



## Cellular lipids and protein alteration during biodegradation of expanded polystyrene by mealworm larvae under different feeding conditions

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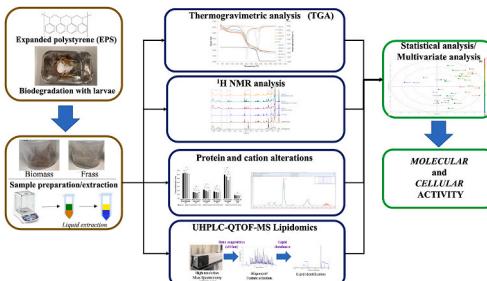
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### HIGHLIGHTS

- Polystyrene is degraded by *Tenebrio molitor* larvae.
- Biodegradation decreases protein biomass.
- PS-containing diets cause increase in apoptosis biomarkers.
- There was a decrease in molecular mass of PS.
- A decrease in mineral content was associated with PS-fed insects.

### GRAPHICAL ABSTRACT



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### ABSTRACT

The present study reports the biodegradation of polystyrene (PS) by mealworm (*Tenebrio molitor*) following different feeding regimes. Changes in lipids and protein were studied to evaluate possible differences in the growth and metabolic pathways of the insects depending on the diets. Thermo-gravimetric analysis of the excretions (frass) revealed a decrease in the molecular mass of the PS polymers. The insects' biomass contained less protein when PS was part of the diet, suggesting that the insects undergo a certain level of stress compared to control diets. The frass also contained lower amount of nitrogen content compared to that from insects fed a control diet. NH<sub>4</sub><sup>+</sup> and other cations involved in biochemical processes were also measured in insects' frass, including potassium, sodium, magnesium, and calcium, combined with a small pH change. The decrease in the mineral content of the frass was attributed to increased cellular activity in PS-fed insects. A higher amount of ceramides and cardiolipins, biomarkers of apoptosis, were also found in association with PS consumption. It was concluded that the insects could metabolize PS, but this caused an increase in its stress levels.

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## 1. Introduction

Polystyrene (PS) is a polymer extensively used in the production of food contact material (FCM) packaging due to its low cost, durability and mechanical properties (ILSI, 2002). However, plastic packaging is associated with negative effects on the environment (Tsochatzis et al., 2020b). Plastic recycling, bio-recycling and biodegradation are critical to achieve a more sustainable, responsible and green society, and efforts are undergoing to ensure a net zero environmental impact by 2030, as defined by the European Commission and the United Nations Sustainable Development Goals (SDG) (European Commission, 2019a, 2019b; Ojha et al., 2020; Stroka et al., 2021).

PS is a polymer that is difficult to decompose, it presents a low recycling rate, and can result in potential accumulation in the environment in the form of microplastics. There is currently an increasing interest in the biodegradation of plastics, and using microbial ecosystems, based on their potential enzymatic activities, which have shown to be able to degrade plastic materials (Przemieniecki et al., 2020; Yang et al., 2018b). Several studies have already reported the ability to degrade PS by insects such as mealworms (*Tenebrio molitor*) (Tsochatzis et al., 2020, 2021; Yang et al., 2015a, 2015b, 2018b), superworms (*Zophobas morio*) (Yang et al., 2020), waxworms (*Galleria mellonella*) (Lou et al., 2020) and *Tenebrio obscurus* (Peng et al., 2019). It has been recently demonstrated that their gut microbiome contributes to plastic biodegradation (Brandon et al., 2021). In the gut, there are some dominant microbial species and a high variety of enzymatic activities, including phosphatases, esterases, leucine arylamidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, chitinase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase (Przemieniecki et al., 2020; Stoops et al., 2016).

It has been shown previously that during plastic biodegradation there is an increased formation and accumulation of long chain free fatty acids (FFAs) in the insects' biomass (Lou et al., 2020; Tsochatzis et al., 2020). The generation of these FFAs was attributed to both enzymatic degradation as well as increased metabolic processes of the intestinal microbiota in waxworms (Lou et al., 2020) and mealworms (Tsochatzis et al., 2020, 2021). The presence of amides has also been reported, in relation to the increase in enzymatic activity and bio-catalytic amide bond formation (Tsochatzis et al., 2020), as a result of the enzymatic activation of amino acids followed by nucleophilic substitution by general amines using a tyrocidine synthetase from *Escherichia coli* (Hara et al., 2018). Further, a recent gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) metabolomics study revealed several bioactive molecules (FFA, amides), coexisting with several hydrocarbons, mostly with longer carbon chains, once again supporting the increased enzymatic and biochemical activity in the larvae intestine during plastic biodegradation (Tsochatzis et al., 2020, 2021).

It is known that different feeding supplementation strategies, including the addition of barley bran and hydration, affect mealworms' growth rate and PS consumption yields, while contributing to the insects' survival and growth (Lou et al., 2021; Tsochatzis et al., 2021). These studies revealed no significant amounts of hydrolysed PS in the insects' intestinal tissue suggesting that the breakdown of PS occurs by enzymatic degradation to styrene monomers or oligomers, thus highlighting the potential of this approach for PS biodegradation (Lou et al., 2020; Tsochatzis et al., 2021). By the same token, several bioactive compounds and traces of alkanes, mostly small carbon chains, were identified during biodegradation. Furthermore, a variation in the levels of glycerol and fatty acids led to the hypothesis that the mealworms undergo significant metabolic stress, mostly due to the reduced amount of available nutrients in PS rich diets (Tsochatzis et al., 2020, 2021). It is therefore necessary to carry out further studies to better understand the effect of different PS-containing diets on the biochemical processes occurring during PS biodegradation.

The scope of this work was to further understand the mechanisms underpinning PS biodegradation by assessing differences in larva lipids

(lipidomics) and protein biomass alterations that could be correlated with cell activity. The latter will underpin and confirm existing differences in larva growth and metabolic pathways, depending on the different studied diets. Thus, the presence of cellular and membrane lipids, linked to important biochemical pathways, was evaluated, and the key compounds identified. Furthermore, biomarkers of insects' well-being were assessed and related to the biodegradation of PS, to identify efficient strategies that may lead to an improvement of this process. To the best of our knowledge, no research exists, highlighting the cell alteration of cellular lipids and protein in mealworms.

## 2. Materials and methods

### 2.1. Chemicals

LC-MS grade methanol, acetonitrile, isopropanol, methyl *tert*-butyl ether (MTBE) and tetrahydrofuran (THF; anhydrous,  $\geq 99.9\%$ ) were purchased from Merck (Steinheim, Germany). For the NMR experiments, chloroform-d (99.8%) and tetramethylsilane (TMS) to lock the signals were provided by Sigma Aldrich (Steinheim, Germany), while the certified qNMR standard Dimethyl sulfone-DMSO<sub>2</sub> (TraceCERT) was supplied by Sigma Aldrich (Steinheim, Germany). Ultrapure water (18.2 MΩ) was produced using a millipore system (Millipore, Bedford, USA). LC MS grade acetic acid, formic acid, ammonium formate and ammonium acetate were purchased from Sigma- Aldrich (Steinheim, Germany). For protein and nitrogen analysis, Ethylenediaminetetraacetic acid (EDTA; 99.9%) and tris (hydroxymethyl) aminomethane (THAM) was purchased from Sigma Aldrich (Steinheim, Germany).

### 2.2. Polystyrene and biodegradation with *Tenebrio molitor* larvae

Expanded PS (EPS) foam packaging (density 0.014 g/cm<sup>3</sup>) was purchased at the local store and was assessed by attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy. Differential scanning calorimetry (DSC) was used to confirm the nature of the material (matching FTIR spectra and a glass transition of 109 °C, confirmed with standards). The PS studied, based on the producer's information, had a number-average molecular weight (M<sub>n</sub>) of approximately 100000 and weight-average molecular weight (M<sub>w</sub>) of 220000, which represents the most common type of PS on the market.

*T. molitor* larvae were reared at Aarhus University, Department of Animal Science where they were fed a diet consisting of a mixture of rolled barley (bran) together with vegetable waste and water. The composition of the bran was: 22% starch, 2.8% soluble fibre, 43.2% insoluble fibre, 14.7% protein, 4.5% fat and 5.6% ash.

