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Pearl millet (*Pennisetum glaucum*) couscous breaks down faster than wheat couscous in the Human Gastric Simulator, though has slower starch hydrolysis

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Consumption of traditional West African pearl millet (*Pennisetum glaucum*) couscous delayed gastric emptying in our recent human study compared to other starch-based foods (white rice, boiled potatoes, pasta). The objective of this study was to determine whether physical properties of pearl millet couscous affect particle breakdown and starch hydrolysis during simulated gastric digestion to understand the basis of the slow gastric emptying. Starch fine structure and viscosity were analyzed for initial millet and wheat couscous samples by high performance size-exclusion chromatography and the Rapid Visco Analyzer, respectively. Couscous samples were subjected to simulated gastric digestion using the Human Gastric Simulator (HGS), a dynamic model of human gastric digestion. Digesta was collected from the HGS at 30 min intervals over 180 min. Particle size and percent starch hydrolysis of couscous in the digesta were evaluated at each time point. The number of particles per gram of dry mass substantially increased over digestion time for millet couscous ($p < 0.05$), while changed little for the wheat couscous samples. Millet couscous showed lower starch hydrolysis per unit surface area of particles than wheat couscous ($p < 0.05$). Slower starch hydolysis was associated with smaller ($p < 0.05$) amylose chain length for millet (839-963 DP) than for wheat (1225-1563 DP), which may enable enable a denser packing of millet starch molecules that impedes hydrolysis. We hypothesize that the slow gastric emptying rate of millet couscous observed in humans may be explained by its slow starch hydrolysis property that could activate the ileal brake system, independent of high particle breakdown rate in the stomach.

Introduction

Increasing prevalence of obesity is indicative of dysregulation in the control of appetite and energy homeostasis. Thus, there is a need for foods with satiating properties that can better promote health. Slow gastric emptying is associated with increased satiety[1,2]. Our recent human study showed that couscous made from pearl millet (*Pennisetum glaucum*), a traditional West African food, substantially delayed gastric emptying rate compared to foods typically consumed as part of the Western diet (white rice, boiled potatoes, and pasta)[3]. However, the underlying cause of the observed difference was not well understood. Previous research has identified the importance of rate of food breakdown in the stomach and suggested that foods which resist breakdown have a slower gastric emptying rate[4–7]. It is also hypothesized that a slow starch hydrolysis property of pearl millet-based foods, including couscous, plays a role in their slow gastric emptying through activation of the ileal brake feedback mechanism in the body[8].

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The emptying of food from the stomach is a complex process, as it depends on food breakdown, physical properties of digesta, and physiological regulation[9–11]. It has been found that food particles larger than approximately 2 mm in size are retained by the pyloric valve through a phenomenon called gastric sieving, which contributes to longer stomach retention times for foods that resist particle breakdown[12]. Accordingly, foods which break down more slowly in the stomach have been found to delay gastric emptying[13]. However, gastric emptying rate is also controlled by the presence of macronutrients, including starch and partially hydrolysed starch, in the distal small intestine, triggering the ileal brake feedback response[14–16].

The objective of this work was to determine how pearl millet couscous, which will hereafter be referred to as millet couscous, breaks down in a simulated gastric environment and whether it is resistant to breakdown; and to determine starch hydrolysis rate related to potential ileal brake activation. In both procedures, millet was compared to wheat couscous. Three types of millet couscous (self-made with large and small final particle sizes as well as a commercial type from Senegal) were studied along with two types of wheat couscous (self-made small and a commercial type). Initial flour particle size was controlled and the final couscous particle size was matched in three types of couscous so that effects due to the differences in particle breakdown, and not differences in flour or initial

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particle size, could be elucidated. Starch fine structural features and rheological properties of initial couscous samples were analyzed.

Materials and Methods

Raw material preparation

Couscous materials. Whole grain pearl millet (*Pennisetum glaucum*) grain was obtained as intact kernels (Alif Group, Dakar, Senegal) to be made into couscous. Commercially prepared millet couscous from Dakar, Senegal was also acquired and evaluated to represent couscous typically consumed in Western Africa (Mme. Deme of Free Work Services, Dakar, Senegal). Wheat flour was obtained from a commercial source (Bob's Red Mill, Milwaukie, OR, USA) and was made into wheat couscous. Commercial wheat couscous was purchased as a comparator (Riviana Foods Inc., Houston, TX, USA).

Couscous pre-processing. Millet grain was decorticated using an abrasive decorticator (15% bran removal). The kernels and bran were separated using a sieve shaker (Smico Corp., Oklahoma City, OK, USA) such that particles greater than approximately 1 mm were retained as decorticated kernels and were milled to flour using a pin mill operated at 5.5 rpm (Alpine, 160 Z, Augsburg, Germany). The millet flour was then separated into different particle size fractions using a sieve shaker (Model RX-24, W.S. Tyler Inc., Mentor, OH, USA). Millet flour particles between 300-495 µm were used to make the millet couscous.

Flour for wheat couscous was obtained commercially, so no decortication or milling was necessary. Wheat flour was separated into the same particle size fractions as the millet flour using the procedure described above. Wheat flour particles between 300-495 µm were used to make the wheat couscous.

Couscous preparation. Couscous was prepared according to a traditional West African method with the expertise of a Senegalese native with more than 15 years of experience preparing couscous. Briefly, flour (500 g wet weight) was weighed and water (283 ± 11 mL) was gradually added as the mixture was rolled continuously by hand into small couscous particulate spheres. These spheres were then passed through a 1.70 mm or 2.36 mm sieve to constitute the small and large couscous samples, respectively. All retained couscous spheres were sieved again before being steamed in a couscoussier for 14 min over boiling water (100-120 ^{o}C). For uniformity and storage, samples were dried at 50 °C for 240 min following steaming. Prior to simulated gastric digestion, all couscous samples were hydrated with water (2.5:1 g couscous: mL water, wet basis) and steamed 10 min, after which they were immediately used for simulated digestion. In total, five types of couscous were used in this study (Table 1).

Table 1. Types of couscous. Source of each couscous is given in addition to its initial (before digestion) particle size.

