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Digestion supplemented with commercial proteases: Evaluation of the fate of gluten immunogenic peptides in pizza

Alessandra Camarca [a,*,1], Giovanni D'Auria [b,1], Vera Rotondi Aufiero [a,c], Chiara Nitride [b], Nicola Giardullo [d], Anna Sapone [e], Daniel Leffler [e,f], Pasquale Ferranti [b], Giuseppe Mazzarella [a,c]

[a] Institute of Food Science, CNR, Avellino, Italy
[b] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy
[c] European Laboratory for the Investigation of Food-Induced Diseases (ELFID), Napoli, Italy
[d] Division of Gastroenterology, S. G. Moscati Hospital, Avellino, Italy
[e] Takeda Pharmaceuticals International Co, Cambridge, USA
[f] Center for Celiac Research and Treatment, Massachusetts General Hospital, Boston, USA

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ABSTRACT

Enzymatic supplements designed to aid in gluten digestion can be found in the market, also in combination with enzymes targeting other macronutrients. Their impact on digestion of gluten sequences that are immunogenic and/or toxic for individuals with Celiac Disease (CeD), remains uncertain, especially within a complex food matrix. This study aims at applying a biochemical and immune-based approach for understanding the effect of such supplements on the degradation of gluten immunogenic peptides in pizza. Plane-pizza was digested using the INFOGEST model, with/without the addition of three over the counter gluten enzyme supplements (S1, S2 and S3). Proteins digestion was monitored in the gastric (G) and gastroduodenal (GD) phases using electrophoresis and primary amines detection. Residual immune-toxic epitopes were quantified by R5- and G12-based ELISA. Immunogenicity of resistant, deamidated peptides, was assayed on CeD-derived intestinal-T cell lines (iTCLs). The highest content of primary amines was determined in presence of S1 and S2 supplements, containing starch-degrading enzymes in addition to dipeptidyl-peptidase-IV (DPP-IV) and other proteases. The most rapid degradation of R5- and G12-immune-toxic epitopes was observed in presence of S3 supplements, containing prolyl-endopeptidase (*An*-PEP). Consistently, iTCLs showed significant IFN-γ decrease toward S3-treated peptides. Nevertheless, none of the supplements was able to abolish iTCLs response, particularly at the end of gastric phase, thus allowing to conclude that they work to different, but potentially limited extent. The developed approach, combining simulated gastric and gastroduodenal digestion, biochemical/immunochemical characterization and functional bioassays, has proven to be a robust tool to assess residual immunoreactivity of enzyme-treated foods.

1. Introduction

Gluten is a viscoelastic network that forms when gluten-containing flours are mixed with water. This network results from the interaction of a complex combination of hundreds of related yet distinct proteins, existing as monomers, oligomers, and polymers. In wheat these storage proteins are traditionally classified into two main groups: alcohol-water soluble α/β-γ- and ω-gliadins, which contribute to the viscosity of the dough, and insoluble LMW and HMW glutenins, which are primarily responsible of its elasticity. In addition to their complexity, gluten proteins are characterized by a unique composition, with very low proportions of some essential amino acids, and high contents (50 % or more) of others, namely glutamine and proline (gliadin and glutenins are also known as prolamins) (Wieser, 2007). Due to the abundance of

Abbreviations: CeD, celiac disease; G, gastric; GD, Gastroduodenal; S, Supplement; DPP-IV, dipeptidyl-peptidase IV; *An*-PEP, Aspergillus Niger prolylendopepidase; NOPA, α-amino nitrogen; tTG2, type 2 Tissue Transglutaminase; tTG2, deamidated molecule (molecule-TG); PT-Glia-TG, deamidated pepsin-trypsin digest of gliadin; iTCLs, gliadin-reactive intestinal T-cell lines; PBMCs, peripheral blood mononuclear cells..

* Corresponding author at: Immuno-Morphology and Cellular Immunology Lab, Institute of Food Science, CNR, Via Roma 64, 83100 Avellino, Italy.
E-mail address: alessandra.camarca@cnr.it (A. Camarca).

[1] The two authors contributed equally to the manuscript preparation.

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these amino acids, certain sequences in gluten are highly resistant to degradation by human gastrointestinal (GI) proteases, thus leading to the persistence of protein fragments of variable size, confirmed by studies conducted on synthetic peptides both in vitro and in vivo *on Sprague- Dawley rats* (Hausch et al., 2002; Piper et al., 2004), as well as by studies on gluten-based meals, using TNO gastrointestinal model (Mitea et al., 2008) and human gastric and duodenal aspirates (Salden et al., 2015).

In genetically predisposed people, a number of proline and glutamine rich peptides produced by hydrolysis of prolamins, are involved in the development and activation of Celiac Disease (CeD). This is a multisystem inflammatory disorder positioned between food hypersensitivity and autoimmunity, that develops in individuals carrying the HLA-DQ2 or -DQ8 molecules. It has been largely demonstrated that specific gluten-immunogenic peptides (GIPs) are able to reach the intestinal lamina propria, where they are subject to deamidation by type 2 Tissue Transglutaminase (tTG2). Especially after this enzymatic modification, GIPs are potent stimuli for lamina propria CD4 Th1 lymphocytes, through the binding of the HLA-DQ2 and -DQ8 molecules. The CD4 Th1 subpopulation is central into the inflammatory process underlying villus atrophy and crypt hyperplasia, typically found in duodenal mucosa of CeD patients (Iversen & Sollid, 2023). Of great importance, to bind the HLA-DQ2 and -DQ8 molecules, GIPs must be at least 9 amino acids long, as demonstrated for several immunogenic sequences identified from α/β-γ- and ω-gliadins and glutenins (Camarca et al., 2009; Sollid et al., 2020).

Currently, the only available therapy for CeD is a lifelong gluten free diet (GFD), since patients risk relapse of symptoms and progression of the disease when exposed to low trace amount of gluten (Catassi et al., 2007). To guarantee the safety of gluten-free products for CeD patients, a threshold of 20 mg/kg for labeling of gluten-free foods is recommended by Codex Alimentarius and legislation (EC Regulation 41/2009 and subsequent 1169/2011). Analytical methods for gluten content in food are mainly based on immunochemical assays, and in particular the Codex Alimentarius type I method is the R5-based ELISA (Codex Standard 234–1999, Codex Alimentarius. FAO/WHO, 2013). This method relies on the R5 monoclonal antibody (moAb), raised against rye secalin, that strongly binds to QQPFP, QQQFP, LQPFP, and QLPFP peptides, occurring in α−/β, ω-, and γ-gliadins (Kahlenberg et al., 2006; Méndez et al., 2005). Besides the R5 method, Morón et al. developed the G12 ELISAs, by raising antibodies to the 33-mer α-gliadin peptide. This moAb recognizes QPQLPY and QPQLPF sequences, that are particularly relevant for celiac disease immune response (Morón, Bethune, et al., 2008; Morón, Cebolla, et al., 2008).

Recently, the oral administration of gluten-degrading enzymes (known as glutenases) has been proposed to assist CeD patients, in particular subjects at GFD in case of accidental ingestion of gluten proteins (Discepolo et al., 2024; Kivelä et al., 2021).

To this scope, exo- and/or endopeptidases, able to degrade polypeptides containing celiac toxic motifs already in the stomach (Wei et al., 2020), are needed. Digestion of immunogenic gluten peptides may also be facilitated by supplementation of starch-degrading enzymes active at acidic pH, as it is known that the digestion of proteins and carbohydrates in starchy foods is synergistic (Kuang et al., 2023). One of the first enzymes investigated for CeD was the dipeptidyl peptidase IV (DPP-IV), which is an X-Pro amino-pepidase from *Aspergillus oryzae*, capable of degrading the celiac toxic motifs (i.e. PQPQLPY, QLPYPQ, PQPQLP). However, this action can only occur in the duodenum since the enzyme is active at pH 7 (Ehren et al., 2009; Janssen et al., 2015). Several enzymes, which are active at physiological stomach pH 3.5, and resistant to the gastric pepsin, have been characterized from different sources, including EP-B2 (Gass et al., 2006), Latiglutenase (previously *ALV003,* Lähdeaho et al., 2014; Syage et al., 2019), Tak-062 (previously Kuma030, Wolf et al., 2015; Pultz et al., 2021), *An*PEP (*Aspergillus niger* prolyl endopeptidase, also known as Tolerase G, Stepniak et al., 2006; Colella et al., 2024) and the recently reported endopeptidase 40 (E40, Cavaletti et al., 2019; Mamone et al., 2023). Some of these enzymes have entered clinical trials, and studies are ongoing to evaluate clinical efficacy in patients with CeD (Discepolo et al., 2024).