In addition to a control diet consisting of 100% bran, three additional dietary regimes were compared to assess the effect of the supplementation of PS in the mealworm' diet. Diet 1 consisted of an addition of PS to bran at a bran:PS ratio of 4:1 w/w (Diet 1), while the other two diets contained a bran:PS ratio of 20:1 (w/w), with (Diet 2) or without (Diet 3) H<sub>2</sub>O every fourth day. The latter aimed to assess whether larvae were prone to metabolize PS with hydration.

The insect growth was measured by placing 75 larvae per treatment (weighing  $78.0 \pm 3.0$  mg/larvae) into a 50 cm<sup>2</sup> cylindrical plastic container, with a density:surface ratio of 1.5 larvae/cm<sup>2</sup>, in a climate-controlled room maintained at 25 °C  $\pm 1$  °C, and a relative humidity of 55  $\pm$  5%. The PS was cut into 2–3 cm cubes and mixed with the bran. The related PS consumption, insects' growth, average weight, and survival rates were monitored as previously reported (Tsochatzis et al., 2021). In brief, insects' growth/survival rate was assessed by removing every 4 days, the dead larvae, counting them and then weighing the biomass. All experiments were performed in duplicate, using identical amounts of polymer/bran diets, for a total time of 15 days (Brandon et al., 2018a). At the end of each experiment, the larvae were collected, separated from the residual biomass, which also contained their frass, and were then stored at –80 °C until further analysis.

### 2.3. Moisture, ash and nitrogen/protein in frass and biomass

The moisture content was measured gravimetrically, according to the official AOAC method (Association of Official Analytical Chemists, 1990) for the insects and frass, to identify any potential differences due to the different diets. In brief, a portion of 300 mg was weighted in pre-weighted metal racks which were then placed in an oven for 6 h at  $100 \pm 0.5$  °C. The crude protein content was determined using a DumaTherm Nitrogen/Protein analyzer (C. Gerhardt GmbH & Co. KG, Königswinter, Germany) with a protein-to-nitrogen conversion factor of 6.25, in the case of mealworms. The N<sub>2</sub> content calibration was performed with EDTA and THAM. The combustion reactor was operated at 979 °C, the reduction reactor at 649 °C and the degassing oven at 299 °C. Data were analyzed using the DumaTherm Manager v4.17 (Gerhardt, Germany) software. All experiments were performed in duplicate for each diet, on both the worm and frass fractions.

### 2.4. Ion chromatography for free cations in the frass

For the ion chromatography analysis an aliquot of 100 mg of frass was dissolved in 1 mL of ultrapure H<sub>2</sub>O overnight, centrifuged at 14000 g for 15 min at 4 °C, and the supernatant was collected for cation analysis. A 1:100 dilution of the sample was performed and filtered through a 0.22 µm nylon filter prior to injection in the ion chromatographic system. For the analysis of cations, a Dionex ICS-6000 ion chromatographic system (Thermo Scientific, USA), equipped with a gradient pump module, with a high-pressure injection valve and a 10-µL sample loop and conductivity detector was used. Data collection was controlled by the Chromeleon 7 software. For the cations, the Dionex Ionpac CS12A analytical column (250 × 2 mm I.D.) and CG12 A-8 µm guard column (50 × 2 mm I.D.) were used with a Cation Dynamically Regenerated Suppressor (Dionex CDRS 600 2 mm). A 10 µL aliquot of sample was injected and eluted at a flowrate of 0.25 mL min<sup>-1</sup>, at a temperature of 25 °C for 15 min. The eluent was mixed with 20 mM methanesulfonic acid, and all the samples were prepared and analyzed in duplicate. Lithium, sodium, ammonium, potassium, magnesium, and calcium were determined and quantified using a set of commercial standards (Dionex Six Cation-I standard) prepared at different concentrations and eluted under the same conditions as above detailed. Of note is the fact that lithium was not detected in any of the analyzed frass samples.

### 2.5. Thermo-gravimetric analysis

A thermo-gravimetric analysis instrument (TGA-2 STAR system, Mettler Toledo, USA), equipped with STAR software, was used to detect thermal changes in the residual PS polymer in mealworm frass. An aliquot of 5.5–6.0 mg of bran and frass samples and 2.5 mg of PS powder was loaded in Mettler Toledo aluminum crucibles with punctured lids and heated from ambient temperature to 600 °C at a heating rate of 10 °C/min under a high-purity nitrogen environment. Because of the foamy nature of PS, it was not possible to load equal amounts of it into the aluminium crucibles. To estimate the degradation of PS a preliminary derivative analysis was carried out to calculate the peak temperature and area at which thermal melting of the PS occurred.

### 2.6. Lipidome

#### 2.6.1. Lipid extraction from tissue samples

The extraction protocol followed the same method as reported for the organic extraction of tissue samples (Vorkas et al., 2018). Briefly, aliquots (75 mg, with a 5% maximum variation) of wet tissue were mixed with the extraction solvent, consisting of methanol/methyl *tert*-butyl ether (MTBE) 1:3 v/v, and loaded in bead beating tubes together with the weighed tissue and/or bead. The samples were treated into a tissuelyzer system, where they were homogenized using a vibration

sequence at 6500 Hz for 40 s. The tubes were then centrifuged at 20,000 g for 30 min at 4 °C and the organic solvent phase was divided in three aliquots of 400 µL each. A pooled sample was prepared by pooling the remaining tissue extracts and decanting an equal volume (25 µL) from each bead-beating tube. The solvent was then evaporated using a vacuum concentrator, at 30 °C, for 2 h. All samples were reconstituted using a solvent mixture of water/acetonitrile (ACN)/isopropanol (IPA) 1:1:3 v/v/v (Pham et al., 2019; Vorkas et al., 2018).

