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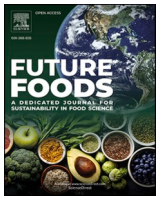

Optimizing *Tenebrio molitor* powder as ingredient in breadmaking: Impact of enzymatic hydrolysis on dough techno-functional properties and bread quality

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ABSTRACT

Edible insects have garnered significant attention due to their potential to provide high-quality proteins, nevertheless, despite the appealing composition, their large-scale utilization still needs improving. In this study, a two-step enzymatic treatment with Alcalase® and Flavourzyme® was set up to improve protein bio-accessibility and digestibility of *Tenebrio molitor* powder (TMP) and a first comprehensive analysis of its impact on bread's nutritional and technological properties was performed. An integrated approach, including the combination of electrophoretic and chromatographic techniques was used to monitor the proteolysis extent. During the treatment, peptide concentration changed in the first few hours of treatments (increasing significantly from 145 to 439 mg/g). A modification of the protein profile was also observed, whereas a 7-fold increase of free amino acids was found by the end of the hydrolysis. The treatment also affected TMP emulsifying capacity, foaming activity, as well as other techno-functional properties. Since these changes led to hypothesizing a positive influence on leavened products volume and nutritional quality, breads fortified with TMP were produced using mixture-process experimental design. Substitution reduced bread dough viscosity, nevertheless, fortified breads had higher protein, lipid, and ash contents than the control, meeting EU nutritional claim requirements for protein content. The use of enzymatic treated TMP provided a higher content of proline and arginine, and a different volatile organic profile, with higher concentrations of amino acids catabolism products (2-methyl-butanal, 3-methylbutanal, and 2-pentylfuran). The fortification also impacted bread visual-textural features (hardness, color) in a more pronounced manner as the inclusion rate increased.

1. Introduction

Over the past three decades, the concerns over the increasing world population, along with the need to enhance the nutritional value of foods, have boosted the global demand for non-conventional protein sources. Indeed, given the significant environmental challenges associated with traditional livestock farming, the consumption of insects, algae, and microorganisms has been proposed as a potential solution to offer more sustainable production cycles, thus ensuring food security (Barret, 2021). Among the various alternatives proposed, edible insects have garnered significant attention due to their potential to provide high-quality proteins (Gkinali et al., 2022; Hong et al., 2020) and one of the most studied and commercially viable edible insect species is *Tenebrio molitor,* commonly known as yellow mealworm (Turck et al., 2021). Although its larvae have been consumed in various cultures for centuries, it is only recently that they have been considered a viable protein source for large-scale production in industrialized nations. In Europe, the use of insects as food has been subjected to increasing regulatory scrutiny. In January 2021, *T. molitor* became the first insect to be approved for human consumption in the European Union under the novel food regulation (Regulation (EU) 2015/2283) thus paving the way to new markets (Turck et al., 2021).

The most appealing trait of *T. molitor* is the protein quantity, which typically ranges between 41 and 66 %, and quality, since it includes high levels of leucine, lysine, and valine, essential amino acids often limiting in plant-based proteins (Gkinali et al., 2022). Besides proteins,

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mealworms are also a good source of fats which, although largely affected by the feed composition (Lawal et al., 2021), are rich in monounsaturated fatty acids. The fiber content ranges between 2 and 19 % and commonly includes chitin that is an indigestible fiber found in their exoskeleton. Additionally, they contain significant amounts of vitamins and minerals, including B-vitamins, iron, zinc, and magnesium (Gkinali et al., 2022).

Nevertheless, despite the appealing composition, few drawbacks among which *i*) consumer acceptance, *ii*) potential allergenicity, *iii*) protein digestibility and *iv*) the technological performances when used as ingredient, limit or delay their large-scale utilization. For instance, cultural and behavioral biases against insect consumption persist and the "ick" factor represents a significant challenge in integrating insects into the regular diet in Western societies (Hartmann et al., 2015). It was suggested that the factors influencing consumer acceptance and buying behavior of insect-containing food fall within *i*) personal factors (socio-demographic, psychological, and familiarity) and *ii*) the elements of the marketing mix (product, price, promotion, and place) (Alhujaili et al., 2023). Yet, to overcome consumers' unwillingness to eat insects, the incorporation into mainstream food products represents a key strategy for increasing consumer acceptance and market penetration. One of the most promising applications is as ingredient in baked goods. Mealworm powder can be used to partially replace wheat flour in bread formulations, enhancing the protein content and nutritional profile of the product (Gkinali et al., 2022; Kotsou et al., 2023), and the potential of *T. molitor* in bread making has been the subject of recent investigation. Results indicate that 5–10 % wheat flour replacement could result in doughs and bread of satisfactory technological characteristics (e.g., volume, softness, color) and improved nutritional properties, nevertheless additions higher than 2 % might not be as preferred by consumers (for a review see Kotsou et al., 2023). Moreover, when referring to the nutritional properties of edible insects and products made thereof, the proximate composition (namely protein content) is the main criterium used to assess its quality and rarely protein digestibility is factored in. Indeed, although the research on the matter is scarce, and it is not clear whether and how digestibility differs from other protein sources, nor among insect species, it has been reported that chitin content affects protein digestibility which, in insects, is lower than most vegetable sources (Longvah et al., 2011; Marono et al., 2015). Similarly, compared to traditional protein sources (e.g., cereals, legumes, milk, and meat), there has been a paucity of research conducted on the physicochemical and techno-functional properties of insect proteins and there is a notable lack of studies examining the impact of processing techniques on these properties (Gravel and Doyen, 2020). Another challenge is the potential allergenicity of insects, which are arthropods like crustaceans. Indeed, there is evidence that individuals with shellfish allergies may also react to insect proteins and cross-reactivity is mainly mediated by tropomyosin and arginine kinase (Ribeiro et al., 2021).

Based on the above considerations, this study aimed at evaluating the effect of treatments with two commercial food-grade enzymatic preparations having protease activity and the potential of *T. molitor* bioprocessed powder as ingredient in bread-making. To the best of our knowledge, no other study has ever extensively studied the effect of enzymatic treated *T. molitor* powder on protein tecno-functional and rheological properties, nor its effect on insect-fortified bread nutritional and technological potential. Enzymatic treatments with proteases could *i*) reduce potential allergenicity by breaking down allergenic proteins (Ribeiro et al., 2021), *ii*) improve the digestibility and nutritional value (Sá et al., 2020) of insect-based foods, *iii*) remove chitin residues from proteins (Kim et al., 2023), and *iv*) modify mealworm techno-functional properties, such as solubility, foamability and emulsifying capacity (Purschke et al., 2018), thus rendering *T. molitor* powder more versatile as a food ingredient. An integrated approach was used to study the nutritional and technological properties of the bioprocessed *T. molitor* ingredient as well as those of the experimental breads made thereof.

2. Material and methods

2.1. Raw material and enzymes

Tenebrio molitor larvae (Broukárna, Ráby, Cechia), having the following proximate composition: moisture, 7 %; proteins, 51 %; lipids, 30 %; fibers, 7 %; ashes, 5 %, were used in this study. Before analyses larvae were grinded into powder using a laboratory mill Ika-Werke M20 (GMBH and Co. KG, Staufen, Germany).

Alcalase® 2.4 L FG (Novozymes, Denmark), a commercial preparation of serine endopeptidase obtained from *Bacillus licheniformis* (mainly subtilisin A; 27.3 kDa) which hydrolyzes amino esters, and Flavourzyme® 1000 L (Novozyme), a blend of endo- and exo-peptidases were employed for the bioprocessing treatment.