Besides these "glutenases", there are numerous dietary supplements already on the market, including single or enzymes mixtures, promising robust gluten digestion. However, their potency for digestion of immunogenic epitopes is not well known (Krishnareddy et al., 2017). Recently, the INFOGEST semi-dynamic digestion protocol has been used to investigate the effect of one supplement on the digestion of proteins from three wheat-based foods. The study showed that supplemental enzymes can effectively accelerate the breakdown of proteins, including toxic gliadin fractions, from the early stage of gastric digestion (Freitas et al., 2022). Nevertheless, this study did not include functional assays to assess residual activity on gut T cells from patients with CeD, making it difficult to assess the biological relevance of these findings. Conversely, these experiments were included in a previous paper investigating five commercial supplements for their ability to digest purified gliadin proteins, by mass spectrometry and activation assays on intestinal gliadin-specific T cell lines (iTCLs) from CeD patients. The authors concluded that the commercial enzyme supplements tested were ineffective in degrading well known immunogenic gluten fragments from α- and γ-gliadins (Janssen et al., 2015). Recently, relative rates of gliadin consumption by 9 dietary proteases supplements, were investigated on a trypsin/pepsin-digested gliadin, by competitive R5-based and Glutentec immunoassays. Only one of these nine enzyme preparations was able to rapidly digest the immuno-reactive gluten epitopes recognized by the two ELISA methods (Tanner, 2021).

In this context, it is important to highlight that, studies examining the effects of enzymes supplementation on gluten detoxification should consider several factors influencing the results. These include: the digestion protocol and the duration of digestion (i.e. simple pepsin/trypsin or physiological digestion model), the starting material (i.e. purified proteins or complex food matrices), the enzyme purity (i.e. pure proteins or in formulation), the qualitative and quantitative detection methods (i.e. mass spectrometry, electrophoresis or immunochemical assays) and the methods for evaluating the immunological properties of the hydrolyzed proteins (immunochemical assays and the specific type of immunoassay, or functional assays).

To address these issues, an efficient analytical platform has been recently applied to investigate the effect of E40-glutenase on digestion of gluten in wheat-derived products (Mamone et al., 2022). The analytical design (reviewed by Mamone et al., 2023) is based on in vitro semi-dynamic digestion by standardized INFOGEST model (Brodkorb et al., 2019; Minekus et al., 2014), followed by extensive characterization of residual gluten peptides by proteomic (liquid chromatography-mass spectrometry, LC-MS/MS) and immunochemical analyses (R5/G12 based ELISA) and finally by validation of the immunostimulatory properties of digested gluten trough functional studies, based on gut mucosal derived T cell lines from CeD patients. Of relevance, in absence of an in vivo model mimicking the entire pathogenetic process, *in vitro* cultured iTCLs represent a unique tool, that has been largely used to functionally validate the effects of detoxification strategies based on gluten modifications (Gianfrani et al., 2007; Ribeiro et al., 2020) or hydrolysis (Stepniak et al., 2006; Mitea et al., 2008; Wolf et al., 2015; Cavaletti et al., 2019; Mamone et al., 2023).

This paper applies a holistic analytical pipeline, to assess the impact of over the counter gluten enzyme supplements on gluten degradation, using plane Neapolitan pizza as a model of gluten-containing food. Unlike previous studies, which mainly focused on individual enzymes or model systems, this work directly compares three supplements with distinct enzymatic composition - two containing complex mixtures of DPP-IV, amylase/glucoamylase and proteases, and one based solely on *An*-PEP- under identical, standardized in vitro digestion conditions (INFOGEST protocol). The digestive process was comprehensively monitored by integrating biochemical assays, with immunochemical detection of celiac toxic motifs and functional readouts via T cell-based

assays, to collect evidence of the breakdown of celiac-toxic motifs after gastric and duodenal digestion. The comparative analysis of the supplements under identical analytical conditions offered a multidimensional perspective on the mechanism and efficacy of gluten hydrolysis/digestion.

2. Materials and methods

2.1. Digestive supplements included in the study

The composition of the commercially available digestive supplements analyzed in this study is reported in Table 1. Two supplements (S1 and S2), included DPP-IV and non-specific proteases from Aspergillus strains or unspecified "Thera-blend" proteases, as well as amylases and glucoamylases. The third enzyme contained exclusively prolyendopeptidase from *Aspergillus niger* (*An*-PEP).

2.2. Production of pizza and digestion by INFOGEST protocol

Neapolitan pizza base was prepared by a local bakery following the Neapolitan pizza Specialty Guaranteed (TSG) specifications (COUNCIL REGULATION (EC) No 509/2006, COMMISSION REGULATION (EU) No 97/2010). The pizza was prepared in two days (biological replicates). Each day a total of 20 g of cooked pizza was randomly collected using sterile scissors, including both the base and the edges. The pizza was then roughly hand-minced and was divided into 5 g samples. The five grams of cooked pizza, quantity as indicated by the standardized model, were digested following the in vitro static oral gastro duodenal (OGD) INFOGEST method (Brodkorb et al., 2019). Simulated human salivary fluid (SSF), simulated human gastric fluid (SGF), and simulated human intestinal fluid (SIF) were prepared according to the harmonized protocol conditions. All digestion steps were carried out in an orbital shaker incubator at 37 °C and 600 rpm.

For the oral phase, 5 g of cooked pizza samples were grossly minced along with the SSF (including human salivary amylase) for simulate the mastication and incubated for 2 min. One pill of supplement and pepsin were dissolved in 10 ml of SGF. Subsequently, boluses were mixed with supplemented SGF, the pH was adjusted to 3 and samples were then incubated for 2 h. Afterwards, SIF was added to the chymes, the pH was raised up to 7.0, and bile salts and porcine pancreatin were incorporated into the mixtures; the duodenal digestion was carried out for further 2 h. The use of one supplement pill is consistent with the manufacturers' recommended conditions for use in cases of accidental or voluntary gluten ingestion. Of note, using a total of 5 g of food, an extreme scenario of enzymatic activity was reproduced, where the enzyme-food ratio was much higher, if compared to those expected in a real meal condition.

Experiments for kinetic curves were performed in duplicates and samples were taken for subsequent analysis at T0, T30, T60 and T120 min of gastric (G) or gastroduodenal (GD) digestion phases. In experiments focused only on T120G and T120GD digestion products, two batches of digestion were performed for each condition. One batch was interrupted after 120 min of gastric digestion. The second batch was in vitro digested up to 120 minutes of the duodenal phase. Immediately after the digestion, soluble fractions were separated from insoluble pellets by centrifuging the samples at 7900 x g for 15 min. The fractions were then stored at −20 °C until required for further analyzes.

2.3. Protein and α-amino nitrogen (NOPA) quantification

Protein quantification of digest fractions was performed by using the EnzytecTM Total Protein kit (Ref: E2620, R-Biopharm Italia, Milan, Italy). The quantity of primary amines derived from the protein digestion was determined with the EnzytecTM Alpha-amino Nitrogen kit (Ref. E2500, R-Biopharm Italia, Milan, Italy). The analyzes were accomplished on the iMagic-M9 (R-Biopharm Italia, Milan, Italy) following the manufacturer's instructions.

2.4. SDS-PAGE analyses

Soluble digests were diluted with 6× Laemmli buffer [Tris-HCl 50 mM pH 6.8, containing 2 % (*w/v*) SDS, 60 % (w/v) glycerol, and traces of bromophenol blue] and 1 M dithiothreitol (DTT) (reaching a final concentration in the sample of 0.1 M). Supplements were powdered in a mortar and 2 mg were then dissolved in 1 ml of 1× Laemmli buffer [Tris-HCl 50 mM pH 6.8, containing 2 % (w/v) SDS, 10 % (w/v) glycerol, and traces of bromophenol blue] and 0.1 M DTT. Samples were boiled for 10 min and 12.5 μg of protein per well was loaded onto a 12 % polyacrylamide gel. Precision Plus Protein Standards (Bio-Rad Laboratories, Inc., Hercules, CA) were used as molecular markers.

The separation was conducted at room temperature (approx. 20 °C), and, after protein migration, gels were fixed in 24 % (w/v) TCA for 16 h and rinsed three times in Milli-Q water with gentle shaking for 5 min. The gels were then stained for 16 h in Coomassie Brilliant Blue G-250 Colloidal staining solution (Bio-Rad Laboratories, Inc., Hercules, CA) and de-stained in Milli-Q water. Gels were then imaged with a commercial scanner.

2.5. Extraction and deamidation of soluble peptides

Soluble fractions at T120G and T120GD, were deamidated following the procedure described elsewhere (Iacomino et al., 2021). At this step, the same volumes were taken from different reactions, to compare the same quantities of initial pizza. Deamidated samples (indicated by the acronym TG) were salted out by C18 solid phase extraction, following manufacturer's instructions (SepPak WAT051910; Waters Corporation, Massachusetts, USA). Afterwards, samples were concentrated with gaseous nitrogen and freeze dried. Before functional assays, samples were resuspended in phosphate buffered saline and the pH was adjusted to 7 with additional HEPES buffer.