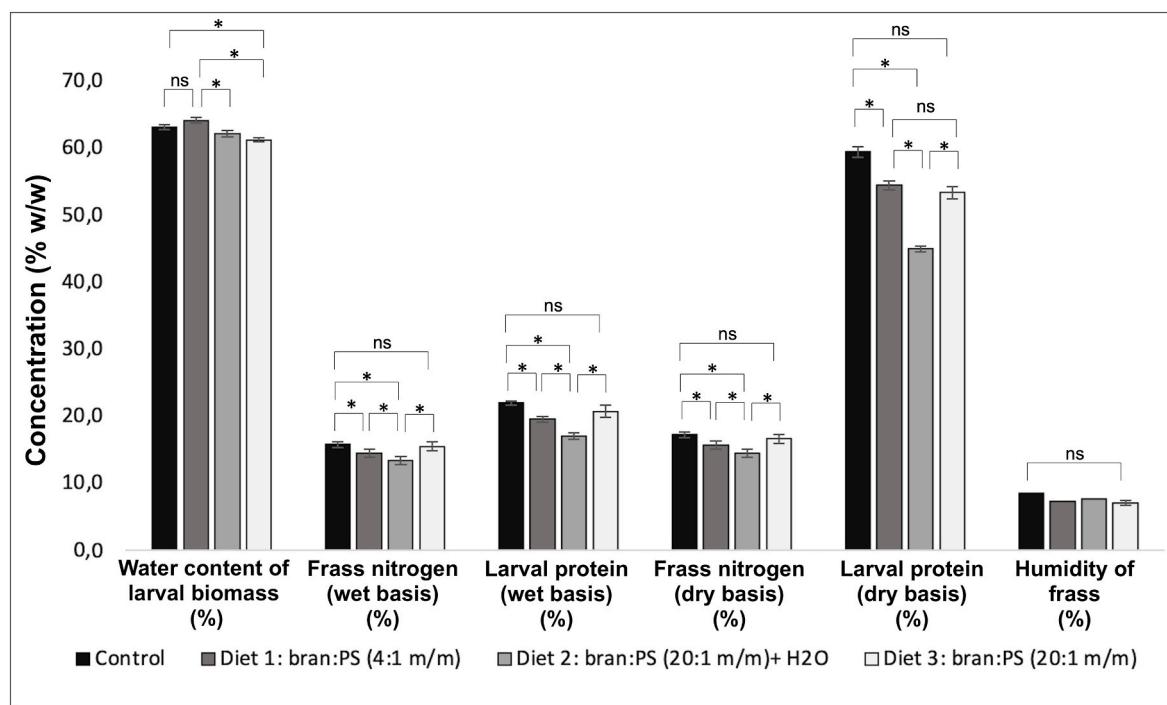
#### 2.6.2. UHPLC conditions

The extracted larvae's tissue (section 2.6.1) was analyzed by a UHPLC system (Agilent 1290) coupled to a quadrupole Time-Of-Flight (TOF) mass spectrometer (Agilent 6540 UHD Accurate-Mass, Agilent, Germany). The analytical column was a Luna C<sub>18</sub> 100 × 2.1 mm, 1.7 µm particle size (Phenomenex, USA). The mobile phase consisted of solvent A (acetonitrile: H<sub>2</sub>O 60:40 v/v) containing 10 mM ammonium formate and solvent B consisting of isopropanol: acetonitrile:water 90:10:1 v/v/v. Both phases A and B contained 0.1% formic acid. The gradient program changed linearly from 50% to 95% B in 25 min followed by an isocratic elution for 4 min. An equilibration time of 1 min was set for the mobile phase (Vorkas et al., 2018). The column was maintained at 55.0 °C with a column oven while the autosampler was kept at 8.0 °C. The injection was set with a partial loop, with needle overfill option and overfill flush of 4 µL. The sample injection volume was 5 µL for positive polarity and 10 µL for negative polarity mode (Vorkas et al., 2018). The following system gradient was used for separating the lipid classes and molecular species (Pham et al., 2019): with a flow rate of 0.2 mL min<sup>-1</sup>. The run started with 30% solvent B for 3 min, followed by 43% for 5 min, then to 50% for 1 min, to 90% for 9 min and finally to 99% for 8 min. The final step (99% B) was kept isocratic for 4 min. The column was re-equilibrated to the starting conditions (70% solvent A) for 5 min prior to each new injection.

#### 2.6.3. High resolution mass spectrometry

Combined with the UHPLC system (section 2.6.2) a high-resolution quadrupole time-of-flight mass spectrometer (QTOF) was used. Hence, dual electron spray ionization (ESI), operating in positive ionization mode, with a 4 kV capillary voltage was used. The source was kept at 325 °C, with nitrogen gas used for both drying (40 psi) and nebulizing (with a flow rate of 10 L min<sup>-1</sup>). The fragmentor, the skimmer and the octopole 1 RF Vpp were set at 170 V, 65 V and 750 V, respectively. The acquisition mode of the TOF-MS was full scan MS mode and was set to acquire data over an *m/z* range of 100–1600 Da at a rate 3 spectra/s.

The system had the possibility of carrying out MS<sup>E</sup> analysis, or, as called in Agilent products, "all ion MS-MS". This technique is useful when screening is needed, as the precursor and product ions of an analyte are simultaneously acquired in a single run, in what is generally considered a data-independent acquisition mode. For these experiments, three scan segments were set in MS mode, alternating the collision energies between 5 and 60 eV. Alternating the collision energies allowed us to acquire simultaneously the precursor ion (segment one) and product ions within a single injection (segment two and three) (Tsochatzis et al., 2020a; Vorkas et al., 2018). Identification was done using the MassHunter software (Agilent, USA), which provides the most probable formula for the ion under investigation, considering that the molecules are formed with the most common elements selected by the analyst (C, H, O, N, Cl, S and Na as adduct) with a resolving power of 35,000 and a MS accuracy ( $\Delta m$ ) of 0.5 ppm. This tool is based on both the accurate mass measurement and the isotopic ratios. Once the different options for the elemental composition of each accurate mass were known, the molecular structures were searched for using both the Agilent MassHunter METLIN Lipids library (Agilent, USA) and online chemical databases (PubChem, ChemSpider).



**Fig. 1.** Humidity (moisture content), nitrogen and protein concentration in the insects' biomass and their respective frass, as a function of the various diets: Control: bran diet only, Diet 1: 4:1 bran:PS, Diet 2 and 3 20:1 bran:PS w/w, Diet 2 also included water. Values are w/w % and represent the average of two independent replicates, bars represent standard errors. All values represent mean  $\pm$  SD, n = 2. Significance (Tukey's test correction; p < 0.05 indicated by asterisk “\*”; no statistical significance indicated by “ns”.

## 2.7. $^1\text{H}$ NMR analysis

### 2.7.1. Extraction and analysis of PS from frass

A solid liquid extraction protocol was applied, following a slightly modified method for the extraction of PS from frass (Wu and Criddle, 2021). Briefly, an amount of 50.0 mg ( $\pm 0.01$  mg) of frass or 20.0 mg ( $\pm 0.01$  mg) of PS material, was weighed in a glass vial, where a volume of 2.0 mL of THF was added. The vial was closed and heated to 55 °C for 2 h. Then the resulting extracts were filtered through 0.45  $\mu\text{m}$  PVDF filter and transferred to a clean 10-mL glass vial. The extract was evaporated to dryness under nitrogen and subsequently reconstituted in 2 mL  $\text{CDCl}_3$  for the NMR analysis. Approximately 600  $\mu\text{L}$  of this solution was then transferred to a 5 mm NMR sample tube (Wilmad, LabGlass, Vineland, NJ, USA). The extractions of PS were done in duplicate and all samples were weighed on a Mettler Toledo (Columbus, OH, USA) digital balance with a  $\pm 0.01$  mg (manufacturer's stated) uncertainty.

### 2.7.2. Instrumental conditions

To confirm the changes in the PS molecular structure, the extracted PS from the mealworm frass were characterized by mono-dimensional  $^1\text{H}$  NMR experiments using Zg30 Bruker experiment sequences. The experiments were performed on a Bruker Avance 600 (nominal frequency 600.13 MHz) (Bruker, Rheinstetten, Germany) equipped with a 5 mm cryo-probe. The spectra were recorded at 298 K using a 90° flip angle, an acquisition time of 8 min 49 s (64k data points) and a total recycling time of 5.0 s. A spectral width of 16 ppm with 64 scans and no sample rotation in the DQD acquisition mode were applied. Prior to Fourier transformation, a 0.5 Hz line-broadening factor was applied, and all spectra were phase- and baseline-corrected using the Bruker Topspin 3.2 software. Chemical shifts ( $\delta$ ) for  $^1\text{H}$  NMR spectra are reported in parts per million (ppm) relative to the TMS internal standard.

## 2.8. Statistical, multivariate and data analysis

All analytical data was processed with Masshunter 10.0 software

(Agilent Technologies, USA), while Mass Profiler Professional (MPP) v.15.0 software (Agilent Technologies, USA) was used as the chemometrics platform for alignment and exploitation of the received MS data. MPP is the only platform that provides integrated identification/annotation of compounds and automated sample classification in combination with MassHunter. Agilent MassHunter Lipid Annotator software (Agilent, USA) was used for rapid and accurate lipid annotations.

A multivariate statistical analysis was performed using Simca-P v17.0.2 software (UMETRICS AB Sweden). Principal component analysis (PCA) and Orthogonal Partial Least Squared Discriminant Analysis (OPLS-DA) was used for modelling the differences between the metabolic profile of control insect samples and the different feeding conditions and for the statistical evaluation of the models. To increase the reliability of the models R2Y (cum) and Q2 (cum) approach was applied [R2Y (cum):total sum of variation in Y explained by the model and Q2 (cum): goodness of prediction]. Variable Importance for Prediction (VIP) scores also were calculated from OPLS-DA models. Only variables with scores >1 and without negative confidence intervals were selected as most significant. Statistical analysis of replicate samples was performed using Microsoft Excel, and further statistical analysis, such as one-way analysis of variance (ANOVA), followed by Tukey comparison tests in all cases, were performed with Minitab 18.0 statistical software (Minitab Inc., State College, PA, USA), to establish and confirm the significance of important variables.

