Using insects in conversion of agricultural by-products and applications of their value-added products

By

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Abstract

Humanity faces two colossal and interwoven challenges: 1) By 2050, the world population may exceed 9 billion people, thereby requiring more food production, and 2) Increased food production will generate more agricultural byproducts from downstream food processing (i.e. stems, seeds, pulp), posing a significant environmental safety and management concern due to associated greenhouse gas emissions. As detritivores and herbivores, the diversity of insect species includes groups highly specialized in their ability to thrive on unique and unbalanced organic substrates, such as, agricultural by-products. Examples of agricultural by-products with relevance to the Davis area include tomato waste (skins, extracted pulp), wine waste (skins, stems, seeds), and almond hulls. In this project and in a rapidly growing body of research literature, two insect species, mealworms (*Tenebrio molitor*) and black soldier flies (*Hermetia illucens*) were studied as potential bioconverters of such locally available agricultural by-products. Insects feeding on agricultural by-products are harvested for protein and fats which may be reintroduced into the food system as animal feed. In addition, insects may be used to produce additional commodities, such as chitinous products, pharmaceuticals, biofuels, lubricants, and fertilizer from their excrement. Consequently, the services rendered from insect-based bioconversion provide marketable solutions for reducing food byproducts that are fiscally manageable, modest both in space and energy requirements, environmentally sustainable, while yielding higher feed conversion ratios than conventional livestock. As such, insect bioconversion is gaining traction both as a research topic and as a business opportunity.

This dissertation addresses questions covering multiple stages across the bioconversion process with the expressed intent on exploring strategies to improve the bioconversion of agricultural by-products into value-added products. Specifically, I ask how variable is individual

insects bioconversion efficiency, how does the microbial community influence performance, and how may insect-based products be reincorporated into agriculture? Chapter 1 is a review of the use and potential of using insects as a tool for food waste management. Chapter 2 is a review on the targeted breeding of insects, diversity of insect bioconverters, and research into insect-gut microbial complexes. Chapter 3 provides a framework to characterize intraspecific phenotypic variation in mass-reared insect populations. *Tenebrio molitor* larvae are compared in three bioassays evaluating variation in feeding efficiency on a novel diet (polystyrene), and diets representative of those used in the insect bioconversion industry. This chapter presents linear regressions of ranked trait responses as a useful tool to quantify and compare intraspecific variation both within and across populations of mealworms. Bioassays reveal that addition of polystyrene in *T. molitor* diets increases larval weight and overall diet consumption. However, feed conversion of larvae is lower and less variable on the polystyrene amended diets than on a standard diet. Chapter 4 describes effects of different rearing environments and their influence on black soldier fly, *Hermetia illucens*, production efficiency on agricultural by-products. This chapter also describes effects of microbial inocula on production efficiency. Main conclusions drawn from chapter 4 are that different rearing conditions favored bioconversion of particular agricultural byproducts, but only have a small effect on microbiota. In contrast, addition of microbial inocula did not significantly improve performance, nor does it alter intestinal and residue microbiota. Chapter 5 describes applications of insect-derived value-added by-products in an agricultural setting. More specifically, legume seeds were treated with chitinous products (raw insect derived chitin, pure chitin, pure chitosan) to determine their effects on mold growth, seed germination, and seedling vigor (biomass). Key findings from chapter 5 were that application of chitinous products significantly suppress mold growth. At higher dosages (5% and 10% by weight), raw insect-

derived chitin adversely affects germination of soybeans, but not of fava beans and black-eyed peas. However, chitinous products do not significantly affect seedling weight. Considering the world-wide availability, low economical cost, and superior efficacy as a mold growth inhibitor, results from this study highlight raw insect-derived chitin as a promising novel fungicide when applied at a low dosage (2.5% by weight).

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Dedication

I dedicate this dissertation to Andrew Fowles

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Chapter 1: Insect-based bioconversion: Value from food waste

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Abstract

Insect bioconversion is gaining traction both as a research topic and as a business opportunity. When insects are mass produced under controlled conditions, they can break down significant quantities of food waste. Further, as the insects consume this waste, they produce multiple valuable commodities, such as insect biomass (proteins, lipids), pharmaceuticals, biofuels, lubricants, and fertilizer from their excrement. This process is called bioconversion and will be a serious contender among food waste treatment options in the coming decades. Here, the authors discuss both the need to increase capacity and to maximize the potential benefits of using insects as bioconverters of food waste. Authors provide both theoretical and practical solutions for expanding insect-based bioconversion to food waste streams.

Introduction

As detrivores and herbivores, the diversity of insect species include groups highly specialized in their ability to thrive on different organic substrates as food sources. Some of these substrates resemble food wastes from agriculture and food processing industries. In the literature, this is referred to as insects-based "bioconversion" and represents an economically viable method for turning large quantities of food waste into valuable materials—including feed for animals (insect biomass as a supplement added to animal feed), food for people, secondary industrial compounds (biofuel, lubricants, pharmaceuticals, dyes, etc.), and the left-over food waste can be used as organic matter and nutrient-rich soil amendments. Consequently, the services rendered from insect-based bioconversion provide marketable solutions for reducing food waste that are fiscally manageable, modest both in space and energy requirements, environmentally friendly, associated with real market/commercial opportunities, and yielding higher feed conversion ratios than

conventional livestock (Li, Zhao, & Liu, 2013; van Huis & Oonincx, 2017). Though a relatively nascent industrial sector, mass production of insects for feed and secondary products is a rapidly growing enterprise with significant potential for growth (Dossey, Morales-Ramos, & Rojas, 2016; van Huis & Oonincx, 2017). Presently, only a few insect species are commercially used for insect-based bioconversion of food waste, with black soldier fly larvae (*Hermetia illucens* L.) being the most commonly used species (Wang & Shelomi, 2017). This is juxtaposed by the immense diversity of insects adapted to a wide range of food sources and therefore likely capable of providing effective bioconversion of a wide range of food waste materials. Considering the diversity of food waste streams generated from numerous crop varieties and their by-products from downstream processing, there appears to be ample opportunity for exploration of optimized combinations of food wastes-to-insect pairings to maximize both bioconversion and insect biomass production. In this chapter, we argue better food waste-to-insect pairings and selective breeding of insects are needed to increase capacity of using insects-based bioconversion of food waste. In addition, we provide both theoretical and practical solutions (businesses), and regulatory hurdles relating to insect-based bioconversion of food waste.

The case for insects- why bioconversion of food waste?

Insect-based bioconversion of food waste is the controlled breakdown of an initial feedstock (food waste) into insect biomass and frass (waste residuals) (Barry, 2004), with the latter consisting of predominantly insect frass and to a lesser extent, shed exoskeletons, dead insect parts, and potentially uneaten feedstock. The process of insect-based bioconversion of food waste mirrors the natural breakdown of organic matter in ecosystems (Lim, Lee, & Wu, 2016). In such systems, naturally occurring insects, earthworms, a wide range of other invertebrates, fungi, and bacteria

colonize and break down food waste, converting the nutrients for their own metabolic and reproductive needs. Under controlled conditions, the species responsible for the decomposition process can be regulated and the ambient conditions can be optimized to favour the growth and bioconversion by the given species performing the service. Importantly, value may be produced at multiple steps in the bioconversion process (Barry, 2004). For instance, value can be gained from the elimination of the initial waste itself (Mutafela, 2015) (disposal fees), sales of insect biomass for food and feed (Anankware, Fening, Osekre, & Obeng-Ofori, 2015), sales from fractionated secondary products (Zheng et al., 2012), and sales of the remaining bioconverted waste for soil amendments (Suantika, Putra, Hutami, & Rosmiati, 2017). Industrial insect rearing can efficiently turn many tonnes of food waste feedstock into valuable products, with some sources suggesting most food waste can be diverted to insect-based bioconversion (Ortiz et al., 2016; Veldkamp et al., 2012). Currently, Agriprotein's South African facility has the capacity to process 250 megagram (Mg wet weight) tonnes of food waste each day, in turn generating 7 Mg of "insect meal" (dried powder from ground insect biomass), 3 Mg of insect oil, and 20 Mg of fertilizer (dry) (www.agriprotein.com). Agriprotein uses black soldier fly for its food waste bioconversion and is looking into using other species as they expand. They are one of several companies in the rapidly expanding insect-based bioconversion sector, with others including Ynsect (www.ynsect.com), Nextalim (www.nextalim.com), UNIQUE (www.gzunique.com.cn), and Alapre (www.insectmeal.com.co).

Commercialization of insect-based bioconversion represents a promising shift in providing alternative options for food waste reduction (Nyakeri, Ogola, Ayieko, & Amimo, 2017; Wang & Shelomi, 2017), as the industrial production of insects requires significant quantities of cheap, reliable feedstock (Ortiz et al., 2016). With supplies of global food waste estimated at 1.3 billion

tonnes and growing (Ambuko, 2014; Food and Agriculture Organization of the United Nations (FAO), 2017), and demands for protein, biofuels, and fertilizers increasing (Parfitt, Barthel, & Macnaughton, 2010), businesses adopting insect-based bioconversion make economic sense (Barry, 2004). Moreover, insect-based bioconversion of otherwise disposable food wastes provides a much-needed link for recirculating nutrients and resources from consumers back into agricultural supply chains. With consumers evermore concerned about the environmental profile of goods, insect-based bioconversion of waste is a marketable asset that may appeal to the sustainably minded customer (D'Souza, Taghian, Lamb, & Peretiatko, 2007). On a philosophical level, the concept of insect-based bioconversion hinges on the notion of completely re-thinking the concept of "food waste". In the Webster's English Dictionary, "waste" is defined as "an unwanted by-product". The FAO makes a further distinction between "food loss" (early stages of the food supply chain) and "food waste" (later in the food supply chain) (FAO, 2017). The concept of insect-based bioconversion means that by-products from one food production system become the input in bioconversion systems, so the concept of "waste" and "loss" really cannot be applied. Thus, the trend trail-blazed by insect-based bioconversion and described in this chapter represents a re-thinking of nutrient and resource flows within and among food production systems, and it is expected to become a critical part of more sustainable food production systems in the 21st century.

Waste-to-insect pairings

While the most commonly used species of bioconverters may be very suitable in some situations, one species cannot adequately capitalize on the immense diversity of food waste streams (Lardé, 1990; Smetana, Palanisamy, Mathys, & Heinz, 2016). Within the diversity of insects, there are undoubtedly species with specific attributes that make them uniquely suited as

bioconverters of a highly specialized food waste. To optimize pairings of insect species and food waste, one must consider a combination of abiotic interactions and functional traits of the insect for handling the waste. Abiotic attributes are non-living chemical and physical characteristics of the food waste (i.e. moisture content, phenolics, nutrient load, etc.). Whereas, functional attributes of insects include: feeding behaviour, morphology (i.e. large mandibles (mouth part) for masticating, soft bodies for moving through substrates, behavioural avoidance of poor egg laying sites), development time, ability to resist diseases, and a range of other attributes. It is the combination of these abiotic and functional attributes that allow some insects to be well suited for bioconversion of waste, while rendering others as maladapted.

For example, vegetative food wastes can be fed to both black soldier fly larvae and mealworm larvae (Li et al., 2013; Manurung, Supriatna, Esyanthi, & Putra, 2016), but this waste is too low in protein content for housefly larvae (Hogsette, 1992). Conversely, restaurant and kitchen wastes containing meat are well suited for housefly and black soldier fly larvae, but are too wet for mealworms, which can get moisture directly from the air and thus perform optimally in drier wastes (Cheng, Chiu, & Lo, 2017). Further, black soldier fly larvae tolerance of wet wastes and high temperatures (from bacterial and colony metabolism), allow them to capitalize on many waste streams (Table 1). But husbandry practices also require specific lighting for breeding, the flies are intolerant to temperature drops, and perform poorly in some low nutrient wastes (beet pulp (Smetana et al., 2016)). This combination of abiotic interactions and functional traits of the flies translate to actual economic trade-offs, as drying food wastes and using special equipment (lights) add costs to commercial operations. As such, considering appropriate waste-to-insect pairings is a significant component in using insects in food waste reduction. Table 1 illustrates examples of appropriate waste-to-insect pairings, while not an exhaustive list, it highlights the

extent to which more insects should be studied for their potential bioconversion performance. Table 1 also includes products of economic value generated from bioconversion, with the inclusion of less commonly used insect species.

Selective breeding

Due to their short life spans, high reproductive rates, and variable genetic expression, insect adaptation (evolution) may occur within economically relevant time scales (Jensen, Kristensen, Heckmann, & Sørensen, 2017). When adaptation is controlled by humans, the process is referred to as artificial selection or selective breeding, and will play an important role in developing/engineering insect lines for bioconverting specific food wastes (Jensen et al., 2017). For example, some by-products of food processing are high in plant defensive chemicals and are largely inedible. These "recalcitrant" food wastes may be high in tannins and phenolics (for instance, the chemicals partially responsible for the specific/unique tastes associated with wine, cranberries, coffee, chocolate, and cinnamon), and are difficult to bioconvert using insects. These chemicals are plant adaptations evolved to repel or even kill herbivores (van Dyk, Gama, Morrison, Swart, & Pletschke, 2013). However, studies focusing on insect-plant defensive interactions have demonstrated insects can be adapted to detoxify these chemicals (Carroll, Klassen, & Dingle, 1998; De Jong & Bijma, 2002). Using selective breeding, insects could be bred to overcome defensive chemicals found in recalcitrant wastes and thus allow for bioconversion of troublesome food wastes (e.g. wine and olive pomace). Other examples of insect breeding may include, improving germlines to increase yields of secondary products (oils and pharmaceuticals) (Li et al., 2012), larger body size (Jensen et al., 2017), and shifts to novel food sources (Alves et al., 2016).

Selective breeding in industrial mass production of insect occurs actively or passively (Jensen et al., 2017). Passive selective breeding involves permitting mated females to self-select waste oviposition (eggs laying) sites across generations. For breeding and bioconversion operations, female self-selection may pose a cost-efficient method for capitalizing on insect instinctive (innate) survival behaviours (Nansen et al., 2016). For example, silkworm "innate recognition templates" is programmed to specific chemical cues that indicate the best food for her offspring even after thousands of years of domestication (Garlapow, Huang, Yarboro, Peterson, & Mackay, 2015). Active selective breeding involves forming separate lines for each waste and using inbreeding, linebreeding, and outcrossing to control gene expression (Jensen et al., 2017). In general, active selective breeding requires more maintenance and containment, and therefore can be cost prohibitive for some operations. However, active selective breeding is more controlled, which may appeal to capital intensive operations, and it represents an opportunity to develop and commercialize specific insect strains. In conclusion, insect breeding for more efficient food waste reduction is still in a preliminary phase academically, which contrasts to the proprietary lines already used by commercial enterprises. Nevertheless, as businesses continue to develop around industrial insect production there will be more funding and research interest in advanced insect breeding programs.

Business Processes

Food waste may be viewed as a problem by some, but others view it as an appealing opportunity for business. The last two decades have seen an explosion of growth in businesses using insects to convert food waste (Table 2). Yet, businesses centred on the mass production of insects have existed for centuries (honey bees, silk moths, lacquer bugs) (van Huis, 2013).

Additionally, many businesses developed in the second half of the 20st century selling insects for biocontrol, medical research, and for supporting the pet trade (Ortiz et al., 2016). Drawing on research and methods developed for mass production of insects for other purposes, new companies are finding significant opportunities producing insects for feed and food. An indispensable component for these businesses is acquisition of inexpensive, abundant, and consistent sources of feedstock, and for many the preferred and economical choice is food waste.

In the following, we describe the basic design of mass production of insects for bioconversion, with different steps for producing valuable materials (Figure 2, steps 1-11). Operations begin with an incoming food waste feedstock (1). Food waste feedstock may require pre-processing before it can be used as feedstock for the given insects (2). Some pre-consumer food wastes like juice pulps are already processed and can therefore go directly into the bioconversion process. Once the feedstock is ready, insect inoculum is added either as eggs or as small immatures (3). For all insect species, most of the growth and bioconversion occurs during the immature stages. To optimize biomass production the ideal harvesting time is during late (well-developed) immature stages. Harvesting/extraction (4) may be done by mechanically sifting immatures from frass, however, some insects have self-extraction behaviours which allow them to be collected by controlling their evacuation routes. The sifted frass may then be further broken down via microbial decay (10) or mixed with additives and packaged as a fertilizer (7). Depending on the business, populations of extracted insects may be sold live (5) or further refined into valuable commodities such as biodiesel, de-fatted insect-meal, pharmaceuticals, etc. (6-7). In addition, each of these steps may require external inputs of electricity, water, labour, etc. (11) It should be noted that there is a range of opportunities provided within the production chain, from high-valued small-volume products to low-value bulk commodities. Below, we briefly review

some of the possible revenue streams from insect bioconversion systems.

Bioconversion to produce fertilizers

The chemical and physical properties of insect-frass used as a fertilizer is compatible to other commercial products (Salomone et al., 2017). For example, in one study the growth rate and chemical composition of cabbages grown using black soldier fly frass was identical to commercial fertilizers (Choi et al., 2009). Similarly, onion production was identical for both insect frass and compost amendments (Zahn & Quilliam, 2017). This may be due to the added ammonia (NH_4^+) from nitrogen in insect frass, which has been shown to increase five-fold relative to the non-fertilized plants (Green & Popa, 2012). In addition, benefits of insect-frass compost include reduction of pathogenic microbes and pesticides (Lalander et al., 2016), However, there are concerns that heavy metals may accumulate in the frass of some insects (Diener, Zurbrügg, & Tockner, 2015).

Bioconversion for biodiesel

Biodiesel is a promising non-fossil fuel, however, concerns about the resources diverted for its production have sparked debate over a reliance on oilseeds, which require large tracts of arable land and impact food prices. Insects are an alternative source for generating precursors for biodiesel (fats and oils), due to immature insect's predisposal for sequestering high energy fat prior to pupating into adults (Manzano-Agugliaro et al., 2012). In addition, food wastes that are naturally high in fat, such as palm oil cake and restaurant waste may be used as a feedstock with

the added benefit of reducing the food waste problem while generating sustainable biodiesel. The methodology for producing biodiesel from insects is similar to producing biodiesel from other biological fat sources (Figure 3) (Tyson & McCormick, 2006).

Fat contents harvested from insects vary between species, food waste source, and development stage – with the larval stage containing the highest fat content (Manzano-Agugliaro et al., 2012). The immatures of many species have fat contents above 25%, with some in excess of 77% (moth, *Phassus triangularis*) (Manzano-Agugliaro et al., 2012). Biodiesel yields can be doubled by first pre-extracting fats from the food waste, then feeding the post-extraction remains (solid residual fraction) to insect immatures that are later harvested (Yang et al., 2012; Zheng, Li, Zhang, & Yu, 2012). Examples of insects used for biodiesel production include; black soldier fly larvae with added microbes (Rid-X) to convert rice straw (30%) and restaurant waste (70%), producing 43.8 g of biodiesel from 1 kg of waste (Zheng et al., 2012); yellow mealworm larvae fed decaying vegetables and dry leaves, producing 34.2 g of biodiesel from 234.8 g of dried mealworm larval biomass (Zheng et al., 2013); yellow mealworms fed fruit waste and palm oil cake (Leong, Kutty, Malakahmad, & Tan, 2016); latrine fly larvae *(Chrysomya megacephala Fabricius*) and common housefly (*Musca domestica)*, fed restaurant waste were ~24% and ~20-35% oil by dry weight, respectively; flesh fly (*Boettcherisca peregrine*) fed solid residual fraction of restaurant waste (~31% oil by weight) (Yang et al., 2012). Finally, indicative of the interwoven utility of insect-based bioconversion, one study found waste corn cobs too lignified for direct consumption by black soldier fly larvae were first fermented anaerobically, then given to black soldier fly larvae to make biodiesel—resulting in 87 L of biogas and 3 g of biodiesel from 400 g of corncobs (Li et al., 2015). In conclusion, many steps in insect-based bioconversion of food

waste can be a used for extraction of fuel sources, providing an alternative to our finite fossil fuel resources.

Bioconversion for food and feed

Human populations are expected to exceed 9 billion before the next century, this will accompany a 60-70% increase in consumption of animal products (Godfray et al., 2010). Insect-based bioconversion of food waste has the potential for supplementing future protein demands and is an extremely underutilized resource (van Huis, 2013). As such, multiple agencies, including the FAO, EU, and USDA encourage the use of insect protein as a logical component for feeding future populations (FAO, 2017; Mlcek, Rop, Borkovcova, & Bednarova, 2014). However, despite their support, current legislative and oversite infrastructure are underdeveloped for human consumption (European Food Standards Agency [EFSA], 2015) (see section 6.0Regulations). Instead, insect protein is entering markets as animal feed, and a growing number of companies use food waste as the feedstock to sustain their operations (Table 2).

For animal feeds, the most well studied and commonly used species are black soldier fly larvae, house fly, mealworms, and crickets. Black soldier fly larvae are an especially lucrative feed source, rich in protein and fat, with faster development than other species used for bioconversion (Wang & Shelomi, 2017). When ground into insect-meal they may be used as a replacement for soya- and fish-meals in many animal feeds. Studies have shown that they are suitable for monogastric animals such as pigs, poultry, freshwater prawns, and some fish species, but not suitable for alligators, some frogs, or ruminants (cows) (Makkar, Tran, Heuzé, & Ankers, 2014). Larvae fed fish offal from processing plants were on average 30% lipid, of which 3% was

omega-3 fatty acids (St-Hilaire et al., 2007). Table 1 lists a wide range of food wastes used as feedstock for black soldier fly larvae (and other insects) processed into animal feed. A life cycle assessment from one pilot bioconversion facility employing black soldier fly larvae for food waste treatment found 10 megagram (Mg) tonnes of food-waste input, generated 0.3 Mg of dried larvae and 3.3 Mg of compost (Salomone et al., 2017). These results are consistent with figures provided from large full-scale operations such as Agriprotein and Nextalim (Table 2).

In animal production, comparison of inputs to outputs of mass is referred to as the Feed Conversion Ratio (FCR), with the inverse being the Conversion of Ingested food (ECI) (Waldbauer, 1968). Low FCR's indicate higher efficiencies and therefore conversion of the food waste into animal biomass. The literature on conventional livestock feed often uses the FCR, which we will also use to compare insects to other livestock. Studies have found the following FCR's for insects: black soldier fly larvae = 1.4-2.6, mealworms = 4.1-19.1, and crickets = 2.3-10.0 (Oonincx, van Broekhoven, van Huis, & van Loon, 2015). In comparison, conventional livestock FCRs are: poultry = 2.3, pork = 4.0, and beef = 8.8 (Wilkinson, 2011). This suggests, it takes a larger quantity of feed to produce a kg of beef or pork than it takes to produce a kg of insects. For example, if 100 kg of restaurant food waste was fed to black soldier fly larvae, chickens, or a cow, the food waste would yield 58 kg of black soldier fly larvae, 25 kg of chicken, or 2.9 kg of beef. It should be noted that the FCR of insects can be highly variable depending on the source feedstock and density of insect populations. However, using average FCR of black soldier fly larvae, we can assess how much income would be generated per unit of food waste. For example, assuming an FCR for black soldier fly larvae of 1.7, a filled refuse truck (21 m^3,) with 50 Mg of food waste, yields ~29 Mg of prepupae (62% moisture content), which can be dried into ~11 Mg of dry larvae (Diener, Zurbrügg, & Tockner, 2009). At the price of 995 €·Mg−1 (1131

$·Mg−1), this would yield € 11,000 ($12,500) (Salomone et al., 2017) each truckload. Insect-based bioconversion of food waste therefore is an appealing opportunity for producing marketable proteins, while simultaneously mitigating the negative impacts of food waste.

Regulations

Commercialization of output materials from insect-bioconversion requires a high degree of confidence in their safety. Due to the novelty of industrially mass-produced insects for food and feed, risks of associated contaminants entering the food chain warrant investigation and oversight. In anticipation of new products making their way into European markets, the European Food Standards Agency (EFSA) has published an opinion on the risk profile of insects as food and feed, concluding that food and feed products should pose no greater threat than products already on the market (EFSA, 2015). Further, the agency highlighted the need for continued research in microbial, chemical, and allergenic hazards, as well as impacts on processing, storage, and environmental hazards (EFSA, 2015). This has been welcoming news for stakeholders of insect derived products, demonstrating increased legitimacy and legislative consistency for the growing economic sector. However, significant legal hurdles remain, for example, the European Union prohibits insect-meal as feed for pigs and poultry, but not aquiculture (Regulation EC No. 999/2001), it is prohibited to use catering waste as feed stock (Regulation EC No. 1069/2009); and insects must be 'slaughtered' off-site (Regulation EC No. 1099/2002). In addition, the United States and European Union considers some insects as 'mini-livestock', thus affording protections against inhumane slaughter (Vantomme, Mertens, van Huis, & Klunder, 2012).

Research into the chemical safety concerns have been mostly positive, for example many insects accumulate chemical contaminants (pesticides, heavy metals, pharmaceuticals, dioxins,

and mycotoxins) below recommended maximum concentrations suggested by the European Commission and World Health Organization (Charlton et al., 2015; Lalander et al., 2016; Purschke, Scheibelberger, Axmann, Adler, & Jäger, 2017). However, examples of toxic heavy metal accumulation have been documented for house fly (i.e. cadmium) (Charlton et al., 2015), blow fly (*Calliphora sp.*) (mercury) (Nuorteva & Nuorteva, 1982), and black soldier fly (lead) (Purschke et al., 2017). Recommended measures ensuring end product safety include monitoring the food waste feedstock, as well as the insects produced (Purschke et al., 2017). In the case of microbial contamination, highly competitive 'pestiferous' species, such as black soldier fly secretes antimicrobial compounds into the wastes they feed in (Park, Chang, & Yoe, 2014; Sheppard, 2007). These secretions limit and can even prevent hazardous pathogens like *E. coli* and *Salmonella* in the waste (Erickson, Islam, Sheppard, Liao, & Doyle, 2004; Lalander, Fidjeland, Diener, Eriksson, & Vinnerås, 2015). These antimicrobial properties are highly beneficial for the bioconversion of municipal food waste, due to the wastes' heterogeneous states of decomposition.

Regulations on producing animal feeds were not designed with insect-meals in mind. As laws come under review, amendments likely will be added to permit more biologically informed oversight. Overall, insects used for food and feed is considered safe (Belluco et al., 2013). This is consistent with insects' role as an integral component of many animals diets, and humans long history of consuming insects both intentionally and inadvertently (Center for Food Safety, & Applied Nutrition, 1995; DeFoliart, 1992).

Conclusion

Insect-based bioconversion of food waste offers an exciting vision for a more sustainable future and for novel paths to sustainable food production and food security. Insect-based bioconversion is particularly exciting because it enables food and feed production in densely

populated areas (urban settings) and therefore goes against the common notion that urban development and food production are antagonistic. After many years of advocating the potential of developing industrial scale operations to tackle food waste (van Huis, 2013; Wang & Shelomi, 2017), insect-based bioconversion companies are now being established and their throughput is reaching scale, becoming profitable, and moving into international markets (van Huis, 2017; Joly, 2019). This next decade will see considerable growth in this sector, bringing jobs, novel commodities, new inputs to the food and feed supply, and ultimately reduction and reuse of food waste streams currently considered problematic. For this vision to materialize, research is needed to find more food waste-to-insect pairings, as well as selective breeding to develop specialized insect strains. Both are needed to increase capacity and to maximize the potential benefits of using insect-based bioconversion of food waste. Risks posed by the development of high-performance insect strains for food waste elimination, such as escape and introduction, are small, as many of the commercial insect species used for bioconversion are naturally occurring globally (mealworms, black soldier fly). Research is needed to bridge the gap between enterprises engaged in insect-based bioconversion and the regulatory agencies keeping us safe. More studies on the safety of insect derived products are likely to lead to biologically informed policy. With the proper checks, insect-based bioconversion of food waste has the potential to serve as a powerful tool to eliminate food waste, create jobs, and provide an environmentally friendly source of protein to help feed our ever-growing global population.

