protein, and calorie contents in the insect-based and traditional soy tempeh samples were similar; suggesting that adding insects into the formulation does not compromise the nutritional value of the tempeh. In fact, although we cannot demonstrate statistical significance, the B-vitamin data supports that the insect formulation may be higher in riboflavin. A larger sample set may help provide more information to establish significance.

2.5.3 Protein digestibility

Fermentation by *Rhizopus oligosporus* has been observed to improve overall protein quality and content. In a recent study that utilized *R. oligosporus* to ferment cassava leaves and babassu mesocarp, researchers observed a 15.8% increase in the amount of crude protein in the substrate and an increase of protein quality (Chebaibi et al., 2019). In another study, researchers observed that fermentation of soybeans by *R. oligosporus* resulted in a reduction of carbohydrates from the unfermented control to an increase in amino acids available (Chong et al., 2022). We observed a similar trend in our results. We saw a 25% decrease in carbohydrates between the 50/50 soybean mealworm control and the 50/50 soybean mealworm tempeh. In addition, we did observe an increase with three essential amino acids, tryptophan (35%), lysine (13%), and methionine (14%), though more samples are necessary to establish significance. Deficiencies in these essential amino acids can result in a variety of adverse health effects. Deficiencies in dietary tryptophan have been associated with dysregulation in the intestinal flora and even heightened inflammatory responses in COVID-19 infections (Qin et al., 2021; Rankin et al., 2023). While the digestibility of the 50/50 mealworm soybean tempeh (0.49) was less than traditional soy tempeh (0.60), the product still contained enough protein to be considered high in protein by the standards of the FDA. However, more testing is needed to establish the significance of the difference in digestibility.

2.6 Conclusions

Overall, the application of tempeh fermentation methods appears to be an effective way to incorporate insect protein into soy-based tempeh. While we observed some variation of nutrient content and texture, the 50/50 mealworm soybean tempeh appears to be similar to that of traditional tempeh. While more tests are needed to establish the significance of the vitamin and macronutrient differences, this study has established that fermentation by *Rhizopus oligosporus* can achieve an insect-based product that is similar to the traditional soy tempeh. Future studies should further examine the *in vivo* absorption and digestibility of these nutrients as well as establish consumer acceptability of an insect-based tempeh product.

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Chapter 3: Comparison of the Iron Bioavailability of Tempeh Made with *Tenebrio molitor* to Beef and Plant-Based Meat Alternatives

3.1 Summary

Iron is an essential mineral that supports several biological functions like growth, oxygen transport, cellular function, and the synthesis of hormones. Insufficient dietary iron can lead to anemia and cause fatigue, cognitive impairment, and poor immune function. Dietary iron can be found in two forms: heme and non-heme. Heme iron is more bioavailable than non-heme iron, which must be converted from ferric to ferrous before it is absorbed by the intestinal lining. Heme iron is typically found in animal-based proteins like chicken, fish, and beef which are good sources of bioavailable iron. Non-heme iron is most commonly found in plant-based foods like lentils, seeds, and spinach. Insects vary in their iron content and may have heme or non-heme forms, depending on their diet. As insects are being explored to address food insecurity and malnutrition in low resource contexts, it is important to understand the bioavailability of iron from insect-based food sources. In this study, we used Inductively Coupled Plasma and Mass Spectrometry (IPC-MS) to compare the amount of soluble and bioavailable iron among five different lab-produced tempeh products and their non-fermented controls, containing two different life stages of the *Tenebrio molitor* beetle. The tempeh products included a soy tempeh, 100%mealworm tempeh, 100% pupae tempeh, 50/50 soy mealworm tempeh, and 50/50 pupae mealworm tempeh, all fermented with *Rhizopus oligosporus* spores. Further, we compared the insect tempeh iron bioavailability with two sources of conventional beef (ground beef and sirloin steaks) and two commercially available plant-based meat alternative products. We used Caco-2 human colonic cells as a model for determining the bioavailability of the iron in each product. Our results showed that

while both the plant-based meat alternative samples and the tempeh samples contained more ($P < 0.0001$) soluble iron than the conventional beef samples, there was no significant difference ($P < 0.0001$) in the amount of ferritin absorbed by the Caco-2 cells among these samples. Furthermore, we observed a substantial increase in the amount of ferritin detected in the three of the insect-based tempeh samples, suggesting that the fermentation process or the presence of insect proteins increased the iron bioavailability.

3.2 Introduction

Iron is an essential mineral that is found naturally in a variety of foods. It is a constituent part of oxygen transport molecules, like hemoglobin and myoglobin, and is essential to muscle metabolism and the maintenance of healthy connective tissue. Iron supports a variety of essential biological functions including physical growth, neurological development, cellular function and hormone synthesis. Insufficient iron uptake can lead to a diminished capacity to transport oxygen to the tissues and organs of the body, resulting in a physiological condition known as anemia. Anemia caused by iron deficiency can significantly affect cognitive function, physical activity, and immune function. It has also been associated with complications during pregnancy and maternal mortality, as well as a variety of other health issues.

While dietary requirements for iron vary depending on age, biological sex, and pregnancy status (Table 3.1), the FDA requires food labels to list their percent daily value iron content based on 18 mg/day. Since iron uptake is regulated through absorption in the intestine, the bioavailability of that iron is key to preventing anemia. Humans, on average, consume between 10 and 15mg of dietary iron per day (Piskin et al., 2022). Foods that are considered a high source of iron must contain at least 20%, or 3.6 mg, of the daily value of iron (Piskin et al., 2022).

Table 3.1: Dietary Recommended Dailly Allowance (RDA) of iron intake for various age groups (adapted from Supplements, 2022).

Life Stage	Iron RDA
Birth to 6 months	0.27 mg/ day
Children 6 months to 13 years	7-11 mg/ day
Teenage boys 14- 18 years	11 mg/ day
Teenage girls 14-18	15 mg/day
Adult men 19-50	8 mg/ day
Adult women 19-50	18 mg/ day
Adults 51 years and older	8 mg/day
Pregnant women	27 mg/ day
Breastfeeding women	10 mg/day

Dietary iron is accessed in two forms: heme and nonheme (Supplements, 2022). Heme iron, a ferrous (Fe^{2+}) iron chelated into a porphyrin ring structure, is typically found in the hemoglobin and myoglobin of animal proteins like red meat, poultry, and seafood. Heme iron is considered more bioavailable than nonheme iron (Carpenter and Mahoney, 1992), largely due to the different pathways the types of iron use to enter enterocytes. Non-heme iron, found predominately in plant-based foods, accessed from the diet can be ferrous or ferric (Fe^{3+}), but ferric iron Fe^{3+} must be converted to Fe^{2+} by duodenal cytochrome B (DCYTB) before it can be transported into the enterocyte through the Divalent Metal Transporter 1 (DTM1) transporter (Lane et al., 2015). Heme iron can bypass this and enter the enterocyte through heme transporters (HCP1). It is estimated that ~ 10-20% of consumed heme iron is absorbed while absorption of non-heme iron sources varies. Iron sourced from dark leafy greens is between 7 and 9%. In contrast, we absorb roughly 4% of the iron contained in grains and 2% of iron found in legumes (Milman, 2020a). Of that, roughly 1 to 2 mg are absorbed in the intestines.

Nonheme iron is found in plants and iron-rich foods like nuts, fortified grains, legumes, and vegetables. Because of this disparity in bioavailability, heme iron is a more significant source of dietary iron than non-heme, plant-based sources of iron (Carpenter and Mahoney, 1992). However, interaction between food components and food preparation methods can directly influence the bioavailability of nonheme iron. For example, Vitamin C can improve the absorption of nonheme iron. In addition, lactic fermentation has been shown to improve iron bioavailability in fermented foods (Scheers et al., 2016) and another study suggested that solid-state fermentation of black-eyed peas improved their iron bioavailability (Chawla et al., 2017). On the other hand, chelating agents found in plant foods like phytates, and polyphenols can reduce the bioavailability of nonheme iron (Hurrell and Egli, 2010a). The bioavailability of iron becomes more nuanced when examining insect-based foods. While it is likely that insects contain both heme and non-heme iron, the primary form of heme iron in insects is found in cytochromes. However, the iron in insects is predominantly present in non-heme forms like ferritin and holoferritin. do not contain heme iron, there is some evidence that the presence of animal protein improves the bioavailability of nonheme iron (Mwangi et al., 2022). Known as the "meat factor," there is evidence to support that peptide containing cysteine can facilitate the absorption of nonheme iron (Hurrell and Egli, 2010b). If this is the case, then we would expect to see greater iron absorption from food sources containing conventional meat and insect-based protein than from plant sources alone.

Understanding iron bioavailability in different food sources is important in contexts where meat is rarely consumed or intentionally omitted from the diet. For example, diets that intentionally omit animal products for reasons of religious observance or motivated by human and planetary health concerns are common and can lead to iron deficiencies. Edible insects may serve as an alternative protein source in these situations, although previous studies are equivocal about their ability to supply bioavailable dietary iron (Hilaj et al. year) In a recent study comparing the mineral content of grasshoppers, crickets, mealworms, and buffalo worms to sirloin beef, the researchers observed significantly higher amounts of soluble iron in the insect samples, but the bioavailability of that iron varied between species. The researchers attributed this to the lack of hemoglobin and myoglobin in most insect species (Latunde-Dada et al., 2016). Therefore, further research is needed to assess iron bioavailability from insect-based foods and compare them with animal protein and other plant-based meat alternative products, whose global sales were roughly $10 billion in 2018, and are expected to increase to $30.92 billion by 2026 (Shurtleff and Aoyagi, 2014) (Zhao et al., 2023).

In this study, we compared the iron bioavailability of fermented and nonfermented tempeh products made with varying ratios of soybean and *Tenebrio molitor* (mealworm) larvae and pupae. Furthermore, we compared the tempeh preparations with conventional beef and two popular plant-based meat alternatives. Little is known about levels of soluble iron or iron bioavailability from *T. molitor,* particularly whether there are differences in iron between larval and pupal stages and whether solid-state fermentation can impact these factors. With regard to the plant-based meat products, they are typically formulated to contain similar levels of iron to their conventional meat counterparts; however, the presence of anti-nutrients like oxalates, phytates, and tannins found in plant tissues may reduce iron absorption (Hurrell and Egli, 2010a). To counter these effects, some manufacturers include vitamin C or are using soy-based leghemoglobin, a plant-based heme containing protein found in the roots of nitrogen fixing plants, to improve iron bioavailability. Here we address differences in soluble iron (based on simulated gastric digests) and iron bioavailability among fermented and non-fermented tempeh preparations containing edible insects, as well as comparing an insect-based formulation to two beef products, and three plant-based meat alternatives (Beyond Burger, Impossible Burger, and lab-based soy tempeh) using a well-established Caco-2 cell culture model. Based on the current literature and attempts to enhance iron bioavailability in the commercial meat alternative formulations, we hypothesize that the presence of animal protein in the insect-based tempeh and the reduction of chelating agents due to *Rhizopus oligosporus* fermentation will increase the bioavailability of the iron contained within the tempeh products, rendering them as rich a source of iron as the beef and plant-based alternative samples.

3.3 Materials and Methods

3.3.1 Mealworm sourcing

All mealworms used were between 18 and 25 mm long and were procured from a commercial producer (Rainbow Mealworms, Campton, California) and fed a diet of wheat bran, lyophilized brewer's yeast, and whole carrots for a minimum of five days prior to processing. The mealworms were then euthanized with liquid nitrogen and stored at – 20 °C until use. Pupae were acquired by allowing the mealworms to enter the pupal stage of their development, separating them from the remaining stock, and euthanizing with liquid nitrogen before storing at – 20 °C until use.

3.3.2 Tempeh production

Five different variations of tempeh were produced in the lab for this project: 100% soy, 100% mealworm (*Tenebrio molitor* larvae), 100% pupae (*Tenebrio molitor* pupae), 50/50 mealworm soy by weight, and 50/50 pupae soy by weight.

100% Soy – 400 g of dry soybeans were soaked in deionized (DI) water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool, the beans were manually broken apart to separate the husk of the bean from the inner flesh. Once the husks were separated, 2ml of distilled white vinegar was added and the beans were mixed for 60 seconds to incorporate. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores (Wira, Pemona, Ca), rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

100% Mealworm – 400g of frozen mealworms were blanched in boiling water for 60 seconds. After blanching, the mealworms were allowed to cool for 20 minutes. Once cool, 2ml of distilled white vinegar was added and the worms were mixed for 60 seconds to incorporate. After mixing, 20g of inoculum containing Rhizopus oligosporus spores, rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

50/50 Mealworm Soy – 200 g of dry soybeans were soaked in DI water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool. The beans were manually broken apart to separate the husk of the bean from the inner flesh. Once the husks were separated, 2ml of distilled white vinegar was added and the beans were mixed for 60 seconds to incorporate. Then 200g of frozen mealworms were blanched in boiling water for 60 seconds. After blanching, the mealworms were frozen once again with liquid nitrogen and pulverized into rice-sized pieces with a food processor. Once pulverized, the mealworms were added to the soybeans and mixed. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores, rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

<u>100% Pupae –</u> 400g of frozen pupae were blanched in boiling water for 60 seconds. After blanching, the pupae were allowed to cool for 20 minutes. Once cool, 2ml of distilled white vinegar was added and the pupae were mixed for 60 seconds to incorporate. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores, rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

<u>50/50 Pupae Soy –</u> 200 g of dry soybeans were soaked in DI water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool. The beans were manually broken apart to separate the husk of the bean from the inner flesh. Once the husks were separated, 2ml of distilled white vinegar was added and the beans were mixed for 60 seconds to incorporate. Then 200g of frozen pupae were blanched in boiling water for 60 seconds. After blanching, pupae were added to the soybeans and mixed. After mixing, 20g of inoculum containing *Rhizopus oligosporus* spores,

rice flour, and soy flour was added, and the substrate was mixed for an additional 60 seconds. The mixture was then placed into six, quart-sized plastic bags, each with five rows of six perforations spaced 1 cm apart. Excess air was removed from each bag, and the samples were placed in an incubator at 30° C and 80% relative humidity (RH) for 24 hours.

3.3.3 Control Preparation

Five different variations of controls were produced in the lab for this project: 100% soy, 100% mealworm (*Tenebrio molitor* larvae), 100% pupae (*Tenebrio molitor* pupae), 50/50 mealworm soy by weight, and 50/50 pupae soy by weight. All mealworms used were between 18 and 25 mm long and were procured from a commercial producer (Rainbow Mealworms, Campton, California) and fed a diet of wheat bran (Star of the West Milling Co. Churchville, NY), lyophilized brewer's yeast, and carrots for a minimum of five days prior to processing. The mealworms were then euthanized with liquid nitrogen and stored at – 20 °C until use. Pupae were acquired by allowing the mealworms to enter the pupal stage of their development, separating them from the remaining stock, and euthanizing with liquid nitrogen before storing at – 20 °C until use.

<u>100% Soy Control</u>– 400 g of dry soybeans were soaked in deionized (DI) water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool. The beans were manually broken apart to separate the husk of the bean from the inner flesh. The soybeans were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V

(Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

<u>100% Mealworm Control</u> – 400g of frozen mealworms were then blanched in boiling water for 60 seconds. After blanching, the mealworms were allowed to cool for 20 minutes. The mealworms were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler (temp) for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

<u>100% Pupae Control –</u> 400g of frozen pupae were blanched in boiling water for 60 seconds. After blanching, the pupae were allowed to cool for 20 minutes. The pupae were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

<u>50/50 Mealworm Soy Control–</u> 200 g of dry soybeans were soaked in DI water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool. The beans were manually broken apart to separate the husk of the bean from the inner flesh. Then 200g of frozen mealworms were blanched in boiling water for 60 seconds. The mealworms were added to the soybeans and mixed. The soybeans and mealworms were then heated in a dry pan at medium-high heat for five minutes. Following

cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

<u>50/50 Pupae Soy Control –</u> 200 g of dry soybeans were soaked in DI water for 24 hours prior to processing. After the soaking period, the soybeans were boiled for 90 minutes, drained, and allowed to cool for 20 minutes. Once cool. The beans were manually broken apart to separate the husk of the mean from the inner flesh. Then 200g of frozen pupae were blanched in boiling water for 60 seconds. The pupae were added to the soybeans and mixed. The soybeans and pupae were then heated in a dry pan at medium-high heat for five minutes. Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

3.3.4 Sample Cooking and Homogenization

All ground and whole muscle products were collected from various suppliers in Fort Collins, CO to ensure different lot production numbers of ground beef (80% lean, 20% fat), sirloin steaks, Beyond Burger, Impossible Burger, and a mushroom-based alternative protein. All products were cooked in a Rational oven (Model No. SCC WE 61; RATIONAL AG, Landsberg am Lech, Germany) on the dry heat setting at 204˚F with ground products being cooked to an internal temperature of 71˚F and 65˚F for whole muscle cuts. Following cooking, products were chilled in a walk-in cooler (temp) for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

All commercial ground and whole muscle products and commercial soy tempeh products were collected from various suppliers in Fort Collins, CO to ensure different lot production numbers of ground beef (80% lean, 20% fat), sirloin steaks, Beyond Burger, Impossible Burger, and a mushroom-based alternative protein. The remainder of the products were prepared in the lab. All beef and commercial alternative protein products were cooked in a Rational oven (Model No. SCC WE 61; RATIONAL AG, Landsberg am Lech, Germany) on the dry heat setting at 95.6˚C with ground products being cooked to an internal temperature of 21.6˚C and 18.3˚C for whole muscle cuts.

