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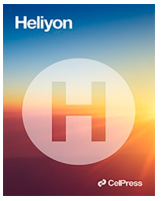

Research article

In vitro simulated study of macronutrient digestion in complex food using digestive enzyme supplement

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ABSTRACT

Digestive enzymes secreted by the body are vital for digestion and nutrient absorption. Enzyme supplements are commonly used to support them in achieving optimal digestion. Herein, the efficacy of digestive enzyme supplement (DigeSEB Super) in digestion of complex food was assessed using INFOGEST simulated static and modified semi-dynamic *in vitro* digestion models. Digestive enzyme supplement was found to assist the endogenous digestive enzymes to disintegrate the food matrix. Hence, it reduced the viscosity of the gastric digesta by 2.75 fold ($p = 0.04$) compared to the control digestion (only endogenous digestive enzymes) during the first hour of digestion. Similarly, enzyme supplement showed statistically higher release of reducing sugars in the gastric digestion ($p \leq 0.05$) indicating improved digestion of carbohydrates. Further, digestion of proteins and fats was also improved in the presence of enzyme supplement. The kinetic aspects of the semi-dynamic model (transient nature of gastric secretions and gradual acidification) was found to alter the macronutrient digestion compared to the static digestion. Thus, semi-dynamic model should be preferred for the *in vitro* studies. Overall, current study demonstrated the potential of a digestive enzyme supplement to improve digestion by aiding digestive action of the endogenous enzymes.

1. Introduction

The digestive enzymes are secreted by the oral, gastric, and intestinal systems in a human body. They are essential for hydrolysis of carbohydrates, proteins, and fats to promote nutrient absorption [1,2]. Several factors such as digestive disorders, stress, ageing, digestive overburdening, various diseases, changing lifestyle may pose challenges in production, secretion, and function of these endogenous digestive enzymes [3]. This could lead to indigestion of food and related health problems such as disrupted gut health, gas, bloating, and malnutrition [3]. Further, it could also weaken the metabolic activities and immunological responses [4]. The supplementation of exogenous digestive enzymes is one of the approaches currently used to assist the endogenous human digestive system for food digestion [5]. They either work individually or in combination with endogenous enzymes for improving macronutrient digestion under normal or compromised health conditions. Currently, several enzyme formulations containing a mixture of exogenous digestive enzymes are available in the market to aid food digestion [6]. Few clinical studies had shown significance of enzyme supplements in improving digestion and treating digestive disorders. In a clinical study, supplementation of N-SORB® (a multi-enzyme complex) for 90 days showed improvement in gut health and gastro-intestinal and metabolic functions [3]. Similarly, oral supplementation of a combination of lactase from *A. oryzae* and yoghurt starter cultures for 4 days displayed an enhancement in lactose digestion in the

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lactose intolerant population [7]. In other investigation, the supplementation of DigeZyme® (a penta-enzyme complex of α-amylase, lactase, cellulase, lipase, and protease) for 60 days showed significant improvement in efficacy parameters, and reduction in gastric and intestinal symptoms [8]. Additionally, the supplementation of proteases from *A. niger* effectively improved the gluten digestion in porridge meal [9]. Ido et al. noticed a prominent reduction in gastric and intestinal symptoms in subjects with non-celiac gluten sensitivity after supplementation of protease blend sourced from papaya and fungi [10].

In spite of the availability of clinical study data, understanding the action of multi-enzyme supplementation needs thorough research in terms of its mechanistic details. *In-vitro* studies would provide those details and also serve as a screening step to evaluate the efficacy. Simulated INFOGEST static and semi-dynamic digestion models are the standardized and worldwide accepted *in-vitro* models [11–13]. Both models include three steps digestion process i.e. oral, gastric, and intestinal phase performed in the presence of simulated fluids and digestive enzymes mimicking the human digestive system [14]. However, these models differ in the gastric phase digestion processing. The addition of gastric juice (enzymes, acid, electrolytes etc.) is at fixed time in the static model, whereas it is added in a sequential manner in the semi-dynamic model that affects the rate of food digestion. The INFOGEST model has been used by many researchers to evaluate the performance of external enzymes such as the digestion of pea, whey, and collagen proteins with SEBrolase (bromelain), Pepzyme AG (acid proteases from *A. niger*), and SEBproX (papain) [15]; lipid hydrolysis and carotenoid bio accessibility using lipases (*R. niveus* and *R. oryzae*) [16]; hydrolysis of proteins in soybean milk with papain [17]; breakdown of immunogenic peptides in gluten with cysteine protease from green kiwifruit [18]. Another study had illustrated the application of an *in vitro* static and dynamic digestion models to understand the milk protein digestion pattern. The results were compared with *in vivo* data i.e. milk protein digestion in pigs. The researchers found that the results of dynamic model exhibited "near real" values [19]. However, implementation of dynamic model is limited by the instrumentation and processing cost. Thus, wise selection of a suitable digestion model is pre-requisite to study the food digestion.

In a current study, the effect of digestive enzyme supplement (DigeSEB Super containing amylase 20,000 SKBU/g, protease 13,000 PC/g, lipase 5 LU/g, cellulase 1000 CMC/g, lactase 1000 ALU/g, and hemicellulase 15,000 XU/g) on complex food digestion was assessed using INFOGEST simulated static digestion model and modified semi-dynamic digestion model. The progress of digestion was monitored in terms of reducing sugars, free sugar profile, degree of hydrolysis, free amino acids, peptide pattern, and free fatty acids. Further, two digestion models (simulated static and modified semi-dynamic) were compared to provide insights on digestion models to be used as a tool to screen the formulations before clinical studies. The diskette was used as a model complex food that contains proteins, carbohydrates, fats, and other nutrients in a proportion that nearly matches the specifications of nutritional requirements suggested by the US FDA.

2. Materials and methods

2.1. Materials

Standard digestive enzymes namely α-amylase from human saliva (300–1500 units/mg), pepsin from porcine gastric mucosa (≥3200 units/mg), pancreatin from porcine pancreas (8 × USP specifications), and bile salts (cholic acid) were purchased from Sigma-Aldrich, USA. Diskettes were procured from local market in Thane (India). The digestive enzyme supplement (DigeSEB Super) was a gift sample from Speciality Enzymes, USA. Analytical standards such as glucose, maltose, fructose, sucrose, and serine were from Sigma Aldrich, USA. The peptides molecular weight standards (Advance BioSEC 130A) were purchased from Agilent. Triolein, diolein, monoolein, and oleic acid standards (HPLC grade) were from NuCheck. All the other chemicals, reagents, solvents, and salts used for the study were of AR grade procured from reliable sources.