2.2. Microbial characterization of insect powder

For the microbiological analysis, 10 g of sample were suspended in 90 mL of sterile sodium chloride (0.9 %, w/v) solution and homogenized with a stomacher. Total mesophilic aerobic bacteria were enumerated on Plate Count Agar (PCA, Oxoid Ltd, Basingstoke, Hampshire, UK) supplemented with 0.01 % cycloheximide (Sigma Chemical Co., USA) under aerobic conditions at 30 °C for 48 h and *Enterobacteriaceae* were cultivated on Violet Red Bile Glucose Agar (VRBGA, Oxoid) at 37 °C for 36 h. Presumptive lactic acid bacteria cell density was determined on De Man Rogosa and Sharpe (MRS) agar (Oxoid), supplemented with 0.01 % of cycloheximide, at 30 °C for 48 h, under anaerobiosis. Yeasts and molds were cultivated on Sabouraud Dextrose Agar (SDA, Oxoid) supplemented with 0.01 % chloramphenicol (Sigma) at 25 °C, through pour and spread plate enumeration, respectively.

2.3. Set-up of the enzymatic treatment

Aiming at enhancing the nutritional potential of *T. molitor* powder, a bioprocessing protocol as proposed by Yoon et al. (2019) with some modifications was used. The two-step enzymatic treatment with two commercial proteases included: *i*) the preparation of slurries by mixing powder and water at a ratio of 25:75 (w/v); *ii*) the pH modification to 8.0 to be in the optimal range of that of the enzymes; *iii*) the addition of Alcalase® (72 mU/g); *iv*) the incubation at 50 °C for 1 h; *iv*) the addition of Flavourzyme® (60 U/g) to the slurries; and *v*) the incubation at 50 °C for 7 h. Aliquots were taken every hour for characterization. Enzymes were deactivated by treating the aliquots at 100 °C for 5 min. Samples were then cooled down and stored at −20 °C.

2.4. Proteolysis evaluation

Aiming at evaluating the effect of enzymatic treatment on the protein fraction, water/salt-soluble extracts (WSE) of the slurries were prepared (Weiss et al., 1993). An integrated approach, including the combination of electrophoretic and chromatographic techniques, was used to characterize proteins, peptides and free amino acids. More specifically, proteins were quantified using the Bradford method (Bradford, 1976). Then, aliquots of the WSE containing circa 15 μg of proteins, were diluted 1:1 with a sample buffer (0.125 M Tris-C1, pH 6.8; 10 % glycerol; 5 % β-mercaptoethanol, and 2 % SDS), treated at 100 °C for 5 min and analyzed by SDS-PAGE as described by Laemmli (1970).

Peptides concentration in WSE was determined by the *o*-phtaldialdehyde method (Church et al., 1983) and their profiles were characterized by RP-FPLC, using a Resource RPC column and an ÄKTA FPLC equipment, with a 20 μL loop and the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Elution gradient was performed at a flow rate of 1 mL/min using a mobile phase composed of water and acetonitrile (CH_3CN), containing 0.05 % trifluoracetic acid as described by Rizzello et al. (2010).

Total free amino acids (TFAA) were quantified by a Biochrom 30+

series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, Cambridge, UK) with a Li-cation-exchange column (4.6 × 200 mm internal diameter), as described by Verni et al. (2021).

For the in vitro protein digestibility (IVPD), slurries were subjected to a sequential enzymatic treatment carried out according to the consensus method developed within a large European framework (COSTAction InfoGest). The digestion procedure consisted of three consecutive phases and all the gastro-intestinal fluids were prepared according to the INFOGEST protocol (Brodkorb et al., 2019). The Bradford method (Bradford, 1976) was used to determine the concentration of the protein in the digested and non-digested fractions. IVPD was expressed as the percentage of the total protein solubilized after enzyme hydrolysis.

2.5. Antifungal activity of bioprocessed insect powder

The antifungal activity of raw and bioprocessed *T. molitor* powder was assayed based on hyphal radial growth rate of fungi. *Penicillium roqueforti* DPPMAF1, belonging to the Culture Collection Culture Collection of DISSPA (University of Bari, Italy) was used as indicator. WSE were sterilized by filtration on 0.22 μm membrane filters (Millipore Corporation, Bedford, MA 01,730) and added (30 % vol/vol, final concentration) to sterile Potato Dextrose Agar (PDA, Oxoid) into Petri plates. Control plates contained PDA supplemented with 30 % (vol/vol) of sterile water. The assay was carried out by placing a 3-mm diameter plug of growing mycelia onto the center of Petri dishes. Plates were incubated aerobically at 25 °C. The radial growth of mycelia was measured 6 days after inoculation. The percentage of growth inhibition was calculated from mean values as follows: percentage of inhibition = [(mycelial growth under control conditions - mycelial growth in the presence of WSE)/mycelial growth under control conditions] × 100.

2.6. Techno-functional properties of insect powders

Based on the characterization performed on each hydrolysis time, three conditions (T0, T3, and T8) were selected for further study of the techno-functional properties which were measured as reported in De Angelis et al. (2024). More specifically, water absorption capacity (WAC), oil absorption capacity (OAC), water absorption index (WAI) and water solubility index (WSI) were determined. WAI was calculated as the ratio of the weight of the sediment to the weight of the ingredient, whereas WSI was expressed as percentage ratio of solid content of the supernatant relative to the weight of the ingredient. The emulsifying capacity (EC), foaming ability (FA) and foam stability (FS) at 10 (FS10) and 20 (FS20) min were measured as described in De Angelis et al. (2024). Each analysis was carried out in duplicate per TMP batch (n = 2).

2.7. Bread making

Bread doughs were produced using wheat flour (WF; SELEX Gruppo Commerciale S.p.A., Trezzano sul Naviglio, Italy) and *T. molitor* powders at T0 (TMP_0), T3 (TMP_3), and T8 (TMP_8), following the experimental design described in section 2.11. The total amount of dry ingredients (WF and TMP) was set at 160 g and the tested proportions of TMP were 0 %, 5 %, and 10 %. The percentages of substitution were chosen to ensure compliance with the maximum limit of TMP set for bread (Reg. (EU) 2017/2470). Wheat flour and TMP were mixed with 2 g of NaCl, 0.8 g of baker's yeast (Lievital, Lesaffre Italia S.p.A., Sissa Trecasali, Italy), and 120 g of water. The leavening was carried out overnight at 4 °C and subsequently at 25 °C for three hours.

Before baking, doughs' viscosity was measured as reported in De Angelis et al. (2024) by a rheometer (HAAKE MARS iQ Air, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a parallel plate geometry with 1 mm gap between the plates. The shear rate ramp test was performed by increasing the shear rate from 1 to 100 s^{-1} at 25 °C. The data were fitted to the Ostwald–de Waele model, according to the following equation:

$$\tau = K \times \gamma^n$$

where τ = shear stress (Pa), K = consistency index (Pa s^n); γ = shear rate (s^{-1}) and n = flow behavior index. Each dough was produced in duplicate (n = 2).

Then doughs were cooked in a static electric oven (Smeg SI 850 RA-5, Smeg SpA, Guastalla, Italy) in porcelain pans at 220 °C for 45 min leaving the container covered with aluminum foil, and for the last 5 min, uncovered. The bread was then left to cool completely to room temperature. Each bread was produced in duplicate (n = 2).

2.8. Biochemical and nutritional characterization of breads

Total free amino acids were quantified as described above whereas the volatile profile of the breads was determined by HS-SPME-GC–MS. About 0.5 g of sample was weighed in a 20 mL vial with the addition of 150 μL of a 1-propanol solution (160 ppm) as internal standard and 4 mL of saturated aqueous solution of NaCl. Volatile Organic compounds (VOC) were then extracted by using a 75 μm Carboxen/polydimethylsiloxane SPME fiber (Supelco, Bellefonte, PA, USA) exposed into the vial headspace for 20 min at 40 °C. After that, VOC were desorbed into the GC (6850 series gas chromatograph coupled to a mass spectrometer 5975 series, Agilent Technologies Inc., Santa Clara, CA, USA) injector for 2 min at 250 °C. The stationary phase was HP-INNOWax and the chromatographic conditions were those already reported in Caponio et al. (2019). Volatile compounds were identified by comparison of their mass spectra with the mass spectra present in the NIST and Wiley libraries and only those with a match quality above 80 % were considered. The quantification was performed by the internal standard method and the results expressed as μg of 1-propanol equivalent per g of sample. The analysis was carried out in duplicate per type of bread (n = 2).

