In Vitro Hydrolysis Study Review
and Claims Substantiation for OPTIZIOME® G-D4
February 8, 2023

1. Introduction

The purpose of this report is to summarize *in vitro* studies of the proprietary **OPTI**ZIOME® G-D4 fungal protease blend and its proteolytic activity on gluten proteins, as compared to clinically tested competitor protease blend products. These data provide support for claims related to gluten digestion.

2. Executive Summary

BIO-CAT developed a proprietary blend of two distinct fungal protease preparations to support gluten digestion. The BIO-CAT branded product containing this protease blend is named **OPTI**ZIOME® G-D4 (Glutenase with Dipeptidyl peptidase-4 activity, hereafter referred to as "G-D4"). G-D4 was studied under acidified or simulated gastric conditions in vitro and shown to digest proteins from whole wheat bread. Altogether, these data support the following claims for G-D4:

- Supports gluten digestion[*,**,†] (can add "wheat" before gluten)
- Helps digest gluten[*,**,†]
- Helps break down gluten[*,**,†]
- Hydrolyzes gluten[*,**,†]

*This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.
**This product is not intended to replace a gluten-free diet or treat or prevent celiac disease.
[†]Based on standard in vitro gastric digestion experiments that showed enhanced amino acid liberation from whole wheat bread

3. Background

Glutens are a group of seed storage proteins found in cereals (Schalk 2017, Lexhaller 2018). Gluten plays a role in aggravating the gastrointestinal (GI) tract in patients with celiac disease. The estimated prevalence of celiac disease is 1% across the general population. Celiac disease is a gluten-induced, immune-mediated enteropathy associated with specific human genetic variants. Celiac disease induces an inflammatory process along the intestinal mucosal lining, and commonly presents with symptoms related to GI distress. NCGS, on the other hand, occurs in otherwise healthy individuals who do not have diagnosed celiac disease or wheat allergy, and experience milder symptoms of GI distress related to consumption of gluten-containing grains (Leonard 2017). The symptoms of NCGS are highly variable, including abdominal bloating, abdominal pain, flatulence, and diarrhea, symptoms also common to irritable bowel syndrome. Other intestinal manifestations may include nausea, acid reflux, mouth ulcers, and constipation (Lebwohl 2015; Volta 2014). One review estimates that NCGS impacts up to 6% of the population in the United States (Igbinedion 2017), while others have reported that NCGS may occur in up to 13% of the population (Molina-Infante 2015).

The innate immune response associated with NCGS is triggered by gluten and more specifically alpha-gliadin, a protein that occurs in the alcohol-soluble fraction of gluten (Sharma 2020). Alpha-gliadin digestion yields a number of well-characterized peptides that can trigger an immune response in humans. Particularly immunogenic in some individuals are peptides arising from digestion of a 33-peptide long, glutamine- and proline-rich peptide commonly referred to as the "33-mer" (Lexhaller 2018). Gliadin digestion can be inspected in vitro by measuring the release of free amino acids, and especially free glutamine (Gln) and proline (Pro).

Enzyme supplementation is a well-established practice to help reduce GI symptoms associated with food intolerance. Examples include the use of beta-galactosidase, or lactase, to help digest lactose and reduce symptoms associated with dairy or lactose intolerance (DiPalma 1989; Lin 1993) and the use of alpha-galactosidase (found in the dietary supplement Beano®) to help digest complex carbohydrates and reduce gas and bloating associated with the consumption of legumes and other high-fiber foods (Ganiats 1994; Di Stefano 2007; Di Nardo 2013).

Likewise, proteases that preferentially or effectively promote the digestion of gluten proteins present as candidate ingredients for dietary supplements to help break down gluten. In particular, proteases with high dipeptidyl peptidase-4 (DPP-IV) activity are predicted to be effective at hydrolyzing peptide bonds around proline, an amino acid that occurs at high levels in gluten proteins and immunogenic gluten peptides. DPP-IV activity is measured utilizing glycine-proline p-nitroanilide as a substrate. Proteases with DPP-IV activity cleave the p-nitroanilide bond and liberate the p-nitroaniline component. This hydrolysate, in turn, linearly absorbs light at 405 nm wavelength. This assay enables the assignment of DPP-IV activity units to a commercial protease.

As such, BIO-CAT developed a proprietary protease blend named G-D4 ("Glutenase" with Dipeptidyl peptidase 4 activity) that targets digestion of gluten. G-D4 is comprised of a protease, [redacted] from *Aspergillus* [redacted], (peptidase), [redacted] from *Aspergillus* [redacted], and tapioca dextrin (**Table 1**). In this report, we show that under acidified conditions simulating gastric pH *in vitro*, an experimental dose of 64 mg of concentrated G-D4 with total 1,100 DPP-IV activity hydrolyzed an estimated 3 g of wheat protein better than control samples without G-D4 based on indirect immunolabeling methods. In a follow up experiment, the equivalent of 1,500 DPP-IV of G-D4 was sufficient to significantly release free amino acids form a whole wheat bread slurry better than control conditions. The statistically significant results from these *in vitro* studies of G-D4 support claims around gluten digestion.