2.2. Methods

2.2.1. In-vitro simulated food digestion protocol

The diskettes were digested by two *in vitro* simulated digestion methods namely a harmonized INFOGEST static method [12] and a modified semi-dynamic method [13]. The effect of digestive enzyme supplement on the macronutrient digestion was studied by using a control containing standard digestive enzymes (given in INFOGEST protocol) and a test containing the standard digestive enzymes along with the digestive enzyme supplement, added at the 0.28 % w/w of food. Enzyme supplement dose was decided based on the previous understanding about enzyme activities and the supplier's application note. For all experiments, the diskettes were ground in mixer grinder to obtain homogenous powder, which was used as a complex food in the digestion model.

2.2.1.1. Digestion by a harmonized INFOGEST static method. Static digestion model encompasses first oral phase (2 min), second gastric phase (2 h), and the last intestinal phase (2 h). Briefly, for the oral phase, the diskette powder (10 g) was mixed with simulated salivary fluid (1.25X, 8 mL), $CaCl_2.(H_2O)_2$ (0.3 M, 50 μL), amylase (1500 U in final volume) and the final volume was made to 20 mL with distilled water. The mixture was incubated at 37 ± 2 °C, 100 rpm for 2 min. For the gastric phase, simulated gastric fluid (1.25X, 16 mL), $CaCl_2.(H_2O)_2$ (0.3 M, 10 μL) was mixed with the solution from oral phase (20 mL), and pH was adjusted to 3.0 with 5 M HCl solution. Pepsin (80000 U in final volume) was added to the mixture, and final volume was adjusted to 40 mL with distilled water. The mixture was incubated at 37 ± 2 °C, 100 rpm for 2 h. Further, for intestinal phase, simulated intestinal fluid (1.25X, 16 mL), $CaCl_2(H_2O)_2$ (0.3 M, 80 μL) were mixed with the solution from gastric phase (40 mL), and pH was adjusted to 7.0 with 5 M NaOH solution. Pancreatin (8000 U in final volume) and bile salts (10 mM in final volume) were added to the mixture, and final volume was adjusted to

80 mL with distilled water. The resultant mixture was incubated at 37 ± 2 °C, 100 rpm for next 2 h. Samples were withdrawn after gastric digestion (gastric digesta) and intestinal digestion (intestinal digesta). The samples were heated at 95 ± 2 °C for 5 min to terminate the reaction, centrifuged at 10000 rpm for 10 min at 25 °C, and obtained supernatants were used for the analysis.

2.2.1.2. Digestion by a modified semi-dynamic method. The semi-dynamic digestion protocol [13] was used with some modifications. During the modified semi-dynamic digestion, the oral phase was performed same as mentioned above. For the gastric phase, food obtained after the oral phase was mixed with the gastric juice (electrolyte, acid, pepsin) in a sequential manner to mimic *in vivo* gastric digestion at 37 ± 2 °C and 100 rpm. Briefly, at 0 min 1.6 mL of simulated gastric fluid (1.25X) and $CaCl_2.(H_2O)_2$ (0.3 M, 1 μL) were added and pH was recorded followed by addition of pepsin (8000 U). After 20 min of incubation, 3.2 mL of simulated gastric fluid (1.25X) and $CaCl_2.(H_2O)_2$ (0.3 M, 2 μL) were added and pH was adjusted to 4.25 with 2 N HCl followed by addition of pepsin (16000 U). At 40 min incubation time, 3.2 mL of simulated gastric fluid (1.25X) and $CaCl_2.(H_2O)_2$ (0.3 M, 2 μL) were added, and pH was adjusted to 2.99 with 2 N HCl followed by addition of pepsin (16000 U). In the last phase (after 90 min), 8 mL of simulated gastric fluid (1.25X) and $CaCl_2.(H_2O)_2$ (0.3 M, 5 μL) were added in the reaction mixture, and pH was adjusted to 1.89 with 2 N HCl followed by addition of pepsin (40000 U). The volume was made up to 40 mL with distilled water and the incubation was continued until 120 min at 37 ± 2 °C and 100 rpm. The digestion in the intestinal phase was carried out in the same manner as described above in the static model. Samples were withdrawn after gastric digestion (gastric digesta) and intestinal digestion (intestinal digesta) phases, heated at 95 ± 2 °C for 5 min to terminate the reaction, centrifuged at 10000 rpm for 10 min at 25 °C, and obtained supernatants were used for analysis.

2.3. Analysis

2.3.1. Effect of digestive enzyme supplement on the viscosity of the food

Separate digestion reactions (control and test) were set up with 50 g of food according to static INFOGEST model to obtain 200 mL reaction mixture at the end of gastric phase. Viscosity was analyzed in gastric phase at 1 h and 2 h interval. Before analysis, samples were equilibrated in water bath to attain 30 ± 2 °C temperature. Viscosity of gastric digesta was measured by the Brookfield viscometer in centipoise (cP) at 30 ± 2 °C. Single sample was analyzed on viscometer for 15 min to obtain stable reading.

2.3.2. Effect of digestive enzyme supplement on the digestion of carbohydrates

The effect of digestive enzyme supplement on the digestion of carbohydrates was assessed by determining the amount of reducing sugars and free sugars released after gastric and intestinal digestion. Total reducing sugars werequantified using DNSA method [20] whereas, free sugars released in the digesta were analyzed by HPLC method [21]. Absolute ethanol (4.5 mL) was added to the digesta (0.5 mL) followed by 16 h incubation at 4 °C. The sample was centrifuged at 1000 rpm for 10 min at 4 °C; supernatant was filtered through 0.45μ filter and analyzed on HPLC with refractive index detector. HPLC analysis was performed on analytical column Shodex Asahipak NH2P-50-4E (4.6 mm ID × 250 mm L); at 30 °C and flow rate 1 mL/min with 75 % acetonitrile as a mobile phase for 30 min. Glucose, fructose, and maltose were used as sugar standards.

