Co-supplementation of a polyethylene diet for improved fitness of *Galleria mellonella* larvae

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ABSTRACT

A growing number of plastivore insects have been discovered that readily consume and biodegrade various petro plastics, including LDPE. The caterpillar larvae of *Galleria mellonella* are capable of breaking down the polymers within 24 hours of ingestion. However, feeding on LDPE as a sole nutrient source is inefficient and detrimentally impacts larval survival, growth, and development. The objective of our study was to improve the fitness parameters and feeding activities of LDPE-fed larvae through the addition of various macro- and micronutrients. Each co-supplementation recovered fitness and consumption to some extent in comparison to pure LDPE; however, artificial sources produced outcomes that were well below those of the caterpillar's natural diet, regardless of the combination. Co-supplementation of LDPE, honeycomb, and corn syrup was the most successful, with larval fitness and consumption approximating their natural diet. Further, GC-MS analyses identified notable differences in their fat body metabolic profiles that may contribute to slower developmental rates. We also assessed the capability of the larvae to eliminate food wastes, which showed promise and could represent a fruitful avenue for future research. Predicated on our findings, we discuss the potential of mass insect farming for plastic remediation and plastivores as part of a circular economy.

Environmental Implication

Every year, over 400 million metric tons of petro plastics are produced, yet only 9% of the resulting waste is recycled and 80% of synthetic waste ends up in landfills or the natural environment. Among the various plastics types, polyethylene is the most commonly used in products due to its elasticity, chemical resistance, and durability. However, these properties that make plastics polyethylene commercially desirable also make the polymers highly resistant to degradation and

they also contain hazardous chemicals that pose a significant environmental threat. This research advances the large-scale application of plastivore insects to reduce plastic pollution.

Introduction

Over 450 million tons of plastics are produced globally each year (Ritchie et al., 2023), with the vast majority ending up in landfills or the natural environment (Evode et al., 2021). Among the various types of petroleum-derived (petro) plastics, polyethylene is the most commonly used. This synthetic polymer, and in particular low-density polyethylene (LDPE, plastic number 4), is used to produce various products (e.g., shopping bags, housewares, clear food wrap, electrical components) due to its elasticity, chemical resistance, and durability (Yao et al., 2022). While these properties of LDPE are commercially desirable, they also make the polymers highly resistant to degradation and pose a significant environmental threat (Evode et al., 2021). As a result, there has been a growing focus on innovative technologies for plastic remediation, including the biodegradation of LDPE. Various environmental microorganisms are capable of degrading LDPE and include diverse bacteria and fungi taxa isolated from freshwater, marine, and terrestrial ecosystems (Morohoshi et al., 2018; Urbanek et al., 2018; Raddadi and Fava, 2019). However, this inefficient process often requires prior physical processing and can take weeks to years to degrade naturally (Orr et al., 2004; Restrepo-Flórez et al., 2014; Yang et al., 2014; Bombelli et al., 2017).

Recently, a variety of "plastivores" spanning six insect orders have demonstrated the ability to consume and biodegrade LDPE (for review, Boctor et al., 2024). In particular, the caterpillar larvae of the greater wax moth, *Galleria mellonella*, can degrade and metabolize LDPE at expedited rates – within 24 hours of ingestion. This lepidopteran is a well-known pest of apiaries globally. Their life cycle encompasses six fast-developing instar stages that feed on honeycomb comprised of beeswax, pollen, and honey (Ellis et al., 2013). They feed voraciously as larvae to acquire the nutrient reserves necessary to subsist during the subsequent non-feeding pupal and adult moth stages (Niemierko and Wlodawer, 1950; Kwadha et al., 2017). It is believed that the chemical similarities between beeswax and petro plastics (i.e., long-chain hydrocarbons) endow *G. mellonella* with the unique capability of biodegrading LDPE (Cassone et al., 2022).

Biodegradation of LDPE by larval *G. mellonella* likely involves contributions from both the animal host and its associated gut microbial consortia (Cassone et al., 2020; LeMoine et al., 2020). The primary mechanism for biodegradation of high molecular weight petro polymers is through enzymatic oxidation and/or hydrolysis (Shah et al., 2008). Oxidation increases the surface area accessible to microbes, and there is evidence of associated enzymes (e.g., phenol oxidases, hexamerins, cytochrome P450s) in the larval saliva and digestive tract (Sanluis-Verdes et al., 2022; Spínola-Amilibia et al., 2023; Son et al., 2024). Although enzymes capable of breaking down the carbon skeleton of plastic polymers through hydrolysis have not yet been identified in *G. mellonella*, esterase enzyme activity has been detected in LDPE-fed larvae (Das et al., 2024). Moreover, a variety of petro plastic degrading enzymes (e.g., cutinases, esterases, lipases, laccases, peroxidases, proteases, ureases) have been identified in environmental microbes (Amobonye et al., 2021; Jin and Jia, 2024; Kim et al., 2024), and bacteria capable of subsisting solely on LDPE as an energy source have been characterized from the digestive tract of larval *G. mellonella* (Cassone et al., 2020). Indeed, the larvae are somewhat unique among lepidopterans in possessing a gut microbiome that can persist and proliferate on certain food substrate (Gohl et al., 2022). Nevertheless, our understanding of the precise mechanisms of biodegradation by *G. mellonella* and other plastivores is not fully resolved.

In general, larval *G. mellonella* fed on polyethylene show reductions in fitness, including decreased survival, growth, and development in comparison to their natural honeycomb diet (Bombelli et al., 2017; Cassone et al., 2020; Cassone et al., 2022; Réjasse et al., 2022). This is accompanied by metabolic and physiological alterations in the animal, including changes in frass consistency and lipid storage maintenance through augmented fatty acid metabolism (Cassone et al., 2020; LeMoine et al., 2020; Cassone et al., 2022). The reduction in fitness is likely attributed to nutritional deficiencies associated with petro plastics as a sole food source (Cassone et al., 2022). Co-supplementation has been shown to recover fitness to some extent, enhance plastic consumption rates, and alter the physiological properties of the larvae (Lou et al., 2020; Mahfooz et al., 2024); however, an optimized dietary regime for large-scale insect biodegradation has not yet been formulated for *G. mellonella* or other plastivores.

As high-throughput insect farming is becoming more commonplace, improved fitness and feeding activities on a co-supplemented plastic diet could represent a fruitful avenue for large-scale implementation of plastivores in plastic bioremediation. Studies undertaken to optimize/improve the diet of plastivores with or without plastic exposure have incorporated natural sources of supplements rather than artificial ones (Kundungal et al., 2019; Lou et al., 2020; Hickin et al., 2021; Mohamed and Amro, 2022; Mahfooz et al., 2024; Ndotono et al., 2024). Moreover, no research has explored the potential of food waste as a functional diet for plastivores, which may be desirable as part of a circular economy model. With this in mind, the objective of our study was to improve consumption and fitness parameters (e.g., survival, growth, and development) of LDPE-fed *G. mellonella* larvae through co-supplementation using both artificial (carbohydrates, protein/amino acids, and vitamins) and natural (honeycomb and corn syrup) sources of macro- and micronutrients. For context, we compared changes in fitness to larvae fed pure LDPE or their natural honeycomb diet. Further, we investigated the potential of larval *G. mellonella* to feed and develop on food waste substrate. Finally, we carried out untargeted metabolomics to evaluate the impacts of co-supplementation on fat body metabolomics for our most successful dietary regime.

2. Materials and Methods

2.1. *Galleria mellonella* colony maintenance

Our *G. mellonella* colony was established in 2017 from larvae provided by The Worm Lady (Lakefield, Ontario), with multiple subsequent infusions from the supplier and local apiaries. The colony is maintained in 2.5 gal Montana glass jars on an ad libitum diet of natural honeycomb from local apiaries containing residues of honey, pollen, and cast bee larval skins. The sourced honeycomb is sterilized at -80 °C for a minimum of 24 h prior to use in colonies. We maintain the colony in portable greenhouse chambers under optimal rearing conditions of 26-28°C, 60-80% relative humidity, and a 24 h dark cycle. Pupae are transferred to 9,425 cm^3 chambers (~100 individuals per chamber) containing crumpled wax paper for oviposition. The adult moths are monitored, and dead moths are removed daily.