Disclaimer: G-D4 has not been evaluated in a clinical trial and is not intended to replace a gluten-free diet or treat or prevent celiac disease.

Table 1. G-D4 formulated to contain (15,000 DPP-IV/g) standardized specification not less than (14,500 DPP-IV/g)

Ingredients	Source Organism	Enzyme Activity (unit)	Lot Used for Testing
[redacted]	*Aspergillus* [redacted]	14,500 DPP-IV/g	[redacted]
	Aspergillus [redacted]	500 DPP-IV/g	
Tapioca dextrin			

DPP-IV, dipeptidyl peptidase-4 activity

4. In Vitro Study Review for Claims Support: Experiment 1

Methods

A single gluten protein source, NUTRIOR™ soluble wheat gluten hydrolysate was obtained from Nouriche Nutrition Ltd (O'Fallon, MO) to test the gluten-digesting activity of G-D4. Fifty grams NUTRIOR™ was added to 450 ml deionized water, and the resulting wheat slurry was adjusted to pH 4 and a final volume of 500 mL. Nouriche product information sheets indicate that NUTRIOR™ contains mimimum 78% crude wheat protein. Approximately 80% of wheat protein is gluten (Geisslitz 2018; Geisslitz 2019), so the resulting 500 mL wheat slurry was approximately 6.2% gluten with approximately 31.2 g gluten proteins:

$$\left[\frac{50\text{ g}}{\text{NUTRIOR}^{\text{TM}}} \times \frac{78\text{ g wheat protein}}{100\text{ g NUTRIOR}^{\text{TM}}} \times \frac{80\text{ g gluten}}{100\text{ g wheat protein}}\right] \div 500\text{ mL} = \frac{6.24\ \%\text{ gluten}}{(31.2\text{ g gluten per }500\text{ mL})}$$

The average daily gluten intake in a Western diet is estimated at 5-20 g/day (Biesiekierski 2017; Hoppe 2017). Note that two typical slices of wheat bread contain about 8 g total gluten. After a meal, gastric volume rises up to 500 mL (Malagelada 1977; Burton 2005; Kwiatek 2009), yielding chyme that is predicted to be at least 1.6% gluten following consumption of a sandwich with two pieces of wheat bread. Thus, the estimated 6.2% gluten slurry used in our experiment reflects a high dietary load of gluten during a meal, compared to two slices of wheat bread.

Six tubes, four per G-D4 experimental group and two per negative controls without enzyme, were prepared to contain 40 mL of the wheat protein slurry containing 3.12 g crude protein, and an estimated 2.5 g gluten proteins. All tubes were allowed to equilibrate in a 37 °C water bath for 10 minutes. Temperature was held constant throughout all experimentation.

The legacy BIO-CAT R&D digestion model is a static gastric phase model utilizing an artificial gastric solution described in 2014 (Donhowe et al.). This gastric solution is based on highly cited publications by Hur et al in 2009 and Versantvoort et al in 2005. The Hur et al publication has been cited by over 200 scientific publications that describe in vitro modeling of nutrient digestion and bioavailability. The simulation typically includes use of an artificial gastric solution that contains porcine pepsin to model typical pepsin levels in the human stomach following a meal. Other standard conditions in the gastric simulation include incubation at human body temperature, continuous stirring to mimic peristaltic contractions, 1-3 hour duration to mimic gastric emptying time, and acidified pH between 1.5 and 5.0. Pepsin, however, was not used in this test in order to gain a better understanding of G-D4 activity alone, without the added endopeptidase activity from pepsin. G-D4 activity, without pepsin, would be an even more compelling result since this gastric simulation is lacking the addition of pepsin endopeptidase. Moreover, the starting substrate was a wheat protein hydrolysate that had already undergone proteolysis during manufacturing.

The gluten slurry digestion experiments included one experimental protease formulation (i.e., G-D4) and one negative control devoid of enzymes. G-D4 was weighed on tared weigh paper and then transferred to tubes using deionized water at 0.64 grams G-D4 per 10 mL.

During preparation, one mL of G-D4 enzyme solution (64 mg of concentrated G-D4 with 1100 DPP-IV activity) was added to 40 mL of the 6.2% gluten slurry. For the negative control, one mL deionized water (instead of enzyme) was added to the slurry. The treated slurry was incubated at 37 ^{o}C and pH 4 for 60 minutes to mimic gastric conditions. After incubation, experimentation was stopped by holding slurry at 90 °C for 15 minutes to inactivate enzymes. Thirty mL samples were collected and frozen for enzyme-linked immunosorbent assay (ELISA).