2.3.3. Effect of digestive enzyme supplement on the digestion of proteins

The effect of addition of digestive enzyme supplement on the digestion of proteins was determined by assessing degree of hydrolysis (DH) and amino acids released after gastric and intestinal digestion. The degree of hydrolysis was determined using OPA method previously explained by Jadhav et al. [15]. Briefly, the sample (25 μL) was mixed with OPA reagent (175 μL; 0.15 g OPA and 0.3 g dithiothreitol (DTT) were dissolved in 11.25 mL methanol, volume was made up to 100 mL with di-sodium tetraborate buffer pH 9.6). The mixture was incubated at 25 °C for exactly 2 min and absorbance was taken at 340 nm. Serine was used as a reference standard (40–200 μg/mL) to determine serine equivalence released from the sample. Degree of hydrolysis was calculated as shown in Eq. (1).

$$\text{Degree of hydrolysis } (\%) = \frac{\text{Serine equivalance of digested sample}}{\text{Total serine equivalence of food}} \times 100 \quad \text{(Eq. 1)}$$

Free amino acids released after digestion were detected using Ninhydrin method [22]. The molecular weight pattern of the released peptides was evaluated using size exclusion chromatography analysis (SEC-HPLC) on a Phenomenex BioSep 5μ SEC-S2000-145A° (300 × 78 mm) column (Agilent Technologies) at 25 °C. The mobile phase was 50 mM phosphate buffer, pH 6.8 (isocratic) at 1 mL/min flow rate and detection was on DAD detector at 214 and 280 nm. Advanced BioSEC 130 A (Agilent Technologies) was used as a reference standard to determine molecular weights of the peptides in the sample.

2.3.4. Effect of digestive enzyme supplement on the digestion of fats

After digestion, the reaction mixture was extracted with petroleum ether (2 times, 5 vol). The obtained organic layer was evaporated using rotary evaporator at 50 ± 2 °C, 300 mbar, and 100 rpm. The mass of the total fat extracted was determined and then dissolved in 10–20 mL neutralized *iso*-propanol. This mixture was titrated against 0.1 N NaOH with phenolphthalein indicator till pink colouration was observed. Burette reading was recorded for calculation of FFA as mentioned in Eq. (2).

$$\text{FFA } (\%\text{ palmitic acid}) = \frac{\text{NaOH consumed} \times 25.6 \times \text{NaoH Normality}}{\text{Mass of test portion (g)}} \times 100 \quad \text{(Eq. 2)}$$

Fat digestion profile was evaluated using size exclusion chromatography analysis. The samples after evaporation were dissolved in 50 mL tetrahydrofuran, and analyzed on HPLC using column PhenogelTM 5 μm 100 A° (size:300 × 78 mm). The mobile phase was 100 % tetrahydrofuran (isocratic), and detection was on RI detector. The standards used were monolein, diolein, triolein, and oleic acid.

2.4. Statistical analysis

All the data were analyzed using GraphPad Prism (version 9.5.1) software. Multiple unpaired *t*-tests were performed for all the parameters to compare difference between control and test. All the experiments were performed in triplicates and the results are expressed as mean ± standard deviation; $p \leq 0.05$ was considered statistically significant unless specified.

3. Results and discussion

Literature is scarce to discuss the potential of enzyme supplements for improving digestion in the presence of endogenous digestive enzymes under *in vitro* conditions. The addition of external enzymes to endogenous digestive enzymes makes the system too complex to understand the actual impact on the digestion process. With the aspect to assess the disintegration and interaction of complex food (diskettes) in the gastrointestinal tract, we have used two *in vitro* digestion models *viz.* static and modified semi-dynamic. INFOGEST static digestion model was used as explained by Brodkorb et al. [12], whereas the semi-dynamic model was modified from the original method described by Mulet-Cabero et al. [13] as per the requirement. The semi-dynamic model was previously developed to overcome the challenges associated with the static model such as providing end-point assessment and dynamic model being expensive and less accessible/feasible at each lab. The semi-dynamic model particularly focussed on the kinetic aspects associated with the gastric phase of digestion, including gradual acidification, fluid and enzyme secretion, and emptying. However, gastric emptying depends on several physiochemical properties of the meal such as composition, viscosity, osmolarity, meal volume, caloric density, physical state of the meal, etc. Further, the digestive condition of the individuals, age, and patho-physiological state also contribute to the gastric emptying. Practically it is difficult to arrange the instrumental requirements as well as to adhere to the real scenario of gastric emptying. Hence, for an ease of operations, we have modified the method of semi-dynamic simulated model where kinetic aspects of gastric phase (gradual acidification and fluid and enzyme secretion) was followed but not the gastric emptying (Fig. 1). In the present study, we have selected diskettes as a complex food that had a balanced composition of macronutrients, which nearly matches to nutritional requirement specifications postulated by the US FDA [23]. The proximate analysis of the diskettes showed 6.59 % moisture, 10.98 % fat, 29.85 % protein, 1.14 % ash, and 58.03 % carbohydrates. The nutrients in the diskettes are mainly from precooked rice flour, casein, sucrose, malt extract, bengal gram, and edible vegetable fat. In the present investigation, we have studied the digestion of diskettes with inherent digestive enzymes in the absence (control) and presence (test) of digestive enzyme supplement (DigeSEB Super). The objective was to evaluate the change in the food disintegration (with respect to viscosity) and macronutrient digestion using two different simulated digestion models.