Breads IVPD was determined as described above whereas starch hydrolysis index (HI) was determined by mimicking the in vivo digestion of starch (De Angelis et al., 2009). Briefly, bread portions, containing 1 g of starch, were treated with amyloglucosidase and the released glucose was quantified with the glucose assay Kit (GOPOD--format, Megazyme) following manufacturer's instructions. The degree of starch digestion was expressed as the percentage of potentially available starch hydrolyzed after 180 min. Control wheat bread was used as reference to estimate the hydrolysis index (HI = 100). The equation: pGI = 0.549 × HI+39.71 proposed by Capriles and Arêas (2013) was used to calculate the predicted glycemic index (pGI).

2.9. Proximate composition of breads

Moisture was measured by a thermogravimetric analyzer (Mod. MAC 110/NP, Radwag Wagi Elektroniczne, Radom, Poland). Protein content was determined according to the method AOAC 2001.11 (Thiex et al., 2002) using a conversion factor weighted according to the actual percentage of WF (N × 5.70) and TMP (N × 6.25) used in the bread formulations. Ash content was determined according to the AOAC methods 923.03 (AOAC, 2006). The determination of the lipid content was carried out following the Randall method (SER 148, Velp Scientifica srl, Usmate, Italy) with diethyl ether as extraction solvent. The total carbohydrate content was determined by difference, subtracting the content of moisture, protein, lipid, and ash to 100. Each analysis was carried out in duplicate per type of bread (n = 2).

2.10. Texture and color analyses of bread

The Texture Profile Analysis (TPA) was carried out on bread slices (thickness 2 cm) using a Z1.0 TN texture analyzer (Zwick Roell, Ulm,

Germany) equipped with a cylindrical stainless-steel probe with a diameter of 36 mm and a 50 N load cell and controlled by the TestXPertII software version 3.41. The conditions of the test were those reported in De Angelis et al. (2024). Hardness (N) and springiness were the parameters considered. The color coordinates L* (lightness, from black to white), a* (from green to red), and b* (from blue to yellow) of the samples were measured using a colorimeter (CM-600d, Konica Minolta, Tokyo, Japan). The color analysis was carried out both on crust and crumb. Six technical replicates were carried out for both TPA and color analyses per each type of bread ($n = 2$).

2.11. Statistical analysis

Data of TMP, doughs and breads analysis were subjected to one-way analysis of variance (ANOVA), followed by the Bonferroni test for multiple comparisons, at a significance level of 5 % using Minitab 17 (Minitab Inc., State College, PA, USA). The Principal Component Analysis (PCA) on the volatiles profile was carried out using the same software on the autoscaled data matrix.

Breads were produced according to a mixtures-process experimental design (Squeoe et al., 2021), considering 2 mixture factors (TMP and WF), and one process categorical factor (the type of insect powder: TMP_0, TMP_3, TMP_8). Then, selected responses (i.e., % kcal derived from proteins, crust luminosity, hardness, and IVPD) were modelled according to the following mathematical model:

$$Y = TMP + WF + Ta + Tb + TMP * WF$$

where Y is the response, and that allows to estimate the linear terms and the quadratic term for the mixture variables (TMP and WF), together with the linear terms for the type of TMP (i.e., Ta and Tb). Design-Expert 12 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to build and analyze the design.

The data obtained from the analysis of breads TFAA were also analyzed using the MetaboAnalyst version 5.0 software (metaboanalyst.ca/; accessed online November 24th, 2024) to obtain a heatmap with hierarchical clustering (Ward's method) based on Euclidean distance. Correlations within the biochemical, nutritional and technological features of bread ($P < 0.05$), assessed based on Pearson's bivariate correlation, were determined with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

3. Results and discussion

3.1. Microbial characterization of raw material

Due to a combination of factors, among which the *i*) neutral pH, *ii*) high water activity, and *iii*) high concentration of nutrients, insect powders are considered a suitable substrate for the growth of a broad range of microorganisms. Hence, aiming at evaluating the microbial load of *T. molitor* powder used in this study, microbial counts were performed. The microbial contamination of these matrices is highly dependent on the production process. For instance, it was suggested that larvae starvation reduces the total counts of all microorganisms, specifically yeasts and total mesophilic anaerobic spore-forming bacteria (Costa et al., 2020) and of course, heat treatment by boiling is sufficient to kill vegetative forms (reducing cell density of 5 Log cfu/g) (Yan et al., 2023). In this study, the larvae powder was characterized by a cell density of 1.61 ± 0.08 and 1.28 ± 0.07 Log cfu/g of yeasts and molds, respectively. Total mesophilic bacteria and presumptive lactic acid bacteria were 3.02 ± 0.08 and 1.59 ± 0.05 Log cfu/g, respectively. Whereas *Enterobacteriaceae* were below 1 Log cfu/g. These data are well below those previously reported by Vandeweyer et al. (2017) when they examined the microbial load of several *T. molitor* larvae and found cell densities of yeasts and bacteria up to 8 Log cfu/g. Nevertheless, the cell densities found in the *T. molitor* powder used in this study are within the microbiological criteria and limits set by the Regulation (EU) No. 2021/882.

3.2. Effect of the enzymatic treatment on the ingredient

3.2.1. Proteins, peptides and amino acids

Although the enzymatic treatment did not modify TMP proximate composition (data not shown), aiming at evaluating its effect on protein, peptides and amino acids profiles, a combination of techniques involving spectrophotometric assays, electrophoresis, and liquid chromatography was used. Protein content, as determined by the Bradford method, was ca. 10 mg/g at T0 and significantly decreased to 6.66 mg/g after the first hour of treatment with Alcalase® (Fig. 1a).

A further decrease was observed after the second hour of treatment, but from T2 to T8, protein content remained stable around 4.3 mg/g. This trend was confirmed by the SDS-PAGE electrophoresis, as shown in Figure S1. Overall, although SDS is a method widely used to separate insect proteins, which are then subjected to liquid chromatography and mass spectrometry to be identified, the complexity of the identification process is determined by the lack of information on insect proteomes. Before treatment the matrix was characterized by circa 20 bands evenly distributed among the gel within the range 10–75 kDa. The most intense bands were at 75, 65 and 37 kDa, nevertheless, most of the bands, although presenting lower intensity than the previous, were observed in the range 10–25 kDa. It was suggested that low MW proteins in *T. molitor* are mainly proteins with anti-freezing properties, such as hemolymph proteins which range from 8.5 to 13 kDa (Ma et al., 2023). After the first hour of treatment, as consequence of the decomposition of high MW bands, similarly to what reported by Purschke et al. (2018) and Yoon et al. (2019), all the bands disappeared, leaving a long smear below 20 kDa. Indeed, Alcalase®, an endopeptidase which breaks peptide bonds from C-terminal amino acids, is considered one of the most effective enzymes for protein hydrolysis, highly efficacious on matrices, both of insect and vegetable origin (Hunsakul et al., 2022; Purschke et al., 2018; Yoon et al., 2019). Flavourzyme®, instead, contains exoproteases cleaving small peptides fragments at the extremities of proteins, leading to a slow, step-wise protein decomposition. The addition of Flavourzyme® from T2 to T8 determined the appearance of two bands around 50 and 15 kDa, but no differences were observed among samples.

A trend inversely proportional to that of proteins was observed for peptides and amino acids content. Indeed, peptides significantly ($P < 0.05$) increased from 145 mg/g of T0 to 220 and 439 mg/g of T1 and T2. From T4 onward peptides concentration remained stable around 600 mg/g (Fig. 1b). These data were confirmed by the FPLC analysis (Table 1) which highlighted similar differences in the total area of the chromatogram. Although the number of peaks detected did not differ among samples, a modification in peptides hydrophobicity was observed. Already from T2, compared to T0, significantly ($P < 0.05$) higher percentages of peptides eluted at the lowest percentage of acetonitrile (from 0 to 46 % of eluent B). On the contrary, peptides eluting at 100 % of acetonitrile, hence more hydrophobic, reached almost 0 % at the end of the treatment (Table 1). Indeed, it was suggested that enzymatic hydrolysis releases more ionized groups, thus producing smaller proteins and peptides, and as a result, increases protein solubility (Ma et al., 2023).

