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Multilevel Assessment of the Antioxidant Potential of Two Edible Insects Following In Vitro Simulated Gastrointestinal Digestion

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Abstract

In recent years, insect-derived peptides have attracted attention for their potential biological activities, particularly antioxidant properties. This study assessed the antioxidant activity of two widely consumed edible insects, *T. molitor* and *A. diaperinus* larvae, using cell-free and cell-based approaches. Whole lyophilized larvae, digestion products from the oral, gastric, and intestinal phases, as well as the <3 kDa permeate fraction (D-P3) derived from the intestinal digestion phase, were evaluated using biochemical antioxidant assays. Overall, digested samples exhibited higher antioxidant capacity than their undigested counterparts. At the cellular level, treatment of LPS-stimulated, PMA-differentiated THP-1 macrophages with *A. diaperinus* D-P3 was associated with increased mRNA expression of genes related to antioxidant defense, including NFE2-like bZIP transcription factor 2 (*NFE2L2*, also known as Nrf2), glutathione-disulfide reductase (*GSR*), superoxide dismutase 1 (*SOD1*), and catalase (*CAT*), whereas *T. molitor* D-P3 preferentially modulated nuclear factor kappa B p50 subunit (*NFKB1*) and nuclear factor kappa B p65 subunit (*RELA*). Overall, these findings indicate that gastrointestinal digestion enhances the bioaccessibility of antioxidant components in both edible insect species while revealing species-specific transcriptional responses under in vitro inflammatory conditions. This multilevel assessment provides mechanistic insight into the antioxidant-related biological activity of digestion-derived insect peptides and supports their further investigation as functional ingredients in food and feed systems.

Keywords: tenebrionidae; yellow mealworm larvae; lesser mealworm larvae; in vitro digestion; bioactive peptides; cellular assays; antioxidant biochemical assays; THP-1

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1. Introduction

The rapid growth of the global population, which is expected to reach 9.7 billion by 2050, together with the progressive depletion of natural resources, poses significant risks to global food security and increases the prevalence of malnutrition, highlighting the urgent need for sustainable food sources [1]. Edible insects have emerged as a promising alternative, as their high nutritional value, low resource requirements, and limited greenhouse gas emissions make them an attractive option for incorporation into the human diet [2].

Previous research has addressed the nutritional profile of edible insects [3], their safety for human consumption [4], and their potential as functional ingredients intended for use in food and feed science and technology [5]. Currently, several edible insect species are legally authorized for placement on the European Union market [6] in various processed forms [7,8]. The global edible insect market is projected to grow by approximately 47% between 2019 and 2026, with the highest expansion rates expected in North America and Europe [9]. Despite this rapid growth, limited data exists regarding two commercially relevant and easily reared Tenebrionidae species, *Tenebrio molitor* L. and *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), particularly with respect to their behavior during gastrointestinal digestion and their capacity to release bioactive components.

Protein-rich foods of both animal and plant origin have been extensively characterized as substrates for the generation of bioactive peptides [10]. More recently, edible insects have been recognized as a novel and efficient protein source for bioactive peptide generation, and several studies have focused on the antioxidant properties of insect protein hydrolysates across various species, using enzymes such as thermolysin, alcalase, and other proteases [11,12]. However, only a limited number of studies have applied the standardized INFOGEST in vitro digestion model, which provides more physiologically relevant insights into nutrient bioaccessibility and potential bioactivity [13].

NFE2-like BZIP transcription factor 2 (NFE2L2) is a key regulator of the cellular response to oxidative stress, as it binds to antioxidant response elements (AREs) and controls the transcription of genes involved in antioxidant defense and cytoprotection [14]. These include crucial downstream enzymes such as heme oxygenase 1 (HMOX1), superoxide dismutase 1 (SOD1), catalase (CAT), glutathione-disulfide reductase (GSR), and NAD(P)H quinone dehydrogenase 1 (NQO1) [15,16]. In parallel, the canonical NF-κB signaling pathway is activated primarily through Toll-like receptors (TLRs) and involves the nuclear factor κB p50 (NFKB1) and p65 (RELA) subunits, whereas non-canonical or atypical NF-κB pathways may provide additional mechanisms for modulating inflammatory and oxidative stress responses [17]. Reactive oxygen species (ROS) can modulate NF-κB signaling in a context-dependent manner, while NF-κB itself plays a dual role in oxidative stress by both responding to redox changes and regulating the expression of genes involved in antioxidant and inflammatory responses [18].

Despite growing interest in edible insects, gaps remain in the understanding of insect-specific protein digestibility, peptide release during gastrointestinal digestion, and the molecular basis of antioxidant activity. Accordingly, the present study addresses these gaps by evaluating the nutritional composition and antioxidant potential of lyophilized larvae of *T. molitor* and *A. diaperinus* using a standardized static in vitro digestion model. To this end, biochemical assays and cell-based approaches were employed to enable a multilevel assessment of antioxidant activity and associated molecular responses.

2. Materials and Methods

2.1. Edible Insects' Culture and Preparation

The Agricultural Zoology & Entomology Laboratory (Athens, Greece) hosts the colonies of the insects used in the experiment, at continuous darkness, 30 °C, and 65% relative humidity [19,20]. For *A. diaperinus*, 75% wheat bran, 25% yeast, and apple slices for additional moisture constitute the rearing medium [21] and for *T. molitor* a mixture of potato pieces and oat bran for extra moisture [22]. *Tenebrio molitor* and *A. diaperinus* larvae used in the experiments measured > 7 mm and 10–14 mm long, respectively. A total mass of 20 g/raw larvae were collected for each species. After the removal from the feed substrate, a 24 h fast was instituted to enable the larvae to expel their intestinal contents. Following the fasting phase, dead larvae were removed via visual inspection.