## 3. Results and discussions

### 3.1. Insect growth and biomass composition

In a parallel study (Tsochatzis et al., 2021), it was reported that the kinetics of degradation of PS (PS/g larvae) increased throughout the experimental period, from 0.4 mg PS/g larvae/day to 1.7 mg PS/g larvae/day, after 15 days. The diet containing additional  $\text{H}_2\text{O}$ , showed an initial lag phase (Tsochatzis et al., 2021). A lower bran:PS ratio provided relatively higher consumption, which was tentatively explained by the

fact that the bran was preferred to that of the PS, although not statistically different from diets including higher PS concentrations (Tsochatzis et al., 2021). Consumption of PS reached 24% of the total PS present after 15 days, for Diet 1 and Diet 2, representing a ratio of bran:PS of 4:1 (w/w) and 20:1 (w/w) with H<sub>2</sub>O, respectively. While in the case of Diet 3 (20:1, no water) significantly lower PS consumption was noticed. In this work, the results were in accordance with these previous findings (Lou et al., 2021; Tsochatzis et al., 2021; Tsochatzis et al., 2021a). In particular, the current study is in alignment with the outcomes reported by Lou et al. who suggested that a diet of up to 7:1 bran:PS ratio (w/w) seems to achieve a balance between survival and growth rates of the mealworms (Lou et al., 2021), and with the fact that hydration in the diet will further support the well-being of the larvae (Tsochatzis et al., 2021).

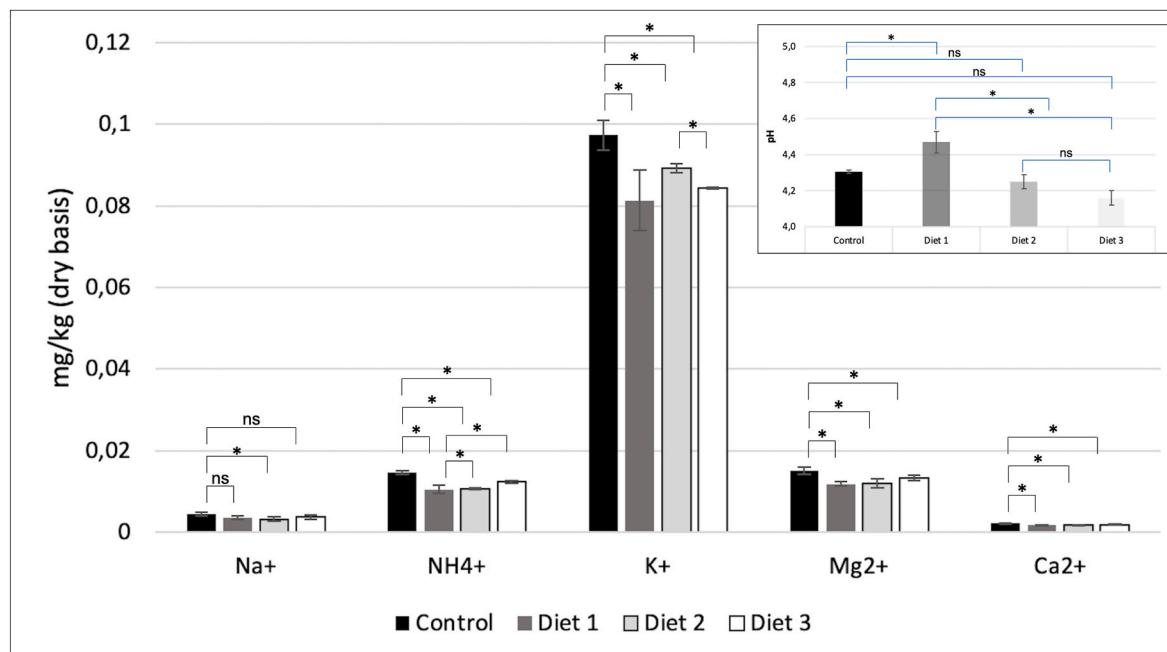
When considering the differences in total mass caused by the degradation of the PS within the entire trial duration, significant degradation rates were observed among the different applied diets over the total feeding period (Fig. S1, Table S1). The results were also supported by the study of the consumption rate (mg/day) during the experimental period (Table S1). In the diet including H<sub>2</sub>O, the initial decrease of PS consumption at Day 4 (3.6 mg/d) grew to much higher values at Day 15 (34.6 mg/d), indicating the adaptation of the larvae's microbiota to the substrate (Tsochatzis et al., 2021; Tsochatzis et al., 2021a). A similar trend was observed with Diet 1 (initial low PS consumption followed by higher values after 15 days) but not for the high bran:PS diet (Diet 3), for which the PS rate showed the opposite trend: a high consumption at day 3, followed by a significant decrease in rate at Day 15. This may indicate a lower rate of adaptation of the microbiota to the provided feed or their reduced health due to malnutrition. In agreement with previous reports, the diet including high PS:bran ratio and H<sub>2</sub>O (Diet 2) showed improved PS consumption, compared to the other diets (without water). Within the 15 days experimental period, the PS consumption ranged from 155 mg (Bran:PS 20:1 w/w; Diet 3) to 225 mg (Bran:PS ad libitum 4:1 w/w, Diet 1) corresponding to 16% to 23% of the total PS, respectively, while the survival of the mealworms fed with PS ranged from 87% (Diet 3) to 92.5% (Diet 1), with none of the treatment diets differing significantly from the control.

No overall differences were noted in this experiment, compared to other comparable studies (Brandon et al., 2018a; Yang et al., 2021) (Fig. S1), and in the present study, no cannibalism was observed during the established experimental period (Table S1). For larvae average weight, Diet 3 showed the highest statistically significant value, followed by Diet 2, and then Diet 1. Only for Diet 2 and 3 the average larvae weight was higher than for the control diet.

Fig. 1 shows the water and protein/nitrogen content of the larval biomass and the moisture content of the excreted frass. The goal was to identify any differences between the different diets and in particular any effects due to hydration. Control and Diet 1 had the highest moisture content, compared to the other two diets, even if in Diet 2 additional H<sub>2</sub>O was provided to the insects. This result supported our hypothesis of a modified metabolic and biochemical activity in PS diets. In case of the frass, the results indicated no statistical difference between any of the treatments (control vs PS containing diets). Thus, the moisture content of the larval biomass was not statistically different between diets, ranging from 63% ( $\pm 0.8\%$ ) for control diet to 64% ( $\pm 0.6\%$ ) for Diet 1. The moisture content of the frass ranged from 8.5% ( $\pm 0.12\%$ ) for control samples to 7.1% ( $\pm 0.11\%$ ) for Diet 3 (Fig. 1).

An ANOVA statistical analysis (Table S1) denoted differences between the different feeding experiments for PS degradation in relation to the Control diet, revealing that Diet 2 (the only one with water added) was always statistically different from the other 2 diets (Diet 1 and 3) and the control. More specifically, Diet 2 presented the lowest nitrogen frass content (14.4% dry basis), the lowest protein content (44.9% dry basis) and an intermediate water content of the larvae biomass (Table S1). Nevertheless, in all experiments the moisture content of the frass (%) proved not to be statistically significant.

There was a clear effect of PS consumption on the protein content in the insects' biomass as well to the amount of nitrogen excreted in their frass. It was expected that, with the increase in insects' growth, there would be an increase in the protein biomass, rather than a decrease. However, the obtained results showed a different behavior. In the insect population fed a control diet, the biomass protein content was 59.4% (dry basis), in accordance with previously reported results (Kourímská and Adámková, 2016; Zhao et al., 2016). Conversely, a decrease was



**Fig. 2.** Concentration of cations in the insects' frass. Values are expressed on a dry basis, as a function of different diets and are the average with standard deviations, in relation to the respective pH. [Control: bran diet only, Diet 1: 4:1 Bran:PS, Diet 2 and 3 20:1 Bran:PS w/w, Diet 2 also included water. All values represent mean  $\pm$  SD, n = 2. Significance (Tukey's test correction; p < 0.05 indicated by asterisk “\*”, no statistical significance indicated by “ns”].

observed in the biomass of insects fed diets containing PS. All these diets showed significantly lower values of protein/dry basis from the control, regardless of the diet treatment. Similar trends were also observed in the frass, where the excreted nitrogen content was lower in PS-containing diets compared to control treatments.