Wheat slurry hydrolysate samples were shipped on ice to Eurofins GeneScan (New Orleans, LA) to perform an R5 ELISA-based quantitative test to determine the presence of gluten (R-Biopharm kit R7001, sandwich method). The ELISA method utilizes a single monoclonal antibody called R5, or “Rye 5”, derived from ethanol-extracted rye injections of BALB/c mice (Sorell 1998; Osman 2001; Valdes 2003). The R5 antibody detects prolamins such as hordeins, secalins and gliadins from barley, rye, wheat and other cereal grains and strongly binds the glutamine-glutamine-proline-phenylalanine-proline (QQPFP) epitope and several homologous epitopes including: leucine-glutamine-proline-phenylalanine-proline (LQPFP), glutamine-leucine-proline-tyrosine-proline (QLPYP), glutamine-glutamine-serine-phenylalanine-proline (QQSFP), glutamine-glutamine-threonine-phenylalanine-proline (QQTFP), PQPFPF, QQPYP, and PQPFP, some of which occur in the 33-mer peptide of alpha-gliadin (Kahlenberg 2006; Thompson 2008). Determination of sample protein content was performed using spectrophotometric (450 nm) readouts. Readouts were tabulated, consolidated and used as raw data for statistical analysis (**Table 2**). Upper and lower limits of detection for quantitative

tests are 80.0 ppm and 3.0 ppm, respectively. Eurofins performed a single R5 ELISA test on each of the 2 different control samples, and the 4 different G-D4 samples.

Table 2. Dataset for statistical analysis[†]

ID	Treatment	ppm Gluten	ID	Treatment	ppm Gluten
CI90734	Control	> 80.0	CI90736	G-D4	14.0
CI93145	Control	78.0	CI93146	G-D4	6.0
			CI93147	G-D4	5.4
			CI93148	G-D4	7.9

[†]Upper and lower limits of detection for quantitative tests are 80.0 ppm and 3.0 ppm, respectively.

Statistical Analysis

Statistical analysis was performed using R® version 3.6.2 (R Core Team, 2020). Figures were produced using the gglot2 package (Wickham, 2016). A t-test was performed to determine significance and an p value of $p < 0.05$ was considered statistically significant. Data were imputed at implied upper limit of detection for the Control treatment. All observations are expressed as mean ± standard deviation (**Table 3**). A pairwise t-test was performed to determine statistical significance (**Table 4**).

Results

The negative control without enzymes did not hydrolyze gluten protein below the 80 ppm upper limit of detection in one sample, and with imputation, the average gluten content across the two control samples was 79.0 ± 1.4 ppm, **Figure 1, Table 3**). G-D4 significantly reduced gluten as compared to the control group (8.3 ± 3.9 ppm vs. 79.0 ± 1.4 ppm, $p < 0.001$, **Figure 1, Table 3, Table 4**). All individual sample-level data are provided in the appendix.

Table 3. Summary of Gluten Hydrolysis

Treatment	Mean ± SD (ppm Gluten)
Control	79.0 ± 1.4
G-D4	8.3 ± 3.9

Table 4. T-test comparison

Comparison	P value
Control – G-D4	< 0.001

Implications

As per the final rule on 'Gluten-Free Labelling of Fermented or Hydrolyzed Foods' 21 CFR 101 as issued by the FDA, the FDA knows of no scientifically valid analytical method effective in detecting and quantifying with precision the gluten protein content in fermented or hydrolyzed foods in terms of equivalent amounts of intact gluten proteins. The regulation as referred to above became effective 13 October 2020. The compliance date of this final rule was 13 August 2021.

Moreover, FDA set a 20 ppm upper limit on gluten content for "gluten-free" labeling of foods (FDA 11 CFR 201). While G-D4 reduced gluten below this 20 ppm gluten from a wheat protein hydrolysate slurry, these results do not provide any substantiation for "gluten-free" labeling of foods that G-D4 may be combined with. In addition, the R5 ELISA method used to test G-D4 is not an FDA approved method to quantify gluten proteins. These data contained herein support general claims around gluten digestion based on in vitro testing under acidified conditions. G-D4 has not been evaluated in a clinical trial and is not intended to replace a gluten-free diet or treat or prevent celiac disease.

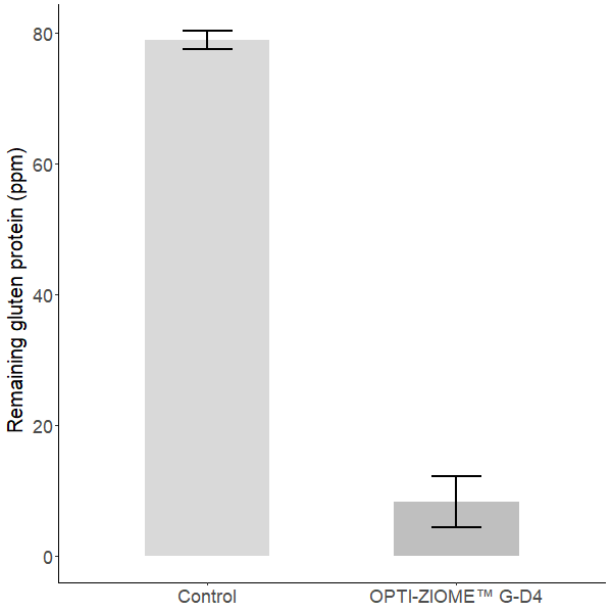

Figure 3. Parts per million of remaining gluten protein following wheat slurry digestion in an *in vitro* gastric model. Negative control (left), G-D4 (right).