Following cooking, the beef and plant-based meat alternative products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in a Robo Coupe BLITZER 6V (Robot Coupe USA Inc., Ridgeland, MS) blender. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

All tempeh products were cut into 25mm cubes and cooked on each side in a dry pan set to medium-high to an internal temperature of 73 ˚C.

Following cooking, products were chilled in a walk-in cooler for 30 min before being homogenized. The homogenization process consisted of freezing products in liquid nitrogen and immediately homogenizing them in Cuisinart 120V spice grinder model DCG-12BC. The samples were then sealed in vacuum bags and frozen at -2˚C until further processing.

3.3.5 Gastric Digestion

One gram of each sample was added to 10 mL of isotonic saline solution (140nM NaCl and 5mM KCl) and vortexed for ten seconds to homogenize. The pH of the solution was adjusted to 2.0 using HCL (1 M). Then, 0.5 mL of 16mg/mL pepsin was added, and the samples were incubated at 37°C for 75 minutes. After the incubation, peptic digestion was terminated by increasing pH to 5.5 with $NaHCO_3$ (1M). Next, 2.5 mL of 7mg/ml bile-pancreatin was added to the samples and the pH was increased to 7.0 with $NaHCO_3$ (1M) to simulate digestion. The solution was increased to 16 mL using isotonic saline solution and the samples were incubated at 37°C for 120 minutes, after which they were centrifuged at 3000 x g for five minutes. The supernatant was then collected for microwave digestion.

3.3.6 Microwave Digestion

Microwave digestion was conducted utilizing a Titan MPS microwave digester prior to ICP analysis. To determine total ferritin content of the gastric digests, samples were prepared by adding 1 mL of gastric digest supernatant to the microwave vessel with 9 mL of nitric acid. The samples were allowed to sit for 15 minutes to allow for an initial reaction. Then, the samples were placed in the microwave for 1 hour. Once cooled, the samples were decanted into 50 mL conical tubes and diluted to 20 mL with milliQ water. One mL of this was added to a 15 mL conical and further diluted to 15 mL with milliQ water to prepare them for ICP analysis.

3.3.7 Inductively Coupled Plasma and Mass Spectrometry (ICP-MS) Analysis

Microwave digested samples of chemical digesta were analyzed by ICP-MS to determine total micronutrient profiles (whole-product samples) and soluble iron (sample digesta). Elemental iron concentration was measured using a NexION 350D mass spectrometer connected to a PFA-ST nebulizer and a peltier controlled quartz cyclonic spray chamber set to 4°C. Elemental concentrations Fe were measured using a NexION 350D mass spectrometer (PerkinElmer) connected to a Type A quartz MEINHARD® concentric nebulizer and a quartz cyclonic spray chamber. Samples were introduced using a SC-2DX autosampler (ESI). Fe was measured in DRC mode using ammonia as the reactive gas. Before analysis, the torch alignment, nebulizer gas flow and the Quadrupole Ion Deflector (QID) were optimized for maximum indium signal intensity. A daily performance check was also run which ensured that the instrument was operating properly and minimized oxide and doubly charged species formation by obtaining a CeO^+:Ce^+ of <0.025 and a Ba^{++}:Ba of <0.03. A calibration curve was obtained by analyzing 7 dilutions of a multi-element stock solution made from a mixture of single-element stock standards (Inorganic Ventures). To correct for instrument drift, a quality control (QC) solution, which consisted of a pooled digested sample, prepared by mixing 1mL of each digested individual sample, was run every 10th sample (Haugen et al. 2000). Calibration was confirmed by a 7-point curve prepared by serial dilution of commercially available single element standard stock solutions. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3 times or 10 times the standard deviation of the blank divided by the slope of the calibration curve respectively and were subsequently corrected for dilution factor (Broccardo et al., 2021) (Gupta, 2011). Final concentrations are given in ng/mg of digested sample. Measured calculations below the LOD were assigned as <LOD.

3.3.8 Caco-2 Cell Assay

The process from sample preparation to Caco-2 cell assay is illustrated in Figure 2. The cell assay utilized the Caco-2 cell line, which is an immortalized human colonic cell line that can mimic the function of intestinal enterocytes. Cells were grown in tissue culture treated flasks and kept in an incubator at 37°C. and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% minimum essential media (MEM), and 1% penicillin. Confluent cells were trypsinized and sub-cultured onto Transwell inserts that were seeded at a density of 2 x 105 cells/ml. Medium was changed every other day for 21 days until cells established monolayers that functionally mimic intact intestinal epithelia. Once established DMEM was replaced with MEM 24 hours prior to adding sample digests. After 24 hours, old MEM was replaced with fresh MEM. Sixty ug of sirloin, ground beef, Beyond Burger, Impossible burger, soybean control, mealworm control, pupae control, mealworm soy control, and pupae control were added to the cells. Due to the low levels of soluble iron in the soybean tempeh samples, 90 ug of the soy samples were added to the Caco-2 cells. Cells were incubated for 2 hours at 37°C and 5% CO_2 and supplemented with an additional 0.5mL of MEM. Cells were then incubated for 22 hours, after which they were washed with PBS, lysed, centrifuged for 4 minutes at 19,000 x g, and the supernatant was collected for determining ferritin levels using ELISA.

3.3.9 Ferritin Analysis

Sample ferritin content was analyzed utilizing a Human Ferritin ELISA Kit (Sigma-Aldrich Saint Louis, MO) according to manufacturer's instructions. Thirty ug of supernatant from the Caco-2 Cell Assay of each sample was added to each well, and the wells were incubated and run on a BioTek Gen5 Microplate reader and Analysis Software. Ferritin levels were normalized to total protein as previously described (cite Martin and Liz's paper- just got accepted in Frontiers in Nutrition).

3.3.10 Statistical Analysis

Data were statistically analyzed using Graphpad Prism version 9.5.1 (733). Data were assessed via One-way ANOVA and Tukey's multiple comparison analysis. A p-value of

<0.05 was considered statistically significant. P-values of 0.080-0.051 were identified as close to significant, indicating that differences may be revealed with a larger sample set.

3.4 Results

3.4.1 Comparison of soluble iron detected in fermented tempeh products and their controls.

Gastric digests of preparations of soy and mealworm larvae or pupae were compared for levels of soluble iron before (control) and after fermentation (tempeh). A Tukey's multiple comparison analysis of the sample set observed a statistically significant difference in soluble iron content between several of the tempeh samples and their controls. As seen in table 3.2, on average, the mealworm control had 469.7 ng more soluble protein than the soy control (P=0.0003).

Table 3.2: Significant differences from a Tukey's multiple comparison analysis of significantly different soluble iron means compared to the soybean control.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Mealworm Control vs. Soy Control	166.6 to 772.7	469.7	0.0003
100% Pupae Tempeh vs. Soy Control	92.70 to 728.4	410.5	0.0079
50/50 Mealworm Soy Tempeh vs. Soy Control	6.123 to 612.2	309.2	<0.0001
50/50 Pupae Soy Tempeh vs. Soy Control	183.5 to 789.5	486.5	0.0072

In addition, significantly higher levels of soluble iron were observed in the 100% pupae tempeh (P=0.0079), the 50/50 mealworm soy tempeh ($P<0.0001$), and the pupae soy tempeh (P=0.0072), suggesting that the addition of the insect protein may be contributing to higher soluble iron levels than the soybean control. However, we observed no statistical difference between the same samples when compared to the soy tempeh, seen below in table 3.3, with the exception of the 50/50 pupae soy tempeh, which had a mean of 328.5 ng of soluble iron more than the soybean tempeh (p=0.0037).

Table 3.3: Significant differences from a Tukey's multiple comparison analysis of the soluble iron contained in the insect-based tempeh when compared to the soybean control.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Soy Tempeh vs. 100% Mealworm Tempeh	-394.0 to 212.0	-91	0.9731
Soy Tempeh vs. 100% Pupae Tempeh	-570.4 to 65.30	-252.5	0.0845
Soy Tempeh vs. 50/50 Mealworm Soy Tempeh	-454.2 to 151.9	-151.2	0.6378
Soy Tempeh vs. 50/50 Pupae Soy Tempeh	-631.5 to -25.46	-328.5	0.0037
Soy Tempeh vs. Soy Control	-145.0 to 461.0	158	0.5793

Figure 3.1 further illustrates the difference in the amount of soluble iron observed across the control and tempeh samples. It should be noted that while most of the samples clustered together in the testing, there is an outlier of the 50/50 pupae soy tempeh that is higher than the others. The reason for this outlier is unclear.

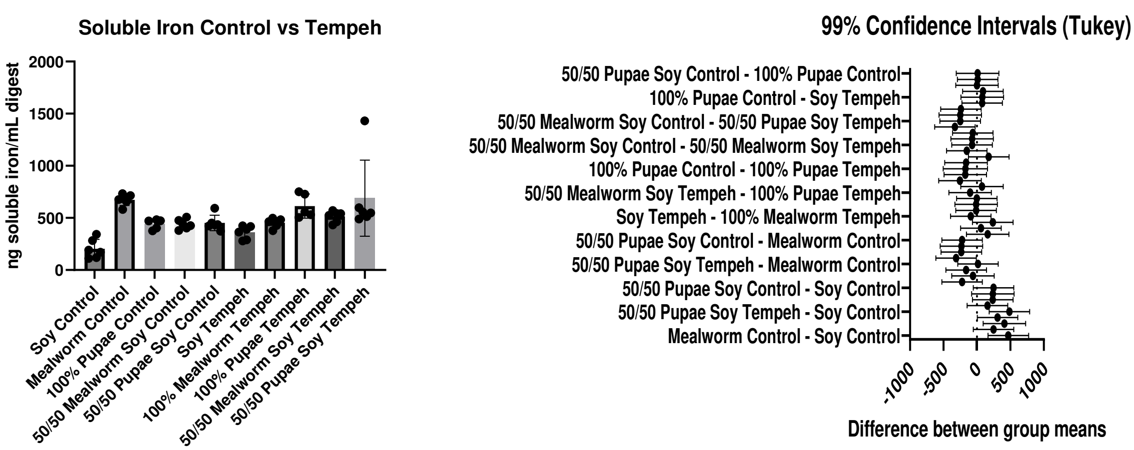
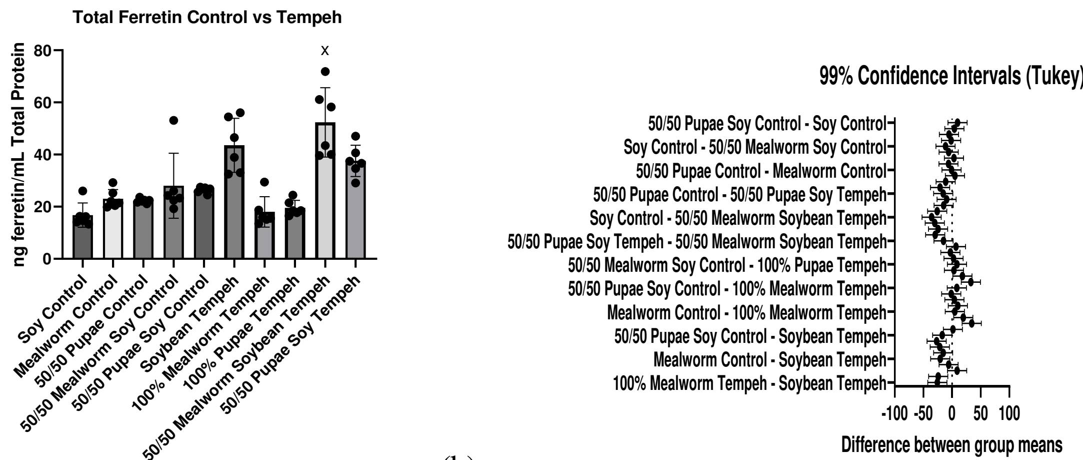


Figure 3.1: A: Multiple Comparison – One-way ANOVA comparing the total Soluble Iron Detected Per Sample between the control samples and the tempeh samples via Inductively Coupled Plasma and Mass Spectrometry (ICP-MS). B: Confidence intervals of soluble iron sample comparison via Tukey multiple comparison analysis.

3.4.2 Comparison of bioavailable iron detected in fermented tempeh products and their controls.

Next, we examined the amount of ferritin/ mg protein absorbed by Caco-2 cells for each of the fermented or unfermented sample preparations. As illustrated in Figure 3.2, ferritin amounts detected appear highest in the 50/50 mealworm soybean tempeh (x) as compared to the other tempehs in the sample set, however, there was no statistical significance found amongst the samples (P>0.05).



(a) (b)

Figure 3.2: One Way ANOVA (a) and Tukey's multiple comparison analysis (b) showing differences in total ferritin content between control groups and fermented tempeh and the confidence intervals of soluble iron sample comparison.

Table 3.4 shows statistically significant differences in bioavailable iron among the tempeh samples when compared to the control. Analysis of the total ferritin content of the control samples vs the tempeh samples revealed improved iron absorption in the soybean tempeh (p<0.0001), 50/50 mealworm soybean tempeh (P<0.0001), and pupae soybean tempeh (P= 0.0005) over the soybean control. In addition, Caco-2 cells absorbed significantly more iron from the 50/50 mealworm soybean tempeh (P<0.001), 50/50 pupae soybean tempeh (P=0.0257), and the soy tempeh (P=0.0006), than their relative controls.

Table 3.4: Significant differences from a Tukey's multiple comparison analysis of the total ferritin absorbed in the insect-based tempeh when compared to the soybean control.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Soy Control vs. Soybean Tempeh	-43.55 to -10.05	-26.8	<0.0001
Soy Control vs. 50/50 Mealworm Soybean Tempeh	-52.35 to -18.85	-35.6	<0.0001
Soy Control vs. 50/50 Pupae Soy Tempeh	-37.55 to -4.054	-20.8	0.0005
Mealworm Control vs. Soybean Tempeh	-37.30 to -3.804	-20.55	0.0006
Mealworm Control vs. 50/50 Mealworm Soybean Tempeh	-46.10 to -12.60	-29.35	<0.0001
Mealworm Control vs. 50/50 Pupae Soy Tempeh	-31.30 to 2.196	-14.55	0.0414
50/50 Pupae Control vs. Soybean Tempeh	-38.06 to -4.570	-21.32	0.0003
50/50 Pupae Control vs. 50/50 Mealworm Soybean Tempeh	-46.86 to -13.37	-30.12	<0.0001
50/50 Pupae Control vs. 50/50 Pupae Soy Tempeh	-32.06 to 1.430	-15.32	0.0257
50/50 Pupae Soy Control vs. 50/50 Mealworm Soybean Tempeh	-42.68 to -9.187	-25.93	<0.0001
50/50 Pupae Soy Control vs. Soybean Tempeh	-33.88 to -0.3869	-17.13	0.0077
50/50 Mealworm Soy Control vs. 50/50 Mealworm Soybean Tempeh	-41.05 to -7.554	-24.3	<0.0001

3.4.3 Comparison of soluble iron detected in fermented tempeh products, conventional beef, and plant-based meat alternatives.

