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Simulated gastrointestinal digestion of two convenience meals using fungal enzyme formulations

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ABSTRACT

Dietary supplements containing microbial enzymes are widely used to support digestion and optimize nutrient absorption, particularly in individuals with functional dyspepsia characterized by impaired gastric emptying and endogenous enzyme secretion. This study aimed to evaluate the *in vitro* digestive performance of two fungal-derived enzyme blends, Poolzyme® MULTI and Poolzyme® DAIRY, on representative convenience meals using the standardized INFOGEST static digestion protocol. Simulated oral, gastric, and intestinal phases of digestion were conducted according to the INFOGEST model. Two food matrices, a fast-food hamburger with fries and a multi-cheese frozen pizza, were incubated under enzyme-free control conditions or with Poolzyme® MULTI or Poolzyme® DAIRY at manufacturer-recommended doses. At the end of the intestinal phase, digesta were collected and analyzed for total free amino acids, branched-chain amino acids (BCAAs), residual lactose, glucose release, and free fatty acids (FFAs) using validated analytical assays. Both enzyme blends significantly enhanced proteolysis compared to controls, as evidenced by increased total free amino acids and BCAA liberation. Poolzyme® DAIRY yielded a dose-dependent reduction in residual lactose in the cheese-based matrix. Poolzyme® MULTI's amylolytic and cellulolytic activities augmented glucose release from carbohydrate-rich foods, while lipolytic activity in both formulations markedly increased FFA liberation. Kinetic profiles indicated accelerated substrate hydrolysis and elevated nutrient bioaccessibility across both meal types. Fungal-derived enzyme blends effectively complement endogenous digestive enzymes, improving macronutrient hydrolysis and bioaccessibility in diverse dietary contexts. These mechanistic insights support clinical observations of symptom alleviation in functional dyspepsia and underscore the potential of targeted enzyme supplementation to optimize digestion of processed convenience foods.

1. Introduction

The use of dietary supplements containing exogenous enzymes to complement the body's digestive enzyme activities has gained increasing interest, particularly for addressing digestive inefficiencies and optimizing nutrient absorption (Ianiro et al., 2016). These dietary supplements utilize enzymes sourced from diverse origins, including: (i) animals (where permitted by local regulations (Precup et al., 2024); e.g., pancreatin from porcine pancreas), (ii) plants (e.g., bromelain from pineapple stem) (iii) and microorganisms (e.g., neutral proteases from *Bacillus* spp. and acid proteases from *Aspergillus* spp.) (Singh et al., 2018)., Among these sources, microbial-derived enzymes have emerged as a preferred choice due to their broad pH activity range, superior stability, and enhanced effectiveness (Roxas, 2008). Additionally, most microbial enzymes can be efficiently produced through cost-effective fermentation and downstream processing from the biomass of wild-type microorganisms (Garvey et al., 2022; Nakamura et al., 1998; Tran Do et al., 2016). For instance, neutral proteases from bacterial genera such as *Bacillus* have been shown to facilitate protein hydrolysis, thereby improving the bioavailability of essential amino acids (Song et al., 2023). Similarly, yeasts, including *Candida* and *Kluyveromyces* spp., are known for their ability to produce lipases, lactase and other enzymes that assist in lipid and carbohydrate metabolism (Chandra et al., 2020; Roxas, 2008). Nevertheless, filamentous fungi represent the

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primary microbial source of enzymes with nutritional applications. These fungal enzymes have been successfully employed to enhance food processing, improve nutrient bioavailability, and support digestive efficiency. For example, enzymes from Aspergillus spp. contribute to protein digestion, making plant-based proteins more bioavailable (Kumitch et al., 2020; Rathi et al., 2024). Additionally, fungal enzymes are widely used in food production to improve flavor, texture, and nutritional quality (Pouris et al., 2024). Another example is fungal phytases, which release bound minerals from plant-based foods, increasing their bioavailability (Singh & Kumar, 2019). Notably, filamentous fungi such as *Aspergillus* spp. and *Trichoderma* spp. are extensively employed as sources of proteases, lipases, cellulases, and lactases, which exhibit optimal activity under physiological conditions in the gastrointestinal tract (Singh & Kumar, 2019; Singh et al., 2019; Gautam & Naraian, 2020).

Functional dyspepsia, characterized by postprandial bloating, early satiety, and upper abdominal discomfort without identifiable causes on endoscopy, is common and affects up to 30 % of the global population (Singh & Kumar, 2019). Given the absence of effective pharmacological treatments for many cases, microbial enzymes have been investigated as potential therapeutic adjuncts for functional dyspepsia. For example, a study demonstrated that supplementation with an acid-resistant microbial lipase significantly reduced postprandial fullness in healthy individuals consuming a high-fat meal, with no significant effects on gastric myoelectrical activity or other upper gastrointestinal symptoms (Levine et al., 2015). Furthermore, a randomized, double-blind, placebo-controlled trial found that a multienzyme complex containing bacterial protease and lipase, as well as fungal amylase, cellulase, lactase, and lipase, significantly improved functional dyspepsia symptoms, including bloating and postprandial distress, compared to placebo (Majeed et al., 2018). Similarly, Ullah et al. (2023) published the results of a recent randomized, double-blind, placebo-controlled clinical trial that assessed the efficacy of a commercially available multi-enzyme blend derived from filamentous fungi. This formulation, containing protease, amylase, lipase, cellulase, and lactase, was tested in patients with functional dyspepsia. Results showed that two months of supplementation significantly improved quality of life, reduced pain severity, and enhanced sleep quality, with no reported side effects (Ullah et al., 2023).

The study by Ullah et al. served as the starting point for the present investigation, which aimed to evaluate the same enzyme blend alongside an alternative formulation containing the same enzymatic components but optimized for dairy product digestion. Specifically, we sought to gain a mechanistic understanding of the efficacy of these enzymes by assessing whether the fungal-derived enzymes collectively enhance the hydrolysis of their respective substrates under physiologically relevant conditions, without specifically testing the activity of each enzyme in isolation. Additionally, we aimed to assess whether these microbial enzymes act additively in combination with endogenous human digestive enzymes.

To achieve these objectives and simulate human digestion under standardized and physiologically relevant conditions, we employed the INFOGEST static *in vitro* digestion model (Brodkorb et al., 2019), which simulates the sequential oral, gastric, and intestinal phases of human digestion under physiologically relevant conditions. Its reproducibility and adaptability to diverse food matrices make it a valuable tool for evaluating nutrient bioaccessibility and the efficacy of functional ingredients.