Couscous type	Source	Initial particle size (mm)
Wheat small	Flour from Bob's Red Mill, Milwaukie, OR, USA Couscous self-made at Purdue University, IN, USA	< 1.70
Wheat commercial	Riviana Foods Inc., Houston, TX, USA	< 2.36
Millet small	Grain from Alif Group, Dakar, Senegal Couscous self-made at Purdue University, IN, USA	< 1.70
Millet large	Grain from Alif Group, Dakar, Senegal Couscous self-made at Purdue University, IN, USA	1.70-2.36
Millet commercial	Mme. Deme of Free Work Services, Dakar, Senegal	< 2.36

Couscous characterization

Rapid Visco Analyzer analysis. Pasting profiles of couscous (small and large millet couscous combined, small wheat combined with an additional large wheat couscous treatment) without the second steaming step, and raw flours, were determined using a Rapid Visco Analyzer (RVA; model RVA-4; Perten Instruments Instrumentvägen 29, SE-126 53 Hägersten, Sweden) using the Standard 1 protocol supplied with the instrument. This protocol consists of holding at 50 °C for 1 min, heating at a rate of 12 °C/min to 95 °C, equilibrating at 95 °C for 2.5 min, cooling at a rate of 12 °C/min to 50 °C, and holding at 50 °C for 2 min. The RVA mixing paddle speed was 960 rpm for the first 10 s and then 160 rpm for the remainder of the experiment. Slurries were made for each sample (3 g dry basis) with water (25 mL for millet flour and wheat flour; 15 mL for millet couscous and wheat couscous).

Starch fine structure. Molecular size and unit chain length distribution of amylose and amylopectin (starch structure) were characterized for all couscous samples along with the flour starting materials for the self-made couscous by high performance size-exclusion chromatography (HPSEC) following the procedure of Roman et al.[17].

Simulated oral and gastric digestion

Simulated saliva formulation. Simulated saliva was prepared according to Bornhorst and Singh[18]. Briefly, mucin (1 g/L, Sigma-Aldrich, St. Louis, MO, USA), α-amylase (from *Bacillus subtilis*, 1.18 g/L, MP Biomedicals, Catalog Number 100447, 160000 BAU/g activity, Santa Ana, CA, USA), NaCl (0.117 g/L, Avantor Performance Materials, Radnor, PA, USA), KCl (0.149 g/L, ThermoFisher Scientific, Waltham, MA, USA) and $NaHCO_3$ (2.10 g/L, ThermoFisher Scientific, Waltham, MA, USA) were mixed in

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deionized water. The concentration of α-amylase was set to reflect its activity in vivo[19].

Simulated gastric juice formulation. Simulated gastric juice was prepared according to Mennah-Govela and Bornhorst[20]. Mucin (1.5 g/L, Sigma-Aldrich, St. Louis, MO, USA), NaCl (8.78 g/L, Sigma-Aldrich, St. Louis, MO, USA), and pepsin from porcine pancreas (1.0 g/L, Sigma-Aldrich, St. Louis, MO, USA) were mixed in deionized water (acidified to pH 1.8 using 3 M HCl). After all components were dissolved, pH was adjusted to 1.8 using 3 M HCl. Pepsin concentration was chosen to provide an activity of 2,000 U/mL in simulated gastric juice[21,22], and pH was set to 1.8 to simulate the fasted pH of gastric juice[23].

Simulated oral and gastric digestion procedure. Following steaming, 300 g cooked couscous was weighed, and 60 mL of simulated saliva was added (0.2 mL/g) and mixed for 30 s to represent the oral phase[24].

Simulated gastric digestions were conducted using the HGS to simulate the peristaltic movement of the human stomach (Fig. 1). The HGS has been described in detail previously[25,26]. Briefly, the HGS utilizes rollers to apply a simulated peristaltic wave (3 contractions/minute) to the food and simulated gastric juice[13,27], which are contained in a flexible plastic bag. The temperature is maintained at 37 °C.

Simulated gastric digestions were initiated immediately after the oral phase by placing the test meal into the HGS which was preloaded with 75 mL gastric juice preheated to 37 °C. A peristaltic pump (Model 13-876-2, ThermoFisher Scientific, Waltham, MA, USA) was used to continuously secrete simulated gastric juice at 2.5 mL/min[12,28]. Simulated gastric digestions were carried out for 180 min, with 90 mL samples of digesta (approximately 100 g wet weight) collected from the simulated pyloric valve every 30 min. After collection, samples of digesta were analyzed for moisture content, pH, particle size, and reducing sugar content. Digestions were conducted in triplicate except for small millet couscous which was done in quadruplicate.

Moisture content

Digesta was collected at each time point and 3 g, weighed into pre-dried aluminum pans, was dried in a vacuum oven (Lindberg Blue M, Thermo Scientific, Waltham, MA, USA) for 20 h at 120 °C (AACCI Method 44-40.01).

pH measurement

Digesta from each time point was placed in a 50 mL conical tube for pH measurement (IQ Scientific IQ150-77 ISFET, Cole-Parmer, Vernon Hills, IL, USA).

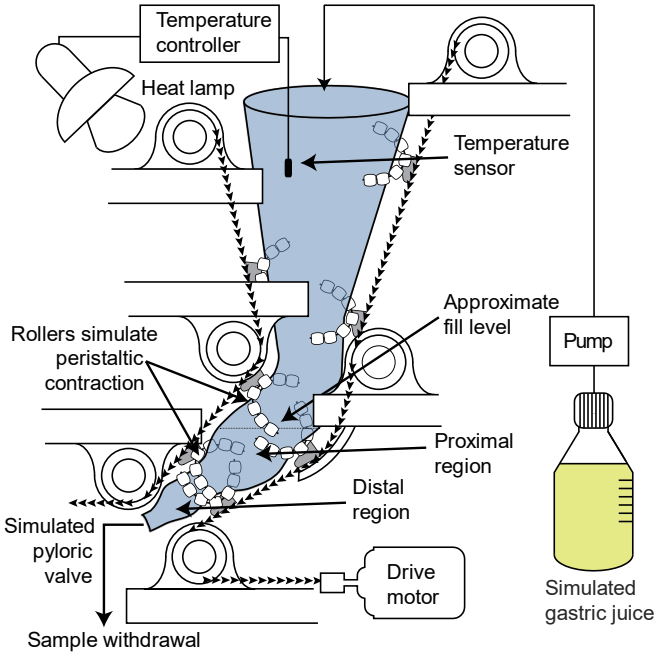

Figure 1. Diagram of the Human Gastric Simulator (HGS).