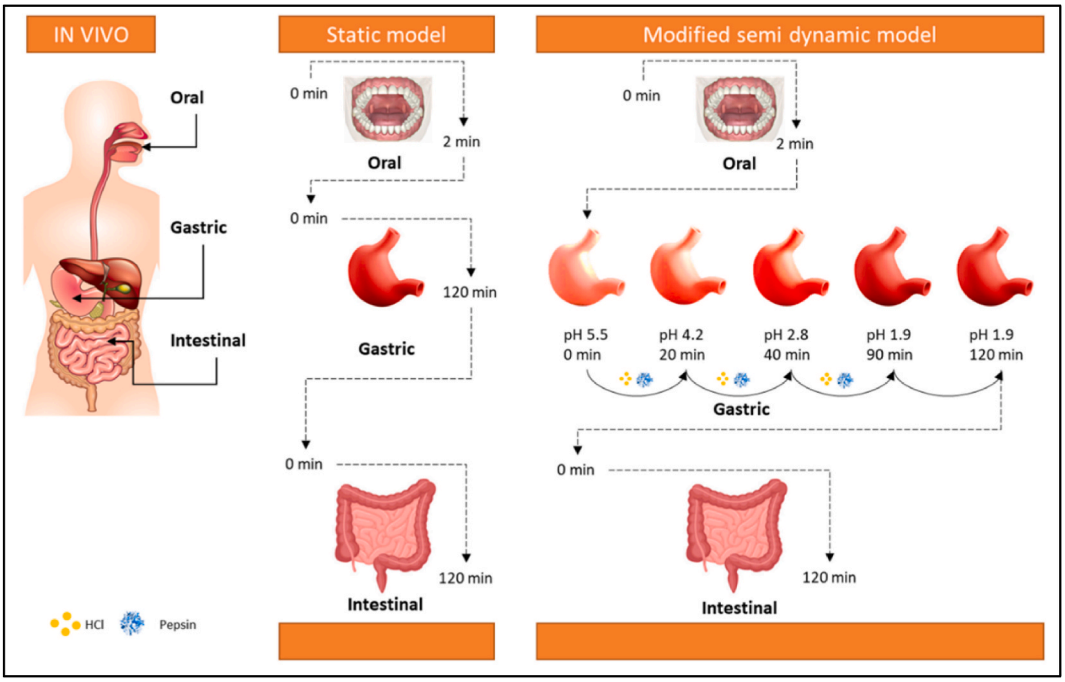

Fig. 1. Schematic representation of static and modified semi-dynamic *in vitro* models of digestion.

3.1. Effect of digestive enzyme supplement on the viscosity of the food

Viscosity is one of the physiochemical properties of the meal affecting gastric emptying rate. The changes in the viscosity of the food matrix (solid food, diskettes) was studied in a static model in the absence (control) and presence (test) of digestive enzyme supplement. A gradual decline in viscosity was recorded with the progression of digestion time (Fig. 2). The initial viscosity of the food after addition of simulated gastric juice was 81.60 ± 2.45 cP. Viscosity of the control set was reduced to 39.95 ± 7.43 cP in 1 h, which further declined to 18.3 ± 0.14 cP after 2 h of gastric digestion. On the other hand, digestive enzyme supplement facilitated food disintegration observed as a sharp decrease in the viscosity. In the first hour, viscosity reduced to 14.55 ± 0.07 cP ($p \leq 0.05$ compared to control) and marginally declined to 13.30 ± 0.0 cP ($p \leq 0.001$ compared to control) in the next hour of gastric digestion. The impact of digestive enzyme supplement on the food matrix degradation was observed in the initial phase of gastric digestion. In the presence of digestive enzyme supplement, the viscosity decreased by 82.17 % whereas endogenous digestive enzymes (control) could decrease viscosity only by 51.04 %. At the end of the gastric phase (2 h), both sets attained similar viscosity as the study was done using a static model and gastric emptying was not considered. The results illustrated that the digestive enzyme supplement could reduce the viscosity of the food which would lead to improved gastric emptying.

Digestive enzyme supplement used in this study contains various hydrolytic enzymes such as amylase, protease, lipase, lactase, hemicellulase, and cellulase. These exogenous enzymes act on complex food matrices individually or synergistically with the body's digestive enzymes and increase nutrient availability. Karthikeyan et al. [24] stated that digestion of maltodextrin was inversely proportional to the initial viscosity of food, and observed 35 % decrease in conversion of maltodextrin to glucose when viscosity increased from 1 mPa s to 15 mPa s. Further, they postulated that the viscosity induced delayed digestion is affected in gastric phase and once the food reaches the intestine its absorption is independent of initial viscosity. In another study, high viscosity peanut butter had shown slower pH decrease, high buffering capacity and less protein and lipid digestion compared to its low viscosity counterpart [25]. Thus, a rapid reduction in viscosity would help in faster digestion of the food under *in vivo* conditions. Other than the type or nature of food, some physiological conditions such as gastroparesis may retard gastric emptying [26]. Digestive enzyme supplements improve food disintegration by their hydrolytic activity. Hence, would be helpful in increasing the rate of gastric emptying, reversing the symptoms of the gastroparesis, and promoting the digestion process for better nutrient absorption.

3.2. Effect of digestive enzyme supplement on the digestion of carbohydrates

Carbohydrates form a major portion of standard diet and contribute around 45–65 % of total energy [23]. Carbohydrates are the primary source of energy and provide 4 calories per gram. Therefore, efficient digestion and absorption of carbohydrates is vital for normal functioning of the body [27]. Indigestion and malabsorption of carbohydrates may cause bloating, gas, flatulence, diarrhea, and abdominal pain. These symptoms may arise due to overeating (higher carbohydrate load), impaired gastric emptying, inability of the colon to reabsorb water, and metabolic characteristics of colonic microbes [28]. We have studied the digestion of carbohydrates in diskette in terms of total reducing sugars released as well as free sugar profile in the absence (control) and presence (test) of digestive enzyme supplement. The diskettes contains 58 % carbohydrates comprising of starches (precooked rice flour, malt extract and bengal gram) and added sugar (sucrose). A steady increase in reducing sugar content was observed in control and test with the time in both the static and modified semi-dynamic digestion (Fig. 3). In the static model, there was a substantial increase in the release of reducing sugars in test to 2145.0 ± 38.18 mg and 2931.47 ± 217.79 mg and in control to 1193 ± 39.32 mg and 2394 ± 115.97 mg from an initial value of 714.97 ± 26.71 mg after 2 h and 4 h of gastro-intestinal digestion respectively (Fig. 3a). The digestive enzyme