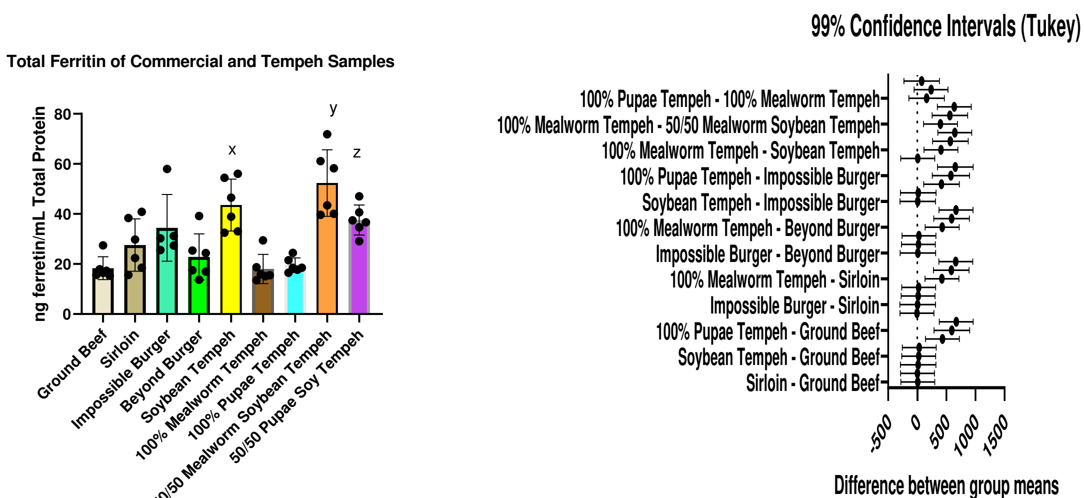
A broader analysis of the soluble iron detected in the tempeh, beef, and plant-based meat alternatives found some significant differences between the samples (Table 3.5).

Table 3.5: Significant differences from a Tukey's multiple comparison analysis of the soluble iron absorbed in the insect-based tempeh when compared to beef and plant-based meat alternatives.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Impossible Burger vs. Ground Beef	-49.84 to 599.2	274.7	0.0488
100% Pupae Tempeh vs. Sirloin	-28.47 to 682.5	327	0.0235
Impossible Burger vs. Beyond Burger	-649.3 to -0.3308	-324.8	0.0099
Soy Tempeh vs. 50/50 Pupae Soy Tempeh	-653.0 to -3.997	-328.5	0.0087
50/50 Mealworm Soybean Tempeh vs. Beyond Burger	-659.5 to -10.50	-335	0.007
100% Pupae Tempeh vs. Ground Beef	25.53 to 706.2	365.9	0.0043
50/50 Pupae Soy Tempeh vs. Sirloin	62.63 to 743.3	403	0.0012
100% Mealworm Tempeh vs. Beyond Burger	-719.7 to -70.66	-395.2	0.0008
50/50 Pupae Soy Tempeh vs. Ground Beef	117.3 to 766.3	441.8	0.0001

The Impossible Burger samples contained significantly more soluble iron than the ground beef samples (P=0.048) but they contained significantly less soluble iron than the Beyond Burger samples (P=0.0099). Amongst the tempeh samples, three pupae-based samples contained significantly more soluble iron than conventional beef products. The 100% pupae sample contained more soluble iron than the sirloin (P=0.0235) and the ground beef samples (P=0.0043). In addition, the 50/50 Pupae Soybean tempeh contained more soluble iron than the ground beef samples (P=0.0001) and the sirloin samples (P=0.0012). When compared to the plant-based meat alternatives, the Beyond Burger samples contained significantly more soluble iron than the 50/50 mealworm soybean tempeh (P=0.007) and the 100% mealworm tempeh (P=0.0008). No significant differences were observed between the other samples within the set (P>0.05).

When comparing iron absorption between the commercial beef and the plant-based meat alternative, and the tempeh samples, significantly more iron was absorbed from the soybean tempeh (x), 50/50 mealworm soybean tempeh (y), and the 50/50 pupae soy tempeh samples (z) as seen in figure 3.3.



(a) (b)

Figure 3.3: One Way ANOVA (a) and Tukey's multiple comparison analysis (b) showing differences in total ferritin content between the commercial products, soybean tempeh, and the 50/50 mealworm soybean tempeh.

3.4.4 Comparison of bioavailable iron detected in fermented tempeh products, conventional beef, and plant-based meat alternatives

Across the samples iron absorption differed, with more iron absorbed from three of the lab-based tempeh samples :the 100% mealworm tempeh, the 100% pupae tempeh, and the 50/50 pupae soy tempeh (Table 3.6).

Table 3.6: Significant differences from a Tukey's multiple comparison analysis of the total ferritin absorbed in the tempeh when compared to the commercial beef and plant-based alternative samples.

Sample Comparison	Confidence Interval	Mean Difference	P Value
100% Mealworm Tempeh vs. Ground Beef	141.9 to 724.8	433.3	<0.0001
100% Mealworm Tempeh vs. Sirloin	132.6 to 715.6	424.1	<0.0001
100% Mealworm Tempeh vs. Impossible Burger	111.5 to 722.9	417.2	0.0001
100% Mealworm Tempeh vs. Beyond Burger	137.4 to 720.3	428.8	<0.0001
100% Mealworm Tempeh vs. Soybean Tempeh	116.7 to 699.6	408.1	<0.0001
100% Mealworm Tempeh vs. 50/50 Mealworm Soybean Tempeh	107.9 to 690.8	399.3	0.0001
100% Pupae Tempeh vs. Ground Beef	289.2 to 900.6	594.9	<0.0001
100% Pupae Tempeh vs. Sirloin	279.9 to 891.3	585.6	<0.0001
100% Pupae Tempeh vs. Impossible Burger	259.5 to 898.0	578.8	<0.0001
100% Pupae Tempeh vs. Beyond Burger	284.7 to 896.0	590.3	<0.0001
100% Pupae Tempeh vs. Soybean Tempeh	264.0 to 875.3	569.7	<0.0001
100% Pupae Tempeh vs. 50/50 Mealworm Soybean Tempeh	255.2 to 866.5	560.9	<0.0001
50/50 Pupae Soy Tempeh vs. Ground Beef	379.4 to 962.3	670.8	<0.0001
50/50 Pupae Soy Tempeh vs. Sirloin	370.1 to 953.1	661.6	<0.0001
50/50 Pupae Soy Tempeh vs. Impossible Burger	349.0 to 960.4	654.7	<0.0001
50/50 Pupae Soy Tempeh vs. Beyond Burger	374.9 to 957.8	666.3	<0.0001
50/50 Pupae Soy Tempeh vs. Soybean Tempeh	354.2 to 937.1	645.6	<0.0001
50/50 Pupae Soy Tempeh vs. 50/50 Mealworm Soybean Tempeh	345.4 to 928.3	636.8	<0.0001

Significantly more iron was absorbed from the 100% mealworm tempeh than all of the commercial beef and plant-based alternative samples ($P<0.0001$). In addition, more iron was absorbed from the 100% mealworm tempeh than the soybean tempeh ($P<0.0001$) and the 50/50 mealworm soybean tempeh ($P=0.0001$). On average, 418 more ng/mg of iron was absorbed from the 100% mealworm tempeh samples than the other samples of significance.

An analysis of the iron absorption of the 100% pupae tempeh revealed significantly more iron absorbed than all the commercial beef and plant-based meat alternatives ($P<0.0001$). In addition, significantly more iron was absorbed from the 100% pupae tempeh than the soybean tempeh and the mealworm soybean tempeh ($P<0.0001$). On average, 580 more ng/mg of iron were absorbed from the 100% pupae tempeh than the other samples of significance. This is an increase of 161 ng/mg over the average iron absorption of the 100% mealworm tempeh when compared to the same samples.

Finally, the 50/50 mealworm soy tempeh provided the most significant amount of iron absorbed across the sample set, providing more absorbed iron than all the commercial beef and plant-based meat alternatives as well as the soybean tempeh and the 50/50 pupae soybean tempeh ($P<0.0001$). With an average of 655 ng/mg of iron absorbed, this is 75 ng/mg more absorption than the 100% pupae tempeh and 237 ng/mg more than the 100% mealworm tempeh when compared to the same samples.

3.5 Discussion

Overall, three factors seem to affect both the total soluble iron and bioavailability of the iron within a sample and the amount of iron absorption from each sample: the presence of soybeans, the presence of insect protein, and whether the samples have been fermented. When compared to the soy control, four samples contained significantly higher amounts of soluble iron: mealworm control ($P=0.0003$), pupae tempeh ($P=0.0079$), 50/50 mealworm soybean tempeh ($P<0.0001$) and the 50/50 pupae soy tempeh ($P=0.0072$). The increased amounts of iron in the mealworm control suggests that insect protein may contain more soluble iron than soybeans alone. The increased iron detected in the mixed insect/soy tempeh also suggests that the addition of insect protein in the fermented product may increase the overall soluble iron content. However, only the 50/50 mealworm soy tempeh demonstrated more soluble iron than the soy tempeh. This suggests that this life stage of the *T. molitor* beetle may be a richer source of soluble iron than the other tempehs made from the *T. molitor* pupae or soybeans alone. These effects appear to be cumulative as there a significant difference between the 50/50 mealworm soybean tempeh and 100% mealworm tempeh ($P<0.0001$), suggesting that the fermented soybeans still contribute a significant amount of iron to the overall product, despite the difference in pupae content.

When compared to the commercial beef samples, The 100% pupae tempeh contained significantly more soluble iron than the ground beef (P=0.0043) and the sirloin samples (P=0.0235). In addition, the 50/50 pupae soy tempeh contained significantly more soluble iron than the both the ground beef (P=0.0001) and sirloin samples (P=0.0012), giving further evidence that the pupal stage of *T. molitor* may contain more soluble iron that the other life stages of the beetle. It may also suggest that the fermentation process may help increase the amount of soluble iron not just in the samples containing soy, but the insect-based samples as well, but further research is needed to confirm.

Despite the significant amounts of soluble iron detected in the fermented pupae samples, there was no significant difference detected between the soluble iron detected in the plant-based meat alternative samples and the fermented pupae samples. However, we did observe significantly more iron in the Beyond Burger samples than the 100% mealworm tempeh (P=0.0008) and the 50/50 mealworm soybean tempeh (P=0.0007). Two factors may account for the differences here. First, this may by confirmation that the pupal stage of *T. Molitor* may contain more iron than the larval stage, though there was no significant difference found between the pupal and larval control samples (P>0.05). Another factor may be the ability of the manufacturer to add exogenous iron to the Beyond Burger product, increasing the overall soluble iron content within the product.

Soybeans are a rich source of iron, but phytates and polyphenols within the soybeans may inhibit the solubility and bioavailability of the iron. When compared against the soybean control, the soybean tempeh had significantly more iron absorbed (P<0.0001). This agrees with previous research indicating that fermentation by *Rhizopus oligosporus* may reduce the chelating agents in soybeans, allowing the iron to be more bioavailable (SUTARDI and BUCKLE, 1985; Fekadu

Gemede, 2014). While the amount of bioavailable iron detected in the soy control was also significantly less than the 50/50 mealworm soybean tempeh ($P<0.0001$) and the pupae soy tempeh ($P=0.0005$), the soy tempeh had significantly more iron absorption than the mealworm control ($P=0.00060$), pupae control ($P=0.0003$), and the 50/50 pupae soy control ($P=0.0077$), suggesting that the addition of insect protein alone is not enough to increase the amount of iron absorbed from the sample.

When compared to the commercial beef and plant-based alternative samples, the Caco-2 cells absorbed significantly more iron from three of the tempeh samples than all the other commercial samples: 100% mealworm tempeh, 100% pupae tempeh, and 50/50 pupae soy tempeh. On average, the Caco-2 cells absorbed 418 ng/mg more iron from the 100% mealworm tempeh than the other commercial beef and plant-base meat alternatives ($P<0.0001$). That number increases to 580 ng/mg more iron absorbed than the commercial samples in the 100% pupae tempeh, giving more evidence to the possibility that the pupae contain more iron than the larval stage of *T. molitor*. It also suggests that the iron contained may be more bioavailable than the T. molitor larvae; however, no significant difference in iron absorption was observed between the 100% mealworm tempeh sample and the 100% pupae tempeh sample ($P>0.05$). In addition, there was also no significant difference in the amount of iron absorbed from the 100% mealworm control and the 100% pupae control ($P>0.05$)

The average amount of iron absorbed increases again with the 50/50 pupae soy tempeh, which absorbed 655 ng/mg more iron that the commercial samples ($P<0.0001$). This is 75 ng/mg more than the 100% pupae tempeh and 237 ng/mg more than the 100% mealworm tempeh. While the pupae appear to be the richest insect-based source of iron within the sample set, the addition of soybeans to the ferments also helps increase the overall amount of bioavailable iron, confirming the assertion that the combination of pupal insect protein, soybeans, and *Rhizopus oligosporus* fermentation provides the most bioavailable iron within the sample set.

Given the disparities in iron uptake, it appears that the potential presence of anti-nutrient factors like phytates, oxalates, and polyphenols in the plant-based meat alternative samples may influence the bioavailability of the ferritin in the samples when compared to three of the tempeh samples. In addition, the fortification of iron in the plant-based alternatives does not increase the cells' ability to utilize the iron in solution, suggesting that the iron content in these products may be less bioavailable, possibly due to potential antagonistic compounds endogenous to the products. Even with the differences in iron uptake between samples, the evidence suggests that all samples in the set can be considered high sources of iron as defined by the FDA.

3.6 Conclusion

The perception that plant-based meat alternatives are healthier than conventional animal protein is driving the growing popularity of these products. The value of this market is expected to grow to $30.92 billion by the year 2026 (Zhao et al., 2023). While plant-based startups like Beyond Meats and Impossible Foods have been leading the industry in the development of plant-based meat alternatives, conventional meat producers including Cargill, JBS, and Tyson are also developing their own products to compete for a share of the market. These products are being formulated to reflect the nutrient composition of conventional meat products, especially in the areas of protein and iron content (Hurrell and Egli, 2010a). The insect-based products evaluated in this study may exist in a space between conventional meat products and plant-based meat alternatives. To meet the nutritional needs of the consumer, it is important to ensure that these products can provide comparable amounts of nutrition as their commercial counterparts. Our research indicates that at least three of the products, 100% mealworm tempeh, 100% pupae tempeh, and 50/50 mealworm soy tempeh are significantly richer in iron than the conventional beef and the plant-based meat alternatives from Beyond Burger and Impossible Meats, and consumers looking to increase their iron intake may look to these products for better iron absorption.

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Chapter 4: Assessing the Consumer Acceptability of Tempeh Containing Mealworm Larvae

4.1 Summary

Edible insect protein has been identified as a possible new animal protein source to help meet the demand of the growing population. Consumer acceptance of edible insects in the West is often hampered by neophobia and disgust. To promote the acceptance of edible insect protein in the global West, it is important to develop new products that present insect protein in a form that is familiar and acceptable to consumers, for example burgers, nuggets, or strips. This project seeks to evaluate the consumer acceptability of an insect and soy-based tempeh product utilizing mealworms, the larval stage of the *Tenebrio molitor* beetle, fermented with *Rhizopus oligosporus*. To assess this, we used an online survey distributed through social media to assess the general perception of entomophagy, or insect-eating, in the general public. We then developed a lexicon for the product description with a trained sensory panel. Finally, we conducted a consumer acceptability study with 40 untrained participants to assess the organoleptic potential of the product when compared to a traditional soy-based tempeh.

We determined that knowledge of insect eating was widespread amongst the sample population, with many of the respondents having previously engaged in insect eating. Vegans and vegetarians were also willing to consume insects, as well as respondents who follow spiritual food practices including kosher and halal traditions. In the untrained assessment, the soy tempeh was preferred over the 50/50 mealworm soybean tempeh, but the majority of respondents reported being more likely to try additional food products containing insect protein.

4.2 Introduction

Developing novel foods is an essential process in the food industry. New foods, such as novel sourcing of proteins, are key to the sustainability of the food system as they relieve the pressures on currently available foods while meeting the nutritional and cultural niches of the consumer (Siddiqui et al., 2022). In the development of novel foods, consumer acceptance is a significant hurtle that must be assessed. Product developers must consider the cultural mores, religious pressures, and consumer perspectives when developing and marketing new products. For a new product to be successful, it must offer a desirable flavor, convenience of use to the consumer, and properties that are beneficial to consumer health. Often, there is a distrust and rejection of new products, particularly if the consumers don’t understand the technology involved in the manufacture of that product or are not familiar with a products constituent ingredients (Albertsen et al., 2020).