Similarly, TFAA modification was linear with the trend observed for peptides and proteins (Fig. 1c). TFAA concentration gradually increased from 21 to 70 mg/g in T0 and T3, respectively, and exceeded 110 mg/g from T6 onward. The most abundant amino acids before treatments were proline (Pro) and arginine (Arg), with 5.74 and 2.66 mg/g, respectively, whereas asparagine (Asn), cysteine (Cys), methionine (Met), and ornithine (Orn) were the least represented with values below 0.12 mg/g (Table S1). The sole treatment with Alcalase® did not change significantly TFAA profile and the overall content, while the combination with Flavourzyme®, determined increases proportional to the time

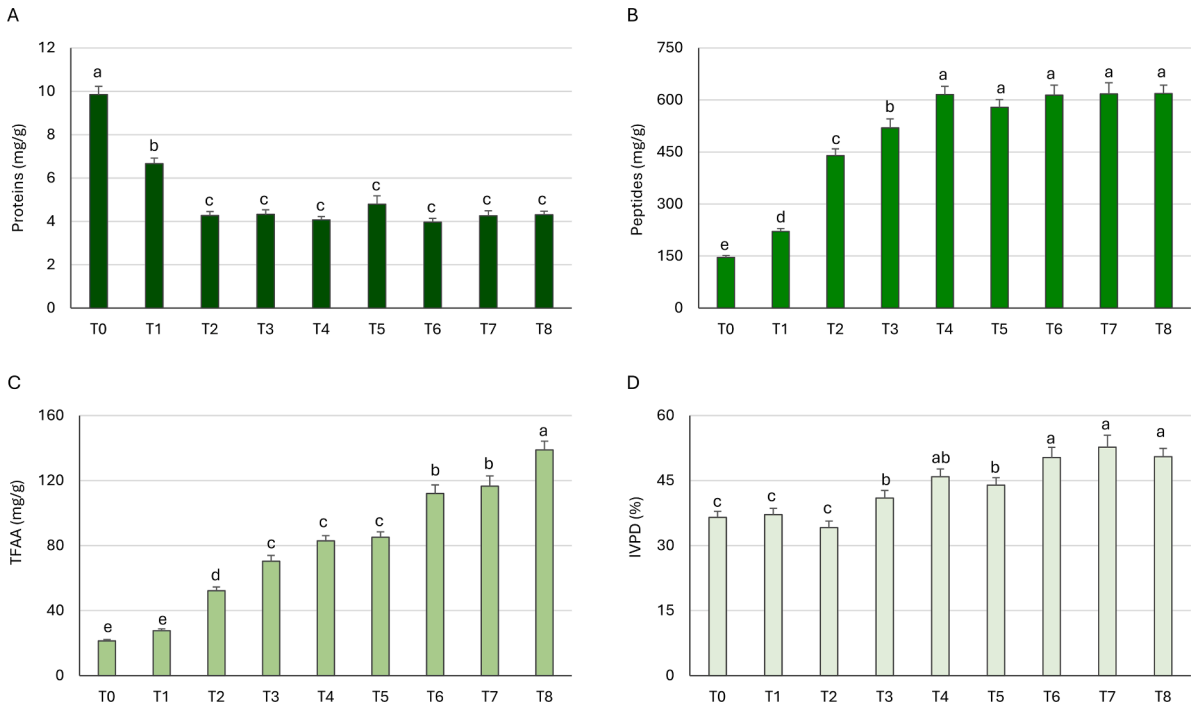

Fig. 1. Concentration of proteins (a), peptides (b), total free ammino acids (c), and *in vitro* protein digestibility (d) of *Tenebrio molitor* powder before (T0) and after (T1-T8) enzymatic treatment with Alcalase® and Flavourzyme®.

Table 1
Area, number of peaks and peaks area of peptides as determined by Reversed Phase Fast Performance Liquid Chromatography (RP-FPLC) of Tenebrio molitor (TMP) before (−0) and after 8 h (−1 to −8) enzymatic treatment with Alcalase® and Flavourzyme®. Peaks area of detected peptides, expressed as percentage of the total area, is separated based on hydrophobicity (elution at Eluent B different percentages).

			Peak area/Total (%)			
	Area (mAU/mL)	N. peaks	0 %B	0–46 % B	46–100 % B	100 % B
TMP_0	2914 ± 114^{e}	43 ± 2^{a}	47.7 ± 1.9^{c}	16.2 ± 0.6^{c}	2.39 ± 0.09^{a}	33.7 ± 1.3^{a}
TMP_1	3332 ± 130^{d}	45 ± 2^{a}	59.2 ± 2.3^{ab}	27.8 ± 1.1^{b}	0.53 ± 0.02^{d}	12.5 ± 0.5^{b}
TMP_2	4226 ± 190^{c}	36 ± 2^{ab}	67.2 ± 3.0^{a}	32.0 ± 1.4^{ab}	0.31 ± 0.01^{e}	0.5 ± 0.0^{d}
TMP_3	5032 ± 252^{b}	42 ± 2^{a}	65.6 ± 3.3^{a}	25.1 ± 1.3^{b}	0.66 ± 0.03^{d}	8.6 ± 0.4^{c}
TMP_4	5939 ± 232^{b}	41 ± 2^{a}	56.5 ± 2.2^{b}	27.7 ± 1.1^{b}	0.81 ± 0.03^{c}	15.1 ± 0.6^{b}
TMP_5	5478 ± 214^{b}	40 ± 2^{a}	64.9 ± 2.5^{a}	25.0 ± 1.0^{b}	1.78 ± 0.07^{b}	8.2 ± 0.3^{c}
TMP_6	7067 ± 473^{a}	32 ± 2^{b}	55.2 ± 2.6^{b}	29.1 ± 1.4^{b}	1.47 ± 0.07^{b}	14.3 ± 0.7^{b}
TMP_7	6241 ± 331^{a}	34 ± 2^{b}	61.5 ± 3.3^{a}	29.4 ± 1.6^{b}	0.49 ± 0.03^{d}	8.6 ± 0.5^{c}
TMP_8	6868 ± 268^{a}	29 ± 1^{b}	59.3 ± 2.3^{ab}	38.9 ± 1.5^{a}	0.86 ± 0.03^{c}	0.9 ± 0.0^{d}

The data are the means of three independent experiments ± standard deviations ($n = 3$).
a–eValues in the same column with different superscript letters differ significantly ($P < 0.05$).

of hydrolysis. At T8, compared to T0, amino acids increased from 1.5- to 17-times, and even higher increases, up to 88-times, were found for Asn, Cys, and Met (Table S1).

The effect of the enzymatic treatment on protein digestibility was also evaluated. Protein digestibility of insect powders has been the topic of much research, and it appears to be a consensus on the fact that IVPD among insect species, which can vary from 45 to 67 %, is lower than that commonly found in most vegetable protein sources (Hong et al. 2020; Marono et al., 2015). In this study, IVPD was slightly below 40 % (Fig. 1d) and remained low for the first 3 hours of treatment. The low protein digestibility in insect powders is often ascribed to chitin, a linear polymer of b-(1–4) N-acetyl-d-glucosamine units structurally similar to cellulose, that interferes with their digestion (Longvah et al., 2011). Nevertheless, as the enzymatic treatment proceeded, IVPD increased reaching 50.47 ± 1.97 at the end of the treatment. Although this aspect was not evaluated in this study and is subject of ongoing investigation, the enzymatic treatment might have reduced TMP potential allergenicity. Indeed, it was observed that high hydrostatic pressure-assisted enzymatic hydrolysis by Alcalase® not only improved the degree of hydrolysis of mealworm protein but also decreased protein allergenicity by 70 % (Boukil et al., 2020). It was also recently demonstrated that Alcalase® can be used to remove protein from chitin and chitosan (Kim et al., 2023) thus explaining the higher IVPD found as the proteolysis continued. It is also possible that the chitosan, normally contained in insect powders, is responsible for the antifungal activity of the ingredient. Indeed, an inhibition of circa 60 % against *P. roqueforti* growth was observed in this study. The lack of differences among samples (from T1 to T8) suggests that such activity cannot be ascribed to the protein fraction, but rather to chitosan whose antifungal activity has been extensively studied for several decades (Velásquez and Avelizapa, 2020).