The alive larvae were rinsed three times using distilled water. Subsequently, they were killed by blanching in boiling water for 1–5 min, followed by cooling down and removal of excess water. Specifically, the blanching of 1 kg of *T. molitor* and *A. diaperinus* larvae was performed in water at 88 °C for 90 s and 93 °C for 150 s, respectively. Blanched larvae were frozen at −20 °C for subsequent analyses. From the same rearing batch and at the same developmental stage, three independent larval pools per insect species were collected. Each larval pool was processed independently, including freeze-drying, grinding and sample preparation (see below), generating three distinct biomass samples per species.

2.2. Proximate Composition Analysis

Proximate analysis of *T. molitor* and *A. diaperinus* larvae was performed to determine protein, crude fat, crude fiber, and ash content, following AOAC standard procedures and Palamidi et al. [23], with minor modifications to the nitrogen-to-protein conversion factor (K_p), which was adjusted for each species instead of using the conventional value of 6.25. This adjustment prevented overestimation of protein content resulting from the high proportion of non-protein nitrogen typically present in insects, thereby ensuring a more accurate proximate composition [24]. All results were expressed on a dry matter basis. Carbohydrate content was calculated as: carbohydrates (%) = 100 − (%protein + %lipids + %ash). Compositional analysis was performed on one representative biological replicate per insect species. Measurements were conducted in duplicate, and results are presented as mean values of technical replicates.

2.3. Simulated Gastrointestinal Digestion and Digestates' Fractionation

Tenebrio molitor and *A. diaperinus* larvae were freeze-dried under the following conditions: −20 °C to 15 °C, with 5 °C increments every 4 h and a maximum shelf-to-sample difference of 10 °C, under a vacuum of 1 mbar. Samples were rehydrated overnight at 4 °C with 5 mL of distilled water to ensure complete solubilization. Each biomass sample was then subjected to in vitro gastrointestinal digestion following a slightly modified INFOGEST 2.0 protocol [25], resulting in three independent digestion experiments per insect species (n = 3 biological replicates from the same rearing batch). All digestion experiments were performed using samples normalized to equal protein content (0.5 g protein per digestion). In vitro gastrointestinal digestion was conducted according to the protocol reported by our group [26], except for the oral phase, which was performed using 0.17 mL of α-amylase for 2 min at 37 °C. At each digestion stage, 5 mL aliquots were collected and stored at −20 °C for subsequent analyses. The protein concentration of the final intestinal digestates was 0.0625 g/mL, and peptide fractions below 3 kDa (D-P3) were obtained exclusively from this phase by ultrafiltration using regenerated cellulose membranes with the corresponding molecular weight cut-off (MWCO). Blank digestates were prepared in parallel by replacing insect samples with water and are denoted as BL-D (full digest) and BL-D-P3 (<3 kDa fraction).

2.4. Biochemical Assays

Biochemical antioxidant assays were applied to undigested samples, as well as to digestates collected at different stages of the in vitro gastrointestinal digestion process. Specifically, analyses were performed on oral-phase, gastric-phase, and final intestinal-phase digestates (total digest), as well as on intestinal-phase fractions obtained by ultrafiltration, including the retentate fraction (>3 kDa) and the permeate fraction (<3 kDa).

2.4.1. Potassium Ferricyanide Reducing Power (P-FRAP)

Reducing power was determined using a microplate modification [27]. Samples were mixed with $K_3Fe(CN)_6$ and PBS, incubated at 50 °C for 20 min, and treated with TCA. After centrifugation (12,000× g, 5 min), the water-soluble sample was combined with $FeCl_3$,

incubated, and absorbance was measured at 700 nm. Ascorbic acid (15.6–500 µM) was used as standard, and results were expressed as mM AA/g protein. Each biological replicate was measured in three technical replicates on a 96-well plate, and the experiment was independently repeated three times using fresh reagents.

2.4.2. Ferric Reducing Antioxidant Power (FRAP)

Reducing activity was measured using a modified FRAP assay [28]. The FeIII-TPTZ reagent (TPTZ, $FeCl_3$, CH_3COONa; 1:1:10) was incubated at 37 °C for 1 h. This solution was then combined with the sample, and absorbance was measured at 590 nm. Ascorbic acid (31.30–1000 µM) served as standard, and results were expressed as mM AA/g protein. Each biological replicate was measured in three technical replicates on a 96-well plate, and the experiment was independently repeated three times using fresh reagents.

2.4.3. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Radical Scavenging Assay (ABTS)

ABTS radical scavenging activity was determined following a modified protocol of Ozgen et al. [29]. Samples were diluted (1:100) to fit the Trolox curve. The radical cation $ABTS^{\bullet+}$ solution (7 mM ABTS, 2.45 mM $Na_2S_2O_8$ in equal volumes) was incubated for 16 h at 25 °C in the dark and subsequently diluted with 20 mM CH_3COONa to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Samples were mixed with the $ABTS^{\bullet+}$ solution, and absorbance was measured at 734 nm. Trolox (3.90–250 µM) was used as a standard, and results were expressed as mM TE/g protein. Each biological replicate was measured in three technical replicates on a 96-well plate, and the experiment was independently repeated three times using fresh reagents.