5. In Vitro Study Review for Claims Support: Experiment 2 (BIO-CAT Technical Report No. BCTR0012)

Methods

The INFOGEST simulation of salivary-gastric (SG) and salivary-gastric-intestinal (SGI) digestion was used to investigate the efficacy of G-D4 on gluten protein hydrolysis from a slurry of whole wheat bread. The INFOGEST protocol has been extensively described elsewhere (Minekus 2014; Brodkorb 2019), and it has been adapted for the study of exogenous enzymes (Garvey et al.). Briefly, the full INFOGEST protocol models three phases of digestion – salivary, gastric, and intestinal. The salivary phase entails mixing 25 g bread slurry with simulated salivary fluid (SSF) and incubating for 2 min with 300 rpm agitation at 37°C at neutral pH in the presence of porcine salivary amylase. The gastric phase entails mixing of the salivary digesta with simulated gastric fluid (SGF) for 2 hrs with 300 rpm agitation at 37°C at a starting pH of 3, in the presence of porcine pepsin. The intestinal phase entails mixing of the gastric digesta with simulated intestinal fluid (SIF) for 2 hrs with 300 RPM agitation at 37°C at a starting pH of 7, in the presence of porcine pancreatin and porcine bile extract.

Note 1,500 DPP-IV G-D4 is recommended per serving of food. To reduce volumes during experimentation, a 1/3 recommended dose of G-D4 (Lot No. OPT2GD4-NE17) corresponding to ~ 500 DPP-IV was added to the gastric digesta 10 min following the start of gastric digestion of 1/3 serving size of whole wheat bread (Nature's Own® 100% Whole Wheat Bread (Lot No. 19147). The 10-min delay until inclusion mimics the dissolution time of a standard hydroxypropylmethylcellulose capsule in the stomach. At the end of the gastric phase, a 10 mL sample was removed and transferred to a conical tube that was placed in a 90°C water bath for 10 min to halt enzymatic activity. The samples were stored at 4°C until further analysis. The remainder of gastric digesta continued to advance to the intestinal simulation, which began with the addition of simulated intestinal fluid (SIF) containing porcine pancreatin and porcine bile extract to the gastric digesta. The intestinal phase proceeded for 2 hrs with 300 rpm agitation at 37°C at a starting pH of 7. At the end of the 2-hour intestinal phase, samples were withdrawn and transferred to conical tubes and placed

in a 90°C water bath for 10 min to halt enzymatic activity. The samples were stored at 4°C until further analysis. Experiments were repeated in triplicate on 3 separate days (n = 3).

NOPA Procedure: Concentration of Primary (Free) Amino Nitrogen

The nitrogen by o-phthaldialdehyde assay (NOPA) was used to evaluate protein digestion and free amino acid nitrogen release, which is used as a marker of amino acid release. In the NOPA assay, the sample's proteins' amino nitrogen groups of free amino acids react with NAC and OPA to form isoindole derivatives. The amount of isoindole derivative formed in the reaction is stoichiometric with the amount of free amino nitrogen (FAN). The isoindole derivative is measured by the increase in absorbance at 335 nm. NOPA results are reported in mg of free nitrogen per g of bread slurry (mg N/g).

High-Pressure Liquid Chromatography (HPLC)

Amino acids were directly measured using an Agilent 1100 Series HPLC with fluorescence detection, using a Zorbax® Eclipse Plus C18 4.6 x 150 mm column at 40°C, and flow rate of 1.5 mL/min (gradient started with 98% 10 mM sodium phosphate:10 mM sodium tetraborate:5 mM sodium azide, pH 8.2 and 2% 45:45:10 acetonitrile:methanol:water and ended with 100% 45:45:10 acetonitrile:methanol:water). Standards and samples were prepared in 0.1 N HCl. Amino acids were derivatized online after buffering using 0.4 M borate buffer at a pH of 10.2, which allows direct derivatization of hydrolyzed proteins and peptides. The primary amino acids were reacted first with OPA using 3-mercaptopropionic acid (3-MPA). The secondary amino acids do not react with OPA but are then derivatized using 9-fluorenylmethyl chloroformate (FMOC). Results are reported in mg amino acid per gram of bread slurry. The results reported here are the average of the values measured from triplicate digestion experiments for G-D4 treated digestive simulations.