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Tables and Figures

Table 1: Some insects used for bioconversion, the different wastes that they can be fed, and the final products.

Species	Organic wastes	Country	Bioconversion output	Reference
Black Soldier Fly (*Hermetia illucens*)	Rice straw, restaurant waste (3:7)	China	Biofuel	(Zheng, et al., 2012)
	Rice straw	Indonesia	Biomass	(Manurung et al., 2016)
	Coffee pulp, husk	El Salvador, Indonesia	Biomass, fertilizer	(Lardé, 1990; Suantika et al., 2017)
	Waste from pears, banana and cucumber (5:3:2)	Sweden	Biomass	(Mutafela, 2015)
	Corn stover	China	Biofuel, soil amendment	(Wang et al., 2017)
	Corncob	China	Biofuel	(Li et al., 2015)
	Sorghum	United States	Biomass	(Tinder et al., 2017)
	Cowpea	United States	Biomass	(Tinder et al., 2017)
	Cassava peel	Indonesia	Biomass	(Supriyatna, Manurung, Esyanthi, & Putra, 2016)
	Vegetable trimmings, spent coffee grounds and tea leaves.	United States, Hong Kong	Biomass	(Cheng, 2016)
	vegetables, peels of yam, cassava, plantain	Ghana	Biomass	(Bonso, 2013)
Housefly (*Musca domestica*)	Restaurant waste (70%) Corn silage, sawdust (30%)	China	Biomass, biofuel, fertilizer	(Niu et al., 2017)
Codling moth (*Cydia pomonella*)	Starch and cheese wastewater sludge	Canada	Biomass	(Brar, Verma, Tyagi, Valéro, & Surampalli, 2008)
Cambodian field crickets (*Teleogryllus testaceus*)	Cassava plant tops, spent grain, mung bean sprout waste, field weeds	Cambodia	Biomass	(Miech et al., 2016)
Yellow Mealworm (*Tenebrio molitor)*	Wheat straw, bruised cabbage leaves	China	Biomass	
	Corn stover	China	Biofuel	(Wang et al., 2017)

Table 2: Examples of insect bioconversion companies. Reduction rates estimated from a Feed Conversion Ratio of 1.7 and 68% moisture content of extracted larvae.

Company	Food waste eliminated	Daily reduction (estimate)	Insects used	Products sold	Country	Founded (year)
Agriprotein	Pre- and post-consumer organic waste	72 Mg	Black soldier fly	Protein feed ingredient (MagMeal™), oil feed ingredient (MagOil™) and compost (MagSoil™)	South Africa	2008
Nextalim	100% traceable EU approved by-products	41 Mg	Black soldier fly	Insect fertilizer, black soldier fly larvae live or dried, black soldier fly larvae defatted proteins and BSF oil	France	2013
Alapre	Organic waste and animal by-products	24 Mg	Black soldier fly	Insect meal and compost sold under the trademark "ENTHOS"	Colombia	2014
Proti-farm	Vegetable by-products	confidential	Lesser mealworm + various	Various applications with focus on food: buffalo's frozen, freeze-dried, grinded (EntoPure)	Netherlands	1978
Entofood	100% vegetal by-products	240 kg	Black soldier fly	Whole insect meal, defatted insect meal, insect oil	Malaysia	2012
Insectum	Former foodstuff including milk and eggs	22 kg	Black soldier fly	Black soldier fly larvae frozen, dried and/or defatted	Lithuania	2016
Nextprotein	Organic inconsumable food matter	4.5 Mg	Black soldier fly	Insect protein meal, oil and fertiliser	France/ Tunisia	2015
Hermetia	Bruised rye	4.3 Mg	Black soldier fly	Dried larvae, insect meal, insect oil, fertiliser	Germany	2006
F4F	Preconsumer organic waste	1.3 Mg	Black soldier fly	Starter feed functional ingredients (fish and poultry), exotic pet snacks, fertilizer and feed development trials	Chile	2014
Innovafeed	Cereal by-products	1.1 Mg	Black soldier fly	Defatted meal and purified fat of black soldier fly larvae	France	2015
Enterra feed	Pre-consumer recycled food products	confidential	Black soldier fly	Whole dried larvae, meal and oil	Canada	2007
Ynsect	Vegetal material only, like cereal by-products	confidential	Mealworm	Protein, oil, frass	France	2011

Figure legends

Figure 1: Two adult black Soldier flies. Adults live only a couple of weeks, while they mate and lay eggs (a). Black soldier fly larvae on restaurant waste (b). Once growing to their full size, larvae exhibit self-extraction behaviours and move away from their food source.

Figure *2*: Typical business process for insect-based bioconversion of food waste. Note that value can be extracted from both the elimination of waste, and downstream materials, such as, insect protein (biomass), oils, frass, and pharmaceutical ingredients. Image is modified from an original design by www.eawag.ch/ and licensed under (CC BY 2.0).

Figure 3: Representative process for production of insect biodiesel.

Figure 1.

Figure 2.

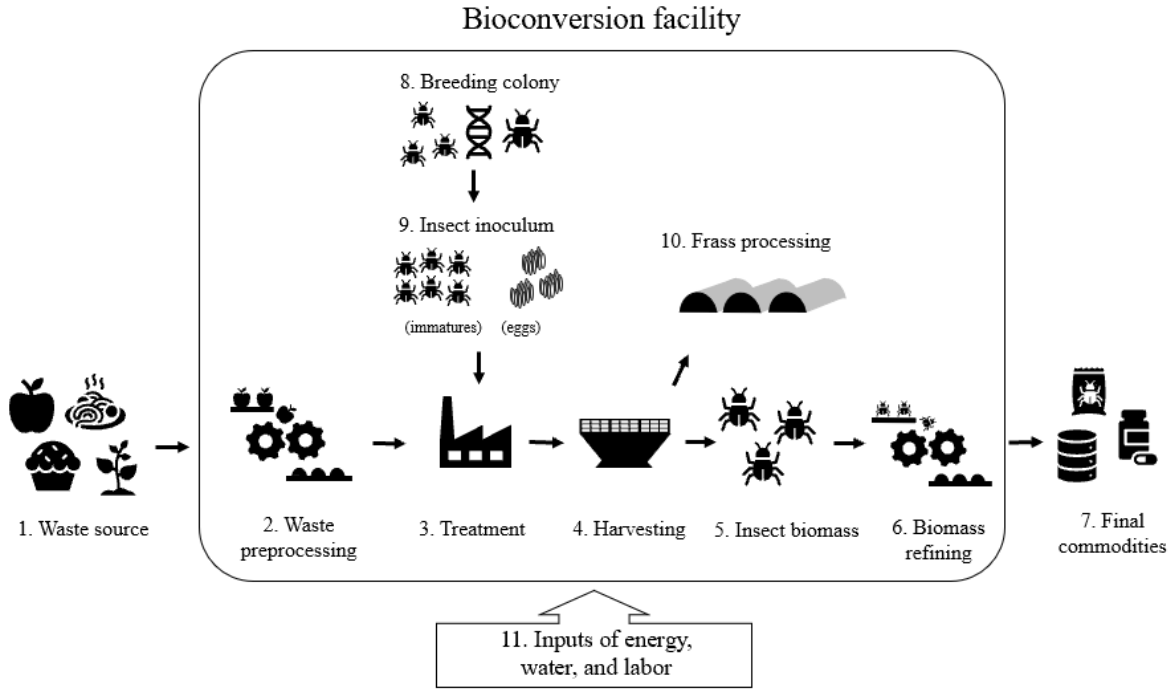
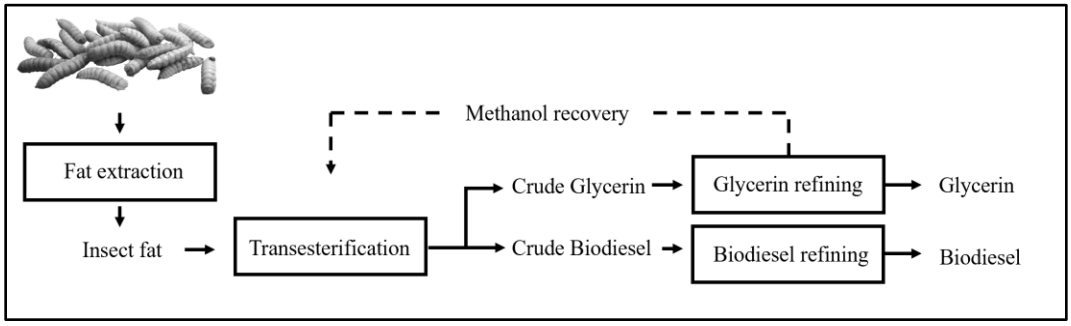

Bioconversion facility
8. Breeding colony
9. Insect inoculum
(immatures)
(eggs)
10. Frass processing
1. Waste source
2. Waste preprocessing
3. Treatment
4. Harvesting
5. Insect biomass
6. Biomass refining
7. Final commodities
11. Inputs of energy, water, and labor

Figure 3.


Methanol recovery
Fat extraction
Insect fat
Transesterification
Crude Glycerin
Crude Biodiesel
Glycerin refining
Biodiesel refining
Glycerin
Biodiesel

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Chapter 2: Artificial selection of insects to bioconvert pre-consumer organic wastes. A review

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Abstract

As the human population continues to grow, so to do the concerns regarding the sustainability of waste management from our food production systems. Faced with limited environmental resources for food production, issues related to food loss and waste are critical in mitigating challenges stemming from projected population growth and long-term food security and sustainability. The potential for using insects to consume organic waste materials and convert them into feed for animal, biofuels, and other valuable secondary products is gaining momentum as both a research discipline and as a business opportunity. Here, this ecosystem service is referred to as "insects as bioconverters of organic waste". Scientific reviews of this topic have mainly focused on the challenges associated with development of commercial scale systems. Here, we address this exciting topic from an artificial selection perspective, as we review and discuss aspects associated with targeted breeding and adaptation of both gut microbial communities and host insects themselves. We describe the "ideal insect bioconverter", insects uniquely equipped to convert wastes into biomass and other valuable secondary products, and we present the current knowledge and existing research gaps towards the development of such organisms. We conclude that: 1) Targeted breeding of insects and their gut microbes can produce tailored insect lineages for bioconversion of specific waste streams. 2) Research is needed to take full advantage of the existing insect diversity to identify new candidate species for bioconversion. 3) Further research into insect-gut microbial complexes will likely provide important insight into ways insects can be used as sustainable bioconverters of highly specialized waste streams.

Keywords: Bioconversion; sustainable agriculture; breeding; adaptive plasticity; entomophagy; microbiome; industrial entomology; organic residues; food waste

1 Introduction

1.1 The problem

It is estimated that by 2030, United States agricultural production and industrial processing of food will generate between 145-602 gigatons of organic waste annually[1,2]. Assuming a population of 359.4 million[3], this amounts to approximately 4.5 kg per day per person. Food wastes are often differentiated as either pre- or post-consumer waste, with the former including waste streams derived from losses incurred during growth, harvest, transport, processing, and storage[4]. Conversely, post-consumer wastes are derived from losses incurred at the consumer level, including over- or inappropriate purchasing, storage, preparation, portioning and cooking[4]. While post-consumer food waste is certainly a global concern, this review treats only pre-consumer organic wastes, as they are often less covered in reviews of insect bioconversion[5–8], do not have the same regulatory and health concerns[9], and are more chemically and physically diverse[4]. Specifically, pre-consumer organic wastes are byproducts in the food supply chain based on materials not designated for human consumption, and they include: 1) Non-marketable but edible food (damaged and misshapen). 2) food spoilage at production sites. 3) Byproducts from primary food processing, including: stems, leaves, hulls, seeds, skins, and pulps generated from cleaning, de-hulling, pounding, grinding, packaging, soaking, winnowing, drying, sieving, and milling. 4) Byproducts from secondary food processing- the cuttings, crumbs, and remains generated from mixing, cooking, frying, molding, cutting, and extrusion[4]. 5) Non-food post-harvest byproducts associated with orchard and field crops- the chips, slash, wood, fibers, and stovers[10]. (Fig.1)

Each combination of crop and its method of production, processing, packaging, storage, and distribution generates a unique set of organic wastes. For example, residuals from pre-consumer processing of fruit and vegetables for juice can include leaves, peels, pulps, and seeds[11], each with

different chemical and physical properties. With over 6000 crop species in production globally[12], and a wide range of processed goods, the diversity of organic waste streams is immense[3]. Despite growing legal restrictions, some pre-consumer organic wastes are still disposed in landfills and considered a problem rather than an economic opportunity[13]. Disposal of pre-consumer organic wastes in compost and landfilling operations generate considerable greenhouse gas emissions and other environmental pollutants[14,15]. Therefore, developing innovative ways to use pre-consumer organic wastes is important for reasons of materials efficiency and product development as well as pollution prevention and economic gain. Currently, approximately about 3.73 billion hectares, a staggering ~75% of the planets arable land, is dedicated to livestock grazing[16], and the demand for meat expected is to grow 58% by 2050[17]. Consequently, there is a dire need for alternative sources of proteins and fats to meet the growing human demand. Insects are a logical and proven choice and focus if the goal is to develop more sustainable waste management practices. Moreover, pre-consumer organic wastes can be consumed as a feedstock by insects, which 'bioconvert' the waste into valuable products[6,18–20].

1.2 The solution

The production of pre-consumer organic wastes may be considered a waste problem, but they also represent potentially significant resources and business opportunities due to their richness in nutrients and active compounds[21]. An illustrative example of this type of transformation is how whey protein from cheese production represented a major problem for the diary industries up until the 1980's, with farmers paying for disposal or reuse as fertilizer. In recent years, the protein powder industry has recognized the value of whey and is now willing to pay for this high-value protein source. Moreover, novel markets and industries may emerge through innovative utilization

of existing organic "waste products" and in the process eliminate waste streams and create jobs and industries. Other examples include use of organic wastes as substrate for mushroom production, compost, energy production, or fillers in animal feed (e.g. insect biomass)[5,13,22–26]. Similar to conventional livestock production, the insects themselves can be commercialized as bulk biomass to be added to animal feed or human food, and/or specific compounds can be extracted from their biomass for industrial, pharmaceutical, or energy (biofuel) purposes, such as, proteins and fats [5,6,27]. In addition, the left-over material [insect molts and feces (frass) and left-over waste material] may be processed and commercialized as high-value soil-amendments. Current insect bioconversion facilities have the capacity to accept as much as 250 tons of food waste per day (www.agriprotein.com), so development and adoption of insect-based waste management solutions is not a thing of the future but unfolding and gaining momentum. A crucial aspect of large-scale use of insects as bioconverters of pre-consumer organic wastes is their "bioconversion rate", which is a quantitative measure of the input:output ratio[28–30]. The bioconversion rate can be measured based on a number of variables, including energy, protein, fat, and a low bioconversion rate implies high efficiency. In livestock nutrition, it is common to calculate the bioconversion rate based on the nutrient or energy content of feed material compared to the nutrient or energy content of meat or milk produced[29]. Such a calculation is partially incomplete, as considerable energy, fertilizer, labor, and other inputs often were used to produce and process the feed materials. We are unaware of any direct comparisons of bioconversion rates of insects and typical livestock animals. That is to accurately compare their conversion rates, the exact same feed material should be given to insects and, for instance, cows or chicken, and their growth in biomass as well as their production (eggs and milk) should be quantified. Without such true comparisons, it is difficult to accurately compare bioconversion rates. Regarding conversion

rates of insects versus traditional livestock, it is also important to emphasize that entire insect bodies can typically be used, while principally only the flesh from vertebrate livestock is commercialized as food. Thus, the proportion of usable biomass (compared to bones, hides, internal organs, etc. in vertebrate livestock) is generally markedly higher for insects. Finally, the protein content of insects, such as houseflies (*Musca domestica*), mealworms (*Tenebrio molitor)*, and crickets (*Acheta domesticus*), is typically 40%-70%[31]. For comparison, the protein content of a whole chicken or cow is typically ~55%, respectively ~40%[32]. Thus, from a bioconversion standpoint, there are strong arguments for focusing on insects as bioconverters of our current and future pre-consumer organic wastes.

In this review, we argue that development, use, and commercialization of tailored/customized insect-microbial systems to specific pre-consumer organic wastes is at the brink of becoming a serious and profitable business sector and productive research discipline. Moreover, we show that insects (and their gut microbials) can and will play a major role in the development of sustainable management plans for pre-consumer organic wastes. We review this exciting area from the perspectives and applications of evolutionary and ecological theory to insect breeding.

Artificial selection

Natural selection may be defined as the process, in which variable and heritable fitness-promoting traits are selected for within a population of a given species to increase the fitness of individuals in the following generations[33]. In nature, complex community interactions drive natural selection, and these interactions are underpinned by spatio-temporal dynamics of the given environment. Consequently, “artificial selection” of insects is defined as deliberate anthropogenic control and manipulation of selection forces to promote a particular evolutionary outcome (optimization of an

insect population to serve as bioconverter of a specific organic waste product)[34,35]. While modern phenotypes (observable traits) of only a few insect species are regarded as the outcome of artificial selection (i.e. domesticated honey bees (*Apis melifera* L), flightless mulberry silkworm (*Bombyx mori* L), and resinous lac bug [*Kerria lacca* (Kerr)][36]), the potential of artificial selection to improve insect lineages has been discussed for decades[37]. In addition, this endeavor is greatly facilitated by copious research and development in the mass rearing of insects[38], with notable examples including production of sterile insects and natural enemies for biocontrol[39], production of medically important species for research, and insect biomass for animal and human consumption[6,40]. However, the recent recognition of insects as potential bioconverters of pre-consumer organic wastes is a new and exciting area. Moreover, progress in use of insects for bioconversion of wastes will benefit, if mass rearing insects is viewed through a particular lens[41], in which evolutionary processes and gut microbe–host interactions play a major role.

1.4 The "ideal insect bioconverter"

As decomposers and herbivores, the diversity of insect species includes groups that are highly specialized in their ability to thrive on different organic substrates and under specialized environmental conditions[42]. Moreover, some natural host substrates resemble pre-consumer organic wastes, in terms of moisture content, digestibility, and nutritional composition[18]. In addition, insect functional diversity (the behavioral and the ecological services they provide) can be exploited to substitute mechanical and/or chemical steps in conventional waste processing[43], such as, using beetles larvae maceration to feed around and remove the seeds. Insect species that exhibit innate biological compatibility with target pre-consumer organic wastes, and/or possess an exploitable functional service, can then be further improved via artificial selection (targeted

breeding). In this way, specific insect species with distinct traits (i.e. physiological, microbial, behavioral, etc.) can be bred to function as the "ideal insect bioconverters" for a target waste stream.

Here, we consider candidate "ideal insect bioconverters", as those that possess as many of the traits listed in Table 1 as possible. Certainly, no incipient bioconverter species or population will possess all these traits initially, but a strain of insects subjected to targeted breeding may ultimately gain a unique potential for bioconversion of a particular waste stream at a large scale[41]. Considering the sizeable literature on insects undergoing rapid adaptation in nature, including adapting to new foods[44,45], ecological communities[46], pesticides[47], and experimental evolution in the laboratory[48], it is reasonable to predict targeted breeding programs could rather rapidly and cost-effectively yield new and significantly improved bioconverters in manageable and economically practicable time frames.

Mealworms and experimental units

1.5 Insect species currently used as bioconverters. At present, only a handful of insect species are used for bioconversion of organic wastes, with the most represented species being[49,50]: crickets, locusts *Locusta migratoria* , black soldier flies *Hermetia illucens,* green bottle flies *Lucilia sericata*, and several mealworms species, including the yellow mealworms *Tenebrio molitor* (see Table 2 for an extended list). Research on the growth performance and feeding conversion of these species suggests they alone are not sufficient to fully capitalize on the high diversity of unique organic wastes available for bioconversion. For instance, the most utilized bioconverter, the black soldier fly (Fig 2), has a well-documented capacity to break down wastes[5,51–53], which evolved in the context of feeding on nutrient-rich decaying biomass. However, studies have shown that black

soldier flies are only marginally-suited for bioconversion of low-nutrient fruit and vegetable pulps[18]. Likewise, researchers found markedly different performance in feeding efficiency and growth rates of three mealworm species, which were reared on four different organic waste diets of variable starch and protein composition[54]. The authors concluded that certain diets may be unsuitable for mealworms due to a lack of essential nutrients, and that mealworms reared on high starch diets (49.8% starch; 10.7% crude protein; 1.8% crude fat) had the lowest growth and waste conversion rates.

Mealworms and experimental units

1.6 The role of gut symbionts. An important consideration in the pursuit of ideal insect bioconverters is the prospect of incorporating modern invertebrate microbiome research into targeted breeding programs of ideal insect bioconverters. Studies have shown that invertebrate symbionts interactions are hyper-diverse and critical in facilitating host exploitation of food resources[55,56], and that gut symbiont community structures correlate with the chemical composition of the host's food source[57]. For instance, in multiple insect species [including fruit flies (*Drosophila spp.*), indianmeal moth (*Plodia interpunctella),* gypsy moth (*Lymantria dispar*), and German cockroach (*Blattella germanica*)] there is a relationship between protein content in an insect's diet and the hosts bacterial diversity[58–61]. While insects are generally considered to be less symbiont rich compared to other animals, such as vertebrates, polyphagous insect species have higher symbiont species richness compared to specialists[55]. One hypothesis possibly explaining the difference in gut symbiont diversity suggests diverse diets do not require particular symbionts, and therefore polyphagous hosts benefit from the diversified metabolic capabilities provided by a wider array of symbionts[59]. From the perspective of developing ideal bioconverters, monitoring

the microbial diversity developing within insect-to-waste pairings will be of high value in the pursuit of optimizing insects as bioconverters.

Experiments discerning how direct manipulations of a host's gut symbiont community alter host performance and efficiency in bioconverting biomass may yield valuable insight into the bioconversion potential of particular interactions[62,63]. Several strategies may be deployed for direct manipulation of gut microbe–host interactions. First, facultative gut symbionts can be transferred horizontally between target bioconverters, to aid in modulating immunity or accessibility of essential amino acids[64]. Second, organic wastes may be inoculated with beneficial companion bacteria. This practice is already used in part to induce oviposition in black soldier fly, where bioconverted substrate is added to fresh media to make an attractant for gravid females to lay eggs[65]. Likewise, agar inoculated with the bacteria isolated from black soldier fly leads to higher rates of female oviposition[66], suggesting volatiles emitted from the microbiota of conspecifics mediates oviposition. While these techniques are not a direct manipulation of the gut-symbionts per se, cues from the bacteria inform female flies of substrates with microbial communities favorable for larval growth. For example, when chicken manure is inoculated with black soldier fly companion bacteria, the adult body length increases, while the development time from hatching to 90% reaching the prepupual stage is reduced by ~5 days (29.00 ± 1.00 d vs. 34.33 ± 3.51 d)[67], both valuable improvements for insect bioconversion enterprises. Finally, as interest in bioconversion advances, a bioconverters symbiont community may be manipulated by inclusion of genetically modified symbionts added for custom-made bioconversion applications. To our knowledge, this final strategy has not yet been used in insects used as bioconverters of pre-consumer organic wastes. However, the strategy has been used to reduce transmission of diseases by biting insects[68], as well as to introduce transgenic gut symbionts to an entire termite colony

from only a few initially inoculated individuals[69]. One could imagine how engineered microbes, perhaps capable of synthesizing more complete amino acid profiles, may assist and add value to insect's bioconverting nutrient deficient pre-consumer wastes, such as almond hulls or tomato pomace. In summary, insect-based bioconversion of pre-consumer organic wastes will benefit from comprehensive strategies, those using microbial surveillance and direct manipulations, that incorporate both the health and composition of insect-symbiont relationships. Furthermore, knowledge derived from livestock breeding and other disciplines will be of tremendous value in this effort.

1.7 Bioconversion outputs

A detailed review by Makkar (2014) cites numerous studies of the chemical constituents of insect meals derived from various pre-consumer organic wastes, and lists the insect meals' nutritional value when consumed by different animal species.[31] In addition, many life cycle assessments and protocols have been developed for these insects species for use as animal feed or secondary products (i.e. pharmaceuticals, lubricants, biodiesels)[38,41,49]. Table 2 includes a compiled review of organic wastes and bioconversion outputs for the most commonly cited bioconverting species, as well as other less commonly cited insects.

Substantive gains from artificial selection and discovery

2.1 Mining insect diversity

Insects are the most hyper-diverse grouping of animals on the planet[70]. Recent estimates put the number of described species at over 1 million[71]. Half of this diversity is captured within the groups

containing the most commonly cited bioconverting species (beetles: 386,500, flies: 155,477, butterflies and moths: 157,338, grasshoppers: 23,855, cockroaches: 7,314). Intuitively, most species will not be enlisted as bioconverters, but use of insects for bioconversion of waste material is a rapidly growing industry, and interest in finding new applications for waste valorization and subsequent sources of sustainable proteins warrant experimentation into new insect-to-waste pairings[72].

Taking into account the remarkable diversity of insects capable in providing bioconversion services versus the dearth of species conventionally used[73], further investigation is warranted into research of additional insect species to assess their potential performance as bioconverters. Such future research will likely elucidate not only additional candidate species for waste bioconversion, but it may lead to identification of exploitable enzymes and microbial symbionts facilitating organic waste bioconversion[72], yielding unforeseen economic and societal benefits[74]. An obvious concern is the rapid decline in insect biodiversity[75]. That is, specialized insect species with unique adaptations to certain host materials that resemble certain pre-consumer organic wastes may be harder to identify, if the current decline in insect biodiversity continues.

2.2 Breeding program design

One of the first considerations when beginning a targeted breeding program for a specific insect bioconverter is to standardize rearing conditions[39,95,96]. This ensures that the phenotypes being quantified, and resulting selection decisions, are the result of genetic difference between individuals and not the environment. Consistency is critical for the program to be reliable and effective, as genetic variation can be masked by environmental influences[78,79]. Moreover, the environment in which breeding trials are performed should be similar to the environment where

large scale bioconversion will take place[80]. For example, in black solider fly bioconversion, a local Chinese strain outperformed foreign strains in both their bioconversion and weight gain efficiency[81], suggesting their adaptations to the local environment impacted bioconversion performance. Similarly, bioconverters express different growth rates and nutritional quality depending on the food waste[54,82,83].

Drawing on practices used in livestock and aquaculture, several breeding program designs may be used to breed insects for bioconversion, including the tandem selection method, independent culling levels, and index selection[76]. Each method's relative efficiency depends on selection intensity, number of traits under selection, the traits relative importance, heritability, and a traits genetic correlation to other desirable traits[84]. Tandem selection selects for one trait per generation, which may alternate between several traits of value. Independent culling selection selects for two or more traits each generation, where individuals meeting or exceeding a measured threshold are permitted to breed. Index selection calculates the estimated breeding value of individuals, pairing couples with high predictive value. Each method comes with its own value and potential drawbacks, generally dictated by their cost and time efficiency. Traits that may be targeted for selection include life history traits (such as fecundity, time to sexual maturity, diapause duration, adult lifespan, etc.), ecological traits (such as tolerance to pathogens or parasites, or stocking density performance) economically relevant traits (fat content, protein content, consumption rate, etc.), and usage and safety traits (such as toxin sequestration, allergenicity). Table 1 provides a list of the many traits that may be targeted for selection, priority in ranking these traits is defined by the breeding programs goal and method of selection.