Particle size analysis

Image analysis was used to determine the size distribution of couscous particles in the digesta according to a previously described method with minor modifications[29]. Following collection of digesta from the HGS, an aliquot of 0.48 ± 0.01 g was dispersed into several petri dishes, each containing 20 mL deionized water. Multiple dishes were used for each sample in order to minimize particle overlap. Lugol's iodine solution (5-10 µL) was added to each petri dish to enhance the contrast between the light field and the particles. Petri dishes were illuminated from underneath using a lightbox (AGPtek HL0163, Brooklyn, NY, USA; color temperature 6000 °K). A reference object (ABFO No. 2 photomacrographic standard reference scale) was included in all images for spatial calibration. One image of each dish was captured using a Canon EOS Rebel SL1 digital camera (18 Mega Pixels, APS-C CMOS sensor, Canon USA, INC. San Jose, CA, USA) that was fixed to a vertical support and triggered using a computer interface. The camera settings were: no flash, 35 mm focal length, aperture F8.0, ISO 100, and shutter speed 0.1 s. Images were analyzed using MATLAB (MathWorks, Natick, MA, USA) to determine the total number of particles in each image and the area of each particle. Particles per gram of dry mass was defined as the number of particles in a sample of digesta divided by the dry mass of the sample, as determined by moisture content analysis. The particle size distribution was analyzed by fitting the cumulative area percentage of the particles in each sample to the Rosin-Rammler model[30]:

$$C_{area} = 1 - \exp\left(-\left(\frac{x}{x_{50}}\right)^{b} \ln(2)\right) \quad (1)$$

Where C_{area} is the cumulative area percentage of each particle (0 to 100%), X_{50} is the median particle area (mm^2), and b is the

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distribution breadth constant (dimensionless). Smaller *b* values represent a broader distribution spread. This model has been used by previous researchers to describe the size changes of solid food particles during oral and gastric digestion using image analysis[4,29,31].

Starch hydrolysis analysis

Aliquots of digesta samples from each time point were centrifuged at 1000 × g, upon which the supernatant was mixed with 0.3 M sodium carbonate (200 µL) and stored until analysis was performed. Reducing sugar content was then quantified using the dinitrosalicylic acid (DNS) method[32] and expressed as percent starch hydrolysis. Percent starch hydrolysis at each time point was then divided by the total area of couscous particles from image analysis to obtain a value of starch hydrolysis per unit area of digested particles ($\%\ starch\ hydrolysis/mm^2$).

Relative gastric emptying of solids from the HGS

Samples of digesta were subjected to gravimetric moisture content analysis as described above to determine the solid matter content. The relative gastric emptying of solids was expressed as the remaining dry matter in the HGS at each time point divided by the initial dry matter. Since the overall emptying rate of digesta (90 mL every 30 min) was held constant for all types of couscous, the relative gastric emptying of solids reflected the solids content of digesta samples that were withdrawn from the HGS. For each type of couscous, the curve of relative gastric emptying of solids was fit to a modified power-exponential model used by previous researchers to fit gastric emptying data[5,33,34]:

$$y(t) = 1 - (1 - e^{-kt})^{\beta} \quad (2)$$

Where $y(t)$ is the percent of initial dry matter retained in the HGS at time t, t is digestion time (min), k is the emptying rate parameter (min^{-1}), and β is the extrapolated y-intercept from the terminal portion of the curve (dimensionless). The fit was conducted using nonlinear least squares in MATLAB (MathWorks, Natick, MA, USA). The half-time for relative gastric emptying of solids was estimated using Equation 3:

$$t_{1/2} = \frac{ln(1-0.5^{1/\beta})}{-k} \quad (3)$$

Where $t_{1/2}$ is the estimated time at which 50% of the dry mass present at time zero was emptied from the HGS (min), and the parameters k and β were from Equation 2.

Statistical analysis

Statistical analysis was conducted using SAS Enterprise Guide 7.1 (SAS Institute, Cary NC, USA). Initial properties of couscous were analyzed using a single factor ANOVA with type of couscous as the factor. Variables that were measured at each time point during simulated gastric digestion (median particle area, particles per gram, moisture content, pH, and starch hydrolysis per unit area) were analyzed using a two factor ANOVA with repeated measures (PROC MIXED). If the F value of the overall model was significant ($p < 0.05$), post hoc tests were conducted using Tukey's HSD and significance was taken at $p < 0.05$.

Results

Couscous properties

Rapid Visco Analyzer analysis. To determine if initial material properties (i.e., viscosity) of couscous could help explain their behavior during simulated gastric digestion, analysis using the Rapid Visco Analyzer (RVA) was conducted. Viscosity profiles obtained using the RVA are shown in Fig. 2. Wheat couscous exhibited an initial increase in viscosity before heating (0-250 s), referred to as cold swelling, while millet couscous did not. Peak viscosity (at ~320 s and 95 °C) was lower for millet couscous than wheat couscous (2791 ± 51 cP and 4084 ± 137 cP, respectively, $p < 0.01$). The final viscosity measured during RVA was 8206 ± 116 cP for wheat couscous, 2761 ± 28 cP for wheat flour, 5793 ± 22 cP for millet couscous, and 3887 ± 11 cP for millet flour. All final viscosity values were statistically different from each other ($p < 0.01$).

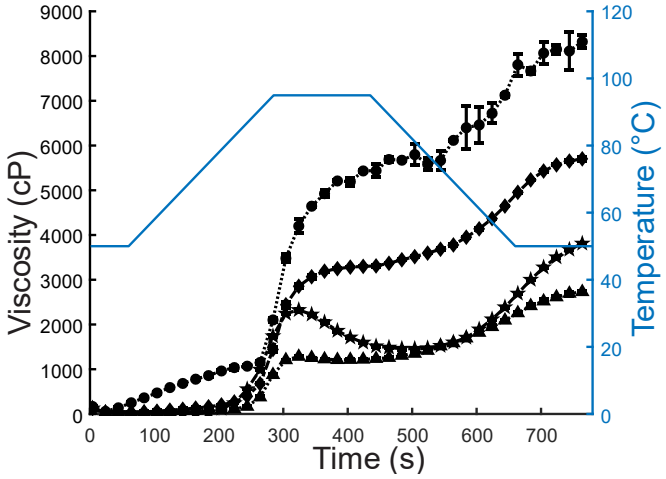

Figure 1. RVA profiles of wheat couscous (●), wheat flour (▲), millet couscous (♦), and millet flour (★). Error bars represent the standard deviation of two runs and in some cases may be too small to be seen. Viscosity was recorded every 4 seconds, but for clarity a symbol has been placed on every fifth data point (every 20 s).