A plausible explanation for the lower protein level observed in insects exposed to PS may relate to microbiota-host physiological interactions under nutritional stress, as recently reported for *Drosophila melanogaster* (Erkosar et al., 2017). It may also be hypothesized that the insects may be using proteins in a number of stress-induced metabolic pathways. The excreted nitrogen content in frass, reflects the breakdown of proteins and nucleic acids in the production of nitrogen-containing compounds, as well as deamination, which will generate  $\alpha$ -keto acids, further oxidized to  $\text{CO}_2$ ,  $\text{H}_2\text{O}$  and  $\text{NH}_3$  (Weihrauch and O'Donnell, 2017). However, for insects, an excess of  $\text{NH}_3$  can be toxic:  $\text{NH}_4^+$  may interfere with neuronal activity, whereas  $\text{NH}_3$  can disturb and damage cells and organelles, including mitochondria (Weihrauch and O'Donnell, 2017). Insects are considered generally as uricotelic, as they excrete nitrogenous compounds with low solubility, such as urea, uric acid, or allantoin, as a means of conserving water. Uric acid, despite the metabolic cost of its synthesis (8 mols ATP/mol uric acid), is the ideal compound for elimination of excess nitrogen at physiological pH (Weihrauch and O'Donnell, 2017). In addition, it is reported that insects may also produce low molecular mass antioxidants, such as urate, when they face oxidative stress. Therefore, synthesis of urate might increase when the insects are exposed to hyperoxia, in an antioxidant response (Weihrauch and O'Donnell, 2017).

Furthermore, in the case of insects, the primary source of uric acid is either the *de novo* synthesis through the uricotelic pathway where protein is the source of nitrogen, or the degradative metabolism of the purines, such as adenine and guanine (Weihrauch and O'Donnell, 2021). Both pathways are correlated with a decrease in protein content. Symbiotic bacteria or fungi can convert dietary or metabolic nitrogenous waste into molecules that can be further metabolized by the insects. Through nitrogen fixation, bacteria use energy to fix inorganic and atmospheric nitrogen ( $\text{N}_2$ ) into organic forms, including essential amino acids or their precursors (Weihrauch and O'Donnell, 2021).

### 3.2. Quantification of cations in insects' frass

In addition to other nutrients such as vitamins, proteins, and fatty acids, minerals are also essential in a large range of biochemical reactions and processes (Lopresti, 2019). Five cations were quantified in the insects' frass as a function of the diet they were fed, namely sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), ammonium ( $\text{NH}_4^+$ ), Magnesium ( $\text{Mg}^{2+}$ ) and calcium ( $\text{Ca}^{2+}$ ). A typical ion chromatogram of the cation analysis of the frass, is given in the supplementary material (Fig. S2). For all the cations, the same trend was observed, and identical to the one identified for both protein content and frass' nitrogen content. It has been reported that *T. molitor* midgut is buffered at pH 5.6 at the two anterior thirds and at 7.9 at the posterior third (Jensen et al., 2020; Moreira et al., 2017). Accordingly, a significant pH decrease in the frass was observed in all the excreted frass from the different diets. No correlation was identified between the different cations, and especially  $\text{NH}_4^+$ , and the variations in pH. The Diet 1 indicated a statistically significant higher pH (4.5) compared to the control (4.3), Diet 2 (4.25) and Diet 3 (4.2) (Fig. 2). Furthermore, the pH of the Control was not statistically different from those of Diets 2 and 3, enhancing the hypothesis that the observed differences in the excretion of ions is not correlated with the pH. The decrease in the frass pH can also be supported by the excretion of organic acids, as previously reported (Tsochatzis et al., 2021) or by other excreted acidic metabolites.

There was a statistically significant decrease (Fig. 2, Table S2) for all cations in diets supplemented with PS compared to the control diet. Diet 1 and Diet 3 were not statistically different from one another, whereas Diet 2 showed lower excreted cations compared to the other two diets.

**Table 1**

TGA peak temperatures (for bran and PS) and peak areas for PS peak, from analysis of the frass from different diets, as well as bran and PS controls.

|  | Peak bran Temperature (°C) | Peak PS Temperature (°C) | Area PS (mg/ $^{\circ}\text{C}$ ) |
|--|----------------------------|--------------------------|-----------------------------------|
| PS   | –                          | 417 ± 0                  | –                                 |
| Bran   | 303 ± 0                    | –                        | –                                 |
| Control  | 288 ± 2                    | –                        | –                                 |
| Diet 1: bran:PS (4:1 m/m)                        | 287 ± 4                    | 428 ± 1                  | 1.58                              |
| Diet 2: bran:PS (20:1 m/m)+ $\text{H}_2\text{O}$ | 291 ± 2                    | 428 ± 1                  | 1.05                              |
| Diet 3: bran:PS (20:1 m/m)                       | 288 ± 1                    | 428 ± 2                  | 2.03                              |

Values are expressed as average with standard deviations (n = 3).

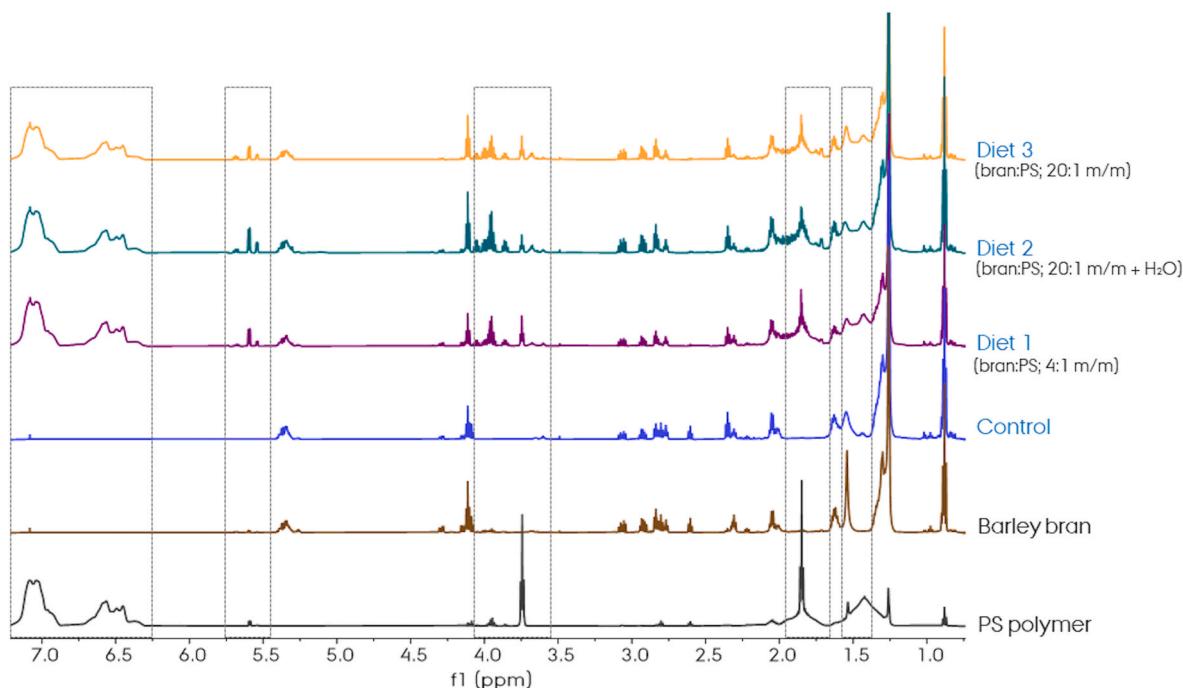
The observed changes in metabolites (cations) concentration might be due to a potential bran dilution in the diet; however, it may be possible to consider that, if any diet dilution occurred, then there should be a lower cation content for Diet 3 than Diet 1, as they differed in the bran: PS ratio. However, this was not the case. Furthermore, Diet 2, with the same bran:PS ratio as Diet 3, showed a decrease in cations compared to the other diets (of note that the values are reported on a dry basis).

The mineral content present in the insects fed the control diet was similar to what was reported for mealworm larvae in the literature. As with vitamins and other essential food nutrients, mineral requirements vary between animal species (Ryan, 1991; Soetan et al., 2010). Magnesium ( $\text{Mg}^{2+}$ ) is an essential mineral used as a cofactor of several biochemical activities, such as cellular energy production processes. Yamanaka et al. (2016) reported that a dysfunctional  $\text{Mg}^{2+}$  homeostasis is involved in various cellular malfunctions, especially in relation to those reactions where mitochondria, the energy-producing organelles are involved. Indeed, mitochondria are major intracellular  $\text{Mg}^{2+}$  reservoirs, as this ion regulates coupled reactions in energy metabolism, i.e. the TCA cycle, the electron transport chain and ADP/ATP translocation (Yamanaka et al., 2016). Therefore, nutritional stress can alter the mitochondrial  $\text{Mg}^{2+}$  concentration, ultimately affecting cellular energy metabolism (Yamanaka et al., 2016). Therefore, the observed reduction in  $\text{Mg}^{2+}$  ions in the excreted frass of insects, may be a sign of the nutritional stress posed to the insects, when PS is present in their diets.