2.2. Co-supplementation feeding trials

2.2.1. Experimental design

A summary of our experimental design for co-supplementation of LDPE is shown in Fig. 1. This entailed a series of larval feeding trials on 100% LDPE powder [Alfa Aesar™ polyethylene powder, low density, 500 microns (A10239; Lot: 10213083)] infused with various artificial and natural sources of macro- and micronutrients and formed into ~1.5 g films using a hydraulic press (trials 1 and 2) or through other methodologies described below (trial 3). Experimental *G. mellonella* were randomly collected from our maintenance cages, and second instar larvae were identified through measurements of body length (mm), body width (mm), head capsule length (mm), head width (mm), and body weight (mg), according to Rahman et al., 2017. Larvae were then placed individually in 50 mL conical tubes containing the respective co-supplemented LDPE treatment or finely ground honeycomb (control) until pupation. We ensured that the experimental tubes were loosely sealed to prevent escape and suffocation. Experiments were carried out through the duration of *G. mellonella*'s life cycle on the control substrate, which varied by trial. Moreover, experimental tubes were aerated daily by opening the lids once for approximately 1 min. Concurrently, fresh treatment films or honeycomb were provided and waste was removed.

2.2.2. Measurements of larval consumption, survival, growth, and development

For each experimental trial (see below), survival was recorded daily, and the probability of survival was adjusted to account for larvae that were lost during the respective trial. Changes in caterpillar mass in mg (i.e., growth) for each treatment/control were conducted prior to the commencement of experiments and subsequently on a weekly basis (unless otherwise noted). During this time, treatment/control films were weighed (mg) to provide a measurement of consumption. Larval growth (i.e., instar development) was also recorded at each time interval using the morphometric measurements described above (Rahman et al. 2017). Exposure of an individual to their respective treatment/control would cease at the onset of pupation; instead, survival and adult emergence were monitored daily. All experimental larvae were at the second instar stage upon commencement of a given trial.

2.2.3 Co-supplemented diet regimes

A total of three co-supplementation dietary regimes for *G. mellonella* were implemented through optimization of a different spectrum of artificial and natural sources of macro- and micronutrients with LDPE, as detailed below.

Trial 1 – sugar supplemented diet. Various concentrations of sugars were formulated by mixing them in equal parts weight (1:1:1:1): fructose (MW = 180.16 g/mol; D(-)- Fructose), glucose (MW

= 198.17 g/mol; D-Glucose Monohydrate), maltose (MW = 360.31 g/mol; D-(+) Maltose Monohydrate), and sucrose (MW = 342.30 g/mol). All sugars were supplied by BioBasic (Markham ON, Canada; product numbers: FB0213, GB0218, MB4950, SB0498). Sugar-LDPE films were then produced by heating the appropriate amount by weight of the sugar mixture and LDPE powder and formed into a film using a hydraulic press. In addition to the honeycomb control and 100% LDPE (no sugar), we produced four different concentrations: 10% sugar mix + 90% LDPE (low sugar, 10%), 50% sugar mix + 50% LDPE (medium sugar, 50%), 82% sugar mix + 18% LDPE (medium-high, 82%), and 90% sugar mix + 10% LDPE (high sugar, 90%). Exposures were conducted in triplicate (n = 10 per chamber) for a total of 180 experimental larvae (30 larvae × 6 treatments/control). Daily survival was assessed over 64 d, whereas consumption, growth, and development were recorded in 4 d intervals over 32 d. Reductions in sample sizes due to pupation (honeycomb, day 20) or larval death (LDPE, day 8; 10% sugar, day 24) resulted in early cessation of some treatments and fitness parameters.

<u>Trial 2 – sugar, protein/amino acid, and vitamin-supplemented diet</u>. In this trial, we endeavored to further optimize a sugar-LDPE diet by artificially incorporating ovalbumin (A-5253, CAS-no. 9006-59-1) as a protein source (Sigma-Aldrich, Burlington, MA), an essential amino acid mixture (Nutricia, Hoofddorp, Netherlands), and vitamins (100% complete max strength multivitamin) formulated by Jamieson (Toronto, ON, CA). The 50% sugar mix + 50% LDPE films were painted with one of two solutions: 1% (w/v) ovalbumin + 1% (w/v) amino acids + 0.25% (w/v) vitamins (PAV-SLF) dissolved in distilled water (ddH_2O); or 1% ovalbumin + 0.25% vitamins (PV-SLF) dissolved in ddH_2O. Exposures were conducted in triplicate (n = 10-15 per chamber), with a total of 100 second instar larvae (30-35 larvae × 3 treatments/control) fed their respective treatment or

the honeycomb control for 49 d, with survival assessed daily and other fitness parameters on a weekly basis.

Trial 3 - Honeycomb and corn syrup supplemented diet and food waste. We assimilated a naturally sourced co-supplementation of LDPE powder with honeycomb and golden corn syrup (Great Value™, Mississauga, ON, CA). Equal parts of the three ingredients were combined to obtain a clay-like consistency. This HC-CS-LDPE mixture was transferred into a mold to set for 24 h at room temperature and then stored at -20 °C. A total of 100 *G. mellonella* larvae were used with the treatment, and honeycomb control run in duplicate (50 larvae × 2 treatment/control). Caterpillars were exposed to their respective food substrates for 42 d, with survival evaluated daily and other fitness parameters on a weekly basis over 4 wks.

Our final treatment was performed concurrently with HC-CS-LDPE and did not include LDPE; rather, it assimilated a food wastes treatment consisting of dehydrated vegetable and fruit scraps. We first obtained a variety of fruit and vegetable boxes from the Real Canadian Superstore® (Brandon, MB, CA) through the Flashfood® application. These were coarsely chopped and desiccated at 52°C for 5 h using a digital food dehydrator (Hamilton Beach, Glen Allen, VA). All dehydrated food substrates were mixed and stored at -4°C until the commencement of experiments. A total of 10 larvae were used in this trial.

2.2.4. Statistical analyses

All statistical testing included only larvae that were alive at given time intervals, and the small number of larvae that were lost during experimentation were also omitted from further analysis. Moreover, data collection for a given individual ceased upon pupation (where applicable), with the exception of survival. The probability of daily survival was analyzed and plotted through

Kaplan-Meier survival analysis (log-rank test) and pairwise multiple comparisons analysis (Holm-Sidak method; $p < 0.05$) using SigmaPlot (version 11.0) (Goel et al., 2010; Rich et al., 2010). Since at least some of the data generated for each trial did not distribute normally, consumption and changes in larval mass among treatments at each time interval were analyzed by Kruskal-Wallis non-parametric tests followed by Dunn's test post-hoc ($p < 0.05$), using the MultNonParam and dunn.test libraries in R (R Development Core Team). As there were no significant differences between replicates within the treatments for all of the trials, treatment data was pooled to maximize statistical power. Analyses were carried out on treatments with ≥9 larvae at a given time interval except for the food waste treatment (≥7 larvae), as the number of experimental specimens was considerably reduced compared to the other trials/treatments.

2.3. Fat body metabolomics

2.3.1. Larval collections

In addition to the feeding trials, we carried out untargeted metabolomics on fat bodies of fifth instar larval *G. mellonella* for both the HC-LDPE-CS treatment and honeycomb control. A total of 20 individuals were used per treatment/control, with five individual fat bodies pooled per sample replicated four times. The targeted tissues were dissected out on an ice-filled sterile petri dish via a lengthwise ventral incision. A new set of sterilized scalpels (size 10 blades) and forceps were used for each individual. Samples were then lyophilized (-40°C at 0.050 mbar) and sent to the Biodiscovery Institute at the University of North Texas for untargeted metabolomics analysis via gas chromatography and mass spectrometry (GC-MS), as described below.

2.3.2. Untargeted metabolomics

Samples (~5-10 mg) were crushed with 5 mm stainless steel beads using a bead beater set to a frequency of 30 Hz/s for 3 min. Homogenized samples were centrifuged (17,000 g) on ice for 30 s at 4°C. Samples were kept on ice until the addition of 5μL of internal standard mixture (20 mM 13C-glycine, 10 mM ampicillin and 20 mM 13C-mannose) and MTBE:methanol extraction solvent (3:1 v/v at -20°C). After a 10 min incubation in the thermomixer (1,500 rpm at 4 °C), the samples were sonicated for 10 min at 4°C. A 500μL water/methanol (9:1 v/v) solution was added to each sample and mixed (1,750 rpm at 4 °C) for 1 min. The samples were centrifuged at 17,000 g for 5 min, after which the lower phase (i.e., polar and semi-polar compounds) were collected (650 μL) and transferred (250 μL) to a 300 μL glass insert. Each glass insert was then placed in a 1.5 mL microcentrifuge tube and sealed with a lid with two poked holes for methanol removal. Methanol was evaporated from the samples at 30 °C via a speed vacuum. The evaporation process was repeated twice more until the remainder of the lower phase product had been exhausted. Samples were immediately flash-frozen in liquid nitrogen and subsequently lyophilized at -83 °C for 3 h.