As the global population increases, the demand of animal protein is also on the rise. To meet this demand, it is important to identify new sources of sustainable protein that minimize the environmental impact of increased production. Edible insects may meet that demand. Around the world, more than 2 billon people eat roughly 2,000 different species of insects. With the majority of these insect eaters being in developing countries, the practice of insect eating has yet to become mainstream in the Global West (Alhujaili et al., 2023). This does not mean that a shift in cultural tastes is not possible. At one time, eating raw fish was considered taboo in the United States. Now, sushi is a popular and acceptable food across the country, largely due to the efforts of scientists and politicians that promoted the practice (Shelomi, 2015).

4.2.1 Drivers for Consumer Acceptance

A variety of factors influence whether a product will be accepted by a target consumer group. Consumers often have limited knowledge about food products, particularly when it comes to nutrition, environmental impact of the food, and the process involved in manufacturing that food (Onwezen et al., 2019). Food is often shared by consumers through social media. While manufacturers can lead a social media marketing campaign, consumer sharing can provide confusing or misleading information that may lead to rejection of novel products. This was observed during the COVID-19 pandemic, where consumers increasingly relied on social media for information (Siddiqui et al., 2022). In addition, food processing techniques meant to extend the shelf life and safety of the product may also affect consumer perception of a product. Irradiation or food additives, which are often perceived as unnatural, may improve the overall quality of the product, but can face rejection by the consumer (Bearth & Siegrist, 2019).

Nutrition is also a significant driver for consumer acceptance. As consumer understanding around the importance of nutrition increases, the demand for nutritionally dense food has also increased (Siró et al., 2008). That being said, consumers sometimes find it difficult to sacrifice the hedonic aspects of food for improved nutrition (Bolha et al., 2020). This suggests that care needs to be taken to preserve the organoleptic expectations of consumers when improving the nutritional content of novel foods.

While research on insect acceptance often cites food neophobia, or fear of new foods, as a cause for rejection of insects as a food source, other research has concluded that it is not the fear of new foods that is a hurtle to insect eating but a fear of insects in general (Moruzzo et al., 2021). In the Western world, insects are often seen as dirty or unhygienic. They are vectors for disease and are often viewed with distrust (Onwezen et al., 2019). These ideas were reinforced in popular media in the United States with shows like Fear Factor and Survivor targeting the notion of disgust while encouraging participants to eat insects on national television.

The notion of disgust is important when developing novel insect-based foods for populations that do not traditionally eat insects. The drivers for disgust can vary. Factors like food abundance, diversity of available foods, perceived safety, and moral norms can all affect the notion of disgust when faced with a novel insect-based product. Current research has shown that consumers are more likely to engage in insect eating if they perceive the insects as tasty and culturally acceptable. In addition, the visibility of the insects within the food product is an important factor for acceptance, with most consumers preferring not to have the insects visible when choosing to debut (Onwezen et al., 2019). While disgust plays an important role in food choice, there are aspects of the reaction that are learned through cultural norms, social pressures, and exposure. Research has shown that conducting events that feature edible insects to novel populations helps combat the perception that insects are dirty and dangerous and facilitate the acceptance of insect eating (Looy & Wood, 2006).

Environmental impact may also play a role in consumers' willingness to engage in insect eating. For many consumers, new products can be received with suspicion, as they are unsure of the long-term environmental impacts of those products, particularly if the sustainability cannot be verified by the target market (Albertsen et al., 2020). The rearing of insect protein has a lower impact on the environment when compared to conventional beef and chicken production which produce 89% more greenhouse gasses than operations rearing insects like crickets (Halloran et al., 2017). In addition, insect rearing requires less space, less water, and fewer waste stream management systems to produce than conventional meat rearing operations. As a result, some consumers may be more willing to debut insect eating if they believe it will reduce the environmental pressures of the current food system (Michel & Begho, 2023).

Currently, there are two main strategies to encourage consumers to adopt insect eating as a regular part of their diet: Sensorial-focused strategies and Marketing and Education- focused strategies. Sensorial-focused strategies are designed to maximize the hedonistic experience of eating insects. These strategies focus on the eating experience, maximizing the flavor, aroma, and mouthfeel of an edible insect product to provide the most positive debut experience for the consumer (Kauppi et al., 2019). This approach targets food acceptance drivers like the overall enjoyment of food, the universal attraction to sweetness, and cultural standards of the target consumer to develop the demand for edible insects (Deroy et al., 2015).

Marketing and education-focused strategies focus on providing information on the environmental impact, nutrition, and cultural norms around insect consumption (Kauppi et al., 2019). Previous studies have indicated that consumers who are informed about the safety and environmental impact of adopting entomophagy respond more favorably to insect-based burgers than those who did not receive the same information (Schouteten et al., 2016). Other research suggests that connecting insect products to foods already acceptable to a community may facilitate consumer acceptance. One researcher suggested that insects can be promoted as a nut alternative as they have similar texture, flavor, and nutritional qualities to nuts that are commonly consumed (Shelomi, 2015). Target marketing can also be advantageous when encouraging people to eat insects. Insects can be marketed as natural foods, targeting consumers who have a distrust of foods perceived to be highly processed. Due to their high protein and low carbohydrate content, insects can also be marketed to bodybuilders who are following the paleo diet trend to help increase acceptance(Ramos-Elorduy, 2009) (Barska, 2014).

This project seeks to examine both the Sensorial-focused and the Marketing and education-focused approaches to determine how to maximize consumer acceptance of edible insect protein. By including the edible insect protein in a versatile food product like tempeh, we can then convert it into a variety of food products that are familiar to the target population, can be flavored to suit the palates of the target audience, and meet the organoleptic expectations of the consumer. To evaluate this, we conducted an online survey to assess the population's exposure to the concept of entomophagy, or insect eating. We made a tempeh made with 50% soybeans and 50% mealworm larvae that was fermented with *Rhizopus oligosporus* and used a trained sensory panel to develop a lexicon describing the organoleptic attributes of that tempeh product. Finally, we conducted an in-person sensory study to evaluate the product's potential for consumer acceptance.

4.3 Methods and Materials

4.3.1Trained Panel Sensory Analysis

A sensory evaluation of the commercial soy and the 50/50 mealworm soybean tempehs was conducted with 6 trained sensory panelists. Both tempeh samples were cubed into 25mm pieces, lightly salted, and fried in canola oil on all sides prior to the evaluation. The trained panelists evaluated the products based on visual appeal, aroma, taste, mouthfeel, and overall appeal. The panel then developed a lexicon that was used during the untrained sensory evaluation (Appendix A).

4.3.2 Electronic Consumer Perception Survey

An Institutional Review Board (IRB) - approved online consumer survey was conducted to gauge consumer perception of edible insects. Participants aged 16 and older were the target of the survey. The survey was released via social media, and responses were collected for 48 hours. The questions for the survey can be seen in Appendix B.

4.3.3 50/50 Mealworm Soybean Tempeh Production

The 50/50 mealworm soybean tempeh for this study was prepared as described in Chapter 2: Nutritional Analysis of Tempeh Products Using *Tenebrio molitor* as a Substrate.

4.3.4 Untrained Sensory Evaluation

Participants were recruited online and in-person for an unspecified "new product taste test" to reduce the potential for self-selection for edible insects. The criteria for participation were adults 18 years or older with no known food allergies. Participants were gathered in a classroom separate from the sensory kitchen. Written consent was obtained, and the study was conducted in accordance with the Declaration of Helsinki under IRB Protocol #4056. After consent forms were signed, they were introduced to the food product being tested. Participants were then notified that they could cease participation in the study at any time, and anyone wishing to stop participation would be asked to fill out a survey describing the reasons why they chose not to continue (Appendix D).

If participants decided to continue with the study, they were provided one of two prompts. The first prompt provided information on traditional soy-based tempeh, how it is made, and the nutritional information of the traditional product. The second prompt provided all of the above information and included information on the environmental impact of conventional beef production and how mealworm production can reduce the environmental impacts of commercial protein (Appendix C). Next, participants were asked to fill out the same consumer perception study that was utilized during the electronic survey (Appendix A). Once that survey was complete, participants were prompted to try the commercial soy tempeh sample. The commercial soy tempeh was cut into 25mm cubes, lightly salted, and fried on all sides in canola oil. Once the panelists tried the soy tempeh, they were prompted to evaluate the tempeh based on appearance, aroma, taste, mouthfeel, and overall appeal, using the lexicon developed by the trained sensory panel as a reference. Participants were also asked questions around their willingness to purchase and consume the soy product.

After the evaluation of the soy product, consumers were prompted to request the sample of 50/50 mealworm soybean tempeh. The mealworm soybean tempeh was prepared in an identical manner as the commercial soybean tempeh. Participants were once again prompted to evaluate the tempeh based on appearance, aroma, taste, mouthfeel, and overall appeal and asked questions around their willingness to purchase and consume the soy product. Once sensory evaluation of both samples was complete, participants provided information on tempeh preference and their willingness to try other insect-based food products in the future. A total of 40 people participated in the untrained sensory evaluation.

4.3.5 Statistical Analysis

Data were statistically analyzed using Graphpad Prism version 9.5.1 (733). Data were assessed via One-way ANOVA and Mixed model Tukey's multiple comparison analysis. A p-value of <0.05 was considered statistically significant. P-values of 0.080-0.051 were identified as close to significant, indicating that differences may be revealed with a larger sample set. Frequency charts and Principal Component Analysis were also produced using Graphpad Prism version 9.5.1 (773). Averages and standard deviations were calculated via Microsoft Excel. Pie charts and radar plots were also produced via Microsoft excel.

4.4 Results

4.4.1 Trained Sensory Analysis

During the visual analysis of the 50/50 mealworm soybean tempeh, the trained panel observed that the tempeh appeared dry and oily with clearly visible insect pieces. The appearance of the insects was noted as off putting by four of the six panelists. The panel agreed that reducing the visibility of the insects would make the product more appealing overall.

Evaluation of the aroma led to a variety of feedback. While the aromatic attributes described in Table 4.1 represent the consensus of the panel, there was some disagreement on the attributes including peanut butter, coffee, and popcorn.

Table 4.1: Lexicon of aromatic attributes identified by the trained sensor panel.

Aroma		
Roasty	Musty	Walnut
Savory	Vegetal	Corn
Grainy	Peanut Butter	Earthy
Nutty	Toasted Sesame	Soil
Umami	Tahini	Nutritional Yeast
Soy Sauce	Grain	Popcorn
Coffee	Bean	Dry Leaves

The mouthfeel of the product (Table 4.2) was universally agreed upon by the trained panelists, with crunchy, crumbly, and chewy identified strongly amongst participants.

Table 4.2: Lexicon of mouthfeel attributes identified by the trained sensor panel.

Mouthfeel		
Crunchy	Dry	Moist
Crumbly	Soft	Chewy

As with the mouthfeel, the panel was unanimous with the assessment of the flavor of the

product (Table 4.3) with salty and bitter being most strongly identified by the group.

Table 4.3: Lexicon of flavor attributes identified by the trained sensor panel.

Flavor		
Bitter	Acidic	Sour
Salty	Sweet	

4.4.2 Online Survey

During the initial response of the online survey, we received over 1,000 electronic responses. However, an analysis of the responses indicated that the survey system may have been affected by bots. Responses that had answers that were provided in strange symbols, appeared in the same language numerous times, or did not answer the questions in a manner that reflect human responses were eliminated. As a result, we were left with 369 viable responses to the online survey.

Even with the elimination of a large number of responses, we were still able to capture a diverse number of respondents, with a wide range of age, education, and income, whose demographics are represented in Figure 4.1. Respondent reported 27 different nationalities, 9 ethnicities. Of the respondents, 38% reported adhering to some food restrictions. Figure 4.2 shows that 10% of respondents reported being vegetarian, 6% reported being vegan, 10% reported pescatarian with another 11% adhering to halal or kosher rules.

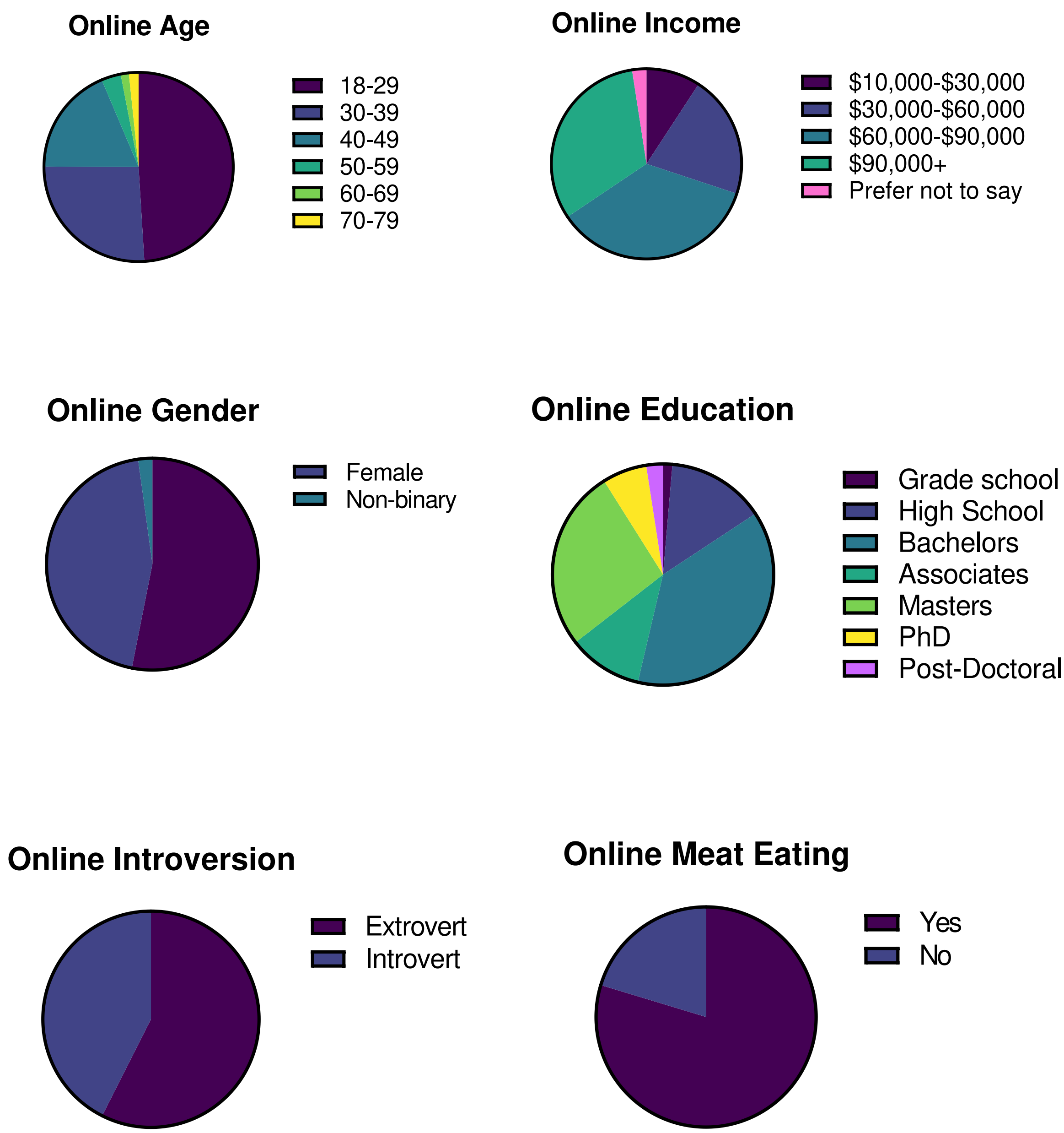

Figure 4.1: Demographic data of the online respondents

Within the responding group, tempeh appears to be well known with 76% of the respondents having knowledge of the product, and 64% having tried it. In addition, tempeh appears to have a visible presence in the places where the respondents live with 65% reporting seeing tempeh in their local grocery stores. Edible insects also appear to be well-known among the respondents with 79% reporting having seen insects as food, and 58% reporting insects being eaten in the home countries. Insects reported observed as food include cicadas, grasshoppers, ants, crickets, centipedes, locusts, cockroaches, silkworms, mealworms, scorpions, spiders, and termites. They were prepared fresh, fried, dried, in a beverage, and in a candy.