In the case of  $\text{Na}^+$  and  $\text{K}^+$ , in insects, the V-ATPase and the  $\text{Na}^+/\text{K}^+$ -ATPase play dominant roles in osmo-regulation and excretion. Furthermore, the branchial  $\text{Na}^+/\text{K}^+$ -ATPase is stimulated synergistically by  $\text{NH}_4^+$  and  $\text{K}^+$  and is involved in transport of ammonia from the hemolymph into the cytoplasm via the basolateral  $\text{Na}^+/\text{K}^+$ -ATPase (Weihrauch and O'Donnell, 2015). The differences in  $\text{Na}^+$  and  $\text{K}^+$  observed in this work, together with the changes in  $\text{Mg}^{2+}$ , may then suggest an enhanced mitochondrial activity in the insects, stimulated by the self-regulating process (homeostasis) induced by the nutritional stress.

### 3.3. Thermo-gravimetric analysis of the frass

A recent study demonstrated no accumulation of PS monomers or oligomers in the biomass of the insects exposed to similar dietary conditions (Tsochatzis et al., 2021). This work extended the knowledge to the determination and identification of the excretion metabolites of PS in the insects' frass. To evaluate the presence of PS in the frass, TGA analysis was carried out under a nitrogen atmosphere. The analysis of the frass of the mealworms fed the different PS diets was carried out by heating the samples from 40 to 600 °C. The obtained curves are reported in the supplementary material (Fig. S3). Table 1 summarizes the peak temperature for the two thermal transitions for bran and PS as well as the peak area for the PS peak. Any difference in the thermo-gravimetry curve may indicate differences in the physical and chemical structure of the polymers present in the samples (i.e. molecular weight changes).



**Fig. 3.**  $^1\text{H}$  NMR and selected chemical shifts for the assessment of oligomers, PS degradation and evaluation of the PS depolymerization.

The thermograms, and, more clearly, the first derivative peaks, indicated three stages during heating. A first peak (at  $\sim 85\text{--}90\text{ }^\circ\text{C}$ ), identified in all samples but for PS, was caused by water and other volatiles losses. A 2nd transition was clearly identified between 287 and  $303\text{ }^\circ\text{C}$  and was attributed to the bran fraction. Thermal degradation of carbohydrates and proteins occurred over a broad range of temperatures (Chen et al., 2018), depending on their composition and nature, and in this case as seen by the bran sample, the main transition was noted at 287 to  $303\text{ }^\circ\text{C}$ . Clearly, this transition peak was hardly visible in the pure PS sample. The third transition, occurring at high temperature, was related to PS decomposition, as clearly demonstrated by the PS control sample. There was a shift in the temperature of transition of PS in the frass samples compared to the neat PS, from a degradation temperature of  $417\text{ }^\circ\text{C}$  (for the neat PS) to  $428\text{ }^\circ\text{C}$ , regardless of feeding regimes. The area of the peak was also the smallest for Diet 2 while the highest for Diet 3. The thermal stability of the polymer blends depends on the nature of the polymer components in the blends. The characteristic temperatures for PS, as reported in the literature are  $401\text{ }^\circ\text{C}$  ( $T_{50}$ ),  $440\text{ }^\circ\text{C}$  ( $T_{100}$ ) and  $403.3\text{ }^\circ\text{C}$  ( $T_p$ ), with a melting point of  $412.3\text{ }^\circ\text{C}$  ( $T_m$ ) (Farha et al., 2020). The shift to higher transition temperatures is related to a decrease in the molecular mass of the polymer ((Kokta et al., 1973; Nishizaki, 1981). This is also the case for other similar polymers like poly ( $\alpha$ -methyl styrene) (Ye et al., 2015).

The data clearly demonstrated that although PS was still present in the insects' excreted frass, there were differences between the PS molecular mass for the different diets. The PS peak in Diet 1, 2 and 3 did not show significant differences in relation to the peak temperature. The diet with high bran-to-PS ratio and the presence of water (Diet 2; bran:PS 20:1+ $\text{H}_2\text{O}$ ) degraded more polymer compared to the other two diets without water (Diet 1; bran:PS 4:1 and Diet 2; bran:PS 20:1), and excreted less amount, as it can be observed from the decreased area of the dTGA peaks. Thus, the decrease in peak area (Diet 2 < Diet 1 < Diet 3) suggested a higher extent of polymer degradation for Diet 2. This reduction in the mass of the polymer and of the degrading monomer (styrene) or PS oligomers in the excreted frass of the insects has been previously reported (Brandon et al., 2018b; Tsouhatzis et al., 2021; Yang et al., 2018b). These results were fully aligned with the results obtained with the  $^1\text{H}$  NMR (Section 3.4). The Diet where the insects were also

given  $\text{H}_2\text{O}$  seemed to be the one with the highest extent of degradation amongst all the treatments, although the decrease in average molecular weight ( $M_n$ ) seemed to be less affected than in the case of Diets 1 and 3.

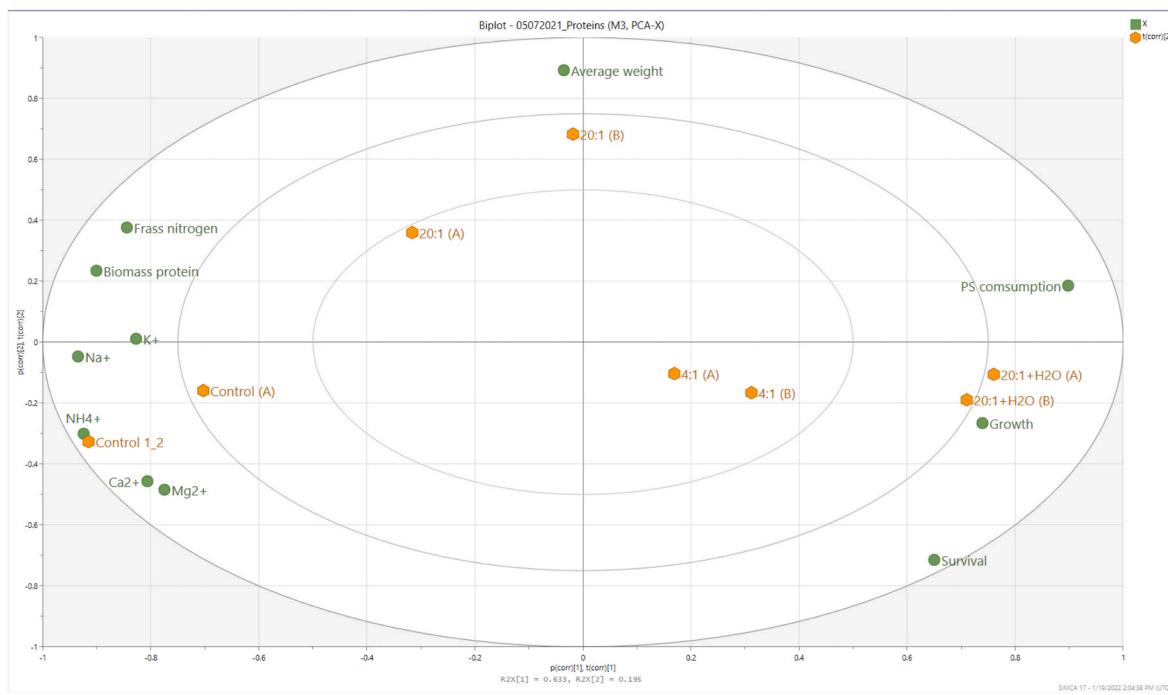
For the latter, previous work has demonstrated the presence of a different microbiome in the presence of PS in the diet (Brandon et al., 2018b; Przemieniecki et al., 2020; Tsouhatzis et al., 2021a). Thus, the differences in the extent of PS degradation may be caused by varying enzymatic activity and microbial activity (microbiome) in insects' gut intestine, due to the combined presence of both bran and  $\text{H}_2\text{O}$ . The latter may induce a higher activity of aerobic microbial strains to decompose the PS (Przemieniecki et al., 2020). Furthermore, the gut microbiota and microflora proved to be different between the different diets (Tsouhatzis et al., 2021a) which consequently led to an effect on the PS consumption, the larvae health and the depolymerization in a different way.