Glass inserts were transferred to GC-MS vials for intracellular metabolite derivatization. A 200 μL methylene chloride (CH_2Cl_2) solution was added, and the biological samples were flushed using nitrogen (at room temperature) for 10 s to remove methylene chloride. Similarly, 50 μL of methylene chloride was added to 20 mg/mL methocyamine HCl in pyridine solution and immediately flushed. All samples were incubated at 40 °C for 90 min at 1,250 rpm. After incubation, 75 μL of MSTFA + 1% TMCS derivative reagents were added to the samples. Samples were then flushed, vortexed, and incubated using the previous settings at 45 min. The derivatized extracts (supernatant) were transferred to a new 250 μL insert for GC-MS analysis.

Gas-chromatography of alkylsilyl derivatives was performed using Thermo Trace 1310 gas chromatograph helium as a carrier gas (flow rate = 1.4 ml/min). The temperature was raised in three steps: 1) the initial temperature was set to 70 °C for 5 min; 2) a ramp was applied at a rate of 3°C/min to raise the temperature to 235 °C; and 3) a second ramp was applied at 6 °C/min to reach the final temperature of 320 °C, which was held for 15 min. A 1 μL aliquot of the derivatives was injected into the column set to 300 °C at a split ratio of 10. An ISQ single quadrupole mass spectrometer coupled to the chromatograph (Thermo Fisher, San Josem, CA, USA) was used for the MS analysis. This analysis was conducted with the electron impact ionization set to positive ion mode over a mass range of 50 amu to 1,100 amu at 300 °C (ion source) and 325 °C (interface temperature).

Picking, identification. and feature alignment was conducted through MS-DIAL (version 4.9.221218). A total of 1,669 features and mass quantifiers were initially picked. After normalization (using the corresponding biological sample weights and internal standards) and qualitative checks, compounds were submitted to the NIST17 library for compound identification/assignment.

2.3.3. Statistical analysis

Untargeted metabolomics data analysis was carried out using MetaboAnalyst 6.0 (Xia et al., 2009; Pang et al., 2024). We normalized the data by sum with square root transformation and autoscaling. Significant metabolites were identified using two-sample t-tests and Wilcoxon rank-sum tests ($p < 0.05$). Data were visualized using two-dimensional partial least squares discriminant analysis (PLS-DA) and hierarchical clustering heatmaps.

3. Results

3.1. *Sugar co-supplemented LDPE diet*

In this trial, LDPE films were infused with increasing concentrations of carbohydrates (i.e., various sugars found in the natural larval diet), and changes in the fitness of second instar *G. mellonella* larvae were evaluated over time relative to a pure LDPE diet. To determine the extent by which any recovery in fitness occurred via co-supplementation, we also compared these treatments to caterpillars fed honeycomb. The probability of daily larval survival over 64 d (Fig. 2A) was significantly higher on augmented sugar concentrations (50%, 82%, and 90%) in comparison to the 10% sugar and LDPE treatments (all comparisons, $p < 0.001$). Larvae fed pure LDPE had mean survival times of <4 d (3.28 ± 0.042), which increased to 13.7 ± 2.2 d with the addition of 10% sugar. Survivability was comparable among the 50%, 82%, and 90% treatments at 25.2 d ± 3.3, 24 d ± 3.4, and 26.7 d ± 3.2, respectively. Despite this recovery, survival did not attain the levels of honeycomb-fed larvae (all comparisons, $p < 0.05$), largely due to greater mortality rates after ~20 d of experimentation.

Although sugar co-supplementation prolonged survival, larval growth and development recovered minimally. Honeycomb-fed larvae routinely developed into pupae or adults during the experimental period, whereas the other treatments did not progress through the third (10%) or fourth (50%, 82%, and 90% sugar) instars (Fig. S1). Similarly, individuals fed honeycomb gained mass throughout the experimental period until pupation (~20d), whereas those provided carbohydrates showed negligible changes (<±2 mg per time interval) regardless of concentration (Fig. 2B). Although this represents a statistically significant improvement over the LDPE treatment, it is markedly lower than the honeycomb-fed larvae (all applicable time intervals/treatments, $p < 0.05$) and not indicative of meaningful growth recovery. Co-supplementation improved consumption to some extent (Fig. 2C), with the total amount of substrate ingested across larvae increasing with carbohydrate concentration: LDPE (151.5 mg), 10% (987 mg), 50% (4,797.1 mg), 82% (12,843.6), and 90% (12,822.3 mg). Although several time intervals showed significant increases in consumption at higher carbohydrate levels, honeycomb-fed larvae consumed more substrate overall (21,367.2 mg) and at each applicable time interval until pupation (all treatments, $p < 0.05$).

3.2. Sugar, protein/amino acid, and vitamin-supplemented LDPE diet

In this trial, we expanded the co-supplementation of LDPE films with carbohydrates also to include proteins/amino acids and/or vitamins to explore whether a more well-rounded artificially sourced nutrient profile would further improve larval *G. mellonella* fitness. We chose to supplement the 50% sugar-LDPE treatment with these additional nutrients because trial 1 indicated larval fitness was generally reduced on lower sugar concentrations (0% and 10%) but comparable on augmented concentrations (50%, 82%, and 90%). Overall, the addition of proteins/amino acids and vitamins did not result in meaningful recovery of larval fitness relative to those supplemented with carbohydrates only. Daily survival over the 7 wk period was comparable to the augmented sugar concentrations ($p > 0.05$) but still significantly lower than those fed their natural diet (Fig. 3A). As before, co-supplementation did not result in larvae progressing to the fourth instar, whereas all honeycomb-fed caterpillars developed into pupae by the end of week 4. Both larval weight gain (Fig. 3B) and consumption (Fig. 3C) were comparable for sugar-LDPE larvae provided protein/amino acids or vitamins only, but significantly lower than those fed honeycomb at each experimental week ($p < 0.001$, all pairwise comparisons). Specifically, the average mass gained on these two artificial treatments never exceeded 2 mg per week, with total consumption between 901 mg (protein/amino acids and vitamins) and 1,478 mg (vitamins).

3.3. Honeycomb and corn syrup supplemented LDPE diet and food waste

In our final trial, we co-supplemented LDPE with natural sources of nutrients, specifically honeycomb and corn syrup. Further, a subset of caterpillars was offered food wastes comprised of various dehydrated fruits and vegetables (no LDPE). Both the naturally supplemented and food waste treatments showed marked improvements in overall larval fitness over the 4 wk experimental period in comparison to the previous trials, with the former exhibiting recovery levels exceeding or in approximation to their natural diet for most parameters (Fig. 4). Indeed, daily survival of naturally supplemented larvae was comparable to that of honeycomb, and consumption was significantly higher over the initial 2 wks of experimentation (both wks, $p < 0.001$). Moreover, changes in mass fluctuated, with naturally supplemented larvae showing comparable gains in the first week and significantly higher gains in the third week ($p = 0.03$). Larvae fed on food wastes did not recover to the same extent, showing significantly lower probability of survival ($p = 0.001$), as well as reduced mass gains (all wks, $p < 0.004$) and consumption in the second and third weeks (both wks, $p < 0.05$) relative to the honeycomb treatment. Both experimental treatments exhibited slower developmental rates than honeycomb-fed larvae (Fig. S2). Specifically, only 5.6% of naturally supplemented larvae reached the sixth-instar and no individuals fed food waste progressed to the sixth instar over a 4 wk period.