Statistical Analysis

FAN, total amino acids (TAA), and individual amino acids were analyzed by unpaired t-test. Normality was assessed by the Shapiro-Wilk test on residuals and by QQ-plot. Homogeneity of variance was confirmed using the Brown-Forsythe test and homoscedasticity plot. The significance level was set at 5% ($p \leq 0.05$) for all analyses. Statistical analyses were performed and figures were generated in GraphPad Prism version 9.2.0 for Windows (GraphPad Software, Inc.; San Diego, California USA).

Results

Gliadin is one of the main components of the protein gluten which is found strictly in wheat products like bread, pasta, and even beer. The two predominant gliadins found in gluten—α-gliadin and γ-gliadin—are made up of 286 and 251 amino acids, respectively. Further examination of the amino acid content of these gliadins shows that there are three primary amino acids, glutamine (Gln), leucine (Leu), and proline (Pro). Because these three amino acids make up more than 50% of gliadin proteins, hydrolysis and the release of smaller peptides and free amino acids would likely render the gliadin protein and related peptides more GI-friendly for consumption in individuals with NCGS or gluten intolerance.

G-D4 was tested in the INFOGEST static in vitro SGI gastrointestinal digestion simulation with a portion of a whole wheat bread slurry corresponding to 1/3 serving of bread and 1/3 the 1500 DPP-IV recommended dose of G-D4, and the results were reported using HPLC analysis for amino acids the NOPA method of measuring free amino nitrogen (FAN).

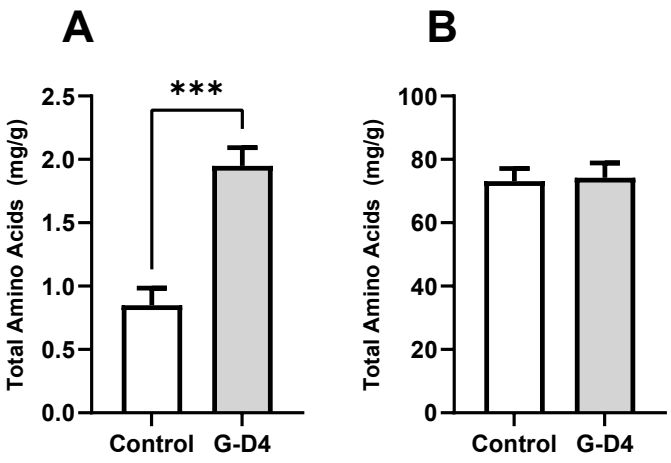

Figure 4. Total amino acid concentrations of gastric (A) and intestinal (B) digestas following simulated digestion of whole wheat bread. Error bars show ± standard deviation (n = 3). The significant difference between groups ($p < 0.001$) is denoted by asterisks.

All 20 amino acids found in dietary proteins (including glutens) were investigated by HPLC. Following salivary-gastric digestion simulation, the average concentration of the total, or sum, of all 20 individual amino acids (TAA) in the gastric digesta was 2.3-fold greater with G-D4 treatment than control with pepsin alone ($p = 0.0007$) (**Fig. 4A**). These data further suggest that G-D4 at a 1,500 DPP-IV dose per serving of bread enhances gastric digestion of glutens before transiting to the intestine. In the intestinal digestas following the full SGI simulation, TAA concentrations were no different between control conditions with pancreatin and G-D4 treatment plus pancreatin (**Fig. 4B**). These data suggest that G-D4 may not effectively hydrolyze gluten better than pancreatic enzymes in the small intestine in individuals with healthy digestive and pancreatic function. However, static modeling of intestinal digestion has extra limitations due to the more dynamic nature of movement through the small intestine compared to the stomach.

Analysis of the concentrations of the most abundant amino acids in gliadins—Gln, Leu, and Pro—in the digestas reasonably serves as a marker of gliadin hydrolysis. Following simulated salivary-gastric digestion, G-D4 promoted 11.6-fold ($p = 0.0047$), 3.3-fold ($p = 0.0044$), and 70% ($p = 0.0023$) greater release of Gln, Leu, and Pro, respectively, compared to controls (**Figs. 5A–C** & **Table 4**). Following total SGI simulation, G-D4-treated digestas showed no significant differences in Gln, Pro, and Leu concentrations compared to control (**Table 4**).