Within the sample set, participants reported a broad array of food restrictions (Figure 4.2). 62% of the respondents reported no food restrictions with vegetarians (10%), pescatarians (10%) and vegans (6%) making up the remaining non-religion specific restrictions.

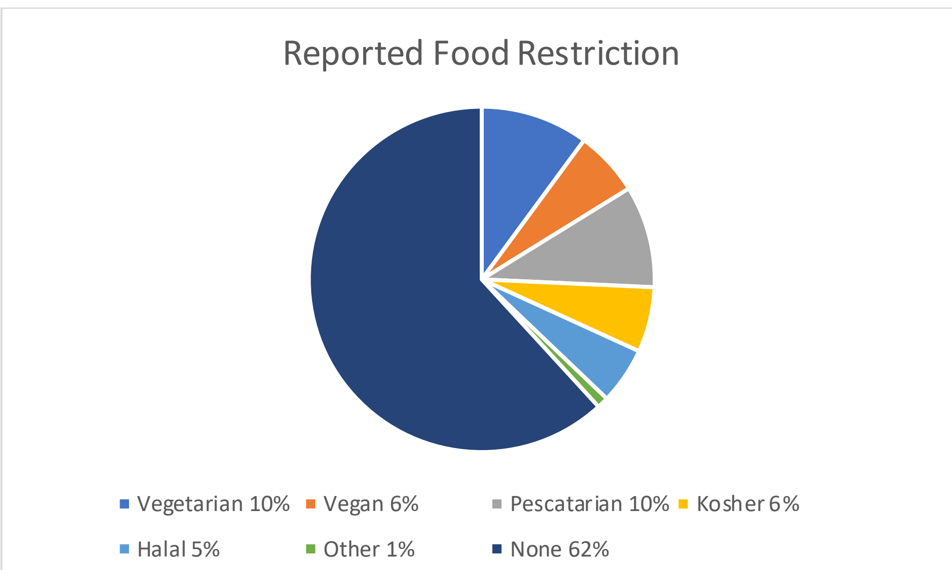

Figure 4.2: Reported food restriction of the respondents.

Among the respondents, 50% reported prior consumption of insects. Among that group, 45% of the vegetarians, 43% of the vegans, and 33% of the pescatarians reported eating insects sometime in the past (Figure 4.3).

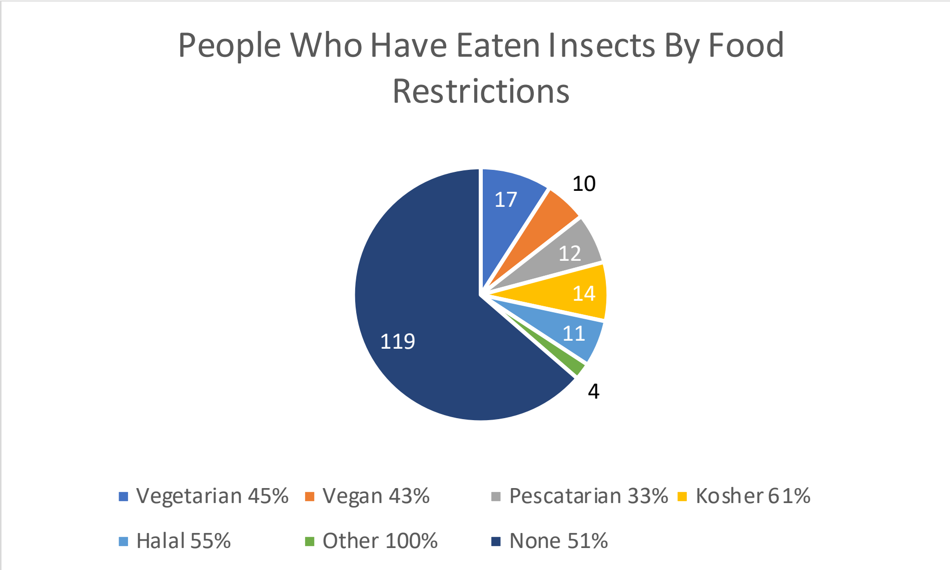

Figure 4.3: Respondents who have reported prior consumption of insects broken down by food restriction.

Among the vegans and vegetarians who reported never eating insects, 54% of the vegans and 67% of the vegetarians reported a willingness to try foods containing edible insects.

When reporting on the likelihood of respondents to engage with edible insects, the responses seem evenly distributed in Figure 4.4. however, there were some responses of significance.

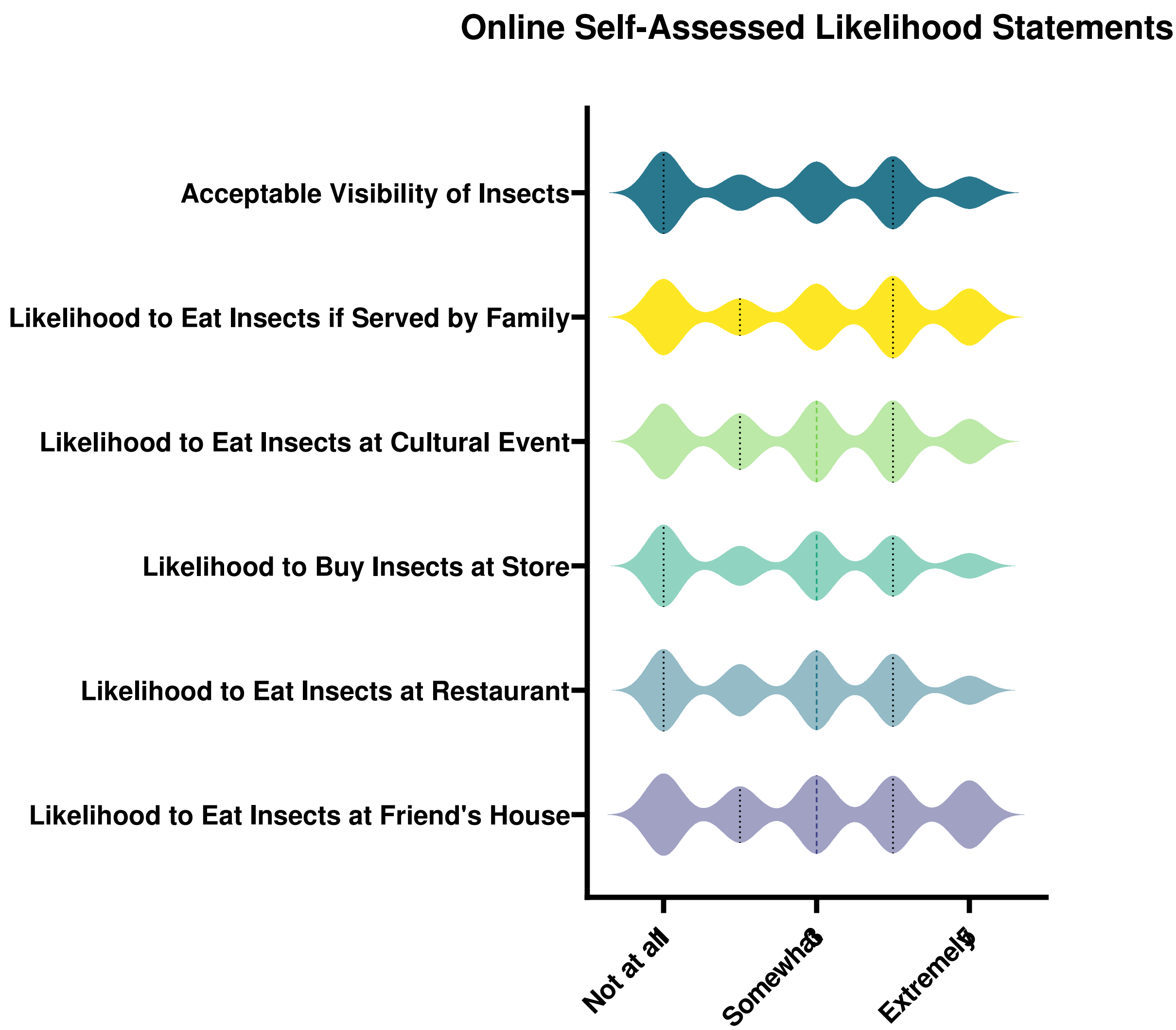

Figure 4.4: Likelihood of respondents to engage with edible insects

Respondents appeared to weigh close social interactions when deciding to eat insects. Respondents were significantly more likely to eat insects if served by a family member than buy them in a store (P= 0.0069) or order them in a restaurant (P=0.0455). In addition, respondents were significantly more likely to eat insects served at a friend's house than purchase insects at the store for personal consumption (P=0.0268).

4.4.3 In Person Sensory Evaluation

The in-person sensory evaluation involved 40 untrained participants recruited from the public, 20 of which received the first prompt (prompt A) providing details around tempeh production and nutrition, and 20 who received the second prompt (prompt B) which included all the information included in prompt A with the addition of environmental considerations around conventional meat production versus the production of mealworm larvae for protein. The participants included 16 males, 33 females, and 1 individual who preferred not to identify their gender. The sample group includes 10 nationalities, six self-identified ethnic groups, and twenty separate cultural heritages. The ages ranged from 19 to 71 with levels of education from associates degree to PhD. While Christians represented the majority of the participants, the samples set was also comprised with Atheists, Agnostics, Buddhists, and Hindus. Several participants also declined to identify their spiritual traditions. Ninety-five percent of participants reported traveling outside of their home countries, with Europe, at 63%, being the continent most visited. While most of the participants have traveled abroad, only 45% of them have lived outside of their home country.

The normal food patterns among the participants varied. While 95% identified themselves as meat eaters, the study did include two vegetarians, one pescatarian, and one vegan. Pork and chicken were the meats most commonly consumed by the sample set. One participant reported adherence to halal rules of eating and several others reported excluding pork and beef from their diets due to cultural and spiritual considerations.

Knowledge of insect eating seemed to vary among participants. While 80% of the participants reported having seen insects as food products, only 23% reported insects being eaten in their home countries. Of the observed insect-based food products reported, processing included fresh, powdered, fried, in a beverage, in a prepared dish, dried, candied, or in some other preparation. In addition, 55% of the participants reported having previously eaten a variety of insects including ants, grasshoppers, cicada chrysalis, crickets, mealworms, scorpions, and termites. The most common preparation experienced for these insects was dried.

Further exploration around the drivers of food choice indicated variation in the importance. As seen in Figure 4.5, spirituality was the least important factor for food choices among the participants, followed closely by cultural considerations.

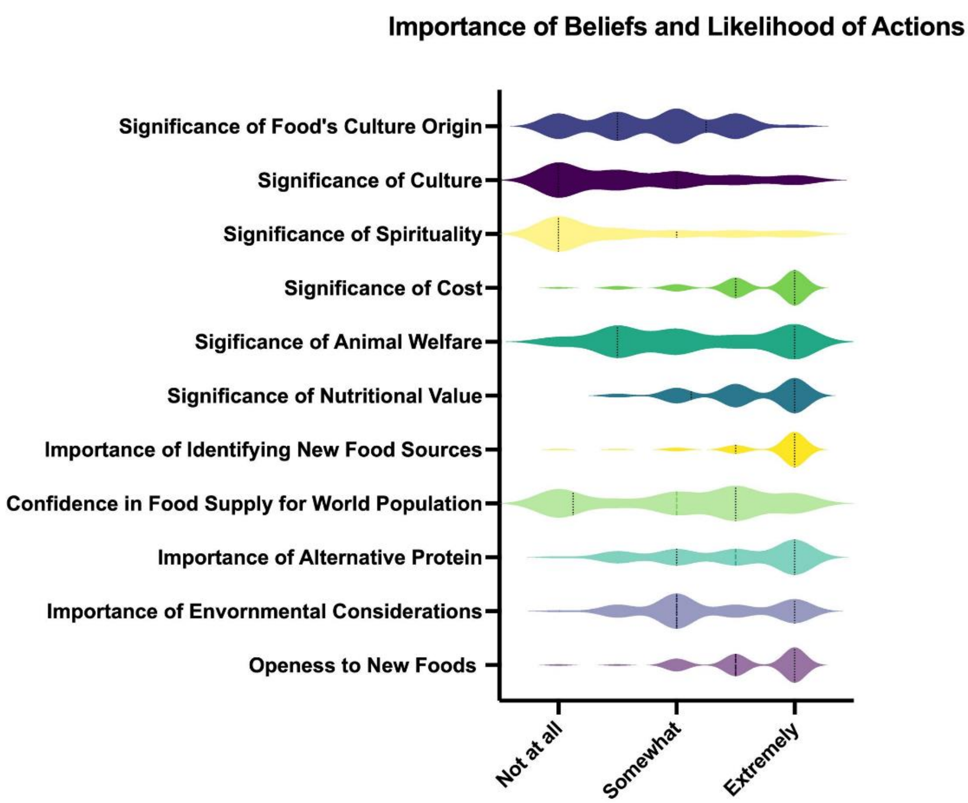

Figure 4.5: Ranked importance of beliefs when making food choices.

The most reported considerations to food choice were cost and nutritional content, but no significance for nutrition could be determined within the sample set ($P>0.05$). As seen in table 4.4, cost was a more significant consideration than people's confidence in the food supply ($P=0.0248$).

Table 4.4: Significant differences from a Mixed Effects Tukey's multiple comparison analysis of significantly different personal values and beliefs when it comes to food choice.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Importance of alternative proteins vs identifying new food sources	-1.280 to -0.01979	-0.65	0.0383
Sigificance of Animal Welfare vs. Significance of Spirituality	0.03820 to 2.247	1.143	0.0375
Nutitional value vs Animal Welfare	0.06640 to 1.678	0.8722	0.0248
Confidence in food suppy vs. Significance of cost	-2.182 to -0.1005	-1.141	0.0218
Openess to New Foods vs. Sigificance of Animal Welfare	0.1444 to 1.650	0.8972	0.0092
Openess to new foods vs confidence in food supply	0.2113 to 1.989	1.1	0.0057
Confidence in food supply vs impotance of identifying new foods	-1.943 to -0.2068	-1.075	0.0057
Environmental considerations vs Significance of food's cultural origins	0.1883 to 1.622	0.9054	0.0047
Environmental considerations vs identifying new food souces	-1.764 to -0.2362	-1	0.0028
Importance of alternative proteins vs Significance of food's cultural origins	0.3737 to 2.137	1.255	0.001
Identifying new food sources vs Animal welfare	0.3631 to 2.081	1.222	0.001
Confidence in food supply vs importance in idnentyfing new foods	-2.425 to -0.4247	-1.425	0.0009
Environmental Considerations vs Signnificance of Perosnal Culture	0.3569 to 1.940	1.149	0.0007
Importance of Alternative Proteins vs Significance of Personal Culture	0.4659 to 2.531	1.499	0.0007

Environmental and animal welfare considerations varied among the sample set. While environmental considerations were significantly more important to participants than the cultural origin of the food (P=0.0047), they were significantly less important than the need to identify new food sources for the growing population (P=0.0028). suggesting that there is a limit to the amount of money the participants were willing to spend to relieve the environmental pressures and moral implications around conventional food production. Despite the main drivers of cost and nutrition when making food choices, the majority of participants reported being open to trying new foods, with openness to new foods being significantly more important than animal welfare (P=0.0092) and confidence in the food supply (P=0.0057). This corresponds with the importance of identifying new food sources, which was significantly more important than animal welfare (P=0.001).

With 95% of the participants reported as meat eaters, 82% reported having tried plant-based meat alternatives. Curiosity was the main driver for trying these alternative protein sources with intermittent periods of vegetarianism and veganism following closely behind. While

88% of the participants were aware of tempeh as an alternative proteins source, only 68% of them had tried it.

As can be seen inf figure 4.6, although curiosity prompted the panelists to try alternative protein sources, the most significant choice amongst the sample set was still conventional meat products (P<0.0001). For the population that did consume plant-based meat alternatives, other plant-based alternatives were chosen at significantly higher rates than tempeh (P<0.0001).