Starch fine structure. Starch fine structure was analyzed using HPSEC (Table 2). There were no statistically significant differences in amylose hydrodynamic radius or amylopectin hydrodynamic radius ($p > 0.05$) between the wheat and millet flours and couscous, but there were significant differences in amylose chain length [degree of polymerization (DP), $p < 0.05$].

Notably, wheat flour had the highest amylose chain length as represented by DP (1563.3 ± 38.5 DP), while small and large millet couscous samples had the lowest amylose chain lengths (839.0 ± 42.2 DP and 865.8 ± 24.9 DP, respectively). All millet samples had smaller amylose chain lengths than wheat samples, although statistically significant differences were not observed across all samples (Table 2).

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Table 2. Starch structural characterization of initial (undigested) couscous as well as for flour starting materials used for self-made couscous types. Values in each column that do not share a letter (abc) represent significant differences ($p < 0.05$) between different types of couscous or flour. If no letter is shown, there were no statistically significant differences.

	Amylose hydrodynamic radius (nm)	Amylopectin hydrodynamic radius (nm)	Amylopectin short chain length (DP)	Amylopectin long chain length (DP)	Amylose chain length (DP)	Molar ratio of long to short amylopectin chains	Amylose ratio (%)
Wheat flour	43.1 ± 3.7	131.5 ± 0.4	13.1 ± 0.1	38.8 ± 0.5	1563.3 ± 38.5^{a}	0.541 ± 0.012	33.5 ± 2.6
Millet flour	28.9 ± 3.8	131.2 ± 1.1	14.7 ± 0.1	38.9 ± 0.2	962.6 ± 26.5^{bc}	0.532 ± 0.002	29.9 ± 2.7
Wheat small	33.5 ± 0.4	130.1 ± 6.7	13.3 ± 0.2	39.1 ± 0.5	1224.9 ± 105.7^{b}	0.550 ± 0.004	34.7 ± 0.2
Wheat commercial	27.5 ± 0.0	133.1 ± 0.0	16.1 ± 3.0	37.2 ± 2.4	1250.5 ± 123.2^{ab}	0.593 ± 0.042	35.6 ± 0.3
Millet small	26.3 ± 7.0	131.9 ± 5.9	14.9 ± 0.1	38.7 ± 0.1	839.0 ± 42.2^{c}	0.540 ± 0.009	33.8 ± 4.1
Millet large	25.0 ± 4.6	132.3 ± 7.4	14.9 ± 0.1	39.1 ± 0.3	865.8 ± 24.9^{c}	0.533 ± 0.004	32.6 ± 3.2
Millet commercial	27.1 ± 0.5	128.2 ± 6.9	14.6 ± 0.2	38.0 ± 1.6	939.0 ± 47.1^{bc}	0.598 ± 0.122	38.2 ± 9.3

Particle size analysis

The Rosin-Rammler model (Equation 1) was fit to the cumulative distribution of particle areas as measured using image analysis. It was found that the Rosin-Rammler function provided a good fit to the data as evidenced by a high coefficient of determination, with a minimum R^2 of 0.945 across all types of couscous and gastric sampling time points, and the average R^2 across all treatments of 0.980. Median particle area, as represented by Rosin-Rammler x_{50} (mm^2), was significantly influenced by digestion time ($p < 0.01$), type of couscous ($p < 0.01$), and their interaction ($p < 0.01$). The particle size of couscous experienced a large and statistically significant decrease throughout the simulated digestion for all types of couscous ($p < 0.05$) (Table 3). For example, x_{50} of commercial millet after 30 s of simulated oral phase was 1.62 mm^2 and decreased to 0.2 mm^2 for the sample that underwent 180 min of simulated gastric digestion in the HGS.

The spread of the particle size distribution is represented by Rosin-Rammler b (dimensionless), where a smaller value of b indicates a wider distribution of particle sizes and a larger value of b indicates a narrower distribution of particle sizes. Rosin-Rammler b was significantly influenced by digestion time ($p < 0.01$), type of couscous ($p < 0.01$), and their interaction ($p < 0.01$) (Table 3). Post-hoc tests showed that statistically significant differences in the value of b between different types of couscous were only present during the first two time points (after 30 s oral phase, and after 30 s oral phase followed by 30 min gastric digestion, respectively). This suggests that the spread of the particle size distribution was different between types of couscous at the beginning of simulated digestion, but that after 60 min of simulated digestion in the HGS the differences in distribution spread between different types of couscous were no longer significant.

Particle size analysis also allowed for quantification of the number of particles per gram of dry mass in the digesta. Particles per gram of dry mass in the digesta was significantly influenced by digestion time ($p < 0.01$), type of couscous ($p < 0.01$), and their interaction ($p < 0.01$) (Fig. 3). For all types of millet couscous there was a large and statistically significant increase in particles per gram of dry mass during simulated digestion. For example, small millet couscous had 7.0×10^3 particles per gram after 30 s of simulated oral phase, but after 180 min in the HGS had 88.8×10^3 particles per gram ($p < 0.05$). Results showed that higher particle breakdown occurred during simulated gastric digestion of millet couscous leading to a substantial and statistically significant increase in the number of particles per gram dry mass, whereas in wheat couscous there was not a statistically significant increase in the number of particles per gram of dry mass.

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Table 3. Median particle size, quantified by Rosin-Rammler x_{50} (Equation 1), and particle size distribution spread parameter, quantified by Rosin-Rammler *b* (Equation 1), for particles in digesta withdrawn from the HGS at different time points and for different types of couscous. All values are means of multiple runs in the HGS (*n = 3* runs, except for millet small which was *n = 4*) ± standard deviation. Values in each column that do not share a letter (abc) represent significant differences ($p < 0.05$) within a certain type of couscous across different digestion times. Values in each row that do not share a letter (zyx) represent significant differences ($p < 0.05$) within a certain time point across different types of couscous. If no letter is shown, there were no statistically significant differences.