2.6. Evaluation of residual gluten content by R5- and G12-based ELISA assays

R5-gluten content was estimated in soluble fractions of digestion reactions (obtained as described in section 2.2), by RIDASCREEN® Gliadin competitive assay (R-Biopharm AG Darmstadt, Germany),

Table 1
Composition of the dietary supplements as stated in the information leaflet. PPI = protease picomole international; HUT (Hemoglobin Unit on a L-Tyrosine basis); DPP-IV = dypeptyl peptidase IV; AP = Alkaline Phosphate; DU = Dextrinizing units (Amylase); AGU = Amyloglucosidase Units (glucoamylase activity units).

		Supplement S1	Supplement S2	Supplement S3
Protease (not specified)	Protease from *Aspergillus oryzae*	30,000 HUT		
	Protease from *Aspergillus melleus*	8.5 AP		
	Protease Thera-blend (G1)		95,000 HUT	
Protease (specified)	Dipeptidyl peptidase (DPP-IV)	500 DPP-IV	1000 DPPU	
	Prolyloligopeptidase from *Aspergillus niger (An*-PEP)			30,000 PPI
Amylase	Amylase from *Aspergillus oryzae*	12,000 DU		
	Amylase Thera-blend		15,000 DU	
Glucoamylase	Glucoamylase from *Aspergillus niger*	20 AGU	15 AGU	

following the manufacturer's instruction, in particular those for liquid food. Briefly, samples were first extracted with 60 % ethanol (1:9, v:v). Afterwards, ethanol extracted fractions, were opportunely diluted and analyzed by ELISA, following the test implementation phase instructions. For gluten digestion kinetics analysis, 100 μl of the soluble fractions, collected at the indicated time-points, were analyzed. For analysis focused on T120G and T120GD samples, 300 μl of the soluble fractions were used. After the extraction phase, samples were diluted as follows: CTR_G 1:20.000, S1_G 1:10.000, S2_G 1:5000, S3_G 1:2000, GD samples all diluted 1:500. Results were elaborated using the RIDASOFT® Win.NET software, as indicated by product instructions.

Soluble fractions were also analyzed by GlutenTox ELISA competitive G12 kit (Hygiena, Spain). To this scope, 300 μl of T120G and T120GD samples were processed following the manufacturer's instructions for liquid food. Samples were first extracted using Universal Gluten Extraction Solution (UGES) provided by the kit (1:4, v:v), then they were analyzed by ELISA assay, using kit reagents/solutions. The same final dilution factors described for RIDASCREEN ELISA assay were applied.

Finally, for measurement of residual G12-immunoreactive peptides in insoluble fractions, pellets obtained from T120G and T120GD digestions (as described in section 2.2), were analyzed by GlutenTox ELISA rapid G12 (Hygiena, Spain), following the manufacturer's instructions for solid/semisolid samples. Briefly, 0.5 g of pellets were mixed with 5 ml of UGES extraction solution and incubated at 50 °C in a water bath for 40 min, to facilitate gluten extraction. Then, the suspensions were centrifugated (2500 ×g, 10 min) and the supernatants were subjected to ELISA determination (final dilution of samples 1:200). Data derived from both G12-based ELISAs were analyzed using Microsoft Excel Software, as indicated by the products instructions.

According to manufacturer's instruction, the limit of detection (LoD) and the quantification ranges of the assays were as follow: RIDASCREEN gliadin competitive, LoD 2.3, range 5–270 ppm of gliadin; GlutenTox ELISA competitive G12, LoD 0.8, range 1.2–50 ppm of gliadin; GlutenTox ELISA rapid G12, LoD 0.2, range 0.8–100 ppm of gliadin.

For all the ELISA assays, gliadin values were calculated based on the specific kit standards and on the applied dilution factors, then converted into gluten by multiplying for a factor of 2. The determined ppm values obtained from raw data (expressed as μg of gliadin per ml of soluble/per g of insoluble digest) were multiplied by the total volumes of soluble fractions/total weight of insoluble fraction, recovered for each reaction, and normalized for 1 g of pizza (expressed as mg gluten/g of pizza).

2.7. Cytotoxicity assay on peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMCs) were obtained from blood of 3 healthy volunteers by Ficoll-Paque PLUS gradient (Cytiva, Uppsala, Sweden) as reported in manufacturer's instructions. 2.5×10^6 cells were stimulated with PHA (1 μg/ml) and IL-2 (20 U/ml), in 250 μl of complete RPMI medium (RPMI 1640 supplemented with 10 % FCS, and 1 % of antibiotics, glutammine, non-essential aminoacids and sodium pyruvate, all provided by Lonza, Belgium), in 96 well cell culture plates. Deamidated G extracts (5 μl) and GD extracts (10 μl) were added, and cells were incubated, at 37 °C/5 % CO2. After 72 h, 50 μl of cell culture medium were analyzed by sandwich IFN-γ ELISA (eBioscience Inc., San Diego, California). For cell viability, 50 μl of cell suspensions were analyzed by CyQuant direct cell proliferation assay (Invitrogen, Thermo Fisher Scientific, Italy). Fluorescence emission (λ_{Ex} 485 nm/ λ_{Em} 528 nm) was detected after 1 h.

2.8. Generation of gliadin-specific intestinal TCLs (iTCLs) and iTCLs activation assay

Jejunal biopsies were obtained from 6 CeD patients, (5F/1M), with a mean age of 36 years (range 16–58). 3/6 CeD patients had overt CeD (villus atrophy-VA) and the remaining had treated CeD (gluten-free diet-GFD). All subjects were enrolled at S.G. Moscati Hospital of Avellino (Italy) (ethical committee number CECN/819 dated 03/21/2018) and gave their full informed consent to the study. iTCLs were generated from intestinal biopsies as previously described (Camarca et al., 2009; Gianfrani et al., 2015). Briefly, mucosal explants were digested with collagenase-A (1 mg/ml final concentration, 90 min, 37 °C), and the isolated cells (0.5×10^5) were suspended in complete X-Vivo medium (X-Vivo15 supplemented with 5 % AB + human serum and antibiotics, all provided by Lonza, Belgium). Autologous PBMCs (1.5×10^5) were irradiated at 3500 Rad using a Gammacell 220 Irradiator system (Nordion, Canada) and added to intestinal cells, concomitantly with 50 μg/ml of tTG2-deamidated Pepsin Trypsin digest of gliadin (PT-Glia-TG, gliadin purchased from SIGMA). On days 7 and 21, iTCLs were re-stimulated with irradiated autologous PBMCs and PT-glia-TG, to expand gluten-specific T cells. IL-2 and IL-15 (R&D System, Minneapolis, MN, United States) were used as growth factors, respectively at 20 U/ml and 10 ng/ml. For functional assays, EBV-transformed B lymphoblastoid cell lines expressing HLA-DQ2.5 ($DQ2.5^{pos}$ EBV-B cells) were used as antigen presenting cells (APC). Specifically, 3×10^4 T cells were co-incubated with 1×10^5 $DQ2.5^{pos}$ EBV-B cells, in 200 μl of complete medium in 96-well plates. The different pizza-digested extracts were added before incubating for 72 h at 37 °C/5 % CO2. Culture supernatants (50 μl) were then collected for determination of IFN-γ by sandwich ELISA. In titration experiments for evaluation of the deamidation effects, not deamidated or tTG2-deamidated peptide extracts from gastric control digestion were added at serial dilutions starting at 20 μl/well. For dose-response experiments with deamidated G and GD digestion extracts, T cells were stimulated with increasing doses of T120G_TG peptides extracts (0.625 to 5 μl), or T120GD_TG (1.25 to 10 μl). GD extracts were used at double compared to G extracts because the soluble volumes after addition of intestinal phase fluids and enzymes are about twice the volume of the gastric phase.

2.9. Statistical analysis

For the evaluation of proteins digestion kinetics, data from α-amino nitrogen and from RIDASCREEN gliadin competitive ELISA analysis at T0, T30, T60 and T120 of G and GD reactions, were processed using the OriginPro 8.0 (OriginLab Corporation, Northampton, MA, USA). Nonlinear growth curve analysis (type 1 sigmoidal logistic curve: $y = a/(1 + \exp.(-k*(x-xc)))$ was used for plotting the NOPA values in function of the time, to obtain the K value. RIDASCREEN R5-gliadin values were plotted in function of time by non-linear exponential curve analysis (Exponential Decay1: $y = A1*\exp.(-x/t1) + y0$), to obtain the t1 value. The slope of lines obtained plotting T0-T30 values in function of time were also calculated as an indication of the initial reaction velocity.