The tandem method selects for one trait per generation (Fig. 3a), and alternates between one of two (or more) traits each generation. However, selection for a particular trait may continue

for several generations before switching to the other trait[85]. While simple and cost effective, this method is considered inefficient due to: 1) selection pressure is relaxed when moving to subsequent traits, and 2) less heritable or economically valuable traits may undergo selection for too few (or too many) generations[86]. However, in some contexts tandem selection may be useful for traits with high heritability[87].

Breeding programs using the independent culling method select for 2-3 traits at once, setting minimum limits for the phenotype of each trait. Individuals falling below these limits are culled from the breeding population, while those reaching or exceeding certain thresholds are mated. This is repeated for each cycle of breeding. Fig. 3b shows selection for two traits; 'days to pupation' (x axis) and 'larval weight' (y axis) for black soldier fly. Note that individuals with high-performing phenotypes for one trait may be culled if not exceeding the threshold for the second trait. Large numbers of individuals are culled, when minimum thresholds are set too high. This should be avoided, as too strong a selection intensity will deplete the genetic diversity of the breeding stock, and slow improvement of target phenotypes. This is not unlike challenges found in conservation biology, which implements the '500' rule, a theoretical minimum viable population size, which balances allelic drift and mutation[88]. Later reviews on minimum viable populations have placed suggested populations at approximately 5000 individuals to be sufficient in preventing the loss of quantitative genetic variation[89]. For insect breeders, this is easily obtainable by leveraging the prolific reproduction of insects to maintaining large colonies during selection. For example, black soldier fly are very fecund, with an average 998 eggs per mass[90]. Therefore, larval colonies, with populations of many hundreds of thousands may be subjected to selection pressures leading to a final breeding colony of ~10,000 individuals. In addition, black soldier fly have relatively large genomes compared to other flies[91], suggesting ample genetic

material for selection[92]. This point is also exemplified in red flour beetles (*Tribolium castaneum*, a relative of mealworms), which exhibited little decrease in genetic gain per generation when selected for pupal weight over 120 generations, eventually accumulating a weight increase 17 standard deviation units from the source population's mean[93]. By leveraging these aspects of insect biology, breeders may find independent culling a relatively easy and productive method to implement compared to other methods like tandem selection and index selection (below).

The index selection method selects for multiple traits each generation, and unlike the other two methods, can be used effectively with more than three traits. Index selection incorporates estimated breeding values (EBV) for multiple traits into a single index of values that are used in making selection decisions. EBV's are multiple regression predictors of an offspring's performance, and are calculated from observations of an individual, or its relatives. Calculating a selection index requires information on genetic correlation, heritability of traits, and the economic value of the phenotype. Unfortunately, these are not well-defined for insect bioconverters considering the 1) correlation of traits and trait heritability need to be resolved for bioconverter species, and 2) uncertainty of regulations and regional markets affect economic values of the phenotypes[31]. However in theory, index selection is never less efficient than independent culling[86], though in some cases it may be no more efficient. Consequently, it is the most used selection system in animal and plant breeding[76,94]. For this to be applied to insect bioconverters 1) meaningful phenotypes need to be measured using standardized data collection methods; 2) economic weight needs to be placed on each phenotype; and 3) logistical frameworks need to be developed for the husbandry of numerous crosses and their subsequent progeny.

2.3 Adaptive phenotypic plasticity

Phenotypic plasticity is the deterministic genetic expression of observable traits (phenotypes) resulting from an organisms genes (genotype) in response to its environment[95]. The same genotype may produce different phenotypes under different unique environmental conditions. Phenotypic plasticity includes changes to an individual's physiology, morphology, behavior, or life history[79]. These changes counter environmental variation to alter fitness either within or between generations[95,96]. Moreover, phenotypic plasticity is ubiquitous across living sexually-reproducing organisms and thought to be commonly adaptive in insects [95].

Quantitative genetic models treat an organism's phenotype (P) as the product of its genetics and the environment (G × E) (see Fig. 4), and genotype-by-environment interactions are well studied in insects[54,79,97]. Likewise, the quantifying genotype by environment are of great interest to insect breeders, because unlike traits emerging from genetic evolution, trait variation due to phenotypic plasticity is not heritable, though it maximizes fitness in variable environments. This is important for insects used in bioconversion, because the transition from ancestral food sources to novel diets of pre-consumer food waste may not necessarily be accompanied with the genes conferring high performance for the new waste. Insects' plastic responses allow them to bridge the gaps temporally, while the adaptive genes for the novel diets of pre-consumer organic waste accumulate in the population. Thus, the ability to convert a new food resource may increase over generations, but often at the expense of adaptation to the ancestral diet (Fig. 5). Some examples of insect adaptive phenotypic plasticity in response to food quality include black soldier fly larvae adjusting energy budgets to prioritize growth and metabolism in response to a diminishing food source[82]. Another example is grasshoppers (*Schistocerca americana*) increasing the relative number of sensory hairs (sensilla) when fed diets supplemented with volatile compounds[98].

Deterministic expression of traits in response to the environment result in trade-offs during development but diversify populations' available evolutionary trajectories[79,99]. Thus, the increased allocation of resources for growth and metabolism in the black soldier flies comes at the cost of self-maintenance, such as, supporting a robust immune defense[100], or increased reproduction[101].

Adaptation via phenotypic plasticity plays a major role in insect development and evolution, and it should be considered an integral component of insect-based bioconversion programs[41,102]. Moreover, insect breeders should assess a population's response to environmental conditions to better select stock for different environments or for robust tolerance to environmental variation. Phenotypic plasticity in insects used for bioconversion needs to be carefully considered for the following reasons: 1) to determine populations variation in response to environments, (e.g. novel food wastes, biotic and abiotic factors), 2) to leverage parent bioresponse and offspring imprinting to identify and amplify better equipped populations. Such as using female oviposition preference between wastes to screen for potentially preadapted offspring, 3) to develop monitoring programs as a means of quality control to assess if variation is due to plasticity, or genetic gains as a result of the breeding program.

2.4 How to Monitor and quantify adaptive phenotypic plasticity

Phenotypic plasticity is measured using "variance partitioning", quantified by the deviation of traits from the mean, for genotypes across different environments[103]. Experimental designs compare individual responses to controlled environmental treatments, using individuals of close relatedness (i.e. full-siblings, clones, back-crossings, etc.), thereby reducing observed variance due to genetics[45,104]. Results are graphically presented as the "reaction norms", which plot plastic responses (e.g. behavior, survival, fecundity, consumption rate, etc.) across multiple

environmental treatments[105,106]. For example, Fig. 5 illustrates variable adaptation of two populations of insects reared in two different environments, in this case diets of either a native or introduced plant species. Here, wild-type insects are far more likely to survive on the native host plant compared to the introduced host species. Conversely, derived-type insects, adapted to and feeding on the introduced plant species perform poorly on their ancestral host. The transition from wild-type to derived-type appears to have naturally occurred over only a few decades[45]. This method of reciprocal rearing therefore may be used to elucidate differences in performance for environments these insect encounter. Similar reciprocal rearing experiments will be used to monitor and quantify the gradual adaptation from breeding of candidate insect bioconverters to a novel target waste stream[107,108]. For example, artificial selection for increased thorax length in fruit fly, and thus larger body size, has been shown to correlate with a drop in larval survival at higher larval densities[109]. Reciprocal rearing for a genotype's response across multiple environments (i.e. stocking densities), will inform how artificial breeding may be shifting optimum rearing parameters of bioconversion operations.

Interestingly, breeders may want to target insect's plasticity itself, whereby treating the robustness or plastic response to environmental conditions becomes part of the breeding program's goals[102]. Some pre-consumer wastes are relatively homogenous and may be bioconverted under highly controlled conditions, thus permitting a more robust phenotype (less plastic) to be sufficient for valorization. However, many wastes undergo a succession of microbial colonization when bioconverted by insects, leading to wide shifts in the temperature and moisture content of the substrates as bacteria and fungi reproduce and metabolize nutrients[110]. Additionally, some models for insect bioconversion have breeding and egg production facilities far from the location bioconversion actually occurs, necessitating insects to

tolerate not only variable environmental conditions, such as temperature and humidity, but also differences in regional crop varieties, which may differ in nutrient quality[111]. Viewed in this way, the plasticity across environment itself may be treated a component within the Estimated Breeding Values used in index selection[112]. In this way, breeding program objectives may be set to maximize phenotypic responses across environments. For example, consider the combined selection for both larger body size and fat content, but under conditions of variable stocking densities. Lower stocking densities permit les per capita competition for resources, and thus a larger body size with more fat content. Here, the increase in fat is presumably the result of larger body size, which comes from the greater availability to food at lower stocking density (i.e. scaling effects). However, from the breeder's perspective, greater food availability should preferably result in insects of the same size, but with their greater fat content being the result of genetic gains rather than environmentally determined plasticity. As previously mentioned, some insects artificially selected for increased body size also experience a drop in larval survival at higher stocking densities[109]. Thus, a breeder may choose to maximize fat content across environments (stocking densities), thereby increasing the output of fat genetically regardless of environmental influences pre-determining size.

Assessing phenotypic plasticity in large scale breeding and bioconversion operations may be economically prohibitive, therefore indirect methods should be used. One approach for capitalizing on the adaptive nature of individuals plasticity is "following the bioresponse" of gravid females (i.e. oviposition preference). For example, vegetable leafminer females (*Liriomyza sativae*) collected on cowpea and tomato show no preference for oviposition on either host when presented each host singly in 24-hour trials in alternating order, and average larval performance (pupal weight) does not differ between hosts[113]. However, individual performance of larvae

relative to siblings' positively regresses on the decision of the mother. Meaning, mothers' preference at the individual level produces offspring better suited for that host, even if the mean oviposition preference suggests no difference. Large scale breeding and bioconversion operations allowing gravid females to self-select may pose a cost-efficient method for capitalizing on adaptive phenotypic plasticity. Furthermore, insects' natal experience has been found to influence later generations' preference for suitable environments. For example, that gravid flies unable to find familiar host plants will oviposit on novel hosts and produce offspring that imprint on the new host that seek these new plants over the ancestral host as adults[114]. This cycle of gravid female bioresponse and natal experience imprinting happens passively in many insect colonies, allowing the population to adapt to their artificial environment[81,107,115,116]. To develop stock for multiple food wastes or stock with robust tolerance to variation, we suggest active monitoring and experimentation on colonies phenotypic plasticity over time.

3 Conclusions

Governmental agencies across Europe, North America and elsewhere are increasingly advocating zero-waste programs, colloquially referred to as circular or bioeconomies[117,118]. A primary challenge in developing such zero-waste programs center on waste disposal and re-use (i.e. recovery of nutrients and valuable compounds)[119]. Conventional sustainable practices, such as composting and biorefining, should include insect bioconverters as mechanisms for managing large quantities of organic waste[19]. Many countries worldwide have active research programs into insects as bioconverters and private companies developing large-scale facilities. Optimization of insects as bioconverters will greatly benefit from ecologically and biologically informed insect-to-waste pairings and the subsequent improvement on insect strains through breeding. Such ventures

will drive novel research and the development of new economic opportunities. Several breeding methods exist for achieving those breeding goals, with tandem and independent culling offering quick and easy improvement of limited traits. Later, more sophisticated and capital-intensive breeding programs will overcome nascent technical and biological obstacles inhibiting breeding, likely leading to the development of selection indices and genome-based selection. Ultimately, visions of a zero-waste future will include insects as waste bioconverters at an industrial scale, with the societal dividends of a plentiful source of proteins for animal feed, as well as lucrative downstream secondary products.

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Tables and Figures

Table 1. *Traits of ideal insect bioconverters*

Biology	*Physical*	High consumption rate
		Rapid development
		Large bodied (at harvest)
		Fecund
		Moisture and heat tolerant
	Ecological	Polyphagous
		Communal
Rearing	*Maintenance*	Operationally scalable
		Large colonies easy to maintain
		Easy to rear/cultivate
		Multiple stages feed on same diet
		Low/negligible susceptibility to diseases
		Low/negligible susceptibility to parasites
	Processing	Life stages easy to separate
		Self-removing behavior and/or easily extracted
Usage	*Functional service*	Mechanical separation
		Toxin sequestration
		Consumes lignin
	Active compounds	High nutritional value
		Rich multiple valuable compounds (lipid, protein, chitin)
		Frass is of value
Safety	*Human*	Hypoallergenic
		Easy to handle/docile
		Not prone to escape
		Does not sting/bite
		Does not transmit diseases to humans.
	Environmental	Non-invasive

Table 2. Conventional bioconverting species with focal organic wastes and bioconverion outputs.

Species	Organic waste	Country	Bioconversion output	Reference
Black Soldier Fly (*Hermetia illucens*)	Rice straw (30%) Restaurant waste (70%)	China	Biofuel	53
	Rice straw	Indonesia	Biomass	54
	Coffee pulp, husk	El Salvador, Indonesia	Biomass, fertilizer	55,56
	Reject material from pears, banana and Cucumber (5:3:2)	Sweden	Biomass	57
	Spent distillers grain	United States	Biomass	62
	Fruits and vegetables	Canada	Biomass	31
	Corn stover	China	Biofuel, soil amendment	59
	Corncob	China	Biofuel	28
	Sorghum	United States	Biomass	60
	Cowpea	United States	Biomass	60
	Cassava peel	Indonesia	Biomass	61
	Vegetable trimmings, spent coffee grounds, and tea leaves.	United States, Hong Kong	Biomass	62
	Vegetables, peels of yam, cassava, plantain	Ghana	Biomass	63
Housefly (*Musca domestica*)	Restaurant waste (70%) Whole plant corn silage, sawdust (30%)	China	Biomass, Biofuel, fertilizer	64
Codling moth (*Cydia pomonella*)	Starch and cheese wastewater sludge	Canada	Biomass	65
Cambodian field crickets (*Teleogryllus testaceus*)	Cassava plant tops, spent grain, mungbean sprouts waste, field weeds	Cambodia	Biomass	66
Yellow Mealworm (*Tenebrio molitor)*	Wheat straw, bruised cabbage leaves	China	Biomass	67
	Corn stover	China	Biofuel	59

Figure legends

Figure 2: Depending on the processing method, pre-consumer food wastes can be quite variable in their final composition. a) freshly pressed white wine pomace. This waste will contain considerable stems and leaves. b) freshly fermented red wine pomace. Higher in alcohol and lower in sugar content, fermented pomaces contain less stems. c & d) samples of white wine pomace (left) and red wine pomace (right) free of stems and leaves, and ready for bioconversion.

Figure 3: Many bioconverters require different rearing parameters throughout their life cycle. a) Black soldier fly larvae growing in almond hulls. Key requirements for this life stage include high moisture content, evacuation of gasses, limited light, access to food. b) Adult black soldier fly in caged enclosure. Key requirements for this life stage include sufficient light for mating displays, ample flying space for aerial copulation, and egg traps with oviposition stimulant.

Figure 4: Theoretical data illustrating how tandem selection (a) or independent culling selection (b) can be used to eliminate individuals below one or two thresholds (dotted line(s)). Some individual (blue dots) are selected for further breeding, while others (red dots) are culled. An advantage of independent culling is that selection pressure is not relaxed since multiple traits may be targeted at once. However, multiple thresholds may eliminate more individuals from subsequent pools of a breeding population, therefore lower thresholds are typically maintained.

Figure 4: An individual's phenotype is a product of its genetics in response to environmental conditions. Change in either the genetics of the organism (genotype) or the environmental conditions the individual's experiences alters which phenotypes are expressed. Phenotypic

expression of traits may include size, fecundity, behavior, lifespan, susceptibility to disease, fat content, etc.

Figure 5: Survival of two different populations of the same species of insects. In nature, the wild-type feeds on an ancestral food source, while the derived-type feeds on an introduced plant species. Although only a few decades have passed since the plants introduction, enough response to selection has occurred such that the phenotype of the insects (survival) differs depending if the two populations are fed either their ancestral or the introduced food. This figure is adapted from results of Carroll et al.[30].

Figure 1.

Figure 2.

Figure 3

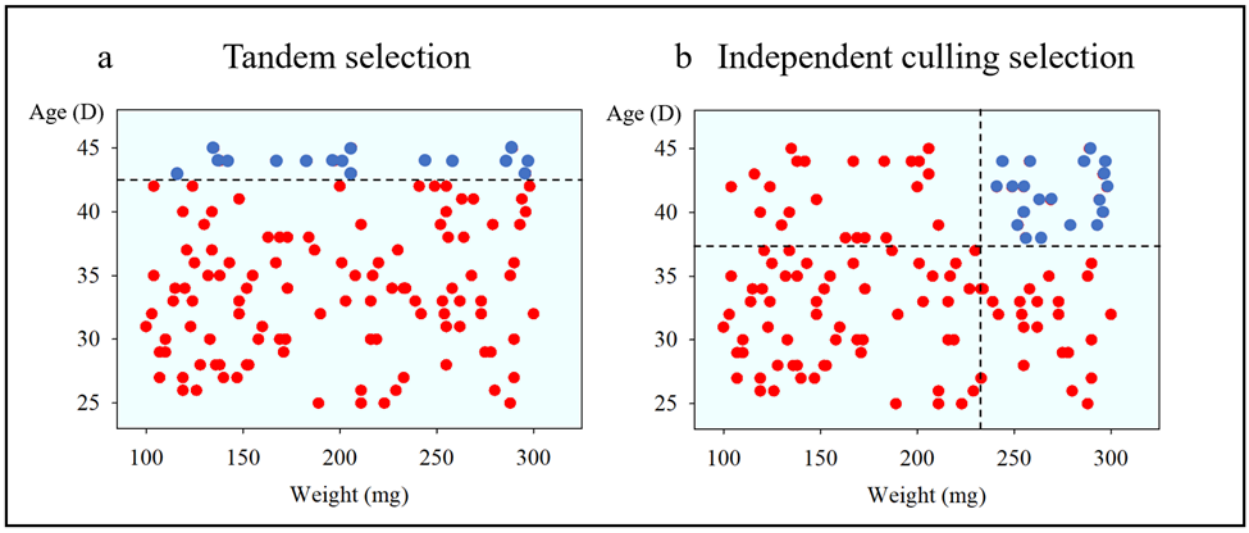


Figure 4.

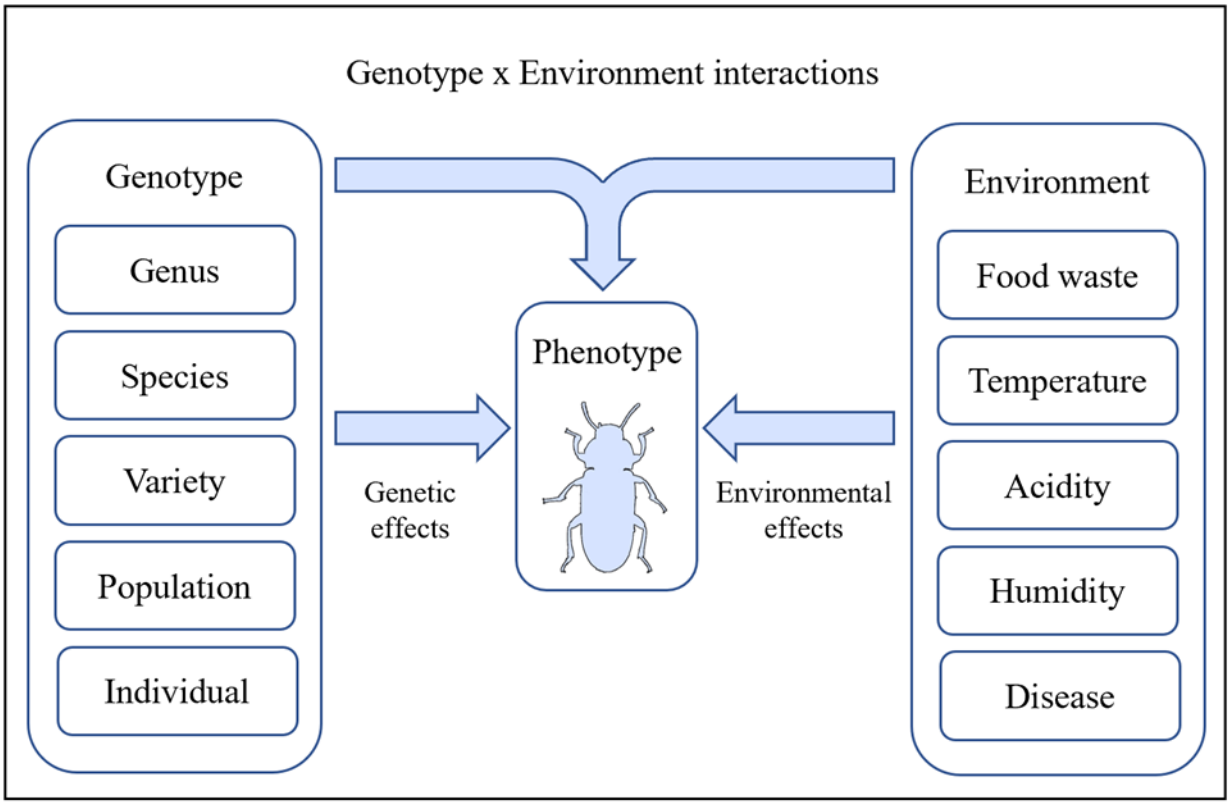
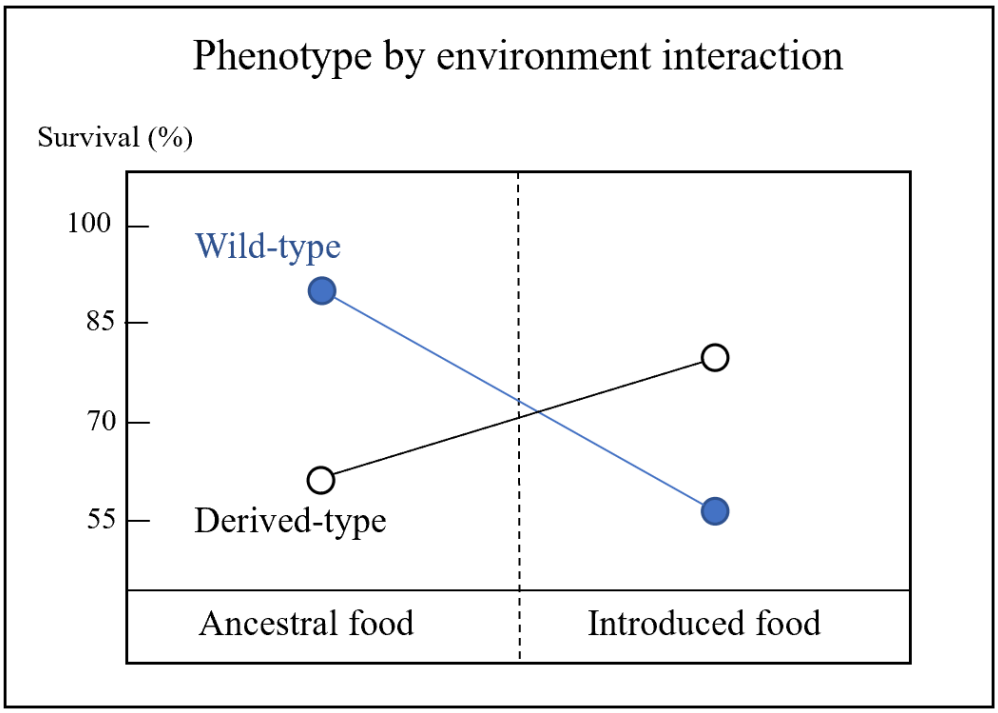


Figure 5.


Phenotype by environment interaction
Survival (%)
100
85
70
55
Wild-type
Derived-type
Ancestral food
Introduced food

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Chapter 3: Intraspecific variation in feed conversion of mass-reared insect populations

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Abstract

The use of insects to bioconvert agricultural byproducts is rapidly gaining momentum as a research interest and business opportunity. As such, many novel diets are being screened for use as feedstock. We argue that performance variability among individuals is an important metric to quantify when screening novel diets for use as feedstock. This study proposes a framework to characterize intraspecific phenotypic variation in mass-reared insect populations. In three bioassays, variation in *Tenebrio molitor* feeding efficiency (FCR) was assessed within (intrapopulation bioassays) and across populations (interpopulation bioassays) fed polystyrene and/or oats, as well as populations fed agricultural byproducts representative of those used in the insect bioconversion industry (food byproduct bioassays). It was concluded that the framework can be used to characterize a single insect population reared under two or more production conditions, or for assessment of two or more insect populations under the same production conditions. Larvae performance in the intrapopulation bioassays revealed that FCR was lower and less variable on the mixed diets than on an oat only diet ($p < .001$). Larvae performance in the interpopulation bioassays revealed "Fluker" mealworms had the greatest variation ($R^2 = 0.91$), followed by Bassets ($R^2 = 0.89$) and Rainbow ($R^2 = 0.83$). Larvae performance in the food byproduct bioassays revealed FCR on diets of 100% White wine (Ww) were significantly different from diets of 100% tomato (To) ($p < .001$) and 25:75 To:Ww ($p < .001$). Further, FCR on the 100% To was significantly different from 50:50 To:Ww. The variation for the FCR response curves from largest to smallest are as follows; Ww ($R^2 = 0.94$) > 50:50 To:Ww ($R^2 = 0.73$) > 75:25 To:Ww ($R^2 = 0.86$) > 100% To ($R^2 = 0.94$) > 25:75 To:Ww ($R^2 = 0.71$). Overall, the proposed framework is of high relevance to the rapidly growing research efforts into insect-based bioconversion of food byproducts and waste streams.

Introduction

Mass-production of insects for bioconversion of food byproducts and other organic waste streams requires monitoring of performance-related factors, including: egg hatch rate, development time, mortality of life stages, disease outbreaks, diet consumption, and feed conversion (Brits, 2017; Ortiz et al., 2016). That is, experimental data have been used to generate frameworks and optimization models to maximize both economic and sustainability aspects of such mass-production systems (Padmanabha et al., 2020). An optimization parameter of paramount importance is the feed conversion ratio, FCR, which is the ratio between diet consumption, Dc, and body weight gain, Wg.

$$FCR = \frac{Dc}{Wg}$$

Numerous studies have evaluated average feed conversion ratios of mass-reared insects (Diener et al., 2009; Oonincx et al., 2015; Ramos-Elorduy et al., 2002; Rehman et al., 2017; Van Broekhoven et al., 2015). These studies have shown that efficiency in feed conversion can vary widely for insects fed different diets. For example, Rehman et al. (2017) found black soldier fly [*Hermetia illucens* L. Dipetera: Stratiomyidae] FCRs ranging 3.2 to 6.2, which is less efficient than reported by Oonincx et al (2015) (1.6 to 2.4). However, Oonincx et al (2015) also reported considerable variability in FCRs for yellow mealworm [*Tenebrio molitor* L. Coleoptera: Tenebrionidae)] (3.8 to 19.1). To our knowledge, individual's intraspecific variation of feed conversion within an insect population has received considerably less attention. Characterization and quantification of intraspecific variation may provide valuable insight into adaptability and performance of insect populations reared on novel and/or suboptimal food byproducts. Furthermore, when an insect population shows considerable intraspecific variation in phenotypic traits, high-performing

individuals may be identified and used as part of breeding programs (Fowles & Nansen, 2019, 2020; Jensen et al., 2017).