The enzyme blends were tested using two realistic, multi-nutrient meals (a fast-food hamburger with fries and a multi-cheese frozen pizza), selected based on macronutrient composition and relevance to real-world dietary habits. These matrices, rich in proteins, fats, complex carbohydrates, and lactose, allowed us to investigate enzymatic activity across all major macronutrient classes. Quantitative analyses focused on protein hydrolysis, lactose breakdown, and the release of free fatty acids and glucose, assessing the ability of fungal enzymes to function throughout digestion and complement endogenous enzymatic activity.

2. Material and methods

2.1. Composition of food supplements based on fungal enzymes

The products employed in this investigation are from Poolzyme®, a series of fungal origin enzyme blends by Giellepi S.p.A. (Milan, Italy). Specifically, Poolzyme® MULTI contains five enzymes to facilitate the digestion of proteins, carbohydrates, fats, lactose, and fibers. In contrast, Poolzyme® DAIRY is a 3-enzyme blend (lactase, protease, lipase) designed to aid those experiencing minor discomfort after consuming dairy (Table 1).

2.2. Test meals

We tested two food items: first, a frozen “white” cheese pizza made with multiple cheeses, including Provolone Valpadana D.O.P. (9.2 %), Asiago D.O.P. (6.6 %), Gorgonzola D.O.P. (6.6 %), Stracchino (3.5 %), Mascarpone (3.5 %), and Parmigiano Reggiano D.O.P. (2.9 %), without tomato. D.O.P. stands for “*Denominazione di Origine Protetta*”, an Italian quality label that guarantees a food product is entirely produced, processed, and prepared in a specific region using traditional methods. The pizza was purchased from a local supermarket and cooked in an electric oven set to 200 °C for 12 min. The second item was a fast-food meal consisting of a hamburger and fries, obtained from a local fast-food chain restaurant.

2.3. In vitro digestion protocol

Both meals described above underwent simulated digestion following the INFOGEST protocol as described by Brodkorb et al. (2019), consisting of an oral, gastric and intestinal phase.

Oral phase. One hundred grams of each meal, sampled to maintain the proportion of ingredients, were homogenized by adding simulated salivary fluid with the following composition: 15.1 mM KCl, 3.7 mM KH2PO4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 1.1 mM HCl, and 1.5 mM $CaCl_2(H_2O)_2$. The mixture was blended for 30 s at medium speed using a Bimby blender (Vorwerk,

Table 1
Enzymatic composition of the two Poolzyme® formulations used in the study and relative activity (expressed per g of lyophilized ingredient with an uncertainty of ±5 %). CU, cellulase unit defined as the amount of activity that produces a relative fluidity change of 1 in 5 min in a defined carboxymethylcellulose substrate under the conditions of the assay (pH 4.5 and 40 °C). SKB, α-amylase dextrinizing unit, defined as the quantity of α-amylase that dextrinizes soluble starch in the presence of an excess of β-amylase at the rate of 1 g per h at 30 °C. HUT, unit of proteolytic (protease) activity, defined as that amount of enzyme that produces in 1 min a hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.10 g per l of tyrosine in 0.006 N hydrochloric acid. ALU, acid lactase unit, defined as the quantity of enzyme that liberates 1 μmol of o-nitrophenol per minute at 37 °C and a pH of 4.5 (based on a 15-min hydrolysis of an O-nitrophenyl-β-D-galactopyranoside substrate). FIP, standardized unit of measurement for lipase enzyme activity as defined by the Fédération Internationale Pharmaceutique; one FIP unit is defined as that quantity of a standard lipase preparation (Fungi Lipase-International FIP Standard) that liberates the equivalent of 1 μmol of fatty acid per minute from the substrate emulsion under the described assay conditions.

Enzyme	Source	Activity		Unit of activity
		DAIRY	MULTI	
Amylase	*Aspergillus oryzae*	–	13000	SKB
Cellulase	*Trichoderma reesei*	–	500	CU
Protease	*Aspergillus oryzae*	83000	67000	HUT
Lactase	*Aspergillus oryzae*	17000	13000	ALU
Lipase	*Rhizopus oryzae*	1000	1000	FIP

Wuppertal, Germany). The suspension was diluted to 200 ml minus the volume needed to add α-amylase from *Aspergillus oryzae* (Merck, Milan, Italy) to achieve a final concentration of 75 U/ml. The pH was adjusted to 7 using 5 M NaOH or HCl. Before initiating the gastric phase, the corresponding capsule containing the specific enzyme blend was added to an aliquot of 5 ml of oral suspension. For samples not treated with enzyme blends, an empty capsule was added.

Enzyme blend addition. Before initiating the gastric phase, the enzyme blends were added to aliquots of the food homogenized in salivary fluid in the form of powder encapsulated in hydroxypropyl methylcellulose (HPMC) capsules, without any addition of excipients. The weights of the enzyme blends in the capsules were adjusted to achieve doses per meal. Specifically, the fast-food meal (hamburger and fries) weighed 410 g, and the enzyme doses were 200 and 400 mg of Poolzyme® MULTI for the whole meal. The frozen cheese pizza meal weighed 380 g, and the enzyme doses were 300 and 600 mg of Poolzyme® DAIRY for the entire portion. Digested samples were also prepared without enzyme blends, along with non-digested samples that were simply diluted 1:8 with deionized water, matching the dilution occurring in the INFOGEST protocol. Empty capsules were added to samples not treated with the enzyme blends. The samples were then incubated at 37 °C for 2 min with continuous shaking.

Gastric phase. To simulate gastric conditions, 4 ml of simulated gastric fluid with 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 15.6 mM HCl, and 0.15 mM $CaCl_2(H_2O)_2$ was added. The pH of the mixture was adjusted to 3.0 using 6 M HCl. Rabbit gastric extract (RGE15; Lipolytech, Marseille, France) was added to reach a final lipase concentration of 60 U/ml. RGE15 also contains pepsin and with porcine pepsin (Merck, Milan, Italy) was used to obtain a final concentration of 2000 U/ml of pepsin units in the gastric phase. The samples were then diluted to 10 ml with deionized water and incubated at 37 °C for 2 h under continuous shaking.

Intestinal phase. Then, 4.25 ml of simulated intestinal fluid (6.8 mM KCl, 0.8 mM KH_2PO4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)6$, 8.4 mM HCl, and 0.6 mM $CaCl_2(H_2O)_2$) were added to the 10 ml of gastric suspension. The pH was adjusted to 7.0 using 6 M NaOH. Porcine pancreatin and bovine bile salts (Merck, Milan, Italy) were incorporated at final concentrations of 100 U/ml of trypsin and 10 mM bile salts, respectively. The total volume was adjusted to 20 ml, and the samples were incubated at 37 °C with continuous shaking for 2 h.