NOPA and total proteins data from T120G and T120GD samples, were compared by analysis of the variance (ANOVA) and the post-hoc Tukey HSD, calculated using R. Statistical.

Results of R5 and G12 ELISA on T120G and T120GD samples, were compared by paired *t*-test, using Microsoft Excel. Differences across S1-S2- S3-treated samples and relative G or GD CTRs were considered significant for $p < 0.05$.

3. Results

3.1. Digestive supplements accelerate protein hydrolysis

Three commercially available digestive supplements have been included in this study, with different composition (Table 1), both in terms of enzyme specificity and activity, as described in the methods section.

The fate of pizza's gluten proteins during 120 min of both gastric (T120G) and duodenal (T120GD) digestion was assessed by electrophoresis, α-amino nitrogen (NOPA) and total proteins determination. Following gastric digestion, samples digested in the presence of the

supplements exhibited a reduced number of proteins bands under 40 KDa, compared to the control (Fig. 1a) suggesting that proteins were broken down more rapidly in the presence of supplements. In addition, the S3 supplement enzymes were found to be more resistant to gastric conditions as compared with the S1 and S2. After complete GD digestion (T120GD) (Fig. 1b) no appreciable differences in proteins patterns were detected by electrophoresis, showing no residual proteins of MW larger than 10 kDa, regardless of the presence of supplements.

At the end of the soluble gastric phase, when quantifying α-amino nitrogen, data clustered into two groups ($p < 0.01$). The S3 treated food showed a comparable level of NOPA to the control (CTR), with higher protein content and lower NOPA. On the contrary, the S1 and S2 showed a lower total protein and a higher α-amino nitrogen content (Fig. 1c and d, respectively), indicating a higher proteolysis during gastric digestion.

Quantification of NOPA was used also to estimate protein digestion kinetics, through measurements of samples collected at T0, T30, T60 and T120 minutes of both gastric (T0G, T30G, T60G, T120G) and gastroduodenal phases (T0GD, T30GD, T60GD, T120GD) (Fig. 1 e-f). Data collected at different timepoint showed a type 1 sigmoidal logistic function and the steepness of the curves (k values, Fig. 1e) showed similar rates between S1 and S2 and between S3 and CTR. The initial velocity, estimated by the slopes of the T0-T30 lines (Fig. 1e), confirmed a higher protein digestion rate for S1 and S2, compared to CTR and S3.

Conversely, the rate of duodenal digestion was comparable across conditions (slopes and k values, Fig. 1f), suggesting that supplements may have less effect during this phase. Thus, the different NOPA values at the end of the duodenal phase reported in Fig. 2, reflect differences already detectable during in the gastric phase.

Overall, the biochemical analysis of in vitro digested pizza showed clear differences between digestive supplements and with the CTR digestion.

3.2. Digestive supplements accelerate gluten hydrolysis

To evaluate the digestion kinetics of gluten during simulated digestion with and without enzyme supplements, residual gluten content in samples at different timepoints was estimated using the RIDASCREEN R5-gliadin competitive ELISA (Fig. 2). Since the ELISA kit employed is optimized for trace gluten detection, substantial serial dilutions were required to avoid exceeding the upper LOQ of the kit, allowing a comparative assessment of the samples.

Overall, we observed lower measured gluten concentrations at the beginning of the duodenal phase compared to the end of the gastric phase. This apparent discrepancy is attributable to methodological factors associated with the transition between simulated digestion stages. In fact, the protocol required a 1:2 dilution of the gastric digest upon addition of simulated intestinal fluid (SIF). In addition, the introduction of SIF may have altered the solubility of gliadin and its derived polypeptides, potentially impacting their detection. Finally, the substantial serial dilutions used could introduce variability when comparing absolute concentrations across phases with vastly different dilution factors. Crucially, the primary objective was to compare supplemented versus control digests within each specific compartment (gastric or duodenal).Kinetic analysis results showed that, already after 30 min of gastric digestion (T30G), all the supplements enhanced R5-peptide hydrolysis, as demonstrated by the descending slope of the lines (T0-T30) compared to CTR (Fig. 2). Based on the measurements of the 4 timepoints, all enzymes fit with a type 1 Exponential Decay with R square of at least 0.98. The S3 data showed the highest negative slope and the lowest constant rate.

In the gastro-duodenal phase, the initial slopes and the constant rates indicate a trend for a faster decrease of R5-peptides in CTR samples, compared to S1 and S2, while for S3 calculation was not possible as all samples were under the limit of quantification of the assay (at the used dilutions). However, the R5-immunoreactive epitope(s) are consistently lower in the presence of all of the enzyme supplements compared to the CTR. These results indicate that, although the enzymes primarily act on gluten under gastric conditions, their effects can influence overall gastroduodenal digestion outcomes as suggested by α-amino-nitrogen determinations.

3.3. R5- and G12-immunoreactive peptides surviving the whole digestion process

To further assess the quantity of immunogenic gluten peptides surviving the digestion process, and to provide comprehensive information about the immunostimulatory potential of gluten digested with and without enzyme supplements, soluble fractions obtained at T120G and T120GD, were assayed by both R5- and G12-based competitive ELISA assays, recognizing different gliadin epitopes. In addition, the residual gluten content was also evaluated in the insoluble fractions by the G12-based sandwich ELISA.

As a first readout of the analysis, data were reported as μg of gluten per ml of soluble/per g of insoluble digest (thereafter indicated as ppm) (Table 2). The results obtained confirm a significant effect of the three supplements on reduction of celiac toxic sequences, as measured by both the R5- and the G12-specific antibodies. This effect was also observed in the insoluble pellet, suggesting that the decrease observed in the soluble fractions was not due to the presence of undigested gluten into the insoluble fraction.

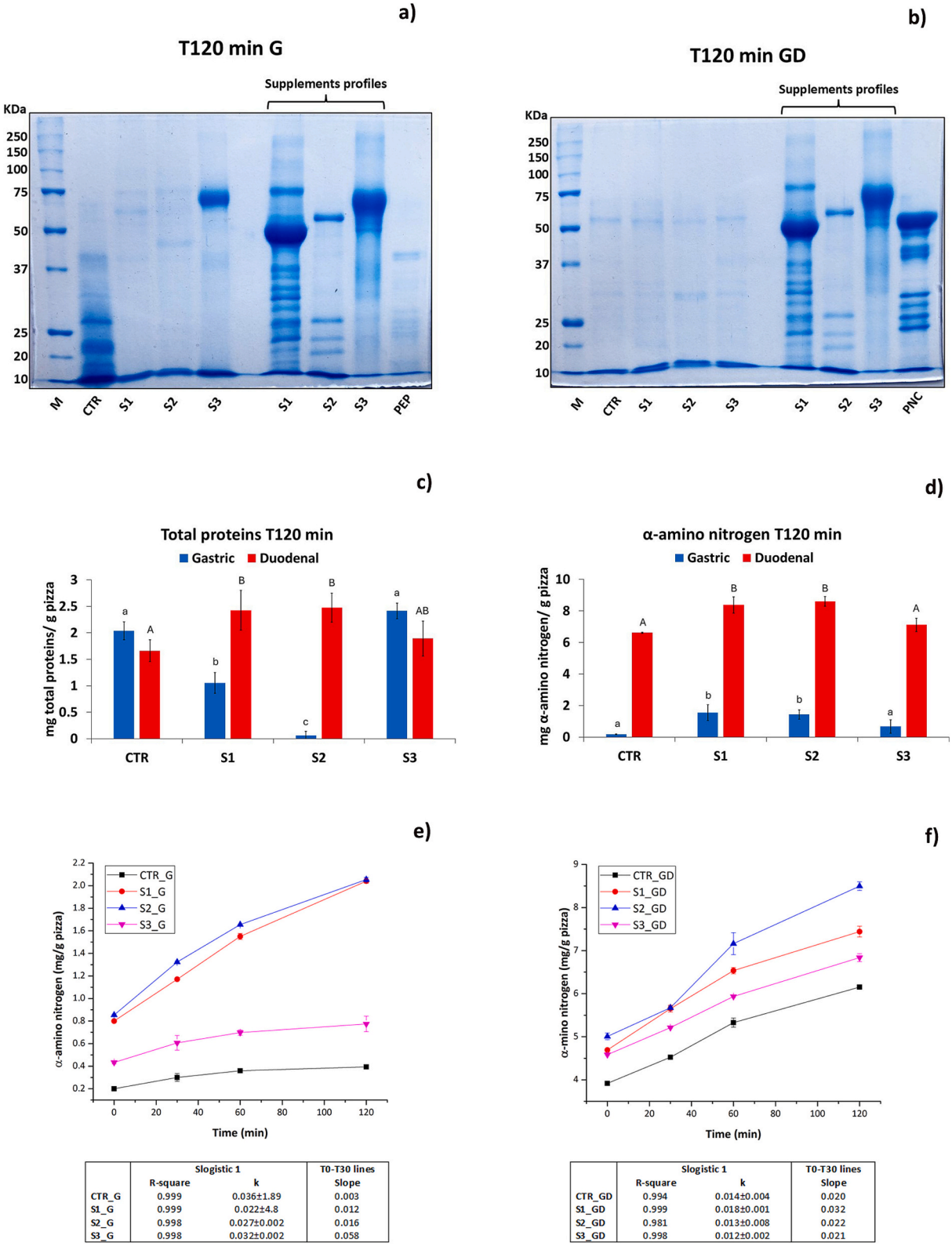
Although experiments were performed under controlled experimental conditions, the ppm of gliadins determined using G12-assay were generally higher when compared to the results of R5-based assay. This result is likely related to differences between the test kits used, including the specificity of the antibody, alongside the calibrant and the extractants (Rzychon et al., 2017). Overall, results showed a consistent hydrolyzing action of the supplements on R5- and G-12 immunoreactive peptides, with S3 being the most efficient.