Since the major changes in terms of proteolysis and protein digestibility occurred after 3 and 8h of treatment, TMP_3, and TMP_8 were selected for further characterization. Although the highest nutritional improvement (liberation of peptides and free amino acids and related IVPD) was provided after 8 hours of treatment, TMP_3 was also chosen to meet halfway possible industrial requirements. Lower time of hydrolysis entails lower production costs; hence 3 hours of hydrolysis was selected as the minimum processing time where significant improvements occurred. TMP_0 was used as reference.

3.2.2. *Techno-functional and rheological properties*

The techno-functional characteristics of the TMP are reported in

Table 2
Techno-functional properties of T. molitor before (TMP_0) and after 3 (TMP_3) and 8 (TMP_8) hours of enzymatic treatment with Alcalase® and Flavourzyme®. WAI, water absorption index; WSI, water solubility index; OAC, oil absorption capacity; EC, emulsifying capacity; FA, foaming activity; FS, foam stability at 10 (FS 10) and 20 (FS 20) minutes, respectively.

	TMP_0	TMP_3	TMP_8
WAC (g/g)	1.49 ± 0.01^a	0.55 ± 0.01^b	0.58 ± 0.05^b
WAI (g/g)	2.88 ± 0.12^a	1.92 ± 0.12^b	1.83 ± 0.12^b
WSI (%)	20.26 ± 0.77^c	41.87 ± 0.04^b	44.11 ± 0.05^a
OAC (g/g)	1.51 ± 0.19^a	1.59 ± 0.03^a	1.59 ± 0.24^a
EC (g/g)	110.38 ± 12.48^b	157.62 ± 9.38^a	185.58 ± 4.49^a
FA (%)	2.50 ± 0.00^b	5.50 ± 0.71^a	5.00 ± 0.00^a
FS10 (%)	100.00 ± 0.00^a	95.83 ± 5.89^a	100.00 ± 0.00^a
FS20 (%)	100.00 ± 0.00^a	95.83 ± 5.89^a	100.00 ± 0.00^a

The data are the means of two independent experiments ± standard deviations ($n = 2$).
a–cValues in the same raw with different superscript letters differ significantly ($P < 0.05$).

Table 2. Biotechnological treatment caused a significant reduction in WAC and WAI. WAC is affected by several factors including amino acid profile, number of charged residues, protein conformation, pH, protein concentration (Bußler et al., 2016), playing a crucial role in functional and sensorial properties of baked goods, and higher values can contribute to the production of softer and moister bread (Du et al., 2014; Lee et al., 2019; Menonet al., 2015). WAC of TMP_0 was similar to that reported in Bresciani et al. (2022) for wheat flour, and to those reported in Du et al. (2014) and Kaur and Singh (2005) for legumes flours. WAI measures the amount of water trapped in the food network and is affected by the properties of macromolecules such as starch and proteins (Du et al. 2014). WAI values were lower than those reported for wheat flour (Bresciani et al., 2022), likely due to the lack of gluten in TMP, or for other ingredients used for protein enrichment of bread such as legume flours, probably due to their higher starch content (Du et al., 2014). WSI, EC and FC were significantly increased by a longer enzymatic treatment. WSI varies mainly as a function of pH, and it was found that TMP had higher solubility values at more alkaline pH (Bußler et al., 2016).

The increase in WSI could be a relevant result because a solubility increase could interfere with the ability of the flour to trigger an IgE mediated reaction (Ribeiro et al., 2018).

The increase in EC after the biotechnological treatment is consistent with what reported by Bußler et al. (2016) and justified by that greater degradation of proteins which leads to an increase in their emulsifying capacity. FA is linked to the surfactant activity of proteins (Kaur and Singh, 2005) and is an important technological parameter. In fact, higher FA corresponds to an improved capacity to retain air and CO_2 (Menon et al., 2015). Therefore, the twofold increase of FA observed with longer enzymatic treatment could positively influence the volume of leavened products. Finally, no significant differences were observed in the OAC, FS10, and FS20 (Table 2).

3.3. Bread dough viscosity

The flow curves of the doughs, prepared according to the experimental plan, are reported in Fig. 2. It emerged that all the doughs showed a typical pseudoplastic behavior, highlighted by a progressive reduction in viscosity as the shear rate increased. The flow behavior index (n) confirms this observation (Table S2) as values between 0 and 1 are indicative of a pseudoplastic material (De Angelis et al., 2024). Thus, there was a tendency of the dough microstructure to align in the direction of the flow as the shear rate increased with a consequent reduction in the viscosity value. By comparing the flow curves, it could be observed as the control bread (CTR) had a generally higher viscosity than the other doughs. The value of K, the consistency index, in Table S2

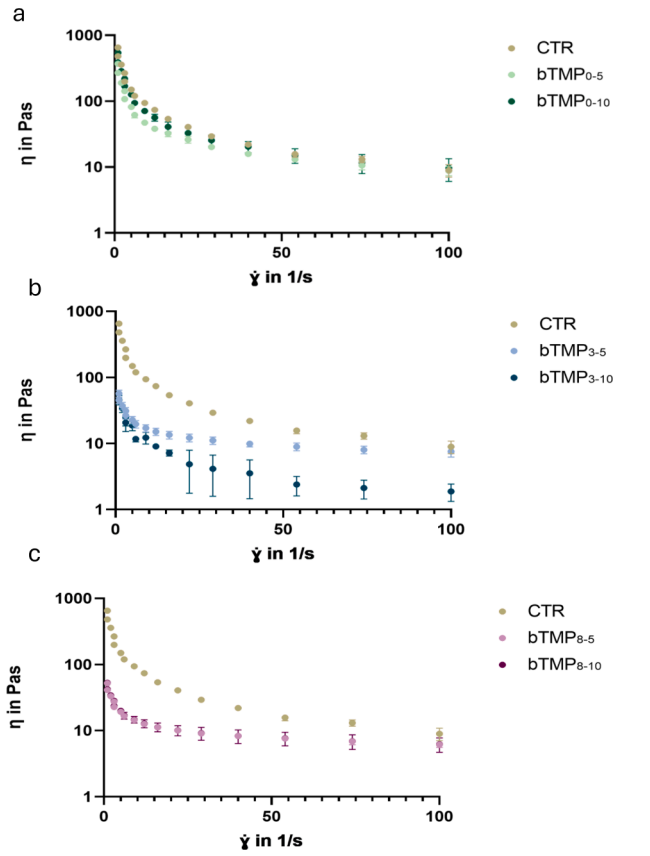

Fig. 2. Apparent viscosity of bread doughs (η, Pa s) vs the shear rate (γ̇, 1/s) of *T. molitor* before ($bTMP_0$; a) and after 3 ($bTMP_3$; b) and 8 h ($bTMP_8$; c) hours of enzymatic treatment with Alcalase® and Flavourzyme®. CTR, control wheat dough; 5 or 10 indicate *T. molitor* percentage of inclusion.

confirms this observation. The partial substitution with TMP always reduced the viscosity. However, the reduction was very limited in the case of the TMP_0. This behavior might be explained in different, complementary, ways. A previous study showed that enzymatic treatments on protein isolates determine a reduction in viscosity (Vogelsang-O'Dwyer et al., 2022) but viscosity can be also influenced by the polysaccharide content. Specifically, chitin, interacting through hydrogen bonds and electrostatic interaction with other macromolecules, determines an increase in viscosity (Kim et al., 2020). Therefore, the reduction observed following the enzymatic treatment could be an indication of a potential degradation of the polysaccharide within insect powders. Finally, this result might be also explained by the greater WAC of TMP_0 compared to the others (Table 2), which substantially reduced the amount of free water, improving the consistency of the dough. The same behavior had in fact been found in a leavened dough where the addition of sodium alginate, capable of retaining water, was able to increase the consistency (Upadhyay et al., 2012). The viscosity of the other doughs was generally lower (Fig. 2, Table S2). A marked effect of the percentage of wheat flour substitution is observed only in the case of TMP_3, suggesting a greater influence of the biotechnological treatment time on dough viscosity. Dough viscosity has a good correlation with bread quality (Dobraszczyk and Morgenstern, 2003). From a practical point of view, changes in dough viscosity are reported to affect volume, texture and crumb porosity of the final products (Yazici and Ozer, 2021; De Angelis et al., 2024). In particular, lower viscosity can lead to greater losses of air/CO_2 resulting in a partial collapse of the product (De Angelis et al., 2024), which might be more compact and harder. de Oliveira and colleagues (2017) reported that higher percentages of insect flour into bread formulation were negatively related with the specific volume while positively related with bread hardness due to

modifications in dough viscoelasticity. Such results are confirmed also in other works (Gaglio et al., 2021; Osimani et al., 2018).