2.5. Cellular Assay and Downstream Gene Analysis

2.5.1. Culturing THP-1 Cell Line and Cell Viability Assay

THP-1 cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in supplemented RPMI-1640 medium, as described in established studies and in accordance with methodologies previously applied by our group [30,31]. THP-1 monocytes were seeded onto culture plates and differentiated into macrophages using PMA (100 ng/mL) for 48 h, followed by a 24 h resting period. For the cell viability assay, THP-1 monocytes were seeded in 96-well plates (5×10^4 cells/well), differentiated into macrophages with PMA as described above, and subsequently exposed for 24 h to increasing protein concentrations (625–6250 µg/mL) of digested insect samples. Cell viability was assessed using the MTT assay by measuring absorbance at 570 nm. For gene expression analyses, THP-1 monocytes were seeded in 6-well plates, differentiated into macrophages with PMA as described above, and treated for 24 h with insect-derived D-P3 or BL-D-P3 (10%), selected based on the preceding cell viability assessment, in the presence of LPS (100 ng/mL). For each biological digestion replicate, cell treatments were performed in triplicate wells.

2.5.2. Quantification of Gene Expression

Following treatment, total RNA extraction (Nucleozol, Macherey-Nagel, Macherey-Nagel, Düren, Germany), DNase treatment (New England Biolabs, Ipswich, MA, USA), reverse transcription (PrimeScript RT reagent kit, Takara Bio, Kusatsu, Shiga, Japan) and qPCR (FastGene IC Green 2X qPCR Universal Mix, Nippon Genetics, Düren, Germany) were carried out according to the manufacturers' protocols. Primers were designed in-house (60 °C annealing), and relative expression was normalized to the housekeeping genes (*RPS18*, *RPL37A* and *B2M*) [30]. Primer details are summarized in Table 1.

Table 1. Oligonucleotide primer sequences, amplicon size and reaction efficiency in qPCR.

Gene (Accession Number)	Primer Direction	Sequence (5′-3′)	Primer Concentration (nM)	Amplicon Size (bp)	Reaction Efficiency (%)
NFKB1 (NM_001382627.1)	Forward Reverse	GCACAAGGAGACATGAAACAG CCCAGAGACCTCATAGTTGTC	300	189	97
RELA (NM_001404662.1)	Forward Reverse	GGACTACGACCTGAATGCTG ACCTCAATGTCCTCTTTCTGC	300	228	105
NFE2L2 (NM_006164.5)	Forward Reverse	GATCTGCCAACTACTCCCA GCCGAAGAAACCTCATTGTC	200	121	90
HMOX1 (NM_002133.3)	Forward Reverse	GCTTCAAGCTGGTGATGG AGCTCTTCTGGGAAGTAGAC	400	112	90
SOD1 (NM_000454.5)	Forward Reverse	CGAGCAGAAGGAAAGTAATGG CCAAGTCTCCAACATGCC	300	194	95
CAT (NM_001752.4)	Forward Reverse	TGCCTATCCTGACACTCACC GAGCACCACCCTGATTGTC	300	137	92
GSR (NM_000637.5)	Forward Reverse	CTTGCGTGAATGTTGGATGTG CACAACTTGGAAAGCCATAATCAG	300	102	110
NQO1 (NM_001286137.2)	Forward Reverse	AACTTTCAGTATCCTGCCGAG AGAATGCCACTCTGGAATATCAC	300	122	98
RPS18 (NM_022551)	Forward Reverse	CTGAGGATGAGGTGGAACG CAAATCCAACAAAGTCTGGCT	300	240	98
RPL37A (NM_000998)	Forward Reverse	AGTACACTTGCTCTTTCTGTGG GGAAGTGGTATTGTACGTCCAG	300	119	106
B2M (NM_004048)	Forward Reverse	GCTATCCAGCGTACTCCA CTTAACTATCTTGGGCTGTGAC	300	285	103

2.6. Statistical Analysis

Statistical analyses were performed with SPSS version 22.0.0 (SPSS Inc., Chicago, IL, USA) and graphs were generated using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). For the digestion procedure, three independent experiments were conducted per insect species. Biochemical assays were performed in triplicate for each biological replicate and repeated across three independent assay days, while cell-based treatments were performed in three wells per condition. Results are presented as means ± standard error of means (SEMs). Data were tested for normality using the Kolmogorov–Smirnov test and for homogeneity of variances using Levene's test. Differences among more than two groups were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's post hoc test, whereas comparisons between two groups were performed using Student's *t*-test. A p value < 0.05 was considered statistically significant.

3. Results

The proximate composition (dry-weight basis) of *T. molitor* and *A. diaperinus* larvae is summarized in Table 2. Protein content was higher in *A. diaperinus* compared to *T. molitor*, whereas *T. molitor* exhibited a higher crude fat content. Crude fiber, ash, and carbohydrate contents were comparable between the two species, at approximately 7.40%, 4%, 19%, respectively. These compositional data were used as the basis for protein normalization in subsequent digestion and cell-based assays.

Table 2. Proximate composition (dry-matter basis) of edible insect larvae (*Tenebrio molitor* and *Alphitobius diaperinus*). Results are represented as mean ± standard deviation (technical variation).

Nutrients (% *w/w*)	*Tenebrio molitor*	*Alphitobius diaperinus*
Protein	39.19 ± 0.19	49.43 ± 0.10
Crude fat	29.56 ± 0.26	20.31 ± 0.11
Crude fiber	7.41 ± 0.18	7.44 ± 0.16
Ash	4.01 ± 0.02	4.01 ± 0.01
Carbohydrate †	19.84 ± 0.37	18.82 ± 0.22

† Carbohydrate = 100 − (protein % + crude fat % + crude fiber % + ash %).

Beyond proximate composition, the antioxidant activity of edible insects was evaluated using three biochemical assays: P-FRAP, FRAP, and ABTS. Samples were normalized to equal protein content for antioxidant activity assays. Following in vitro digestion, both insect species exhibited a marked increase in antioxidant capacity, with the highest values consistently observed in intestinal digestates (Figure 1A–C). In the P-FRAP assay (Figure 1A), permeate fractions (<3 kDa) displayed significantly higher activity than retentate fractions (>3 kDa) (p = 0.011), whereas these differences were largely attenuated in the FRAP and ABTS assays (Figure 1B,C). Species-specific differences were limited to undigested samples in the P-FRAP assay (Figure 1A), where *A. diaperinus* exhibited significantly higher activity than *T. molitor* (p < 0.001). No additional species-dependent differences were observed following digestion across any of the assays (Figure 1A–C).