Since the soluble volumes and insoluble weights recovered varied across simulated digestion conditions, the residual R5- and G12-immunoreactive peptides were normalized per g of digested pizza to enable a more accurate comparison of the enzymes' gluten-hydrolyzing potential (Fig. 3).

After normalization, the results of the R5-competitive ELISA (Fig. 3a-b) confirmed the data from the kinetic curves, showing a significant reduction in R5-immunoreactive peptides content in the soluble fraction of gastric digests. The most notable reductions were observed with the addition of S3 (98.7 %) and S2 (96.4 %) compared to CTR gastric digestion. Although the S1 supplement also led to a significant reduction in R5-peptides, its effect was less pronounced (75.9 % vs. CTR_G). At the end of gastroduodenal digestion, S1 and S2 resulted in a moderate reduction in R5-immunoreactive peptides (51.7 % and 51.2 %, respectively, vs. CTR_GD), while treatment with S3 resulted in a more substantial decrease (84.3 % vs. CTR_GD).

As shown before, the measurement of the residual gluten toxic G12-motifs revealed substantial differences compared to the R5-based evaluations, mainly regarding the action of S1 (Fig. 3 c-d). Unlike R5 motifs, where all enzymes led to a decrease in the gastric phase, the G12 motifs were substantially reduced by S2 and S3 compared to control (77.9 and 98.8 % decrease vs CTR_G, respectively), but were not significantly affected by S1 during the gastric phase. At the end of gastroduodenal digestion, the G12-results were comparable to the R5-results (37.9 %, 42.3 % and 96 % reduction vs CTR_GD, for S1, S2 and S3, respectively).

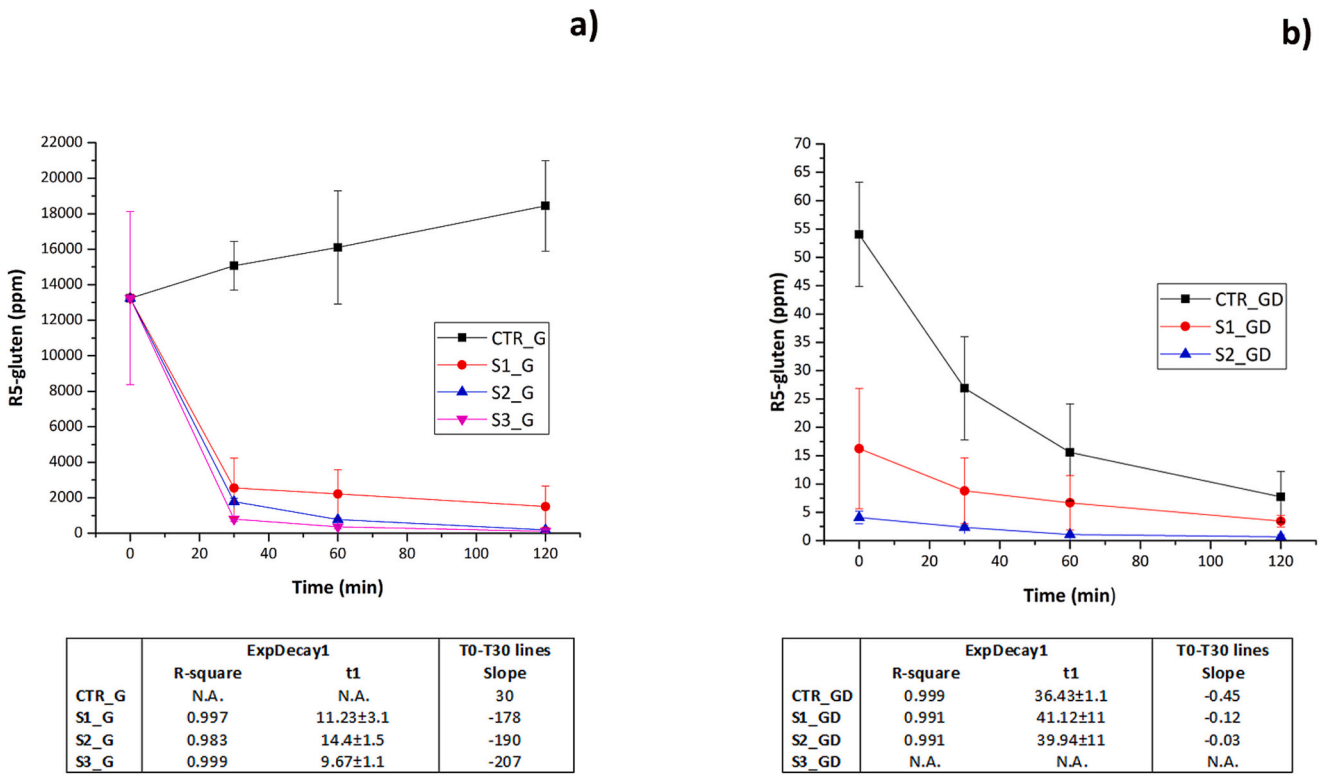
Finally, insoluble material was analyzed to assess the presence of undigested gluten proteins. To this scope, sandwich ELISA format (G12-based) was used, since the analysis of proteins and large polypeptides requires using antibodies recognizing at least two binding sites for a more specific detection. Obtained results (Fig. 3 e-f) were consistent with the observations made on soluble digests by G12-based ELISA. In particular, S1, S2 and S3 decreased the G12-detectable motifs by 79.6, 81.1 and 62.1 %, in gastric phase, and by 66.5, 78.1 and 88.6 % in intestinal phase, respectively. This strongly supports that the observed



	Slogistic 1		T0-T30 lines
	R-square	k	Slope
CTR_G	0.999	0.036±1.89	0.003
S1_G	0.999	0.022±4.8	0.012
S2_G	0.998	0.027±0.002	0.016
S3_G	0.998	0.032±0.002	0.058

	Slogistic 1		T0-T30 lines
	R-square	k	Slope
CTR_GD	0.994	0.014±0.004	0.020
S1_GD	0.999	0.018±0.001	0.032
S2_GD	0.981	0.013±0.008	0.022
S3_GD	0.998	0.012±0.002	0.021

Fig. 1. Evaluation of proteins digestion by commercial supplements. (**a-b**) SDS-PAGE under reducing conditions of the soluble gastric (G) (**a**) and soluble gastro-duodenal (GD) digests (**b**). **(c-d)** Quantification of total proteins (**c**) and primary amines (**d**). Bars with different letters are significantly different at $p < 0.01$ by analysis of the variance (ANOVA) and the post-hoc Tukey HSD test. **(e-f)** Evaluation of proteins digestion kinetics. Mean values and SD of biological duplicate experiments were reported for α-amino nitrogen detection at T0, T30, T60 and T120 minutes of G (**e**, upper panel) and GD (**f**, upper panel). K values are obtained from type 1 sigmoidal logistic curve fitting of data. The slope of lines obtained plotting T0-T30 values, were also calculated as indication of the initial reaction velocity (**e-f** lower panels).



	ExpDecay1		T0-T30 lines
	R-square	t1	Slope
CTR_G	N.A.	N.A.	30
S1_G	0.997	11.23±3.1	-178
S2_G	0.983	14.4±1.5	-190
S3_G	0.999	9.67±1.1	-207

	ExpDecay1		T0-T30 lines
	R-square	t1	Slope
CTR_GD	0.999	36.43±1.1	-0.45
S1_GD	0.991	41.12±11	-0.12
S2_GD	0.991	39.94±11	-0.03
S3_GD	N.A.	N.A.	N.A.