After *in vitro* digestion, the enzymes were inactivated by heating the samples to 90 °C for 10 min.

During a mock INFOGEST assay, under agitation, the capsules were found to open in less than 2 min and were fully dissolved within 5 min.

The INFOGEST experiments were conducted on three different aliquots of the same meal for each condition.

2.4. Protein hydrolysis measurement

The degree of protein hydrolysis in the samples was assessed through the quantification of the free terminal nitrogen (FTN) groups using the Primary Amino Nitrogen (PANOPA) Assay kit from Megazyme (Bray, Ireland), following the manufacturer's instructions for microplate analysis. Briefly, the samples were serially diluted 1:2 with deionized water to obtain absorbance readings within the kit's linear range. A calibration curve was generated using the standard solution provided with the kit. All measurements were performed in triplicate. The FTN groups in the samples reacted with N-acetyl-L-cysteine and o-phthaldialdehyde, resulting in the formation of isoindole derivatives (amino nitrogen + N-acetyl-L-cysteine + o-phthaldialdehyde → isoindole derivative). The amount of isoindole derivative produced in this reaction is stoichiometrically proportional to the quantity of FTN present. The isoindole derivatives were quantified by monitoring the absorbance increase at 340 nm using a Powerwave XS2 automated spectrophotometer (Biotek, Milan, Italy). Data were expressed as mg of reactive nitrogen per g of food sample.

2.5. Lactose quantification

Following the centrifugation of samples at 13000 g for 10 min at room temperature, the analysis of lactose degradation was conducted using the Lactose & D-Galactose (Rapid) Assay kit by Megazyme (Bray, Ireland). This protocol employs spectrophotometry to quantify lactose and galactose in the samples. The underlying principle of this method involves the hydrolysis of lactose to D-galactose and D-glucose by Aspergillus niger β-galactosidase at pH 5.0, as outlined in a modified version of Association of Official Agricultural Chemists (AOAC) Official Method 984.15 for lactose in milk. The resulting D-galactose undergoes interconversion of α- and β-anomeric forms catalyzed by galactose mutarotase (GalM). Subsequently, β-D-galactose is oxidized to D-galactonic acid in the presence of β-galactose dehydrogenase (β-GalDH) at pH 8.6. The stoichiometric amount of NADH formed in this reaction correlates with the quantity of lactose; finally, the produced NADH is measured through the increase in absorbance at 340 nm. All lactose and galactose concentrations are expressed on a wet-weight (as-is) basis, *i.e.*, mg per 100 g of the initial food prior to digestion and without moisture correction.

2.6. Amino-acid profile determination

One g of sample was vortexed in 20 ml of water, centrifuged at 6000×*g* for 10 min at 4 °C, and the supernatant was transferred into a 50 ml volumetric flask. The residue was extracted with 20 ml of water, treated as described above, and the final volume was adjusted to 50 ml with water. Amino acid derivatization using AccQ•Tag reagents (Waters S.p.A., Sesto San Giovanni, Italy) was conducted according to the manufacturer's protocol and as previously reported (Gardana et al., 2018). Liquid chromatographic separation was performed on an Acquity UPLC system (Waters) coupled with an eLambda DAD (Waters). The injection volume was 2 μl. The derivatives were separated on an AccQ•Tag Ultra column (1.7 μm BEH, 100 mm × 2.1 mm) maintained at 50 °C. The flow rate was 0.7 ml/min. Data were acquired in the range of 200–450 nm, and the chromatogram was integrated at 260 nm. The stock solution contained a 2.5 mM concentration of each amino acid, except for cysteine at 1.25 mM. Calibration curves were prepared in the range of 2.2–51 μg/ml.

2.7. Quantification of sugars

Glucose, fructose, and sucrose standards were from Sigma-Aldrich (St. Louis, MO, USA). Two hundred mg of the sample were dispersed in 100 ml of sterile deionized water, and the suspension was sonicated for 10 min, centrifuged at 1000×*g* for 5 min at room temperature, and the supernatant was recovered. The residue was extracted with 50 ml of water and treated as described above. The supernatants were combined, and the final volume was adjusted to 200 ml with acetonitrile (Merck, Darmstadt, Germany). The sugar content was assessed using a UHPLC Vanquish model Flex (Thermo Fisher Scientific S.p.A., Rodano, Italy) coupled to a Q-Exactive Focus Orbitrap (Thermo Fisher Scientific) equipped with a HESI-II probe for ESI. The column used was a BEH amide C18 (150 mm × 2.1 mm i.d., 1.7 μm; Waters), and the flow rate was 0.2 ml/min. One μl was injected. The column and sample were maintained at 25 °C and 20 °C, respectively. The eluents were 0.02 % NH4OH in water and 0.02 % NH4OH in acetonitrile (75:25, v/v). The operative conditions were as follows: spray voltage 3.0 kV, sheath gas flow rate 40 (arbitrary units), auxiliary gas flow rate 10 (arbitrary units), capillary temperature 275 °C, capillary voltage −95 V, S lens −50 V, and heater temperature 275 °C. The analytes were identified in negative ESI mode by fullscan acquisition (m/z 100–1500 u), using an isolation window of ±2 ppm. The AGC target, injection time, and mass resolution were 1 × 106, 100 ms, and 70 K, respectively. The MS data were

processed using Xcalibur software (Thermo Fisher Scientific). Peak identity was ascertained by evaluating both the accurate mass and retention time. Calibration curves were prepared in the range of 2–50 μg/ml.