3.4. Bread characterization

3.4.1. Proximate composition

The proximate composition of the breads is reported in Table 3. The protein, lipid and ash content were significantly lower in CTR compared to the TMP breads. For these parameters, it emerged an effect related to TMP percentage of inclusion rather than the enzymatic treatment.

The total energy value slightly increased in the TMP breads respect to CTR. However, while the latter did not fulfill the requirements of the Regulation (EC) No 1924/2006 about nutritional claims for proteins, all fortified breads reached, at least, the limit of 12 % of energy provided by proteins and thus could be labelled as "source of protein" whereas those made with 10 % of TMP, could be labelled as "high protein". Bread fortification has been explored by a number of authors, using legumes, pseudocereals, insects, dairy and fish products (Prieto-Vázquez del Mercado et al., 2022). Evidently, protein content highly varies based on the type and amount of flour used, ranging from 15 to 40 g/100 g and the related "source of protein" claim is almost always reached. Overall, the energy content of the bread produced in this study was lower than that of fortified bread commonly found in the literature and, with lower fortification rates compared to those commonly reported, the same claim, if not higher, was achieved.

The experimental results were used to model the response (e.g., % kcal from proteins) along the entire defined experimental domain and the results are presented in Figure S2-A. Mathematical modeling showed how the kcal % from proteins changes according to the mixture. The analysis of variance did not highlight (data not shown) any significant effect of the type of TMP (i.e., after 0, 3, or 8 h of biotechnological treatment) thus the results presented are the average of the experiments carried out with all the TMP. From the figure, it could be easily concluded that a substitution of about 2 % was already enough to reach the claim "source of protein" and that there is a wide range of ingredients ratios that allows to fulfill the requirements for that claim (roughly from 2 % to 8 % of TMP). Above 8 % the products could be labelled as "high protein". Thus, for what concerns the production of a bread with proteins-related nutritional claims, it emerged that *i*) the extent of the enzymatic hydrolysis of TMP did not matter but *ii*) only the percentage of addition should be considered, and given the wide range of formulations possible *iii*) the amount of TMP to be used is a matter of a tradeoff between the nutritional, organoleptic and all the other characteristics that one wishes to consider.

3.4.2. Free amino acids and VOC profile

Control bread contained 560.2 ± 38.1 mg/kg of total free amino acids with Asn, Alanine (Ala), aspartic (Asp) and glutamic (Glu) acids being the most abundant, with concentrations of 78, 57, 76 and 56 mg/kg, respectively, whereas Met, Orn, and Lysine (Lys) were the limiting amino acids, with values below 4 mg/kg. The addition of raw insect powder increased TFAA content which reached 1.29 and 1.94 g/kg when 5 and 10 % TMP were added, respectively. Amino acids that increased the most were Pro and Arg (up to 12- and 6-fold respectively). According to the ingredients used (TMP_3 or TMP_8) and the percentage added (5 or 10 %), the addition of enzymatic treated insect powder determined higher increases, up to 4.97 g/kg in TMP_8. The permutation analysis clearly distinguished control breads (either with or without *T. molitor* powder), from those containing the enzymatic treated TMP (Fig. 3). Indeed, the bioprocessed ingredient provided a completely different profile to bread amino acids, with Ala, valine (Val), leucine (Leu), tyrosine (Tyr), Lys, Arg, and Pro being the most abundant and values up to 36-fold higher compared to that of CTR.

The volatiles profile of the breads is reported in Table S3. The identified compounds were in accordance with those reported in other studies (Gaglio et al., 2021; Keil et al., 2022). Most of the volatiles are carbonylic compounds produced from different routes such as fermentation, Maillard reaction, and lipid oxidation. The addition of TMP modified the pattern of such profiles but depicting a definitive effect of the percentage of substitution or of the kind of TMP used is a hard task. Hence, to help with the exploration of the dataset, a PCA was carried out

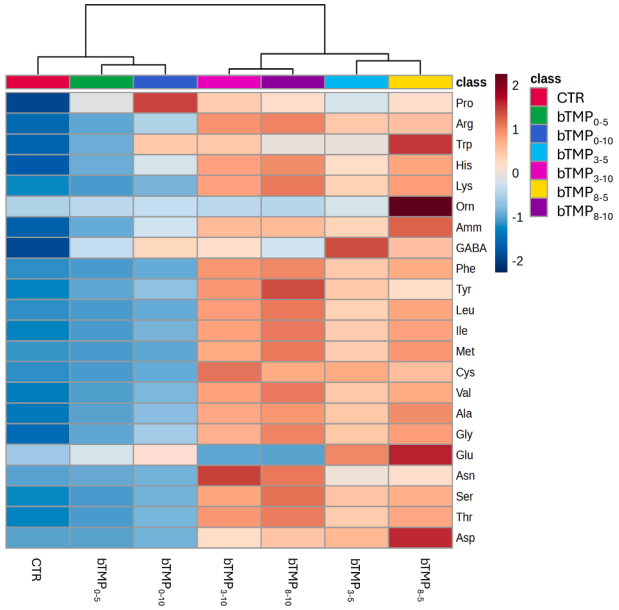

Fig. 3. Hierarchical clustering heatmap of total free amino acids profiles of the experimental breads containing 5 (−5) and 10 % (−10) of *Tenebrio molitor* powder before ($bTMP_0$) and after 3 ($bTMP_3$) and 8 h ($bTMP_8$) enzymatic treatment with Alcalase® and Flavourzyme®. CTR, control bread obtained with wheat flour. The color scale-bar ranged between low (blue) to high (brown) concentrations that were used to perform the clustering (Ward's method) based on Euclidean distance.

Table 3
Nutritional composition (g/100 g d.w.b.) and energy value of the experimental breads containing 5 (−5) and 10 % (−10) of Tenebrio molitor powder before ($bTMP_0$) and after 3 ($bTMP_3$) and 8 h ($bTMP_8$) enzymatic treatment with Alcalase® and Flavourzyme®. CTR, control bread obtained with wheat flour.

	Proteins	Lipids	Ashes	Carbohydrates	Energy (kcal)	% kcal from proteins
CTR	9.85±0.70^{c}	1.25±0.13^{d}	1.61±0.03^{d}	87.28±0.70^{a}	213	10
$bTMP_{0-5}$	17.12±0.39^{b}	2.31±0.19^{c}	2.40±0.02^{b}	78.17±0.41^{b}	214	17
$bTMP_{0-10}$	21.35±1.03^{a}	3.69±0.31^{a}	2.66±0.04^{a}	72.29±1.35^{c}	216	21
$bTMP_{3-5}$	16.01±0.64^{b}	2.54±0.21^{bc}	2.11±0.07^{c}	79.34±0.73^{b}	222	16
$bTMP_{3-10}$	21.78±0.84^{a}	3.52±0.30^{ab}	2.43±0.06^{b}	72.26±0.85^{c}	221	21
$bTMP_{8-5}$	16.32±0.78^{b}	2.53±0.68^{c}	2.33±0.07^{b}	78.83±1.38^{b}	220	16
$bTMP_{8-10}$	22.40±0.48^{a}	3.59±0.13^{a}	2.38±0.09^{b}	71.64±0.50^{c}	222	22

The data are the means of two independent experiments ± standard deviations ($n = 2$).
a–dValues in the same raw with different superscript letters differ significantly ($P < 0.05$).
d.w.b., dry weight basi.