Fig. 2. Evaluation of R5-gliadin peptide digestion kinetic. 100 µl of the soluble fractions collected at the indicated timepoints from gastric (**a**) or gastroduodenal (**b**) digestion, were processed as indicated for liquid food following the manufacturer's instructions. R5-gluten values (µg of R5-gluten/ml of soluble fractions) were calculated using the RIDASOFT® Win.NET software and reported as mean and SD of duplicate digestion experiments (upper panels). Fitting of data was performed using OriginPro 8.0 software.

Table 2
Residual gluten content after G and GD digestion by R5 and G12 immunoassays. 300 µl of the soluble fractions of G120 and GD120 samples were diluted as described in method section, and assayed by RIDASCREEN gliadin competitive ELISA (R5-competitive) or by GlutenTox ELISA competitive G12 kit (G12-competitive), following the manufacturer's instructions. Insoluble pellets (0.5 g) from G120 and GD120 samples, were analyzed by GlutenTox ELISA rapid G12, following the manufacturer's instructions. Data derived from RIDASCREEN assay were processed by RIDASOFT® Win.NET while values from GlutenTox assays were analyzed by Microsoft Excel. µg of R5 or G12 gliadins per ml of soluble or per g insoluble fractions, were multiplied by factor 2, to obtain gluten ppm, as recommended by manufacturers. Mean and SD of duplicate digestion experiments are reported. $^{\S}p < 0.05$ vs relative CTR, $^{\dagger}p < 0.05$ vs S1, $^{\ddagger}p < 0.05$ vs S2, calculated by paired t-test. RIDASCREEN gliadin competitive ELISA Intra-assay and Inter-assay variability: 2.9 % and 1.1 %, respectively. GlutenTox ELISA competitive G12 Intra-assay and Inter-assay variability: 0.66 % and 6 %, respectively. GlutenTox ELISA rapid G12 Intra-assay and Inter-assay variability: 2.8 % and 9 %, respectively.

	R5/G12-detected gluten (ppm)		
	Soluble fraction (µg/ml)		Insoluble fraction (µg/g)
	R5-competitive	G12-competitive	G12-sandwich
CTR_G	19,460 ± 763	37,701 ± 698	813 ± 261
S1_G	2175 ± 20§	20,610 ± 269§	317 ± 114
S2_G	359 ± 20§†	4288 ± 85§†	230 ± 40
S3_G	135 ± 14§†‡	229 ± 6.4§†‡	433 ± 133
CTR_GD	27.6 ± 0.17	268 ± 5	23.1 ± 5.8
S1_GD	12.8 ± 0.04§	160 ± 0.37§	8.2 ± 2.3
S2_GD	12.8 ± 2.3	147 ± 4.5§	6.4 ± 3
S3_GD	4.2 ± 1.5§‡	10.5 ± 1.4§†‡	3.2 ± 4.3§

changes in abundance of immunogenic gluten peptides was due to proteolysis rather than a lack of gluten solubility.

In conclusion all the enzymes demonstrated lower levels of G12- and R5-reactive immunogenic gluten peptides compared to control. However, the amount of immunoreactive peptides detected is variable, depending not only on the supplement composition, but also on the detection method, the digestion phase, and the type of samples (soluble/insoluble).

3.4. Residual immunogenic activity of digested pizza extracts on gliadin-specific intestinal T cell lines

Polyclonal, gliadin specific intestinal TCLs were generated from duodenal mucosa of CeD patients and assayed for their response to deamidated commercial gliadin as a control (Supplementary fig. 1a). Then, they were used to compare the immunostimulatory properties of peptides derived from digestion of pizza, under the conditions described above.

The soluble peptides extracts were first deamidated by tTG2 treatment, then were used to stimulate iTCLs. The effectiveness of the soluble peptides extract method and of the deamidation reaction, was ascertained by dose-response experiments performed with CTR_G extracts, before and after deamidation, showing much stronger immunogenic potentials of the deamidated samples (Supplementary fig. 1b). In addition, possible cytotoxic effects that could have affected iTCLs response, were excluded by in vitro cytotoxicity assays on PBMCs. Results showed that, under incubation with deamidated extracts from supplement-treated samples, neither IFN-γ production nor cell viability were significantly different from results observed with CTR_G_TG or CTR_GD_TG extracts (Supplementary fig. 2). Nevertheless, a certain toxicity was observed for S1_GD sample ($p = 0.05$ compared to CTR, Supplementary fig. 2a).

Next, the immunostimulatory activity of the deamidated extracts obtained after digestion in presence or absence of the supplementary enzymes, was compared in dose-response experiments (Fig. 4 a-b). IFN-γ

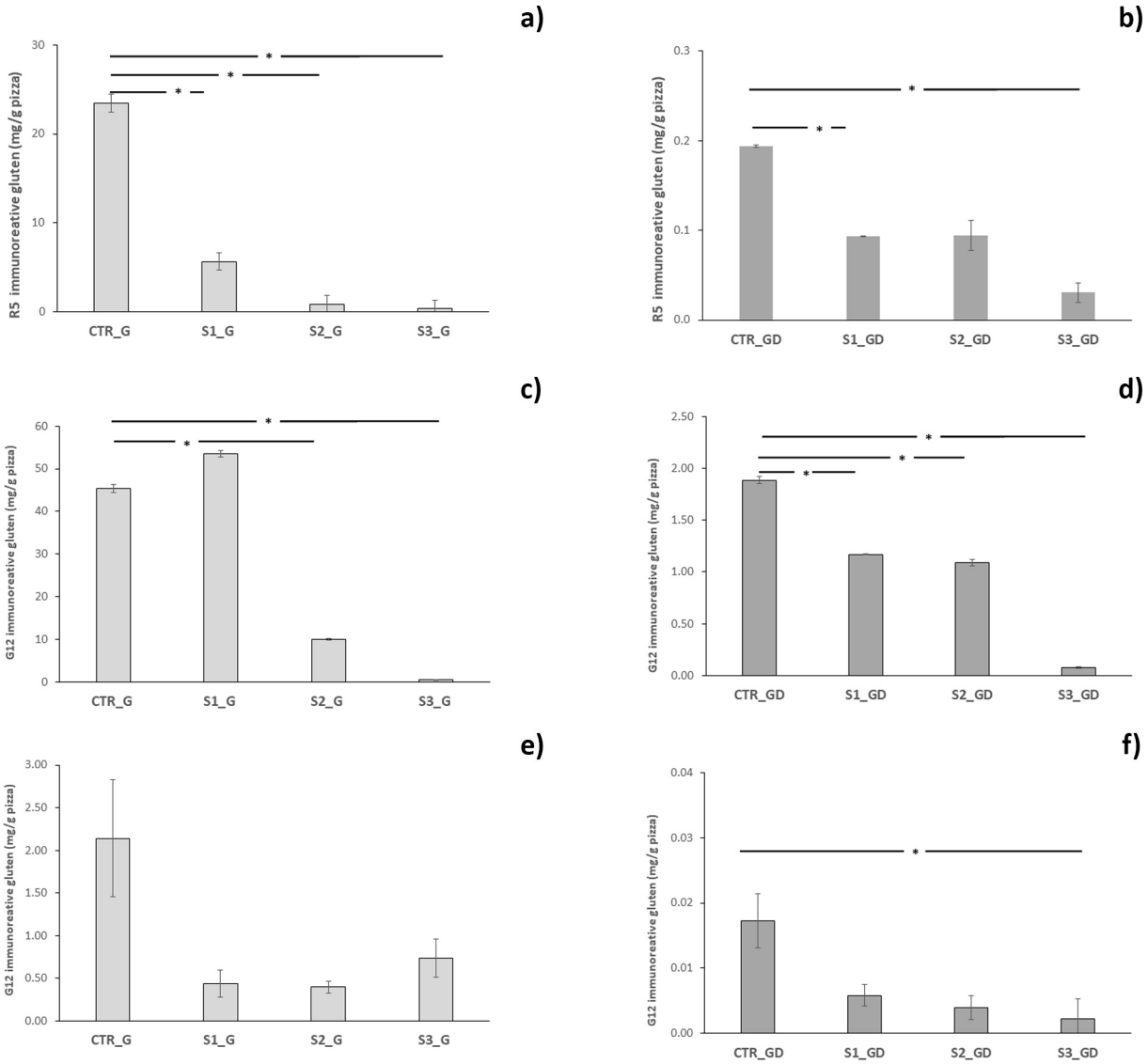


Fig. 3. Evaluation of gluten peptides amount at the end of G and GD digestion. Ppm values obtained as described in Table 2 were multiplied by the total volumes of soluble/total weight of insoluble fractions recovered for each reaction, then normalized for 1 g of pizza**.** Results are shown as mean ± SD of duplicate digestion experiments (mg of R5-gluten/g of pizza). * $p < 0.05$ by paired *t*-test. **a-b)** Residual R5-immunoreactive soluble peptide after gastric (**a**) or gastro-duodenal (**b**) digestion. **c-d)** Residual G12-immunoreactive soluble peptide after gastric (**c**) or gastro-duodenal (**d**) digestion. **e-f)** Residual G12-immunoreactive peptide after gastric (**e**) or gastro-duodenal (**f**) digestion in insoluble pellet.

production revealed that samples derived from gastric digestion in the presence of S1 had stimulatory capability similar to the CTR_G digest, whilst S2 and S3 treated samples stimulated iTCLs to a lower extent (Fig. 4a). At the end of gastroduodenal digestion, the soluble fraction from CTR_GD digestion retained the ability to stimulate the iTCLs, although with lower potency compared to CTR_G, as expected. Under the action of the three supplements, no residual immunogenicity was found at the end of the GD digestion phase.