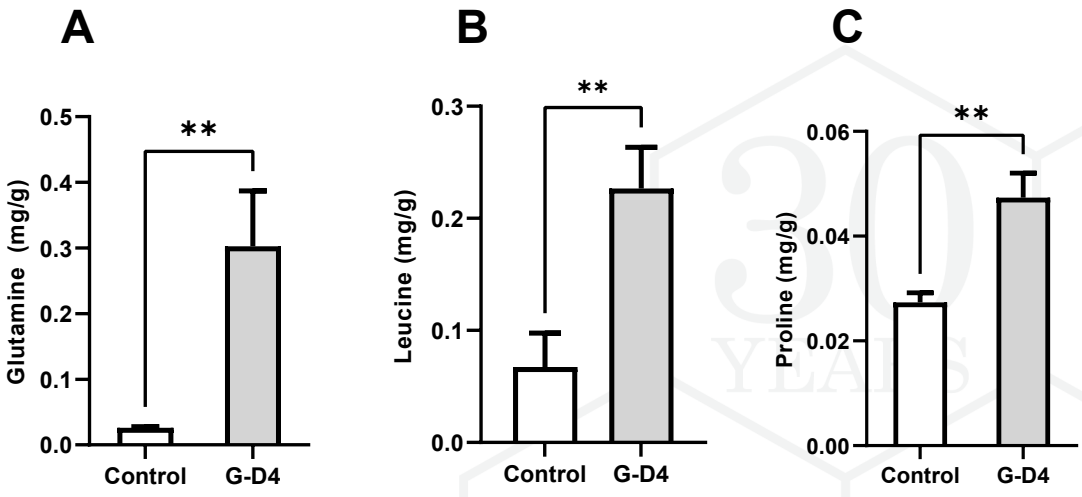

Figure 5. Glutamine (A), leucine (B), and proline (C) concentrations of gastric digestas following simulated digestion of whole wheat bread. Error bars show ± standard deviation (n = 3). Significant differences between samples are denoted by asterisks. **, $p < 0.01$; ***, $p < 0.001$.

Table 4. Amino acid and FAN concentrations of gastric and intestinal digestas following simulated digestion of whole wheat bread.[a]

Analyte	Digesta	Digesta concentrations		p value
		Control	G-D4	
TAA (mg/g)				
	Gastric	0.85 ± 0.14	1.95 ± 0.14	0.0007
	Intestinal	73.12 ± 3.99	74.25 ± 4.61	0.7657
Glutamine (mg/g)				
	Gastric	0.03 ± 0.00	0.30 ± 0.08	0.0047
	Intestinal	5.41 ± 0.29	5.84 ± 0.44	0.2221
Leucine (mg/g)				
	Gastric	0.03 ± 0.00	0.05 ± 0.00	0.0044
	Intestinal	5.76 ± 0.32	5.83 ± 0.43	0.8216
Proline (mg/g)				
	Gastric	0.03 ± 0.00	0.30 ± 0.08	0.0023
	Intestinal	0.59 ± 0.25	0.67 ± 0.27	0.7209
FAN (mg N/g)				
	Gastric	0.03 ± 0.03	0.32 ± 0.03	0.0003
	Intestinal	6.46 ± 0.53	6.71 ± 0.84	0.6862

[a]Data are mean ± standard deviation (n = 3 per group). Statistical analysis performed by individual unpaired t-tests. Significant differences denoted by $p < 0.05$.
Abbreviations: G-D4, **OPTI**ZIOME® G-D4 tested at the equivalent of 1,500 DPP-IV protease activity pers serving of bread; FAN, free amino nitrogen; TAA, total amino acids.

Switching gears from amino acid analysis to FAN analysis by spectrophotometry, G-D4 treatment resulted in 9.4-fold higher average gastric digesta FAN concentration compared to control ($p = 0.0003$, **Fig. 7A** & **Table 4**). Intestinal digesta FAN concentrations were comparable between G-D4 and control (**Fig. 7B** & **Table 4**).