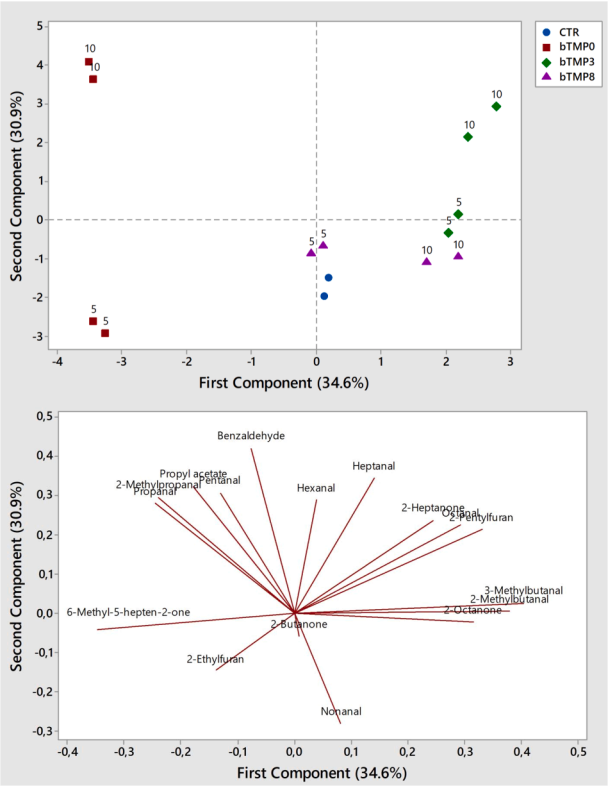

Fig. 4. Score plot and loading plot of the PCA carried out on the data of the volatile composition of the breads. In the score plot, the labels (5 and 10) indicate the percentage of substitution of *Tenebrio molitor* powder before ($bTMP_0$) and after 3 ($bTMP_3$) and 8 h ($bTMP_8$) enzymatic treatment with Alcalase® and Flavourzyme®. CTR, control bread obtained with wheat flour.

(Fig. 4).

Overall, it could be observed that the volatile profile of breads containing TMP_3 and TMP_8 was closer with a limited effect of the percentage of TMP inclusion, while it was much more different respect to that of breads containing TMP_0. In particular, the former samples were characterized by higher content of 2-methylbutanal, 3-methylbutanal, 2-octanone, 2-heptanone, octanal and 2-pentylfuran. 2-methylbutanal and 3-methylbutanal could have originated from the Strecker degradation of branched-chain amino acids (Keil et al., 2022) and the higher concentration could be related to the more intense catabolic effect of the longer enzymatic treatment. Indeed, positive and significant ($P < 0.05$) correlations were found between 3-methylbutanal and Leu, isoleucine (Ile), and Val (Figure S3). Similarly, higher values of 2-pentylfuran have been associated with more intense thermal reactions, such as the Maillard reaction and the caramelization process, which, in turn, could have been triggered by the more intense proteins degradation of the longer enzymatic treatment. Hence, in both cases, larger proteins degradation resulted in a higher concentration of precursors. In fact, 2-pentylfuran positively and significantly ($P < 0.05$) correlated with most amino acids (Figure S3). Differently, breads containing TMP_0 stood out for the higher concentration of 6-methyl-5-hepten-2-one, reported to have a green citrus-like odor notes (Gaglio et al., 2021) and suggested to have a possible positive impact on the product. Differently, the percentage of inclusion had higher impact on the concentration of aldehydes (benzaldehyde, propanal, pentanal, octanal, nonanal, heptanal) as well as propyl acetate, 2-pentylfuran, 2-ethylfuran, and 2-heptanone.

It could be argued that such changes in the volatile profile impact breads aroma and flavor perception and, in the end, their possible acceptability by consumers. In general terms, bread can represent one of the most suitable products for insect inclusion as it undergoes fermentation and cooking, both processes that improve the aroma and mitigate the negative notes linked to the insects (Perez-Santaescolastica et al., 2022). Overall, breads were characterized by volatiles with positive odor notes (Perez-Santaescolastica et al., 2022). Some aldehydes from oxidation, however, were mostly found as TMP percentage increased (e.g., 10 %). It can be inferred that $bTMP_3$ and $bTMP_8$ might be characterized by more intense notes of buttery, toasty, fatty, floral, sweet or roasted (linked to the higher amount of 2-methylbutanal and 3-methylbutanal) and pleasant aromas such as sweet, green and fruity due to 2-pentylfuran (Perez-Santaescolastica et al., 2022). On the other hand, the limited development of aroma precursors in $bTMP_0$ might be responsible for a less pleasant overall aroma. In fact, the important role of precursors in the mitigation of undesirable odors typical for edible insects has been highlighted (Perez-Santaescolastica et al., 2022). Based on these considerations, the enzymatic treatment might be seen, collaterally, as an aroma enhancing process. Nonetheless, in a more general perspective, it should be taken into account that numerous factors affect aroma and flavor perception, starting from the perception threshold of different volatiles, moving to their concentration and odor activity value, until the interaction with those originated from other ingredients present in the formulation.

3.4.3. *In vitro protein and starch digestibility*

Aiming at evaluating the effect of insect powder supplementation and the enzymatic treatments on bread protein digestibility, breads were subjected to the INFOGEST procedure, which simulates gastro-intestinal digestion according to a consensus method developed within a large European framework (Brodkorb et al., 2019). Control bread had an IVPD of 62.77 ± 2.64 % and the addition of TMP_0, regardless of the percentage, did not modify ($P > 0.05$) protein digestibility. Since protein digestibility of the raw ingredient was lower than that of control bread and wheat flour, a lower IVPD could have been expected (Table S4). It is possible that the heat treatment occurred during baking increased protein bio-accessibility. Indeed, it was found that cooking treatments can modify protein digestibility (Mancini et al., 2021). On the contrary, IVPD increases in breads fortified with treated insect powders. Mean values of 69.9 and 74.8 % were found in breads added of TMP_3 and TMP_8, respectively. Figure S2-B shows the average response modelled according to the experimental design from which a parabolic-like trend could be observed. According to the postulated model, the maximum IVPD value is reached between 5 % and 7.5 % of TMP addition whereas, with higher substitution level the value remains almost constant or starts decreasing.

Breads starch hydrolysis index was also quantified with a multi-step enzymatic treatment mimicking the in vivo digestion (De Angelis et al., 2009). The analysis estimates the amount of carbohydrates that raises glucose levels in the blood once the bread is ingested. As shown in Table S4, the predicted glycemic index was lower in fortified breads (from 83.8 to 91.1 and inversely proportional to the amount added), compared to CTR (94.6). This is most likely due to the higher content of fibers and protein, provided by the insect powder, which are known to influence macronutrient digestion and absorption during the gastrointestinal transit, and thus bread glycemic index (D'Amico et al., 2023). Nevertheless, a further decrease was observed in breads fortified with enzymatic treated insect powder. It is indeed possible that amino acids, known to increase insulin secretion in vivo (Borczak et al., 2018), released during the incubation and present in higher concentration in such breads (Fig. 3), affected starch hydrolysis index and consequentially, the predicted glycemic index. Indeed, a strong ($P < 0.05$) negative correlation was found between HI and almost all amino acids (Figure S3).

3.4.4. *Bread visual-textural properties*

Table 4 reports the color and textural characteristics of the products measured by instrumental analyses. By adding TMP, bread color tended towards brown notes, similar to that of wholemeal bread. In fact, TMP inclusion led to a progressive reduction in the brightness of the crust,

Table 4
Color and textural characteristics of the experimental breads containing 5 (−5) and 10 % (−10) of Tenebrio molitor powder before ($bTMP_0$) and after 3 ($bTMP_3$) and 8 h ($bTMP_8$) enzymatic treatment with Alcalase® and Flavourzyme®. CTR, control bread obtained with wheat flour.