2.8. Free fatty acids quantification

Acetonitrile, ammonium formate, tertbutyl methyl ether (TBME), formic acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, 1,2-dioleoyl-glycerol, and 1,2-dipalmitoyl-glycerol were obtained from Sigma-Aldrich. 2-Propanol and ethanol were purchased from VWR International (Bruchsal, Germany). The lipid extraction was performed by adding 10 ml of a chloroform:methanol solution (3:1, v/v) to 2 g of the sample. The mixture was vortexed for 1 min and centrifuged at 1650×*g* for 5 min at room temperature. The chloroform phase was recovered, and the residue was reextracted twice using the same chloroform:methanol solution. The combined chloroform phases were evaporated to dryness under a nitrogen stream. The dried extract was then resuspended in 1 ml of an ethanol:tetrahydrofuran solution (1:1, v/v), followed by centrifugation at 6000×*g* for 2 min at room temperature. The resulting supernatant was transferred to a vial for analysis by UHPLC-HR-MS. The UHPLC-MS system consisted of a Vanquish Flex (Thermo Fisher Scientific) connected to an HR-MS Orbitrap model Q-Exactive Focus (Thermo Fisher Scientific), equipped with a HESI probe. Chromatography was performed on a 1.8 μm HSS T3 C18 column (150 mm × 2.1 mm) with ACN containing 0.1 % HCOOH (A) and 1 mM NH4HCOO in 2-propanol (B). The gradient was as follows: 0 % B (0–5 min), 5–20 min at 20 % B, 20–42 min to 75 % B, 42–55 min at 75 % B, increased to 100 % A at 55–60 min, and kept constant at 100 % A for 5 min. The flow rate was 0.4 ml/min, and the column and sample temperatures were 45 °C and 20 °C, respectively. The injection volume was 5 μl.

Detection was performed in the ESI− mode from 0 to 9.1 min for the analysis of FA and MG, and in the ESI + mode from 9.1 to 55 min for the analysis of DG and TG. The total ion current chromatogram was recorded in scan mode in the range of m/z 100–1500 u. The operative conditions were as follows: spray voltage 3.5 kV, sheath gas flow rate 50 (arbitrary units), auxiliary gas flow rate 20 (arbitrary units), capillary temperature 350 °C, capillary voltage −95 V, S lens −100 V, and heater temperature 320 °C. The analytes were identified in negative ESI mode by fullscan acquisition (m/z 100–1500 u), using an isolation window of ±2 ppm. The AGC target, injection time, and mass resolution were 1 × 106, 100 ms, and 70 K, respectively. The MS data were processed using Xcalibur software (Thermo Fisher Scientific). Peak identity was ascertained by evaluating the accurate mass and retention time. Calibration curves were prepared in the range of 0.5–20 μg/ml.

2.9. Statistical analysis

All experiments were conducted in triplicate. Data were analyzed using Prism 10 (GraphPad Software, CA, USA). Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$.

2.10. Declaration of generative AI in the writing process

During the preparation of this work the authors used the AI-powered language model ChatGPT-4 (https://chat.openai.com/) in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

3. Results

3.1. Protein hydrolysis in tested meals after simulated digestion

Protein hydrolysis was assessed by quantifying free terminal nitrogen (FTN) groups in the food matrices, serving as an indicator of proteolytic activity from the fungal enzymes contained in the Poolzyme® blends. In undigested samples, FTN levels were 0.19 mg/g in the multi-cheese pizza and 0.068 mg/g for the fast-food meal. After *in vitro* digestion using the standardized INFOGEST protocol (control condition: pepsin, gastric lipase, and pancreatin), FTN values increased significantly, reaching 2.43 mg/g for the pizza and 1.60 mg/g for the fast-food meal. The addition of Poolzyme® enzyme blends to the control digestion system resulted in a further significant increase in FTN levels, supporting the hypothesis that these formulations enhance proteolytic activity beyond endogenous enzymes alone. For Poolzyme® DAIRY, FTN concentrations increased to 3.37 mg/g at the lower dose and to 3.49 mg/g at the higher dose. However, the difference between the two concentrations was not statistically significant, although both values were significantly higher than the control. In contrast, Poolzyme® MULTI showed a dose-dependent effect, with FTN values rising from 1.94 mg/g at the lower concentration to 2.21 mg/g at the higher concentration, with statistically significant differences observed between all tested conditions (Fig. 1).

These results demonstrate that both fungal-derived enzyme blends improve protein digestion in realistic multi-nutrient meals, with Poolzyme® MULTI exhibiting a more evident dose-dependent effect.

3.2. Lactose reduction

The activity of the β-galactosidase enzyme in the Poolzyme® enzymatic blends was tested by measuring residual lactose in food after simulated digestion. Lactose was below the detection limit in all fast-food meal samples (data not shown). In contrast, multi-cheese pizza samples contained measurable amounts of lactose. In the undigested pizza sample, lactose content was 458 mg per 100 g of food, which unexpectedly decreased to 360 mg/100 g following digestion with the standard INFOGEST protocol (21 % reduction; Fig. 2(a)). Since lactose quantification with this method relies on measuring free galactose before and after hydrolysis by Aspergillus niger β-galactosidase, galactose quantification was analyzed to clarify the observed lactose reduction. Galactose content (Fig. 2(b)) did not decrease post-INFOGEST digestion, indicating that lactose reduction was not attributable to lactase/β-galactosidase activity within the INFOGEST reagents. Instead, we hypothesize that interference by the INFOGEST digestion matrix, possibly due to bile salts or other components, might have occurred. Supporting this hypothesis, a similar lactose reduction was observed when pure lactose was incubated solely in the intestinal phase of INFOGEST digestion, again showing an apparent reduction in quantifiable lactose while free galactose was not detected (data not shown).

Supplementation with Poolzyme® DAIRY significantly reduced lactose concentration in a dose-dependent manner, decreasing to 242 mg/100 g and 177 mg/100 g at lower and higher enzyme concentrations, respectively (Fig. 2(a)). This was accompanied by a corresponding dose-dependent increase in galactose concentration (from 38 mg/100 g to 161 mg/100 g and 204 mg/100 g at lower and higher enzyme concentrations, respectively; Fig. 2(b)).

These findings confirm the effective dose-dependent enhancement of lactose hydrolysis by the fungal-derived enzyme blend, suggesting its potential utility in complementing endogenous digestive activity and improving carbohydrate digestion in dairy-rich foods.

3.3. Modification of branched-chain amino acid (BCAA) concentration

The proteolytic activity of the Poolzyme® enzymatic blends was further demonstrated by an increase in free branched-chain amino acids