To further investigate the antioxidant potential of *A. diaperinus* and *T. molitor*, THP-1 macrophages were treated with the <3 kDa digestion-derived fraction (D-P3), and gene expression was assessed by qRT-PCR. Prior to gene expression analysis, THP-1 cells were exposed for 24 h to a range of D-P3 concentrations (0.625–6.25 mg protein/mL) to evaluate potential cytotoxic effects. The MTT assay indicated that none of the tested concentrations exhibited cytotoxicity (Figure 2).

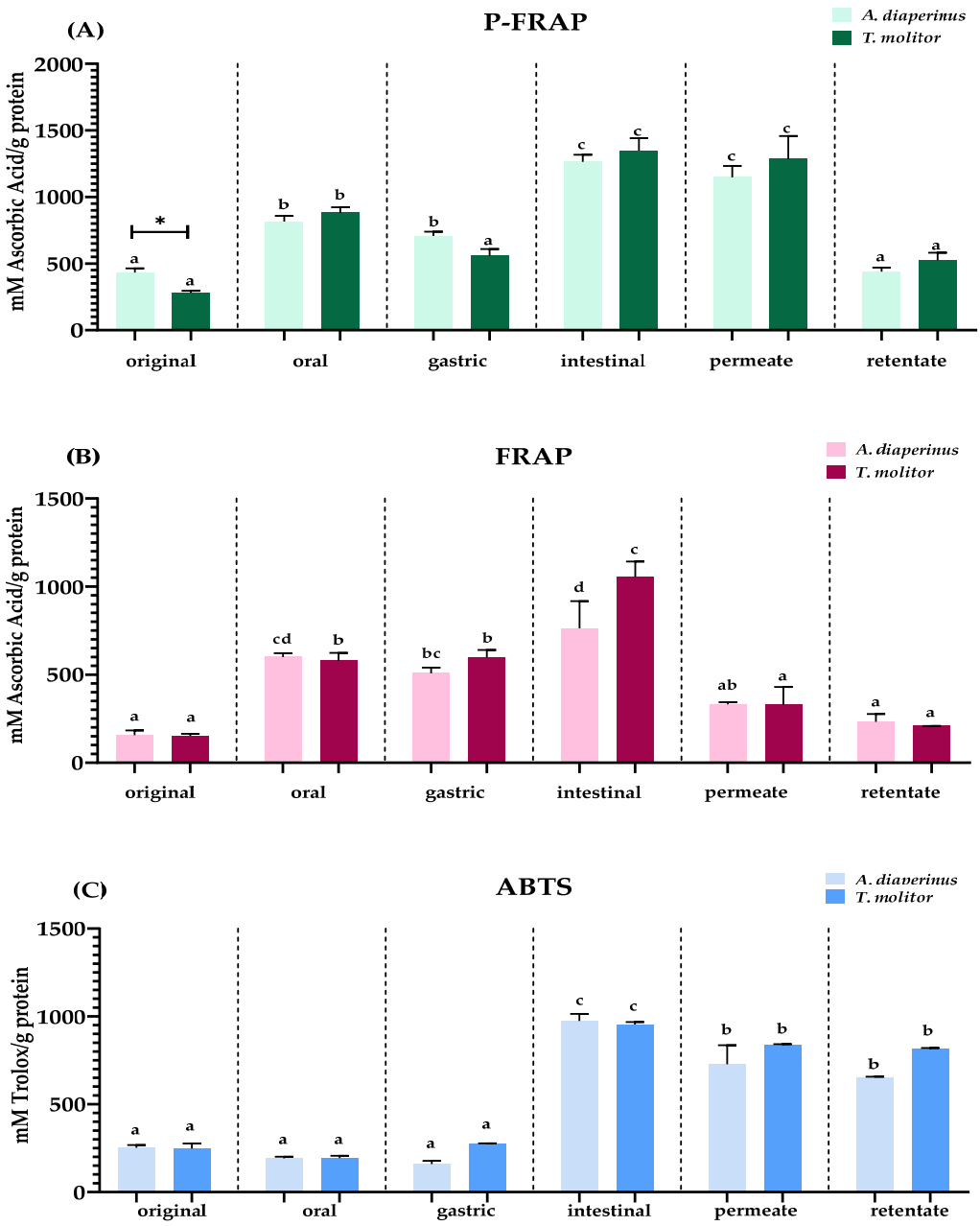


Figure 1. Antioxidant activity of edible insect larvae before and during digestion, as well as of two digested fractions (<3 kDa and >3 kDa), assessed using (**A**) P-FRAP, (**B**) FRAP, and (**C**) ABTS assays. Different letters (a–d) indicate statistically significant differences among digestion stages within each insect species ($p < 0.05$), whereas asterisks (*) denote significant differences between insect species within the same digestion stage.