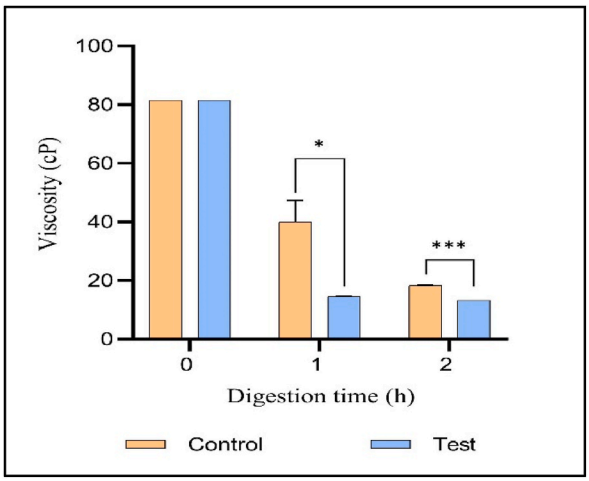

Fig. 2. Viscosity (cP) of diskette in the presence (test) and absence (control) of digestive enzyme supplement during gastric digestion using static digestion model. Values represented as mean ± standard deviation. * and *** represents significant difference between control and test at $p \leq 0.05$ and $p \leq 0.001$ respectively.

supplement increased the release of reducing sugar by 79.80 % ($p \leq 0.01$) and 22.47 % over the control in 2 h and 4 h of gastro-intestinal digestion respectively. On the other hand, in the modified semi-dynamic model, a noticeable increase in reducing sugars by 40.93 % ($p \leq 0.01$) and 9.40 % after 2 h and 4 h of gastro-intestinal digestion was observed in the presence of digestive enzyme supplement (Fig. 3b). The reducing sugar released in the test and control was statistically different in the gastric phase but not in the intestinal phase as the presence of pancreatin in the intestinal phase overruled the effect of digestive enzyme supplement. The significant increase in the carbohydrate digestion in the gastric phase due to the digestive enzyme supplement was in good agreement with the reduced viscosity of the food.

The diskettes contain the carbohydrates sourced from Bengal gram, which has a slow digestibility in the body, and the proteins present in Bengal gram may also interfere in the carbohydrate digestion process [29,30]. Here, the digestive enzyme supplement helped to overcome these challenges and improved the carbohydrate digestion. The comparison of static and modified semi-dynamic models showed that the digestive enzyme supplement could improve the reducing sugar released in the gastric digestion phase by 79.80 % and 40.93 % respectively as compared to control. The results obtained in two models showed variations. Though the static model is easy to implement, the modified semi-dynamic model is close to the real conditions of the gastric phase and hence need to be taken into account while designing further study. Carbohydrate digestion pattern in the digested samples (control and test) were analyzed on HPLC to obtain free sugar profile (glucose, fructose, and maltose) from the gastric and intestinal digesta (static digestion model). In gastric phase, fructose increased from 4.64 ± 0.14 mg to 36.17 ± 2.56 mg (7.79 folds $p \leq 0.01$); glucose increased from 26.15 ± 1.93 mg to 87.40 ± 6.95 mg (3.34 folds $p \leq 0.01$) and maltose increased from 49.72 ± 4.3 to 123.82 ± 8.55 mg (2.49 folds $p \leq 0.01$) after addition of digestive enzymes supplement (Fig. 3c). During intestinal digestion, digestive enzymes supplement was able to improve fructose content from 4.41 ± 1.22 to 45.76 ± 2.88 mg and glucose from 98.09 ± 3.57 to 157.57 ± 12.33 mg that corresponds to 10.3 folds ($p \leq 0.01$) and 1.60 folds ($p \leq 0.05$) improvement respectively (Fig. 3d). The supplement could not show further value addition in the release of maltose in the intestinal phase may be due to the release of maximum maltose in the gastric phase itself. Similar results were obtained when a multi enzyme blend (BC-006) was added under simulated gastric digestion to digest a complex meal consisting of chicken, green peas, mashed potatoes, and butter. The carbohydrate digestion was measured as a free glucose that increased up to 14.1 folds in gastric phase [5]. The increased digestion of carbohydrate owed to the digestive enzymes (amylase,