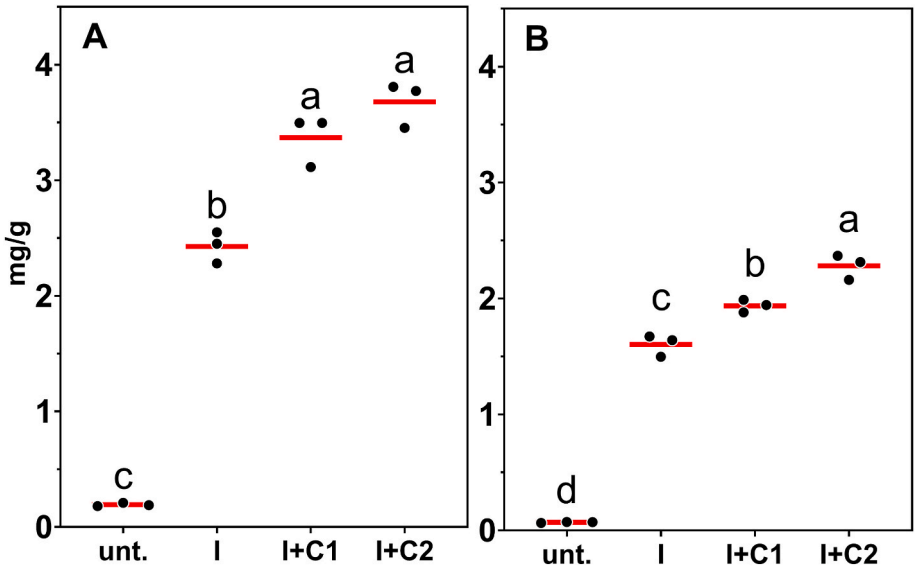

Fig. 1. Overall protein hydrolysis assessed using Primary Amino Nitrogen (PANOPA) Assay, expressed as mg of free terminal nitrogen groups per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

(BCAAs) after simulated digestion, compared to the control condition. The BCAA data presented here are based on the sum of the amino acids leucine, isoleucine, and valine, as determined by UHPLC amino acid profile analysis. The baseline concentration of free BCAAs was 1.5 mg/g in the fast-food meal and 1.2 mg/g in the multi-cheese pizza, respectively (Fig. 3). *In vitro* digestion using the INFOGEST protocol induced a substantial increase in free BCAAs, particularly in the fast-food meal, where levels rose up to 9.4 mg/g while they did not increase significantly for the pizza meal. Supplementation with the enzyme blends further elevated BCAA concentrations in both meals. For Poolzyme® DAIRY, levels increased significantly to 5.2 mg/g and 5.3 mg/g at the lower and higher doses, respectively. In the case of Poolzyme® MULTI, BCAA levels were significantly increased over the control only at the highest tested dose, reaching 11.5 mg/g (Fig. 3).

These findings confirm that both fungal-derived enzyme blends enhance the release of BCAAs from the tested food matrices.

3.4. Changes in free essential amino acid levels

The proteolytic activity of the Poolzyme® enzymatic blends was further confirmed by quantifying the release of free essential amino acids (EAAs). These were calculated as the sum of the following amino acids (phenylalanine, valine, tryptophan, threonine, isoleucine, methionine, histidine, leucine, and lysine) identified and quantified by UHPLC after simulated digestion. Baseline concentrations of free EAAs were 1.3 mg/g in the multi-cheese pizza and 1.6 mg/g in the fast-food meal. In the INFOGEST control condition, a modest increase was observed for the pizza, reaching 3.3 mg/g, while a substantial increase to 17.7 mg/g was detected in the fast-food meal. Supplementation with the enzyme blends further elevated free EAA concentrations. Specifically, Poolzyme® DAIRY increased EAA levels to 9.2 mg/g, while Poolzyme® MULTI reached 18.3 mg/g. At the higher concentration of Poolzyme® MULTI, a slight but statistically significant increase to 21.3 mg/g was observed, whereas Poolzyme® DAIRY reached 9.6 mg/g (Fig. 4). Notably, several individual EAAs were significantly increased following digestion with Poolzyme® DAIRY (Supplementary Fig. S1).

These results confirm that the fungal-derived enzyme blends enhanced protein hydrolysis and promoted the release of EAAs during simulated digestion of the model meals.

3.5. Release of sugars from carbohydrate hydrolysis

To assess the amylase and cellulase activity of Poolzyme® MULTI, mono-, di-, tri-, and tetrasaccharides were quantified in the fast-food meal before and after simulated digestion. The initial glucose concentration was 10.4 mg/g, which increased to 24.7 mg/g following standard INFOGEST digestion. Supplementation with Poolzyme® MULTI led to a further increase in glucose levels: 27.6 mg/g at the lower enzyme concentration and 28.7 mg/g at the higher concentration. All changes were statistically significant compared to the control, except for the difference between the two enzyme doses. Fructose levels remained unchanged across all digestion conditions. In contrast, significant increases in di-, tri-, and tetrasaccharide concentrations were observed after digestion compared to the undigested sample, whereas no significant differences emerged among the enzyme-treated conditions. With the exception of fructose, all sugars showed statistically significant differences between undigested and digested samples (Fig. 5).

These findings indicate that Poolzyme® MULTI effectively enhanced carbohydrate hydrolysis, as evidenced by the increased release of free glucose from the food matrix.

3.6. Increase in free fatty acids

Free fatty acid (FFA) levels were measured before and after simulated digestion to assess lipase activity. In undigested samples, baseline FFA concentrations were low, with 1.6 mg/g in the multi-cheese pizza and 2.10 mg/g in the fast-food meal. Following digestion with the standard INFOGEST protocol, FFA levels increased significantly, reaching 5.8 mg/g and 8.2 mg/g in the pizza and fast-food meal, respectively. The addition of enzyme blends resulted in a substantial further increase in FFAs. For Poolzyme® DAIRY, FFA concentrations rose to 21.4 mg/g at the lower dose and to 22.5 mg/g at the higher dose, although the difference between these two values was not statistically significant. In contrast, Poolzyme® MULTI induced a more pronounced, dose-dependent response, with FFA levels increasing to 24.1 mg/g at the lower concentration and to 26.1 mg/g at the higher dose. This difference