	$x_{50}(mm^2)$					***b*** **(dimensionless)**				
Digestion time (min)	**Wheat small**	**Wheat commercial**	**Millet small**	**Millet large**	**Millet commercial**	**Wheat small**	**Wheat commercial**	**Millet small**	**Millet large**	**Millet commercial**
0.5	2.09 ± 0.73^{a,yx}	3.77 ± 0.11^{a,z}	1.45 ± 0.09^{a,w}	2.39 ± 0.30^{a,y}	1.62 ± 0.52^{a,xw}	1.33 ± 0.03^{a,yx}	1.72 ± 0.36^{a,z}	1.47 ± 0.12^{a,zy}	1.06 ± 0.26^{x}	1.09 ± 0.21^{x}
30	0.75 ± 0.19^{b,y}	2.27 ± 0.63^{bc,z}	0.15 ± 0.02^{b,y}	0.16 ± 0.02^{b,y}	0.42 ± 0.10^{b,y}	1.10 ± 0.15^{ab,z}	0.82 ± 0.02^{b,zy}	1.03 ± 0.05^{b,zy}	0.99 ± 0.04^{zy}	0.79 ± 0.01^{y}
60	0.56 ± 0.06^{b,y}	1.53 ± 0.41^{de,z}	0.14 ± 0.02^{b,y}	0.15 ± 0.03^{b,y}	0.25 ± 0.05^{b,y}	1.08 ± 0.14^{ab}	0.84 ± 0.07^{b}	1.09 ± 0.01^{b}	1.10 ± 0.05	0.88 ± 0.03
90	0.59 ± 0.03^{b,y}	1.61 ± 0.65^{ce,z}	0.16 ± 0.03^{b,y}	0.14 ± 0.03^{b,y}	0.23 ± 0.04^{b,y}	1.07 ± 0.07^{ab}	0.90 ± 0.17^{b}	1.02 ± 0.04^{b}	1.11 ± 0.03	0.90 ± 0.04
120	0.57 ± 0.09^{b,y}	2.50 ± 0.34^{b,z}	0.13 ± 0.03^{b,y}	0.12 ± 0.03^{b,y}	0.19 ± 0.01^{b,y}	1.11 ± 0.10^{ab}	0.86 ± 0.11^{b}	1.03 ± 0.05^{b}	1.10 ± 0.05	0.92 ± 0.05
150	0.76 ± 0.21^{b,y}	1.80 ± 0.84^{bcde,z}	0.27 ± 0.15^{b,y}	0.11 ± 0.03^{b,y}	0.19 ± 0.04^{b,y}	1.01 ± 0.10^{ab}	0.92 ± 0.33^{b}	0.90 ± 0.12^{b}	1.09 ± 0.08	0.89 ± 0.04
180	0.85 ± 0.19^{b,zy}	1.06 ± 0.76^{de,z}	0.18 ± 0.12^{b,x}	0.17 ± 0.07^{b,x}	0.20 ± 0.09^{b,yx}	0.98 ± 0.06^{b}	0.81 ± 0.08^{b}	1.02 ± 0.25^{b}	0.95 ± 0.09	0.85 ± 0.08

Starch hydrolysis in the HGS

Reducing sugar content in the HGS was measured in the liquid phase of centrifuged digesta over the course of the simulated digestion and the percent of starch hydrolysis was divided by the total area of particles at the same time point; the resulting values were expressed as percent starch hydrolysis per unit area of digested particles. Starch hydrolysis per unit area in the HGS was significantly influenced by digestion time ($p < 0.01$) and type of couscous ($p < 0.01$), but not their interaction ($p > 0.05$) (Fig. 4). There was a significant increase in percent starch hydrolysis in the total digestion time per unit area of digested particles for all types of couscous ($p < 0.05$), except for small millet couscous ($p > 0.05$).

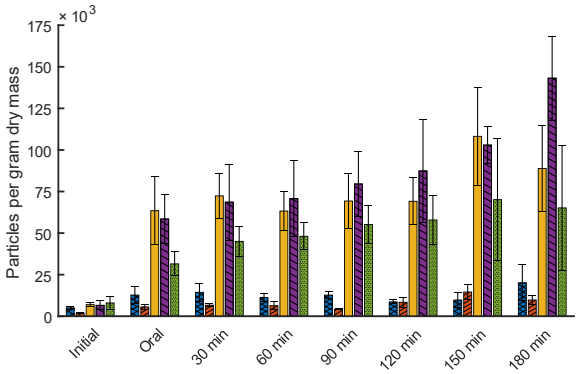

Figure 3. Particles per gram dry matter of for digesta of small wheat couscous (▨), commercial wheat couscous (▨), small millet couscous (▨), large millet couscous (▨), and commercial millet couscous (▨) during vitro gastric digestion (initial = no digestion; oral = 30 s simulated oral digestion; 30-180 min = corresponding simulated gastric digestion time). Error bars represent standard deviation of either three or four digestion in the HGS.

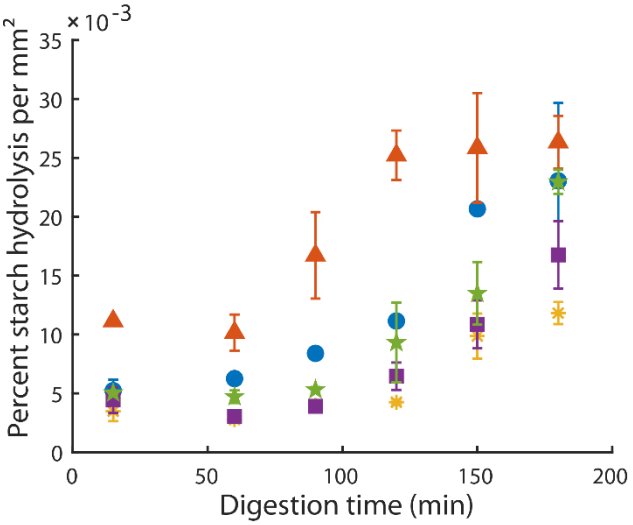

Figure 4. Percent starch hydrolysis per unit area of particles in the HGS for small wheat couscous (●), commercial wheat couscous (▲), small millet couscous (✳), large millet couscous (■), and commercial millet couscous (★). Error bars represent the standard error of three runs except for millet small (four runs).