Finally, data were validated on 6 different iTCLs (Fig. 4 c, d). Results confirmed S1 supplementation reduced the iTCLs response only at the end of GD digestion (87 % decrease vs CTR_GD, $p = 0.024$), whilst at the end of G phase, a very limited (12.5 % decrease vs CTR_GD, p = ns) effect was observed. S2 treatment leads to a moderate reduction (25 % decrease vs CTR_G, 69 %, decrease vs CTR_GD, p = ns). Finally, S3 addition resulted in a more significant IFN-γ reduction at the end of G digestion (55 % decrease vs CTR_G), which was further reduced after GD digestion (93 % decrease vs CTR_GD).

Overall, data from iTCLs assay demonstrate that the addition of supplements to the digestion of 5 g of pizza, results in varying degrees of reduction in immunoreactivity. However, substantial elimination of immunostimulatory potential is only seen at the end of GD digestion, by which time it is expected that gluten peptides would have had ample time to interact with the proximal small intestinal mucosa. In contrast, after 2 h of digestion at G condition, none of the supplements were able to abolish immunogenicity.

4. Discussion

Oral enzymatic therapy based on endopeptidase containing supplements has been proposed to reduce adverse reactions to gluten in patients with CeD, in particular to assist patients at gluten free diet who inadvertently may consume these proteins (Discepolo et al., 2024; Kivelä et al., 2021). In addition to specific glutenases currently being studied in clinical trials (Discepolo et al., 2024), a growing number of enzyme supplements are already available without prescription to consumers, representing a potential hazard for patients (Krishnareddy et al., 2017). Very few studies have investigated their hydrolytic efficacy on gluten proteins, particularly in a real-word food matrix (Freitas et al.,

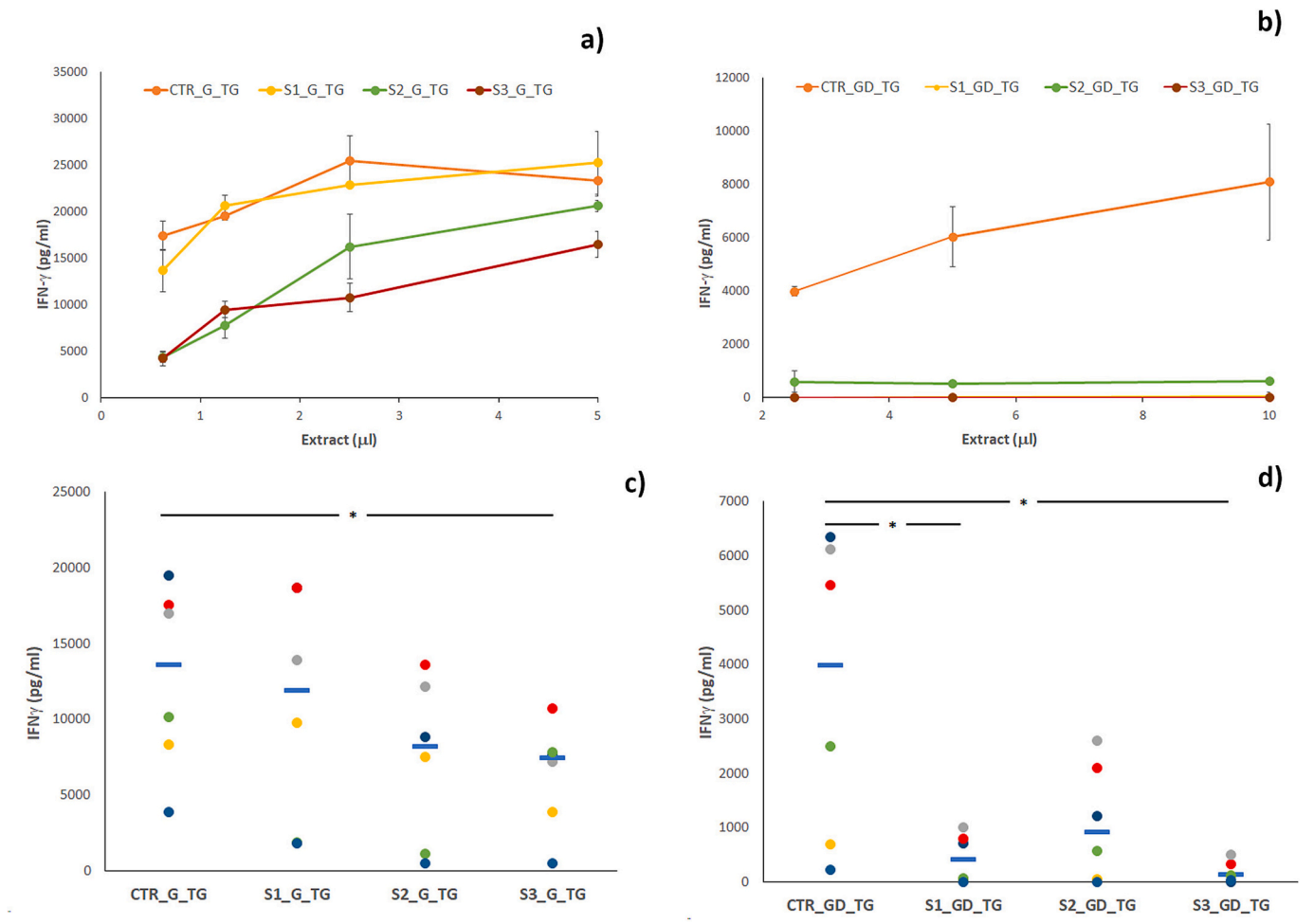


Fig. 4. Immunogenic activity of digested pizza extracts on gliadin-specific intestinal T cell lines. **a-b)** Response of iTCL#1_GFD to increasing doses of deamidated G and GD digestion extracts. T cells (3×10^4) were stimulated with deamidated G **(a)** or GD peptides extracts, at the indicated doses (**b**). Irradiated DQ2.5^{pos} EBV-B cells (1×10^5) were used as APC. Culture supernatants (50 µl) were collected after 72 h for determination of IFN-γ by sandwich ELISA. Mean ± SD of duplicate wells is reported. **c-d)** Response of polyclonal iTCLs to deamidated G or GD peptides extracts. T cells were stimulated as described in (**a**) with deamidated G (5 µl/well, panel **c**) or deamidated GD peptides extracts (10 µl/well, panel **d**) followed by IFN-γ evaluation. Bars represent median values of $N = 6$ iTCLs. * $p < 0.05$ by paired *t*-Test vs relative CTRs.

2022; Janssen et al., 2015; Tanner, 2021).

From a methodological point of view, the evaluation of the effects of gluten digestion, in relation to its immunological properties for CeD and other gluten-related disorders, needs to account for a series of factors that can influence results, such as the food matrix, processing and structure (Costantini et al., 2022; Marengo et al., 2022), the in vitro digestion protocol (Mamone et al., 2015), the detection methods (Bruins Slot et al., 2016; Rzychon et al., 2017) and the type of in vitro, ex-vivo or in vivo functional assays (Camarca et al., 2009; Dotsenko et al., 2023; Gianfrani et al., 2015; Mandile et al., 2017; Stoven et al., 2013).

In this context, we have used a holistic approach, based on the harmonized INFOGEST digestion protocol, evaluation of wheat protein hydrolysis by biochemical analysis, estimation of potential toxicity by R5 and G12 immunoassays and functional assessment of residual stimulating capacity on gut T cells, to address the fate of gluten after digestion of a standardized pizza, by commercial digestive supplements with different compositions.