3.4. Untargeted metabolomics

Given co-supplementation of LDPE with honeycomb and corn syrup largely restored *G. mellonella* larval fitness, we next explored whether HC-LDPE-CS altered the fat body metabolic profile in comparison to a natural honeycomb diet. This organ was targeted for investigation as it is a major metabolic hub of insects, accumulating energetic reserves during growth and development (Czaja-Topińska and Klekowski, 1970; Ellis et al., 2013). In total, 290 compounds were identified through GC-MS analysis. The PLS-DA shows a clear distinct clustering of samples between treatment and control (Fig. 5), indicating that diet altered the metabolic profiles of the caterpillars. Overall, we identified 24 compounds that significantly differed in their abundance between the diets (Fig. 6). Notably, HC-LDPE-CS larvae showed deficiencies in essential amino acids (nicotinic acid), fructose, as well as important metabolic precursors and intermediates (myoinositol and cystathionine). Conversely, HC fed animals had lower fat body content of ethanol phosphate, tryptophan, methionine and homocysteine.

4. Discussion

A variety of insects have been identified in recent years that readily consume and degrade LDPE, including the caterpillar larvae of *G. mellonella* (Bombelli et al., 2017; Cassone et al., 2020). However, the ingestion of the petro plastic as a sole nutrient source is predictably detrimental to feeding activities and larval fitness, including reductions in consumption, survival, growth, and development (Cassone et al., 2022). Therefore, our study aimed to recover larval feeding and fitness to levels approximating their natural honeycomb diet through co-supplementation of LDPE with various macro- and micronutrients. Fats were consistently absent from the dietary regimes despite beeswax (the major constituent of honeycomb) being largely composed of fatty acids and hydrocarbons (Tulloch, 1980). This is because the larvae maintain their lipid reserves on an LDPE diet through elevated fatty acid metabolism of the polymer long hydrocarbon chains (LeMoine et al., 2020; Cassone et al., 2022). Although all the co-supplementations we assessed recovered fitness to some extent in comparison to pure LDPE, only one (honeycomb and corn syrup) produced outcomes akin to a natural caterpillar diet. Notably, recovery from this diet indicates that LDPE and the associated biodegradation process does not adversely affect larval consumption or fitness; thus, large-scale rearing of *G. mellonella* on an optimized, co-supplemented diet could have intriguing potential in plastic bioremediation.

Carbohydrates are the primary source of energy for insects (Lee et al., 2004; Chen and Fadamiro, 2006). Additionally, they are involved in lipid storage, the production of some non-essential amino acids, and have been reported as feeding stimulants (Aherne and O'Brien, 2002; Dursun, 2009; Bernklau et al., 2018; Kilci and Altun, 2020). Larval *G. mellonella* predominately acquire carbohydrates from honey embedded in honeycomb, which is produced by bees from foraged flower nectar (Nicolson et al., 2022). Therefore, we supplemented LDPE with differing concentrations of carbohydrates ranging from 10% to 90%, with 82% approximating the total carbohydrate composition of honey (Khan et al., 2007; Vallianou et al., 2014). Since honey is primarily composed of glucose and fructose and, to a lesser extent, sucrose, maltose, and some other monosaccharides and disaccharides (Kamal and Klein, 2011; Hossain et al., 2023), we formulated these four sugars into LDPE films. Increasing carbohydrates prolonged larval survival and improved consumption, with the latter corroborating that sugars serve as feeding stimulants for *G. mellonella*. Nonetheless, growth and development lagged, and overall consumption and fitness were well below a natural honeycomb diet. It is possible that the addition of other carbohydrates or more optimized sugar ratios (e.g., higher fructose and glucose) could further improve larval fitness parameters, but meaningful recovery is likely not achievable from artificially sourced carbohydrates only.

Honeycomb contains pollen residues collected by bees, which largely satisfies the needs of *G. mellonella* for protein/amino acids and vitamins (Winston, 1987; Hoover and Ovinge, 2018). Therefore, we simulated the larval high energy-to-protein diet (Kwadha et al., 2017; Wojda et al., 2020) by infusing these nutrients into the carbohydrate-LDPE films. However, their inclusion did not improve larval consumption or fitness beyond that of sugars only. From a broader perspective, it is unlikely that LDPE co-supplementation through artificial means could produce outcomes that rival a natural diet, even with further optimization. The only published studies showing significant recovery of polyethylene-fed *G. mellonella* provided nutrients from natural sources, such as beeswax, honey, and wheat bran/germ (Lou et al., 2020; Mahfooz et al., 2024). Indeed, we fully restored larval feeding activities and most fitness parameters through the addition of honeycomb and corn syrup. Even food waste – another natural source – showed far better potential than the artificial diets, albeit LDPE was not included in that substrate.

The nutritional quality of food is correlated with *G. mellonella* fitness (Krams et al., 2015), and our findings suggest artificial sources are inferior. This may, in part, be attributed to naturally sourced diets being inherently more complex and, thus, may contain specific nutrients that are beneficial to *G. mellonella*, even in minute concentrations. For instance, pollen contains ~250 substances that would not be feasible to fully replicate artificially (Nogueira et al., 2012; da Silva et al., 2014). However, it should be noted that some studies have replaced aspects of the natural diet to produce similar larval fitness outcomes. A diet consisting of wheat flour, corn flour, milk, baking powder, yeast powder, honey, and sorbitol resulted in a similar growth performance as honeycomb (Metwally et al., 2012). Hickin et al. (2021) analyzed 17 ingredient variations and found the inclusion of torula yeast was more effective for larval growth than pollen from honeycomb. To this end, the food wastes diet we provided *G. mellonella* was carbohydrate-rich, and it would be interesting to evaluate whether the addition of protein-based food waste could yield notable improvements.

Although co-supplementation of LDPE with honeycomb and corn syrup largely recovered larval feeding activities and fitness, there were notable differences in comparison to a natural diet. Development was slower, and this was true of all our experimental trials. This may be indicative of some degree of dietary stress, perhaps due to the presence of LDPE, as insects are known to extend molt times in order to survive until conditions become more favorable for metamorphosis (Suzuki et al., 2013). Regardless, slower developmental rates may, in fact, be desirable for large-scale use of *G. mellonella* in bioremediation, as it allows for longer feeding durations and presumably more plastic consumption overall. In terms of physiology, there were changes in the fat body metabolic profiles of feeding larvae. The fat body of insects is a dynamic organ somewhat analogous to the human liver and adipose tissues, with functional roles in fuel storage, immunity, endocrinology, and detoxification (Arrese and Soulages, 2010). Naturally, co-supplemented larvae showed enrichment of various compounds, including hexose-P, tryptophan, ethanol phosphate, homocysteine, methionine sulfoxide, and pentose. At least one of these is likely associated with LDPE breakdown (i.e., ethanol phosphate), with the bulk representing metabolite intermediates and precursors central to metabolic homeostasis. Conversely, amphetamine, methyl galactoside, cystathionine, myo-inostitol-4, inosine, nicotinic acid, and fructose-2 were among the metabolites in deficit. Considering the interrelations between some of these compounds, it appears that LDPE consumption directly impacts metabolic processes. Indeed, tryptophan is a precursor of nicotinic acid, and homocysteine and methionine can be metabolized into cystathionine. Similarly, myo-inositol is an important growth-promoting factor of animals (Chhetri, 2019). Thus, LDPE metabolism might be responsible for dysregulation of metabolic pathways that may contribute to slower developmental rates. In addition, both fructose and myo-inositol are well known regulators of lipid homeostasis, and therefore might be provide intriguing avenues to explore considering the maintenance of lipid stores in LDPE fed larvae (LeMoine et al., 2020; Bu et al. 2022; Inci et al. 2023). Future, more targeted studies are needed to understand the biological consequences (if any) of these metabolic changes. Moreover, investigation into the subset of unknown metabolites that are unique to LDPE larvae may yield key insights into the biodegradation process that have thus far eluded researchers.