We propose a model framework, in which bioassays with individual insects (i.e. >50) are performed and their phenotypic trait responses (e.g. feed conversion, but it could also be development rate, disease resistance, heat tolerance, or any other performance-related factor) are ranked in descending order. The framework can be used to characterize a single insect population reared under two or more production conditions, or for assessment of two or more insect populations under the same production conditions. Figure 5 describes the proposed framework, and as an illustrative example, we present theoretical phenotypic trait responses of one insect population reared on two different diets, A and B. Moreover, insect individuals reared on diet A show low/negligible intraspecific variation, while individuals from the same population but reared on diet B show distinct intraspecific variation (Figure 5a). Based on ranking individuals trait performance in descending order, a regression line can be fit to the actual data, and intercept and slope may be used as quantitative indicators of intraspecific variation within the given insect population. Moreover, the intercept denotes the potential maximum phenotypic trait response, and the slope can be used to calculate four important characteristics of the given insect population: 1) The proportion of insect individuals which are within a certain range from the potential maximum phenotypic trait response; 2) Or conversely, the potential minimum phenotypic trait response. 3) The difference between maximum and minimum phenotypic trait response is an indication of the intraspecific variation within the given insect population. 4) The proportion of "zero" or "negative" performers in a population. Zero would indicate no change, for instance in body weight, during the course of the assessment, and negative would imply an actual loss in performance (i.e. body weight). Figure 5b only shows theoretical linear regression fits, and four different outcomes are

presented. Red lines denote scenarios in which insect populations show a low level of intraspecific variation. Thus, it would seem reasonable to conclude that further selection/breeding would only yield limited improvement of phenotypic trait response, as a large proportion of individuals in the insect population are performing close to the maximum trait potential. The two black lines represent regression outcomes, which indicate strong potential for selection/breeding, and an outcome similar to the dotted black regression fit suggests both high maximum potential and a high degree of intraspecific variation. The solid black line implies that the insect population includes a high proportion of zero performers. Such an outcome, with high proportion of zero performers and low maximum potential, may be interpreted as the diet not being nutritionally suitable, so either improvement of the diet would be needed, or the same diet could be bioassayed with a different insect population.

In this study, we used the model framework described in Figure 5 to quantitatively evaluate intraspecific variation of feed conversion in mealworms [*Tenebrio molitor* L. Coleoptera: Tenebrionidae)]. In bioassays with individual mealworms of the same population (referred henceforth as "intrapopulation" bioassays), we quantified their feed conversion on three diets: 100% oats, 100% polystyrene, and 70:30 oat:polystyrene. In a second bioassay (interpopulation bioassays), we evaluated feed conversion of individual mealworms from three separate populations reared on diets of 70:30 oat:polystyrene. In a third bioassay (food byproduct bioassays), we evaluated feed conversion of individual mealworms from one population of mealworms reared on five proportional mixtures of processed tomato (To) and white wine (Ww) byproducts at ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (To:Ww). Based on these three bioassays with individual meal worms. These three bioassays were chosen to look at variation within (intrapopulation bioassays) and across populations (interpopulation bioassays), as well as

on diets representative of the insect bioconversion industry (food byproduct bioassays). We hypothesized that: 1) On a balanced and well-suited diet, we would predict comparatively high maximum and minimum response, a shallow slope, and no zero performers. (vice versa for unsuitable/unbalanced diets); 2) High performance (indicated byby maximum or minimum response) may be similar for a wide range of diets. In other words, we are predicting that, unless a diet is highly unsuitable/unbalanced, it will generally allow at least some insect individuals to perform well; 3) Similarly, distinct populations fed the same diet should have comparatively similar high performance, but slope and zero performers may differ.

The proposed framework is considered relevant to studies of insect performance more broadly, when reared on different hosts/diets and/or under different abiotic conditions. Moreover, this study is of high relevance to the rapidly growing research efforts into insect-based bioconversion of food byproducts and waste streams.

Materials and Methods

Mealworms and experimental units

Mealworms were purchased from three separate companies. (Rainbow Mealworms, Compton, USA; Fluker's Cricket Farm, Port Allen, USA; and Bassett Cricket Ranch, Visalia, USA). Larvae were held at 27^{o}C, 60-75% RH, and 12:12 light:dark. To standardize individual's size, larvae were passed through 1.68 mm and 2.38 mm sieves (W.S Tyler standard Sieve Co; USA), those between 0.01 g and 0.05 g were used in the experiment. Larvae were weighed individually at the onset of experiments, with a target weight of ~0.03 g.

Individual larvae were held in transparent polystyrene 2.5-dram vials (Thorton plastics, Salt Lake City, USA) with an inside diameter of 15.9 mm and height of 38.1 mm. Each container received a single larva, diet mix, and water source (Figure 6A). The water source was made from 1 ml centrifuge tubes filled with deionized water and dammed with cotton swabs (Q-tip, Unilever, USA). Vials remained uncapped, to avoid excess moisture and possible mold growth. Individual containers were held in an environmentally controlled growth chamber at 27^{o}C, 60-75% rH, and 12:12 dark:light (Figure 6C).

In the third feeding trial, we evaluated our model framework's utility in comparing intraspecific variation for a population of mealworms (Rainbow Mealworms) fed diets representative of what may be screened for use at insect bioconversion operations in California. Here, we examined two food production byproducts: white wine pomace (UC Davis experimental winery, Davis, California) and tomato pomace (Campbell's Soup Company, Dixon, CA). Individual mealworms were reared on a 1 gram diet at ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (To:Ww).

Experiments

Three separate feeding trails were performed; In the first feeding trial we evaluated the effect of a highly novel diet on a single population (intrapopulation variation of the "Rainbow Mealworms" brand). We used two food materials: rolled oats from a local supermarket (Quaker Oats Company, USA) and Polystyrene from shipping insulation (polystyrene, Uline, USA). Individual mealworms were reared on one of three diets: oats only (500 mg), Polystyrene only (200 mg), or oats (500 mg) and Polystyrene (200 mg). Not all the diet offered would be consumed over the course of this experiment, and larvae fed *ad libatum*.

In the second feeding trial, we evaluated the effect of the source population on trait response using mealworms from three separate companies (interpopulation variation of larvae from Rainbow Mealworms, Fluker's Cricket Farm, Bassett Cricket Ranch). All three populations were reared on diet mixtures of oats (500 mg) and Polystyrene (200 mg).

In the third feeding trial, we evaluated feed conversion of individual mealworms from one population of mealworms reared on five proportional mixtures of processed tomato (To) and white wine (Ww) byproducts at ratios of 100:0, 75:25, 50:50, 25:75, and 0:100 (To:Ww). Diets were selected for being representative of agricultural byproducts used as feed in the insect bioconversion industry.

For all three feeding trials, food materials were ground and sifted (1 mm, sieve #18; W.S Tyler standard Sieve Co; USA) so that the diet particle size was above 1 mm. 60 individual mealworm larvae were monitored weekly during their development. After ~50 days, diets were sifted using a 1mm sieve to remove frass before the final diet and larval weight were recorded. For mixed polystyrene diets, weights of remaining polystyrene and oats were segregated and recorded separately. Mixed tomato and white wine pomaces were not separated but rather weighed as a whole.

Model framework

For each phenotypic trait response examined, individual performance was ranked in descending order. Each rank was divided by the number of individuals being examined and ordered along the X axis. The Y axis represents the value for each ranked individuals' phenotypic response. For example, out of 60 individuals starting a trial, 58 individuals survive the bioassay and are used in the final analysis. Thus, once ranked in descending order 1-58, each individuals' rank is divided

by 58 and multiplied by 100. This allows normalization of the data to a shared range of 1-100, even if the different treatments result in a variable number of usable replicates. A linear regression is then fit to these data sets to generate the response curves.

Statistical analysis

Analysis of variance (ANOVA) accompanied with Tukey's post-hoc tests were used to test the effect of diet and source population on feed conversion, weight change, and consumption. Normality of rearing trait response between the different groups was assessed using the Shapiro-Wilk normality test. A p-value < 0.05 was chosen to denote significance. This analysis was conducted using R version 4.0.2 (R Core Team, 2020). Packages downloaded included "ggplot2", "ggpubr", "tidyverse", "broom", "AICcmodavg". Linear regression of response curves and R^2 values were calculated in excel (Microsoft Corp., Seattle, USA).

Results

Intrapopulation variation bioassay

FCR on the polystyrene diet was significantly different from both the oat and mixed diets (*p <.001*) (Figure 3). FCR for the oat and mixed diets did not significantly differ from one another (*p >.05)*. On average the FCR was the highest for the mixed diet (3.68), followed by the oat diet (3.29) and polystyrene diet (-2.48) (**Error! Reference source not found.**). In addition, mixed diets u nderwent the greatest weight gain with larvae on average gaining 0.06 g, followed by the oat diet at 0.031g, and polystyrene only at -0.025 g, meaning they lost weight over the course of the experiment. For the FCR curves (**Figure 7**), maximum response for oat and mixed diets were similar at 4.93 and 4.82, respectively. This contrasts with the poor performance for mealworms on

the styrofoam diet (-0.55), which was due to weight loss over the duration of the experiment. Minimum FCR response for the oat, mixed, and styrofoam was 1.7, 2.6, and -4.34, respectively. The slope of the polystyrene diet was the steepest (-0.04, $R^2 = 0.97$), followed by the oats (-0.03, $R^2 = 0.84$) and mixed diet (-0.02, $R^2 = 0.84$). All individuals in the polystyrene diet treatment had negative FCRs, leading to all individuals considered "zero performers" (see Fig. 1a). This was due to very low average polystyrene consumption for the polystyrene only diet (1%) compared to the mixed diet (54%), which differed significantly from the other two diets ($p < 0.001$). In contrast, neither oat nor mixed diets contained zero performers.

Interpopulation variation bioassays

FCR the Rainbow mealworms were significantly different from both Bassetts and Fluker populations ($p < .01$) (Figure 4). FCR for the Bassetts and Fluker mealworms did not significantly differ from one another ($p > .05$). Average FCR was highest for Rainbow mealworms at 3.66, followed by Fluker and Bassetts at 3.01 and 2.94, respectively. Bassetts mealworms gained the most weight on average at 0.11 g, followed by Fluker and Rainbow at 0.08 g and 0.06 g, respectively (see **Error! Reference source not found.**). For the FCR response curves (**Figure *8***), Rainbow and Fluker shared the largest maximum FCR response at 4.75, followed by Bassetts at 4.2. Minimum FCR response for Rainbow, Bassetts, and Fluker were 2.6, 1.6, and 1.3, respectively. The slope of the Fluker mealworms was the steepest (-0.04, $R^2 = 0.91$), followed by the Bassets (-0.03, $R^2 = 0.89$) and Rainbow (-0.02, $R^2 = 0.83$). No population contained zero performers.

Food byproduct bioassays

FCR on the 100% White wine (Ww) was significantly different from 100% tomato (To) ($p < .001$) and 25:75 To:Ww ($p < .001$) (Figure 5). While FCR on the 100% To was significantly different from 50:50 To:Ww. FCR for the other diet combinations did not significantly differ from one another ($p > .05$). On average the FCR from highest to lowest was as follows; 100% Ww > 50:50 To:Ww > 75:25 To:Ww > 25:75 To:Ww > 100% To, at 5.8, 4.8, 4.4, 3.5, 3.0, respectively (see **Error! Reference source not found.**). Each diet combination resulted in an average increase of w eight, with the highest to lowest as follows; 50:50 To:Ww > 25:75 To:Ww > 75:25 To:Ww >100% To >100% Ww, at 0.061g, 0.048g, 0.035g, 0.03g, 0.023g, respectively. For the FCR response curves (**Error! Reference source not found.**) 100% Ww had the greatest maximum response at 11.41, while both 100% To and 75:25 To:Ww both shared the comparatively lower maximum FCR at 6.99 and 6.95, respectively. For the minimum FCR, only 100% Ww, 75:25 To:Ww, and 50:50 To:Ww were positive at 0.54, 0.22, 0.08, respectively. Consequently, 100% To had 11.37 "zero" performers. The slopes for the FCR response curves from largest to smallest are as follows; Ww (-0.1087, $R^2 = 0.94$) > 50:50 To:Ww (-0.10, $R^2 = 0.73$) > 75:25 To:Ww (-0.09, $R^2 = 0.86$) > 100% To (-0.08, $R^2 = 0.94$) > 25:75 To:Ww (-0.07, $R^2 = 0.71$).

Discussion

The purpose of this study was two-fold, first was to explore the intraspecific variability in phenotypic trait response for a key performance trait of interest (feed conversion) in a commonly used commercial insect; second was to propose a framework to quantitatively compare intraspecific variability of phenotypic trait response in insects. This was done by assessing insect performance under three separate bioassays that looked at response variability within and across

insect populations, as well as, between an array of diet mixtures. For our model insect, we used *T. molitor* due to their popularity within the insect production industry and consume food byproducts, as well as, ubiquity to mineralize and digest polystyrene (Yang et al., 2018).

Insect-based bioconversion provides a link for recycling the nutrients from low-value food byproducts back into agricultural supply chains (Alemu et al., 2017; Van Huis et al., 2015; Vandermeersch et al., 2014). These products include the stems, seeds, skins, and inedible fibers that accompany downstream food processing. (Fowles & Nansen, 2020). These byproducts are not a negligible resource considering 5 million tons of low value food byproducts are produced every year in California alone (Amon et al., 2012), and the FAO estimates 1.3 billion tons are wasted globally (FAO, 2017). However, not every food byproduct may serve as a balanced and well-suited diet, and thus mixed diets are necessary to produce insect biomass (Gold et al., 2020; Palma et al., 2018). Moreover, there has been considerable interest in using *T. molitor* to bioconvert plastic waste in recent years (Yang et al., 2018). Therefore, this study provides a timely framework for characterizing and quantifying intraspecific variation that results from novel diet formulations (but may be used more broadly) and may provide valuable insights into potential adaptability and performance of insect populations reared on novel and/or suboptimal food byproducts.

Intrapopulation variation bioassays

Although mealworms will readily consume polystyrene, as a synthetic material, polystyrene remains a truly novel diet-substrate. The bioassays revealed both the oat and mixed diets resulted in similar maximum and minimum responses, whereas individuals fed the polystyrene diet had a negative FCR due to weight loss (while still consuming their diet). In addition, neither the optimal diet (oats) or mixed diet resulted in so called “zero performers”, whereas all individuals fed the

styrofoam were counted as such. The slope of the response curves, a measurement of the intraspecific variation, was greater on polystyrene diets than on either the oat or mixed diets. We therefore accept the hypothesis that, on a balanced and well-suited diet, individuals have a comparatively high maximum and minimum response, shallow slope, and no zero performers.

The inclusion of polystyrene decreased intraspecific variability in larvae fed the mixed diet compared to an oat only diet. Lower variability in FCR for larvae feeding on inert hydrocarbons makes biological sense. For example, Yang et al. (2015) demonstrated that larval consumption of polystyrene contributes more to maintaining respiration than contributing to weight gain. Thus, even high polystyrene consumption may only marginally contribute to mass accumulation (i.e. lipids). As decomposers and detritivores, many insects have evolved to consume various low nutrient and/or recalcitrant diets, such as manure and woody vegetable matter (Gold et al., 2018), which may underpin the ubiquity of *T. molitor* to readily consume and bioconvert polystyrene (Yang et al., 2018). However, in the initial 2010 experiment first documenting *T. molitor* consuming polystyrene, a mixed diet yielded larvae of the highest weight (Gao et al., 2010), yet this fact was seemingly understated (one sentence). Our study confirmed this phenomenon for both intra- and interpopulation bioassays. Higher weight gain and FCR have different implications should mealworms be employed as bioconverters of polystyrene waste or food byproducts. Larvae gain more weight on diets that include polystyrene but are less efficient at doing so compared to those fed non-synthetic diets over the same period. In other words, higher FCR means more "units" of mixed feed are consumed for comparable weight outputs. While potentially unattractive to an insect producer cutting costs using food byproducts as feed, a high FCR may be attractive from a polystyrene waste management perspective. However, concerns remain over microplastic laden frass at industrial scales (He et al., 2020; Peng et al., 2020).

Interpopulation variation bioassays

Each of the populations used in this study came from commercial mealworm producers. We do not know their antecedents' standard diets, nor how inbred the populations are. This makes the three populations good candidates to compare interspecific variation between distinct populations. In an insect mass production setting, such comparisons may take place when validating the success of a breeding program or comparing efficiencies between populations held at separate facilities. Regarding our bioassays, Bassetts and Flucker showed similar FCRs on the mixed polystyrene diets, and their performance was distinct from Rainbow mealworms ($p < .01$). Perhaps counterintuitively a high FCR means the insect is less efficient at converting diet into biomass. For example, a FCR of 8 would require 8 parts of diets to produce one part of biomass. So, we look to the low FCRs within the population to find the highest performers. Bassetts and Fluker have FCR minimum responses of 1.6 and 1.3, whereas the Rainbow mealworms FCR is twice as large at 2.6. Therefore, we reject the hypothesis that distinct populations fed the same diet should have comparatively similar high FCR performance.

Food byproducts

Much research has been done identifying compatible/incompatible matches between insects and food byproducts (Palma et al., 2018; Ramos-Elorduy et al., 2002; Rehman et al., 2017). A businesses success may rely on maximizing affordability of a food byproduct and outputs of insect biomass (Ireri et al., 2019). As part of this study, we sought to apply our comparative framework to mixtures of white wine and tomato processing byproducts. These byproducts were selected due

to their abundance and low-cost in California's Central Valley. Specifically, for the food byproduct bioassays we asked if there is variation in maximum performance across multiple diets and

In our bioassays we found that all diets except 100% To were very similar (<1). Likewise, none had zero performers. In contrast, despite having the lowest average FCR, 100% To also had both the highest slope (variation) and multiple zero performers (~11), which resulted from a significant portion of individuals losing weight over the experiment. However, both To and Ww diets may lack consequential nutrients and may be highly unsuitable/unbalanced for complete development. Both 100% To and 100% Ww treatments resulted in mealworm that weighed less on average than mixtures of the two, with 50:50 To:Ww resulting in the highest average weight. If so, we would accept the hypothesis that high performance (here indicated by minimum FCR response) is similar for a wide range of diets, baring those that are highly unsuitable/unbalanced.

Model framework

Using our model framework, we can quantitatively compare the intraspecific variation in phenotypic trait response between (and within) different populations to garner new insights not gleaned from standard approaches**.** Typically, in insect production, performance is assessed from comparing averages of large subsamples of a population. This differs from how traits are monitored in larger livestock, like cattle and pigs, which are comparatively massive and easier to monitor. However, monitoring the group's performance alone presents a problem. When insects are grown under highly controlled conditions on standardized diets, like those found in mass-rearing facilities, trait plasticity is controlled (Sørensen et al., 2012). Therefore, observable variability may be attributed to intraspecific genetic variation. However, when evaluating insect feeding efficiency on new diets, intraspecific variation due to non-heritable plastic responses to

the new environment (diet) is indistinguishable from the variation resulting from their genetics (i.e losses of prior “genetic improvements”). This is further complicated when FCRs are measured from the group, rather than individuals undergoing an adaptive challenge. Conventional livestock breeding solves this complication with monitoring individuals within ‘contemporary groups’, in which individuals undergoing artificial selection are compared to individuals not undergoing selection that are raised under similar conditions and diets (Crump et al., 1997). Populations reared at mass-reared insect operations are orders of magnitude larger than those of conventional livestock, and thus translating conventional livestock breeding strategies require novel assays and frameworks to better assess insect populations. In this study we provide an example of translating methods from animal husbandry to insect production by monitoring individual larval performance and presenting a novel way to quantify and assess the results. For example, when comparing trait response curves, Rainbow mealworm’s larvae is much steeper at -0.0093, covering a range of 100% to 4%, compared to Bassetts at -0.047 covering a range of 79% to 28%. Further, ~30% of the Rainbow larvae perform better than even the best performing Bassetts larvae. Steep slopes for traits like diet consumption may suggest a greater adaptability to the novel diet offered in this study, potentially an indicator of a well of adaptive and maladaptive genetic response. Notably, this study did not measure heritability of the traits under observation, though the authors recognize heritability calculations are critical to animal breeding. Rather, this study applies a new framework similar to practices used in animal breeding to demonstrate translation of some of these practices in mass reared insects.

The utility in the proposed comparison framework allowed us to visually differentiate the clear stepwise increase along the slope of Basset trait response relative to Fluker, a pattern also present in the populations’ weight changes. Therefore, we accept the hypothesis that using our

model framework, we may quantitatively compare the intraspecific variation of phenotypic trait response between and within different populations. While authors' commentary on the biological underpinnings of particular phenomena would be speculative, we believe the utility of the proposed framework to identity such patterns is a demonstration of its merit. Moreover, the frameworks applicability in assisting the documentation and analysis of intraspecific variability in phenotypic trait response could improve mass-rearing operation's standardization of insect biomass, identify latent adaptability, or quantify genetic gain across generation of selectively bred populations.

Conclusions

This experiment revealed that the addition of polystyrene into the diet of mealworms increases larval weight and the overall consumption of their diet. However, the feed conversion of larvae was lower and less variable on the mixed diets than on an oat only diet. This means more polystyrene is being consumed per unit of insect mass produced. The use of mealworms as a tool for mitigating polystyrene pollution and increasing its sustainable footprint is an attractive proposition. However, during the experiment some polystyrene passed through the larvae and was excreted in their frass. These micro-particles of polystyrene could represent a potential pollution concern should the practice be scaled up to an industrial scale.

Linear regression of ranked trait response was a useful tool to quantify and compare intraspecific variation both within and across populations of mealworms. In this experiment we only compared feed conversion which include measurements of consumption and weight change. However, the proposed method may be used to compare variation of many other phenotypic trait responses. Thus, this method may complement the evaluation of an insect breeding programs specifically

which may be comparing multiple phenotypic trait responses between a ‘contemporary groups’ and populations undergoing artificial selection. The use of insects to bioconvert waste products is rapidly gaining momentum as a research interest and business opportunity (Fowles & Nansen, 2020). Therefore, this study is both timely and relevant in presenting a framework to characterize and quantitatively evaluate intraspecific variation in response to these novel diets.

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Tables and Figures

Table 1: Framework metrics for each bioassay

Bioassay	Population	diet	y=mx+b	R^2	slope	maximum	minimum	zeros	mean
Intra-population	Rainbow	70:30 Oat:PS	y = -0.022x + 4.8165	0.8394	-0.022	4.8165	2.6165	0	3.7165
		100% Oats	y = -0.0317x + 4.9369	0.8444	-0.0317	4.9369	1.7669	0	3.3519
		100% PS	y = -0.038x - 0.5489	0.9687	-0.038	-0.5489	-4.3489	100	-2.4489
Inter-population	Rainbow	70:30 Oat:PS	y = -0.0212x + 4.7567	0.8285	-0.0212	4.7567	2.6367	0	3.6967
	Basset	70:30 Oat:PS	y = -0.0262x + 4.294	0.8938	-0.0262	4.294	1.674	0	2.984
	Flucker	70:30 Oat:PS	y = -0.034x + 4.7525	0.909	-0.034	4.7525	1.3525	0	3.0525
Food byproduct	Rainbow	100% White Wine	y = -0.1087x + 11.41	0.9365	-0.1087	11.41	0.54	0	5.975
		25:75 Tom:Wine	y = -0.0673x + 6.9515	0.7093	-0.0673	6.95	0.22	0	3.585
		50:50 Tom:Wine	y = -0.0971x + 9.7949	0.7375	-0.0971	9.7949	0.0849	0	4.9399
		75:25 Tom:Wine	y = -0.0918x + 9.1775	0.8617	-0.0918	9.1775	-0.0025	0	4.5875
		100% Tomato	y = -0.0789x + 6.9926	0.9423	-0.0789	6.9926	-0.8974	11	3.0476

Figure legends

Figure 5: Theoretical phenotypic trait responses to diet A (optimal diet) and diet B (sub-optimal diet). Note individuals are ranking in descending order. Regression lines are used as quantitative indicators of intraspecific variation.

Figure 6: Experimental units. A) Individual mealworms and diet treatments. B) Multiple vials of polystyrene only diets. C) Each treatment held within a growth chamber.

Figure 7: Intrapopulation feed conversion *for the three bioassays.* Analysis for *feed conversion* was performed using ANOVA. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Figure 8: Intrapopulation feed conversion for the three bioassays. Analysis for feed conversion was performed using ANOVA. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

Figure 1.

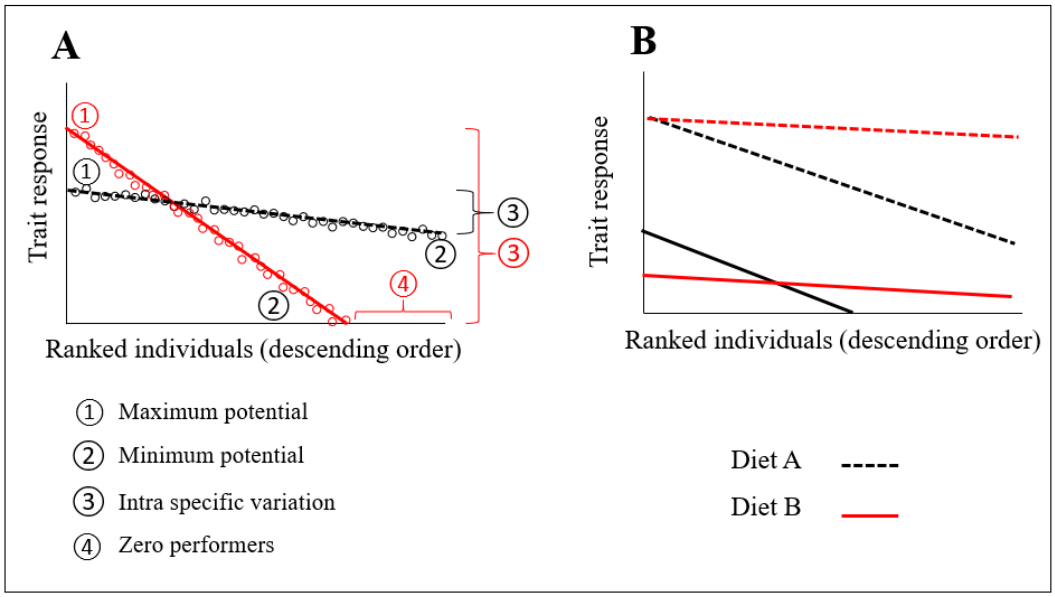


Figure 2.

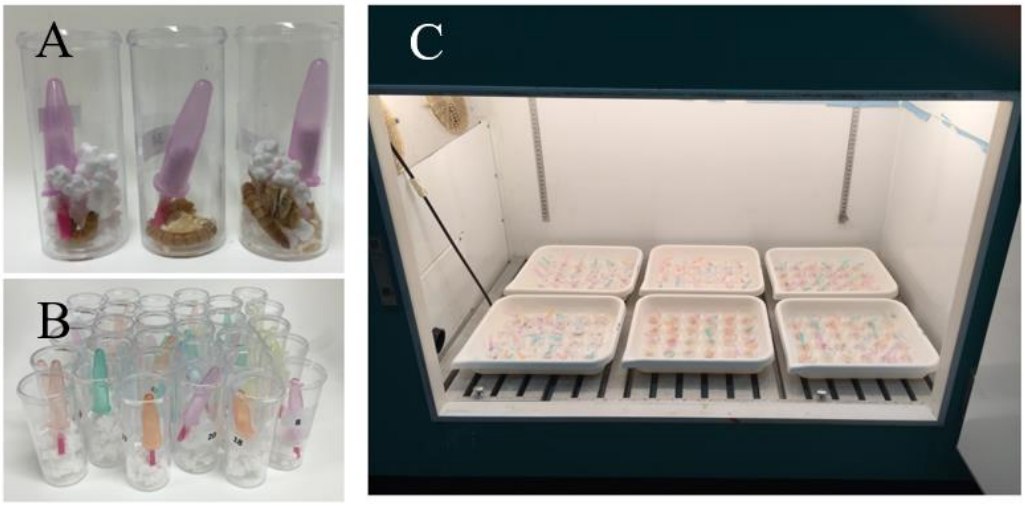


Figure 3.