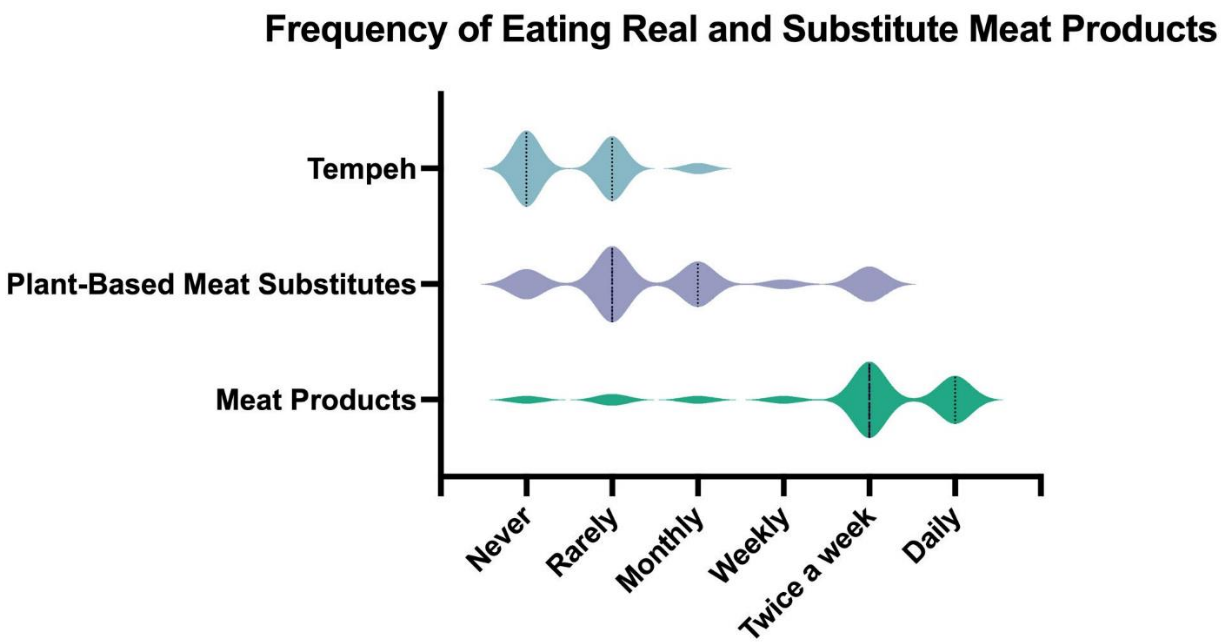

Figure 4.6 Frequency of alternative protein consumption by sensory panel

In the self-reporting of likelihood to make food choices under varying conditions, we observed no significant likelihood to restrict diet based on environmental conditions (P>0.05). As seen in Figure 4.7, participants' willingness to make food under prescribed conditions varied across the sample set.

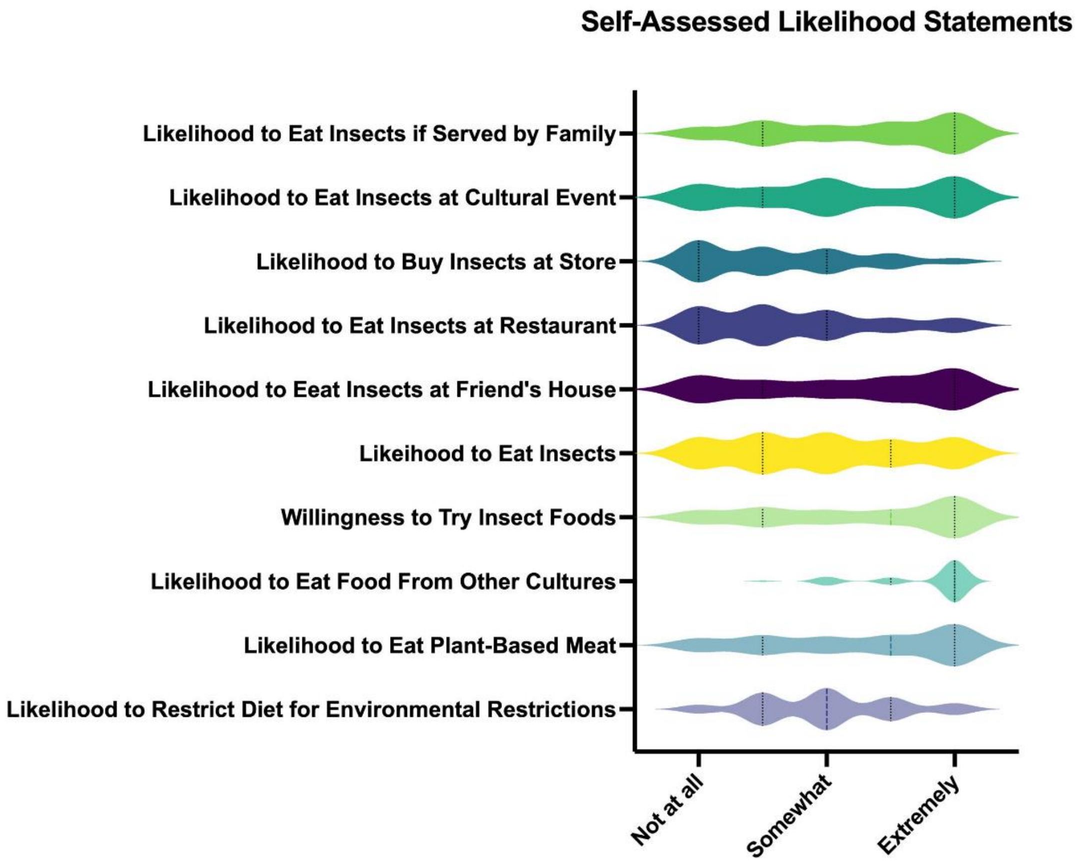

Figure 4.7: Frequency of self-assessed likelihood statements around food choices.

Drivers for participant's likelihood to try insects under different circumstances are illustrated in table 4.5.

Table 4.5: Significant differences from a Mixed Effects Tukey's multiple comparison analysis of significantly different self-assessed likelihood statements when around food choice.

Sample Comparison	Confidence Interval	Mean Difference	P Value
Restrict diet for environmental considerations vs. buy Insects at a store	-0.07160 to 1.427	0.6777	0.1046
Likelihood to eat insects at a friend's house vs. likelihood to buy insects at a store	0.07917 to 1.488	0.7838	0.0194
Likelihood to eat insects vs. likelihood to eat insects in a restaurant	0.05414 to 0.8150	0.4346	0.0148
Willingness to try insects vs. likelihood to eat insects	0.08825 to 1.043	0.5654	0.0101
Likelihood to eat insects vs. likelihood to buy insects at a store	0.1346 to 1.113	0.6238	0.0044
Likelihood to eat plant-based meat vs. likelihood to eat insects in a restaurant	0.2408 to 1.786	1.014	0.0031
Likelihood to eat insects in a restaurant vs. likelihood to eat insects at a cultural event	-1.239 to -0.1666	-0.7027	0.0031
Likelihood to eat insects vs likelihood to eat insects served by family members	-0.9409 to -0.1359	-0.5384	0.0024
Likelihood to eat plant-based meat vs. likelihood to eat foods from other cultures	-1.740 to -0.2600	-1	0.002
Likelihood to eat insects at a friend's house vs. likelihood to buy insects at a store	0.2763 to 1.670	0.973	0.0014
Likelihood to eat foods from other cultures vs. likelihood to eat insects served by family members	0.3515 to 1.730	1.041	0.0004
Likelihood to eat foods from other cultures vs willingness to try insects	0.3544 to 1.673	1.014	0.0003
Likelihood to eat foods from other cultures vs. llikelihood to eat insects at a friend's house	0.4360 to 2.023	1.23	0.0003
Likelihood to eat plant-based meats vs. likelihood to buy insects at the store	0.4459 to 1.960	1.203	0.0002

While participants were significantly more likely to eat a plant-based meat alternative than to buy insects at the store to eat (P=0.00020), overall, participants were significantly more willing to try insects than they were to make insect eating a regular part of their diets (P=0.0101). While participants were significantly more likely to eat insects than to order them in a restaurant (P=0.0148) or buy them in a store (P=0.0014), social consideration appear to be an important driver in the participants' willingness to engage in entomophagy. Participants were significantly more likely to eat insects at a friend's house than buy them on their own at the store (P=0.0194) or order them at a restaurant (P=0.0194). Participants were also more likely to eat insects at a cultural event than to eat insects at a restaurant (P=0.0031). They were also significantly more likely to eat insects at a family member's house than they were to eat them on their own (P=0.0024). While social considerations are significant in some cases, participants were significantly more likely to eat foods from another culture than to eat insects (P=0.0003). They were also significantly more likely to eat foods from other cultures than eat insects served by family members (P= 0.0004) or friends (P=0.0003).

When comparing demographics to self-reported food choice preferences, whether someone has lived abroad appears to be the most significant factor to determining whether or not they will debut insect eating. Participants who lived abroad were significantly more likely to be open to trying new foods (P=0.0008), eat plant-based meats (P=0.0004), and eat foods from other cultures (P=0.0001). In addition, they were also significantly more likely to try insects (P=0.0002), eat insects at a cultural event (P=0.0006), at a friend's house (P=0.0008), and at the house of a family member (P=0.0002). Participants who reported insect-eating in their home country were also significantly more likely to be open to new foods (P=0.0021) but no significance was found in their willingness to eat insects under any of the circumstances described ($P>0.05$). Participants who identified as open to new foods were significantly more likely to eat insects at a friend's house ($P=0.0199$), but there was no significant likelihood to eat insects in other situations ($P>0.05$).

A principal component analysis of the self-reported, seen in Figure 4.8, indicates that there is a close association between participants who are likely to eat plant-based meat alternatives and people who would eat insects at the home of a family member, a friend's home, a restaurant, and a cultural event. The decision to debut insect eating in these settings is not strongly associated with environmental considerations, education, age, or cost.

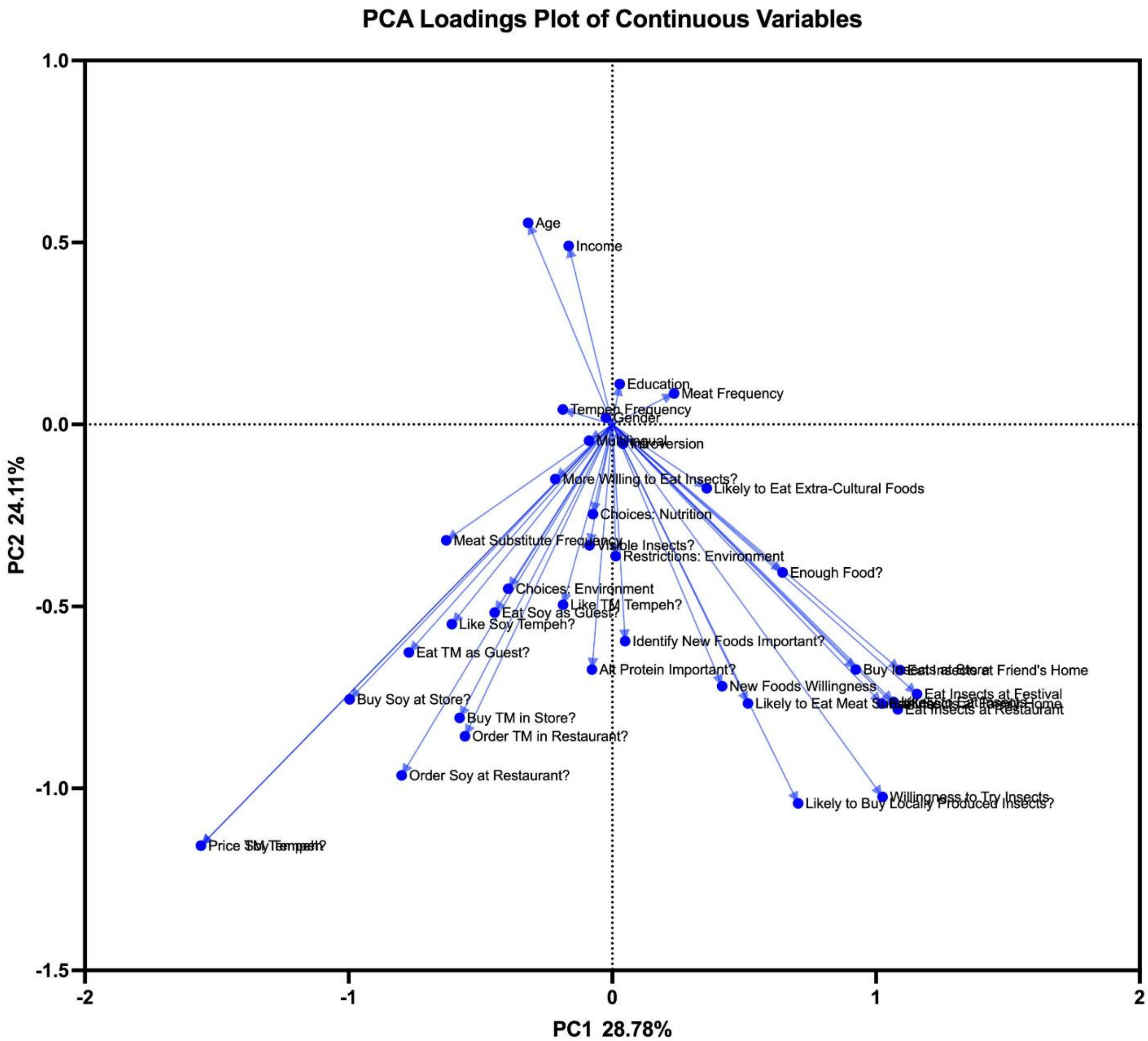

Figure 4.8: Principal Component Analysis (PCA) Loadings Plot of Continuous variables.

Despite the variety in the willingness to debut insect eating under the circumstances described by the initial survey, 36 out of 40 participants chose to debut the 50/50 mealworm soybean tempeh. When asked if the soy tempeh was visually appealing, 86% of the participants said the product was appealing, 8% said no, and 5% said they found it somewhat appealing. In contrast, 50% of the participants found the 50/50 mealworm soybean tempeh visually appealing, 39% found it unappealing, and 11% found it somewhat appealing.

During the evaluation of the aroma of the tempehs, 84% of the participants found the aroma of the soy tempeh to be appealing with 2% reporting a somewhat appealing aroma and 11% reporting the aroma to be unappealing. The predominant aromas identified by the participants were nutty, umami, earthy, and pleasant. When evaluating the 50/50 mealworm soybean tempeh, 68% of the participants found the aroma of this tempeh to be appealing, 12% found it somewhat appealing, and 21% found it unappealing. The predominant aromas identified by the groups were nutty, meaty, umami, and smoky.

When asked to rate the flavor of the soybean tempeh, 68% of the participants found the flavor appealing, 6% found it somewhat appealing, and 26% found it unappealing. The main flavors identified by the group were salty, umami, pleasant, and bitter. Flavor evaluation of the 50/50 mealworm soybean tempeh found that 61% of the participants regarded the flavor as appealing, 9% found it somewhat appealing, and 30% found it unappealing with the predominant flavors identified as salty, umami, bitter, and pleasant, just like the soybean tempeh.

Aromatic analysis of the tempehs revealed the aromas between the tempeh to be similar to the panelists. During the assessment, 74% of the participants found the texture of the soybean tempeh appealing while 3% found it somewhat appealing and 23% found it unappealing. Panelists identified the texture as crunchy, chewy, crumbly, and pleasant. For the 50/50 mealworm soybean tempeh, 72% of the participants found the texture appealing, 13% found it somewhat appealing, and 16% found it unappealing. The group identified the texture of this tempeh as chewy, crunchy, crumbly, and pleasant, just like the soybean tempeh.

Final assessment of the tempeh showed that 91% of the participants felt the soybean tempeh had the best appearance. In addition, 71% said the soybean mealworm had the best aroma. The flavor of the tempehs were rated equally with 47% favoring the flavor of the soybean tempeh and 47% favoring the flavor of the 50/50 mealworm soybean tempeh. the soybean tempeh was rated best overall with 79% of the participants favoring it over the 50/50 mealworm soybean tempeh. Despite the overall preference for the soybean tempeh, 79% of the participants indicated that they were more likely to try other insect-based products in the future, with 9% reporting no change in likelihood. From the written feedback, participants most commonly sighted the appearance of the mealworms as the reason for favoring the soy tempeh over the 50/50 mealworm soybean tempeh, though there were not enough responses to establish significance.