Figure 3 (Figure 3a–h) depicts the mRNA expression of key antioxidant inflammation-related genes in LPS-stimulated, PMA-differentiated THP-1 macrophages following treatment with the D-P3 fraction. Treatment with *A. diaperinus* D-P3 significantly upregulated *NFE2L2* ($p = 0.009$), *CAT* ($p = 0.013$), and *GSR* ($p = 0.021$) (Figure 3c, Figure 3f and Figure 3g, respectively), indicating a transcriptional response associated with cellular antioxidant defense, while treatment with *T. molitor* D-P3 upregulated *NFKB1* ($p = 0.009$) and *RELA* ($p = 0.040$) (Figure 3a and Figure 3b, respectively) and downregulated *HMOX1* ($p = 0.049$) (Figure 3d), suggesting differential regulation of genes involved in oxidative stress and inflammatory signaling. Expression of *SOD1* was significantly increased in response to both insect-derived D-P3 treatments compared to BL-D-P3 ($p = 0.004$) (Figure 3e), whereas no significant changes

were observed for *NQO1* expression (Figure 3h). Notably, *A. diaperinus* D-P3 induced significantly higher expression of *GSR* and *CAT* compared to *T. molitor* D-P3 (Figure 3f,g), indicating species-specific transcriptional modulation of oxidative stress-related pathways.

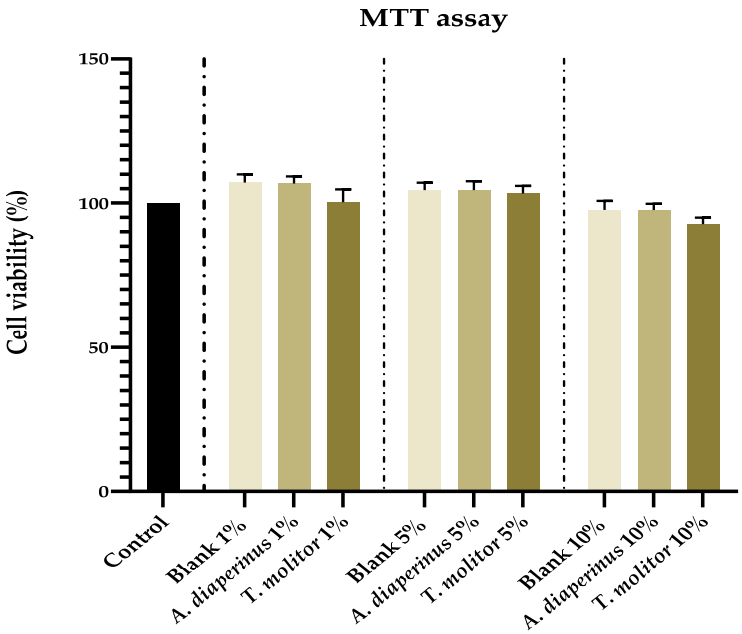


Figure 2. Effect of increasing concentrations of the <3 kDa digestion-derived fraction (D-P3) on the viability of LPS-stimulated, PMA-differentiated THP-1 macrophages, as assessed by the MTT assay after 24 h of exposure. D-P3 was applied at 1%, 5%, and 10% (*v*/*v*), corresponding to 0.625, 1.25 and 6.25 mg protein/mL, respectively. Data are expressed as mean ± SEM (n = 3).

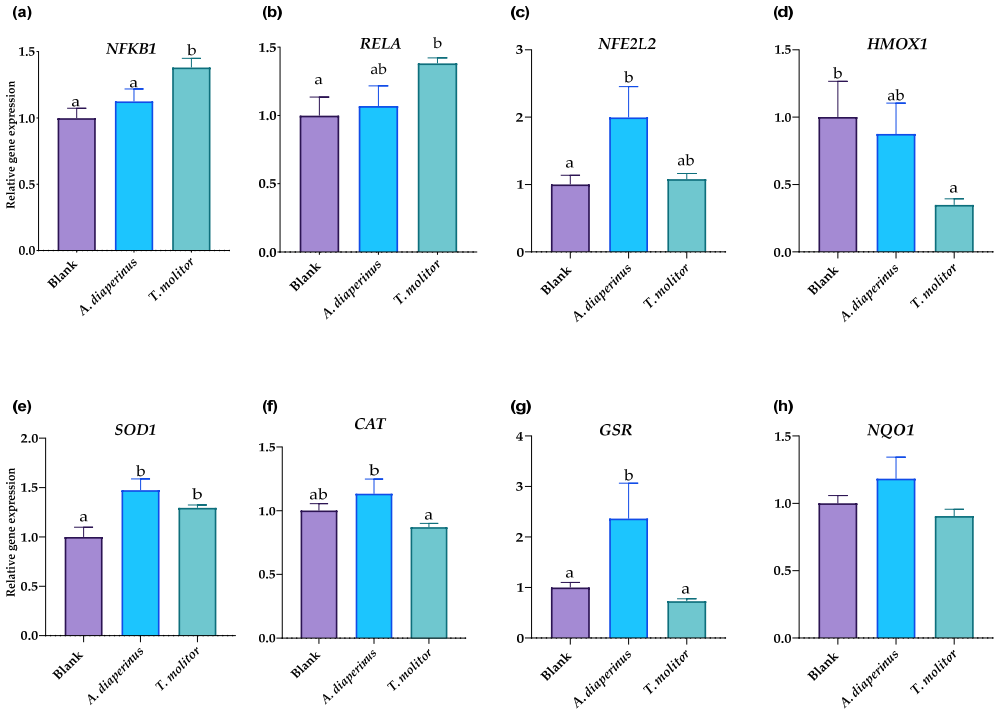


Figure 3. Effects of the <3 kDa digestion-derived fraction (D-P3) from *Alphitobius diaperinus* and *Tenebrio molitor* on mRNA expression in LPS-stimulated, PMA-differentiated THP-1 macrophages. Relative mRNA expression of (**a**) *NFKB1*, (**b**) *RELA*, (**c**) *NFE2L2*, (**d**) *HMOX1*, (**e**) *SOD1*, (**f**) *CAT*, (**g**) *GSR*, and (**h**) *NQO1* is shown. Bars labeled with different letters indicate statistically significant differences ($p < 0.05$).