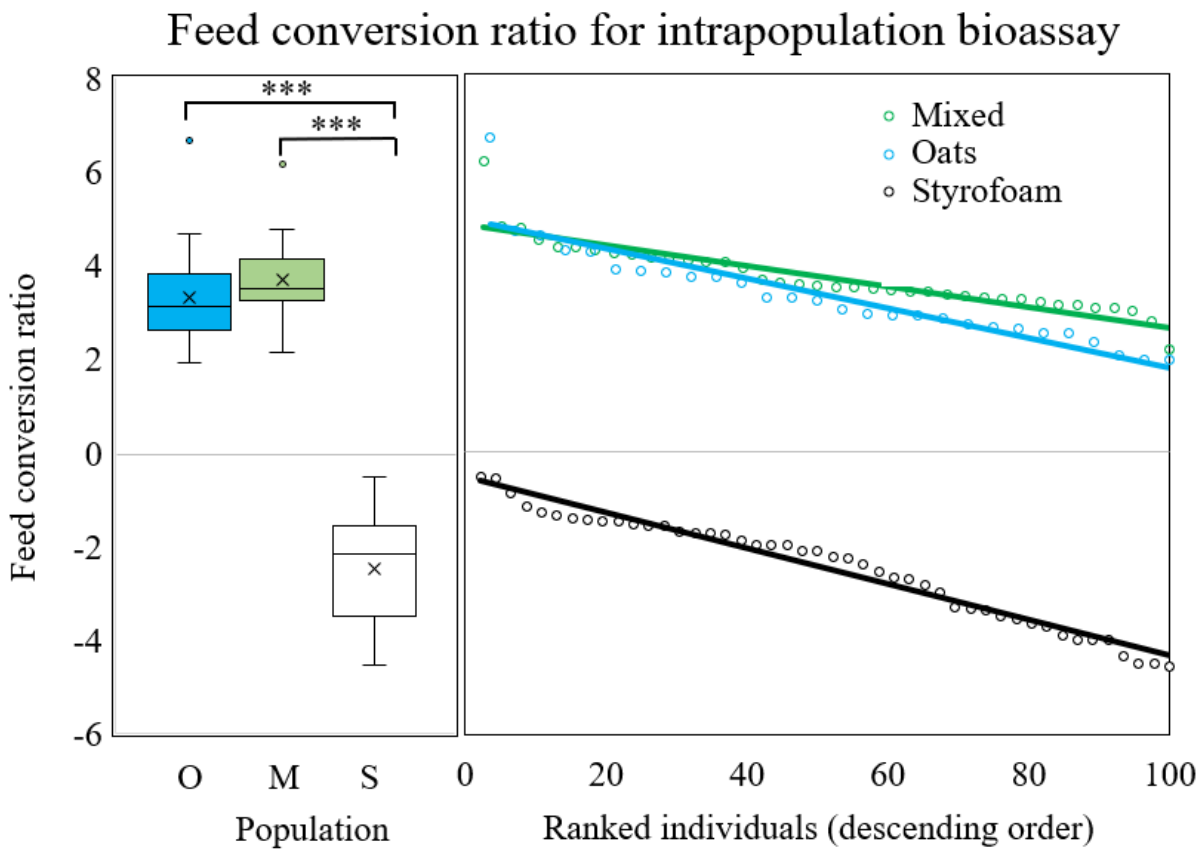

Feed conversion ratio for intrapopulation bioassay
Feed conversion ratio
8
6
4
2
0
-2
-4
-6
***
***
O
M
S
Population
Mixed
Oats
Styrofoam
0
20
40
60
80
100
Ranked individuals (descending order)

Figure 4.

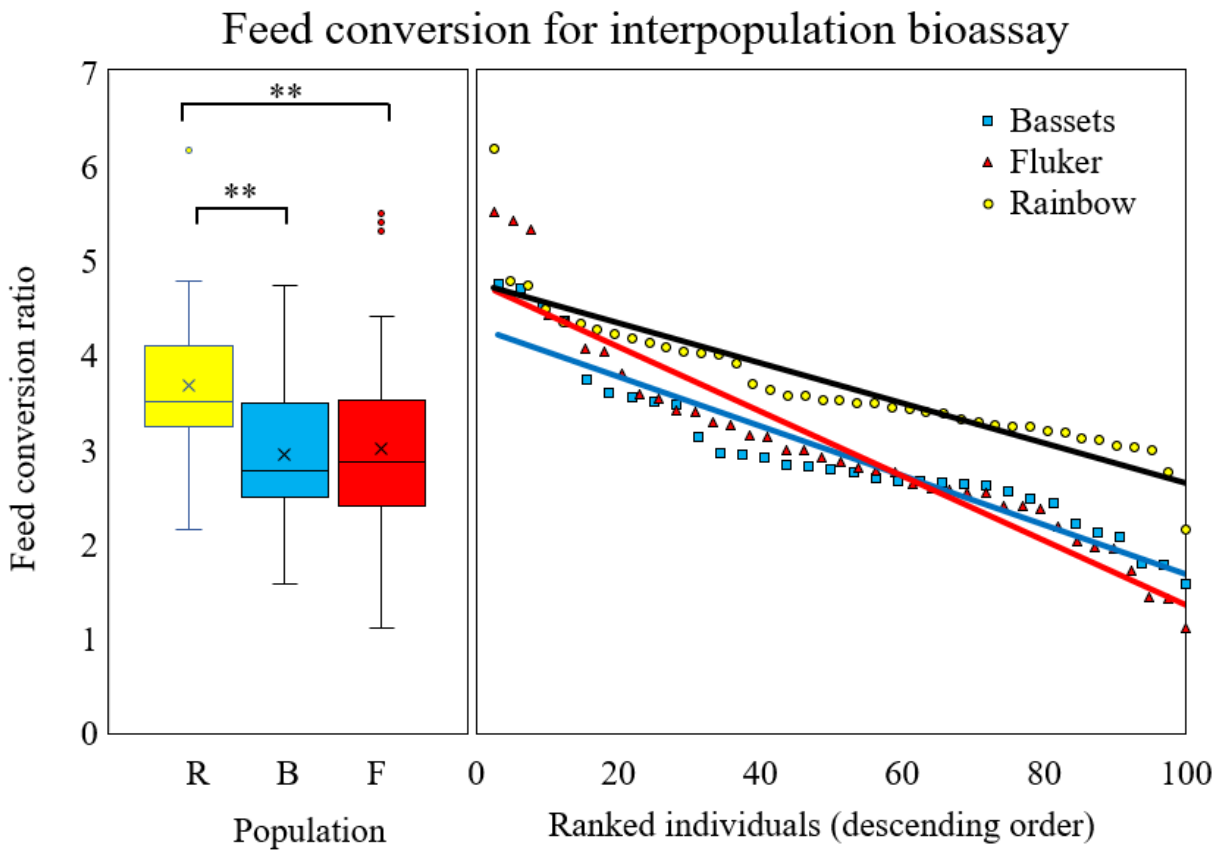
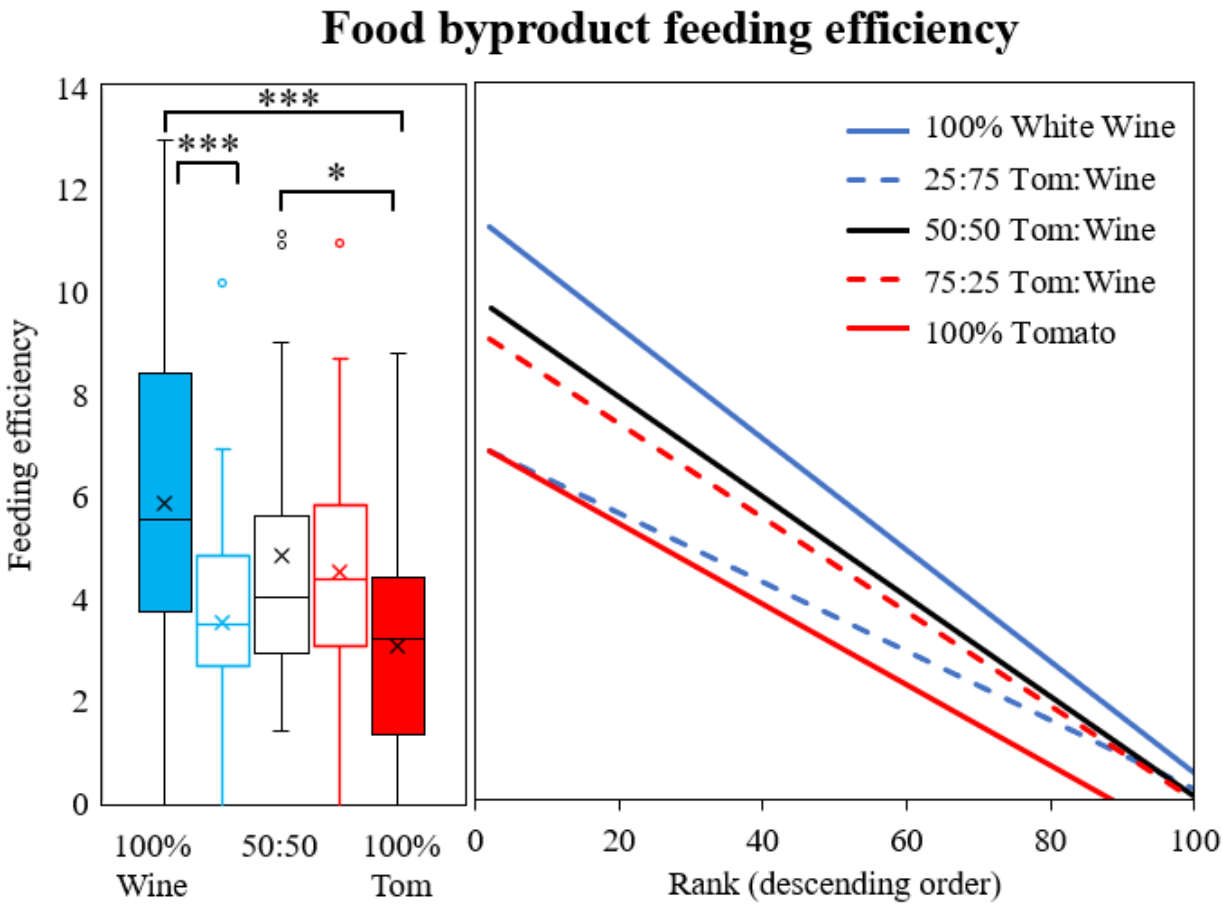

Feed conversion for interpopulation bioassay
Feed conversion ratio
Population
R
B
F
**
**
Bassets
Fluker
Rainbow
Ranked individuals (descending order)
0
20
40
60
80
100
0
1
2
3
4
5
6
7

Figure 5.


Food byproduct feeding efficiency
Feeding efficiency
14
12
10
8
6
4
2
0
***
***
*
100%
Wine
50:50
100%
Tom
0
20
40
60
80
100
Rank (descending order)
100% White Wine
25:75 Tom:Wine
50:50 Tom:Wine
75:25 Tom:Wine
100% Tomato

Chapter 4: Effects of rearing system design and microbial inoculants on black soldier fly growth and microbiota when reared on agri-food byproducts

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Abstract

Black soldier fly larvae (BSFL) are widely used in recycling and upcycling of nutrients of agri-food by-products, but low and inconsistent BSFL bioconversion performance has been identified as a key challenge. The aims of this research were two-fold: 1) validate an existing rearing system design, and 2) assess whether a microbial inoculum derived from the rearing residue increases rearing performance. In controlled bench-scale experiments, BSFL were reared on tomato pomace (TP) and white wine pomace (WWP), along with food waste as control substrate. The two aims were assessed based on the following response variable: larval mass, substrate reduction, residue properties (i.e. pH, temperature, moisture content), and microbiota community composition. Higher BSFL mass (5.1 mg dry mass) at harvest on WWP and substrate reduction on TP (11.7 % dry mass) in the closed that in the open system confirmed the potential of closed systems for earing performance improvements of agri-food by-products. The rearing system also seemingly affected the residual moisture content and temperature, but only had a small effect on microbiota. Performance improvements by the closed rearing system design may be outweighed by insufficient aeration with pasty substrates and higher operational efforts, such as aeration and larval separation from high-moisture residues. In contrast to the rearing system design, addition of the residue-derived microbial inoculum did not result in improved performance, nor did it alter intestinal and residue microbiota. Missing performance improvements could have been due to absent or low numbers of probiotic bacteria. The success of microbial substrate supplementation could be improved by studying effects of larval-associated microbes and developing cultivation methods that selectively amplify the beneficial (yet unknown) members of the microbial community. Our investigations aimed to increase the valorization of low-value agri-food by-products in BSFL rearing.

Keywords *Hermetia illucens*, bioconversion, feed, food waste, microbiota

1. Introduction

Recycling and upcycling of nutrients in agri-food by-products is important for sustainable waste management in food systems (Willett et al., 2019). Currently, several agri-food by-products are only partially utilised, leading to nutrient and resource being discarded as waste and potentially causing adverse environmental impact (Chen et al., 2020; Gustavsson et al., 2011). An emerging approach to upcycling of agri-food by-products is their conversion into insect biomass to be used as raw materials for food and feed (Barragán-Fonseca et al., 2017), biotechnology (Hahn et al., 2019), cosmetics (Almeida et al., 2020), and pharmaceutical (Vilcinskas, 2013) productions.

The black soldier fly (BSF), *Hermetia illucens* L. (Diptera: Stratiomyidae) is a promising insect species for nutrient recycling and upcycling (Gold et al., 2018). However, studies involving the rearing of BSF larvae (BSFL) on some of the most abundant and affordable agri-food by-products (e.g. damaged and discarded fruits and vegetables, fruit and vegetable pomace, maize straw, and almond hulls) showed low or inconsistent rearing performance (i.e. larval growth, bioconversion rate, and substrate reduction (Gold et al., 2018; Lalander et al., 2019). The performance determines the affordability and environmental impacts of BSFL-based bioconversion systems (Smetana et al., 2019). Thus, further innovations and insights into specific aspects of BSFL rearing are urgently needed to increase rearing performance and promote the adoption of insect-based bioconversion of agri-food by-products.

Previous studies improved rearing by optimising the nutrient provision, larval densities, feeding rate, and feeding regime (e.g. one-time vs. multiple) (Barragán-Fonseca et al., 2018; Diener et al., 2009; Gold et al., 2020a). Palma et al. (2018) introduced the first method for BSFL cultivation in closed containers with aeration. This system design supported BSFL growth, but they did not establish whether such a system is comparable or superior to existing rearing methods

in open beds, buckets, or bins. Altered exchange of water, air, and volatile organic compounds between open and closed systems could influence larval behaviour, microbiota, and residue temperature and pH. These parameters are generally considered influential for the rearing performance (Callegari et al., 2020; Meneguz et al., 2018; Raimondi et al., 2020).

Rearing performance has also been improved by inoculating substrates with pure-culture bacteria (Kooienga et al., 2020; Rehman et al., 2019; Somroo et al., 2019; Yu et al., 2011) or defined bacterial mixtures (Callegari et al., 2020; Mazza et al., 2020). Certain fly-, soil-, or manure-associated bacteria (e.g. *Bacillus natto, Bacillus subtilis, Lactobacillus buchneri,* and *Kocuria marina*) reduced the development time and increased the larval growth and substrate reduction. However, the cultivation of pure bacterial cultures alongside insect rearing is practically challenging because of the required laboratory capacities. A simpler method is to use the previously converted residue or residue-concentrate as the inoculum. During growth, some bacteria are excreted by larvae, becoming more abundant in the residue (i.e. substrate and frass (Gold et al., 2020c; Raimondi et al., 2020). It is hypothesised that these microbes contribute to the substrate decomposition and larval growth (Bruno et al., 2019; Chen et al., 2017; Gold et al., 2020c). Consequently, similar to the fermentation of foods (e.g. sauerkraut and sourdough (Kim et al., 2018), the addition of microbes to the substrate of the next rearing cycle could improve the rearing performance.

The aims of this research were to validate the rearing system design proposed by Palma et al. (2018) and assess whether a microbial inoculum derived from the rearing residue increases rearing performance. These objectives address the possible solutions for the low or variable performance of BSFL reared on many agri-food by-products. We hypothesised that: the rearing system alters the residue properties and microbiota, thereby altering the performance; and residue-

derived inoculums increase the rearing performance. In controlled feeding experiments, BSFL were reared on tomato pomace (TP) and white wine pomace (WWP), and the larval mass, substrate reduction, residue properties (i.e. pH, temperature, and moisture content), and microbiota were determined. By investigating rearing conditions and inoculating substrates with microbes, this research sought to increase the valorisation of low-value agri-food by-products in BSFL rearing.

2. Materials and Methods

2.1. Agri-food by-products

BSFL were reared on two agri-food by-products prevalent in the California Central Valley, USA, along with one control substrate. TP consists mainly of crushed skins and seeds, and was collected from the Campbell Soup Supply Company (Dixon, CA, USA). WWP mainly comprised unfermented skins, pulp, seeds, and stems, and was collected from the UC Davis Teaching and Research Winery (Davis, CA, USA). As BSFL usually grow best on food waste (Gold et al., 2020a; Lalander et al., 2019), food waste (DFW) collected from supermarkets and enzymatically digested by California Safe Soils (Sacramento, CA, USA) (Jinno et al., 2018) was used as a high-performance control. Following their collection in non-sterile containers, all substrates were frozen and stored at -20 °C until the start of the feeding experiments.

Prior to feeding experiments, the wastes were thawed at 4 °C for 24 h and Milli-Q water was added to elevate the substrate's moisture content to the typical range (60-80%) for BSFL digestion (Dortmans et al., 2017; Gold et al., 2020a). Milli-Q quantities (0.45 mL/g TP; 0.35 mL/g WWP) were selected based on the perceived absorption capacity of the substrate. The moisture content was increased from 63 to 71% for TP and 60 to 65% for WWP. The DFW had a moisture

content of 68%. WWP was also homogenised with a kitchen blender to increase the palatability by BSFL.

The substrate's gross nutrient composition, moisture content, and pH were determined using standard procedures (AOAC, 2006; AOAC, 2005; AOAC, 1997; see Supplementary Material for detailed method references). The pH was determined in a solution with 1 g of sample and 9 mL of Milli-Q water (Millipore Sigma, Bedford, MA, USA). Moisture content was determined as the gravimetric loss while drying at 80 °C for 24 h. Nitrogen was determined by combustion and the protein was estimated by multiplying the nitrogen value with waste-specific factors. Based on a review of the factors by Mariotti et al. (2008), a factor of 4.4 was used for both TP and WWP based on the results for vegetables and mushrooms, and that of 5.4 was used for DFW based on the results for meat, fish, cereals and vegetables. The lipids were estimated by extraction with ethyl ether. Fibre fractions, including amylase-treated neutral (NDF) and acid detergent fibre (ADF), were assessed by treating samples with neutral and acid detergents. Hemicelluloses were estimated as the difference between NDF and ADF, and ADF was assumed to be a reliable estimate of cellulose and lignin content. Ash was determined based on the gravimetric loss during combustion at 550 °C for at least 3 h.

2.2. Experiments

Two experiments were conducted to assess the influence of the rearing system (Experiment 1) and the addition of a residue-derived inoculum (Experiment 2). In the first experiment, BSFL were reared on each substrate in parallel in the open (Gold et al., 2020a) and closed rearing systems (Palma et al., 2018). The open rearing system comprised a plastic container (diameter: 9 cm; height: 14 cm) covered with a paper towel. The closed rearing system comprised a sealed plastic

bag (approximately 1,500 ml) supplied with compressed humidified air at 40 ml/min, or 0.7 ml/min/g dry mass (DM).

In the second experiment, BSFL were reared on substrates that included a microbial inoculum produced from the residue of the first experiment. BSFL were also reared in parallel in the open and closed systems to validate the results of the first experiment. DFW was excluded from the second experiment as the first experiment confirmed the satisfactory performance of larvae reared on this substrate and did not require further improvement.

The production of the microbial inoculum followed an approach similar to that commonly used for producing pure bacterial cultures. Three to ten grams of residue from the experiment was stored at 4 °C for 24 h and mixed with 40 mL sterile phosphate buffered saline (PBS) in a 50 mL falcon tube at room temperature (21 °C) for 20 min. Large particles were removed with a 40 µM sterile cell strainer (Corning, New York, NY, USA), and the filtrate was diluted 100-fold. Three replicates of the filtrate (1 mL) were incubated at 30 °C overnight in a sterile nutrient broth (5 ml, Difco Nutrient Broth, Becton, Dickinson and Company, Le Pont de Claix, France) accompanied by continuous shaking (120 rpm; Max4000, Thermo Scientific, Waltham, MA, USA). One millilitre of this culture was added to 9 mL of nutrient broth and incubated for another 4 h. Triplicate cultures were pooled, and the total viable counts (TVC) were enumerated by a single dilution series on triplicate agar plates as described below.

The microbial inoculum (10^9 TVC/mL) was added to each substrate with the Milli-Q water used to increase palatability (see description of the rearing substrates) immediately prior to the feeding experiments with BSFL. The inoculum was dosed in TP at 3 ml/100 g DM for TP. Based on these results, the dose was increased to 10 ml/100 g DM for WWP. In the control group, the inoculum was sterilised by autoclaving before its addition to the substrates.

2.3. Fly larvae rearing

The BSFL used in the two experiments were obtained from a colony operated at UC Davis since April 2018. The hatched larvae were fed *ad libitum* with poultry feed (60% moisture content; Purina Mills LLC, Purina Layena Pellets and Crumbles, Grey Summit, MI, USA) to 0.8-1.1 mg DM/larva. Thereafter, the larvae were manually separated from the poultry feed residue. Three to four replicates were prepared for each treatment (i.e. rearing system and microbial inoculum) with approximately 200 larvae per replicate. At the beginning of the feeding experiment, BSFL were placed on 60 g DM substrate and reared in an incubator (Isotemp 637D, Fisher Scientific, Waltham, MA, USA) at 28 °C. The rearing duration was selected based on the larval mass on DFW. As the larvae on WWP were considerably smaller when harvesting those on DFW, the rearing duration was extended to facilitate the larval-residue separation and accurate determination of the performance metrics. BSFL were reared for 6 days on TP and DFW, and 9-10 days on WWP. Temperature was automatically recorded every 10 min in the substrate/residue (DS1922L iButton, Maxim Integrated, San Jose, CA, USA). At the end of the experiment, containers/bags were removed from the incubators, and a residue sample was collected to measure the pH, TVC, and moisture content. Larvae were manually separated from the residue, rinsed with tap water, and counted. Larvae were stored at -20 °C before the determination of larval dry mass and DNA-based sequencing.

2.3. Rearing performance metrics

Larval mass and substrate reduction were evaluated as the rearing performance metrics. Larval DM was determined for each biological replicate by dividing the DM of all larvae by the larval number. Substrate reduction was determined for each biological replicate using Equation 1,

as the ratio of residual DM ($residue_{mass}$) to that of the total substrate DM ($substrate_{mass}$) provided at the beginning of the experiment.

$$\text{Substrate reduction (\% DM)} = \left(1 - \frac{residue_{mass}\ (g\ DM)}{substrate_{mass}\ (g\ DM)}\right) \times 100 \quad (1)$$

The residual DM was determined by correcting the residue mass removed from each biological replicate to determine the moisture content. Larval DM and residue moisture content were determined after drying in a laboratory oven at 80 °C for 24-48 h.

2.3. Microbial numbers and bacterial communities

Larval mass and substrate reduction were evaluated as the rearing performance metrics. Larval DM was determined for each biological replicate by dividing the DM of all larvae by the larval number. Substrate reduction was determined for each biological replicate using Equation 1, as the ratio of residual DM ($residue_{mass}$) to that of the total substrate DM ($substrate_{mass}$) provided at the beginning of the experiment.

$$\text{Substrate reduction (\% DM)} = \left(1 - \frac{residue_{mass}\ (g\ DM)}{substrate_{mass}\ (g\ DM)}\right) \times 100 \quad (1)$$

The residual DM was determined by correcting the residue mass removed from each biological replicate to determine the moisture content. Larval DM and residue moisture content were determined after drying in a laboratory oven at 80 °C for 24-48 h.

2.3. Downstream data analysis

Data were analysed in R (version 3.6.2; R Core Team, 2020). The hourly mean was calculated from the raw temperature readings of the residue. We abstained from statistical analyses among the different treatments for all parameters due to the small number of biological replicates

(n=3-4). Instead, we analysed the results and calculated descriptive statistics (e.g. median, mean, and standard deviation). Heatmaps of bacterial communities were created in 'ampvis2' (Andersen et al., 2018) after the conversion of reads into percent abundance per sample. Alpha diversity (i.e. Chao1 and Shannon index) and beta diversity were calculated using 'phyloseq' (McMurdie and Holmes, 2013). The unweighted pair group method with arithmetic averages (UPGMA) using weighted UniFrac distances of ZOTUs was applied to cluster samples based on the (dis)similarity of bacterial communities. Robust clusters of similar residue/intestinal bacterial communities were identified using the three-step protocol proposed by García-Jiménez et al. (2019). First, the number of clusters with the highest silhouette width score was identified using the 'fviz_nbclust' function in 'factoextra' package (Kassambara and Mundt, 2020). Second, the robustness of this clustering was confirmed using the 'prediction strength' function in the 'fpc' package (threshold > 0.80; Hennig, 2020). Third, the Jaccard score was calculated using the 'clusterboot' function (threshold > 0.75). The UPGMA-UniFrac clustering was visualised in a two-dimensional plane after the principal coordinate analysis (PCoA) of bacterial communities.

3. Results

3.1. Effects of rearing system (Experiment 1)

Considering the mean and standard deviation, the closed rearing system had a better performance in terms of the larval growth on WWP and the substrate reduction on TP (Figure 9). Larval mass on WWP in the closed and open systems were 20.4 (0.5) and 15.3 (0.4) mg DM, respectively. Substrate reduction on TP were 58.6 (1.7) and 46.9 (0.8) % DM in the closed and open systems, respectively. In two replicates, DFW reduction was notably lower in the closed system compared to the open system.

The rearing system also seemingly affected the residual moisture content (Figure 10**Error! Reference source not found.**) and temperature (Figure 11). Considering the results for both experiments, the mean residue moisture content was 7.5-12.5% higher in the closed system for TP, 25.6-50.4% higher for WWP, and 17.1% higher for DFW. The residue temperature was higher in the open system compared to the closed system for DFW and TP, but not WWP. The median temperatures in the open and closed systems were 34.8 and 30.8 °C for DFW and 35.3 and 31.2 °C for TP, respectively.

Microbiota associated with larvae and rearing residues can influence the growth and substrate reduction. To evaluate the impact of the rearing system on these performance metrics, we determined the intestinal and residual bacterial communities. Considering all samples, sequencing using extracted DNA produced 9,439,368 reads, with an average of 86,600 reads/sample and 2,204 ZOTUs. Rarefaction curves (see Supplementary Material) demonstrate that the samples were sequenced to an extent sufficient to approximate the true diversity. As these results do not provide any precise information about the microbial numbers, the TVC in the residue was additionally estimated, which was similar between systems (**Error! Reference source not found.**.