While, taken as a whole, the groups had similar assessments of the aroma, flavor, and texture of the tempehs, there were some interesting differences between the assessment of the prompts. Figure 4.9 shows differences between the aroma, flavor, and mouthfeel of the soy and 50/50 mealworm soybean tempehs between the prompts.

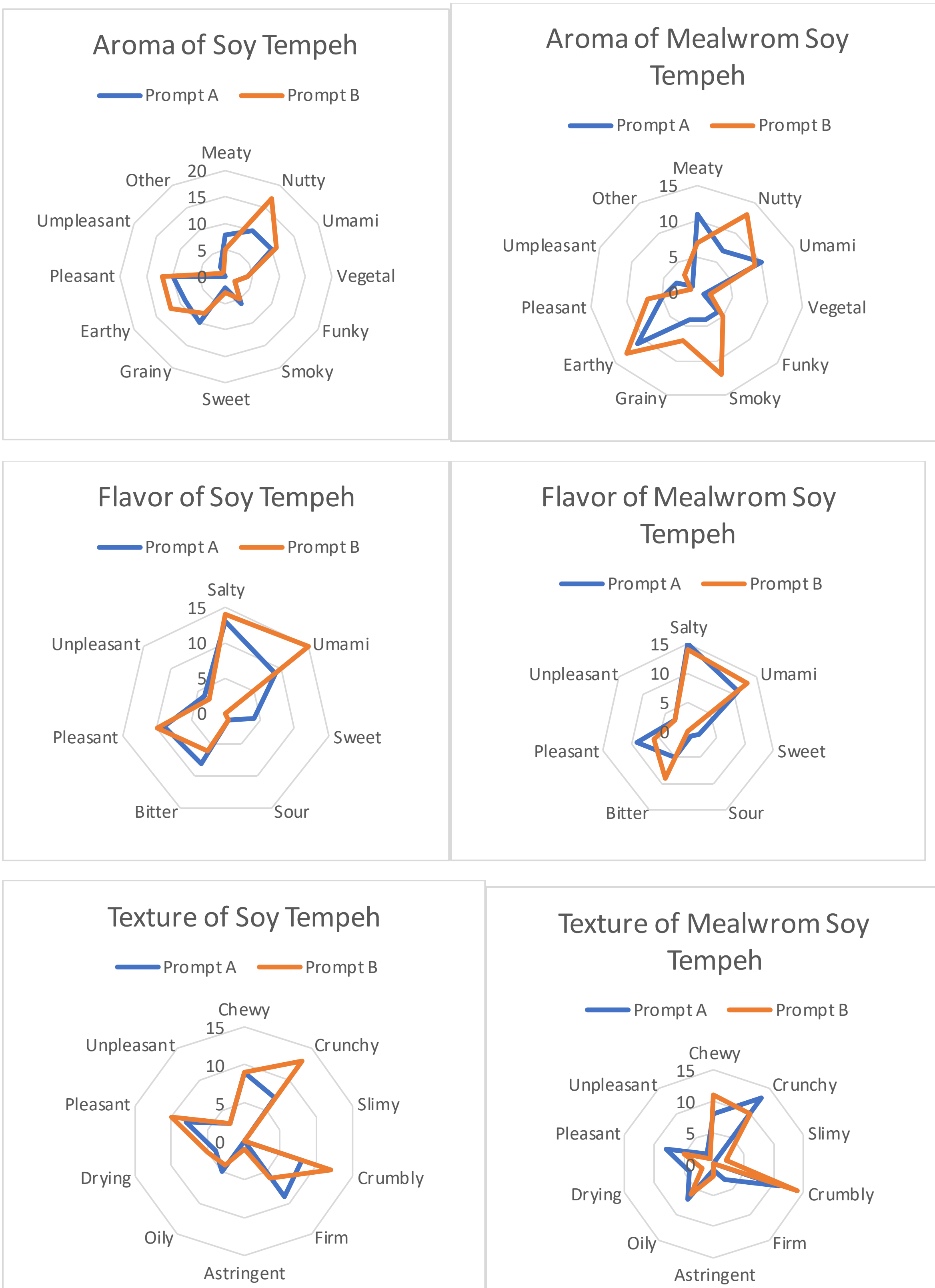

Figure 4.9: Spider graph indicating the difference in the assessment of aroma, flavor and mouthfeel of the soy tempeh and the 50/50 mealworm tempeh divided by prompt.

During the aromatic assessment, participants in the prompt B group strongly identified a nutty aroma in both the soybean tempeh and the 50/50 mealworm soybean tempeh. The prompt B group also identified smokey as a major component of the 50/50 mealworm soybean tempeh, while prompt A did not identify that attribute with the same frequency. While we see these divergent trends across the assessment of flavor and texture as well, there was no statistical difference between the organoleptic experiences of both thempehs as assessed by both prompts ($P>0.05$).

Of the six people who chose not to debut the 50/50 mealworm soybean tempeh, 2 chose not to debut during the initial survey and did not fill out opt out forms, one rejected immediately after the initial survey, one rejected upon smelling the product, one rejected upon seeing the product, and one rejected upon the first taste of the product. The reasons for rejection varied with the most common response being that they just could not bring themselves to eat insects. Other reasons included cultural restrictions, religious prohibitions, not wanting to eat tempeh at all, the texture and appearance of the product, and feeling sick when the product was presented. One participant indicated that though they regularly ate insects in their country of origin, they were unfamiliar with the *Tenebrio molitor* larvae and did not want to eat an unfamiliar insect. Demographics of the participants who opted included an even split between male and female and included individuals from North American, Africa, and Asia.

4.5 Discussion

While edible insects are not widely recognized as a mainstream source of protein in the global west, our data show that a large percentage of the population are not only aware of the practice but have participated in insect-eating at least once in their lifetime. With 79% of online respondents reporting having seen insects in a food product, and 58% having consumed insects, it is reasonable to conclude that insect-eating is not an entirely novel concept amongst the population. That being said, it is important to note that more than 75% of the respondents in the online survey reported being under the age of 40. This is important to note as previous research has found that older people contain less knowledge about insect consumption that younger populations. The same study also found that although 46.6% of the participants had a negative perception of eating insects, 77.7% were willing to try them, indicating that despite some respondents' aversion to edible insects, other factors were driving their willingness to debut (Caparros Megido et al., 2014). Another study concluded that members of generation Y (people born between 1982 and 1994) were more likely to experience food neophilia, or an excitement at the prospect of encountering a new food (Okumus et al., 2021). With 75% of participants belonging to generation y or younger, it is reasonable to associate the broad knowledge and exposure to insect eating with the age of the sample group.

We anticipated environmental considerations to be a major driving factor to edible insect debut. However, we observed no significant relationship between environmental considerations and the choice to consume insects. While there are some studies that indicate that consumers who assess the environmental impact of their food choices may be more likely to engage in entomophagy, we did not observe any significant difference in rate of debut in the group that received the environmental impact prompt(Hartmann et al., 2015; Menozzi et al., 2017; Tan et al., 2015).

Given the low numbers of product rejection, we were unable to identify any demographic indicators for willingness to consume insects. This is confirmation of a previous study on consumer acceptance of where researchers were unable to link demographic variables to willingness to consume. That same study also suggested that providing information on the benefits of entomophagy may help drive acceptance (Hartmann & Siegrist, n.d.). While we did provide environmental impact information to half the participants, we were unable to link that information to the rate of debut or likelihood to try edible insect products again.

Within the in-person panel, 95% reported eating meat, some of which reported adhering, or trying to adhere, to a vegetarian or vegan lifestyle but still incorporating meat periodically in their diets. With 90% of participants reported trying plant-based meat alternatives, the frequency of consumption among the panelists was low. This suggests that among the meat eaters in the panel, alternative protein sources are more of a novelty than a food product that is regularly integrated into their diets. While the panel's openness to new foods may prompt them to try an alternative protein product out of curiosity, factors like cost, nutrition, and familiarity may be more important when making food choices.

Appearance of the insects in the food product was a widely reported consideration during the in-person tempeh assessment. While we were unable to establish the significance of appearance during the evaluation ($P>0.05$), multiple comments left on the evaluation sheets indicated that the appearance of the mealworms in the product was off-putting to participants. This coincides with previous research indicating new consumers of insect products would prefer the insects to not be visible (Mandolesi et al., 2022).

Previous research has suggested that neophobia, or the fear of new foods, if often driving rejection of edible insects (de Carvalho et al., 2020)(Dobermann et al., 2017). This term is often paired with the notion of disgust. While the majority of respondents and participants reported either seeing or? experiencing edible insect product, it is reasonable to conclude that there is an awareness of the practice of insect eating. While neophobia may have affected the overall preference of the soy tempeh over the 50/50 mealworm soybean tempeh, it does not appear to affect the willingness to consume edible insect products in the future. With 90% of the in-person participants choosing to debut the 50/50 mealworm soybean tempeh, and 79% reporting that they were more likely to try edible insect products again, neophobia does not appear to be a significant factor for rejection. Amongst those who rejected the product, disgust appears to be the driving factor for failure to debut. This does not mean that neophobia was not observed among the sample group. One participant who rejected the product reported eating insects regularly growing up. When reporting the reason for rejection, they reported being unfamiliar with mealworms as a food source and being uncomfortable trying them without further knowledge about them.

Finally, vegans and vegetarians in the study appeared to be open to the concept of eating insect protein. In the online survey, 43% of vegans and 45% of vegetarians had previously consumed edible insect protein at least once prior to participation. Of the remainder, 54% of vegans and 67% of vegetarians who had not previously tried edible insect protein reported a willingness to debut the food. In the in-person consumer acceptability study, 100% of the vegans and vegetarians chose to debut the 50/50 mealworm soybean tempeh. These results contrast with previous research that vegans in particular are less likely to engage in insect eating than vegetarians and omnivores (Pantuso, 2019). The literature is sparce on the willingness of vegans and vegetarians to debut insect eating, and this study did not have enough vegan or vegetarian participants to establish significance. More research involving vegan and vegetarian willingness to debut edible insect food is needed to determine if these food products would be acceptable to those populations.

4.6 Conclusion

Although the 50/50 mealworm soybean tempeh was rated less favorably than the traditional soy tempeh, they were rated equally for flavor. This suggests that there is a potential for this product to be commercially viable. It should be noted that the sample preparation for this study was not typical for tempeh production. Study samples were served lightly salted without any other seasoning. Tempeh is traditionally served heavily spiced and often in a sauce. Further research is needed to determine if preparing the 50/50 mealworm soybean tempeh in a more traditional fashion may improve the overall assessment of the product. That being said, 75% of the participants indicated that they were as likely or more likely to try another insect-based food product, suggesting that this product was effective in promoting comfort with insect eating. Future research should include sensory studies with this product transformed into foods more familiar to the western palate, like hamburgers and nuggets.

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Chapter 5: Reflection

In the Western world, entomophagy often viewed with disgust. From an early age, we are taught that insects are dirty, vectors for disease, and indicators of death and decay. This is not without reason, as the animal that causes the most deaths globally is the mosquito, a flying parasitic insect that happens to be a vector for disease-causing viruses and several plasmodia that cause malaria. Insects are associated with dengue fever, chagas disease, and the bubonic plague. We see insects feasting on decaying animal flesh, eating holes in the healthy tissue of our cats and dogs, and rolling up balls of feces to feed to their young. Insects cause painful bites and stings. They shoot caustic chemicals from their bodies, and they burrow into our skin to access the nutrition flowing through our veins. It is safe to say that insects have a stigma in the west that is a barrier to acceptance, but stigma does not mean permanent exclusion from the modern food system. Lobster was once considered an undesirable food for the poor, but now its consumption is associated with wealth and decadence. The Patagonian toothfish was once a cheap fish sold as bycatch by the commercial fishing industry before overfishing caused it to be rebranded as Chilian sea bass. Now, Chilean sea bass in found in fine dining restaurants all over the world. To move past the stigma around edible insects, it is necessary to push accessible edible insect species through a similar rebranding, but the question that remains is how.

More comprehensive research on the effective branding of insects as food is needed to better understand the barriers to acceptance in the West. While neophobia is often identified as primary main reason people will not embrace edible insects, our research shows that knowledge of insect eating is common with many people choosing to debut insect eating out of culinary curiosity. If our sample set is representative of the population, this means that many people are encountering edible insects but are either choosing not to seek them out regularly or do not have a reliable option to do so. A variety of reasons may account for this sporadic consumption. Lack of availability of insect food products, lack of knowledge around preparation, and cost of available products may also be affecting regular consumption.

To assess the impact of availability, future research is needed to assess the role of availability in the consumer's willingness to try insect-based foods. At present, to gain access to edible insects, one must either forage for the insects themselves, visit a place where consuming insects is commonplace, or order insect-based products online, but would that change if insect products were commonly available in local grocery stores? Would food manufacturers be able to access the curiosity that drives many people to debut insect eating if the products could be found next to traditional food products in a grocery store? Common placement of insect-based food products would reduce the burden of procurement and move these products into the familiar.

Having the products readily available and accessible does not address the issue of lack of knowledge around preparation. Over the last 20 years, Western culture has moved professional chefs out of the kitchen and into the media space. The presence of chefs in popular culture presents an opportunity to distribute knowledge around insect preparation to a broader audience. Future research around the ability of chefs in the media to develop confidence to debut edible insects and home insect preparation may help insect-based food producers to rebrand the practice of eating insects. Research around the role of social media platforms like TicTock, Instagram, and Facebook on developing food curiosity through knowledge transfer, frequency of exposure, and communication nutritional benefits may help producers develop effective media campaigns to promote their products.

Once the products are available, and the public is regularly exposed to insect eating, it is important that these products are at a price point that is accessible to the average consumer. To help the cost of insect protein production, it is important to increase the scale of insect production for human consumption. The commercial meat industry may be important partners to explore the increase of edible insect production. By participating in the rearing of insect protein, commercial meat producers may be able to reduce the environmental impact of their operation, practice more conservative land management, and increase the profitability of their operations. Given the high feed to weight conversions of edible insects like mealworms, and their ability to utilize waste streams like spent grain and produce waste, commercial meat producers may be able to produce more high value protein with fewer resources. Commercial meat producers also have access to feed and space to rear insects, but research is needed to determine if conventional animal feeds are effective in rearing edible insects. Research is also needed to assess the profitability of shifting a portion of commercial meat producer's operation to edible insect rearing. Research around the environmental impact of that shift is also necessary to assess how much of a shift is needed to have an impact on the environmental stressors of commercial meat production. If the insect-based food industry can engage to commercial meat industry to increase the supply edible insects, that may make the cost of these products more accessible to the average consumer.

Finally, once the industry has addressed accessibility, knowledge, and cost, the products need names that separates them from the negative associations of insects in Western culture. Market studies are needed to assess new potential names for edible insects. Can producers rebrand scorpions as 'desert crabs', water bugs as 'Siamese crayfish', or mealworms as 'molitos' to increase consumer acceptance of the insects as food? Identifying names that will bypass consumers' stigma and notions of disgust is essential for the rebranding of these products.

The edible insect industry in the West faces a lot of challenges, but the potential for this industry to add variety and diversity to the modern food system is vast. This study examined the application of a single food processing technique on one of the more than 2,100+ edible insects identified to date. If edible insect producers can successfully rebrand their products to reduce stigma, increase knowledge and exposure, and reduce cost, then the industry may be able to strengthen the food system's ability to meet the food demands of the world's growing population.

Appendices

Appendix A

<u>Lexicon</u>

Roasty	Vegetal	Grain	Acidic
Savory	Crunchy	Bean	Sweet
Grainy	Crumbly	Walnut	Sour
Nutty	Dry	Corn	Soft
Umami	Bitter	Earthy	Moist
Soy Sauce	Peanut Butter	Soil	Chewy
Coffee	Toasted Sesame	Nutritional Yeast	Popcorn
Musty	Tahini	Salty	Dry Leaves

Appendix B

Online prompt:

We are developing a new food product at Colorado State University and your feedback is needed to assess its marketability. Participants must be at least 16 years of age to fill out the online survey. Click here to take this brief survey so that we ma better understand consumer perceptions of novel foods. The first 100 people to compete the survey will receive a link for a $10 gift card.