4. Discussion

The proximate composition of *T. molitor* and *A. diaperinus* larvae determined in the present study is largely consistent with previously reported values, although some discrepancies were observed. Specifically, the crude protein content of *T. molitor* larvae in the present study was 39.20% on a dry-weight basis, which is lower than the average crude protein content of approximately 52.40% reported in the literature (range: 47–60.20%) [31–33]. This difference can be primarily attributed to the application of a species-specific nitrogen-to-protein conversion factor rather than the conventional factor (6.25), which has been shown to systematically overestimate protein content in insects. In this respect, Boulos et al. [34] demonstrated for *T. molitor* larvae that species-specific conversion factors substantially reduce apparent protein values by accounting for non-protein nitrogen, thereby yielding lower but more accurate protein estimates. Accordingly, higher protein values commonly reported in the literature-such as those reported by Ghosh et al. [32] (53.20%) and Ravzanaadii et al. [33] (46.40%)—are consistent with the use of the conventional conversion factor (kp = 6.25) or with studies in which the applied conversion factor is not explicitly specified [31,35]. When considered in this methodological context, the lower protein values observed in the present study are consistent with values expected when insect-specific conversion factors are applied. Additional variability may arise from differences in rearing conditions, diet, and developmental stage, as discussed in the literature [31].

T. molitor larvae exhibited a crude fat content of 29.60% on a dry-weight basis. This value is close to the average crude fat content of approximately 30.80% reported in the literature, although substantial variation has been documented depending on processing conditions, such as raw versus vacuum-cooked samples [36]. The ash content observed in the present study (4%) falls within the previously reported range (2.86–4.08%), suggesting relatively stable mineral content across similar life stages and feeding regimes [37]. At the same time, dietary composition has been shown to influence larval body composition and growth, potentially contributing to variability in mineral-related parameters [38].

In the present study, the crude carbohydrate content of *T. molitor* larvae was 19.80% on a dry-weight basis. This value falls within the range of carbohydrate contents previously reported for *T. molitor* larvae [33,39], although it is higher than the crude carbohydrate content of 11.40% reported by Son et al. [39], who also provided a detailed characterization of the carbohydrate fraction of *T. molitor* larvae, identifying chitin and chitosan as the predominant components [40]. Chitin is of particular interest due to its reported contribution to gastrointestinal health, acting as an indigestible dietary fiber with potential prebiotic and immunomodulatory effects [37]. Overall, the carbohydrate levels observed in the present study reflect the strong influence of dietary carbohydrate availability on larval composition. Crude fiber content (7.40%) was consistent with previously reported values for *T. molitor* larvae [33], further supporting the role of feeding conditions in shaping carbohydrate-related fractions.

Furthermore, in this study, *A. diaperinus* larvae exhibited a crude protein content of 49.40% on a dry-weight basis, which is comparable to values reported in the literature, which typically range between 50% and 65% and confirms the high protein content of this species [41]. The crude fat content observed in the present study (20.30%) also falls within the range previously reported for *A. diaperinus* larvae (13–29%), with an average value of approximately 24% [42]. Several studies have reported higher protein and lower fat contents in *A. diaperinus* compared to *T. molitor* [24,43], although considerable variability and some discrepancies have been documented across studies [44]. The ash content observed in the present study (4%) is consistent with previous reports [45], while carbohydrate content (18.80%) was comparable to published values of approximately 21.80% [46]. Crude fiber content (7.40%) was within the typical range of 5–7% of dry weight reported for *A. diaperinus*

larvae [41]. Although insects represent a relatively modest source of dietary fiber compared to plant-based foods, even these amounts are considered beneficial for human health, contributing to satiety and digestive function [47].

Taken together, both *A. diaperinus* and *T. molitor* larvae are recognized for their high protein and lipid contents and their nutritional potential relative to conventional plant- and animal-derived sources [24,48]. Although compositional analysis in the present study was conducted on a single biological replicate, the results provide an indicative characterization that is consistent with previously reported values for insect-derived biomasses and offer appropriate compositional context for the observed bioactivities.

Beyond their nutritional value, edible insect proteins represent a promising source of natural antioxidants, with hydrophobic and aromatic residues, specific amino acid sequences, and low-molecular-weight peptides contributing to radical-scavenging and metal-chelating activities. In particular, digestion-derived peptides—especially those of low molecular weight—have been reported to exhibit strong antioxidant potential [49]. However, antioxidant activity is known to be modulated throughout the digestive process and is influenced by peptide size, composition, and the analytical assay applied. Consequently, both low- and higher-molecular-weight peptides, as well as non-peptidic compounds, may contribute to antioxidant effects, underscoring the complex and assay-dependent nature of these bioactivities. Within this context, the present study showed that the only statistically significant difference between the two insect species was observed using the P-FRAP assay and was restricted to the undigested samples. This observation supports the notion that antioxidant responses are strongly dependent on both the digestion phase and the methodological approach employed, rather than being consistently detectable across all assays. The P-FRAP assay is particularly sensitive to reducing compounds, as it primarily evaluates Fe^{3+}-reducing capacity through single-electron transfer mechanisms, while it is less responsive to antioxidants acting via hydrogen atom transfer pathways, such as thiols and certain polyphenols [50]. This methodological specificity may partly explain the species-specific differences observed in the present study, whereas other assays, including FRAP and ABTS, are based on different reaction mechanisms and therefore may respond to distinct classes of antioxidant compounds [51]. Consistent with this interpretation, similar assay-dependent patterns have been reported for protein hydrolysates from *Hermetia illucens* (L.) (Diptera: Stratiomyidae), where higher-molecular-weight fractions (>30 kDa) exhibited greater antioxidant activity, highlighting that antioxidant capacity does not necessarily correlate exclusively with low molecular weight [52].