Several considerations related to our experimental design must be taken into account when forming conclusions from our study and for the broader application of *G. mellonella* in plastic bioremediation. In comparison to consumption rates reported from shopping bags (Bombelli et al., 2017; Cassone et al., 2020), the larvae did not feed as efficiently on LDPE films. A major advantage of our methodology is that the LDPE offered was pure, whereas all commercially prepared LDPE contain small amounts of stabilizer that prevents oxidation during processing (Briassoulis et al., 2004). It is conceivable that some of these additives determinately impact larval fitness, which would be challenging to delineate from our feeding trials. It should also be emphasized that consumption rates were not adjusted for LDPE content. For instance, caterpillars consuming equal amounts of 10% and 90% carbohydrate films would have consumed 9-fold more LDPE in the former treatment, assuming the larvae exhibited non-selective feeding behaviors. Moreover, consumption measurements were based on changes in the mass of the food substrate pre- and post-time intervals, and this approach may not be entirely accurate. Finally, supplementation could alter the community structure of the insect gut fauna, including microbes that play a fundamental role in the LDPE biodegradation process (Cassone et al., 2020). The influence of LDPE co-supplementation on the microbiome of *G. mellonella* is largely unresolved. There is evidence that co-supplementation and, in general, diet shape the larval gut microbiomes (Lou et al., 2020; Gohl et al., 2022); however, another study found a diet of LDPE and beeswax did not alter the core bacterial assemblages (Noël et al., 2022). Additional research is needed to better resolve the influence of co-supplementation on LDPE biodegradation efficacy.

In conclusion, nutrition – or the lack thereof – plays a crucial role in insect growth, development, survival, and ultimately reproductive success. The findings from our study indicate that *G. mellonella* larvae fed LDPE can sustain fitness parameters and consumption rates that are comparable to their natural honeycomb diet if provided with an appropriate combination of nutrient supplements. This could have intriguing implications in petro plastic bioremediation through mass insect rearing, as the larvae are highly tractable in laboratories/facilities, have high reproductive capacity, require a small footprint relative to biomass, and are voracious feeders. It may even be possible to develop dietary regimes that improve outcomes beyond that of their natural diet, which should be a focus of future research. Moreover, the formulation must be taken into account. Our study co-supplemented nutrients by mixing them into LDPE powder or embedding them into plastic films; however, a large-scale application would require a more efficient delivery mechanism (e.g., spray). The potential for insect farming is not limited to petro plastic elimination, as our food wastes experiment showed promise and could represent a fruitful avenue upon further optimization. However, an underlying issue with the large-scale use of live animals is the daunting surplus of insect biomass that is produced. Future studies are needed to evaluate the potential implementation of *G. mellonella* as part of a circular economy, such as a food source in aquaculture for commercially desirable fishes. Even if the results are promising, a thorough investigation would be required to ensure none of the metabolic by-products produced by the larvae on a given food substrate are detrimental to the environment or downstream organisms. In addition, it is unknown whether residual microplastics remain from LDPE breakdown, which must be addressed. Another research area for plastic biodegradation currently being undertaken is *ex vivo*; a thorough understanding of how plastivores and their microbiome work synergistically and the specific products/by-products each contributes could potentially allow for the development of tools/large-scale re-engineering approaches outside the animal. Overall, our study advances our understanding of LDPE co-supplementation to recover the fitness and feeding activities of plastivores.

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FIGURE CAPTIONS

Fig. 1. Experimental design for the three feeding trials. Individual second instar *Galleria mellonella* larvae were fed with LDPE supplemented with either artificial or natural sources of macro and micronutrients until pupation. Additionally, we assessed the feasibility of using *G. mellonella* for food waste bioconversion. During exposure period, larval survival, growth, development, and consumption measurements were performed. During the pupation period,

survival and adult emergence were recorded and no food substrate was provided. SLF = sugar-LDPE film; HC-LDPE-CS = honeycomb, LDPE, and corn syrup; PAV = protein, amino acid, and vitamin.

Fig. 2. Fitness parameters of *Galleria mellonella* larvae fed on LDPE film supplemented with various concentrations of sugars (10%, 50%, 82%, or 90%), pure LDPE, or honeycomb (HC, control). Shown are (a) Kaplan-Meier (log-rank test) for probability of survival; (b) weight gain (mg); and (c) consumption (mg). Weight gain and consumption values represent averages per treatment of all live individuals at a given time point. Asterisks denote statistically significant differences ($p < 0.05$) in mass or consumption among treatments for a given time interval, based on Kruskal-Wallis test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Different italicized letters indicate statistical differences ($p < 0.05$) between pairwise treatments, based on log-rank test (survival) or Dunn's test (mass and consumption).

Fig. 3. Fitness parameters of *Galleria mellonella* larvae fed on LDPE film supplemented with 50% sugars (1:1) with the infusion of proteins/amino acids/vitamins (PAV-SLF); or 50% sugars (1:1) with the infusion of vitamins only (PV-SLF). A honeycomb (HC) treatment served as the control. Shown are (a) Kaplan-Meier (log-rank test) for probability of survival; (b) weight gain (mg); and (c) consumption (mg). Weight gain and consumption values represent averages per treatment of all live individuals at a given time point. Asterisks denote statistically significant differences ($p < 0.05$) in mass or consumption among treatments for a given time interval, based on Kruskal-Wallis test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Different italicized letters indicate statistical differences

($p < 0.05$) between pairwise treatments, based on log-rank test (survival) or Dunn's test (mass and consumption).

Fig. 4. Fitness parameters of *Galleria mellonella* larvae fed on LDPE naturally supplemented with honeycomb and corn syrup (HC-LDPE-CS); or dehydrated fruits and vegetables (food waste). A honeycomb (HC) treatment served as the control. Shown are (a) Kaplan-Meier (log-rank test) for probability of survival; (b) consumption (mg); and (c) weight gain (mg). Weight gain and consumption values represent averages per treatment of all live individuals at a given time point. Asterisks denote statistically significant differences ($p < 0.05$) in mass or consumption among treatments for a given time interval, based on Kruskal-Wallis test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Different italicized letters indicate statistical differences ($p < 0.05$) between pairwise treatments, based on log-rank test (survival) or Dunn's test (mass and consumption).

Fig. 5. Partial least squares-discriminant analysis (PLS-DA) of fat body metabolites identified in *Galleria mellonella* larvae fed LDPE with honeycomb and corn syrup (HC-LDPE-CS) or honeycomb (HC). The PLS-DA reveals distinct groupings of HC-LDPE-CS and honeycomb.

Fig. 6. Hierarchical clustering heatmap of the 24 fat body metabolites that are significantly enriched or in shortfall ($p < 0.05$) in *Galleria mellonella* larvae fed LDPE with honeycomb and corn syrup (HC-LDPE-CS) in comparison to a honeycomb (HC) diet. The scale bar indicated $\log_2$-fold change differences for a given metabolite between samples.

Fig. S1. Average individual molt frequency of larval *Galleria mellonella* fed on sugar-LDPE films (SLFs) at various carbohydrate concentrations (10%, 50%, 82%, or 90%), pure LDPE, or honeycomb (control). An increased average number of molts was observed with an increased carbohydrate concentration.

Fig. S2. Development of larval *Galleria mellonella* instars (L2-L6) fed (A) LDPE co-supplemented with honeycomb and corn syrup (HC-LDPE-CS); (B) honeycomb (HC); or (C) food wastes.

[a],[b]Significant differences between HC and HC-LDPE fourth instars.

[c,d]Significance differences between HC and HC-LDPE sixth instars.

CRediT authorship contribution statement

Paola Geromino: Investigation, Methodology, Formal analysis, Writing – original draft. Ivan Drahun: Visualization, Writing – review & editing. **Christophe LeMoine**: Conceptualization, Methodology, Supervision, Funding, Writing – review & editing. **Bryan Cassone:**

Conceptualization, Methodology, Formal analysis, Data curation, Visualization, Supervision, Funding, Writing – original draft.

Declaration of competing interest

The authors declare no known competing interests.