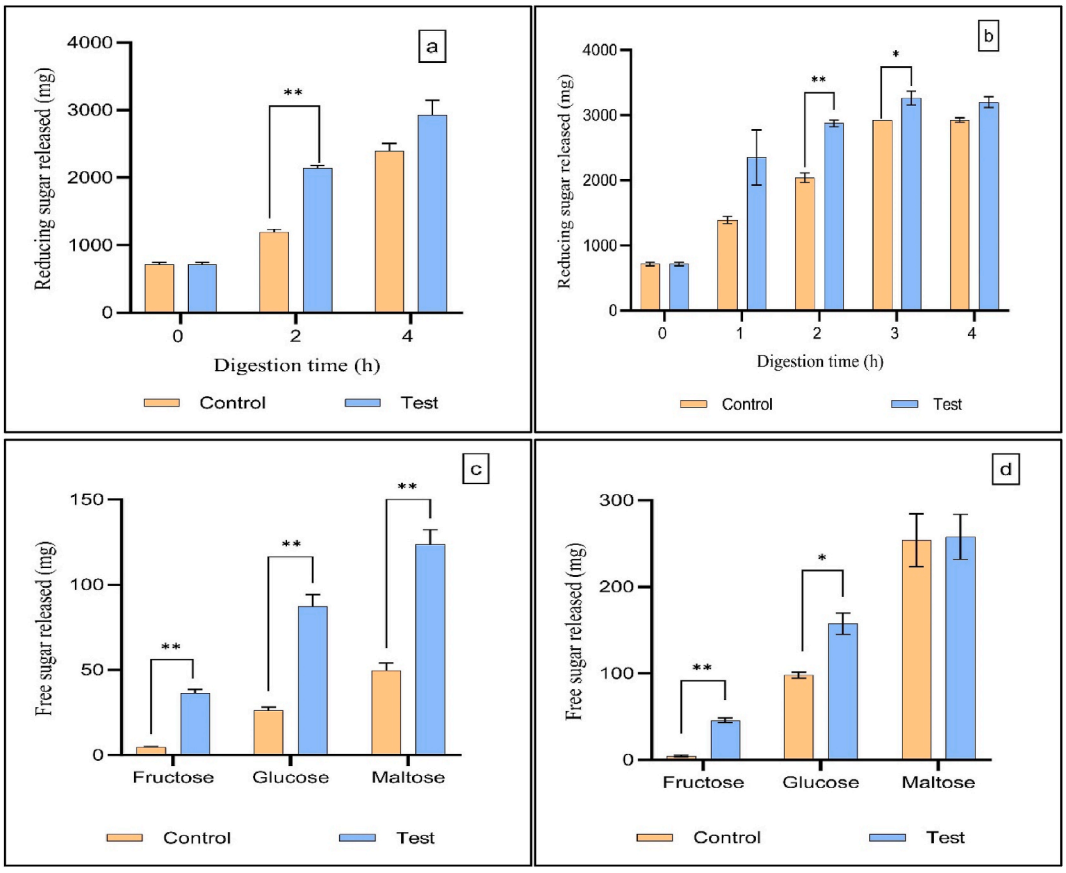

Fig. 3. Reducing sugar released (mg) from diskette during gastrointestinal digestion using (a) static and (b) modified semi-dynamic digestion model in the presence (test) and absence (control) of digestive enzyme supplement. Free sugar released (mg) from diskette in (c) gastric digesta and (d) gastro-intestinal digesta obtained in the presence (test) and absence (control) of digestive enzyme supplement using static digestion model. Values represented as mean ± standard deviation. * and ** represents significant difference between control and test at $p \leq 0.05$ and $p \leq 0.01$ respectively.

cellulase, hemicellulose, and lactase) present in the supplement.

3.3. Effect of digestive enzyme supplement on the digestion of proteins

Complete protein hydrolysis and absorption of amino acids/peptides is crucial for maintaining homeostasis [31]. In order to perform biological functions, protein digestion initiates in the gastric phase by the action of pepsin and ends after the action of pancreatic proteases, elastases, and peptidases in the intestinal phase [32]. However, malfunctioning of the digestive system may lead to protein indigestion. The common symptoms for protein indigestion are vomiting, nausea, diarrhea, abdominal pain, stomach cramping, gas, and bloating. The diskette contains nearly 29.85 % proteins mainly from the ingredients such as casein, bengal gram, malt extract etc. We have studied the digestion of proteins from the diskette in terms of degree of hydrolysis and free amino acids released under static and modified semi-dynamic models in the absence (control) and presence (test) of digestive enzyme supplement. When the static digestion model was implemented, in the gastric phase control set exhibited 6.63 ± 0.51 % DH while addition of digestive enzymes supplement resulted in 7.38 ± 0.05 % DH i.e. 11.24 % increase in protein hydrolysis (Fig. 4a). Similarly, a modified semi-dynamic digestion model showed an increase in protein digestion by 6.74 % (10.39 ± 0.39 % DH *vs*. 11.09 ± 0.53 % DH) in gastric phase in the presence of digestive enzymes supplement (Fig. 4b). The results indicated that the enzyme supplement assisted the endogenous digestive enzymes to improve protein digestion. The progress in protein hydrolysis was in agreement with the decrease in food viscosity during the first hour of digestion. The impact of digestive enzyme supplement was not observed in the intestinal phase in both static and modified semi-dynamic digestion models pertaining to the presence of pancreatin. Gadani et al. [33] documented similar results after hydrolysis of whey protein, raw whey protein isolate, and plant protein with proteases from plant and fungal sources, and reported improved degree of hydrolysis of proteins over the control. Nath et al. [20] reported a rise in hydrolysis of soybean milk proteins after treatment with increasing concentration of papain. In this study, the profile of free amino acids was in accordance with the protein hydrolysis observed in terms of DH. In static model, control reaction yielded 219.83 ± 6.32 mg and 1367.87 ± 28.84 mg of free amino acids after gastric and intestinal digestion respectively. Under similar experimental conditions,

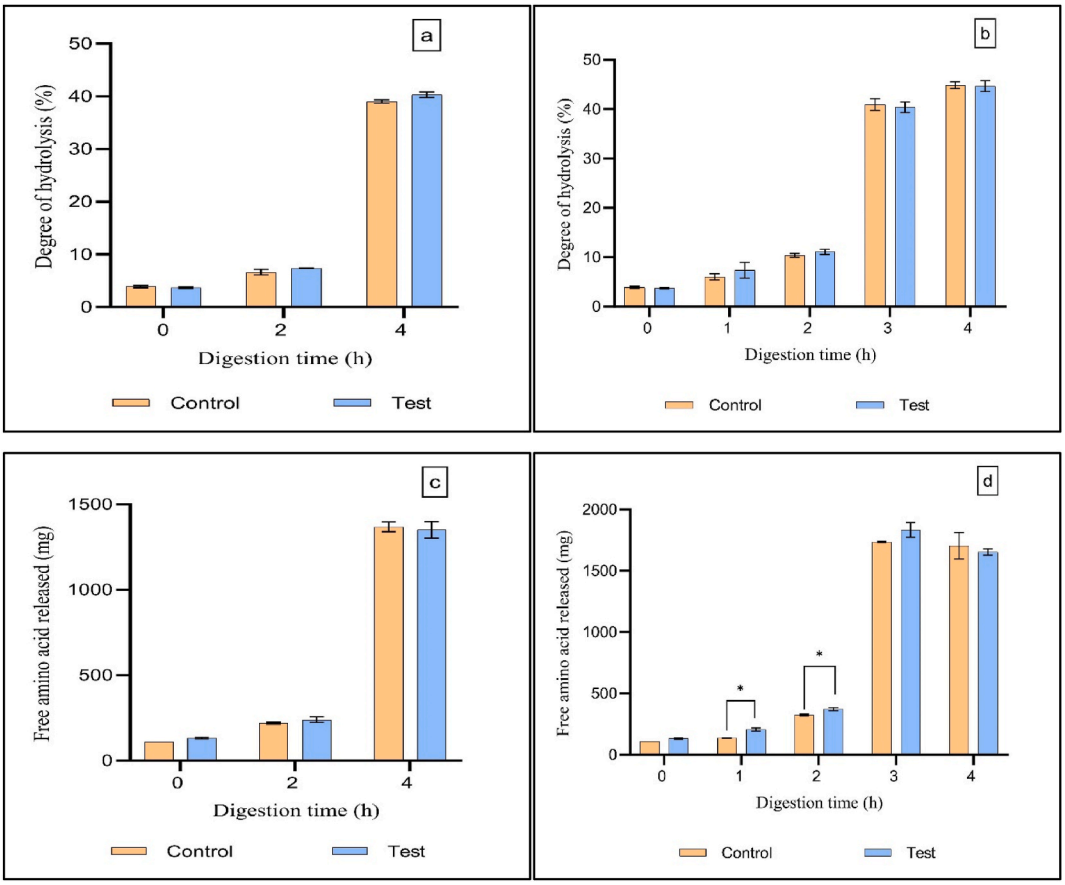

Fig. 4. Degree of Hydrolysis (%) of diskette during gastrointestinal digestion using (a) static and (b) modified semi-dynamic digestion model in the presence (test) and absence (control) of digestive enzyme supplement. Free amino acids released (mg) from diskette during gastrointestinal digestion using (c) static and (d) modified semi-dynamic digestion model in the presence (test) and absence (control) of digestive enzyme supplement. Values represented as mean ± standard deviation.