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At the final timepoint of the simulated gastric digestion (180 min), percent starch hydrolysis per unit area for large millet couscous (16.8 ± 5.0 $\times 10^{-3}$ $\%/mm^2$) and for small millet couscous (11.8 ± 1.9 $\times 10^{-3}$ $\%/mm^2$) were significantly lower ($p < 0.05$) than for commercial wheat couscous (26.3 ± 3.9 $\times 10^{-3}$ $\%/mm^2$).

Moisture content

Moisture content (dry basis) was significantly influenced by digestion time ($p < 0.01$), but not by type of couscous ($p > 0.05$) (Table 4). Moisture content of digesta was not significantly different between the five types of couscous from oral phase up to 150 min of simulated gastric digestion. Moisture content of digesta after 180 min ranged from 4.09 ± 0.58 g moisture/g dry mass (wheat small) to 7.62 ± 1.79 (millet small) g moisture/g dry mass.

Table 4. Moisture content (dry basis, g water/g dry mass) and pH of digesta withdrawn from the HGS at different time points and for different types of couscous. All values are means of multiple runs in the HGS (*n = 3* runs, except for millet small which was *n = 4*) ± standard deviation. Values in each column that do not share a letter (abc) represent significant differences ($p < 0.05$) within a certain type of couscous across different digestion times. Values in each row that do not share a letter (zyx) represent significant differences ($p < 0.05$) within a certain time point across different types of couscous. If no letter is shown, there were no statistically significant differences.

	Moisture content (g moisture/g dry mass)					pH				
Digestion time (min)	Wheat small	Wheat commercial	Millet small	Millet large	Millet commercial	Wheat small	Wheat commercial	Millet small	Millet large	Millet commercial
0.5	0.84 ± 0.09^{b}	0.82 ± 0.03^{b}	0.8 ± 0.09^{b}	0.9 ± 0.1^{c}	0.89 ± 0.05^{c}	6.38 ± 0.51^{a}	6.44 ± 0.32^{a}	6.29 ± 0.09^{a}	6.48 ± 0.05^{a}	5.83 ± 0.17^{a}
30	2.66 ± 0.35^{ab}	2.93 ± 0.22^{ab}	1.8 ± 0.04^{b}	1.81 ± 0.04^{c}	1.86 ± 0.08^{c}	4.88 ± 0.17^{bc}	5.01 ± 0.15^{bc}	5.35 ± 0.13^{b}	5.23 ± 0.09^{bc}	4.98 ± 0.06^{ab}
60	2.36 ± 0.11^{ab}	2.83 ± 0.23^{ab}	1.7 ± 0.06^{b}	1.68 ± 0.05^{c}	1.74 ± 0.12^{c}	5.36 ± 0.16^{b}	5.14 ± 0.26^{b}	5.63 ± 0.10^{ab}	5.63 ± 0.11^{ab}	5.29 ± 0.07^{ab}
90	2.5 ± 0.18^{ab}	3.01 ± 0.09^{ab}	1.93 ± 0.6^{b}	1.76 ± 0.11^{c}	1.66 ± 0.07^{c}	5.30 ± 0.43^{b}	4.92 ± 0.32^{bc}	5.63 ± 0.24^{ab}	5.62 ± 0.06^{ab}	5.36 ± 0.10^{a}
120	2.92 ± 0.12^{ab}	3.45 ± 0.1^{ab}	2.26 ± 0.45^{b}	2.03 ± 0.2^{c}	2.41 ± 1.08^{c}	4.76 ± 0.80^{bcd}	4.58 ± 0.19^{bcd}	5.37 ± 0.22^{b}	5.43 ± 0.14^{bc}	4.96 ± 0.48^{ab}
150	3.61 ± 0.21^{ab}	4.2 ± 0.11^{a}	5.15 ± 1.83^{a}	3.24 ± 0.44^{c}	3.2 ± 0.31^{bc}	4.69 ± 0.38^{bcd}	4.18 ± 0.11^{cd}	3.88 ± 0.94^{c}	4.49 ± 0.41^{c}	4.37 ± 0.24^{b}
180	4.09 ± 0.58^{a,y}	5.16 ± 0.29^{a,zy}	7.62 ± 1.79^{a,z}	7.53 ± 1.82^{b,z}	6.14 ± 1.53^{ab,zy}	4.21 ± 0.25^{cd}	3.84 ± 0.25^{d}	2.91 ± 0.32^{d}	2.87 ± 0.39^{d}	3.24 ± 0.32^{c}

pH

pH of digesta was significantly influenced by digestion time ($p < 0.01$), but not by type of couscous ($p > 0.05$) (Table 4). The pH of digesta decreased throughout the simulated digestion due to the secretion of additional gastric juice. For example, the pH of small millet couscous was 6.29 ± 0.09 after 30 s of simulated oral phase and decreased to 2.91 ± 0.32 after 180 min simulated gastric digestion.

Relative gastric emptying of solids from the HGS

Relative gastric emptying of solids in the HGS was fit to Equation 2, and results are shown in Fig. 5. The model provided a good fit to the data as evidenced by high coefficient of determination (R^2 minimum = 0.997) (Table 5). The emptying rate parameter, k (min^{-1}), was significantly influenced by couscous type ($p < 0.05$). Large millet couscous had a significantly higher k value than commercial wheat couscous ($p < 0.05$). The extrapolated y-intercept from the terminal portion of the curve, β, was not significantly influenced by type of couscous ($p > 0.05$). The half-emptying time $t_{1/2}$ (min) was significantly influenced by type of couscous ($p < 0.01$). The highest half-emptying times were for commercial wheat couscous and small wheat couscous (240 min and 189 min, respectively). This reflects the slower emptying of solids from the HGS during simulated gastric digestions of commercial wheat couscous and small wheat couscous. It is important to note that these results indicate the emptying of solids as the overall gastric emptying rate of digesta was held constant at 3 mL/min for all types of couscous. The more rapid emptying of solids from millet couscous coincided with a greater degree of breakdown as described in the previous section, indicating that the HGS measures gastric emptying based on particle breakdown.