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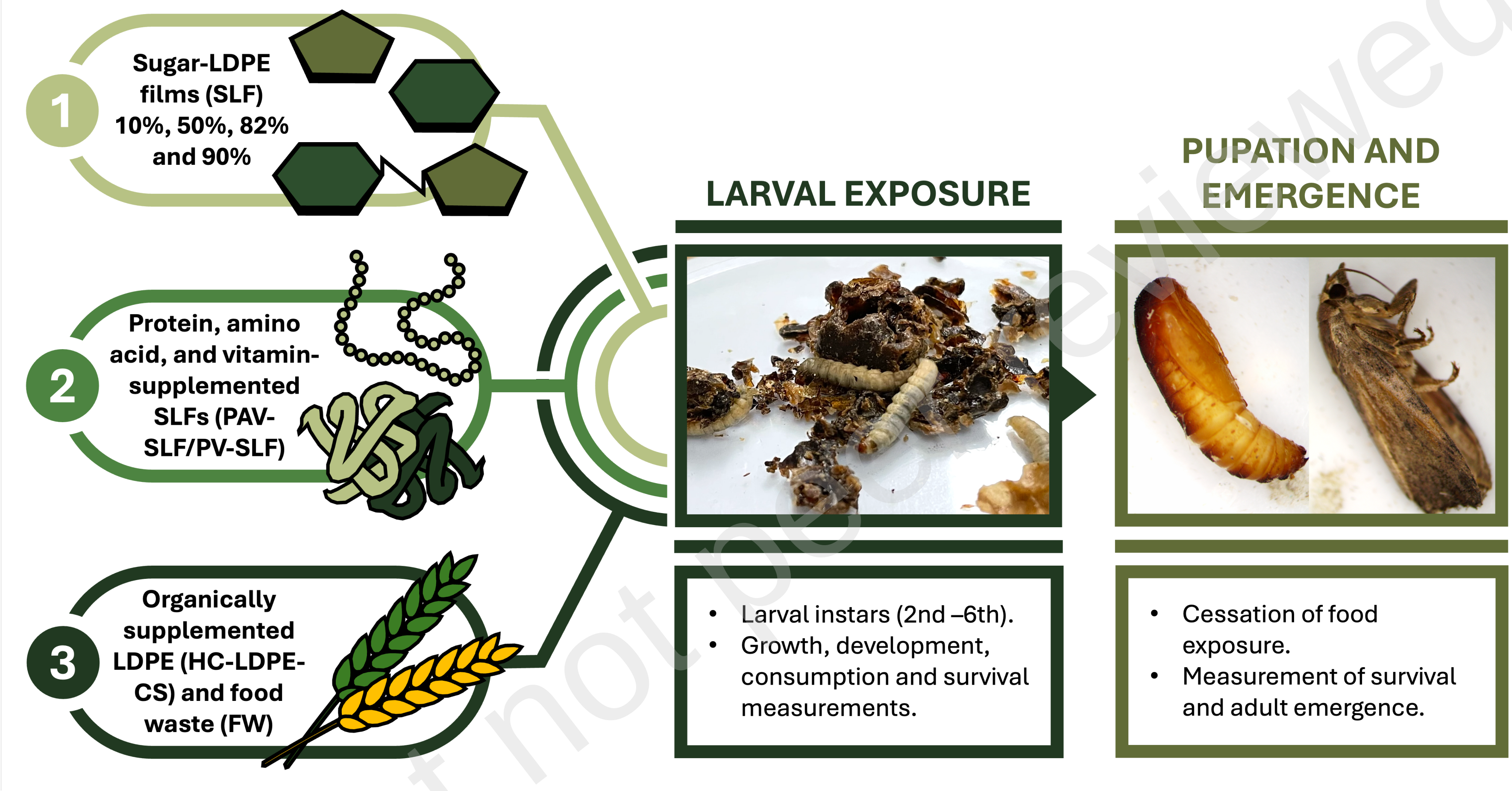


1 Sugar-LDPE films (SLF) 10%, 50%, 82% and 90%

2 Protein, amino acid, and vitamin-supplemented SLFs (PAV-SLF/PV-SLF)

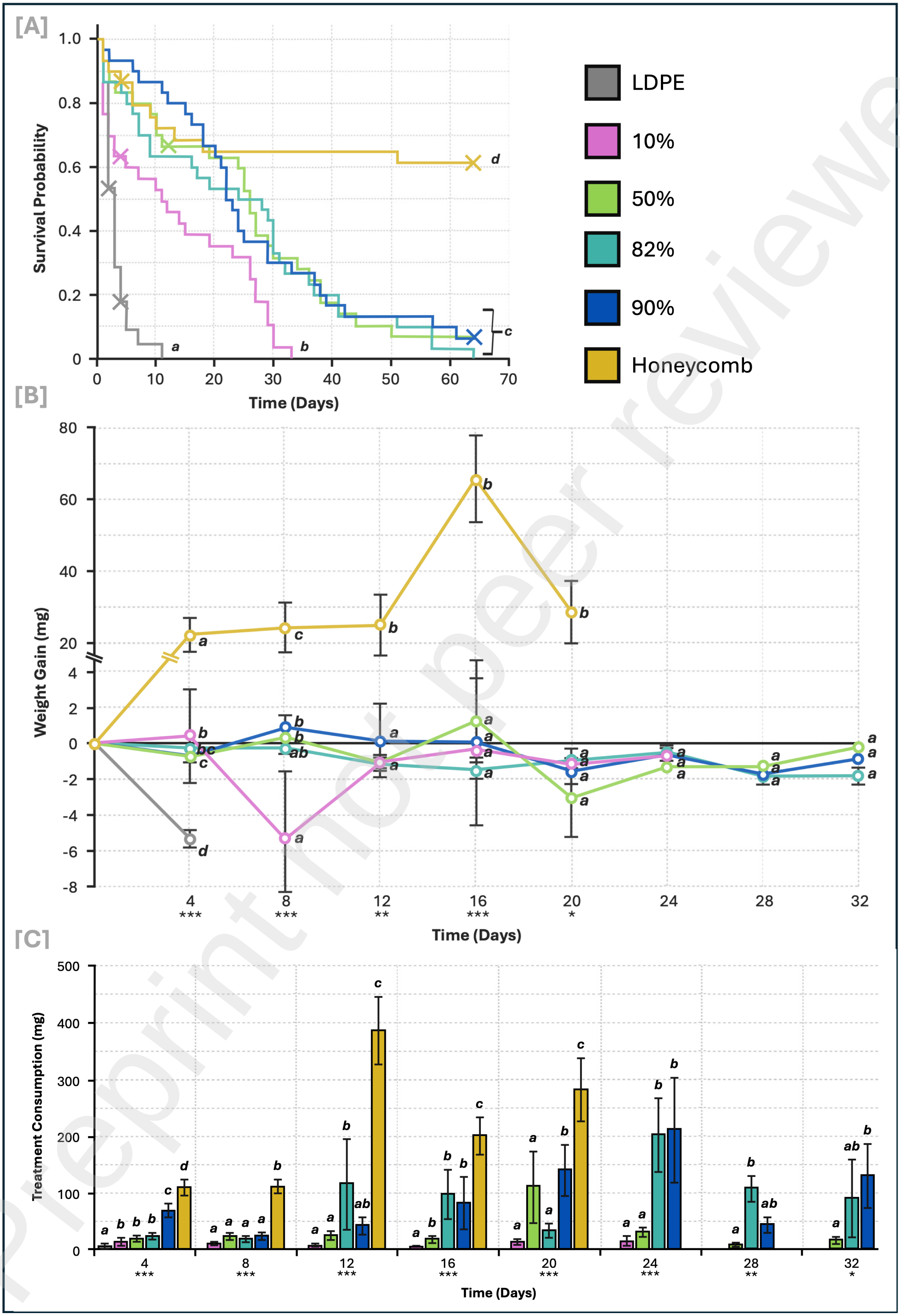
3 Organically supplemented LDPE (HC-LDPE-CS) and food waste (FW)

LARVAL EXPOSURE

- Larval instars (2nd –6th).
- Growth, development, consumption and survival measurements.

PUPATION AND EMERGENCE

- Cessation of food exposure.
- Measurement of survival and adult emergence.