digestive enzyme supplement addition resulted in free amino acid release of 238.87 ± 16.28 mg and 1351.16 ± 48.08 mg; that corresponded to 8.69 % increase in gastric phase (Fig. 4c). Modified semi-dynamic model showed more promising results in terms of amino acid liberation. Statistically significant improvement in amino acid release was observed by 50.95 % (136.95 ± 1.34 mg *vs.* 206.80 ± 12.26 mg; $p \leq 0.05$) and 15.14 % (323.70 ± 8.49 mg *vs.* 372.70 ± 12.79 mg; $p \leq 0.05$) during the first and second hour of gastric digestion (Fig. 4d). Intestinal protein digestion (in terms of free amino acids) did not differ between control and test set.

Size exclusion chromatography was performed to decipher protein digestion in terms of peptide profile. Molecular weights were assigned to peptides by correlating to retention time of protein standards. The addition of digestive enzyme supplements resulted in altered peptide profile in gastric and intestinal phase (Fig. 5a and b). Some peptides were found to decrease with respect to control (11.55 kDa, 8.67 kDa, 7.43 kDa, 7.36 kDa, and 6.10 kDa) indicating their hydrolysis by external proteases. Action of external protease also caused increase in concentration of peptides with molecular weights 7.37 kDa, 6.96 kDa, 6.59 kDa, 4.60 kDa, 3.26 kDa, and 0.78 kDa. Further, a new class of peptides with molecular weight of 5.59 kDa formed in the presence of digestive enzyme supplement. On the other hand, after the intestinal digestion, there was an increase in the concentrations of peptides with a molecular weight of 7.39 kDa, 7.36 kDa, 7.17 kDa, 6.63 kDa, and 0.95 kDa, while a decline in peptides with molecular weight of 6.90 kDa. Here a new class of peptides with molecular weight of 7.08 kDa formed in the presence of digestive enzyme supplement. Digestive enzyme supplement aids protein digestion process by improving overall hydrolysis, free amino acid liberation, and formation of different types of peptides. Jadhav et al. [15] reported similar improvement in protein digestion in the presence of different proteases (acid protease, papain, and bromelain). Increased protein digestion in gastric phase would lead to quick transfer of digested peptides to the intestine resulting in enhanced absorption of peptides [34]. The increase in the release of peptides and amino acids from the diskettes in presence of digestive enzyme supplement could be due to cumulative action of the endogenous and exogenous proteases.

3.4. *Effect of digestive enzyme supplement on the digestion of fats*

The diskette contains nearly 11 % fat majorly consisted of monounsaturated and saturated counterparts. In the static digestion model, digestive enzymes supplement could contribute to increased fatty acid release in the intestine by 16.76 % may be due to the working mechanism of external lipase at alkaline conditions (Fig. 6a). Fat digestion was more in the modified semi-dynamic model compared to the static model in case of control set. This may be due to improved carbohydrate and protein digestion under modified semi-dynamic models (Fig. 6b). Digestive enzyme supplement improved fat digestion by 10 % and 54.5 % after 1 h and 2 h respectively in gastric phase though not statistically significant ($p \geq 0.05$). These results were confirmed by analysing digested samples on size exclusion chromatography with respect to quantification of free fatty acids (FFA), monoacylglycerol (MAG), diacylglycerol (DAG), and triacylglycerol (TAG) to have a clear insights of the fat digestion profile. The SEC profile of the intestinal digesta of the static digestion model displayed an increase in TAG, DAG, and FFA by 15.09 %, 9.22 %, and 6.44 %, respectively after addition of digestive enzyme supplement (Table 1). Along with improving fat digestion, digestive enzyme supplement support food matrix disintegration to increase the release of macronutrients from the matrix as seen by increased TAG under intestinal phase. Further, higher release of DAG and FFA represented elevated fat digestion. The variations in the release of free fatty acids from fats in the diskettes in absence (control) and presence (test) of digestive enzyme supplement owed to the variations in the catalytic sites of pancreatin and lipolytic enzymes. The endogenous pancreatic lipases are known to specifically break the tails with acyl groups present at the SN-1 and SN-3 sites of triglycerides, and then release free fatty acids and SN-2 monoglycerides [35]. On the other hand, the microbial lipases present in digestive enzyme supplements are known to act on the tails with acyl groups present at all the sites, and subsequently release free fatty acids and glycerol [36]. Earlier studies also reported an increase in the release of fatty acids after hydrolysis with exogenous lipases. Petry and Mercadante [37] documented an improvement in the hydrolysis of β-cryptoxanthin ester and micellarization during *in vitro*