Alpha diversity metrics (i.e. Chao 1 and Shannon Index) show a similar species richness and evenness of the intestinal and residual bacterial communities between the two systems (Figure 12). Small differences in the mean species richness and evenness (i.e. Shannon Index) between systems were measured for the intestinal bacterial community on TP (Figure 12A), and the residual bacterial community on TP and DFW (Figure 12B). (Dis)similarities in the bacterial community between the open and closed systems were further explored by hierarchical clustering (UPGMA) and multidimensional scaling (PcoA) using weighted UniFrac distances to account for the

phylogenetic relatedness between ZOTUs. These analyses showed separate clusters between the open and closed rearing systems for the intestinal bacterial community on DFW (Figure 12A) and the residue bacterial community on DFW and WWP (Figure 12B). Overall, the distance between clusters, indicating the dissimilarity between bacterial communities of the open and closed systems, was small. The largest difference between the systems was observed in the intestinal bacterial community on DFW. When the microbial inoculums were added to the substrate (Experiment 2), no effect of the rearing system on the process performance (**Error! Reference s ource not found.**), residue temperature (see Supplementary Material), and bacterial community (Figure 14) was observed.

3.2. Effects of residue-derived bacterial inoculums (Experiment 2)

The inoculums derived from the residue of the first experiment had a much lower bacterial community richness than the residue from the first experiment. The mean community richness decreased from the residue to the inoculum, from 963 to 310 for TP and from 292 to 189 for WWP. The bacterial community was dominated (relative abundance > 5%) by members of the genera *Acinetobacter*, *Lysinibacillus*, *Myroides*, and *Vaccocus* in the TP inoculum, and *Acinetobacter* and members of the family *Enterobacteriaceae* in the WWP inoculum (Figure 5).

The addition of the residue-derived inoculum to the substrate did not influence the rearing performance or residue properties compared to the addition of the sterile inoculum (Figure 10 and**Error! Reference source not found.Error! Reference source not found.**). Moreover, the in oculum did not influence the bacterial numbers and diversity; the richness and community (Figure 14) TVC (n is the number for biological replicates with countable plates) in the treatment (microbial inoculum) and control (autoclaved microbial inoculum) were 8.5 (n=1) and 9.0 (0.5) $\log_{10}$/g (n=4) for TP, and 9.1 (0.1) (n=4) and 9.5 (0.0) $\log_{10}$/g (n=2) for WWP, respectively. Our

clustering approach identified two clusters, all TP and WWP samples. The distance of samples demonstrates that addition of the inoculum to the substrate increased the bacterial community variability among samples of the same treatment and rearing system type compared to the first experiment (Figure 14.

3.3 Effect of the substrate

The substrate type had a considerably larger influence on all metrics measured in this study than the rearing system and residue-derived microbial inoculation to the substrate. DFW and TP were the most abundant in protein and lipids and had similar microbial numbers (Table 3). DFW had the lowest cellulose and lignin contents, and TP contained little ash. WWP had the lowest pH, and much lower microbial numbers than TP and DFW.

Low microbial numbers in WWP presumably resulted in very few reads from gene sequencing to estimate the bacterial communities in the substrate before BSFL rearing. TP and DFW differed in terms of the community richness and composition (Figure 5). TP had a rich and diverse community dominated by species from nine bacterial classes. In contrast, few highly abundant genera (i.e. *Bacillus*, *Lactobacillus, and Leuconostoc*) characterised DFW.

TP had a rearing performance comparable to that of DFW. Larval mass and substrate reduction (pooled results for both rearing systems) were 44.3 (1.7) mg DM and 52.8 (6.4) % DM for TP, and 50.6 (4.7) mg DM and 52.0 (5.3) % DM for DFW. Despite the longer rearing duration, the larval mass and substrate reduction were lower in WWP, showing values of 17.8 (2.8) mg DM and 36.1 (1.8) % DM, respectively.

Considering the alpha and beta diversity metrics, the substrate apparently affected the intestinal and residual bacterial richness and community. Similar to the substrate, the intestinal and residue bacterial communities were the richest when the substrate was TP. Community richness

was comparable between WWP and DFW (Figure 12 and Figure **14**). UniFrac distances and heatmaps demonstrate the unique bacterial communities between the intestine and residue for the same substrate, sharing a few taxa at the family level (Figure 16A). Among the substrates, the intestinal and residual bacterial communities also differed, with few shared taxa at the genus level (Figure 16D). Intestinal samples shared members of *Dysgonomonas*, *Enterococcaceae*, and *Enterococcus*, and residue sample shared members of *Glutamicibacter*.

4. Discussion

The goal of this study was to explore potential solutions to increase the performance of BSFL on abundant and affordably sourced agri-food by-products that represent a challenge and opportunity in valorisation. Specifically, we aimed to: 1) validate whether the novel rearing system designed by Palma et al. (2018) for almond hulls is beneficial for BSFL rearing, and 2) assess whether a microbial inoculum derived from the rearing residue increases rearing performance. We hypothesised that both the rearing system and the introduction of residue-derived inoculums could increase the rearing performance.

4.1. Rearing system design

We found that the rearing system design influenced the performance (Figure 9). BSFL reared in the closed system on WWP were 5.1 mg DM heavier than those in the open system, and the TP reduction in the closed system was 11.7% DM higher than that in the open system. Surprisingly, higher WWP larval mass and TP substrate reduction did not result in a higher WWP substrate reduction and TP larval mass. An advantage of the closed system seems to be that the

sealed bags and humidified airflow maintain a residual moisture content (Figure 10, 71-77% for TP and WWP) in the optimal range (70-80%) for BFSL (Dortmans et al., 2017). The slightly reduced larval mass on WWP in the open system could be due to the low residual moisture content (Figure 10, 42%), which decreased the WWP palatability by BSFL. The higher TP reduction in the closed system is surprising, as the median temperature in the residue was 4 °C lower (Figure 11) than that in the open system (Figure 11). The lower temperature in the closed system can be explained by the continuous aeration of the substrates with ambient temperature air. As an increase in the residual temperature presumably increases the activity of larval digestive enzymes (Bonelli et al., 2019), one could expect higher TP reduction in the open system. A possible explanation for the higher TP reduction in the closed system could be the increased aeration compared to the open system, resulting in enhanced larval/bacterial substrate decomposition (Palma et al., 2018). It remains unclear, however, as to why this effect in the substrate reduction between systems was not observed on WWP or when the residue-derived inoculum was added to the TP substrate (**Error! R eference source not found.**6). A disadvantage of the closed system is the insufficient aeration of pasty substrates, such as DFW. This was indicated by the increase in anaerobic bacteria of the family *Peptostreptococcaceae* (Slobodkin, 2014) in the intestinal bacterial community accompanied by a septic smell. This could explain the notably lower substrate reduction in two of the three replicates for the closed system compared to the open system. Considering these drawbacks and the higher operational resource requirements (e.g. aeration, closing of containers, harvesting), the industrial applicability of the closed system remains unclear.

4.2. Residue-derived inoculums

Our method of incorporating the residue-derived inoculums back into the substrate did not improve the rearing performance. This is in contrast with previous studies that showed clear improvement in rearing efficiencies with the addition of pure-culture bacteria or defined bacterial mixtures (Kooienga et al., 2020; Rehman et al., 2019; Somroo et al., 2019; Xiao et al., 2018; Yu et al., 2011), and even the rudimentary use of fermentate is ubiquitous in accelerating the fermentation of foods. A possible explanation for this result is that the residues did not include probiotic bacteria. We expected that fly-associated bacteria from the genera *Lactobacillus*, *Bacillus*, *Dysgonomona*, *Morganella*, *Proteus*, and/or *Enterococcus* were abundant in the residues on all substrates (Ao et al., 2020; Bruno et al., 2019; Gold et al., 2020c). Typical intestinal bacteria belonging to *Enterococcus* were indeed present in the DFW residue, and *Dysgonomona and Providencia* were present in WWP residues along with the family *Enterobacteriaceae*, to which the *Proteus spp.* and *Morganella spp.* belong (Figure 13). However, the abundance of these genera was < 6%, being absent in the TP residue in either system. Previous researchers have also reported variable bacterial communities in residues and the abundance of intestinal bacteria (Wynants et al., 2019), that were attributed to different initial substrate bacterial communities and nutrient contents, as well as operating parameters (e.g. feeding rate; Gold et al., 2020c; Wynants et al., 2019). A further possible explanation for our results is the insufficient replication of the residual bacterial community by the applied cultivation method. For example, *Dysgonomonas* considered supporting the hemicellulose digestion was reduced in abundance (Bruno et al., 2019). Bacterial abundance in the TP (*Acinetobacter*, *Lysinibacillus*, *Myroies*, *Vagococcus*) and WP (*Acinetobacter*, *Enterobacteriaceae)* inoculums (Figure 13) did not elicit any apparent positive effect on the larval growth and substrate reduction (**Error! Reference source not found.**).

Even though our addition of the residue-derived inoculum resulted in no apparent increase in the performance, our results are not completely unexpected. Performance improvements in BSFL rearing have also been absent or minimal in other studies. Callegari et al. (2020) isolated intestinal bacteria and showed a positive influence on the larval growth after the addition of *Escherichia coli* and *Bacillus licheniformis* to the substrate, but not *Stenotrophomonas maltophilia*. Similarly, Kooienga et al. (2020) observed that the growth rate increased with *Arthrobacter* AK19 and *Rhodococcus rhodochrous* 21198, but the addition of *Bifidobacterium breve* to the substrate had adverse effects. Similarly, Mazza et al. (2020) inoculated chicken manure with pure-culture bacteria and bacterial mixtures isolated from eggs and digestive tracts. Four out of seven bacteria influenced the larval mass by less than ± 2%, and three out of nine bacterial mixtures decreased the larval mass. Several questions remain regarding how the inoculation of substrates can reliably improve the rearing performance; however, variable results can be partially explained by the different digestive/metabolic capacities of microbes and variable nutritional requirements of BSFL depending on the operational rearing parameters. It remains to be confirmed as to which of the added bacteria colonise the residue or digestive tract, and whether viable bacteria are responsible for the reported improvements. Kooienga et al. (2020) recently showed that despite the growth improvements by *R. rhodochrous* 21198 and *Arthrobacter* AK19, only the latter colonised the larval digestive tract. Our study was the first to use sterile inoculums instead of sterile water as a negative control. Autoclaving the bacterial inoculum could have increased the digestibility by BSFL and could explain the higher larval mass of the control compared to the TP treatment. Future studies should isolate members of the potentially beneficial taxon (i.e. *Lactobacillus, Bacillus*, *Dysgonomona*, *Morganella*, *Proteus*, and/or *Enterococcus)* and elucidate their true potential to

influence the mass-rearing performance in bench and industrial-scale experiments. All previous studies on the substrate inoculation in BSFL rearing have focused on pure-culture bacteria or defined bacterial mixtures. Recirculating the bacteria using the residue could be improved by optimising the cultivation conditions (e.g. medium and oxygen conditions) and doses.

4.3 Rearing substrates

Our results show that the substrate type, namely, the substrate composition, including the nutrients, pH, bacterial numbers, and community, as well as metrics of palatability not quantified in this study, had a larger effect on BSFL rearing than the rearing system (Figure 9) or the addition of residue-derived inoculums (**Error! Reference source not found.**). The nutrient composition (i.e. protein and lipid contents, Table 3) and rearing performance metrics confirmed that despite the enzymatic digestion process, DFW is a high-performing BSFL substrate. This was expected, as DFW was known to be promising as pig feed (Jinno et al., 2018) . Additionally, DFW was high in *Lactobacillus* and *Bacillus*, which previously had positive effects on the larval growth (i.e. *Bacillus natto, Bacillus subtilis, Lactobacillus buchneri*; Rehman et al., 2019; Somroo et al., 2019; Xiao et al., 2018; Yu et al., 2011). Despite a much lower nutrient content than DFW, and with a high content of cellulose and lignin (44.8% DM, Table 3), TP showed a rearing performance comparable to that of DFW (Figure 9). Food wastes, such as DFW, frequently have the highest BSFL rearing performance (Gold et al., 2020a; Lalander et al., 2019). The low rearing performance of WWP could be due to the low protein (9.7% DM, Table 3) and high fibre (34.2% DM) contents. Additionally, potential insecticidal and bactericidal properties of secondary metabolites in WWP (i.e. phenolic acids; Katalinić et al., 2010) could have also affected the larval growth and microbiota (Isibika et al., 2019; Pavela, 2011). Finally, pasteurising prior to BSFL rearing by the

companies providing the DFW and TP substrates could have also increased the digestibility of DFW and TP by BSFL (Jinno et al., 2018). In comparison, WWP that was mechanically pressed at a winery was not subjected to heat treatment prior to use. However, despite the pasteurisation, both substrates had high microbial numbers and bacterial community richness (Table 3, .

Figure 15).

5. Conclusions

Efficient rearing of BSFL on agri-food by-products requires solutions to improve the performance. This study examined whether the rearing system and the addition of residue-derived inoculums increased the performance of TP and WWP. The closed rearing system had an equal or superior performance compared to the conventional open system. Research on the sufficient aeration of pasty rearing substrates and the efficient larval harvest from high-moisture residues is indispensable before the onset of industrial BSFL rearing in closed systems. Returning potentially beneficial microbes with an inoculum made from the residue did not impact the performance, residue properties, and microbiota. This approach could be improved by studying the effects of larval-associated microbes and developing cultivation methods that selectively amplify the beneficial members of the microbial community.

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Tables and Figures

Table 1: Total viable counts (TVC, log_{10}/g) in the residue of the open and closed rearing systems.

	Open system	Closed system
TP	8.0 (0.3)‡	7.9 (0.1)‡
WWP	> 9.5 (0.0)°*	9.4 (0.2)*
DFW	7.8 (0.1)†	7.7 (0.4)†

in parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n=2

*n=2, †n=3 and ‡n=4
°counts above countable range
TP: tomato pomace
WWP: white wine pomace
DFW: digested food waste

Table 3: Nutrient composition, pH, moisture content, and bacterial counts of the rearing substrates (n=1).

	pH	Protein	Lipids	Ash	Cellulose & lignin	Hemi-celluloses	TVC
	-	%DM	%DM	%DM	%DM	%DM	log_{10}/g
TP	5.8	15.7	14.1	3.2	44.8	1.6	8.4
WWP	4.7	9.7	9.4	7.7	34.2	20.7	4.2
DFW	5.8	33.8	12.1	24.6	11.5	57.7	7.0

TP: tomato pomace
WWP: white wine pomace
DFW: digested food waste
TVC: total viable counts

Figure Legend

Figure 9: Effect of the rearing system on the larval mass, substrate reduction and pH (Experiment 1). Means (horizontal lines), standard deviations, and results per biological replicate (n=3-4, filled circles=closed system, hollow circles=open system) are displayed. DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace.

Figure 10: Effect of the rearing system (Experiments 1 and 2) and microbial inoculation (Experiment 2, o=sterile inoculum, +=inoculum) on the residue moisture content. Means (horizontal lines), standard deviations, and results per biological replicate (n=3-4, filled circles=closed system, hollow circles=open system) are displayed. DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace.

Figure 11: Effect of the rearing system on the residue temperature. Horizontal lines represent the median temperatures for all replicates between the open and closed system. DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace.

Figure 12: Effect of the rearing system on the (A) intestinal and (B) residual bacterial community alpha and beta diversity metrics. Beta diversity is illustrated by the Principal coordinate analysis (PCoA) of bacterial communities based on the weighted UniFrac dissimilarity. Samples (n=3-4) were clustered with the unweighted pair group method with arithmetic averages (UPGMA). DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace

Figure 13: Bacterial communities of (A) TP and (B) WWP bacterial inoculums and residues used for their production. Heatmaps of the top 15 genera of grouped samples based on the relative abundance of ZOTUs. Relative abundances are the mean of replicate samples (n=3-4 for the residue, n=2 for the inoculum), rounded off to one digit. If no clear assignment to a genus was possible, the family assignment is shown along with the ZOTU. DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace.

Figure 6: Effect of the bacterial inoculation (+=inoculum, o=sterile inoculum) on the larval mass, substrate reduction, and residue pH (Experiment 2). Means, standard deviations, and results per biological replicate (n=3-4) are displayed. DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace

Figure 14: Effect of the microbial inoculation (o=sterile inoculum, +=inoculum) on (A) intestinal and (B) residual bacterial community alpha and beta diversity metrics. Beta diversity is illustrated by the Principal coordinate analysis (PCoA) of bacterial communities based on weighted UniFrac dissimilarity. Samples (n=3-4) were clustered with the unweighted pair group method with arithmetic averages (UPGMA). DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace.

Figure 15: DFW and TP bacterial community. Heatmaps of the top 10 genera in both substrates based on the relative abundance of ZOTUs rounded off to one digit. DFW: digested food waste; TP: tomato pomace.

Figure 16: Bacterial communities in larvae and residues reared on DFW, TP, and WWP on the (A) phylum, (B) family, and (C, D) genus levels. Heatmaps of the most abundant ZOTUs among the grouped samples. Relative abundances are the mean of replicate samples (n=3-4) rounded off to one digit. If no clear assignment to a genus was possible, the family assignment is shown along with the ZOTU. DFW: digested food waste; TP: tomato pomace; WWP: white wine pomace.

Figure 1.

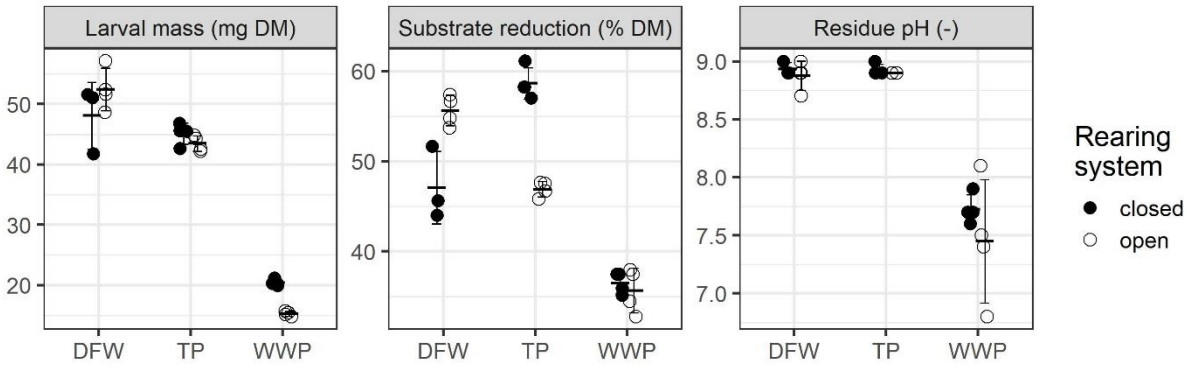


Figure 2

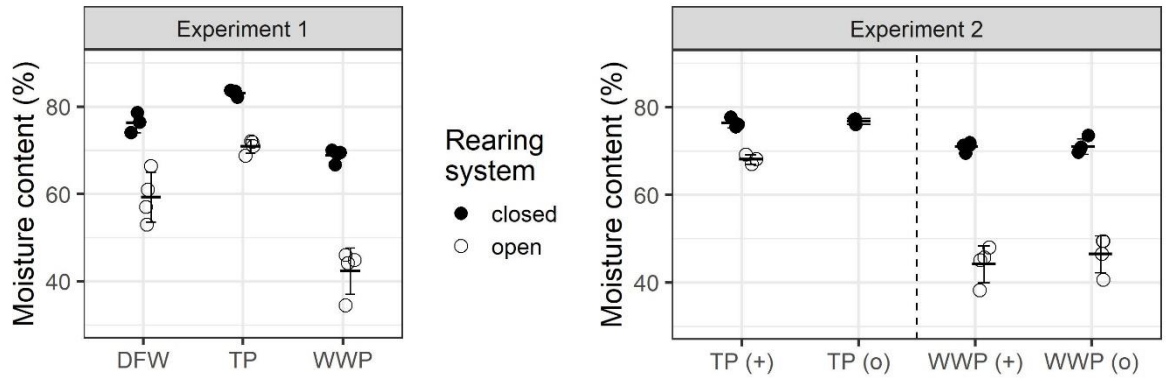


Figure 3

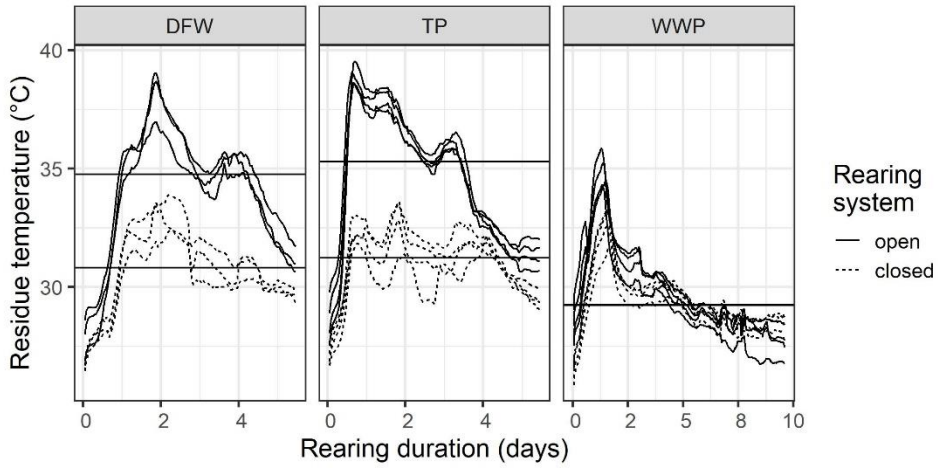


Figure 4

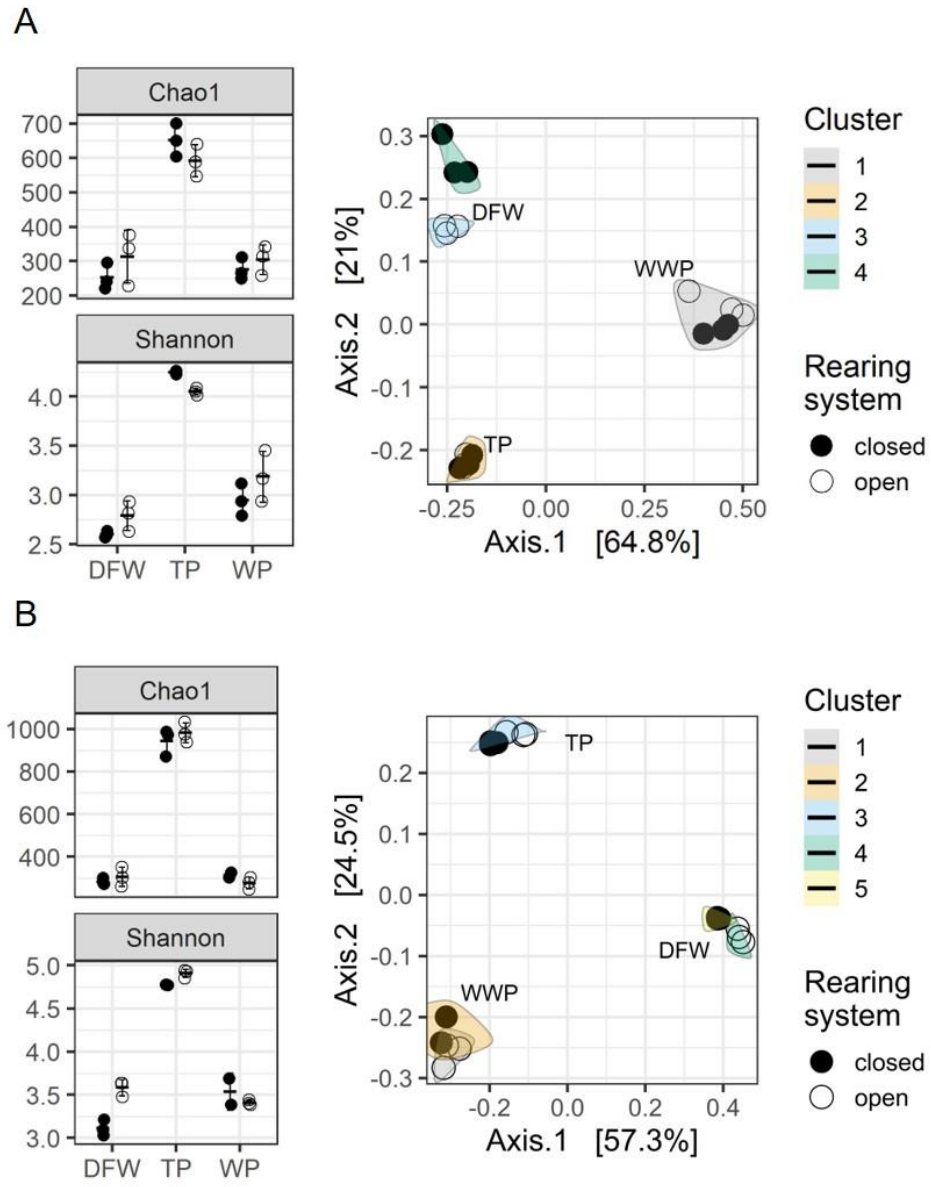

A
Chao1
700
600
500
400
300
200
Shannon
4.0
3.5
3.0
2.5
DFW
TP
WP
Axis.2 [21%]
0.3
0.2
0.1
0.0
-0.1
-0.2
DFW
WWP
TP
-0.25
0.00
0.25
0.50
Axis.1 [64.8%]
Cluster
1
2
3
4
Rearing system
closed
open
B
Chao1
1000
800
600
400
Shannon
5.0
4.5
4.0
3.5
3.0
DFW
TP
WP
Axis.2 [24.5%]
0.2
0.1
0.0
-0.1
-0.2
-0.3
TP
DFW
WWP
-0.2
0.0
0.2
0.4
Axis.1 [57.3%]
Cluster
1
2
3
4
5
Rearing system
closed
open

Figure 5.

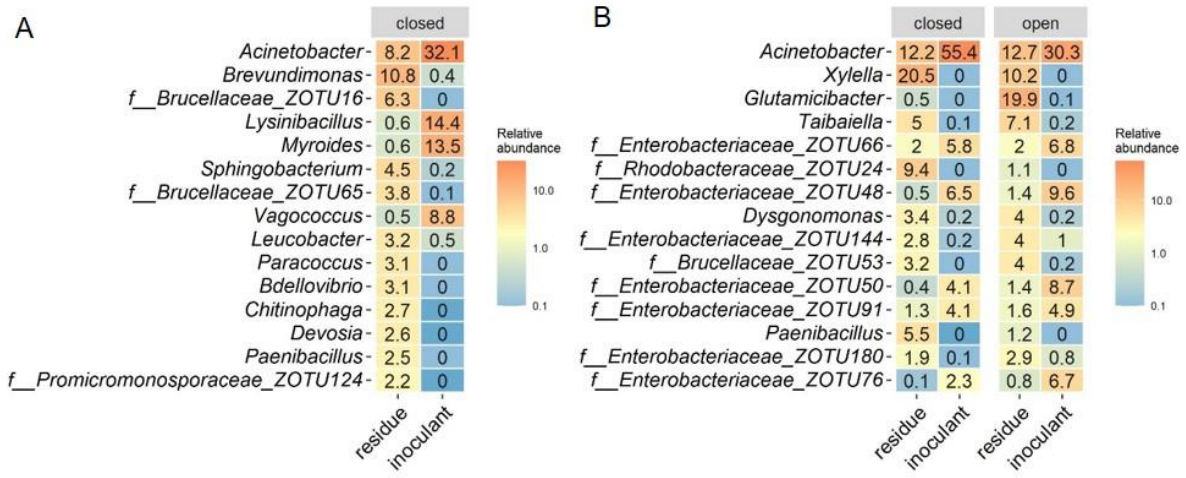


Figure 6.