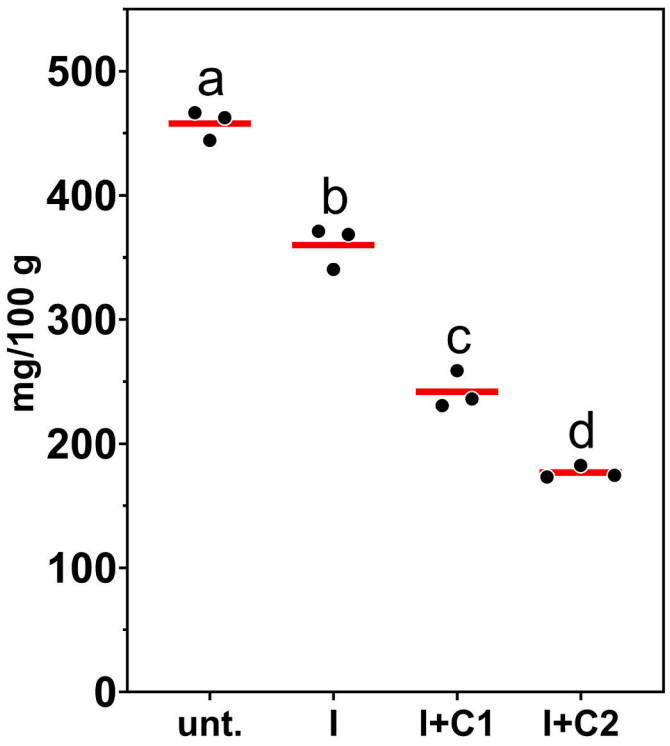

Fig. 2. Lactose (panel a) and galactose (panel b) quantification in "white" pizza samples. All concentrations are on a wet-weight basis (mg per 100 g of initial food). Un, undigested food (no INFOGEST); I, food after *in vitro* digestion without Poolzyme® DAIRY; I + C1, food after *in vitro* digestion in the presence of the lowest amount of Poolzyme® DAIRY (one capsule corresponding to 300 mg of Poolzyme® DAIRY); I + C2, food after *in vitro* digestion in the presence of the highest amount of Poolzyme® (two capsules corresponding to 600 mg of Poolzyme® DAIRY). Each condition has been tested in three independent experiments using aliquots of the same meal. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

was statistically significant (Fig. 6).

Overall, both enzyme blends significantly enhanced lipolysis, as evidenced by the increased release of free fatty acids, confirming their efficacy in promoting triglyceride digestion during simulated gastrointestinal conditions.

4. Discussion

This study evaluated the *in vitro* digestive activity of two fungal-derived enzyme blends, Poolzyme® MULTI and Poolzyme® DAIRY, using the INFOGEST protocol to simulate the human digestive process. This approach provides a mechanistic basis for interpreting the results of a previous clinical study showing improvements in quality of life and a reduction in digestive symptoms in individuals with functional dyspepsia consuming Poolzyme® MULTI (Ullah et al., 2023). Our study aimed to support these findings by providing a detailed analysis of the enzymatic properties of the formulations and their impact on macronutrient digestion.

The selection of the two test meals (hamburger with fries and frozen white pizza) was driven by the need to evaluate enzymatic activity on a food matrix containing a diverse combination of macronutrients. The hamburger with fries represents a meal rich in proteins, fats, and complex carbohydrates, whereas the frozen white pizza was chosen for its lactose and casein protein content, making it ideal for testing the specificity of the Poolzyme® DAIRY formulation. These convenience meals are commonly consumed in Western countries, including Italy (Scarsi et al., 2024), and their nutritional profile may influence digestion, particularly in individuals with functional dyspepsia (Amerikanou et al., 2023).

The INFOGEST static *in vitro* digestion model was chosen for its reproducibility and physiological relevance. It has been validated through inter-laboratory studies demonstrating uniform digestion profiles, particularly for proteins (Egger et al., 2019). Despite its advantages, INFOGEST has limitations. Being a static system, it does not simulate gradual enzyme secretion, gastric emptying, or peristalsis, factors that influence digestion kinetics. Unlike *in vivo* digestion, where enzymes and bile salts act dynamically, static models may underestimate nutrient release rates (Rathi et al., 2024). Studies comparing INFOGEST with dynamic models, such as DIDGI®, indicate similar endpoint digestion but distinct kinetic profiles, with dynamic systems more accurately replicating amino acid release patterns (Egger et al., 2019). In this study, we focused on endpoint digestion while acknowledging the limitation of not capturing intermediate kinetics. Another drawback is the lack of absorption and post-absorptive metabolism, meaning the model assesses bioaccessibility rather than bioavailability. Another potential limitation is that, although pancreatin supplies essential digestive enzymes, it lacks certain human-specific cofactors (such as colipase) that are required for optimal lipase activity and efficient lipid digestion (Lowe, 2002). However, the presence of fungal lipases in Poolzyme® likely compensated for this limitation. The incorporation of brush-border enzymes in recent INFOGEST adaptations has improved physiological relevance (Egger et al., 2017), but the model remains a simplification that does not account for hormonal regulation or microbiome interactions. Nonetheless, INFOGEST has demonstrated strong qualitative agreement with *in vivo* findings, making it a valuable screening tool before committing to costly *in vivo* studies (Egger et al., 2019). While its static nature imposes constraints, it remains a reliable model for controlled digestive studies.

The beneficial effects of Poolzyme® MULTI and Poolzyme® DAIRY in breaking down complex meals align with a growing body of evidence supporting the efficacy of exogenous digestive enzymes in enhancing nutrient digestion. Our findings indicate that supplementing the standard digestive mixture with fungal-derived enzymes significantly improved protein proteolysis, starch hydrolysis, and triglyceride lipolysis compared to endogenous enzymes alone. These enhancements are consistent with the results of Rathi et al. (2024), who assessed a multi-component enzyme blend (DigeSEB Super, containing amylase, protease, lipase, cellulase, lactase, and hemicellulase) on a diskette-shaped complex food model that contains proteins, carbohydrates, fats, and other nutrients in a proportion that nearly matches the specifications of nutritional requirements suggested by the US FDA. Their *in vitro* digestion study demonstrated that enzyme supplementation accelerated food disintegration and increased nutrient release under INFOGEST conditions. Notably, they reported a 2.75-fold faster reduction in gastric digesta viscosity, indicating a more rapid breakdown of the food matrix structure (Rathi et al., 2024). In addition, Rathi et al. observed that enzymatic supplementation led to a substantial increase in reducing sugars (indicative of enhanced starch hydrolysis), as well as higher levels of free amino acids, peptides, and free fatty acids, confirming improved protein and fat digestion (2024b). These results closely mirror our data, where Poolzyme® MULTI promoted the release of BCAAs and EAAs from the hamburger meal, while Poolzyme® DAIRY increased free fatty acid levels and reduced the residual lactose in the pizza meal. Additional studies further corroborate the advantages of enzyme supplementation. For instance, Calvo-Lerma et al. (2019) investigated the optimal pancreatin dosage for various meals, demonstrating that increasing exogenous lipase significantly enhanced fat digestion, as evidenced by greater free fatty acid release. Similarly, proteolytic enzymes from diverse sources have been shown to enhance protein digestion in mixed meals. Furthermore, their findings emphasize that enzyme efficacy depends on factors such as meal composition.