We demonstrated that the applied analytical pipeline was effective in discriminating between enzymes with different glutenase activities, directly on food matrix. Indeed, SDS-PAGE supplied indication of protein hydrolysis (including gluten and other wheat proteins, as well as enzymes themselves) and allowed for discrimination of supplements containing gastric-resistant enzymes. Subsequently, evaluation of NOPA release allowed to estimate the kinetics of proteins hydrolysis, suggesting that the presence of microbic amylases and glucoamylase (in S1 and S2) has an obvious effect on total proteins digestion. These results agree with previous reports demonstrating that starch degrading enzymes, being active at acid pH values (pH 3.5–5.0), can improve protein digestion (Ragunathan & Swaminathan, 2005; Smith et al., 2015). On the other side, at the end of gastric digestion, the higher NOPA content correlated to a lower total protein, further suggesting higher proteolytic activity for S1 and S2, compared to S3 and CTR. Notably, although S1 and S2 exhibited the highest rates of proteolysis, S3 was the sample that showed the lowest indicators of gluten toxicity (R5, G12, and IFN-γ). This suggests that a higher degree of proteolysis is not necessarily connected with reduced gluten toxicity.

When we assessed kinetics of gluten hydrolysis using R5-based competitive ELISA assays, all the supplements enhanced R5 peptide hydrolysis, compared to CTR digestion, and no clusters were identified. Kinetics from the intestinal phase suggested that, despite DPP-IV in S1 and S2, digestion in this phase seems not to be accelerated, under our experimental conditions. Nevertheless, gluten hydrolysis continued also at intestinal condition, and supplemental enzymes still affected the final amount of R5-peptide, likely because they reduced the load of proteins coming from the gastric phase, and increased exposure of proteins to INFOGEST proteases.

Further characterization of peptides resistant to gastric or gastro-duodenal digestion, was conducted in parallel experiments, stopped at T120G and T120GD, avoiding possible bias deriving from sampling at different timepoints from the same batch. In this phase, the residual gluten content was determined not only by R5-competitive ELISA, but also by G12-based ELISA, in both competitive and sandwich formats. In this respect it is important to notice that, according to its specificity (Kahlenberg et al., 2006; Méndez et al., 2005), R5 reacts with high sensitivity against several γ-gliadin peptides immunogenic for CeD patients (listed in Sollid et al., 2020), but has a reduced sensitivity to epitopes of α-gliadin 57–89 peptide (33-mer immunodominant peptide) (Moreno et al., 2016). At the contrary, the G12 antibody, recognizes sequences (Morón et al., 2008a; Morón et al., 2008b), that are present in most α-and ω-gliadin-derived celiac T cell epitopes (Sollid et al., 2020). In addition, while the competitive ELISA format has been optimized for hydrolyzed gluten, the sandwich ELISA format, requiting two antibodies binding sites, is more selective for the quantification of undigested/partially digested gluten proteins (Galera et al., 2023).

The overall results showed that enzymes vary significantly in their ability to digest immunogenic gluten peptides, with the *An*-PEP containing supplement S3 showing the highest peptide detoxification activity, as detected by both ELISA methods. Supplements S1 and S2, although showing high proteolytic activity in biochemical analysis, showed higher residual levels of gluten immune-toxic sequences.

In general, gluten ppm values calculated from G-12 ELISA were higher compared to R5 detected values. In addition, for some samples, R5 and G12 assays provided completely different results, as found for S1_G samples, for which G12 reveals no decrease compared to CTR, in contrast to R5 results. Such differences could be explained by intrinsic differences of the two ELISA methods and/or by different proteolytic activity of the supplementary enzymes on the specific epitopes detected by the assays. Indeed, a wide variation in the results between different commercially available test kits (Bruins Slot et al., 2015; Diaz-Amigo & Popping, 2012) has been reported. Several factors have been discussed to explain such differences, including variations in sample extraction protocols, antibodies specificities and cross-reactivity, interference with antibody binding and calibration procedures (Rzychon et al., 2017; Slot, van der Fels-Klerx, Bremer, & Hamer, 2016; Diaz-Amigo & Popping, 2012). Data from G12-sandwich were of particular importance, since they excluded that results on soluble fractions were distorted by undigested gluten present in the amylaceous material, abundant especially in the S3-treated samples, as S3 does not include supplementary amylase/glucoamylase.To validate ELISA-derived results and to address weather digested gluten peptides were still able to stimulate gluten-specific CD4 T cells, both in terms of quality and quantity, CeD-derived iTCLs were used. From a qualitative point of view, IFN-γ production data demonstrated that soluble extracts contained peptides long enough to bind DQ2 molecules and stimulate T cells, especially at the end of G phase. From a quantitative point of view, at T120G, all soluble extracts were able to activate TCLs, even when diluted up to 200 fold in culture medium (Fig. 4). Although these findings are difficult to translate to in vivo condition, they suggest that a few grams of pizza could be still immunogenic for CeD patients, even if supplementary enzymes were used.

More in detail, data derived from iTCLs assays confirmed relatively higher activity of S3 compared to S1 and S2. Of note, despite we observed a relationship between the decrease in gluten quantification via immunoassay and the IFN-γ production by iTCLs, there was not a direct correlation in terms of percentage decrease obtained with the different methods. This discrepancy could be explained by different specificities among the R5 and G12 antibodies, and the epitopes recognized by polyclonal iTCLs (Sollid et al., 2020). Indeed, we have previously reported that iTCLs may be focused on one/few immunodominant peptides or can be largely heterogeneous, reflecting the diversity of gliadin-specific clones present in the mucosa and/or the number of clones that expands in vitro (Camarca et al., 2009). Even though in this study the pattern of specific immunogenic peptides recognized by iTCLs has not been determined, iTCLs are more likely polyclonal because they were used soon after the expansion (after 2–3 stimulation rounds).

More specifically, results showed that S3-treatment was able to strongly reduce the IFN-γ production, in comparison to CTR, especially at the end of GD digestion. Importantly, we also demonstrated that this reduction was not due to a cytotoxic effect of the peptides-extract on lymphocytes. At the contrary, results obtained with S1_GD and S2_GD samples were different, despite from immunochemical analysis these samples were similar to each other. Differences could be explained by a certain toxicity observed for S1_GD but not for S2_GD.

Concerning the gastric digestion, we observed a significant reduction of the IFN-γ production only in presence of S3. Nevertheless, even in this sample, response of iTCLs was reduced but still present. Differences between our results and previous studies reporting that *An*-PEP abolished T cell response in vitro (Mitea et al., 2008; Stepniak et al., 2006), could be explained considering that the specific formulation used in this work has never been studied, in particular on a food matrix.

Overall, limitations of the study are mainly due to the fact that, while commercial ELISA kits are commonly used to study gluten digestion, they require dilution steps that may introduce some degree of quantitative uncertainty. Additionally, the individual response of iTCLs can vary, depending on their peptide specificity profiles and the relative dominance of gliadin-reactive versus non-specific T cell clones into the culture. Nevertheless, since both immunochemical and T cell based assays were used to compare supplemented versus control digests, and not for absolute quantification, the relative comparisons reported remain valid.

5. Conclusions

The methodological approach presented in this study has proven to be a robust in vitro tool to assess the ability of enzyme supplements/glutenases, to mitigate immune toxicity of gluten peptides, within a complex food matrix.

Since some differences were found within the two immunochemical methods and between them and iTCLs activation, we suggest using more than one assay, for unraveling the immunogenicity of digested food.

Although all tested supplements show varying degrees of efficiency depending on their composition, none was able to fully eliminate the immunogenic potential, measured by immunochemical and/or T cell based assays, of an amount as small as five grams of pizza, in particular at the end of gastric digestion, suggesting caution for use in patients with CeD.

CRediT authorship contribution statement

Alessandra Camarca: Writing – original draft, Methodology, Investigation, Conceptualization. **Giovanni D'Auria:** Writing – original draft, Methodology, Investigation. **Vera Rotondi Aufiero:** Methodology, Investigation. **Chiara Nitride:** Methodology, Investigation. **Nicola Giardullo:** Methodology, Investigation. **Anna Sapone:** Conceptualization. **Daniel Leffler:** Writing – review & editing, Conceptualization. **Pasquale Ferranti:** Writing – review & editing, Funding acquisition, Conceptualization. **Giuseppe Mazzarella:** Writing – review & editing, Funding acquisition, Conceptualization.

Ethical statement

The study was approved by "Campania Nord" Ethical Committee of S.G. Moscati Hospital, Avellino, Italy (Register number CECN/819 dated 03/21/2018).

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Declaration of competing interest

The authors declare that Takeda Pharmaceuticals is not involved in the production of the enzymes tested in the study. Dan Leffler is an employee of Takeda and holds stock and/or stock options in the company. He was involved in the conceptualization of the study and in manuscript review and editing. At the time of the study conceiving and conduction Anna Sapone was employed at Takeda Pharmaceuticals. She was involved in the conceptualization and interpretation of data. The remaining authors declare that the research was conducted in the absence of any financial, personal interest or belief that could have affected their objectivity during the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2025.117027.

Data availability

No data was used for the research described in the article.

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