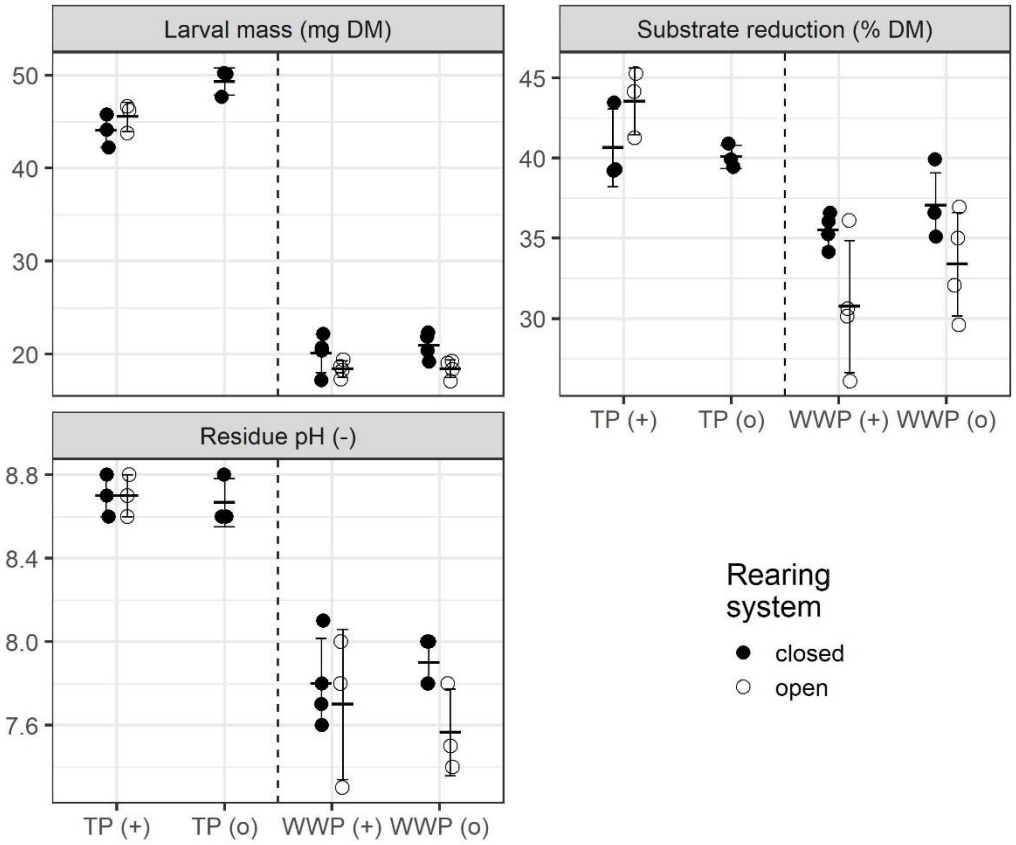


Figure 7

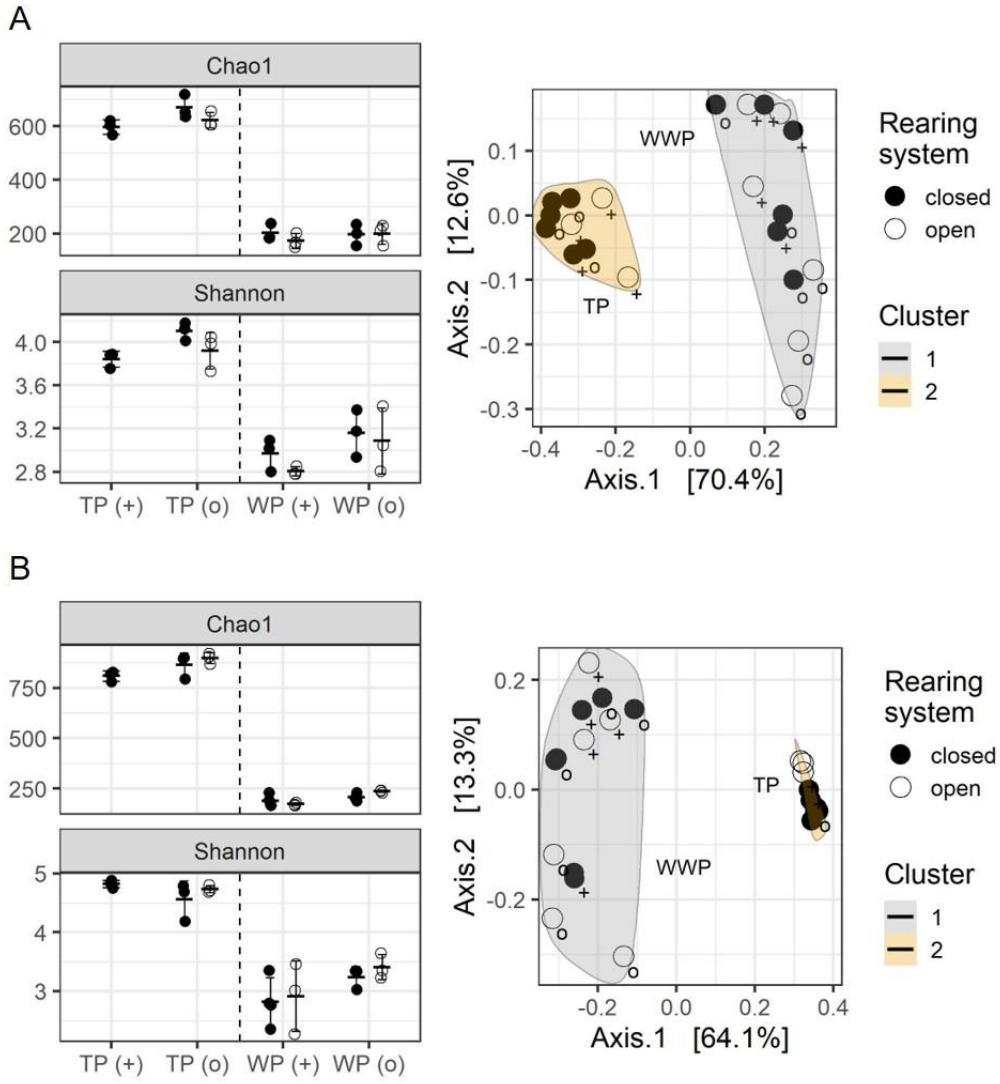

A
Chao1
600
400
200
Shannon
4.0
3.6
3.2
2.8
TP (+)
TP (o)
WP (+)
WP (o)
WWP
TP
Axis.2 [12.6%]
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-0.2
0.0
0.2
Axis.1 [70.4%]
Rearing system
closed
open
Cluster
1
2
B
Chao1
750
500
250
Shannon
5
4
3
TP (+)
TP (o)
WP (+)
WP (o)
Axis.2 [13.3%]
0.2
0.0
-0.2
TP
WWP
-0.2
0.0
0.2
0.4
Axis.1 [64.1%]
Rearing system
closed
open
Cluster
1
2

Figure 8

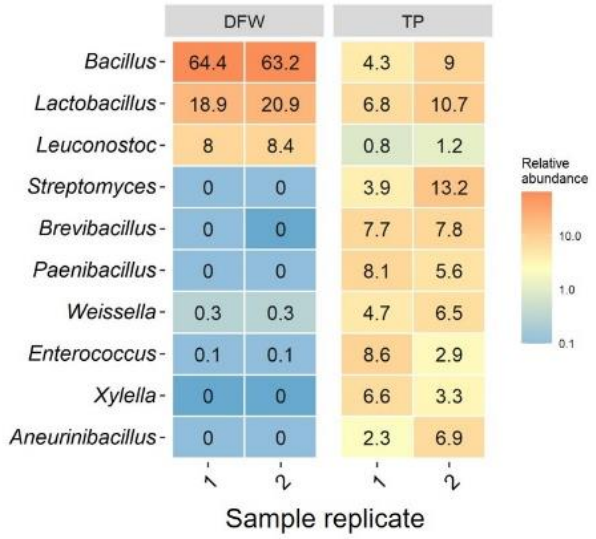
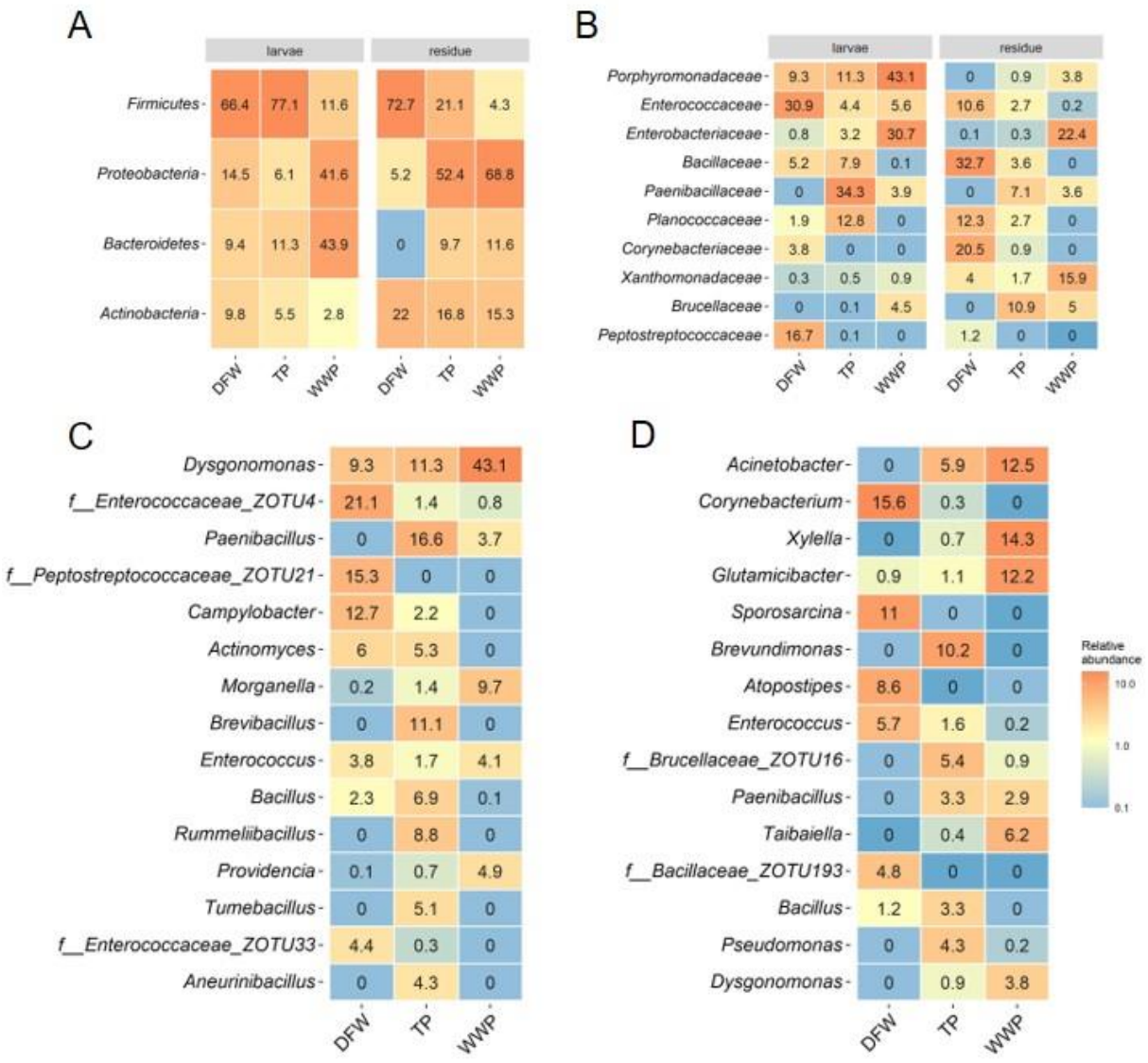

DFW
TP
Bacillus
Lactobacillus
Leuconostoc
Streptomyces
Brevibacillus
Paenibacillus
Weissella
Enterococcus
Xylella
Aneurinibacillus
64.4
63.2
4.3
9
18.9
20.9
6.8
10.7
8
8.4
0.8
1.2
0
0
3.9
13.2
0
0
7.7
7.8
0
0
8.1
5.6
0.3
0.3
4.7
6.5
0.1
0.1
8.6
2.9
0
0
6.6
3.3
0
0
2.3
6.9
1
2
1
2
Sample replicate
Relative abundance
10.0
1.0
0.1

Figure 9


A
larvae
residue
Firmicutes 66.4 77.1 11.6 72.7 21.1 4.3
Proteobacteria 14.5 6.1 41.6 5.2 52.4 68.8
Bacteroidetes 9.4 11.3 43.9 0 9.7 11.6
Actinobacteria 9.8 5.5 2.8 22 16.8 15.3
DFW TP WWP DFW TP WWP
B
larvae
residue
Porphyromonadaceae 9.3 11.3 43.1 0 0.9 3.8
Enterococcaceae 30.9 4.4 5.6 10.6 2.7 0.2
Enterobacteriaceae 0.8 3.2 30.7 0.1 0.3 22.4
Bacillaceae 5.2 7.9 0.1 32.7 3.6 0
Paenibacillaceae 0 34.3 3.9 0 7.1 3.6
Planococcaceae 1.9 12.8 0 12.3 2.7 0
Corynebacteriaceae 3.8 0 0 20.5 0.9 0
Xanthomonadaceae 0.3 0.5 0.9 4 1.7 15.9
Brucellaceae 0 0.1 4.5 0 10.9 5
Peptostreptococcaceae 16.7 0.1 0 1.2 0 0
DFW TP WWP DFW TP WWP
C
Dysgonomonas 9.3 11.3 43.1
f__Enterococcaceae_ZOTU4 21.1 1.4 0.8
Paenibacillus 0 16.6 3.7
f__Peptostreptococcaceae_ZOTU21 15.3 0 0
Campylobacter 12.7 2.2 0
Actinomyces 6 5.3 0
Morganella 0.2 1.4 9.7
Brevibacillus 0 11.1 0
Enterococcus 3.8 1.7 4.1
Bacillus 2.3 6.9 0.1
Rummeliibacillus 0 8.8 0
Providencia 0.1 0.7 4.9
Tumebacillus 0 5.1 0
f__Enterococcaceae_ZOTU33 4.4 0.3 0
Aneurinibacillus 0 4.3 0
DFW TP WWP
D
Acinetobacter 0 5.9 12.5
Corynebacterium 15.6 0.3 0
Xylella 0 0.7 14.3
Glutamicibacter 0.9 1.1 12.2
Sporosarcina 11 0 0
Brevundimonas 0 10.2 0
Atopostipes 8.6 0 0
Enterococcus 5.7 1.6 0.2
f__Brucellaceae_ZOTU16 0 5.4 0.9
Paenibacillus 0 3.3 2.9
Taibaiella 0 0.4 6.2
f__Bacillaceae_ZOTU193 4.8 0 0
Bacillus 1.2 3.3 0
Pseudomonas 0 4.3 0.2
Dysgonomonas 0 0.9 3.8
DFW TP WWP
Relative abundance
10.0
1.0
0.1

Chapter 5: Effects of chitinous products on mold growth, germination rate, and seedling weight of legume seeds

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ABSTRACT

Pathogenic molds, such as white mold (*Sclerotinia*), are known to adversely affect germination and seedling weight of crops, including legumes. In this study, seeds of three legume species [soybeans (*Glycine max*), black-eyed peas (*Vigna unguicula*), and fava beans (*Vicia faba*)] were treated with one of three chitinous products [1) raw insect-derived chitin (RIDC) from shed exoskeletons of yellow mealworms (*Tenebrio molitor*), 2) chitin, and 3) chitosan-derived from chemically rarified crustacean exoskeletons]. Legume seeds were investigated due to their high potential in absorbing chitinous products. We assessed treatment effects on mold growth, seed germination, and seedling weight. In total, results from 2,228 legume seeds were obtained. Overall, we found: 1) presence of white mold growth: control seeds (73%), RIDC (26%), chitin (10%), and chitosan (42%), 2) germination rate: control seeds (58%), RIDC (53%), chitin (76%), and chitosan (77%), and 3) individual seedling dry weight: control seeds (50 mg), RIDC (48 mg), chitin (51 mg), and chitosan (47 mg). From this data the following general trends were identified. Both dosage of chitinous products and legume species were found to influence results, but all three chitinous products significantly suppressed mold growth. At higher dosages (5% and 10% by weight), RIDC adversely affected germination rate of soybeans, but not of fava beans and black-eyed peas. Across chitinous products and dosages applied, no significant effects on seedling weight were observed. Considering the world-wide availability, low economical cost, and superior efficacy as a mold growth inhibitor, results from this study highlight RIDC as a promising novel fungicide when applied at a low dosage (2.5%, by mass). This study is part of on-going research into mass-rearing of insects as bioconverters of agricultural waste streams. The long-term goal is to promote sustainable waste management practices and to identify commercial markets of insect-derived products, including RIDC.

INTRODUCTION

Chitin, a neutral, nontoxic polymer of repeating *N*-acetyl glucosamine monomers[1] is the second most abundant naturally occurring polysaccharide on the planet.[2] Total annual production of chitin exceeds 1 billion tons,[3] of which the majority is produced by fungi and arthropods.[4] In 2016, United Nations estimated 7.5 million tons of chitinous waste was sourced from crustaceans alone[5]. Additionally, by 2030 insect-derived chitinous waste from mass-rearing of insects for food and feed is expected to increase by 28% to reach over 700,000 tons.[6] Consequently, increasing supply of chitin-containing waste materials has spurred interest in its medical, industrial, and agricultural applications.[7] Chitin's innate rigidity and non-polarity makes it insoluble in water. However, heat and chemical alteration of chitin yields its water-soluble derivative, chitosan.[1,8,9], which has been extensively studied.[10] As examples, chitosan has been studied based on its potential insecticidal[9] and fungicidal[11,12] properties, and also as a possible fertilizer.[13] Additionally, important cereal grain crops including wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) exhibited increased germination rates when seeds were primed with chitosan.[14–16] Guan et al.[14] speculated this effect was due to the promotion of chitinolytic bacteria, which release chitosanases. These enzymes degrade chitosan and release amino substituents on the second carbon of chitosan, allowing amino groups to become readily available for plants to absorb.[10] Thus, adding chitosan to crop growth media may promote beneficial chitosanase-releasing microbes.[4,10,17] Additionally, chitosanases degrade chitosan found in fungal cell walls,[4] which may explain fungicidal properties of the *O*-(decanoyl) chitosan derivative on gray mold (*Botrytis cinereali*).[11] While chitosan has been shown to stimulate plant growth and defense, few studies directly compare effects of chitosan and chitin,[18,19] and we are unaware of studies describing possible effects of chitin derived from insect mass production. Though chitin polymers were

discovered prior to chitosan, the insolubility of chitin has resulted in less studies into agricultural applications compared to chitosan.[3]

The present study investigated potential agricultural uses of three chitinous products: 1) raw insect-derived chitin (henceforth RIDC) from shed exoskeletons of yellow mealworms (*Tenebrio molitor*), 2) chitin and 3) chitosan, both derived from chemically rarified crustacean exoskeletons. Specifically, we investigated effects of these chitinous products on mold growth, germination, and seedling weight when applied to seeds of three legume crops: soybeans (*Glycine max*), black-eyed peas (*Vigna unguicula*), and fava beans (*Vicia faba*). Legume seeds were investigated due to their high potential in absorbing chitinous products[20]. We hypothesized that chitinous products significantly inhibit mold growth and increase germination rates and seedling dry weight of all three legumes. This study is part of an on-going applied research effort into ways to promote both environmental sustainability and commercial potential of insect mass production on agricultural waste streams.

MATERIALS AND METHODS

Chitinous products

Three chitinous products, one 'raw' and two 'pure' were used in this experiment. RIDC from shed yellow mealworms exoskeletons was obtained from Beta Hatch Inc. (Seattle, WA) and stored at -20 $^{\circ}$C until further preparation. Crustacean-derived chitin (CAS: 1398-61-4) and chitosan (CAS 9012-76-4) were purchased from Fisher Scientific™ (Waltham, MA) and stored at -20 $^{\circ}$C until further preparation. Each chitinous product was mechanically ground using a blender (Kitchenaid, Benton Harbor, MI, USA) and passed through a 1 mm sieve (#18 W.S Tyler standard

Sieve Co; Mentor, OH, USA). All three chitinous products were prepared in the following dilutions in DI water (by weight): 0 (control), 2.5, 5.0, and 10%.

Mold growth and seed germination bioassays

Soybeans, black-eyed peas, and fava beans were purchased from a local supermarket (Davis Food Co-op, Davis, CA, USA). Preliminary experiments found that 100% of seeds of all three legume species exhibited mold growth one week after inoculation with a supernatant collected from soaked seeds of the respective species. A white mold was the only fungus observed in this study. The developing mold was tentatively identified as the cosmopolitan and highly destructive necrotrophic fungus, *Sclerotinia.* Identification was based on emergence of spore-bearing apothecial discs when infected seeds were transferred to soil media[21,22] (Figure 2A).

Legume seeds were submerged in 50 ml DI water, and hand-stirred for 60 seconds. Individual seeds were transferred to sterile petri dishes (Fisherbrand, Waltham, MA, USA) lined with black filter paper (Ahlstrom, Helsinki, Finland) to enhance mold visibility (Figure 17B). Each petri dish received 3 ml of either 0 (DI water), 2.5, 5.0, and 10% of RIDC, chitin, or chitosan. Inoculated seeds were kept under controlled ambient conditions at (27 $^{\circ}$C, 75% RH) and total constant darkness for 72 hours. Both mold growth and germination were assessed based on dichotomous evaluations (i.e. presence/absence). Moreover, mold was considered present when mycelium was growing directly on the seed itself (Figure 17C). A seed was considered germinated if radicle protrusion was clearly visible (Figure 17D).

Seedling weight bioassay

To test effects of chitinous products on seedling weight, we measured the above "ground" dry weight of three-week-old seedlings subjected to 1-hour seed priming pretreatments. Seed priming

is defined as a pre-sowing treatment to physiologically condition plants prior to radicle protrusion[23]. Hydro-priming is submersion of seeds in fluid to promote accelerated and uniform germination, and is widely applied among legume species including chickpea[24] (*Cicer arietinum*), mungbean (*Vigna radiata*),[25] lentil (Lens culinaris)[26], fava bean[27]. To quantify the efficacy of chitinous seed priming treatments, seedling dry weight was chosen as a variable likely to exhibit the most difference between treatments and which is also used by the Association of Official Seed Analysis [28].

Individual seeds were hydro-primed via submersion in solutions of 0 (control), 2.5, 5.0, or 10% (by weight) of RIDC, chitin, or chitosan for one hour. For each combination of legume species, chitinous product, and dosage, 30 seeds were planted into individual cells of seedling trays. Each cell contained ~50 ml of sterilized plant potting media, and seedling trays were kept under controlled ambient conditions at (27 ˚C, 75% RH) and 12:12 light:darkness. Each seed cell received 3 ml of their respective treatment solution as initial watering. After 3 days, DI water was added to containment trays under all seedling trays to supply water to developing seedlings through the remainder of the study. After three weeks, seedlings were cut at soil level and dried at 60 ˚C for 48 hours before being weighed individually. Similar to assessments performed by commercial seed inspection laboratories and international standards[28], only seeds that sprouted above ground shoots were included in the dry weight bioassays.

Seedling development varied among legume species This was at least partially linked to differences in species-specific ontology (germination times). As example, after the first week most black-eyed peas had visible cotyledons compared to none of the fava beans (though root development was taking place). Accordingly, we did not perform statistical comparisons of species but focused exclusively on dosage responses.

Data analysis

All statistical analysis was performed using R v3.6.1 (The R Foundation for Statistical Computing, Vienna, Austria). Principal component analysis (PCA) was applied to the results of the three bioassays to determine the relationship between mold growth, germination, seedling weight, chitinous products, dosage response, and legume species. R libraries used for PCA analysis included ‘devtools’, ‘ggbiplot’, ‘factominer’, ‘factoextra’, “ggforce”. To test our hypothesis on data from dichotomous evaluations (mold and germination bioassays), contingency tables were constructed for each legume species-dosage combination and multiple two-tailed Z-tests were performed to compare each species-dosage combination’s similarity to the controls. For seedling weight bioassays, we performed pairwise comparisons of average seedling dry weight based on one-way analysis of variance (ANOVA) and Tukey's tests. In addition, dosage responses were assessed using Pearson's Chi-square goodness-of-fit tests comparing similarity among dosages (i.e. for soybean-chitin combinations did 2.5% = 5% = 10%), as well as Pearson's correlation coefficient for dosage and response value. Significant differences were tested at the 0.05 level.

RESULTS

Multivariate data visualization

In a PCA biplot of all data (2,228 observations), the two principal axes, PCA1 and PCA2, accounted for 50.02% of the total variance (Figure 18). With about half of the total variance explained by the two principal axes, the PCA can be used to identify underlying trends. Mold growth was aligned along the principal axis, PCA1, and seedling weight was aligned along the second axis, PCA2. As distances between points in Figure 18 denote level of association/disassociation, it is seen that chitosan was positively associated with mold growth in

fava beans, and that mold and chitin were negatively associated (opposite direction from center, 0,0). In addition, RIDC was negatively associated with germination, especially of black-eyed peas. Size, shape, and location of colored ellipsoids denote scattering of observations within each treatment, and it is seen that the ellipsoid of untreated legume seeds is the smallest, which suggests observations were most consistent. It is also seen that the ellipsoid is biased towards "mold", which implies high propensity of mold growth. The ellipsoid of RIDC is considerably larger than those of other treatments, which suggests more variable responses among individual seeds subjected to that treatment. In the PCA, the variable denoting seedling weight was associated mostly with black-eyed peas, which suggests that variation in dry weight was most pronounced in response to that legume species and to a lesser degree an effect of chitinous products and/or their dosage. In general, the PCA revealed that main sources of variance were associated with the two response variables, mold growth and seed germination, and sizes of ellipsoids suggested product-specific levels of variance. The PCA also demonstrated unique associations and responses among legume species and chitinous products. In the following, we examine individual trends associated with each of the three agriculturally significant responses measured.

Mold growth

Averaging across dosages and species revealed control seeds had a higher average presence of mold growth (72.68 ± 0.45%) than RIDC (25.71 ± 0.44%), chitin (9.60 ± 0.42 %), and chitosan (42.26 ± 0.50%) (Table 4). Thus, chitin treatments resulted in the greatest reduction in the presence of mold (also indicated by the PCA), while chitosan show the least mold growth inhibition potential (Figure 19). Only chitin elicited significant reduction in mold growth relative to the control for all dosage combinations of legume species and dosages tested. ($p <. .001$; Table 5). No dosage response (i.e. difference between responses among low, medium, high dosage) was

detected for chitin or chitosan in any of the three legumes—suggesting the lowest dosage tested was sufficient to inhibit mold growth (Table 6**: Comparison of mold growth, germination, and seedling weight responses to chitinous products**). For RIDC, negative dosage responses were detected for black-eyed peas and fava beans with higher dosages conferring significantly lower mold growth (both $p < .001$). Chi-square goodness-of-fit tests showed that at each dosage, the three chitinous products elicited significantly different mold responses from one another (Table 7).