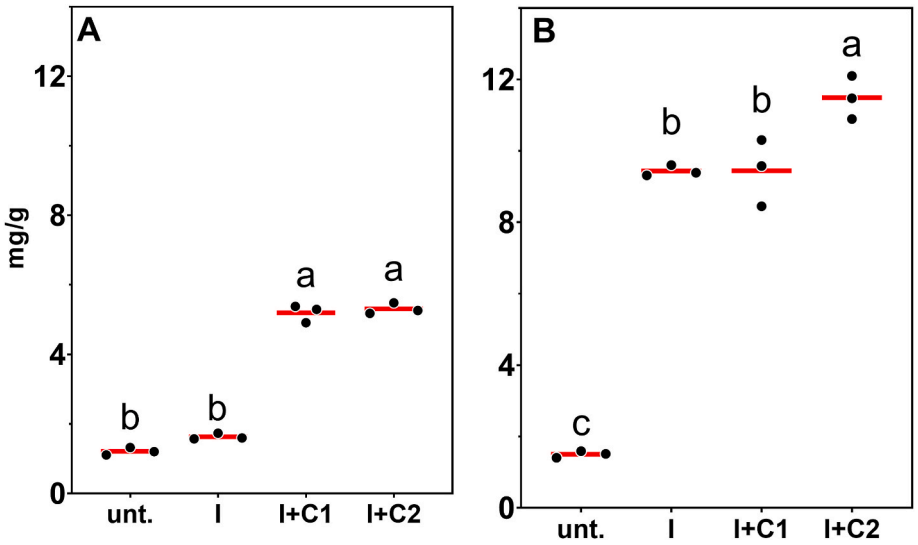

Fig. 3. Free branched chain amino acids (BCAA) in tested meals before and after INFOGEST simulated digestion. Data are expressed as mg of BCAA per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

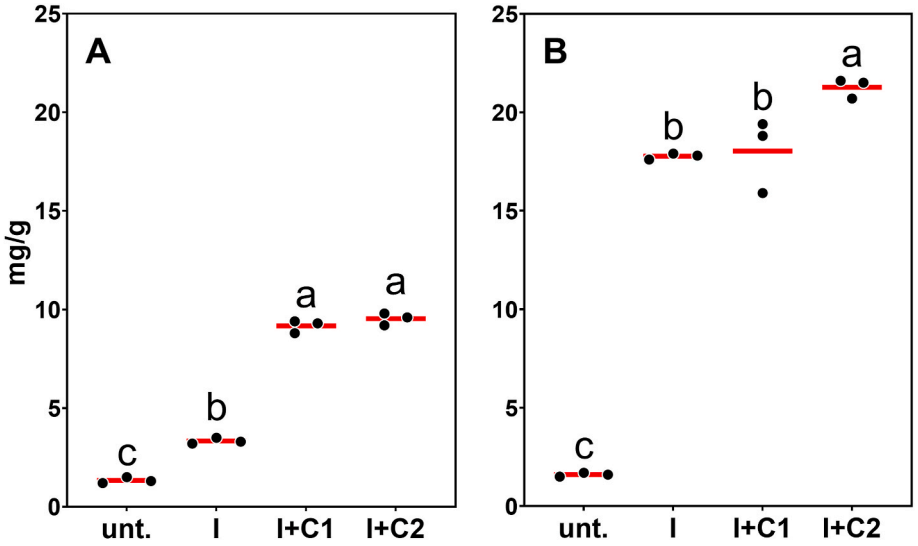

Fig. 4. Sum of the concentration of free essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in tested meals before and after INFOGEST simulated digestion. Data are expressed as mg of EAA per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI-enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

Although supplementation increased free BCAAs and EAAs, these analytes accounted for <20 % of total meal nitrogen, indicating partial hydrolysis comparable to that occurring physiologically in the proximal small intestine (Trommelen et al., 2021), confirming that the enzyme blends promoted bioaccessibility without inducing over-hydrolysis.

Freitas et al. (2022) reported that fungal protease supplementation improved the bioaccessibility of essential amino acids from gluten digestion, though their study focused on individual food items rather than complete meals, as in our case. Likewise, Garvey et al. (2022) demonstrated that adding microbial proteases to an *in vitro* digestion significantly increased free amino acid release. Their study, which applied the INFOGEST static model to a meal composed of chicken, green peas, and mashed potatoes with unsalted butter, reported enhanced macronutrient hydrolysis, paralleling our observations. The concordance between our findings and those of Garvey et al. is particularly noteworthy, as both studies employed real-world mixed meals, underscoring the ability of multi-enzyme supplements to effectively process complex macronutrient matrices.

Our findings also revealed improved starch conversion to sugars, consistent with literature on multi-enzyme supplements containing