[A]
Survival Probability
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
Time (Days)
a
b
c
d
LDPE
10%
50%
82%
90%
Honeycomb
[B]
Weight Gain (mg)
80
60
40
20
4
2
0
-2
-4
-6
-8
4
***
8
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12
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16
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20
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24
28
32
Time (Days)
a
b
c
d
bc
ab
[C]
Treatment Consumption (mg)
500
400
300
200
100
0
4
***
8
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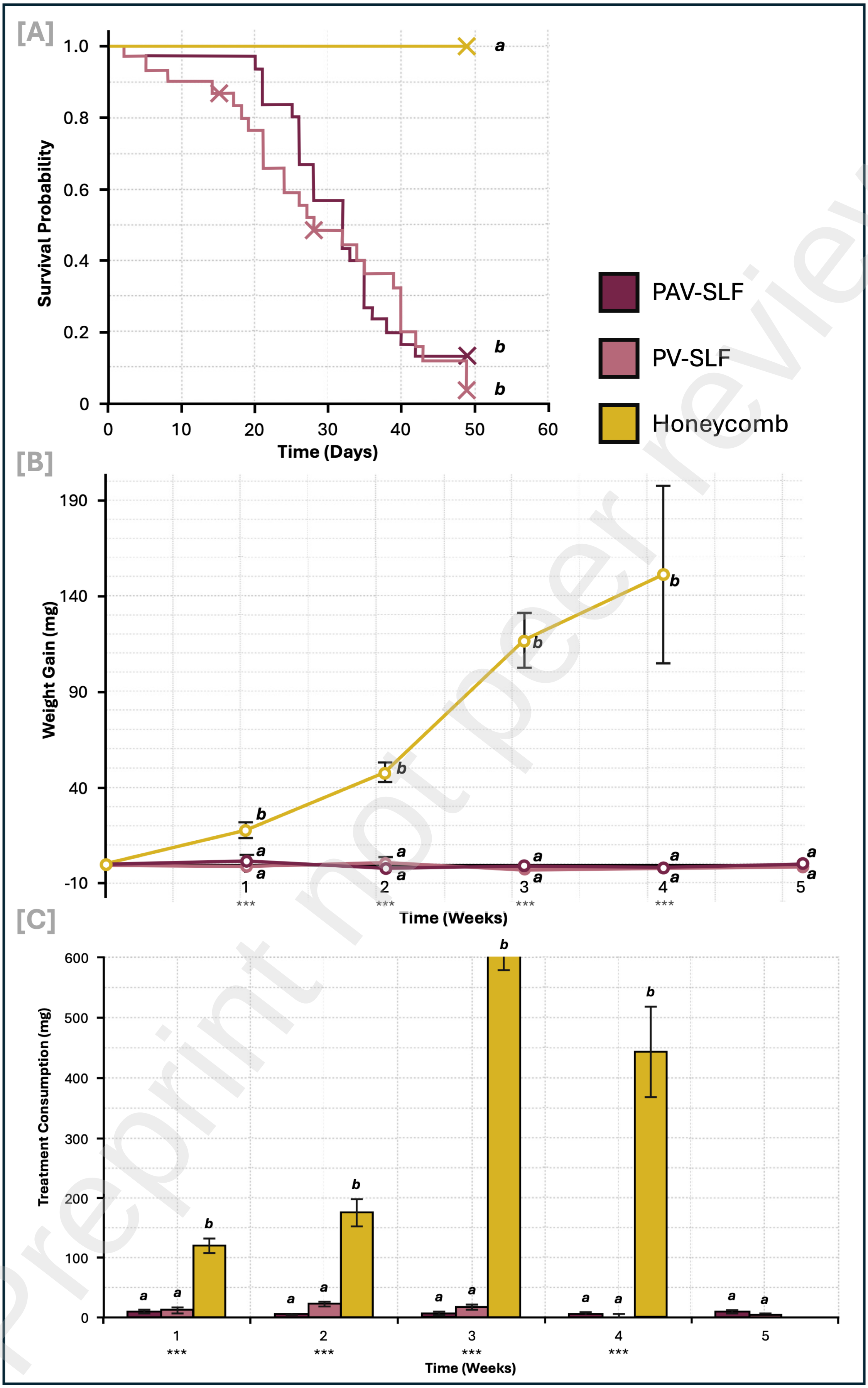
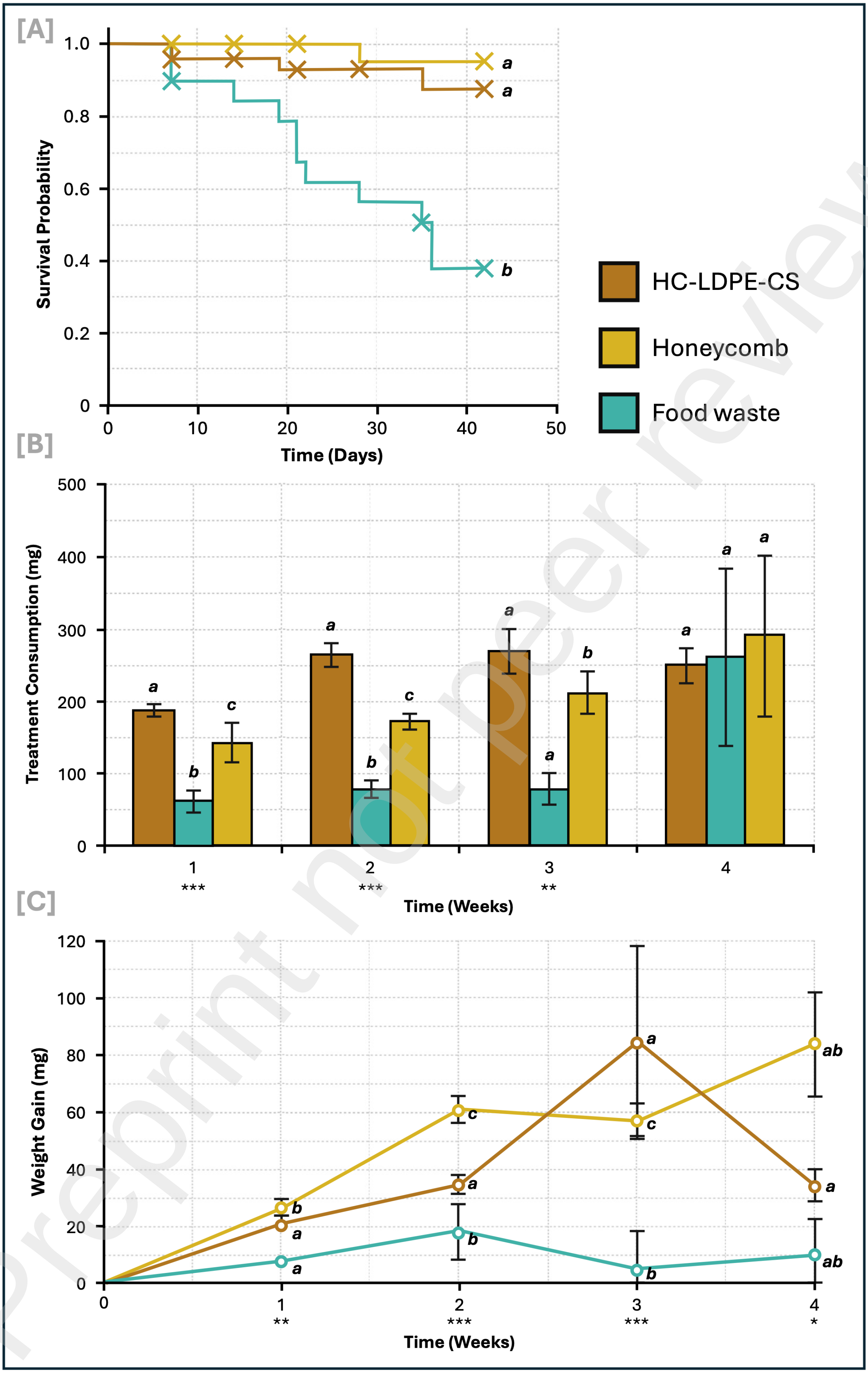
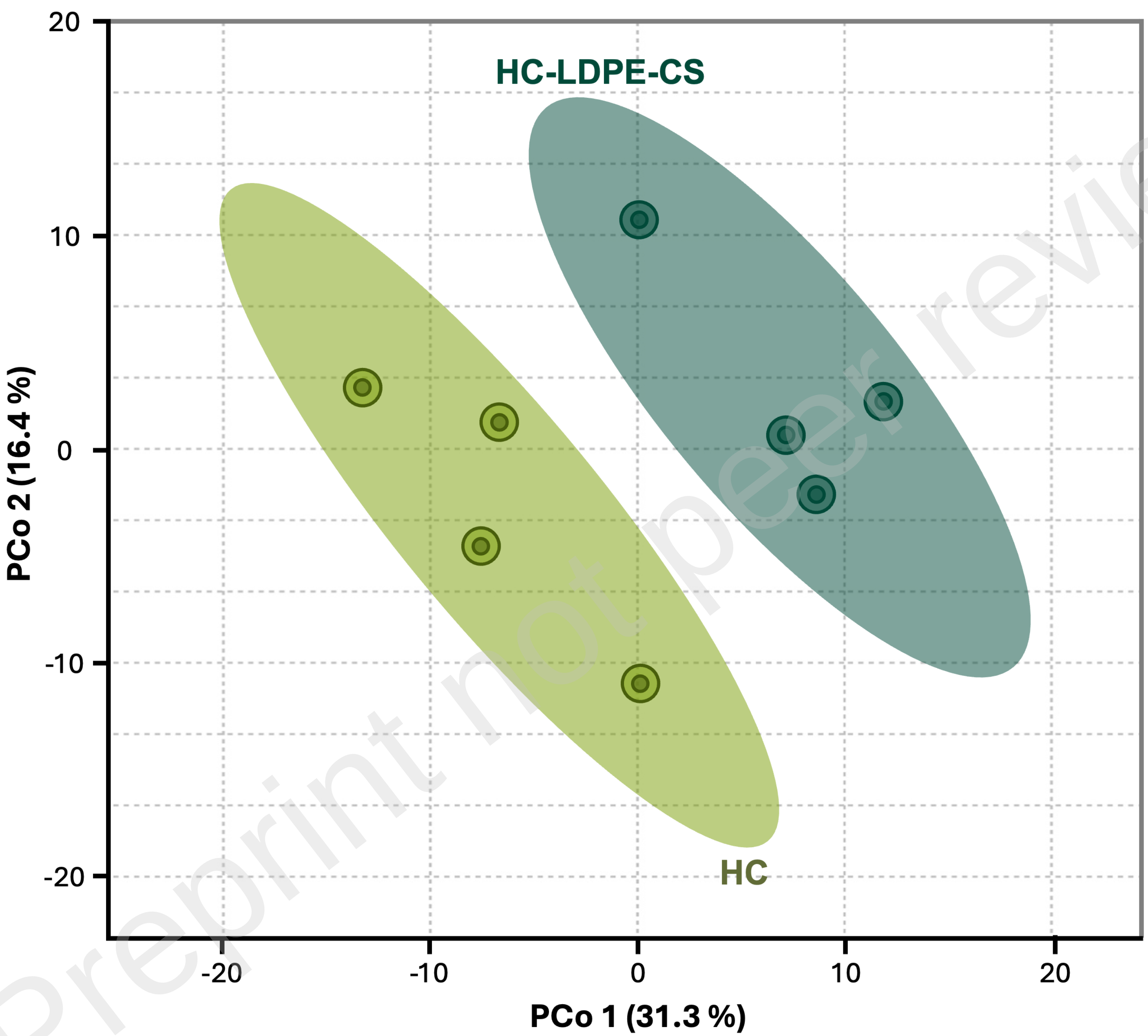
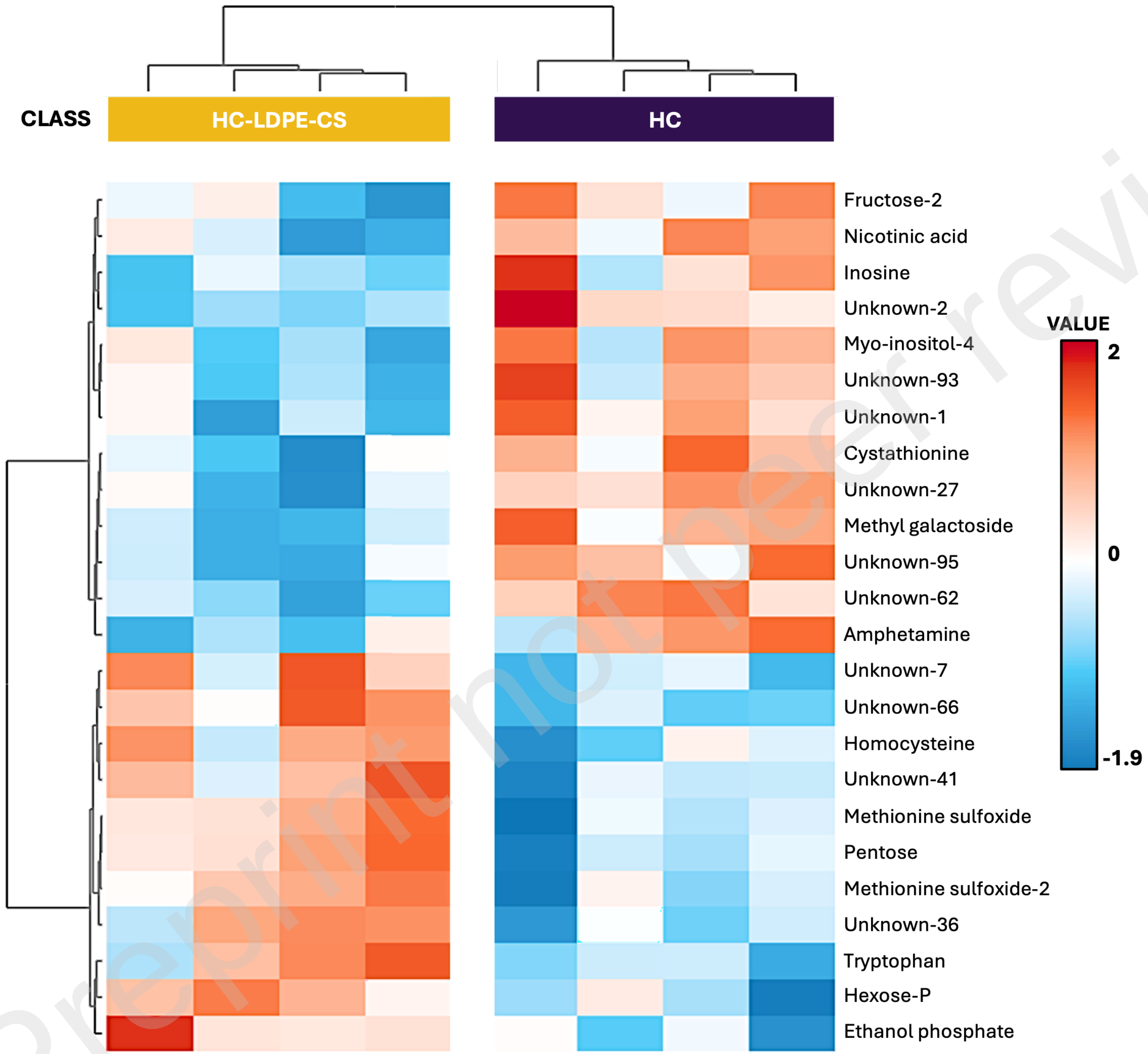
d
ab