Seed germination

Averaging germination rates across dosages and species revealed greater germination rates for controls (57.89 ± 0.49 %) than RIDC (52.75 ± 0.50%), but less than chitin (76.27 ± 0.77%) and chitosan (77.40 ± 0.42%) (Table 4). Germination rates of untreated seeds varied considerably among legume species: black-eyed peas = 70%, fava beans = 52%, and soybeans = 47% (Figure 19). For chitin and chitosan treatments, no clear trends were detected. However, at higher dosages (5% and 10%), RIDC adversely affected germination of soybeans ($p < .001$), but not fava beans and black-eyed peas.

Seedling weight

Across dosages and species, average control seedling weight (45.59 ± 0.03 mg) was not significantly different from those treated with RIDC (47.97 ± 0.03 mg), chitin (51.26 ± 0.03 mg), and chitosan (47.41 ± 0.03 mg) (Table 4). A positive dosage response was detected in soybeans using chitosan ($p < .01$), whereas a negative dosage response was found in fava beans using chitin ($p < .05$; Table 6). No difference in weight was detected for black-eyed peas.

DISCUSSION

A recent increase in numbers of companies and investments into mass production of insects has led to a surplus of insect-derived chitinous byproducts.[6] Supply of chitinous byproducts from insect mass production is set to increase significantly over the coming decades as humans address issues concerning food insecurity, with much of the growth occurring in developing countries.[29] Moreover, commercially available chitosan from crustacean shells have limited potential for industrial acceptance due to seasonality and high processing costs.[30] Consequently, with growing production and saturation of existing market, there is a need for identification of novel and commercially viable usages of chitinous byproducts. Demonstrating mold growth suppression from insect-derived chitinous byproducts may support development of novel biodegradable fungicides with new modes of action that may control fungicide-resistant pathogens. Such use would be beneficial to organic farming operations, as well as edible post-harvest seed treatments.[31]

In this study, effects of chitinous products on mold growth, germination, and seedling weight were investigated. We focused on how an unprocessed raw insect-derived product compared with two highly processed products (chitin and chitosan) with known agricultural benefits. In addition, we explored how dosage may influence the effects of the chitinous products. In absence of chitinous treatments, 73% of control seeds experienced mold growth (Table 4). For comparison, only 36% and 18% of legume seeds treated with RIDC or chitin showed signs of mold growth, respectively. Chitosan was the least efficacious fungicide of the three chitinous products used. Overall, this study demonstrated that at a low dosage RIDC performed well at reducing prevalence of mold, but was not found to have a significant effect on seed germination or seedling weight.

Mold growth

Sclerotinia is among the most destructive and widespread fungal diseases in bean plants[21] Quality losses in bean yield can range from 30 to 100% in conditions favorable for the mold (cool and humid).[32] The fungus overwinters as sclerotia in plant debris and soil, and is also known to spread by both internally and externally infected seeds.[33] Spores infect above and below ground tissue causing a characteristic white mold on necrotic tissue. *Sclerotinia* is known to spread via transport of dried beans and can even internally infect seeds[34,35]. In this study, *Sclerotinia* was identified by characteristic sclerotia and apothecia that developed from infected seeds transferred to soil media. Except for 2.5% RIDC and 5% chitosan, all other legume species and dosage combination significantly decreased the prevalence of mold growth for all three legumes species relative to their controls. Therefore, our data corroborate the hypothesis that all three chitinous products decrease the prevalence of mold growth. These results were consistent with previous findings with the use of chitin and chitosan inhibiting mold[16,36], however to our knowledge this is the first study to use RIDC.

Possible mechanisms underpinning reduction in mold growth on germinating seeds were considered beyond the scope of this study. However, we speculate three potential processes may, at least partially, explain our results. First, chitinous products may act as nonlethal cues that induce a defense response in seeds. For example, *Arabidopsis thaliana* has cell surface receptors where chitin acts as a ligand to initiate an induced immunity pathway (pathogen-associated molecular pattern, PAMP)[37]. Mentlak et al. found rice blast fungus (*Magnaporthe oryzae)* overcomes the same chitin-triggered PAMP defenses by secreting effector proteins that bind to chitin, thus perturbing the plants initial ability to recognize the invading fungi and prevent infection[38]. Moreover, gene knockdown of either the plants chitin elicitor binding protein (CEBiP) or the

fungal effector protein (Slp1) shifts the balance in likelihood of infection. Costs for maintaining gene-for-gene interactions that mediate pathogenicity between plant and fungus have been identified in various plant-pathogen systems[39,40]. Maintaining defensive metabolites balances opportunity costs in allocating energy away from other vital biological function[26]. In this present study, applications of chitinous products may have induced upregulation of chitinases or other defensive metabolic pathways at the expense of seedling weight. This scenario would explain the positive association between seedling weight and dosage for fava beans treated despite similar germination rates relative to the control. Regarding non-consistent effects of chitinous products on weight among legume species, cultivated crops differ in their coevolution and thus their sensitivities to cues from fungal pathogens. Screening for legume varieties that display resistance to *Sclerotinia,* as well as the biological processes underlying resistance is the subject of much research.[21]

Second, reduction in mold growth may be due to the promotion of chitinolytic bacteria, which release chitinase and chitosanases when they feed.[14] These enzymes may deter the growth of mold, which use chitin and chitosan as structural proteins in the cell wall. This would be consistent with the research by Chang et al.[41], which used a supernatant produced from a medium of the chitinolytic bacteria *Bacillus cerreus* and crustacean shell powder to reduce the prevalence of three fungi (*Fusarium oxysporum, Fusarrium solani,* and *Phthium ultimum).*

Third, more fungal spores may have initiated development on the suspended chitinous material than on the seed coat. Fungal spores are highly susceptible to the specific spatial architecture of surfaces, which create microclimates that influence their effectiveness in colonization[42]—including a surfaces hydrophobicity. *Sclerotiorum* has been shown to grow on hydrophobic surfaces such as waxy leaf epicuticles[43]. Chitin's innate rigidity and non-polarity make it insoluble

in water, whereas chitosan is water soluble[8]. In the present study, the seed receiving chitin treatments exhibited less mold growth than chitosan treatments. Although speculative, this may be explained by *Sclerotiorum* spores preferentially adhering to chitin's hydrophobic surfaces, although more research is required to confirm this.

In this study, the lowest dosage tested (2.5%) was sufficient to reduce prevalence of mold by 58% (average among all chitinous products and species), with further increases in dosages not correlating with a change in efficacy. RIDC is not purified and may vary markedly in its chitin and chitosan content (18-40%).[44] Dosages of chitinous products used in this present study were relatively high (2.5-10% by weight) compared to previous studies (0.01%).[45] Two studies using comparable dosages of chitosan similarly also reported fungicidal activity. For example, Benhamou et al.[46] demonstrated fungicidal properties for chitosan against *Fusarium oxysporum* with tomato (*Solanum lycopersicum*) seeds treated with 10% chitosan agar, and Cheah et al. [47] used dosages 1-4% to reduce the size of established *Sclerotiorum* in half.

Combining data from all dosages and legume species, this study revealed that chitin treatment (Table 4) was twice as effective a fungicide as RIDC, with mold growth present on 36% of the seeds treated with RIDC products compared to 18% for chitin products (Figure 19). Chitosan was the least efficacious fungicide of the three chitinous products used, which contradicts a previous *in vitro* study comparing chitosan and chitin that concluded chitosan the superior of the two at eliminating fungi.[45] It is noteworthy that absent any chitinous treatment, 73% of the control seeds experienced mold growth. Therefore, although chemical grade chitin was most efficacious, RIDC nonetheless reduced the occurrence of mold growth in half. Furthermore, considering the ease in preparation, economical cost, and superior efficacy compared to chitosan— RIDC seems a promising novel fungicidal material.

Seed germination

Due to lack of clear trends, we reject the hypothesis that chitinous products increase germination rates of tested legumes. This is surprising since prior studies using cereal grain (wheat, rice, and maize) yielded increased germination rates when seeds were primed with chitosan.[14–16] We speculate different germination rates in response to product dosages may be linked to fertilizer effects from biodegradation of chitinous polymers by chitinolytic bacteria. chitin and chitosan were obtained from chemically purified crustacean exoskeletons, whereas RIDC was obtained from molted exoskeletons and did not undergo any chemical purification. Research shows that mealworm shed exoskeletons (the source of our RIDC) are made up of 18% chitin by weight.[44] Chitinolytic bacteria would have nearly five times more available chitinous polymers to consume in the purified products. Bacterial chitinolytic enzymes catalyze the hydrolysis of chitin and chitosan by binding to the amino terminal. This process releases ammonia-derived compounds,[48] which would provide an nitrogen source to the germinating seeds. Wiwat et al.[49] found that in a medium containing 0.3% chitin, *Bacillus circulans* cultivated at 35 ^{o}C produced chitinase continuously during the exponential phase of growth and dosages peaked near the fifth day. In our study, we examined germination on the third day, at higher dosages and bioassays were performed at 28 ^{o}C. These important differences in experimental design may at least partially explain why our results did not show a clear fertilizer effect for germination, suggesting a different mechanism is influencing germination. As future research, efficacy of RIDC should be validated *in sutu* under field or soil conditions where resident chitinolytic bacteria can break down RIDC. Further, we stress that improvement and optimization in delivery and preparation of RIDC products are a potential line of research to reduce the present gap in performance.

Regarding dosages, a negative germination response was found for RIDC applied to black-eyed peas (Figure 19). A dosage response was not found for chitosan or chitin for any of the legume species tested. At low dosages, germination for legume seeds treated with RIDC was not significantly different from control seeds.

Seedling weight

Average seedling dry weight of treated seeds was not significantly different from controls for either black-eyed peas or soybeans (Table 7). Regarding fava bean, most combinations of dosage and chitinous products resulted in significantly lower average seedling dry weight than the controls, with the exceptions being 2.5% and 5% Chitin. Rather, the data suggests that seedling dry weight was most influenced by the seed species, with black-eyed peas weighing the most, followed by soybeans and fava beans (Figure 19). This is because each species has different development rates, with fava beans germinating best at cooler tempertures[28]. Notably, the root systems of fava beans were observed (though not measured) to be visually larger than the other two legume species with roots growing into the bottom water reservoir tray. This was the case for plants with no visible above "ground" shoots. Further experiments should quantify effects of chitinous seed priming on subterranean plant development, particularly considering subterrain fungi are known to closely associate with various legumes[50].

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TABLES AND FIGURES

Table 4: Average mold growth, germination rate, and seedling weight across dosages and legume seed species

		Chitinous products		
Response	**Control**	**RIDC**	**Chitin**	**Chitosan**
Presence of mold (%)	72.68 ± 0.45	25.71 ± 0.44	9.60 ± 0.42	42.26 ± 0.50
Germination rate (%)	57.89 ± 0.49	52.75 ± 0.50	76.27 ± 0.77	77.40 ± 0.42
Seedling weight (mg)	45.59 ± 0.03	47.97 ± 0.03	51.26 ± 0.03	47.41 ± 0.03

Overview of combined results from all dosages and legume species. Presence of mold is higher for the controls than chitinous products.

Table 5: Legume responses to increasing dosages of chitinous products.

			Chitinous products								
		Control	RIDC			Chitin			Chitosan		
Response	Legume	0%	2.5%	5%	10%	2.5%	5%	10%	2.5%	5%	10%
Mold growth (%)	Black-eyed peas	*82*	*30****	*9****	*5****	*8****	*5****	*3****	*30****	*44****	*52****
	Fava beans	*66*	*61*	*39****	*22****	*32****	*15****	*17****	*42**	*52*	*44**
	Soybeans	*69*	*23****	*15****	*18****	*3****	*3****	*0****	*44***	*38***	*33****
Germination rate (%)	Black-eyed peas	*71*	*69*	*74*	*53*	*75*	*87***	*98****	*100****	*89**	*74**
	Fava beans	*52*	*44*	*56*	*62*	*60*	*80****	*80****	*62*	*67*	*81***
	Soybeans	*48*	*38*	*18****	*23***	*73***	*63*	*70***	*74***	*75***	*74***
Seedling weight (mg)	Black-eyed peas	*58*	*60*	*59*	*69*	*64*	*60*	*68*	*62*	*56*	*56*
	Fava beans	*34*	*20***	*29**	*69***	*34*	*27*	*39**	*24**	*30**	*29***
	Soybeans	*42*	*31*	*37*	*44*	*37*	*38*	*41*	*27*	*41*	*47*

Analysis for mold growth and germination rate was performed using multiple two-tailed Z-tests. Analysis for seedling weight was performed using ANOVA. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ compared to control.

Table 6: Comparison of mold growth, germination, and seedling weight responses to chitinous products

Response	Legume	Chitinous products		
		RIDC	Chitin	Chitosan
Mold growth (%)	Black eyed peas	$\chi2 = 23.24$***	$\chi2 = 1.05$	$\chi2 = 2.83$
	Fava beans	$\chi2 = 26.19$***	$\chi2 = 4.22$	$\chi2 = 0.53$
	Soybeans	$\chi2 = 0.77$	$\chi2 = 0.86$	$\chi2 = 0.71$
Germination rate (%)	Black eyed peas	$\chi2 = 8.68$*	$\chi2 = 8.79$*	$\chi2 = 8.44$*
	Fava beans	$\chi2 = 5.50$	$\chi2 = 5.45$	$\chi2 = 2.72$
	Soybeans	$\chi2 = 4.52$	$\chi2 = 1.03$	$\chi2 = 0.01$
Seedling weight (mg)	Black eyed peas	$F = 1.05$	$F = 0.47$	$F = 0.32$
	Fava beans	$F = 0.28$	$F = 3.32$*	$F = 0.04$
	Soybeans	$F = 1.69$	$F = 0.42$	$F = 5.47$**

Analysis for mold growth and germination rate was performed using Pearson's Chi-square goodness-of-fit test. Analysis for seedling weight was performed using ANOVA. Tests evaluated similarity between each dosage (i.e. for the soybean-chitin combinations does 2.5%=5.0%=10%). * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ compared to control.'

Table 7: Comparison of mold growth, germination, and seedling weight responses to dosages of chitinous products

Response	Legume	Dosage		
		2.5%	5.0%	10%
Mold growth (%)	Black eyed peas	$\chi2 = 8.06$*	$\chi2 = 24.87$***	$\chi2 = 44.68$***
	Fava beans	$\chi2 = 17.51$***	$\chi2 = 14.12$***	$\chi2 = 12.68$**
	Soybeans	$\chi2 = 111.21$***	$\chi2 = 10.93$**	$\chi2 = 7.21$*
Germination rate (%)	Black eyed peas	$\chi2 = 10.91$***	$\chi2 = 4.73$	$\chi2 = 25.55$***
	Fava beans	$\chi2 = 4.12$	$\chi2 = 6.63$*	$\chi2 = 6.03$*
	Soybeans	$\chi2 = 13.24$**	$\chi2 = 25.28$***	$\chi2 = 23.93$***
Seedling weight (mg)	Black eyed peas	$F = 0.17$	$F = 1.25$	$F = 1.81$
	Fava beans	$F = 2.97$	$F = 0.91$	$F = 0.19$
	Soybeans	$F = 1.70$	$F = 0.23$	$F = 0.69$

Analysis for mold growth and germination rate was performed using Pearson's Chi-square goodness-of-fit test. Analysis for seedling weight was performed using ANOVA. Tests assessed similarity between chitinous products (i.e for soybean-dosage combinations does 10% Chitin = 10% Chitosan = 10% Raw insect-derived chitin). * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ compared to control.

Figure Legends

Figure 17: Images from mold and germination bioassays. A) Emerging apotheca from white molds, B) Preparation of seeds, C) White mold growth on seed, D) Germination of seed with radicle protruding.

Figure 18: PCA (Principal component analysis) biplot of bioassays. PCA employed to determine the relationship between mold growth, germination, chitinous products, dosage response, and legume species (2,228 observations). 'BEP' represents black-eyed pea, 'FAV' represents fava bean, 'SOY' represents soybean. Ellipsoids indicate 95% coverage of chitinous product's data.

Figure 19: Effects of chitinous product dosages on each legume species. Black lines indicate RIDC, blue lines indicate chitin, red lines indicate chitosan. Controls are 0%. A) Mold growth; B) Germination; C) Seedling dry weight. Significance level among treatments presented in table 2. Seed weight used continuous data; error bars are for standard error (n = 30). Mold and germination data used dichotomous evaluations (presence or absence) which did result in standard error (n > 35).

Figure 20: Correlation coefficients between: (a) RIDC, (b) Germination, or (c) Seedling weight and responses in black eyed peas, fava beans, soybeans. The right side of the x-axis indicates greater (a) mold growth, (b) germination, (c) or seedling weight.

Figure 1

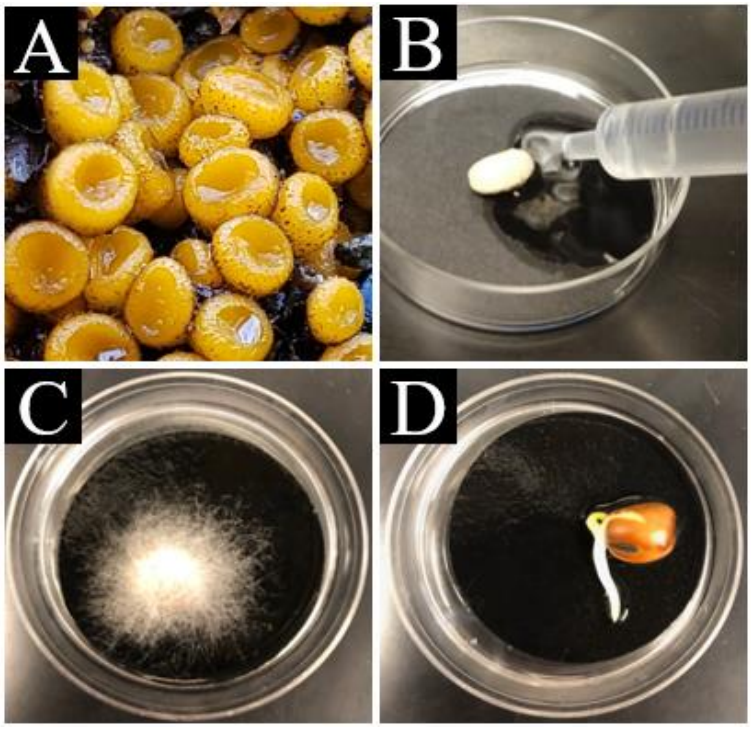


Figure 2

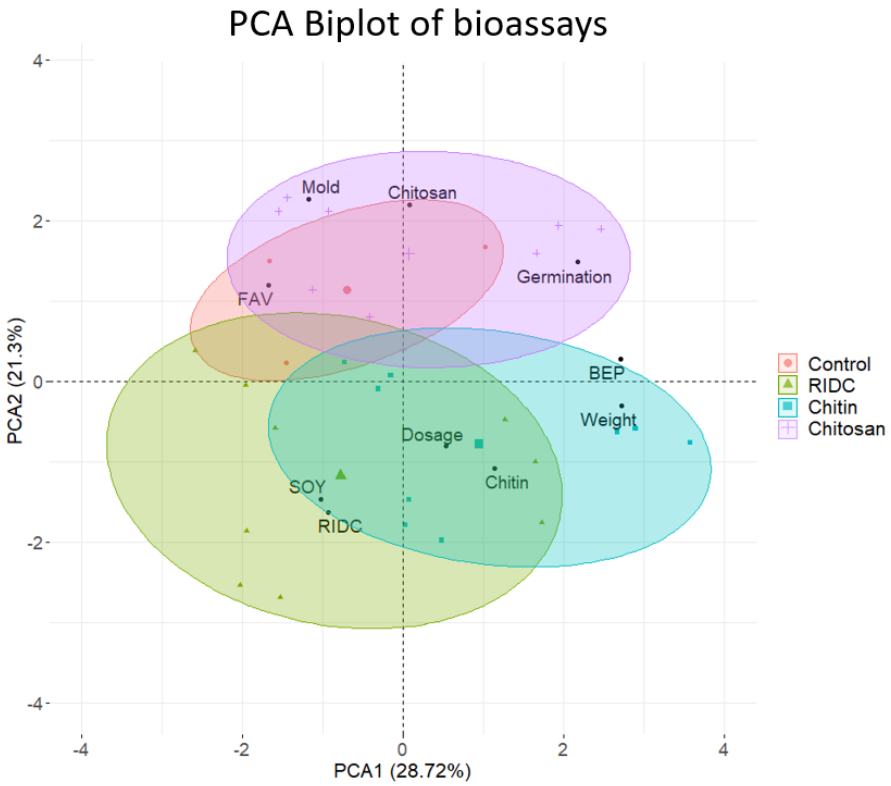

PCA Biplot of bioassays
PCA2 (21.3%)
PCA1 (28.72%)
4
2
0
-2
-4
-4
-2
0
2
4
Mold
Chitosan
Germination
FAV
BEP
Weight
Dosage
Chitin
SOY
RIDC
Control
RIDC
Chitin
Chitosan

Figure 3

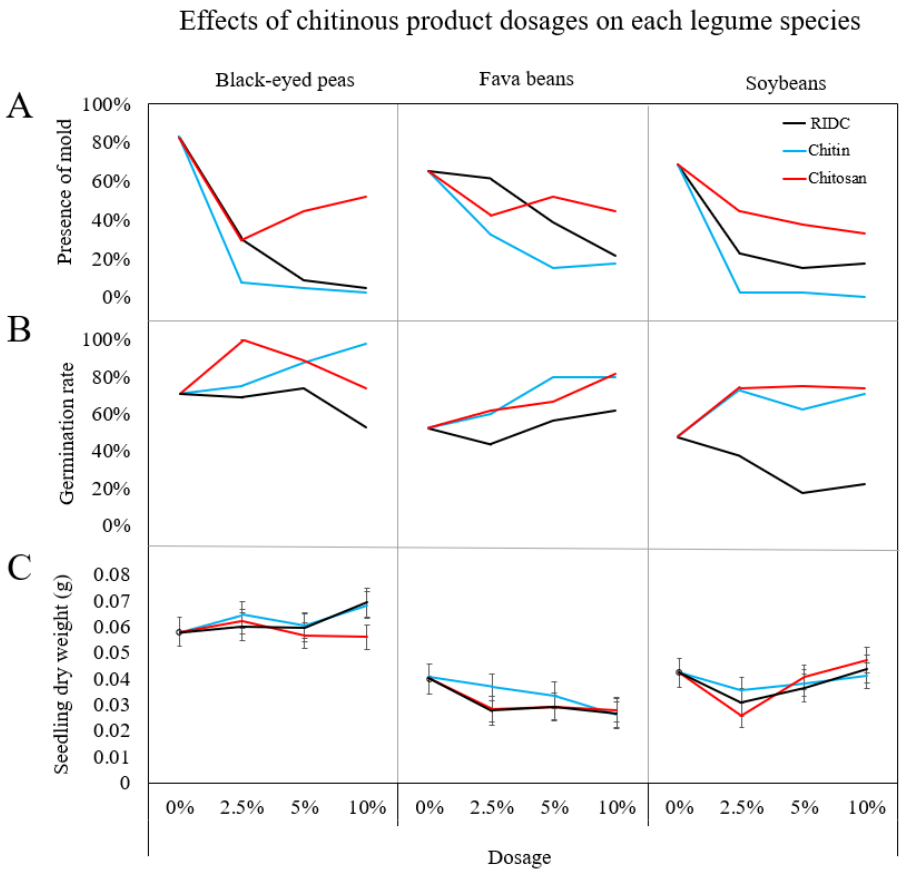
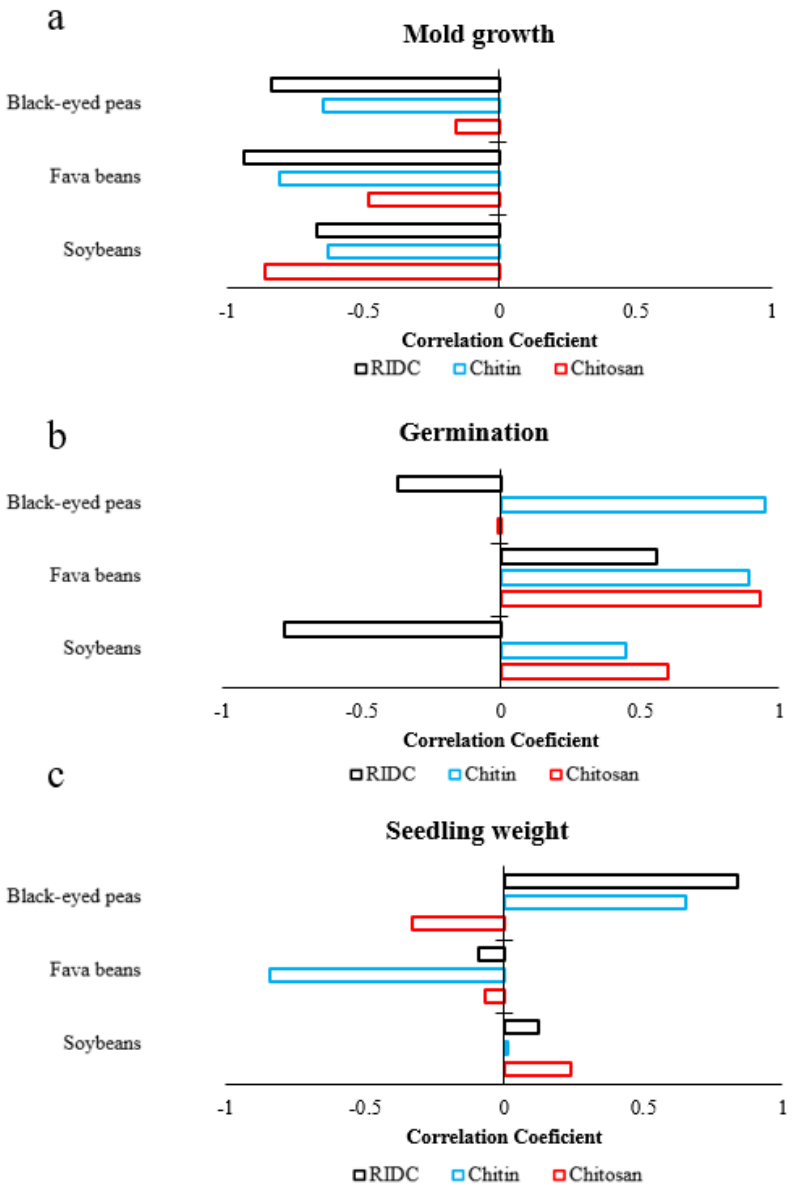

Effects of chitinous product dosages on each legume species
Black-eyed peas
Fava beans
Soybeans
A
B
C
Presence of mold
Germination rate
Seedling dry weight (g)
100%
80%
60%
40%
20%
0%
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
RIDC
Chitin
Chitosan
0%
2.5%
5%
10%
Dosage

Figure 4


a
Mold growth
Black-eyed peas
Fava beans
Soybeans
-1
-0.5
0
0.5
1
Correlation Coeficient
RIDC
Chitin
Chitosan
b
Germination
Black-eyed peas
Fava beans
Soybeans
-1
-0.5
0
0.5
1
Correlation Coeficient
RIDC
Chitin
Chitosan
c
Seedling weight
Black-eyed peas
Fava beans
Soybeans
-1
-0.5
0
0.5
1
Correlation Coeficient
RIDC
Chitin
Chitosan