Given that low-molecular-weight peptides are generally more readily absorbed and often exhibit enhanced antioxidant potential due to greater exposure of reactive amino acid residues, recent studies have focused on permeate fractions to obtain physiologically relevant insights. In this context, a recent study [53] evaluated the defatted, digested *T. molitor* and *Zophobas atratus* (F.) (Coleoptera: Tenebrionidae) larvae D-P3, along with the corresponding soy-derived permeate and BL-D-P3. The *T. molitor* permeate displayed consistently strong antioxidant activity across DPPH, ABTS and FRAP assays, surpassing both the plant-based fraction and the blank control, highlighting the functional potential of low-molecular-weight insect-derived peptides.

The current findings are in line with previous reports demonstrating that digestion enhances the antioxidant potential of insect-derived peptides. In this way, Flores et al. [54] reported a 4-fold increase in ABTS activity of *T. molitor* protein extracts following digestion. This is consistent with the current results, which showed a similar fold increase in antioxidant activity using the same assay. Additionally, Zielińska et al. [55] reported strong metal chelation and reducing power in digested edible insects, indicating effective antioxidant activity. Similarly, Ma et al. [13] found higher ABTS inhibition in gastrointestinal than gastric

digestates of *A. diaperinus* and *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), attributed to the formation of smaller peptides during digestion.

Over the past decade, several studies have highlighted that insect-derived peptides exhibit antioxidant activity, with their stability and efficacy influenced by factors including heat treatment, extraction methods, insect species, and life stage, and showing differences compared to peptides from other invertebrates and plant sources [49,55–58]. Beyond in vitro assays, fortification of foods with insect powders has shown practical potential. Recently, Rochetti et al. [59] evaluated the incorporation of insect powders as meat extenders at 5% *w*/*w* in beef burgers. The antioxidant activity of the cooked, insect-fortified burgers was assessed during in vitro digestion, revealing higher antioxidant activity compared to control burgers. Similarly, the incorporation of 10% *A. diaperinus* powder into pork-based hybrid hams resulted in higher antioxidant values compared to control hams [60]. In addition to these natural effects, the functional properties of edible insects can be further improved through biofortification strategies, such as selenium enrichment in *T. molitor*, which may provide additional antioxidant benefits and nutritional value [61].

To gain deeper insight into the mechanisms involved in antioxidant defense and to explore the potential role of edible insect-derived peptides under conditions of low-grade inflammation, mRNA levels of selected antioxidant- and inflammation-related genes were quantified. Initially, the cell viability of D-P3 fractions from both edible insects was assessed, and no cytotoxic effects were observed, as cell viability remained above 90% across all tested concentrations. Based on these findings, subsequent experiments were conducted using D-P3 at a maximum concentration of 6.25 mg protein/mL, corresponding to the upper range of concentrations previously reported in similar in vitro studies [62–64].

Differential gene expression analysis in THP-1-derived macrophages indicated that the two insect-derived peptide fractions may elicit distinct redox-regulatory and metabolic responses. Treatment with *A. diaperinus* D-P3 resulted in upregulation of NFE2L2 and its downstream targets (CAT, GSR, and SOD1), suggesting activation of the Nrf2/ARE signaling pathway, which plays a central role in maintaining cellular redox balance, regulating glutathione metabolism, and supporting mitochondrial and metabolic homeostasis in macrophages [65,66]. In contrast, *T. molitor* D-P3 preferentially induced *NFKB1* and *RELA*, consistent with redox-sensitive NF-κB activation under LPS-stimulated conditions. NF-κB signaling interacts bidirectionally with oxidative stress pathways, contributing to inflammatory responses as well as metabolic reprogramming, while the limited induction of Nrf2-dependent genes (*HMOX1*, *CAT*, *GSR*) indicates a weaker antioxidant transcriptional response compared to *A. diaperinus* [18,67]. Such an imbalance between NF-κB and Nrf2 signaling is well documented, as NF-κB can functionally antagonize Nrf2-mediated antioxidant defenses through transcriptional and co-activator competition, thereby prioritizing inflammatory over cytoprotective programs [68]. Overall, these findings indicate that *A. diaperinus* digestates preferentially promote transcriptional responses associated with antioxidant defense pathways [69], whereas *T. molitor* digestates are more closely associated with NF-κB–mediated inflammatory transcriptional patterns.

Previous studies have shown that protein hydrolysates from edible insects exhibit various bioactivities. Riolo et al. [70] demonstrated that *H. illucens*-derived hydrolysates reduced intracellular ROS in H_2O_2-induced L-929 cells in a dose-dependent manner, accompanied by increased *Nrf2* expression and nuclear translocation. Despite these findings, dose- response analyses exploring the quantitative relationship between active components and biological efficacy remain largely unexplored for edible insect-derived protein digests. Accordingly, the present study employed a single, physiologically relevant concentration (10% D-P3), selected based on preliminary cell viability screening to ensure the absence of

cytotoxic effects. Future studies incorporating dose–response designs will be essential to further elucidate concentration-dependent effects and cellular responses.

Similarly, two novel antioxidant peptides, PFCPK and ADFW, isolated from *H. illucens* larvae, were shown to reverse oxidative stress-induced downregulation of Keap1/Nrf2 pathway genes, including *Nrf2*, *Nqo1*, *Gpx*, and *Sod2*, in zebrafish embryos [71]. These findings are in line with this study, as *A. diaperinus* D-P3 modulated similarly the gene expression involved in oxidative stress. Gonzalez-de la Rosa et al. [72] reported that *T. molitor*-derived oligopeptides exhibited markedly stronger radical-scavenging activity compared to their corresponding non-digested hydrolysates and significantly reduced the expression of key pro-inflammatory genes, including *TNF-α*, *IFN-γ*, and *IL-6*, following simulated gastrointestinal digestion in LPS-activated Caco-2 cells. In the present study, peptide fractions were evaluated as digestion-derived permeates without prior compositional profiling, with emphasis placed on the overall biological activity of the D-P3 fraction rather than on individual peptide sequences. While this digestion-centered approach allows the assessment of bioaccessible peptide mixtures under physiologically relevant conditions, it does not permit direct attribution of the observed bioactivities to specific peptides. Future studies integrating detailed compositional characterization, such as LC-MS/MS-based peptide profiling and complementary computational approaches, will be required to establish direct links between individual peptide sequences and their biological functions.