### 3.4. $^1\text{H}$ NMR analysis and PS degradation

#### 3.4.1. PS oligomers and PS mass excretion in frass

The  $^1\text{H}$  NMR spectra of the frass from different treatments revealed that in the chemical shifts ( $\delta$ ) region between 3.9 and 4.2 ppm there was a series of new signals, which were absent in the PS spectra. These new peaks can be attributed to PS-derived oligomers, based on existing  $^1\text{H}$  NMR information from a previous study (Brandon et al., 2018b). Hence, the integration of this region suggests that Diet 2 (inclusion of  $\text{H}_2\text{O}$  and bran:PS ratio 20:1 m/m) was associated with a different distribution of PS monomers compared to the other diets, namely, a lower excretion/formation of PS oligomers in the frass, compared to Diets 1 and 3, which presented higher PS oligomers mass fractions, based on the absolute areas (Fig. 3).

Furthermore, in order to assess the PS mass excreted in the frass, signals from different spectral regions were selected, normalized to the TMS reference signal and subsequently integrated in all the spectra (Fig. 3). Due to a partial overlapping of signals for the bran derived compounds in the frass with those of PS, the area of these signals from the control sample (PS free) were removed from all the spectra. Hence, the areas which were representing the PS residues in the frass, for all the different studied diets were not included in the integration. The integration of the areas, either for the signals at 7.235–6.295 ppm, at



**Fig. 4.** PCA biplot of insects' biodegradation identifying various measured parameters (growth, survival, PS consumption, average weight), cations, frass nitrogen and protein concentration in the biomass.

3.779–3.699 ppm, or at 1.519–1.291 ppm, from the NMR spectra proved to be fully aligned with what is reported previously with the TGA. Thus, a higher amount of polymer was found to be excreted in Diet 1 and 3 compared to that for Diet 2 (bran:PS 20:1+H<sub>2</sub>O). These results, together with the results from TGA, supported the initial hypothesis that less polymer was excreted in the frass, following in the order of Diet 2 < Diet 1 < Diet 3.

#### 3.4.2. PS depolymerization

Although gel permeation chromatography (GPC) might be considered a method of choice to assess molecular weight distribution in frass, it should be noted that the presence of metabolites in the frass might influence this assessment. Thus, for the calculation of the depolymerization, the <sup>1</sup>H NMR was used. The areas were normalized to the area of the broad signals between 6.3 and 7.3 ppm, corresponding to the aromatic protons from PS (Brandon et al., 2018a). The chemical shifts at  $\delta$  1.983–1.687 ppm were also characteristic of the PS molecules, and, therefore, the area derived from these regions were compared for the 3 different diets. A specific trend was observed, based on the respective reduction in area of these <sup>1</sup>H NMR signals, compared to that of the initial PS polymer signal; this trend indicated that the molecular weight of the PS was reduced by 8.2% for Diet 2, while the other diets indicated a larger reduction of 23.8% and 24.8% for Diets 1 and 3, respectively.

The results for the diets with no inclusion of H<sub>2</sub>O, thus, Diet 1 (4:1 Bran:PS m/m) and Diet 3 (20:1 bran:PS) were in full accordance with previous studies that used PS polymers with similar molecular weight distribution ( $M_w = 200000$  Da/ $M_n = 100000$  Da, respectively), for PS plastic depolymerization of maximum 20–25% (Brandon et al., 2021, 2018a; Yang et al., 2021, 2018b, 2018a, 2015a, 2015b), depending on the suppression of gut microbiota (e.g. when feeding only with plastics, or after administering antibiotics).

Hence, although the depolymerization can be considered as an indication of polymer biodegradation, it can also be related with PS consumption, the accumulation of compounds in the insects' biomass, and the observed presence of PS oligomers residues, where the latter can be considered an indicator of polymer fragmentation.

Therefore, the decrease in the average molecular weight of the frass

from Diet 2 (PS-fed with high bran:PS ratio and H<sub>2</sub>O) indicated that differences occur in the gut microbiome with diet regimes, and this has an impact on the rate of biodegradation of PS. These results highlighted a higher degradation rate in the insects' gut, more targeted, that would result in the excretion of increasingly non-degraded polymer (limited PS oligomers) (Tsochatzis et al., 2021a).

Furthermore, these results support the hypothesis raised by Lou et al. for Greater Wax Moth larvae (*Galleria mellonella*), where larvae might develop biodegradation mechanisms to break down the polymer branches and extremities, producing smaller molecules and/or CO<sub>2</sub> while leaving the higher molecular weight parts untouched (Lou et al., 2020). This hypothesis can be supported by the comparable behavior of Diets 1 (4:1, no water) and 3 (20:1, no water), where in the absence of H<sub>2</sub>O, identical depolymerization rates were found.

#### 3.5. Multivariate analysis

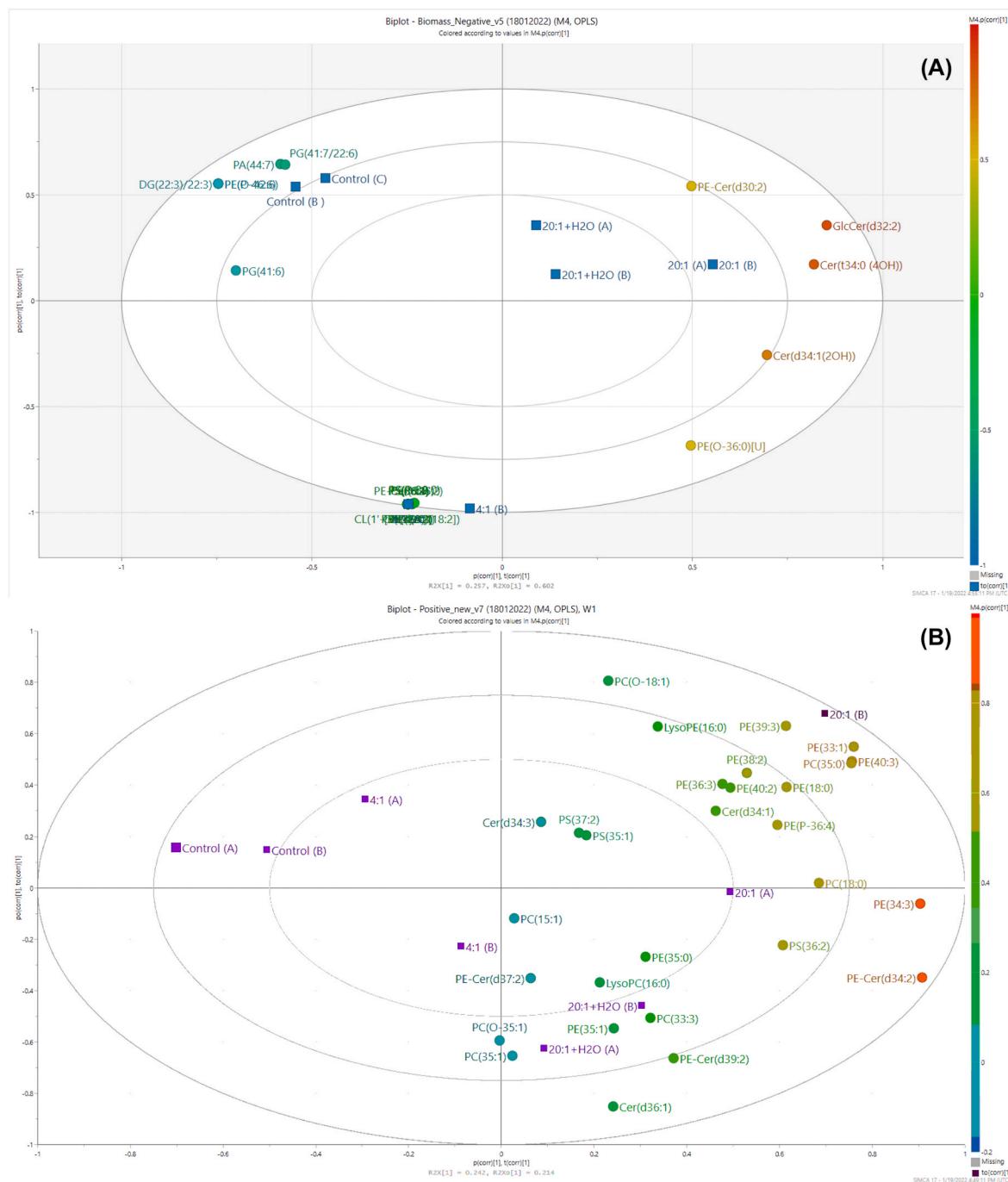
##### 3.5.1. Biodegradation, protein and cations

The results collected from PS biodegradation (insects' growth, survival rate, average weight, PS consumption), biomass protein, frass nitrogen and cation concentrations were evaluated by Principal Component Analysis (PCA), presenting a R<sub>2X</sub> of 0.828 and a Q<sub>2</sub> of 0.638, indicating a good fit of the model (Fig. 3).