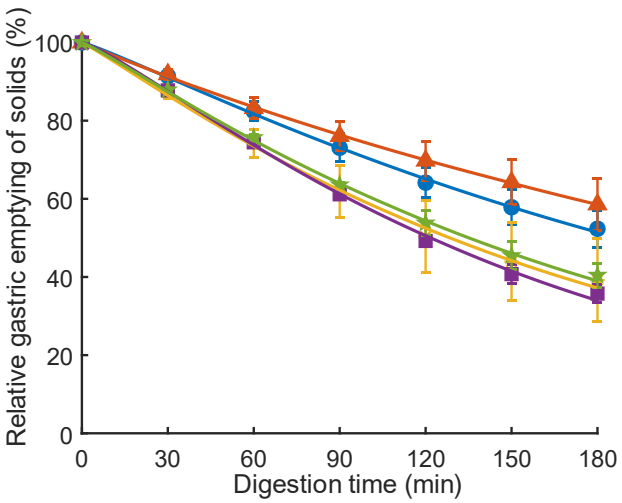

Figure 5. Relative solids remaining in the HGS (%) for small wheat couscous (●), commercial wheat couscous (▲), small millet couscous (✳), large millet couscous (■), and commercial millet couscous (★). Error bars represent the standard deviation of three digestions except for small millet couscous(four digestions). Lines represent the model fit (Equation 2).

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Table 5. Parameters of the modified power-exponential model (Equation 1) for gastric emptying. Values in each column that do not share a letter (abc) represent significant differences (p < 0.05) between different types of couscous.

	$k \times 10^3 (min^{-1})$	β	$t_{1/2}\ (min)$	R^2
Wheat small	4.20 ± 0.59^{ab}	1.14 ± 0.07	189 ± 25^{ab}	0.999 ± 0.00
Wheat commercial	2.97 ± 0.86^{bc}	0.99 ± 0.07	240 ± 49^{a}	0.999 ± 0.00
Millet small	5.28 ± 2.12^{ab}	1.06 ± 0.19	146 ± 29^{bc}	0.997 ± 0.003
Millet large	7.03 ± 0.60^{a}	1.25 ± 0.06	122 ± 6^{bc}	0.998 ± 0.001
Millet commercial	5.76 ± 1.09^{ab}	1.13 ± 0.15	137 ± 10^{bc}	0.998 ± 0.001

Discussion

In this study, the Human Gastric Simulator was used to quantify the physical and chemical breakdown of couscous samples during simulated gastric digestion. Previously, other starch-based carbohydrates, such as white and brown rice, have been studied for their physicochemical breakdown properties during gastric digestion[6,35,36]. Millet couscous and other products made from millet have previously shown slow gastric emptying and digestion properties in vivo[3,37] and in vitro[38,39], yet there is a lack of knowledge of the basis of this response. The objective of this work was to determine how pearl millet couscous breaks down in a simulated gastric environment and the starch hydrolysis of the gastric breakdown products.

Initial properties of couscous samples and flour (for self-made couscous) were characterized by RVA analysis and HPSEC. RVA analysis is a rheological method to characterize suspensions containing starch and water during heating and cooling[40]. Notable factors with impact on peak viscosity include the extent of amylose leaching, amylose-lipid complex formation, starch granule swelling, friction between swollen granules, and competition for free water between leached amylose and remaining ungelatinized granules[41,42]. For the current study, lower water amount was used for couscous samples than for flour samples (25 mL for flours, 15 mL for couscous), because couscous samples were pregelatinized and thus did not absorb water and swell like the intact starch granules in flours. Furthermore, millet couscous did not show a cold swelling property, which could be indicative of either immediate breakdown in the RVA or incomplete gelatinization of starch in millet couscous after the initial 14 min steaming period of preparation. However, it is important to note that for simulated digestions, all couscous samples were steamed an additional 10 min immediately prior to experimentation. Final viscosities from RVA results were compared due to relevance to foods prepared for consumption. Previous researchers found final viscosity to be the most sensitive measurement of intermolecular interactions in maize starch[43]. Results indicated that final viscosity was higher for millet flour than for wheat flour (3887 ± 11 vs. 2761 ± 28 cP, p < 0.01). Given its higher viscosity, starch granules in millet flour exhibited greater amylose retrogradation than those in wheat flour[43]. No significant amylose-lipid complex formation was evident for any of the samples as determined by differential scanning calorimetry (data not shown), so this would not have impacted the RVA profiles. In contrast to flour samples, wheat couscous exhibited higher final viscosity than millet couscous (8206 ± 116 vs. 5793 ± 22 cP, p < 0.01), indicating higher breakdown of millet couscous to smaller particles. Accordingly, millet couscous appeared paste-like, whereas wheat couscous particles remained intact.

HPSEC was used to determine starch fine structural features, as it was hypothesized that they could help explain the slower starch hydrolysis rate that was found for millet samples. Millet samples had smaller amylose chain length (839-963 DP) than wheat samples (1225-1563 DP) (Table 2). The higher amylose chain lengths obtained for flours compared to couscous could indicate amylose leaching during the couscous making process (with the exception of commercial couscous products for which flour starting materials could not be obtained). Amylose of intermediate chain length (667 DP) was previously shown to exhibit a higher propensity to form intermolecular interactions[44]. Although the amylose chain lengths measured for millet samples were somewhat larger than 667 DP, it can be hypothesized that they also could exhibit increased intermolecular interactions, causing denser matrices that impede starch hydrolysis[45]. To the authors' knowledge, starch fine structural characteristics have not been previously elucidated for the particular type of pearl millet studied (grown in Senegal), however, the characteristics for wheat are similar to those found by Martinez et al.[46]. The lack of significant differences in hydrodynamic radius for amylose and amylopectin indicates that these starch structural characteristics do not independently explain the differences observed in starch hydrolysis.

Along with initial property measurements of couscous, the particle size of couscous in digesta emptied from the HGS was assessed. It was found that all types of couscous experienced a statistically significant reduction in particle size throughout simulated digestion, however, breakdown of millet couscous was substantial and significantly (p < 0.05) higher than that of wheat couscous. For example, after 180 min simulated gastric digestion, large millet couscous particles had a median size of 0.17 mm^2, whereas commercial wheat couscous particles had a median size of 1.06 mm^2 (p < 0.05). These differences may be due to the presence of gluten in wheat[47], which acts to provide structure in many gluten-containing foods, and may help wheat-based foods resist breakdown. Previous research has shown a decrease in particle size of white and brown rice during gastric processing in vitro[48] and in vivo[4]. To the authors' knowledge, the particle breakdown of couscous has not previously been studied in vitro or in vivo.