[A]
1.0
0.8
0.6
0.4
0.2
0
Survival Probability
0
10
20
30
40
50
60
Time (Days)
a
b
b
PAV-SLF
PV-SLF
Honeycomb
[B]
190
140
90
40
-10
Weight Gain (mg)
b
b
b
b
a
a
a
a
a
a
a
a
a
a
1
2
3
4
5
***
***
***
***
Time (Weeks)
[C]
600
500
400
300
200
100
0
Treatment Consumption (mg)
a
a
b
a
a
b
a
a
b
a
a
b
a
a
1
2
3
4
5
***
***
***
***
Time (Weeks)


[A]
Survival Probability
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time (Days)
a
a
b
HC-LDPE-CS
Honeycomb
Food waste
[B]
Treatment Consumption (mg)
500
400
300
200
100
0
a
b
c
a
b
c
a
a
b
a
a
a
1
***
2
***
3
**
4
Time (Weeks)
[C]
Weight Gain (mg)
120
100
80
60
40
20
0
0
b
a
a
c
a
b
a
c
b
ab
a
ab
1
**
2
***
3
***
4
*
Time (Weeks)


HC-LDPE-CS
HC
PCo 2 (16.4 %)
PCo 1 (31.3 %)
20
10
0
-10
-20
-20
-10
0
10
20


CLASS
HC-LDPE-CS
HC
Fructose-2
Nicotinic acid
Inosine
Unknown-2
Myo-inositol-4
Unknown-93
Unknown-1
Cystathionine
Unknown-27
Methyl galactoside
Unknown-95
Unknown-62
Amphetamine
Unknown-7
Unknown-66
Homocysteine
Unknown-41
Methionine sulfoxide
Pentose
Methionine sulfoxide-2
Unknown-36
Tryptophan
Hexose-P
Ethanol phosphate
VALUE
2
0
-1.9