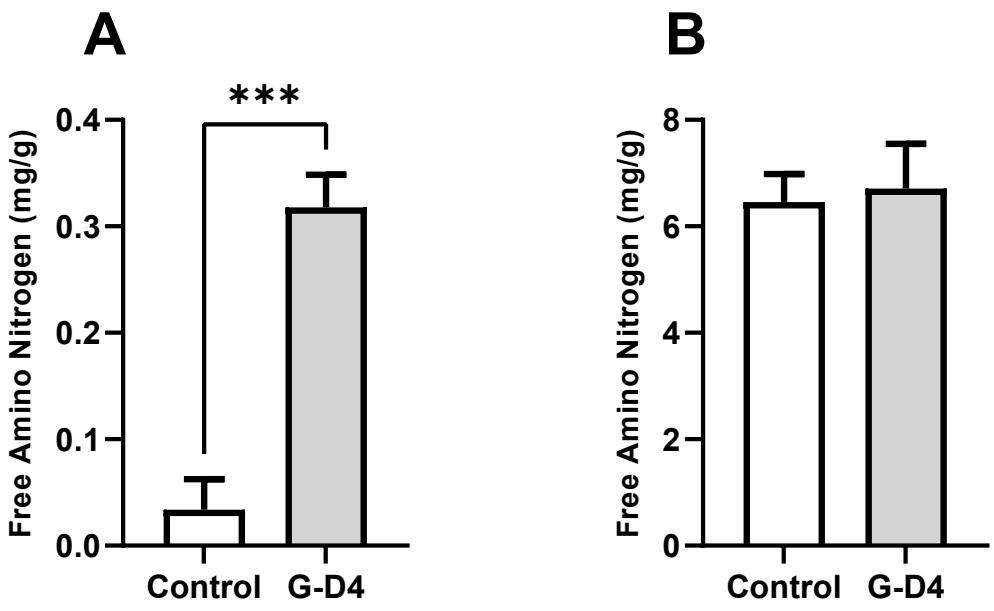

Figure 7. Free amino nitrogen concentrations of gastric (A) and intestinal (B) digestas following simulated bread digestion. Error bars show ± standard deviation (n = 3). ***, $p < 0.001$.

Conclusions

Altogether, these data suggest that G-D4 at the recommended 1,500 DPP-IV dose per serving outperforms pepsin control in multiple salivary-gastric simulations to support gluten digestion, based on enhanced liberation of total amino acids, glutamine, leucine, proline, and free amino nitrogen from glutens found in whole wheat bread.

6. Review any R&D laboratory results generated that will support claim substantiation

Claim	Results	Reference
Supports gluten digestion*,**,† (can add "wheat" before gluten) Helps digest gluten*,**,† Helps break down gluten*,**,† Hydrolyzes gluten*,**,†	A 1,100 DPP-IV dose of **OPTI**ZIOME® G-D4 was added to 40 mL of a 7.8% wheat protein slurry (at least 3.12 g crude protein, estimated 2.5 g gluten total) and incubated at 37 ºC for 60 minutes at a pH of 4 to model gastric pH. Hydrolysates were shipped to and tested at Eurofins with the R5 ELISA sandwich method. A negative control without enzymes (79.0 ± 1.4 ppm) alone did not hydrolyze gluten protein below the 80 ppm upper limit of detection. G-D4 reduced gluten abundance to 8.3 ± 3.9 ppm. G-D4 significantly reduced gluten abundance compared to the control group (8.3 ± 3.9 ppm vs. 79.0 ± 1.4 ppm, $p < 0.001$).	See Section 4 (BIO-CAT Data on File)
Supports gluten digestion*,**,† (can add "wheat" before gluten) Helps digest gluten*,**,† Helps break down gluten*,**,† Hydrolyzes gluten*,**,†	Following simulated salivary-gastric digestion of whole wheat bread, the average concentration of the total, or sum, of all 20 individual amino acids in the gastric digesta was 2.3-fold greater with **OPTI**ZIOME® G-D4 treatment than control with pepsin alone ($p = 0.0007$). G-D4 also promoted 11.6-fold ($p = 0.0047$), 3.3-fold ($p = 0.0044$), 70% ($p = 0.0023$), and 9.4-fold ($p = 0.0003$) higher average gastric digesta concentrations of glutamine, leucine, proline, and free amino nitrogen, respectively, compared to controls.	See Section 5 (BIO-CAT Data on File, BCTR0012)

*This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease.

**This product is not intended to replace a gluten-free diet or treat or prevent celiac disease.

†Based on standard in vitro gastric digestion experiments that showed enhanced amino acid liberation from whole wheat bread

7. Dietary Supplement Claims Framework

The Dietary Supplement Health and Education Act (DSHEA) states that a dietary supplement may bear certain statements on its label or in its labelling if the claims meet certain requirements.

Permitted structure/function statements are described in 21 CFR 101.93(f) as those statements that:

- Describe the role of a nutrient or dietary ingredient intended to affect the structure or function in humans;
- Characterize the documented mechanism by which a nutrient or dietary ingredient acts to maintain such structure or function; or
- Describe the maintenance of 'normal' or 'healthy' structure or function.

The final rule concluded that statements that mention a body system, organ, or function affected by the supplement using terms 'stimulate', 'maintain', 'support', 'regulate', or 'promote' can be appropriate when making structure/function claims.

Such claims are allowed provided such statements do not suggest disease prevention or treatment or use for a serious health condition or do not imply such disease relationships in any other manner (e.g. pictures on label, name of product). If the label or labelling of a product marketed as a dietary ingredient bears a disease claim, the product will be subject to regulation as a drug (with exception of FDA-approved health claims).

DSHEA requires manufacturers to meet three requirements for placing a structure/function claim on a supplement label:

- Substantiation that the claim is truthful and not misleading[1],
- Notification to FDA within 30 days of marketing the supplement with the claim.
- A disclaimer on the supplement label, directly adjacent to or linked to the structure function claims by a unique symbol (e.g. an asterisk) – 'This/these statement(s) has/have not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, or prevent any disease'.

Since BIO-CAT is an ingredient manufacturer, claims will most likely be not included on product labels. However substantiated claims can be present on promotional material/product information sheets.