	Sample	CTR	$bTMP_{0-5}$	$bTMP_{0-10}$	$bTMP_{3-5}$	$bTMP_{3-10}$	$bTMP_{8-5}$	$bTMP_{8-10}$
Crust	L*	53.53 ± 0.90^{a}	50.70 ± 0.28^{b}	48.69 ± 0.62^{c}	46.82 ± 0.64^{d}	45.23 ± 0.53^{d}	46.50 ± 0.57^{d}	38.35 ± 1.71^{e}
	a*	9.70 ± 0.76^{ab}	10.59 ± 0.94^{a}	10.01 ± 0.82^{ab}	8.81 ± 0.65^{b}	9.15 ± 0.67^{ab}	6.82 ± 0.60^{c}	10.12 ± 0.85^{ab}
	b*	29.51 ± 0.54^{a}	$30.89 \pm 1.81a$	25.41 ± 0.45^{b}	24.39 ± 1.42^{bc}	23.48 ± 1.57^{bc}	22.20 ± 1.21^{cd}	19.74 ± 1.58^{d}
Crumb	L*	68.28 ± 0.83^{a}	50.28 ± 0.58^{d}	50.77 ± 0.66^{cd}	50.55 ± 1.99^{cd}	54.61 ± 0.75^{b}	54.30 ± 0.47^{b}	52.33 ± 0.47^{c}
	a*	0.51 ± 0.04^{e}	9.38 ± 0.42^{a}	9.21 ± 0.33^{a}	9.18 ± 0.40^{a}	6.50 ± 0.38^{c}	7.20 ± 0.49^{b}	3.42 ± 0.29^{d}
	b*	19.52 ± 1.38^{d}	26.42 ± 0.49^{b}	28.61 ± 0.48^{a}	27.27 ± 1.53^{ab}	23.87 ± 1.67^{c}	21.51 ± 0.50^{d}	26.29 ± 0.70^{b}
	Hardness (N)	15.43 ± 0.79^{d}	11.78 ± 0.71^{e}	17.43 ± 0.81^{c}	21.03 ± 0.63^{b}	20.80 ± 0.64^{b}	22.05 ± 0.92^{b}	26.70 ± 1.08^{a}
	Springiness	0.86 ± 0.05^{c}	0.93 ± 0.04^{a}	0.91 ± 0.01^{abc}	0.92 ± 0.04^{ab}	0.86 ± 0.02^{c}	0.87 ± 0.02^{bc}	0.92 ± 0.02^{abc}

The data are the means of two independent experiments ± standard deviations ($n = 2$).

that could be linked to the naturally dark color of the ingredient as well as to a more intense Maillard reaction because of the higher protein content provided. Figure S2-C shows the average response modelled according to the experimental design, from which it could be easily caught how the L* value decreased proportionally to the inclusion of TMP. Moreover, the type of TMP had a significant effect, the reduction in luminosity being lower when TMP_0 was added compared to the other ingredients. Breads containing TMP_3 and TMP_8 at 5 % also presented significantly lower values of the red index while the yellow index was significantly lower in all the fortified products, except breads containing TMP_0 at 5 %. With respect to the crumb, it was confirmed that the CTR had the highest brightness and also the lowest value of a* and b*.

[a–e]Values in the same raw with different superscript letters differ significantly ($P < 0.05$As per the structure of the products, the inclusion of TMP, in general, significantly increased the hardness, in a more pronounced way as the enzymatic treatment length increased. This seems consistent with previous results and other research works (de Oliveira et al., 2017; Gaglio et al., 2021; Osimani et al., 2018). In fact, doughs containing TMP_0 showed higher viscosity (Fig. 2, Table 4), similar to CTR, and consequently it can be supposed that doughs prepared with the native *T. molitor* powder can trap more efficiently air and CO_2, undergoing a higher volume increase and, in the end, less compactness and hardness respect to breads fortified with TMP_3 and TMP_8. The springiness of fortified breads was in line with the CTR. Figure S2-D shows the overall trend of the hardness modelled according to the experimental design. The average response increased by increasing the inclusion of TMP. However, a distinct effect of the type of TMP was highlighted, being hardness only to a limited extent affected by TMP_0 respect to the other ingredients.

4. Conclusion

The introduction of *T. molitor* as a sustainable protein source represents a significant step forward in addressing the global protein challenge. Its environmental benefits, coupled with its high nutritional value, make it a promising alternative to traditional animal and plant proteins. However, exploiting TMP full potential as a food source requires overcoming several challenges, including consumer acceptance, regulatory hurdles, and food innovation processes.

In this study an enzymatic treatment with proteases was set up to improve TMP protein bio-accessibility and digestibility. Besides releasing peptides and amino acids, the treatment also affected its emulsifying capacity, foaming activity, as well as other techno-functional properties. Notwithstanding the necessity for adequate technological transfer, the proposed treatment is both cost-effective and readily implementable on an industrial scale. Enzymes are extensively utilized in industrial settings due to their ability to *i*) generate high yields, *ii*) demonstrate consistent activity and reproducibility, and *iii*) be produced in a cost-effective manner. Since these changes led to hypothesizing a positive influence on leavened products volume and nutritional quality, breads fortified with TMP were produced using mixture-process experimental design. Cleary as TMP amount increased so did protein content, nevertheless, the use of enzymatic treated TMP provided a higher amount of FAA and a different VOC profile, richer in FAA catabolism products (2-methylbutanal, 3-methylbutanal, and 2-pentylfuran). As expected, the impact of TMP on bread visual-textural features (hardness, color) was more pronounced as the fortification rate increased, nevertheless, mathematical modeling can help selecting the conditions representing the compromise between nutritional quality and organoleptic acceptance. The wide range between 2.5 % and 7.5 % of TMP addition ensures, in our experimental conditions, the fulfilment of the claim “source of protein”. Considering the evolution of IVPD, hardness and crust luminosity, chosen as key parameters, a 2.5 % substitution seems to represent a satisfactory compromise, which allows to keep color and structural characteristics closer to the control bread and significantly improve bread nutritional properties. Moreover, by extending the length of the biotechnological treatment, hence increasing the concentration of aroma precursors, a positive impact on the final bread aroma might be foreseen, even at such low percentages of substitution.

Previous works tested different levels of insect-derived ingredient addition in bread formulations (Amoah et al., 2023) but without generalizing the effect of substitution (i.e., without modelling any responses). Hence, suggestions were provided but limited to the experiments performed. On the other hand, the main advantage of following a design of experiment approach lies on the possibility of expanding the results to other cases not tested directly (Leardi, 2013; Squeo et al., 2021). Consequently, it has been possible to identify 2.5 % as a promising level of substitution that might minimize the cost/benefit ratio of TMP usage. Such a percentage, in fact, guarantees the fulfilment of the nutritional claim, but is still quite low to suppose a limited effect on the aroma, the acceptability by consumers, and to the other critical quality parameters. Nonetheless, if one aims to reach the “high protein” claim, with all the benefits entailed, it appears clear that some other features should be sacrificed or as often happens in large-scale productions, the use of processing aids must be considered. Overall, it becomes a matter of strategic choices dictated by the aims pursued by each food operator. This study represents a first comprehensive report on the effect of enzymatically treated TMP, on bread nutritional and technological properties, nevertheless, ongoing research into the processing and enhancement of *T. molitor* products will be crucial for addressing these challenges and optimizing the use of mealworms in the food industry.

Ethical Statement - Studies in humans and animals

The authors declare that Ethical Statement is not required since the study does not involve humans, animals nor sensory analysis.

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CRediT authorship contribution statement

Michela Verni: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Giacomo Squeo:** Writing – original draft, Investigation, Formal analysis, Data curation. **Giuseppe Perri:** Formal analysis. **Chiara Demarinis:** Data curation. **Carlo Giuseppe Rizzello:** Validation, Resources. **Francesco Caponio:** Validation, Resources, Conceptualization. **Luigi Faino:** Formal analysis. **Erica Pontonio:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Erica Pontonio reports financial support was provided by European Union. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100665.

Data availability

Data will be made available on request.

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