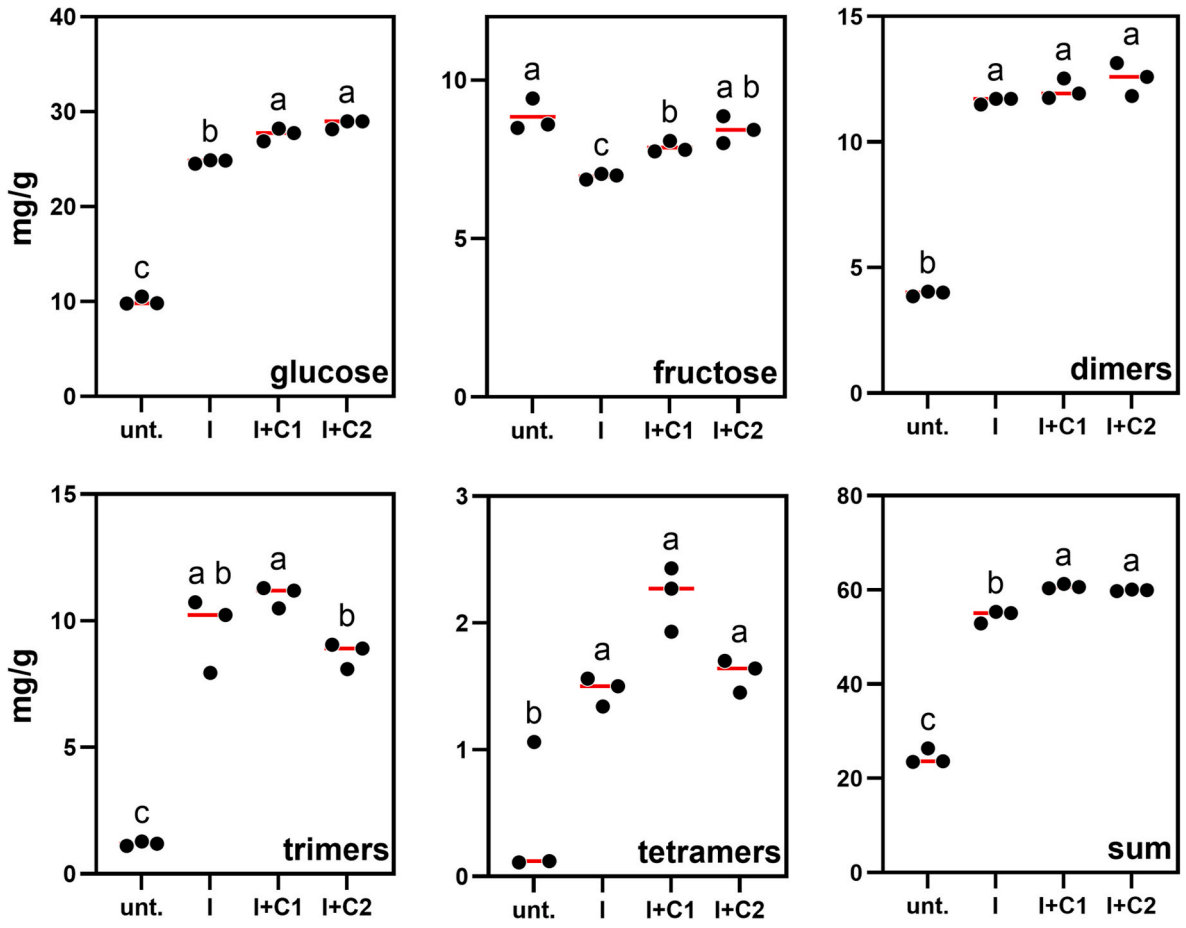

Fig. 5. Sugars quantified in the fast-food meal before and after the INFOGEST simulated digestion. Data are expressed on a wet-weight basis, as mg of sugar per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). For all conditions, three independent replicates were performed, and the median is shown as a red bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

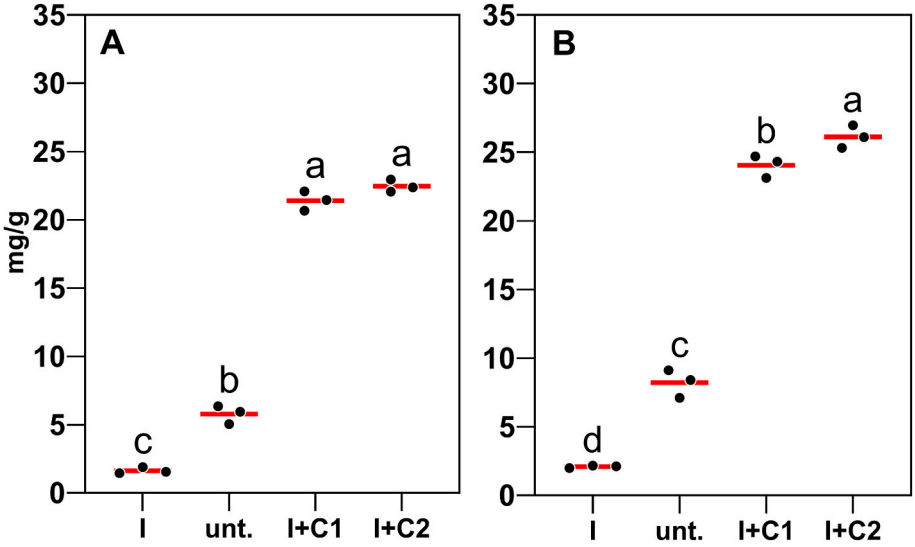

Fig. 6. Free fatty acids in tested meals before and after INFOGEST simulated digestion. Data are expressed as mg of fatty acid per gram of test meal. The data are shown for the untreated (unt.) meal, INFOGEST (I) with no addition of enzyme blend, and INFOGEST with enzyme blend-treated samples at the low dosage (I + C1) and at the high dosage (I + C2). Panel A presents data for the pizza test meal with the Poolzyme® DAIRY enzyme blend, while panel B presents data for the hamburger meal with the Poolzyme® MULTI enzyme blend. For all conditions, three independent replicates were performed, and the median is shown as a bar. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. Post-hoc Compact Lettering Display (CLD) was assigned with a significance threshold of $p < 0.05$. Significant differences between samples are indicated by different lower-case letters.

amylase. Regarding lactose digestion, Poolzyme® DAIRY effectively compensated for the absence of lactase in the pancreatin mix, fully hydrolyzing lactose in a dairy-containing meal, an effect well-documented in lactase supplementation research (Mazhar et al., 2024).

Finally, *in vivo* studies reinforce the relevance of enzyme supplementation. A clinical trial on DigeZyme® (a multi-enzyme complex) using a standardized test meal demonstrated improved protein digestibility and reduced gastrointestinal symptoms over 60 days (Majeed et al., 2018). While healthy individuals typically exhibit sufficient digestive capacity, enzyme supplementation appears particularly advantageous in cases of functional dyspepsia, pancreatic enzyme insufficiency, or after consuming large, nutrient-dense meals. This aligns with Ullah et al. (2023), who reported that Poolzyme® MULTI significantly improved gastrointestinal symptoms and quality of life in individuals with functional dyspepsia. Our study provides a mechanistic foundation for these observations, illustrating how exogenous enzymes enhance nutrient release during digestion.

5. Conclusions

This study strengthens the scientific foundation supporting microbial enzyme supplementation for digestive enhancement. Both Poolzyme® MULTI and Poolzyme® DAIRY significantly improved macronutrient digestion under simulated gastrointestinal conditions. Notably, these enhancements were achieved without modifying the digestion model parameters, emphasizing the intrinsic efficacy of the enzyme blends.

Future research should explore enzyme supplementation in diverse dietary contexts, including plant-based diets with high fiber or anti-nutritional factor content, to extend the applicability of this approach beyond the tested meal types. Controlled clinical trials remain essential to translate *in vitro* findings into tangible gastrointestinal health benefits.

CRediT authorship contribution statement

Robin Duncan: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Giacomo Mantegazza:** Methodology, Investigation, Conceptualization. **Claudio Gardana:** Methodology, Investigation, Formal analysis, Data curation. **Fabio Angelini:** Writing – review & editing, Formal analysis, Conceptualization. **Rosario Russo:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Simone Guglielmetti:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability statement

Data are available upon reasonable request from the corresponding author.

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Declaration of competing interest

R.R. is employed by Giellepi S.p.A. and was not involved in the data analysis or interpretation of results. R.D.'s doctoral fellowship is co-funded by Giellepi S.p.A.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107283.

Data availability

Data will be made available on request.

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