8. References

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[1] See https://www.fda.gov/food/food-labeling-nutrition/label-claims-conventional-foods-and-dietary-supplements

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9. Approvals

Report Approved By (R&D): **(signature):** [redacted]	**Report Approved By (Regulatory):** **(signature):** [redacted]
Print Name: Sean M. Garvey	**Print Name:** Robert Boyd
Date: 08 February 2023	**Date:** 08 February 2023
Position: Director of R&D	**Position:** Director of Compliance

10. Revision History

V1.1
08FEB2023

Revisions were applied to the following sections:

In Vitro Study Review for Claims Support: Experiment 2
New in vitro data with amino acid outcomes were included to complement the prior ELISA outcome.

APPENDIX

Table A1. Dataset for statistical analysis[†]

ID	Treatment	ppm
CI90734	Control	> 80.0
CI93145	Control	78.0
CI90736	OPTI-ZIOME™ G-D4	14.0
CI93146	OPTI-ZIOME™ G-D4	6.0
CI93147	OPTI-ZIOME™ G-D4	5.4
CI93148	OPTI-ZIOME™ G-D4	7.9

[†]Upper and lower limits of detection for quantitative tests are 80.0 ppm and 3.0 ppm, respectively.

DATA REPORT OF ANALYSIS

Figure A1. CI90734 - Control

REPORT OF ANALYSIS

Customer: **Bio-Cat, Inc.**
9117 Three Notch Rd.
Troy, VA 22974
ATTN: Julia Parker

Date Received: 12/23/20
Report Date: 12/30/20

‡Description: C-20201221
Lab Number: CI90734
Commodity: WHEAT GLUTEN SLURRY

Analysis	Result	Unit	MDL	Analyzed	Reference
Gluten (ELISA) - Wheat Rye & Barley	>80	ppm	3.0	12/30/20	R7001

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

Testing Cert. 1940.01

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Figure A2. CI93145 - Control

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REPORT OF ANALYSIS

Customer: **Bio-Cat, Inc.**
9117 Three Notch Rd.
Troy, VA 22974
ATTN: Julia Parker

Date Received: 01/12/21
Report Date: 01/18/21

‡Description: C
Lab Number: CI93145
Commodity: WHEAT SLURRY

Analysis	Result	Unit	MDL	Analyzed	Reference
Gluten (ELISA) - Wheat Rye & Barley	78	ppm	3.0	01/18/21	R7001

‡The results shown in this report relate solely to the item submitted for analysis.

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Figure A3. CI90736 – G-D4

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REPORT OF ANALYSIS

Customer: **Bio-Cat, Inc.**
9117 Three Notch Rd.
Troy, VA 22974
ATTN: Julia Parker

Date Received: 12/23/20
Report Date: 12/30/20

‡Description: G4-20201221
Lab Number: CI90736
Commodity: WHEAT GLUTEN SLURRY

Analysis	Result	Unit	MDL	Analyzed	Reference
Gluten (ELISA) - Wheat Rye & Barley	14	ppm	3.0	12/30/20	R7001

‡The results shown in this report relate solely to the item submitted for analysis.

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Figure A4. CI93146 – G-D4

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REPORT OF ANALYSIS

Customer: **Bio-Cat, Inc.**
9117 Three Notch Rd.
Troy, VA 22974
ATTN: Julia Parker

Date Received: 01/12/21
Report Date: 01/18/21

‡Description: G4 R1
Lab Number: CI93146
Commodity: WHEAT SLURRY

Analysis	Result	Unit	MDL	Analyzed	Reference
Gluten (ELISA) - Wheat Rye & Barley	6.0	ppm	3.0	01/18/21	R7001

‡The results shown in this report relate solely to the item submitted for analysis.

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Figure A5. CI93147 – G-D4

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REPORT OF ANALYSIS

Customer: **Bio-Cat, Inc.**
9117 Three Notch Rd.
Troy, VA 22974
ATTN: Julia Parker

Date Received: 01/12/21
Report Date: 01/18/21

‡Description: G4 R2
Lab Number: CI93147
Commodity: WHEAT SLURRY

Analysis	Result	Unit	MDL	Analyzed	Reference
Gluten (ELISA) - Wheat Rye & Barley	5.4	ppm	3.0	01/18/21	R7001

‡The results shown in this report relate solely to the item submitted for analysis.

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Figure A6. CI93148 – G-D4

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REPORT OF ANALYSIS

Customer: **Bio-Cat, Inc.**
9117 Three Notch Rd.
Troy, VA 22974
ATTN: Julia Parker

Date Received: 01/12/21
Report Date: 01/18/21

‡Description: G4 R3
Lab Number: CI93148
Commodity: WHEAT SLURRY

Analysis	Result	Unit	MDL	Analyzed	Reference
Gluten (ELISA) - Wheat Rye & Barley	7.9	ppm	3.0	01/18/21	R7001

‡The results shown in this report relate solely to the item submitted for analysis.

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