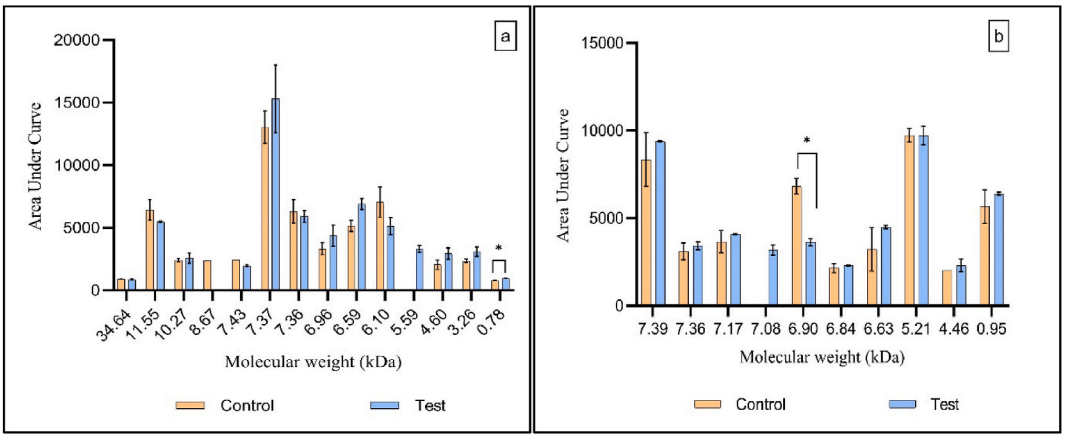

Fig. 5. Molecular weight (kDa) distribution of peptides in (a) gastric digesta and (b) gastro-intestinal digesta obtained in the presence (test) and absence (control) of digestive enzyme supplement using static digestion model. Values represented as mean ± standard deviation. * represents significant difference between control and test at $p \leq 0.05$.

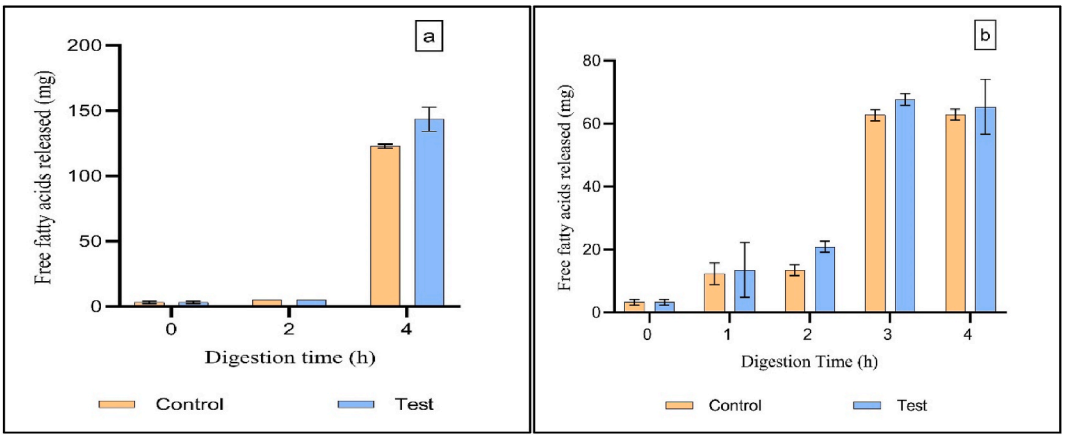

Fig. 6. Free fatty acids released from diskette in the presence (test) and absence (control) of digestive enzyme supplement using (a) static and (b) modified semi-dynamic digestion model. Values represented as mean ± standard deviation.

Table 1
Fat components (mg) released from diskette matrix in the presence (test) and absence (control) of digestive enzyme supplement using static digestion model.

Component	Fat (mg)		Increase in fat (%)	*p* value
	Control	Test		
TAG	291.27 ± 28.74	335.22 ± 31.02	15.09	0.28
DAG	116.87 ± 27.80	127.65 ± 17.02	9.22	0.69
FFA	206.34 ± 19.23	219.63 ± 3.29	6.44	0.44

TAG: Triacylglycerol, DAG: Diacylglycerol, FFA: Free fatty acids.

digestion of mandarin and peach pulps with gastric lipase or cholesterol esterase. Similarly, Iddir et al. [16] found a significant increase in lipid hydrolysis of different food matrices (spinach, tomato juice, and carrot juice) with lipases (*R. niveus*, *R. oryzae* and rabbit gastric extracts) followed by improved carotenoid bio accessibility.

To the best of our knowledge, the current study is the first scientific report to demonstrate the effect of a digestive enzymes supplement on the macronutrient digestion with both static and modified semi-dynamic models. The results obtained by two different simulated models vary in many parameters such as reducing sugars released, degree of hydrolysis, amino acids released, and free fatty acids released. The incorporation of kinetic aspects of gastric phase in modified semi-dynamic models may have contributed to the variations observed in the results of two models. Considering the close relevance of this model to the real scenario (i.e. transient nature of gastric secretions and gradual acidification) and the results deviations from the static model, the modified semi-dynamic, semi-dynamic, or dynamic models should be considered for *in vitro* studies. The extrapolation of the obtained data would be helpful in designing prospective clinical study.

4. Conclusions

The digestive enzyme supplement assisted the endogenous digestive enzymes to reduce the food matrix viscosity and to increase the release of reducing sugars, free amino acids, and fatty acids enabling improved food digestion. The results obtained by static and modified semi-dynamic models vary in parameters of macronutrient digestion suggesting the necessity of using semi-dynamic model in the *in vitro* studies. This study highlighted that the digestive enzyme supplement could improve the digestion during the gastric and intestinal phase by aiding the digestive action of the endogenous enzymes. Hence, the oral consumption of digestive enzyme supplement could benefit the individuals in achieving optimum digestion and may help in relieving food related gastrointestinal distress.

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Data availability statement

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical statement–studies in humans and animals

This article does not include any studies with human participants or animals performed by any authors.

CRediT authorship contribution statement

Abhijit Rathi: Writing – review & editing, Visualization, Methodology, Conceptualization. **Sneha Potale:** Writing – review & editing, Formal analysis, Data curation. **Rutuja Vaze:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Abhijeet B. Muley:** Writing – review & editing, Writing – original draft, Visualization. **Swati Jadhav:** Writing – review & editing, Visualization, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Swati Jadhav reports a relationship with Advanced Enzyme Technologies Limited that includes: employment. Abhijit Rathi reports a relationship with Advanced Enzyme Technologies Limited that includes: employment. Sneha Potale reports a relationship with Advanced Enzyme Technologies Limited that includes: employment. Rutuja Vaze reports a relationship with Advanced Enzyme Technologies Limited that includes: employment. Abhijeet Muley reports a relationship with Advanced Enzyme Technologies Limited that includes: employment. All the named authors are paid employees of Advanced Enzyme Technologies which has a corporate affiliation with Specialty Enzymes and Probiotics, USA. Specialty Enzymes and Probiotics had provided study material (DigeSEB Super) but had no role in study design and actual conduct of the study.

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