Upon accessing the link:

Thank you for agreeing to take this online survey. Your feedback will help us understand the potential marketability of a new food product being developed. All responses are submitted anonymously and there will not be used for personal identification. There are a total of 38 questions. Please take your time and answer the questions to the best of your ability. The first 100 people to complete the survey will receive a code for a gift certificate.

1. What is your age?
2. What is your Gender? Female, Male, Non-Binary, Other, Prefer not to say.
3. What is the highest level of education you have completed? Some grade school, High school, Associates Degree, Bachelors, Master's Degree, PhD, Post-Doctoral, Prefer not to say.
4. What is your household income? $10,000-$30,000; $30,000-$60,000; $60,000-$90,000; $90,000+
5. What is your Nationality? (Nationality is typically identified as the country of origin or independently governed cultural group within a country).
6. What is your ethnicity (Race)? Black, White, Hispanic, Asian, etc. Prefer not to say. Please indicate all that apply.
7. What is your cultural heritage? (Cultural heritage is usually identified by the cultural group from which you draw your traditions, social rules, and etiquette.)
8. Please identify your spiritual tradition: Atheist, Agnostic, Christian, Buddhist, Hindu, Judaism, Muslim, Other, Prefer Not to Say.
9. Do your consider yourself an introvert or an extrovert?
10. Do you speak languages other than English?
11. Are there any foods that are restricted or forbidden in your Spiritual or cultural traditions? If so, Please List
12. Do you follow any dietary traditions? (Check all that apply) Vegetarian, Vegan, Kosher, Halal, Pescatarian, Keto, Other, None
13. Do you personally adhere to the food restrictions of your cultural or spiritual traditions?
14. Please list any non-medicine related allergies you have:
15. Have you ever traveled outside of your home country?
16. If yes, please identify the continents you have visited: Africa, Antarctica, Asia, Australia, Europe, North America, South America.

17. Have you ever lived outside of your home country?
18. If yes, please identify the continents you have lived in: Africa, Antarctica, Asia, Australia, Europe, North America, South America.
19. Do you eat meat (including fish and seafood)? If not, why (Skip to question 22)?
20. If you eat meat, what kinds of meat you consume? What kinds of meat do you eat (Check all that apply)? Beef, Chicken, Pork, Lamb, Rabbit, Duck, Fish, Shellfish, Game Meat, Other, I don't eat meat.
21. How often do you eat meat? Never, Rarely, Once a month, A couple of times a month, Once a week, Several times a week, every day.
22. Have you ever eaten plant-based meat products? If so, why?
23. If yes, how often do you eat plant-based meat products?: Never, Rarely, Once a month, A couple of times a month, Once a week, Several times a week, every day.
24. Do you eat products containing soy?
25. Have you heard of tempeh?
26. Have you seen tempeh in your local grocery store?
27. Have you ever eaten Tempeh? Yes/No
28. If yes, How often do you eat Tempeh?: Never, Rarely, Once a month, A couple of times a month, Once a week, Several times a week, daily.
29. Do people eat insects in your home country?
30. Do people eat insects in your country of residence?
31. Have you ever seen insects as a food product?
32. If yes, in what form (Check all that apply)? Fresh, Powdered, Fried, in a beverage, in a prepared dish, dried, pickled, in a candy, other
33. Have you ever eaten insects?
34. If yes, what insects did you eat?
35. How were those insects prepared (Check all that apply) Fresh, Powdered, Fried, in a beverage, in a prepared dish, dried, pickled, other
36. If you have eaten insects, how likely are you to try them again: Not at all, Not likely, I might try them, somewhat likely, very likely, does not apply.
37. If you have never eaten insects, how likely are you to try them: Not at all, Not likely, I might try them, somewhat likely, very likely, does not apply.
38. If you would not eat insects, please indicate why. I have never had the chance, insects are gross, it is not permitted within my religion, it is not permitted within my culture, I follow a dietary practice that does not allow me to eat insects.

Thank you for taking our online survey. If are interest in participating in the In-person sensory test, you can register at the link below. Participants must be able to read and speak English, get themselves to Colorado State University, be at least 18 years of age at the time of the study, and have no know food allergies.

Appendix C

The Prompts below will be provided as informational pamphlets with pictures of the products.

Prompt A: Tempeh is a traditional plant-based fermented food from Indonesia. It is made from legumes, soybeans, and fermented with a fungus called *Rhizopus oligosporus* whose network of roots bind the soybeans together and make the nutrients within the soybeans easier for the body to utilize. According to the USDA, a 100g serving of tempeh contains approximately 23g of protein, 11g of fat, and 8g of carbohydrates. It is also a source of iron, calcium, magnesium, phosphorous, potassium, manganese, and selenium. It also provides thiamine, riboflavin, niacin, vitamin B6, pantothenic acid, and folate. (https://fdc.nal.usda.gov/fdc-app.html#/food-details/174272/nutrients)

Prompt B: Tempeh is a traditional plant-based fermented food from Indonesia. It is made from legumes, soybeans, and fermented with a fungus called *Rhizopus oligosporus* whose network of roots bind the soybeans together and make the nutrients within the soybeans easier for the body to utilize. According to the USDA, a 100g serving of tempeh contains approximately 23g of protein, 11g of fat, and 8g of carbohydrates. It is also a source of iron, calcium, magnesium, phosphorous, potassium, manganese, and selenium. It also provides thiamine, riboflavin, niacin, vitamin B6, pantothenic acid, and folate. (https://fdc.nal.usda.gov/fdc-app.html#/food-details/174272/nutrients)

With the recent UN announcement that the global population has exceeded 8 billion people, the need for identifying and developing sustainable and nutritious sources of food is increasing. When compared to conventional beef production, mealworms have significantly less impact on the environment overall. Every kilogram of beef requires 25Kg of feed, 15,000 L of water and 29 square meters of land to be reared. In contrast, every Kg of mealworms requires 2.6 Kg of feed, no direct water, and approximately .5 Kg of space. In addition, every Kg of beef produces approximately 5.4 Kg of raw manure to manage whereas mealworm's efficient digestive systems produce no raw manure, allowing their waste to be applied directly to farmland without costly management. With their high feed conversion ratio, low water usage rates, limited space requirements, and manageable waste streams, edible insects are a viable option to meet the protein demands of the growing population while relieving the environmental costs to expanding the conventional meat systems.

Please answer the Survey Questions below to the best of your ability. Once you have finished answering these questions, you may proceed to the sensory room.

39. What is your age?
40. What is your Gender? Female, Male, Non-Binary, Other, Prefer not to say.
41. What is the highest level of education you have completed? Some grade school, High school, Associates Degree, Bachelors, Master's Degree, PhD, Post-Doctoral, Prefer not to say.
42. What is your household income? $10,000-$30,000; $30,000-$60,000; $60,000-$90,000; $90,000+
43. What is your Nationality? (Nationality is typically identified as the country of origin or independently governed cultural group within a country).
44. What is your ethnicity (Race)? Black, White, Hispanic, Asian, etc. Prefer not to say. Please indicate all that apply.
45. What is your cultural heritage? (Cultural heritage is usually identified by the cultural group from which you draw your traditions, social rules, and etiquette.)
46. Please identify your spiritual tradition: Atheist, Agnostic, Christian, Buddhist, Hindu, Judaism, Muslim, Other, Prefer Not to Say.
47. Do your consider yourself an introvert or an extrovert?
48. Do you speak languages other than English?
49. Are there any foods that are restricted or forbidden in your Spiritual or cultural traditions? If so, Please List
50. Do you follow any dietary traditions? (Check all that apply) Vegetarian, Vegan, Kosher, Halal, Pescatarian, Keto, Other, None
51. Do you personally adhere to the food restrictions of your cultural or spiritual traditions?
52. Please list any non-medicine related allergies you have:
53. Have you ever traveled outside of your home country?
54. If yes, please identify the continents you have visited: Africa, Antarctica, Asia, Australia, Europe, North America, South America.
55. Have you ever lived outside of your home country?
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58. If you eat meat, what kinds of meat you consume? What kinds of meat do you eat (Check all that apply)? Beef, Chicken, Pork, Lamb, Rabbit, Duck, Fish, Shellfish, Game Meat, Other, I don't eat meat.
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61. If yes, how often do you eat plant-based meat products?: Never, Rarely, Once a month, A couple of times a month, Once a week, Several times a week, every day.
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65. Have you ever eaten Tempeh? Yes/No
66. If yes, How often do you eat Tempeh?: Never, Rarely, Once a month, A couple of times a month, Once a week, Several times a week, daily.
67. Do people eat insects in your home country?
68. Do people eat insects in your country of residence?

69. Have you ever seen insects as a food product?
70. If yes, in what form (Check all that apply)? Fresh, Powdered, Fried, in a beverage, in a prepared dish, dried, pickled, in a candy, other
71. Have you ever eaten insects?
72. If yes, what insects did you eat?
73. How were those insects prepared (Check all that apply) Fresh, Powdered, Fried, in a beverage, in a prepared dish, dried, pickled, other
74. If you have eaten insects, how likely are you to try them again: Not at all, Not likely, I might try them, somewhat likely, very likely, does not apply.
75. If you have never eaten insects, how likely are you to try them: Not at all, Not likely, I might try them, somewhat likely, very likely, does not apply.
76. If you would not eat insects, please indicate why. I have never had the chance, insects are gross, it is not permitted within my religion, it is not permitted within my culture, I follow a dietary practice that does not allow me to eat insects.

1-5 ranking, 1 being not at all, 5 being definitely (Will vary per question)

1. How likely are you willing to try new foods?
2. Are environmental considerations important when making food choices?
3. How likely are you to restrict your diet based on environmental considerations?
4. Are alternative protein products important?
5. Do you think there is enough food to feed all the people in the world?
6. Is it important to identify new food sources to support the growing global population?
7. How likely are you to eat plant-based meat products?
8. How likely are you to eat foods from other cultures or ethnicities?
9. Is nutritional value a significant consideration when making food choices?
10. Rank the order of importance when making food choices. Cost, Nutrition, Flavor, Environmental Impact, Animal Rights, Religious Adherence, Cultural Adherence
11. Is animal welfare a consideration when making food choices?
12. Is cost a consideration when making food choices?
13. Is religion/spirituality a consideration when making food choices?
14. Is your culture a consideration when making food choices?
15. Is the food's culture of origin a consideration when making food choices?
16. Would you be willing to try a food product containing insects?
17. How likely are you to eat insects?
18. If you were served insects at a friend's house, how likely would you be to try them?
19. If you saw an insect dish on a menu at a restraunt, how likely would you be to try it?
20. If you saw an insect-based product in the grocery store, how likely would you be to try it?
21. If you saw insects being served at a community event, like a cultural festival, how likely would you be to try them?
22. If a member of your family prepared insects for a meal, how likely would you be to try them?
23. If you are a vegetarian, how likely would you be to try a dish made from insects?
24. If you are a vegan, how likely would you be to try a dish made from insects?

25. If you were to eat an insect-based product, how visible can the insects be for you to be willing to try them? Not at all, slightly visible, fully visible.
26. Would you be more likely to buy an insect product if it were produced locally?

Thank you for completing the initial survey portion of the study. You may continue on to the sensory kitchen for the product testing portion of the study.

Sensory Questions

1. Is the Soy Tempeh visually appealing?
2. Is the Mealworm Tempeh visually appealing?
3. Is the aroma of the Soy Tempeh appealing?
4. Please describe the Aroma of the Soy Tempeh – (Check All that apply)
    a. Meaty
    b. Nutty
    c. Moldy
    d. Fruity
    e. Umami (Savory)
    f. Vegetal
    g. Funky
    h. Smoky
    i. Sour
    j. Mildew
    k. Sweet
    l. Grainy
    m. Earthy
    n. Pleasant
    o. Unpleasant
    p. Floral
    q. Fruity
    r. Other

5. Is the aroma of the Mealworm Tempeh appealing?

6. Please describe the Aroma of the Mealworm Tempeh – (Check All that apply)
    a. Meaty
    b. Nutty
    c. Moldy
    d. Fruity
    e. Umami (Savory)
    f. Vegetal
    g. Funky

h. Smoky
i. Sour
j. Mildew
k. Sweet
l. Grainy
m. Earthy
n. Pleasant
o. Unpleasant
p. Floral
q. Fruity
r. Other

7. Is the mouthfeel of the Soy Tempeh appealing?

8. Please describe the mouthfeel of the Soy Tempeh – (Check All that Apply)
    a. Chewy
    b. Crunchy
    c. Slimy
    d. Crumbly
    e. Firm
    f. Astringent
    g. Oily
    h. Drying
    i. Pleasant
    j. Unpleasant

9. Is the mouthfeel of the Mealworm Tempeh appealing?

10. Please describe the mouthfeel of the Soy Tempeh – (Check All that Apply)
    a. Chewy
    b. Crunchy
    c. Slimy
    d. Crumbly
    e. Firm
    f. Astringent
    g. Oily
    h. Drying
    i. Pleasant
    j. Unpleasant

11. Is the Flavor of the Soy Tempeh appealing?

12. Please rate the flavor of the Soy Tempeh – (Check all that apply)

a. Salty
b. Umami (Savory)
c. Sweet
d. Sour
e. Bitter
f. Pleasant
g. Unpleasant

13. Is the Flavor of the Mealworm Tempeh appealing?

14. Please rate the flavor of the Soy Tempeh – (Check all that apply)
    a. Salty
    b. Umami (Savory)
    c. Sweet
    d. Sour
    e. Bitter
    f. Pleasant
    g. Unpleasant

15. Do you like the Soy Tempeh?
    a. I don’t like it at all
    b. I somewhat like it
    c. I like it
    d. I like it a lot
    e. I think this product is excellent

16. Do you like the Mealworm Tempeh?
    a. I don’t like it at all
    b. I somewhat like it
    c. I like it
    d. I like it a lot
    e. I think this product is excellent

17. Would you order the Soy Tempeh at a restaurant?
    a. Yes, definitely
    b. Maybe
    c. I don’t know
    d. Probably not
    e. Definitely not

18. Would you order the Mealworm Tempeh at a restaurant?
    a. Yes, definitely
    b. Maybe
    c. I don’t know
    d. Probably not
    e. Definitely not

19. If someone served the Soy Tempeh to you at their home, would you eat it?
    a. Yes, definitely
    b. Maybe
    c. I don't know
    d. Probably not
    e. Definitely not

20. If someone served the Mealworm Tempeh to you at their home, would you eat it?
    a. Yes, definitely
    b. Maybe
    c. I don't know
    d. Probably not
    e. Definitely not

21. Could you see yourself buying soy tempeh at a grocery store to cook at home?
    a. Yes, definitely
    b. Maybe
    c. I don't know
    d. Probably not
    e. Definitely not

22. Could you see yourself buying mealworm tempeh at a grocery store to cook at home?
    a. Yes, definitely
    b. Maybe
    c. I don't know
    d. Probably not
    e. Definitely not

23. If you were to buy a pound the Soy Tempeh, how much would you be willing to pay for it?
    a. $0, I would not buy it
    b. $3
    c. $5
    d. $7
    e. $9

24. If you were to buy a pound the Mealworm Tempeh, how much would you be willing to pay for it?
    a. $0, I would not buy it
    b. $3
    c. $5
    d. $7
    e. $9
25. Which tempeh had the best aroma?
26. Which tempeh had the best appearance?

27. Which tempeh had the best flavor?
28. Which tempeh had the best mouthfeel?
29. Which tempeh was best overall?
30. Has consuming the mealworm tempeh made you more or less willing to try other insect products?

Appendix D

To be completed by people who opt-out of the study

1. When did you choose to leave the study?
    1. After the introduction
    2. After completion of the initial survey
    3. Before entering the sensory room
    4. Upon Smelling the product
    5. Upon Seeing the product
    6. Upon the first taste of the product

2. Please indicate the reasons why you were unable to complete the sensory study (Check All that Apply):

a. I cannot bring myself to eat insects.
b. My culture does not allow me to eat insects.
c. My religion/spiritual practice does not allow me to eat insects.
d. I do not eat tempeh.
e. The product did not smell appetizing.
f. The product did not look appetizing.
g. The product did not taste good.
h. The texture of the product is not appealing.
i. I felt sick when I saw the product.

3. Would you be willing to try to complete the study at a different date?

Additional Comments?