The different diets clearly clustered in different areas of the PCA diagram. The decrease in content of cations, proteins and nitrogen in the frass, was clearly related to the presence of PS and its biodegradation, suggesting an increased molecular, enzymatic and cellular activity. Diet 2 (+hydration) showed the highest PS consumption yields amongst the treatments, as well as higher insects' growth and survival rates, due to the proper hydration and the potential enhancement of gut microbial activity (Tsochatzis et al., 2021). The highest insects' average weight was obtained with the high bran/PS diet ratio (20:1) without water (Diet 2; Fig. 3), due to the higher survival rates and better insects' metabolic condition (Tsochatzis et al., 2021).

##### 3.5.2. Lipidome

The lipidome of the mealworms fed with different diets was also



**Fig. 5.** OPLS-DA biplot of identified membrane lipids with: (A) negative ESI and (B) positive ESI ionization. Predictive loading values [ $p(\text{corr})$ ] in the OPLS-DA, are described with colors, where dark blue represents lowest and dark red the highest correlation values (color scale is given on the right side of the biplots). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

extracted and analyzed. Mealworms' lipid composition consisted of neutral (glycerolipids) and polar (glycerophospholipids, cardiolipins and sphingolipids) lipids; the latter being the main focus of this study. Among them, phosphatidylethanolamine (PE), phosphatidylcholines (PC), their two lyso forms: lyso-phosphatidylcholine (LPC), lyso-phosphatidylethanolamine (LPE), as well as phosphatidylserines (PS) and ceramides (Cer) were detected using positive (+) ESI ionization (see Table S3, Fig. S4). On the other hand, negative (-) ESI ionization revealed the presence of additional polar lipids, namely phosphatidylinositol (PI), phosphatidic acid (PA) and cardiolipins (CL) (see Table S3, Fig. S5). PS and PE were the highest in terms of number of molecular species followed by Cer, CL, PI. These results are in accordance with

results of other authors who reported PE as the highest in mealworms lipids (Gowda et al., 2021).

A multivariate analysis of the lipids presented in relation to the various diets provided insights into the cellular activity of the mealworms during the biodegradation of PS. OPLS-DA biplots (Fig. 4) clearly revealed that all feeding strategies integrating PS, affected the insects' lipidome. This was attributed to molecular stress, based on the cellular and membrane lipids identified with both ESI (+) and ESI (-). For the population of lipids measured with ESI(+) the R2Y was 0.953 and the Q2 was 0.872, with the prediction model based on one predictive and five orthogonal components. On the other hand, for the lipids identified with ESI (-) the R2Y was 0.971 and the Q2 was 0.932, while the

prediction model was based on one predictive and one orthogonal component. In both models, the results indicated very good total sum of variations, fitting and goodness of prediction ( $Q^2 > 0.8$ ), confirmed also by the permutation plot and CV-ANOVA. Furthermore, significant analytes were further assessed by examining Variable Importance for Prediction (VIP) scores as calculated from OPLS-DA models. Only variables with VIP scores  $> 1$  were selected as most significant and reported for both ionization modes in Table S3, for (ESI+) and (ESI-), respectively. As can be observed in the biplots shown in Fig. 5A and B, there was an increased presence of PE and ceramides in the mealworms fed with PS supplemented diets, especially in those fed with diet 1 (bran:PS; 4:1 m/m) and 2 (bran:PS; 20:1 m/m). The latter can be observed from the predictive loading values (p (Corr)), indicating higher values for Diets 1 and 2 (no H<sub>2</sub>O present) (Fig. 5A and B). The corresponding p (corr) of key lipids, demonstrated that the identified lipids (PS, PE followed by Cer, CL, PI) significantly contributed to the classification and discrimination between the studied diets of the mealworms. Although the reason behind this increase in PE is at this point not known, it is possible to relate this *de novo* ceramide synthesis to mitochondrial function. Indeed, this has been shown to be activated for the surveillance of mitochondrial function if they have been disabled due to genetic or drug-induced treatments (Watts and Ristow, 2017). Moreover, ceramides are believed to be generated during nuclear and mitochondrial apoptosis cascade reactions (Slotte and Ramstedt, 2007), and can be diffused through the membrane to activate stress pathways that promote inflammation (Watts and Ristow, 2017).

Interestingly, cardiolipins (unique phospholipids also known as diphosphatidylglycerol, which are almost exclusively localized in the mitochondrial inner membrane) were detected in higher concentrations in the lipidome of the samples fed Diet 1 (bran:PS; 4:1 m/m) in comparison with the rest of the samples. Cardiolipins are known to be key components in reactions related to energy metabolism and an increase in their biosynthesis and lipid remodelling is reported as a response to environmental variations such as thermal or salinity stress (Luévano-Martínez and Kowaltowski, 2015). The present results showed that the lipids identified at the highest levels in PS diets are those involved in cell metabolism. Therefore, the data would suggest that the supplementation of PS to mealworms diets upregulated the CL synthesis as a response to cell stress during PS degradation.

The significant differences observed between PS supplemented and the control diet (See Fig. 5) can be related to the stress the insects were subjected to during biodegradation of PS. This seemed to occur to a higher extent in the case of the diets without H<sub>2</sub>O (Diet 1 and Diet 3), compared to the diet with H<sub>2</sub>O (Diet 2). This is in full agreement with previous studies (Tschatzis et al., 2020; Tschatzis et al., 2021). Some of the lipids identified are known to be related to apoptosis, often linked to inflammation and cell death.

However, it shall also be noted that the presence of H<sub>2</sub>O during degradation is beneficial for not just the insects or the biodegradation *per se*. As shown in Fig. 4, the lipidome in the case of this diet (Diet 2; bran:PS 20:1 +H<sub>2</sub>O) was closer to the control (normal/regular diet), despite the fact that a higher survival rate and PS consumption rate was observed.

#### 4. Conclusions

The present study reports the modifications in protein, nitrogen, and lipid populations, during biodegradation of polystyrene (PS) by mealworms (*Tenebrio molitor*), as a function of different PS content in their diet. Thermo-gravimetric analysis confirmed that PS was metabolized, by revealing differences in the molecular weight of the polymer in the frass excreted by the mealworms. Furthermore, the amount of protein in the insect biomass decreased in diets supplemented with PS. Similarly, a lower level of excreted nitrogen was measured in the frass which was attributed to a higher retention of nitrogen due to oxidative stress conditions. The latter activity was verified by the identification of NH<sub>4</sub><sup>+</sup> and

other cations involved in biochemical processes, such as potassium, sodium, magnesium, and calcium, without significant alterations in the pH. The identified decrease in excreted minerals indicated an increased cellular activity for mealworms fed a diet containing PS while their lipidome indicated the presence of lipids related to cell apoptosis. It was concluded that the presence of H<sub>2</sub>O, combined with a high amount of feed, was beneficial to both the insects and the rate of degradation of PS, resulting in less excreted polymer in their frass, while being less depolymerized. Moreover, these results, in correlation with cation measurements and protein/nitrogen content in biomass or in frass, supported the hypothesis that cellular and enzymatic activity is modified due to the incorporation of PS to the insects' diet. To the authors knowledge this is the first study reporting variations in metabolites involved in biochemical processes, like apoptosis and homeostasis, in mealworm larvae during the biodegradation of expanded polystyrene.

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#### Disclaimer

The manuscript does not represent nor meant to represent an EFSA (European Food Safety Authority) opinion. The authors declare no competing financial interest.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

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