Beyond Tenebrionidae species, protein hydrolysates from *Protaetia brevitarsis* (Lewis) (Coleoptera: Scarabaeidae) larvae have also been reported to reduce intracellular ROS levels through the induction of Nrf2-mediated antioxidant enzymes, including *CAT* and *HMOX1*, in AML12 liver cells [73]. Similarly, Cho & Lee [74] demonstrated that an alcalase hydrolysate (<1 kDa) from *T. molitor* protected AML12 liver cells against ROS-induced cytotoxicity by activating the Nrf2 pathway, leading to upregulation of antioxidant genes including *CAT*, *HMOX1*, and those involved in glutathione synthesis. In contrast, the present study showed that *T. molitor* D-P3 only upregulated *SOD1* expression, suggesting a more limited activation of Nrf2-dependent antioxidant pathways under the experimental conditions applied. Notably, Cho & Lee [74] primarily used hydrolyzed edible insect proteins as starting material, AML12 liver cells, and H_2O_2 as a stimulus, with comparisons made against untreated cells. In contrast, the present study adopted a digestion-centered design, with comparisons performed against a blank digest (BL-D-P3) rather than untreated cells. This approach provides a more physiologically relevant and conservative framework for evaluating digestion-derived effects, although it may reduce the magnitude of observable differences in gene expression. Importantly, BL-D-P3 may retain residual bioactivity due to the presence of digestive enzymes and bile salts, potentially influencing cellular responses. Therefore, comparisons against BL-D-P3 represent a rigorous strategy, allowing for the identification of insect-specific effects beyond those of the digestion matrix itself. Collectively, these findings underscore that variations in hydrolysis level, incubation time, cell type, stimulus concentration, and treatment conditions can significantly affect the antioxidant effects of insect-derived products in vitro.

While the INFOGEST protocol provides robust insight into peptide bioaccessibility following gastrointestinal digestion, only a limited number of studies have incorporated intestinal epithelial models, such as Caco-2 monolayers or advanced co-culture systems (e.g., Caco-2/THP-1) [75,76]. Such models would enable the evaluation of transepithelial peptide transport, barrier-associated responses, and epithelial-level antioxidant effects as a complementary approach for a more comprehensive assessment, thereby extending the physiological relevance of the current findings. Overall, the present study highlights the antioxidant potential of insect-derived peptides under controlled in vitro conditions; however, these effects may not directly translate to in vivo systems. Consequently, future

animal and human studies are necessary to evaluate bioavailability, absorption, and efficacy, thereby supporting the potential application of edible insects in food and feed systems.

5. Conclusions

In conclusion, gastrointestinal digestion enhances the antioxidant capacity of both insect species, with D-P3 contributing substantially to the observed bioactivity. The increase in antioxidant activity following digestion across multiple biochemical assays indicates improved bioaccessibility of insect-derived antioxidant components. At the cellular level, *A. diaperinus* D-P3 was associated with increased mRNA expression of genes related to antioxidant defense, whereas *T. molitor* D-P3 elicited a more limited transcriptional response. These observations are restricted to in vitro transcriptional outcomes, and further studies incorporating functional assays and in vivo models are required to clarify their biological significance.

Overall, the findings support the potential of edible insects as sources of digestion-derived peptides with antioxidant-related biological activity and provide a basis for further investigation into their application as functional ingredients in food, nutraceutical, and animal nutrition contexts.

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Abbreviations

The following abbreviations are used in this manuscript:

AA	Ascorbic acid
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
$ABTS^{\bullet+}$	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
AOAC	Association of official analytical chemists
ARE	Antioxidant response element
B2M	Beta-2-microglobulin
BL-D	Blank digestate (digestion with water instead of sample)
BL-D-P3	Blank digested fraction below 3 kDa (digestion with water instead of sample)
CAT	Catalase
CH_3COONa	Sodium acetate
D-P3	Digested fraction below 3 kDa
DPPH	2,2-Diphenyl-1-picrylhydrazyl
$FeCl_3$	Iron chloride
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power

GSR	Glutathione-disulfide reductase
HMOX1	Heme oxygenase 1
K_p	Nitrogen-to-protein conversion factor
$K_3Fe\ (CN)_6$	Potassium ferricyanide
LPS	Lipopolysaccharides from *Escherichia coli* O111:B4
MWCO	Molecular weight cut-off
Na_2SO_8	Sodium persulfate
NADPH	Nicotinamide adenine dinucleotide phosphate
NFE2L2	NFE2-like BZIP transcription factor 2
NFKB1	Nuclear factor Kappa-B p50 subunit
NQO1	NAD(P)H quinone dehydrogenase 1
PBS	Phosphate-buffered saline
P-FRAP	Potassium ferricyanide reducing antioxidant power
PMA	Phorbol 12-myristate 13-acetate
qRT–PCR	Quantitative reverse transcription polymerase chain reaction
RELA	Nuclear factor Kappa-B p65 subunit
RPL37A	Ribosomal protein L37a
RPS18	Ribosomal protein S18
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1
TCA	Trichloroacetic acid
TLR	Toll-like receptor
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine

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