Starch hydrolysis per unit area of digested particles increased over time for all types of couscous (p < 0.05). To control for increases in surface area that occurred due to particle breakdown in the HGS, starch digestion was reported as percent

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starch hydrolysis per unit area of digested particles. Percent starch hydrolysis per unit area at the final time point in digestion was significantly lower ($p < 0.05$) for large millet couscous (16.8 ± 5.0 ×10^{-3} $\%/mm^2$) and small millet couscous (11.8 ± 1.9 ×10^{-3} $\%/mm^2$) than for commercial wheat couscous (26.3 ± 3.9 ×10^{-3} $\%/mm^2$). Interestingly, starch hydrolysis per unit area increased by only 4% in the final hour of simulated gastric digestion for commercial wheat couscous, whereas there was an 88% increase over the same time period for large millet couscous and 85% increase for commercial millet couscous. This suggests that the millet starch became more susceptible to hydrolysis in the last hour of digestion, whereas the wheat couscous starch was fully accessible by the end of the second hour of digestion. This finding suggests that changes to millet couscous resulting in an increased rate of starch hydrolysis occur only after two hours of simulated digestion. Previous researchers have found that millet has a slow starch hydrolysis property[37–39], lending support for the current results. Since pH profiles were not significantly different among the types of couscous, differences in hydrolysis between different types of couscous cannot be attributed to differences in residual salivary α-amylase activity during simulated gastric digestion. Thus, differences in starch hydrolysis were due to differences in chemical and/or structural composition of the couscous, as well as differences in their breakdown during digestion. Notably, intermediate amylose chain lengths (841-970 DP) for millet samples could have allowed for increased intermolecular interactions[44] which might have promoted the formation of denser matrices that are more resistant to hydrolysis[45]. Previous researchers have proposed that microstructural and starch granule features of starchy foods can influence starch hydrolysis[49].

pH of the digesta decreased over time for all types of couscous due to the secretion of gastric juice during simulated digestion. Due to the constant secretion rate of gastric juice, the lack of significant differences in pH between the different couscous samples indicates that they had similar buffering capacity. The relatively high pH values (highest value among treatments at 30 min = 6.48; highest value at 180 min = 4.21) throughout gastric digestion create an environment where activity of salivary α-amylase could be partially retained.

Relative gastric emptying of solids was analyzed by calculating the percentage of initial dry matter that remained in the HGS at each time point. Since the overall emptying rate was held constant at 3 mL/min, differences in relative gastric emptying of solids reflected the solids content of samples withdrawn from the HGS, and the relative breakdown of particles that occurred. Curves representing relative emptying of solids were fit to a modified power-exponential model (Equations 2 and 3) which was used to describe the emptying profile in terms of a kinetic value, k (min^{-1}), an extrapolated y intercept, β, and an emptying half-time, $t_{1/2}$ (min). It was found that large millet couscous emptied the most rapidly, with k value of 7.03 min^{-1} and $t_{1/2}$ of 122 min. Commercial wheat couscous emptied more slowly than large millet couscous, with k value of 2.97 min^{-1} and $t_{1/2}$ of 146 min ($p < 0.05$). Gastric emptying half-times in this study (122-240 min) were comparable in magnitude to those reported by previous researchers for in vivo gastric emptying of brown and white rice meals using the growing pig as a model for the adult human (229 and 227 min, respectively)[5]. In our recent human study conducted in Mali, we found that millet couscous had very slow gastric emptying time (5.3 h)[3]. It appears that the discrepancy between gastric emptying times of millet couscous measured in vivo and in the HGS reflects that gastric emptying in vivo is affected by factors other than particle breakdown solely. In the HGS, millet couscous broke down into smaller, more numerous particles than wheat couscous and had a more rapid gastric emptying rate of solids. However, physiological feedback and control mechanisms of gastric emptying in vivo are not reproduced by current in vitro models. Thus, the results of this study lend support to the hypothesis that physiological controls of gastric emptying may better explain the slow gastric emptying of millet couscous in vivo, and that hydrolysis-resistant particles of millet couscous reaching the distal small intestine may activate the ileal brake. The ileal brake mechanism has been shown to delay gastric emptying rate in a dose-dependent manner[50] and when administered as a preload[51]. Of note, higher delay in gastric emptying has been related to greater length of small intestine exposure to glucose[52]. In this study, percent starch hydrolysis of couscous was expressed as per unit surface area of particles, hence increased surface area by greater particle breakdown of millet couscous still did not increase starch hydrolysis. Thus, millet particles exiting the stomach could reach the distal small intestine, potentially activating the ileal brake and contributing to the long gastric emptying times for these foods[3]. It is also possible that millet couscous, which broke down into smaller particles, could have experienced strong particle-particle interactions, forming a viscous paste in the stomach that slowed gastric emptying in the human study, but was not measured in the HGS. Meals higher in viscosity have been shown to delay gastric emptying rate in vivo[53,54]. An area of future research is the measurement of viscosity of foods in the stomach as they undergo breakdown. This study indicates there are future opportunities to understand the mechanisms of gastric emptying and the means by which it is controlled by the body, which could be investigated using a combination of in vivo and in vitro methods.

Conclusions

In this study, three types of millet couscous and two types of wheat couscous were digested using the Human Gastric Simulator, a device which simulates the peristaltic motion, continuous secretion of gastric juice, and intermittent emptying of the human stomach. It was found that millet couscous broke down into smaller and more numerous particles than wheat couscous. Millet couscous samples also showed lower percent starch hydrolysis per unit area of digested particles. It is hypothesized that the slow gastric emptying rate of millet couscous observed in humans may be explained by slow hydrolysis in the small intestines that activates the ileal brake

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mechanism. It was suggested that densely packed starch matrices exist in gastric processed millet couscous particles that are slowly digesting to reach the ileum. This study provides new understanding of the slow gastric emptying of millet couscous, which could provide strategies to make processed foods with this quality for appetite control and extended nutrient (energy) delivery to the body after food consumption.

Conflicts of interest

There are no conflicts to declare.

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